Divergent Pharmacological Activity of Novel Marine-Derived Excitatory Amino Acids on Glutamate Receptors

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ABSTRACT
Kainate receptors show a particular affinity for a variety of natural source compounds, including dysiherbaine (DH), a potent agonist derived from the marine sponge Dysidea herbacea. In this study, we characterized the pharmacological activity and structural basis for subunit selectivity of neodysiherbaine (neoDH) and MSVIII-19, which are natural and synthetic analogs of DH, respectively. NeoDH and MSVIII-19 differ from DH in the composition of two functional groups that confer specificity and selectivity for ionotropic glutamate receptors. In radioligand binding assays, neoDH displayed a 15- to 25-fold lower affinity relative to that of DH for glutamate receptor (GluR5) and GluR6 kainate receptor subunits but a 7-fold higher affinity for kainate (KA2) subunits, whereas MSVIII-19 displaced [3H]kainate only from GluR5 subunits but not GluR6 or KA2 subunits. NeoDH was an agonist for kainate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in patch-clamp recordings; in contrast, MSVIII-19 acted as a potent antagonist for homomeric GluR5 receptor currents with weaker activity on other kainate and AMPA receptors. Neither neoDH nor MSVIII-19 activated group I metabotropic GluRs. Homology modeling suggests that two critical amino acids confer the high degree of selectivity between the dysiherbaine analogs and the GluR5 and KA2 subunits. In summary, these data describe the pharmacological activity of two new compounds, one of which is a selective GluR5 receptor antagonist that will be of use for understanding native receptor function and designing more selective ligands for kainate receptors.

The primary mediators of excitatory transmission in the brain are glutamate receptors, which play key roles in the physiology and pathology of the brain. Glutamate activates a variety of both ionotropic and metabotropic receptors. The

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ABBREVIATIONS: NMDA, N-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; GluR, glutamate receptor; mGluR, metabotropic glutamate receptor; KA, kainate; DH, dysiherbaine; LBD, ligand binding domain; MSVIII-19, (2S,3aR,4aR,6aR,7aR,8R,S)-6-[2-(1(2)OH-tetrazole-5-yl)ethyl]decahydroisoquinoline-3-carboxylic acid; neoDH, neodysiherbaine; EPSC, excitatory postsynaptic current; IPSC, inhibitory postsynaptic current; IPSC, excitatory postsynaptic current; QX-314, 2-(triethylamino)-3,5-dihydroxyphenylglycine; ATPA, (S)-2-amino-3-(3-hydroxy-5-tert-butylisoxazole-4-yl)propanoic acid; LY293558, (3S,4aR,6R,8aR)-6-[2-(1(2)OH-tetrazole-5-yl)ethyl]decahydroisoquinoline-3-carboxylic acid; LY382884, (3S,4aR,6R,8aR)-6-(4-carboxyphenyl)methyl-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxylic acid; UBP296, (3R,3aR)-(2-carboxybenzyl)willardiine.
synthesized, and characterized as a glutamate receptor agonist with 5-fold selectivity for evoking currents from neuronal kainate receptors compared with AMPA receptors (Sasaki et al., 1999; Sakai et al., 2001a). DH also had weak agonist activity on the group I metabotropic receptor mGluR5. The very high affinity that DH exhibited for the GluR5 and GluR6 subunits compared with the KA2 subunit proved useful as a tool for elucidating the functional role of individual subunit activation within heteromeric kainate receptors (Swanson et al., 2002). DH is a rigid analog of glutamate structurally distinct from other high-affinity agonists such as kainate and domoate, and recently other structurally related compounds were shown to have biological activity (Sasaki et al., 1999; Sakai et al., 2001a).

Binding of glutamate and other compounds that act competitively on glutamate receptors occurs at the ligand binding domain (LBD), which is composed of the noncontiguous S1 and S2 regions of subunit proteins. The S1 region precedes the first membrane domain, and the S2 region is contained within an extracellular loop between the second and third membrane domains (Stern-Bach et al., 1994). The crystal structure of the GluR2 subunit LBD was determined in complex with a number of ligands as well as the apo form; these seminal findings produced the first testable models for structural correlates of receptor binding and function (Armstrong et al., 1998; Armstrong and Gouaux, 2000). Homology models based on the GluR2 structures have provided insight into which residues are important for the selectivity of various compounds on kainate receptors (Pentikäinen et al., 2003). The utility of modeling interactions between ligands and kainate receptor binding site domains was validated by recent crystallographic resolution of an initial set of domain-ligand complexes (Mayer et al., 2005; Nanao et al., 2005; Naur et al., 2005) that closely matched those predicted by homology modeling.

