

Differential Activation of Individual Subunits in Heteromeric Kainate Receptors

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Summary

Neuronal kainate receptors are assembled from subunits with dissimilar specificities for agonists and antagonists. The composite biophysical behavior of heteromeric kainate receptors is determined by inter-subunit interactions whose nature is unclear. Here we use dysiherbaine, a selective kainate receptor agonist, to show that GluR5 subunits assembled in heteromeric GluR5/KA-2 kainate receptor complexes can gate current without concomitant activation of their partner KA-2 subunits. A long-lasting interaction between dysiherbaine and GluR5 subunits elicits a tonic current from GluR5/KA-2 receptors; subsequent cooperative gating of KA-2 subunits can be elicited by both agonists, such as glutamate, and some classically defined antagonists, such as CNQX. This study demonstrates that each type of subunit within a heteromeric kainate receptor contributes a distinct conductance upon activation by agonist binding, and therefore provides insight into the biophysical function of ionotropic glutamate receptors.

Introduction

Ionotropic glutamate receptors are multimeric channel-forming proteins that exhibit complex physiological behavior upon activation by receptor agonists. In AMPA and kainate receptors (KARs), each subunit within a receptor complex is thought to have a binding site for glutamate formed from two discontinuous regions of the subunit protein (Stern-Bach et al., 1994; Armstrong et al., 1998). Until recently, it has been difficult to determine how the occupancy of binding sites on individual receptor subunits correlated with the single-channel and

macroscopic physiology of AMPA and kainate receptors. A significant advance in understanding the mechanistic basis for AMPA receptor function was made in two studies that demonstrated that the multiple single-channel conductance levels observed for native and recombinant AMPA receptors arose from a concentration-dependent activation of individual subunits comprising the multimeric receptors (Rosenmund et al., 1998; Smith and Howe, 2000). Surprisingly, the same behavior was not observed with native KARs (Smith and Howe, 2000), suggesting that gating mechanisms in AMPA and KARs might differ fundamentally.

In contrast to AMPA receptors, which have relatively uniform pharmacological sensitivity, KARs are formed from subunits with markedly dissimilar pharmacological properties (Hollmann and Heinemann, 1994; Dingledine et al., 1999). Kainate receptors composed of GluR5, GluR6, or GluR7 subunits form functional homomeric and heteromeric channels that respond to agonists (with the exception of glutamate) with distinct channel kinetics and sensitivity (Egebjerg et al., 1991; Sommer et al., 1992; Schiffer et al., 1997; Cui and Mayer, 1999; Paternain et al., 2000). The KAR subunits KA-1 and KA-2 do not form functional homomeric ion channels, but rather modify the pharmacological and physiological properties of the channel-forming KAR subunits when coexpressed in heteromeric complexes. For example, GluR6/KA-2 receptors respond to the agonists AMPA and (S)-5-iodowillardiine (5-IW), whereas homomeric GluR6 receptors do not contain a binding site for these compounds and therefore are insensitive to their agonist activity (Herb et al., 1992; Swanson et al., 1998).

This “conferring” of novel pharmacological sensitivity by the KA-2 subunit has not been explained mechanistically. At least two models may be imagined. First, inter-subunit interactions may physically alter the binding domain of normally insensitive GluR6 subunits to create a novel binding site (e.g., for AMPA or 5-IW). This alteration then would allow all the subunits within the receptor complex to respond to the agonist. Alternatively, GluR6 may remain insensitive, with the current observed during activation of GluR6/KA-2 receptors arising solely from binding and gating of KA-2 subunits. This second hypothesis requires that kainate receptor gating occurs in a way similar to that of AMPA receptors; that is, it postulates gating by individual receptor subunits. We have obtained evidence to support the latter model. In this report, we use a highly selective KAR agonist, the marine toxin dysiherbaine (DH), and site-directed mutagenesis of KAR subunits to demonstrate preferential activation of only the GluR5 subunits within a heteromeric GluR5/KA-2 KAR complex. These results therefore support the idea that occupancy of binding sites on individual KAR subunits activates distinct channel conductance and kinetic properties.

Results

Dysiherbaine Is a High-Affinity Agonist for Homomeric Kainate Receptors

Dysiherbaine (DH) was characterized previously as a potent epileptogenic amino acid that activates both

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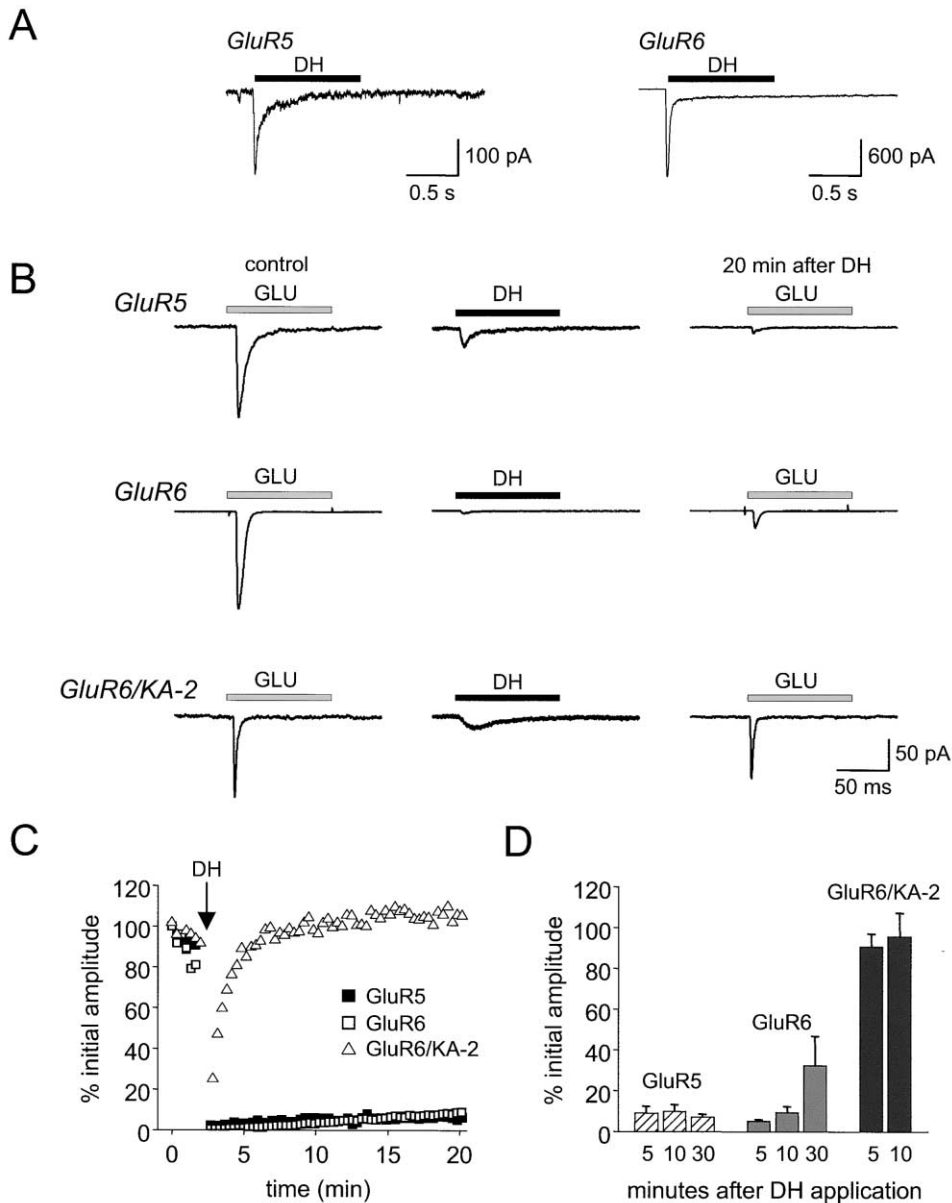