To correlate structural elements of DH-related marine toxins with their specificity on ionotropic glutamate receptors, we characterized the pharmacological action of two new compounds, neodysiherbaine (neoDH) and MSVIII-19 (Fig. 1). NeoDH is a natural analog of DH that is produced in much lower quantities by the marine sponge Dysidea herbacea (Sakai et al., 2001a). MSVIII-19, in contrast, is a synthetic analog lacking two critical functional groups that likely confer specificity and selectivity for kainate receptors (Sasaki et al., 1999). Neither neoDH nor MSVIII-19 demonstrates affinity for rat brain NMDA receptors (Sakai et al., 2001a; K. Shimamoto, personal communication). When injected into mice, neoDH elicits seizure behavior similar to that of DH, whereas MSVIII-19 evokes initial brief seizures that are followed by an extended period of unconsciousness (Sasaki et al., 1999). We report here that neoDH is similar to DH in its pharmacological action on kainate receptors, albeit with slightly different binding affinities for individual receptor subunits. In contrast, MSVIII-19 is a selective antagonist for GluR5 kainate receptors with no activity on GluR6 kainate receptors. MSVIII-19 also inhibits activation of recombinant and native AMPA receptors with much lower potency. To facilitate an understanding of structural elements that confer the specificity of neoDH and MSVIII-19 for a subset of kainate receptors, we modeled the ligand binding domain of GluR5 and KA2 subunits and predict that the large difference in affinity might be accounted for by two residues in the S2 domain of the kainate receptor subunits.

**Materials and Methods**

**Electrophysiology.** HEK293 cells were transfected with receptor cDNAs (0.05–0.3 μg) in combination with enhanced green fluorescent protein cDNA for the visualization of transfected cells. A ratio of 1:6 [GluR6(Q)] or 1:3 GluR5(R) to KA2 cDNA was used for expression of heteromeric receptors. GluR1, GluR2, and GluR4 AMPA receptor cDNAs were flip isoforms. Patch-clamp recordings were made 24 to 72 h after transfection. The internal solution contained 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 was composed of 150 mM NaCl, 2.8 mM KCl, 2 mM CaCl₂, 1.0 mM MgCl₂, and 10 mM HEPES (pH adjusted to 7.3 with NaOH). Patch electrodes were pulled from thick-walled borosilicate glass (Warner Instruments, Hamden, CT) with a final resistance of 1.5 to 2.5 MΩ after fire polishing. Fast application of drugs was achieved through a three-barrel glass tube mounted on a piezo-bimorph, as described previously (Swanson et al., 1997). Briefly, cells were lifted from coverslips to provide a laminar flow across the cells. Fast application of drugs was achieved by applying TTL pulses controlled by pClamp9 (Axon Instruments Inc, Union City, CA). Whole cell patch-clamp recordings were performed with an Axopatch 200B amplifier (Axon Instruments Inc.). The holding potential for all patch-clamp recordings was −70 mV. In general, only a single cell was recorded per coverslip of GluR5 or GluR6 receptor-expressing cells in the neoDH experiments because of the long duration of action of this compound.

Horizontal hippocampal slices (0.35 mm) were made from P30 to P40 129SvEv mice as described previously (Ito et al., 2004). Whole cell patch-clamp recordings of AMPA-EPSCs were made from CA1 pyramidal cells while stimulating Schaffer collateral inputs with a monopolar glass electrode in the stratum radiatum. The internal solution for the EPSC recordings consisted of 95 mM CsF, 25 mM CsCl, 10 mM Cs-HEPES, 10 mM Cs-EGTA, 2 mM NaCl, 2 mM Mg-ATP, 10 mM QX-314, 5 mM tetrathylammonium-Cl, and 5 mM 4-aminopyridine, with the pH adjusted to 7.3 with CsOH. AMPA-EPSCs were recorded in the presence of D-(-)-2-amino-5-phosphonopentanoic acid (25 μM), bicuculline (10 μM), and picrotoxin (50 μM) at a basal stimulation of 0.05 Hz. Inhibitory postsynaptic potentials were recorded from CA1 pyramidal neurons after monopolar stimulation in the stratum radiatum. The internal solution for IPSC recordings was identical to that for EPSC recordings, with the exception that it contained 120 mM CsCl rather than CsF/CsCl. Paired pulse stimulation used two pulses delivered at a 40 ms interval. IPSCs were recorded in the presence of D-(-)-2-amino-5-phosphonopentanoic acid (25 μM) and 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (50 μM). Data were acquired and analyzed using pClamp9 software (Axon Instruments Inc.), Origin 6.0 (MicroCal Software, Northampton, MA), and Prism 4 (GraphPad Software Inc., San Diego, CA).

**Calcium Imaging.** Two to 3 days after transfection of HEK293 cells with mGluR1 or mGluR5 cDNAs, cells were incubated in 5 mM

![Fig. 1. Chemical structures of dysiherbaine and its analogs neodysiherbaine and MSVIII-19. The C8 and C9 carbons are indicated.](image)
FurA-2/AM (Molecular Probes, Eugene, OR) for calcium imaging as described previously (Sakai et al., 2001b). Cells were visualized on an inverted microscope (Nikon Eclipse TE200) equipped with a 12 digital monochrome cooled charge-coupled device camera (Roper Coolsnap HQ) in combination with a DG-4 illumination and rapid excitation-filter-changer (Sutter Instrument Company, Novato, CA). Control of the system was carried out using Metafluo (Universal Imaging Corporation, Downingtown, PA). 

Results

Kainate from Recombinant Kainate Receptors. DH is a high-affinity, subunit-selective ligand for kainate receptors that binds GluR5 subunits with much higher affinity than KA2 subunits (Sakai et al., 2001b; Swanson et al., 2002). Recently neoDH and MSVIII-19, two biologically active structural analogs of DH, were characterized in preliminary behavioral assays (Sasaki et al., 1999; Sakai et al., 2001a). NeoDH contains a hydroxyl group at the C8 ring position rather than the methyleneamine substituent found in DH (Fig. 1). MSVIII-19 lacks functional groups at the C8 and C9 positions. Ligand binding experiments from rat brain membranes suggested that these substituents were critical determinants of affinity for ionotropic glutamate receptors (Sasaki et al., 1999; Sakai et al., 2001a).

To test the role of the C8 and C9 side groups in conferring affinity for kainate receptor subunits, we initially characterized the ligand binding affinity of neoDH and MSVIII-19 for the GluR5, GluR6, and KA2 subunits. Varying concentrations of neoDH and MSVIII-19 were used to displace [3H]kainate from membranes prepared from receptor-expressing COS-7 cells (Table 1; Fig. 2). Table 1 summarizes the Ki values calculated from the average displacement curves for the compounds on each kainate receptor subunit. For the purpose of comparison, we included the Ki values for DH in Table 1 (Sakai et al., 2001b) and representative displacement curves for DH in the graphs in Fig. 2 (Swanson et al., 2002). As shown in Fig. 2, neoDH displaced [3H]kainate most potently from GluR5 receptors, with a Ki value of 7.7 ± 1.3 nM, compared with 33 ± 9.0 nM and 0.6 ± 0.1 μM for GluR6 and KA2 subunits, respectively (n = 3-4 for each concentration). NeoDH was a lower-affinity ligand than DH for GluR5 and GluR6 kainate receptors by 15- to 25-fold (Swanson et al., 2002), consistent with a weaker potency for seizure induction in mice (Sakai et al., 2001a). NeoDH exhibited a markedly lower affinity for the KA2 subunits compared with GluR5 or GluR6 subunits, but compared with DH was of ~10-fold higher affinity for KA2 subunits (Table 1). MSVIII-19 displayed a unique profile on kainate receptors compared with DH and neoDH. This synthetic analog displaced [3H]kainate from GluR5 kainate receptor subunits with a Ki value of 128 ± 21 nM (Table 1; Fig. 2) but did not significantly displace the radioligand from either GluR6 or KA2 kainate receptor subunits at concentrations as high as 100 μM. MSVIII-19 also weakly displaced [3H]AMPA from GluR1, GluR2, and GluR4 AMPA receptor subunits (dis-
placement with 100 μM MSVIII-19 44 ± 3, 31 ± 4, and 18 ± 9%, respectively; n = 3–4 assays for each subunit; data not shown). These data demonstrate that substitution of the C8 methylamine group with a hydroxyl group in neoDH produces mixed effects on kainate receptor subunit affinity, whereas loss of both C8 and C9 functional groups eliminates affinity for all but the GluR5 kainate receptor subunit.

**NeoDH Is an Agonist for AMPA and Kainate Receptors.** To determine whether neoDH acts as an agonist for AMPA and kainate receptors, we carried out patch-clamp recordings from HEK293 cells expressing representative recombinant AMPA and kainate receptors. NeoDH (50 μM) elicited nondesensitizing currents from GluR4 AMPA receptors (Fig. 3A) similar to those evoked by either kainate or DH. The compound elicited desensitizing currents in cells expressing homomeric GluR5 and GluR6 kainate receptors (Fig. 3A). Because the homomeric kainate receptors remained in a desensitized state for prolonged periods after application of neoDH, we assessed the relative affinity of the interaction between neoDH and kainate receptors by measuring the recovery of glutamate responses after a brief application of neoDH (10 μM for 5 s). The rate at which the

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**Fig. 2.** Displacement of [3H]kainate from kainate receptors by DH (squares), neoDH (circles), and MSVIII-19 (triangles). [3H]Kainate was displaced from membrane preparations of GluR5 receptors (A), GluR6 receptors (B), and KA2 receptors (C) expressed in COS-7 cells. Glutamate (1 mM) was used to determine nonspecific binding. Curves are fit with a one-site competition curve. A representative set of data for displacement by DH is shown and is consistent with affinities determined previously (Sakai et al., 2001b; Swanson et al., 2002). n = 3 to 4 at each concentration for neoDH and MSVIII-19. Kᵢ values calculated from these curves are presented in Table 1.