Figure 1. Activity of Dysiherbaine (DH) on Homomeric and Heteromeric Kainate Receptors

(A) DH acts as an agonist on homomeric GluR5 (left trace) and GluR6 (right trace) kainate receptors expressed in HEK293 cells. DH (100 μ M) was applied for 1 s. (B) Time course of recovery of glutamate-activated currents after application of DH. Traces show representative currents during 100 ms applications of glutamate ("control," 10 mM, gray bars), 5 s applications of DH (10 μ M), and glutamate again 20 min after DH application. Glutamate currents were evoked from GluR5 (top), GluR6 (center), and GluR6/KA-2 (bottom) receptors. (C) The graph shows the percent peak amplitudes of test glutamate currents normalized to the initial control amplitudes (before application of DH) for the experiments shown in (B) (GluR5, closed squares; GluR6, open squares; GluR6/KA-2, open triangles). (D) The histogram shows the averaged data for the normalized amplitude of the peak glutamate currents 5, 10, or 30 min after application of DH. Columns show mean \pm SEM.

AMPA and kainate receptors in cultured neurons (Sakai et al., 2001). Additionally, in radioligand binding assays, DH was shown to displace [3 H]kainate from recombinant GluR5 or GluR6 receptors with very high affinity. In our current experiments, we initially determined the activity of DH on recombinant kainate receptors formed from the subunits GluR5, GluR6, KA-1, and KA-2. We first examined currents elicited by DH on recombinant GluR5, GluR6, and GluR6/KA-2 kainate receptors tran-

siently expressed in HEK293 cells. DH (100 μ M) evoked desensitizing inward currents from cells expressing homomeric GluR5 or GluR6 kainate receptors (Figure 1A). These results also suggested that the interaction between DH and the homomeric receptors was of particularly high affinity, because application of DH desensitized the receptor for extended periods. To investigate the time course of the interaction between DH and kainate receptors, we measured the recovery of responses

evoked by glutamate after an application of DH (Figures 1B and 1C). After initial control glutamate applications (10 mM, 100 ms), DH (10 μ M) was applied for 1 s; this was followed by further glutamate applications at 20 s intervals. Representative glutamate and DH currents are shown in Figure 1B. The time courses of recovery of glutamate-activated currents are shown normalized to control amplitudes in Figure 1C. Prior DH application irreversibly and completely occluded any subsequent activation of homomeric GluR5 receptors by glutamate for at least 45 min. DH produced a similar but less profound blockade of glutamate-evoked currents mediated by GluR6 receptors, which returned to $32\% \pm 14\%$ of pre-DH amplitudes after 30 min ($n = 3$). In contrast, heteromeric GluR6/KA-2 receptors recovered to $90.4\% \pm 6.4\%$ of pre-DH amplitudes 5 min after application of DH ($n = 5$). The recovery of glutamate currents relative to control amplitudes for these kainate receptors at selected time points after DH application is summarized in Figure 1D. The most straightforward interpretation of this data is that DH forms an extremely stable closed-channel complex with homomeric kainate receptors, but not heteromeric GluR6/KA-2 kainate receptors.

Component Subunits in Heteromeric GluR5/KA-2 Receptors Respond Differently to Dysiherbaine

In contrast to the three types of kainate receptors shown in Figure 1 (GluR5, GluR6, and GluR6/KA-2), DH elicited complex channel behavior from heteromeric GluR5/KA-2 receptors (Figure 2). Glutamate currents arising from GluR5/KA-2 receptors before application of DH ("control") were rapidly desensitizing (τ_{des} of 2.1 ± 0.1 ms, $n = 20$, Figure 2A). Subsequent application of 10 μ M DH evoked a desensitizing inward current (τ_{des} of 349 ± 85 ms, $n = 9$). Surprisingly, a long-lasting inward current slowly developed when cells were returned to control solution (Figure 2B). The long-lasting current consistently arose *after* removing DH from the bathing solution—regardless of the duration of the DH application. This tonic current had a time course of activation (after removal of DH) of 25.2 ± 2.9 s ($n = 9$) and a mean amplitude of 48 ± 11 pA (range 9–199 pA, $n = 17$), which was $32\% \pm 5\%$ of the peak glutamate current in the same cells. The current amplitude typically decayed back to baseline levels after 10 to 20 min. The presence of the DH-induced tonic current, after removal of the agonist from the external solution, suggested that the interaction between DH and GluR5/KA-2 receptors was very stable.

We then applied agonists, such as glutamate, to GluR5/KA-2 receptors after the development of the DH-evoked tonic current. Glutamate elicited a transient inward current followed by rapid reduction of the tonic DH current to an amplitude similar to the baseline "holding" current *before* application of DH (i.e., an apparent positive displacement of the current, illustrated by the dotted lines in Figure 2C). The onset of the glutamate-induced reduction in current was rapid ($\tau = 2.1 \pm 0.2$ ms, $n = 18$). After removal of glutamate, the stable DH current re-emerged with a time course of 22.3 ± 1.8 s ($n = 5$, Figure 2D). The tonic current was similar in amplitude before and after application of glutamate. Because glutamate did not attenuate the tonic current (by displacing

DH), these data demonstrate that high-affinity DH interaction with one type of binding site gives rise to the tonic current, whereas a distinct binding domain(s) remain accessible to glutamate.

A Model for the Action of Dysiherbaine on GluR5/KA-2 Receptors

We developed a model to provide a mechanistic explanation for our results (Figure 2E). This model proposes that GluR5/KA-2 receptors can be activated at two pharmacologically separable sites. Note that while the model accounts for the actions of DH on heteromeric GluR5/KA-2 receptors, our initial experiments did not allow us to come to any conclusions regarding the location of sites on the receptor subunits. The proposed relationship between DH and glutamate currents and the occupancy of these binding sites is shown in the cartoon in Figure 2E. Initial DH binding to both sites results in transient activation (state 1) and desensitization of the receptor (state 2). After removal of DH, it continues to bind to the high-affinity site (represented by the green box), which produces the prolonged steady-state current (state 3). The second, lower affinity binding site (pink box) remains available to interact with agonists, resulting in transient activation (state 4) and complete desensitization of the entire receptor complex (state 5). After removal of agonist, the receptor returns to state 3, in which stably bound DH continues to elicit the steady-state current (e.g., Figure 2D).

We consider four observations to be supporting evidence for presence of multiple functional sites as shown in Figure 2E. First, activation of the receptor during the DH steady-state current by glutamate or other agonists does not compete with and displace the high-affinity DH binding that gives rise to the tonic current. Second, the high-affinity site can be selectively activated with a very low concentration of DH (100 nM), which produces a slowly rising steady-state current without the desensitization apparent at higher agonist concentrations (data not shown). Third, GluR5/KA-2 receptors can be further activated during the prolonged DH current. Glutamate application in the presence of the stable DH current invariably evoked a small inward current before the onset of desensitization (see Figure 2C, illustrated as element "4" in Figure 2E). This transient inward current had a similar voltage- and glutamate concentration dependency as that of the peak nondesensitized current of the "naïve" receptor (before DH application). The amplitude of the transient peak current elicited by glutamate was $23\% \pm 3\%$ of the peak glutamate current before application of DH ($n = 14$). Fourth, the rate of glutamate desensitization (before DH application) and the rate of glutamate-induced rapid "positive" displacement to baseline amplitudes of the tonic DH current were indistinguishable (τ values of 2.1 ± 0.2 ms and 2.0 ± 0.2 ms, $n = 16$ and 15, respectively). We also compared the rates of desensitization and positive displacement of the DH steady-state current observed with both AMPA (250 μ M) and DH (10 μ M) and found that these also were not significantly different (AMPA: τ values of 8.4 ± 1.5 ms for desensitization and 7.0 ± 1.0 ms for displacement, $n = 4$ and 3; DH: τ values of 349 ± 85 ms for desensitization and 389 ± 93 ms for displacement, $n = 9$ and 8, respec-