**Fig. 3.** NeoDH acts as a high affinity agonist for recombinant AMPA and kainate receptors. A, individual currents evoked by application of neoDH (50 μM) to GluR4 (top), GluR5 (middle), and GluR6 (bottom) receptors. B, recovery of control glutamate responses after application of neoDH. Individual traces on the left depict an initial current evoked by glutamate (10 mM), and the traces on the right depict the response to glutamate 10 min after a 5-s application of neoDH (10 μM). Currents were evoked from GluR5 (top), GluR6 (middle), and GluR6/KA2 (bottom) receptors. C, percentage of initial amplitudes graphed against time of test applications. Test application currents were normalized to the initial control glutamate (10 mM) responses (before neoDH application at time indicated). Data points represent the mean ± S.E.M. for three separate experiments. D, NeoDH differentially activates GluR5 and KA2 subunits within the GluR5/KA2 heteromeric receptor. Trace on the left represents a control response from the 100-ms application of glutamate (10 mM). Middle trace represents the response seen during a 5-s application of neoDH (10 μM). As is seen with DH, a tonic current develops after removal of neoDH; this current arises due to high-affinity interaction with the GluR5 subunit and consequent partial activation of the heteromeric receptors. Scale bars, 100 pA and 5 s. Inset shows the response on a condensed time scale. The trace on the right represents the response to glutamate (10 mM) after application of neoDH. The apparent positive deflection is due to desensitization of the entire receptor complex back to the pre-neoDH baseline current amplitude (dotted line) (for a detailed description of this phenomenon, see Swanson et al., 2002).
receptors can be reactivated by glutamate will be determined predominantly by the stabilization of the desensitized state of the receptor and the affinity of neoDH for the kainate receptor subunits. Figure 3B shows representative glutamate-evoked currents before (left) and 10 min after (right) application of neoDH. As was observed with DH, glutamate currents did not recover to a measurable degree after application of neoDH to GluR5 subunit-expressing cells (Fig. 3B, top row). GluR6 and GluR6/KA2 receptors recovered to control glutamate current amplitudes within the 10 min period after neoDH application. The rate of recovery of glutamate-evoked currents after neoDH application was analyzed by normalizing glutamate current amplitudes to the initial control amplitudes (Fig. 3C). GluR5 homomeric receptors displayed a very slow recovery of glutamate sensitivity after neoDH application relative to GluR6 homomeric kainate receptors and GluR6/KA2 heteromeric kainate receptors, which exhibited single exponential rates of recovery of 1.9 ± 0.4 and 0.6 ± 0.2 min, respectively (n = 3–4 for each receptor type; Fig. 3C). Twenty minutes after application of neoDH, currents elicited from GluR5 homomeric kainate receptors recovered to <15% of control responses. These data demonstrate that neoDH is indeed an agonist of recombinant AMPA and kainate receptors with a particularly high affinity for homomeric GluR5 subunits, which neoDH activates and traps in a closed, desensitized state.

We found previously that DH has a complex action on heteromeric GluR5/KA2 receptors (Swanson et al., 2002). DH binds and activates subunits within the heteromeric complex differentially by virtue of its highly divergent affinity for GluR5 and KA2 subunits. Application of DH to GluR5/KA2 receptors elicited a peak current that desensitized to a proportionally small equilibrium current; unusually, removal of the agonist was followed by the emergence of a tonic inward current arising from stable, selective activation of the GluR5 subunits. The tonic current typically was observed for >20 min after removal of DH, reflecting the very slow rate of unbinding from the GluR5 subunits. Because DH did not remain bound to KA2 subunits, subsequent glutamate application elicited a peak desensitizing response (from activation of KA2 subunits) followed by a complete desensitization of the heteromeric receptors to the baseline current present before DH application.

In our current experiments, we determined whether neoDH had similar actions on heteromeric GluR5/KA2 receptors despite the lower affinity of neoDH for GluR5 subunits observed in displacement assays. Glutamate (10 mM) was applied to GluR5/KA2 receptor-expressing cells to determine the control current amplitude, followed by an application of neoDH (10 μM; 5 s). Representative traces from a single recording are shown in Fig. 3D. Glutamate activated a rapidly desensitizing current with very fast kinetics in control recordings. Similar to DH, neoDH elicited an initial peak desensitizing response that was then followed by a slow inward tonic current upon removal of agonist. Glutamate application in the presence of the tonic current induced a transient peak current followed by a rapid return to the baseline control current amplitude (Fig. 3D, dashed line). This behavior is similar to that observed with DH; however, we noted that the duration of the tonic current was much shorter with neoDH (<5 min) than with DH (>20 min), consistent with a somewhat lower affinity for the GluR5 subunit. In summary, these data demonstrate that neoDH is a potent agonist for kainate receptors with a shorter duration of action than DH, consistent with a somewhat lower affinity for GluR5 and GluR6 subunits. The higher affinity for KA2 subunits observed in displacement assays was not manifested in an obvious difference in physiological responses of GluR5/KA2 or GluR6/KA2 receptors to the marine toxins.