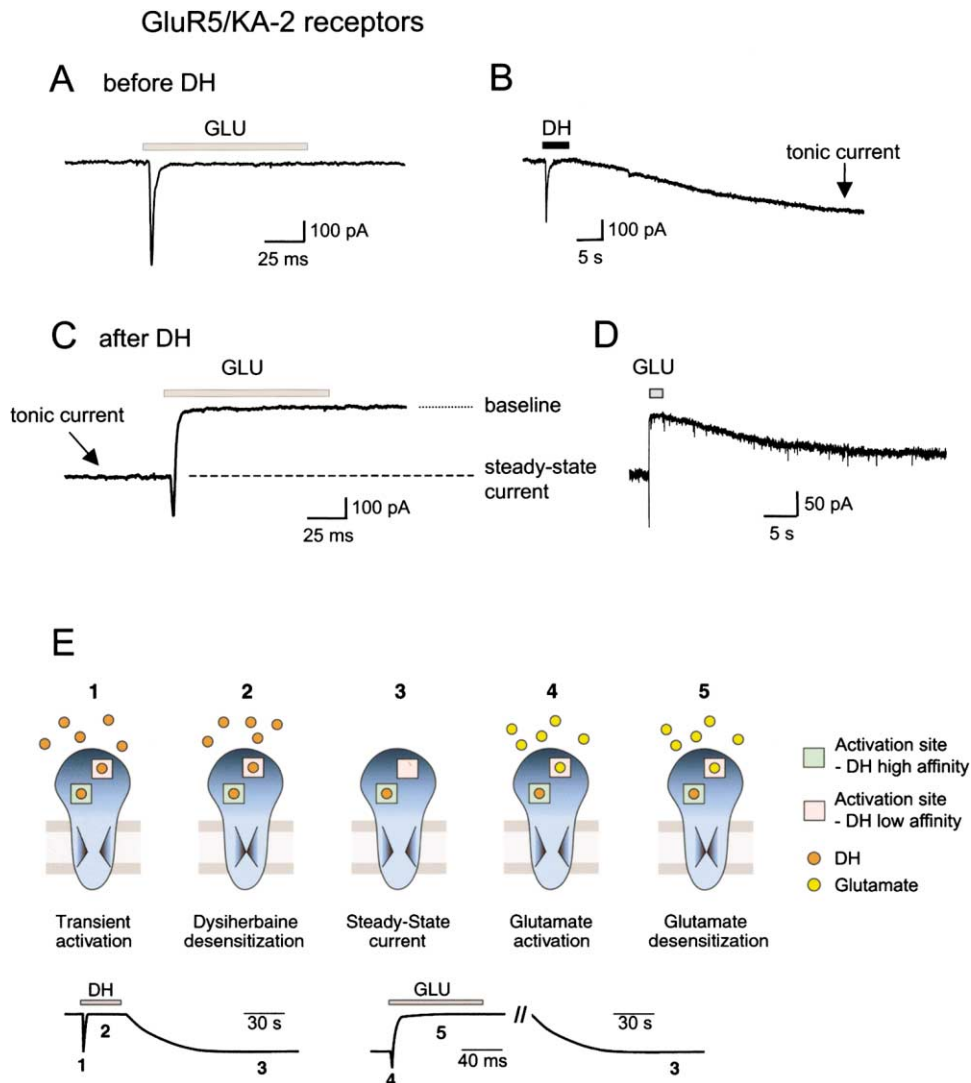


Figure 2. GluR5/KA-2 Receptors Have Multiple Sites and Modes of Activation

(A) Glutamate (10 mM, 100 ms, gray bar) evoked a rapidly desensitizing current from GluR5/KA-2 receptors that were not exposed previously to DH. (B) DH (10 μ M, 5 s, black bar) acts as an agonist on GluR5/KA-2 receptors. After cells are returned to control solution, a long-lasting inward current slowly develops. (C) Applications of glutamate after DH suppressed the steady-state DH current back to pre-DH baseline amplitudes. The dotted line shows the baseline current before DH application, and the dashed line shows the amplitude of the steady-state current after DH. (D) After removal of glutamate, the steady-state DH current returns with a time course of 22 s, demonstrating that the two activities are not competitive. (E) A hypothetical model relating occupancy of two distinct binding sites to activation and desensitization of GluR5/KA-2 receptor complexes. Heteromeric kainate receptors composed of GluR5 and KA-2 subunits are shown as single molecules, within the gray plasma membrane, with two distinct binding sites for DH—high- (green) and low-affinity (pink). The position of the triangular gates within the receptor represent permeant or nonpermeant conformations of the channel. The numbered states correspond to labeled components of the receptor currents shown in the lower left part of the panel. DH binds to both activation sites of the receptor, resulting first in channel opening and a peak transient current (identified as 1 on the idealized DH trace below the cartoons), then a slower onset of desensitization (state 2). After removal of DH from the bathing solution, it remains tightly bound to the high-affinity site, but not the low-affinity site, resulting in long-lasting steady-state current (state 3). Subsequently, glutamate can bind to the available low-affinity site and cause a transient peak current (state 4) followed by desensitization of the entire receptor complex (state 5). Upon removal of glutamate, the tonic DH current returns as the receptors recover from glutamate desensitization, and the receptor returns to state 3.

tively). The high correlation between rates of desensitization of naïve receptors and the displacement of the tonic DH currents provides strong support for the hypothesis that the current displacement arises from receptor desensitization. Thus, we conclude that the tonic DH current arises from partial activation of the heteromeric receptor complex; binding of agonists at a separate site further activates then desensitizes the receptors.

Independent Activation of Subunits in Other Heteromeric Kainate Receptor Complexes

Can we detect independent activation sites in other heteromeric kainate receptor assemblies? As shown earlier, GluR6/KA-2 receptors exhibited a much shorter lived interaction with DH (Figures 1B and 1C). However, re-examination of glutamate-activated currents evoked within 1 min of DH application revealed a small positive displacement relative to baseline amplitudes similar to

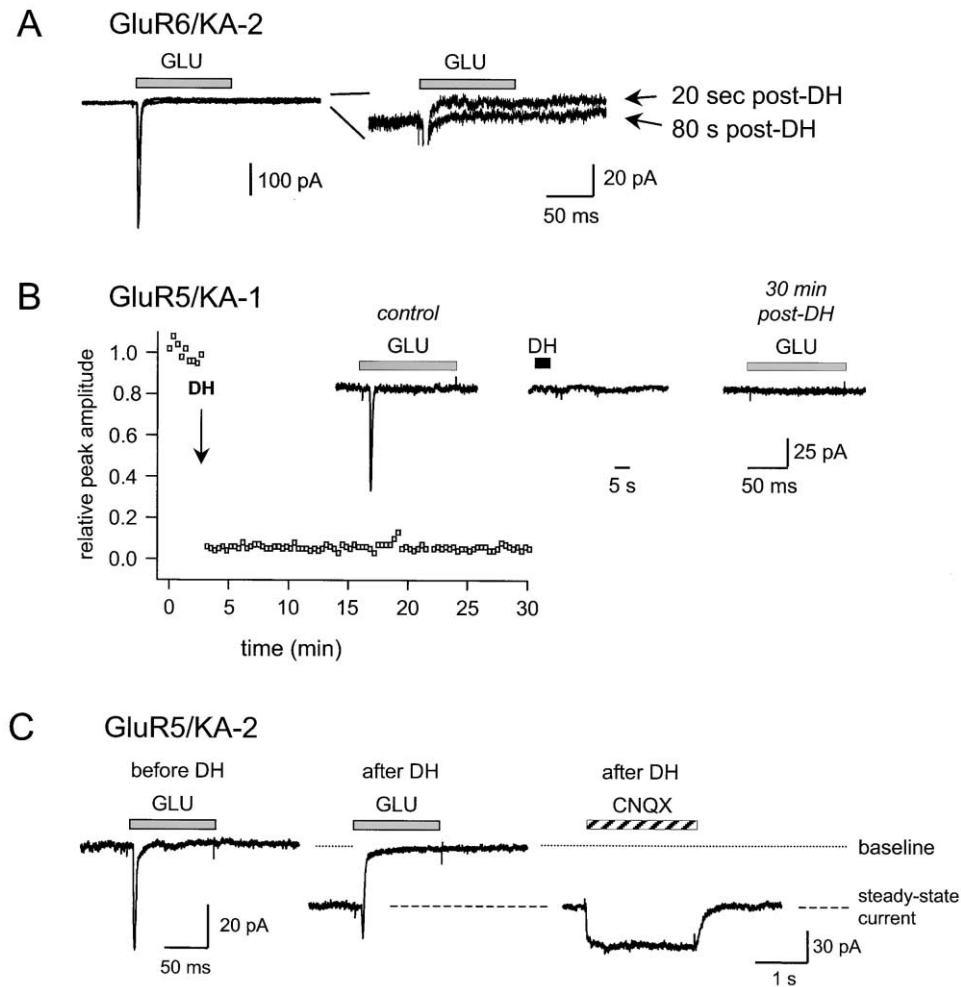


Figure 3. Multiple Activation Sites Are Detectable in KA-2 Subunit-Containing Receptors, and CNQX Acts as an Agonist at the Lower Affinity Site

(A) *Left panel:* Examples of GluR6/KA-2 currents evoked by glutamate (10 mM) 20 s and 80 s after application of DH. *Right panel:* on an expanded scale, a positive displacement of the current is evident at the earlier time point after DH application. The DH-activated steady-state current decays rapidly with this receptor combination; consequently, the second form of activation is not detectable at 80 s. The peak glutamate current has been removed for clarity and the baseline currents were normalized to more clearly illustrate the time-dependent glutamate inhibition. (B) GluR5/KA-1 receptors respond to DH like homomeric GluR5 receptors. Inset traces show representative currents from whole-cell recording during fast application of 10 mM glutamate before (control) and at 30 min after application of DH (10 μ M, 1 s, black bar). DH evokes a very small inactivating current from GluR5/KA-1 receptors, but subsequent glutamate applications do not elicit detectable peak currents. The graph shows the relative peak amplitudes of the glutamate current normalized to the initial control amplitudes for the experiment shown in the current traces. (C) CNQX acts as an agonist at the lower affinity binding site. Representative responses to glutamate before and after DH application are shown to illustrate the baseline and steady-state amplitudes for this recording. Application of CNQX (50 μ M) in the presence of the steady-state DH current elicits a nondesensitizing inward current.

that seen in GluR5/KA-2 receptors (Figure 3A). The DH-activated GluR6/KA-2 current was detectable only for a brief period of time (<1 min), presumably reflecting the more rapid dissociation of DH from the high-affinity activation site of this heteromeric receptor. These experiments demonstrate that DH differentiates multiple activation sites in receptors containing either GluR5 or GluR6 subunits with the KA-2 subunit.

The KA-2 subunit is highly homologous to the KA-1 receptor subunit (Werner et al., 1991; Herb et al., 1992), and these receptor subunits confer similar functional properties when assembled with the channel-forming subunits GluR5, GluR6, or GluR7 (Herb et al., 1992; Partin et al., 1993; Schiffer et al., 1997). We next tested if het-

eromeric kainate receptors containing KA-1 subunits exhibited the long-lasting DH current (Figure 3B). We found that, in contrast to GluR5/KA-2 receptors, GluR5/KA-1 receptors were irreversibly inactivated by DH (Figure 3B). A prolonged steady-state current was not observed. Less than 10% recovery of glutamate currents was observed for up to 30 min after application of DH ($n = 4$). We conclude that domains found on the KA-2 subunit, but not the KA-1 subunit, interact with GluR5 subunits to produce DH-induced tonic current.

CNQX Acts as an Agonist on KA-2 Receptors

We tested whether the well-characterized antagonist CNQX could occlude the action of glutamate on the

GluR5/KA-2 lower affinity site (after application of DH). Surprisingly, in the presence of the DH tonic current, application of 50 μ M CNQX elicited a reversible non-desensitizing *inward* current with a mean amplitude of 24 ± 7 pA ($n = 6$, Figure 3C). No CNQX-activated current was observed before application of DH (data not shown), nor did application of 0.25% DMSO, the solvent used for CNQX, elicit a current after exposure to DH. DNQX, a closely related analog of CNQX, elicited similar currents from GluR5/KA-2 receptors (data not shown). These data therefore show that under some conditions, CNQX is an agonist for GluR5/KA-2 receptors.

Identification of Receptor Subunits that Form the High- and Low-Affinity Binding Sites for Dysihherbaine

The model in Figure 2 proposes two distinct activation sites in GluR5/KA-2 receptors that can be differentiated by the agonist DH, but does not identify which subunits within the heteromeric receptor complex contribute the high- and low-affinity binding sites. We next attempted to refine our model by testing the hypothesis that the two functional binding sites were formed from the S1-S2 binding domains of the individual kainate receptor subunits, as is the case for other AMPA and kainate receptor subunits (Stern-Bach et al., 1994; Armstrong et al., 1998; Abele et al., 1999; Armstrong and Gouaux, 2000). Because of the marked difference in the duration of the steady-state DH current between GluR5/KA-2 and GluR6/KA-2 receptors, we postulated that the high-affinity DH binding site resided on GluR5 subunits, whereas the low-affinity DH binding site (at which CNQX acts as agonist) is formed by the KA-2 subunit. To explore the contributions of individual subunits to the behavior of the GluR5/KA-2 receptor, we made use of site-directed mutants of kainate receptor subunits (Swanson et al., 1997).

Substitution of a serine for an asparagine in the GluR6 subunit at residue 721 (mutant R6(N721S)) produces a receptor that responds to some agonists with the pharmacology of a GluR5 receptor (Swanson et al., 1997). Similarly, we found that homomeric and heteromeric receptors containing the R6(N721S) subunit responded to DH as though they contained GluR5 receptor subunits (Figure 4A). Currents evoked by glutamate from homomeric R6(N721S) receptors previously exposed to DH (10 μ M, 1 s) showed no recovery of glutamate-evoked currents for up to 30 min ($3.4\% \pm 1.8\%$ of control current amplitudes, $n = 4$). Application of DH to heteromeric R6(N721S)/KA-2 receptors elicited a desensitizing inward current followed by a prolonged steady-state current after removal of DH, similar to GluR5/KA-2 receptors (Figure 4A). The time courses of the actions of DH and glutamate on R6(N721S)/KA-2 receptors were similar to those of GluR5/KA-2 receptors; for example, desensitization rates of R6(N721S)/KA-2 receptors upon application of glutamate or DH (τ_{des} of 2.6 ± 0.1 and 331 ± 52 ms, $n = 16$ and 11 , respectively) were similar to that of GluR5/KA-2 receptors. Finally, in the presence of the stable DH-induced current, CNQX evoked a non-desensitizing current from R6(N721S)/KA-2 receptors (23 ± 7 pA, $n = 3$, Figure 4B). Similar results were observed when a threonine was substituted for N721 in

GluR6 (data not shown). These data demonstrate that residue 721 on GluR5 and GluR6 is likely to be a primary determinant of receptor gating following DH binding to the high-affinity site on heteromeric kainate receptors.