MSVIII-19 Is an Antagonist of Kainate and AMPA Receptors. We applied MSVIII-19 to HEK293 cells expressing representative recombinant kainate and AMPA receptors to determine whether this compound also exhibited agonist activity. MSVIII-19 (50 μM) application did not elicit currents from homomeric GluR4, GluR5, GluR6 receptors or heteromeric GluR5/KA2 receptors (representative glutamate and MSVIII-19 traces are shown in Fig. 4A). Instead, we found that MSVIII-19 reduced glutamate-evoked currents from both kainate and AMPA receptors with a wide range of potencies. Representative glutamate (10 mM) currents for GluR5, GluR6, GluR5/KA2, and GluR6/KA2 kainate receptors in the absence and presence of MSVIII-19 are shown in Fig. 4B. At a concentration of 10 μM, MSVIII-19 reduced glutamate currents elicited from GluR6/KA2 receptors by ~20%, inhibited GluR5/KA2 receptors by ~60%, and completely occluded activation of GluR5 receptors. Strikingly, a very high concentration of MSVIII-19 (100 μM) did not inhibit GluR6 kainate receptor currents (Fig. 4). Concentration-response curves for inhibition of peak glutamate currents from these receptors yielded IC \(_{50}\) values of 23 nM for GluR5, 1.9 μM for GluR5/KA2, and ~190 μM for GluR6/KA2 kainate receptors. MSVIII-19 (100 μM) did not inhibit GluR6 kainate receptors to a detectable degree (n = 3–4 for each MSVIII-19 concentration on the receptors; Fig. 4B). MSVIII-19 also inhibited GluR1/GluR2 and GluR4 AMPA receptors, albeit with lower potency than GluR5-containing kainate receptors. Representative glutamate-evoked currents in the absence and presence of 10 μM MSVIII-19 are shown in Fig. 5A. Concentration-response data for MSVIII-19 inhibition of these AMPA receptors yielded IC \(_{50}\) values of 8 μM for GluR1/GluR2 and 23 μM for GluR4 AMPA receptors (n = 3–4; Fig. 5B). In summary, these data demonstrate that MSVIII-19 is a selective non-NMDA receptor antagonist that shows particular affinity for inhibition of GluR5-containing kainate receptors.

To test whether MSVIII-19 had an inhibitory action on neuronal AMPA receptors similar to that observed with recombinant AMPA receptors, we recorded EPSCs from CA1 pyramidal neurons during stimulation of Schaffer collateral inputs. CA1 pyramidal neuron AMPA receptors are composed of heteromeric GluR1/GluR2 and GluR2/GluR3 receptors (Wenthold et al., 1996). Paired stimulations were delivered at a basal frequency of 0.05 Hz; representative current traces in the absence and presence of MSVIII-19 (10 μM) are shown in Fig. 6A. An IC \(_{50}\) of 16 μM (n = 3) was calculated from the concentration-response relationship for inhibition of the first EPSC by MSVIII-19. The similarity of the neuronal and recombinant IC \(_{50}\) values demonstrates that MSVIII-19 has a roughly equivalent activity on AMPA receptors comprised of a variety of different subunits. The paired pulse ratio was not altered in the presence of subsaturating concentrations of the antagonist.

The behavioral effects of MSVIII-19 could be consistent with potentiation of inhibitory transmission (Sasaki et al.,...
For that reason, we also tested the effect of MSVIII-19 on neuronal GABA<sub>A</sub> receptors by recording IPSCs in CA1 pyramidal neurons elicited by stimulation in the stratum radiatum. MSVIII-19 (10 or 20 μM) seemed to show slight inhibition of IPSCs, but this was not significantly different from control amplitudes and was more likely due to modest rundown of the IPSCs (Fig. 6B). The paired pulse ratio of IPSCs during MSVIII-19 application (0.67 ± 0.18; n = 8) was not different from control (0.75 ± 0.17; n = 8). These data show that MSVIII-19 does not exhibit activity on native postsynaptic GABA<sub>A</sub> receptors in the hippocampus.

NeoDH and MSVIII-19 Do Not Activate Group I Metabotropic Glutamate Receptors. We previously reported the high concentration of DH (100 μM) selectively activated mGluR5 but not mGluR1 (Sakai et al., 2001b). To determine the pharmacological profile of neoDH and MSVIII-19 on group I mGluRs, we imaged intracellular calcium signals elicited by activation of recombinant mGluR1 and mGluR5 expressed in HEK293 cells. (R,S)-DHPG (100 μM), a selective group I mGluR agonist used as a positive control, elicited responses from Fura-2/AM-loaded mGluR1 and mGluR5 receptor-expressing cells (Fig. 7). We also reproduced our previous results in which DH (100 μM) was shown to selectively activate mGluR5 (n = 23; Fig. 7, A and B, top). In contrast, neoDH (100 μM) and MSVIII-19 (100 μM) did not elicit calcium signals in DHPG-responsive cells expressing either group I receptor (n = 4–7; Fig. 7, A and B, middle and bottom). MSVIII-19 did not seem to reduce calcium signals from mGluR1- or mGluR5-expressing cells when coapplied with DHPG (data not shown). These results demonstrate that modification of the C8 functional group in DH eliminates the low-affinity agonist activity (at 100 μM) on group I mGluRs.

Discussion

The pharmacological properties of neoDH and MSVIII-19 demonstrate that functional groups at C8 and C9 are critical determinants of specificity and activity. Substitution of a hydroxyl for methylamine at C8 reduces the affinity of neoDH for GluR5 and GluR6 subunits but slightly increases affinity for the KA2 subunit. NeoDH remains an agonist for AMPA receptors but lacks the weak agonist activity on mGluR5 receptors exhibited by DH. Thus, the methylamine-
receptor type and MSVIII-19 concentration). Curves were generated using a homomeric GluR4 AMPA receptors. B, concentration-inhibition curves (gray columns) are shown for heteromeric GluR1/GluR2 receptors and KA2 because ATPA is similar to DH in size and subunit structure, rather than recent kainate receptor and KA2-ATPA structure, providing us with an opportunity to study microsomal GluR4 AMPA receptors expressed in HEK293 cells. Control responses were evoked by a 100-ms application of 10 mM glutamate (black columns). Responses in the presence of glutamate and 10 μM MSVIII-19 (gray columns) are shown for heteromeric GluR1/GluR2 receptors and MSVIII-19 to elucidate possible subunit interactions (Mayer, 2005; Nanao et al., 2005; Naur et al., 2005). GluR6 complexed with agonists were reported recently (Mayer, 2005; Nanao et al., 2005; Naur et al., 2005). In addition to GluR2 AMPA and NR1 NMDA receptor subunits, the crystal structures of LBDs for GluR5 and GluR2 structures (Pentikäinen et al., 2003) accurately predicted LBD-ligand interactions (Mayer, 2005; Nanao et al., 2005). Docking of DH and analogs resulted in binding modes similar to those observed for the agonists ATPA and 5-fluorowillardiine, which do not bind to the GluR6 subunit (Mayer, 2005; Naur et al., 2005). Docking of DH and analogs resulted in binding modes similar to those observed for the α-amino, α-carboxylate, and γ-carboxylate groups of GluR2-bound L-glutamate (PDB code 1Rj; Armstrong and Gouaux, 2000). The hydroxyl-group at C9 on DH and neoDH accepts a hydrogen bond from the main-chain NH group of Glu705. The hydroxyl-group at C9 on DH and neoDH accepts a hydrogen bond from the main-chain NH group of Glu705 (Fig. 8A, left), leading to the higher affinity for GluR5 compared with AMPA or KA2 subunits, which have a methionine in the equivalent position (Fig. 8A, right). Ser708 in GluR5 binds hydrophobic groups of DH and therefore enlarges the binding cavity to accommodate DH, whereas steric occlusion by Met708 would explain the strikingly low affinity of DH for KA2 subunits. Similarly, structural and modeling data suggest that the relative size of the GluR5 binding pocket plays a critical role in conferring selectivity of the agonists ATPA and 5-fluorowillardiine, which do not bind to the GluR6 subunit (Mayer, 2005). Ile650 in KA2 also is predicted to reduce the size of the binding cavity, obstructing DH binding by steric hindrance (Fig. 8A, right), whereas GluR5-7 subunits have a less bulky valine. Finally, Pro478 in GluR5 is positioned to stabilize the C8 methyl group in DH by hydrophobic interactions.

NeoDH would bind to GluR5 in an orientation similar to hydroxyl substitution has small but significant effects on activity of these excitatory amino acids, but the general pharmacological profile is maintained, consistent with similarities in behavioral effects (Sakai et al., 2001a). In contrast, removal of the C8 and C9 groups greatly alters pharmacological activity. MSVIII-19 is an antagonist for GluR5-containing kainate receptors with little detectable affinity for GluR6 or KA2 receptor subunits. MSVIII-19 also inhibits AMPA receptor activation with low potency. The unusual behavioral effects of MSVIII-19—induction of a hypoactive state at low doses (Sasaki et al., 1999)—therefore might be caused by a combination of GluR5 kainate and AMPA receptor inhibition in the central nervous system. This pharmacological characterization of neoDH and MSVIII-19 yields insight into the importance of the C8 and C9 side groups in conferring affinity for glutamate receptors and suggests potential sites of modification to generate compounds with novel activities.