We also made mutations in the KA-2 subunit to determine if interactions with threonine 705, the KA-2 equivalent of asparagine 721 in GluR6, influenced the lower affinity response to glutamate or CNQX. The corresponding residue was shown to interact directly with DNQX in a GluR2 S1S2 crystal structure (Armstrong and Gouaux, 2000). Two substitutions, to an asparagine (KA2(T705N)) or a serine (KA2(T705S)), were made for threonine 705, and the resultant KA2 mutants were expressed in combination with GluR5 subunits. The glutamate desensitization rate was unaffected by these mutations, and GluR5/KA2(T705S/N) receptor assemblies responded to DH with the prolonged current after removal of the agonist. We tested both combinations of heteromeric receptors for sensitivity to CNQX, and found that while it elicited a non-desensitizing current from GluR5/KA-2(T705S) receptors, GluR5/KA-2(T705N) receptors were insensitive to CNQX ($n = 4$, Figure 4C). Receptors comprised of two mutant subunits, R6(N721S)/KA2(T705N), were similarly insensitive to CNQX ($n = 3$). Furthermore, GluR5/KA2(T705N/S) receptors "recovered" to the tonic DH current amplitude more rapidly after application of glutamate. This rate was 1.8 ± 0.2 s for GluR5/KA-2(T705N) ($n = 5$) and 8.4 ± 1.5 s for GluR5/KA-2(T705S) receptors ($n = 5$) (compared to 22.3 s for GluR5/KA-2 receptors). There were no apparent differences in the durations of the steady-state DH current with the KA-2 mutants. In summary, these data support the interpretation that KA-2 is the subunit on which CNQX acts as an agonist and localize residue 705 as one important determinant of KA-2 current kinetics.

Binding Studies Support the Existence of High- and Low-Affinity DH Binding Sites

Our model postulates that GluR5/KA-2 receptors contain two binding sites with markedly different affinities for DH. Previously, we reported that DH displaced [3 H]kainate binding to GluR5 receptors with a K_i value of 0.5 nM (Sakai et al., 2001). In similar experiments to determine the DH binding affinity for the KA-2 subunit, we found that DH displaced [3 H]kainate with a significantly higher K_i value of 4.3 ± 0.8 μ M ($n = 5$, Figure 4D). These data show that the DH binding affinity for GluR5 and KA-2 subunits differs by at least four orders of magnitude, and support the interpretation that the functionally observed high- and low-affinity sites for DH reside on the GluR5 and KA-2 subunits, respectively.

Discussion

By virtue of its unusually high affinity for a subset of kainate receptor subunits, DH provides insight into the biophysical operation of this family of ionotropic glutamate receptors. The action of DH on GluR5/KA-2 and GluR6/KA-2 receptors, together with site-directed mutagenesis of the receptor subunits, provides support for a model of receptor function in which individual subunits can be activated within the multimeric receptor complex. Thus, agonists for heteromeric kainate receptors

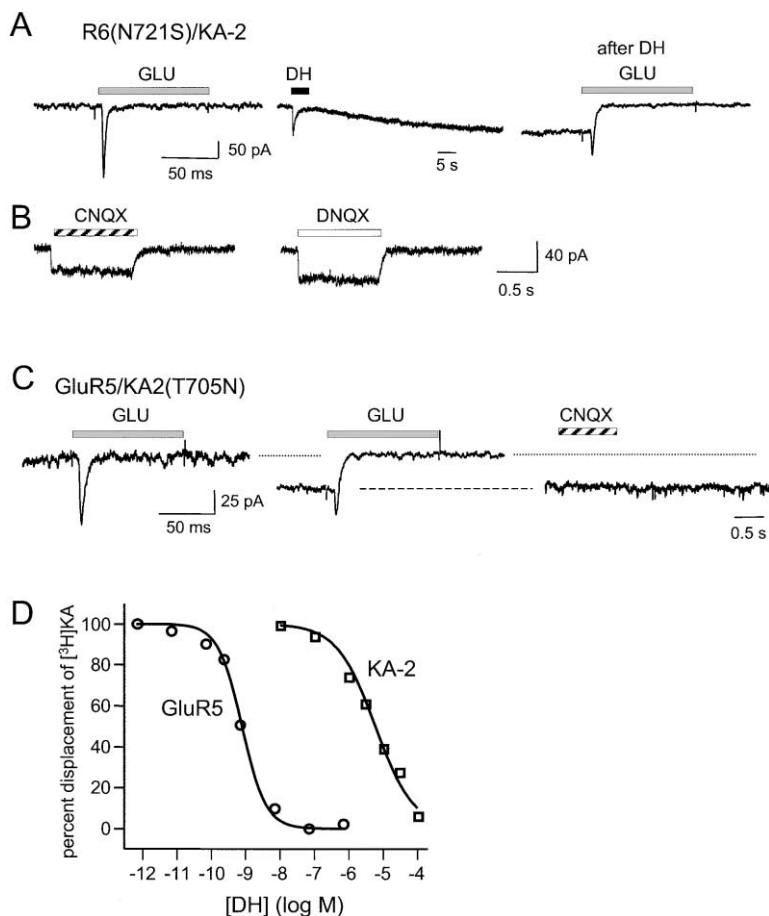


Figure 4. Localization of the High- and Low-Affinity Binding Sites to GluR5 and KA-2 Subunits, Respectively, Using Site-Directed Mutagenesis