Molecular Modeling of Receptor-Ligand Interactions. The divergent pharmacology of DH, neoDH, and MSVIII-19 provides us with an opportunity to study microenvironments within the LBDs of individual kainate receptor subunits. In addition to GluR2 AMPA and NR1 NMDA receptor subunits, the crystal structures of LBDs for GluR5 and GluR6 complexed with agonists were reported recently (Mayer, 2005; Nanao et al., 2005; Naur et al., 2005).

We modeled the complexes between LBDs and DH, neoDH, and MSVIII-19 to elucidate possible subunit interactions that confer specificity for these compounds (Fig. 8). The GluR2-ATPA structure, rather than recent kainate receptor structures, was used to model ligand interactions with GluR5 and KA2 because ATPA is similar to DH in size and subunit selectivity. As well, modeling of kainate receptors using GluR2 structures (Pentikäinen et al., 2003) accurately predicted LBD-ligand interactions (Mayer, 2005; Nanao et al., 2005; Naur et al., 2005). Docking of DH and analogs resulted in binding modes similar to those observed for the α-amino, α-carboxylate, and γ-carboxylate groups of GluR2-bound L-glutamate (PDB code 1Rj; Armstrong and Gouaux, 2000). The hydroxyl-group at C9 on DH and neoDH accepts a hydrogen bond from the main-chain NH group of Glu705. The C8 methylamine in DH forms hydrogen bonds with the Glu705 carboxylate and Ser708 of the GluR5 LBD (Fig. 8A, left), leading to the higher affinity for GluR5 compared with AMPA or KA2 subunits, which have a methionine in the equivalent position (Fig. 8A, right). Ser708 in GluR5 is small (like Thr708 in GluR6) and therefore enlarges the binding cavity to accommodate DH, whereas steric occlusion by Met708 would explain the strikingly low affinity of DH for KA2 subunits. Similarly, structural and modeling data suggest that the relative size of the GluR5 binding pocket plays a critical role in conferring selectivity of the agonists ATPA and 5-fluorowillardiine, which do not bind to the GluR6 subunit (Mayer, 2005). Ile650 in KA2 also is predicted to reduce the size of the binding cavity, obstructing DH binding by steric hindrance (Fig. 8A, right), whereas GluR5-7 subunits have a less bulky valine. Finally, Pro478 in GluR5 is positioned to stabilize the C8 methyl group in DH by hydrophobic interactions.
that of DH (Fig. 8B, left). There are two likely reasons for the reduction in binding affinity: 1) the hydroxyl at C8 of neoDH cannot donate hydrogen bonds to both Ser708 and Glu705, and 2) the favorable hydrophobic interaction between the C8 methyl group and Pro478 is absent (Fig. 8B, left). In the KA2 subunit, Met708 could accommodate the C8 hydroxyl group of neoDH, unlike the bulkier methylamine in DH, leading to increased affinity for neoDH relative to DH (Fig. 8B, right).

MSVIII-19 is missing polar groups on C8 and C9 and therefore cannot bind as stably to the receptor subunits as DH and neoDH. Water molecules may play critical roles during MSVIII-19 binding by occupying positions equivalent to methylamine of DH and hydroxyl group of neoDH. Similarly, the C9 hydroxyl group in DH and neoDH could be replaced with a water molecule. Inclusion of two water molecules in LBD-MSVIII-19 docking models results in unfavorable interactions and a more open conformation, consistent with antagonist activity. Water molecule w2 (Fig. 8C) might donate a hydrogen bond to the γ-carboxylate group of MSVIII-19; similar interactions are observed for the GluR2-glutamate structure (Armstrong and Gouaux, 2000). Unfavorable interactions between MSVIII-19 and Asn686 in GluR6 and Ile650 in KA2 could explain the dramatic reduction in affinity for these receptor subunits. In summary, distinct pharmacological activities of DH analogs on kainate receptors correlate well with predictions based on molecular modeling and on structures of kainate receptors (Mayer, 2005; Nanao et al., 2005; Naur et al., 2005), underscoring the importance of a small number of residues within the LBD in shaping the unique binding properties of these compounds.