(A) A site mutation of the GluR6 subunit converts the DH sensitivity to that of GluR5 receptors. Heteromeric R6(N721S)/KA-2 receptors exhibit rapidly desensitizing responses to glutamate (100 ms, 10 mM, left trace). DH application (10 μM, 5 s) elicits a long-lasting steady-state current like that of GluR5/KA-2 receptors (center trace). The amplitude of the steady-state DH current was 150 ± 34 pA ($n = 16$). In the presence of the steady-state DH current, glutamate elicits a transient peak current and then desensitizes the entire receptor complex (right trace). (B) After treatment with DH, CNQX (50 μM) and DNQX (50 μM) elicited inward currents from R6(N721S)/KA-2 receptors. (C) Mutation of threonine 705 to an asparagine in the KA-2 subunit eliminates CNQX agonist activity. Glutamate evokes currents that rapidly desensitize with time course identical to that of GluR5/KA-2 receptors (left trace). After application (and removal) of DH, the steady-state current resulting from continued occupation of the high-affinity binding site develops, and subsequent applications of glutamate desensitize the GluR5/KA2(T705N) receptors (center trace). Application of CNQX (50 μM) does not evoke an inward current (right trace). (D) Displacement of [³H]kainate binding from membranes containing KA-2 receptor subunits. A range of concentrations of DH was used to displace [³H]kainate from KA-2 subunits. The K_i value was calculated as 4.9 μM for the example shown. DH displacement of [³H]kainate from GluR5 subunits is shown for the sake of comparison and is taken from Sakai et al. (2001).

will elicit currents whose kinetic properties are dependent on the efficacy of activation of each type of component subunit in the receptor complex as well as the degree to which kinetic parameters are dominated by a particular receptor subunit. Kainate receptor subunits are heterogeneous in their pharmacological specificities, as evidenced by the unusual responses to DH, and therefore this model has implications for the design of drugs that target kainate receptors in the CNS.

The model of receptor function that incorporates our site-directed mutagenesis and binding results is detailed in Figure 5. It represents an extension of the car-

toon from Figure 2 with experimentally defined binding sites localized to subunit proteins. We propose that the high-affinity binding site for the agonist DH resides on the GluR5 subunit (or GluR6 subunits mutated at residue 721) and the low-affinity site is formed by the KA-2 subunit. While this model, in which the high- and low-affinity binding domains are contained within individual subunits, represents the most straightforward interpretation of our mutagenesis results, we recognize that possibilities that are more complex could be imagined. The prolonged current after DH application arises from continued occupation of the high-affinity agonist binding site

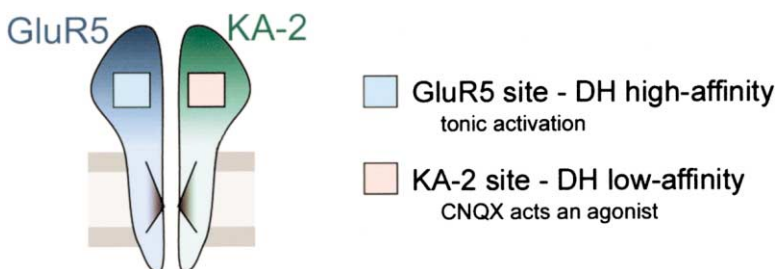


Figure 5. A Cartoon Extending Our Model from Figure 2 to Incorporate Results from Site-Directed Mutagenesis of the GluR5/6 Subunits (at Residue 721) and KA-2 Subunits (at Residue 705)

Tonic current from GluR5/KA-2 receptors results from stable binding to and selective activation of GluR5 subunits by DH. Subsequent binding by glutamate to the low-affinity site on KA-2 subunits causes a small transient inward current, followed by a desensitization of the entire GluR5/KA-2 receptor complex (see idealized currents in Figure 2E). CNQX acts as an agonist on the KA-2 subunit after tonic activation of the GluR5 subunits by DH.

on GluR5 subunits. In the presence of this prolonged current, agonists can access the unoccupied KA-2 binding site and cause a transient current followed by desensitization of the entire receptor complex. Re-emergence of the prolonged DH current is likely determined by the rate of recovery from desensitization and unbinding of agonist from the low-affinity binding site on the KA-2 subunit. Most surprisingly, CNQX acts as a nondesensitizing agonist on the KA-2 subunit, but only when the GluR5 subunits first are activated by DH. The dissimilar pharmacology of GluR5 and KA-2 subunits reveals that each type of subunit can contribute an independent agonist-activated conductance.

Our results suggest that mechanisms of gating in kainate receptors are similar to recent proposals for the activation of AMPA receptors (Rosenmund et al., 1998; Smith and Howe, 2000). Rosenmund and coworkers used a nondesensitizing mutant of the GluR3 AMPA receptor to show that the relative proportion of individual single-channel conductance states depended on the number of agonist molecules bound to receptor subunits; for example, they observed a higher proportion of openings to larger conductances at higher agonist concentrations. From these data, they inferred that binding of an agonist molecule to a single receptor subunit (in a tetrameric complex) produced an associated conductance; as more subunits were activated, the single-channel conductance became larger. Subsequent examination of cerebellar AMPA and kainate receptor single-channel currents by Smith and Howe (Smith and Howe, 2000) supported a similar mode of operation for native AMPA receptors. In contrast, kainate receptors did not appear to behave in the same way, which was surprising considering their structural and functional similarity to AMPA receptors. Single-channel conductance states did not correlate with agonist concentration, suggesting that kainate receptors (in cerebellar granule neurons) might have a gating mechanism distinct from that of AMPA receptors. In our study, the differential pharmacological profile of kainate receptor subunits allowed us to demonstrate at a macroscopic level that these receptors have subunit-associated conductances. Therefore, AMPA and kainate receptors are likely to share a common gating mechanism.

While our data demonstrate that activation of a subset of subunits can occur within a heteromeric receptor complex, other aspects of channel gating, in particular desensitization, are dependent on interaction between subunits. The observation that DH produces a sustained current from GluR5/KA-2 but not homomeric GluR5 receptors demonstrates that the gating of the GluR5 subunits (in heteromeric receptors) is influenced by the presence of KA-2 subunit. The location of the domains on KA-2 that effect the change in GluR5 kinetics is unknown, although it is worth noting that many residues that alter desensitization in AMPA and kainate receptors are located in regions that may form intersubunit interfaces (Armstrong and Gouaux, 2000). Glutamate desensitization of the DH tonic current in GluR5/KA-2 receptors demonstrates that agonist binding to one subunit can produce a dominant desensitization of distinct subunits in the heteromeric complex. Related observations were made in previous studies that examined the pharmacological properties of GluR5/GluR6 heteromers (Cui

and Mayer, 1999; Paternain et al., 2000). For example, Cui and Mayer (1999) found that the currents evoked by the GluR5-selective agonist (S)-5-iodowillardiine desensitized less in GluR5/GluR6 receptors compared to homomeric GluR5 receptors. Similarly, Paternain and coworkers observed that activation of heteromeric GluR5/KA-2, GluR6/KA-2, and GluR5/GluR6 receptors with selective agonists elicited currents with differing desensitization kinetics. It is possible that the complicating effect of desensitization on the gating kinetics of heteromeric kainate receptors accounts for the apparently concentration-independent behavior of kainate receptors in single channel recordings by Smith and Howe, as was previously suggested by these authors (Smith and Howe, 2000).

This aspect of kainate receptor function is intriguing in light of the different pharmacological sensitivities of the two related groups of proteins that associate to form kainate receptors (GluR5/GluR6/GluR7 subunits versus KA-1/KA-2 subunits). By inference from mRNA expression patterns, many, if not all, neuronal kainate receptors will contain at least one member of each group in heteromeric channel complexes (the vast majority of those will contain KA-2 rather than KA-1 subunits). We have identified a compound, DH, which binds to GluR5 and GluR6 subunits with an affinity four orders of magnitude greater than its affinity for KA-2 subunits, which may facilitate the future exploration of the subunit composition of neuronal kainate receptors. Other compounds, such as AMPA, have an inverse selectivity profile and bind with high affinity to KA-2 subunits but with very low affinity to GluR5 or GluR6 subunits; similarly, AMPA will elicit currents from heteromeric GluR6/KA-2 receptors but not homomeric GluR6 receptors. By analogy with our understanding of how we think DH interacts with KARs, AMPA would be selectively activating KA-2 subunits to produce currents from GluR6/KA-2 receptors.

CNQX provides a third, more unusual example of selective activity on heteromeric GluR5/KA-2 receptors. In this case, the compound acts as an antagonist on one subunit, GluR5, but as an agonist on the partner KA-2 subunits. The observation that agonist activity was eliminated by changing a single amino acid in the KA-2 subunit, T705, as well as earlier results in which CNQX was shown to be a potent agonist on recombinant GluR1 AMPA receptors containing the Lurcher mutation (A636T) (Taverna et al., 2000), underscore the conclusion that single amino acids critical for receptor gating can determine how receptor ligands are defined pharmacologically. We note, however, that the CNQX agonist activity thus far is detectable only under a rather unusual set of conditions—previous activation of partner GluR5 subunits. Two possible explanations may account for the absence of a CNQX-evoked current before application of DH: first, the antagonist activity of CNQX on GluR5 subunits may “lock” the heteromeric receptors in a state unfavorable for gating, and thus dominate the agonist activity on KA-2 subunits. Alternatively, KA-2 subunits in heteromeric receptors may require cooperative activation of GluR5 subunits before agonist binding can elicit gating. Site-directed mutations on GluR5 or KA-2 that selectively eliminate the affinity for CNQX may distinguish between these possibilities.

In demonstrating that individual subunits assembled into heteromeric glutamate receptors can respond to agonists in markedly dissimilar ways to give rise to unexpected receptor behavior, our results add a level of complexity on to the design of drugs that target kainate receptors. With its nearly ubiquitous expression in the brain, the KA-2 subunit is likely a constituent of most neuronal kainate receptors. Understanding the pharmacological specificity of this subunit and functional contributions to channel behavior therefore will be important for interpreting the neuronal response to drugs designed to alter glutamate receptor function. These results demonstrate that pharmacological targeting of heteromeric kainate receptors should consider each type of contributing subunit as a distinct site for therapeutic drug discovery.

Experimental Procedures

Transfection and Electrophysiology

Transfection of HEK293 cells (American Type Culture Collection, Manassus, VA) and whole cell patch-clamp analysis of recombinant kainate receptors were carried out as described previously (Swanson et al., 1997). Cells were maintained in DMEM media supplemented with 100 μ g/ml penicillin, 100 μ g/ml streptomycin, and 10% FCS. One day before transfection, cells were split to low density on glass coverslips coated with 100 μ g/ml poly-D-lysine and collagen. Transfection of receptor cDNAs was by standard calcium-phosphate precipitation with 1 μ g cDNA per coverslip for 3–8 hr at 37°C and 8% CO₂. All receptor subunits were co-transfected with a CD8 antigen-containing plasmid (0.2 μ g/coverslip). To ensure that the currents arose from heteromeric, but not homomeric, receptors, we co-expressed the KA-1 or KA-2 subunits with a GluR5 subunit that contained an arginine at the Q/R editing site, GluR5(R); homomeric GluR5(R) receptors do not activate detectable whole-cell currents in single expressing HEK293 cells.

Electrophysiological recordings were made 1–3 days after transfection and following incubation of cells with polystyrene beads coated with anti-CD8 antibody (Dynal Inc., Lake Success, NY). The internal solution was composed of 110 mM CsF, 30 mM CsCl, 4 mM NaCl, 0.5 mM CaCl₂, 10 mM HEPES, and 5 mM EGTA (adjusted to pH 7.3 with CsOH). The external bath solution contained 150 mM NaCl, 2.8 mM KCl, 2 mM CaCl₂, 1.0 mM MgCl₂, and 10 mM HEPES (pH was adjusted to 7.3 with NaOH). To achieve rapid agonist application exchange, cells were lifted from the coverslip into a laminar solution stream that was displaced by a piezo-bimorph under the control of pClamp 8 software (Swanson et al., 1997). Whole-cell patch recordings were made with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Data were acquired and analyzed using pClamp 8 software (Axon Instruments, Foster City, CA) and Origin 6.0 (OriginLab Corp. Northampton, MA) and are given as mean \pm SEM. Electrical artifacts arising from translation of the piezo-bimorph were removed in the figures in the interest of clarity. Dysiherbaine was isolated as described previously (Sakai et al., 2001). Glutamate and other compounds were purchased from Sigma-RBI (St. Louis, MO). The GluR5(Q) and GluR5(R) cDNAs were generously donated by Dr. Peter Seeburg (Heidelberg, Germany).

Equilibrium Binding

Membrane preparations and [³H]kainate displacement assays were performed as described previously (Swanson et al., 1997). The range of [³H]kainate concentrations used in this study were 9–16 nM. Non-specific binding was defined in the presence of 1 mM glutamate.

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