MSVIII-19: A Novel Kainate Receptor Antagonist. GluR5-selective antagonists have recently been developed with similar, but lower affinity, pharmacological properties to MSVIII-19. The competitive decahydroisoquinoline antagonists LY293558, LY377770, and LY382884 show varying degrees of selectivity for GluR5-containing kainate receptors compared with AMPA receptors; all three compounds exhibit little or no affinity for GluR6 or GluR7 subunits (Bleakman et al., 1996b; O’Neill et al., 1998; Simmons et al., 1998; Smolders et al., 2002; Alt et al., 2004). LY293558 is roughly equipotent for inhibition of AMPA and GluR5 kainate receptors (IC50 of ~2 μM) (Bleakman et al., 1996a; O’Neill et al., 1998), whereas LY382884 potently inhibits GluR5-containing kainate receptors but shows little affinity for AMPA receptors, making this compound a highly selective GluR5 antagonist.
antagonist (O’Neill et al., 1998; Simmons et al., 1998; Bortolotto et al., 1999; Smolders et al., 2002). A similar decahydroisoquinoline exhibited /H11022 300-fold selectivity for GluR5 kainate receptors in a preliminary characterization (Filla et al., 2002). More recently, a commercially available willardiine derivative, UBP296, was shown to selectively inhibit GluR5 receptors with an IC50 of 3.5 /H9262 M, whereas only weakly inhibiting AMPA receptors (More et al., 2004). Noncompetitive GluR5 receptor antagonists with varying degrees of selectivity and potency have been described recently as well (Valgeirsson et al., 2003; Christensen et al., 2004). The pharmacological selectivity of MSVIII-19 is perhaps most similar to LY377770, which has IC50 values of 13 and 0.2 to 0.7 μM for inhibition of AMPA receptors and GluR5 receptors, respectively (Smolders et al., 2002; Alt et al., 2004). In comparison, MSVIII-19 is at least a 10-fold more potent inhibitor of GluR5-containing receptors, with an IC50 of 23 nM, compared with other kainate receptor antagonists.

GluR5-selective kainate receptor antagonists are the focus of interest for their potential therapeutic uses because they have exhibited efficacy in in vivo and in vitro pathological models. Kainate receptors likely play a prominent role in nociceptive transmission because they are the primary type of ionotropic glutamate receptor expressed in C-fiber dorsal root ganglion trigeminal ganglion neurons (Huettner, 1990; Sahara et al., 1997). Consistent with this hypothesis, antag-

Fig. 8. Homology modeling of DH, neoDH, and MSVIII-19 with kainate receptor ligand binding domains. A, interaction between DH and GluR5 (left) would be stable because of a hydrogen bond between Ser708 and the C8 methylamine group of DH and the surface complementarity between DH and Val650. The water molecules (w1–w3) that are important for ligand binding in the modeled complexes are shown. In contrast, DH would fit poorly into the KA2 LBD (right) because of Ile650 and the replacement of Ser708 with a less favorable methionine in the KA2 subunit. B, NeoDH would bind the GluR5 LBD with a similar conformation as DH (left), but the loss of the amino group at C8 accounts for the slightly lower binding affinity. In contrast, Met708 in the KA2 subunit would be accommodated in a more energetically favorable conformation because the C8 group is less bulky (right). Ligands are shown with green carbon and the shape of the binding cavity with green surface. C, modeling of MSVIII-19 (green carbon atoms) and the GluR5 LBD (gray carbon atoms) necessitates the placement of two water molecules, w1 and w2, such that the compound does not interact as stably as DH, likely accounting for the reduction in binding affinity for GluR5 receptors.
onists acting on GluR5-containing kainate receptors reduce pain-related behaviors in formalin and capsaicin models of hyperalgesia in rats (Sang et al., 1998; Simmons et al., 1998), capsaicin-induced hyperalgesia and allodynia in humans (Sang et al., 1998), and postoperative pain (Gilron et al., 2000). GluR5-selective antagonists also have potential for therapeutic use in the treatment of migraine. In preclinical research, a produg of a GluR5 decahydroisoquinoline antagonist reduced protein extravasation in the dura and activation of microglia in the nucleus caudalis (two in vitro models of acute migraine) (Filla et al., 2002). The nonselective AMPA/kainate receptor antagonist LY293558 exhibited clinical efficacy for alleviation of migraine pain and other migraine-associated symptoms in a recent proof of concept trial (Sang et al., 2004). The kainate receptor-selective antagonists LY377770 and LY382884 prevent induction and maintenance of epileptiform behavior by pilocarpine in both hippocampal slices and intact animals and thus have anticonvulsant activity (Smolders et al., 2002). This anticonvulsant property generalized to a number of other animal models of epilepsy, including stimuli train-induced bursting in vitro hippocampal slices, in vivo 6-Hz corneal stimulation, and the maximum electroshock model (Smolders et al., 2002; Barton et al., 2003). In contrast, seizures elicited by inhibition of GABAergic transmission with picrotoxin were not attenuated by treatment with either LY377770 or LY382884 (Smolders et al., 2002). Finally, LY377770 was neuroprotective in global and focal models of ischemia, suggesting that GluR5 receptors might contribute to ischemic brain damage (O’Neill et al., 1998).

In summary, the novel structures and pharmacological activities of natural and synthetic disuberyhane-related molecules make them useful for understanding the critical components of ligand-receptor interactions that confer selectivity and affinity for particular kainate receptor subunits. We suggest that these compounds represent a new template for future development of kainate receptor ligands with therapeutic utility.

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