Pharmacological Properties of the Potent Epileptogenic Amino Acid Dysiherbaine, a Novel Glutamate Receptor Agonist Isolated from the Marine Sponge Dysidea herbacea

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ABSTRACT

Dysiherbaine (DH) is a marine sponge-derived amino acid that causes seizures upon injection into mice. In this report we investigate the behavioral effects and characterize the pharmacological activity of DH. DH induced convulsive behaviors in mice with ED50 values of 13 pmol/mouse, i.c.v. and 0.97 mg/kg, i.p. In rat brain synaptic membranes DH displaced binding of [3H]kainic acid (KA) and [3H]a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) with Kᵢ values of 26 and 153 nM, respectively; in contrast, DH did not displace the N-methyl-D-aspartate (NMDA) receptor ligand [3H]CGS-19755. DH displaced [3H]kainate from recombinant GluR5 and GluR6 kainate receptor subunits expressed in HEK293 cells with Kᵢ values of 0.74 and 1.2 nM, respectively. In whole-cell voltage-clamp recordings from cultured rat hippocampal neurons, DH evoked inward currents from both AMPA and KA receptors with EC50 values of 9.7 μM and 210 nM, respectively. AMPA receptor currents were blocked by GYKI 53655, whereas KA receptor currents were blocked by 6-cyano-7-nitroquinoline-2,3-dione (CNQX). Surprisingly, in calcium imaging experiments we found that DH also activated recombinant mGluR5 receptors but did not activate mGluR1 receptors. DH did not activate glutamate transporters or γ-aminobutyric acid A (GABA_A) receptors. These results indicate that DH is a potent non-NMDA-type agonist with very high affinity for KA receptors, as well as a subtype-selective mGluR agonist. DH possesses the most potent epileptogenic activity among the amino acids yet identified. This novel excitatory amino acid may prove useful for evaluating the physiological and pathological roles of non-NMDA receptors, especially KA receptors, in the central nervous system.

1-Glutamate acts as the principal excitatory neurotransmitter in the mammalian central nervous system via ionotropic and metabotropic glutamate receptors. Ionotropic glutamate receptors (iGluRs) are classified into the NMDA and non-NMDA subtypes, and non-NMDA receptors are further divided into AMPA and KA subtypes. Molecular cloning studies demonstrated that iGluRs are encoded by at least six NMDA (NR1, NR2A–2D, and NR3A), four AMPA (GluR1–4), and five KA (GluR5–7, KA1, and KA2) receptor genes (Na- kanishi, 1992; Seeburg, 1993; Hollmann and Heinemann, 1994). Metabotropic receptors (mGluRs) are encoded by eight distinct genes classified into three groups: group I receptors (mGluR1, 5) are coupled to phosphoinositide (PI) hydrolysis and calcium mobilization signal transduction pathways, whereas group II (mGluR2, 3) and group III (mGluR4, 6–8) receptors are negatively linked to cAMP formation [reviewed by Conn and Pin (1997)]. Ionotropic and metabotropic GluRs are widely distributed in the vertebrate brain and play integral roles in excitatory neurotransmission. In addition, these receptors are thought to be involved in higher brain mechanisms such as memory formation, learning, pain transmission, and several neuronal disorders [reviewed by Conn and Pin (1997) and Bleakman and Lodge (1998)].

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ABBREVIATIONS: iGluR, ionotropic glutamate receptors; mGluR, metabotropic glutamate receptor; DH, dysiherbaine; DOM, domoic acid; KA, kainic acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA, N-methyl-D-aspartic acid; CGS-19755, 1-(cis-2-carboxypiperidine-4-yl)-propyl-1-phosphonic acid; trans-ACPD, 1-aminoacycloptanate-trans-1,3-dicarboxylic acid; GYKI 53655, 1-(4-aminophenyl)-3-methylcarbamoyl-4-methyl-7,8-methylenedioxy-3,4-dihydro-5H-2,3-benzodiazepine; CNQX, 6-cyano-7-nitroquinoline-2,3-dione; CPCCoEt, 7-(hydroxymethyl)cyclopropa[b]chromen-1α-carboxylate ethyl ester; (S)-DHPG, (S)-3,5-dihydroxyphenylglycine; EAA, excitatory amino acid; HEK, human embryonic kidney; SYM 2081, (2S,4R)-4-methylglutamate; GABA_A, γ-aminobutyric acid A; PI, phosphoinositide; MDCK, Madin-Darby canine kidney.
Understanding the complex roles that iGluRs play in physiological and pathological processes in the brain has been facilitated by the isolation of selective pharmacological compounds. Differentiation of NMDA and non-NMDA (AMPA and KA) receptors has been possible for a number of years because of the divergent pharmacological profiles of these distinct receptors families. However, pharmacological separation of neuronal currents mediated by AMPA and KA receptors has been more difficult. Recent development of AMPA and KA receptor-selective agonists and antagonists have aided in the detection and characterization of neuronal KA receptors, which appear to play roles in synaptic transmission distinct from those of AMPA and NMDA receptors [reviewed by Chittajallu et al. (1999)].

Several important classes of GluR ligands have been isolated from marine organisms (Laycock et al., 1989). The non-NMDA receptor agonists KA and domoic acid (DOM) have been of particular interest because of their potent epileptogenic properties. KA-induced seizures have long been used as a model for human temporal lobe epilepsy (Ben-Ari, 1985). DOM, a causative agent of amnesiac shellfish poisoning (Perl et al., 1990), is the most potent excitatory amino acid (EAA) yet characterized (Stewart et al., 1990). The seizurogenic activity of these compounds has suggested that EAAs may be useful not only as pharmacological tools but also as lead compounds for therapeutic agents of neurological disorders (Krogsgaard-Larsen and Hansen, 1992; Bleakman and Lodge, 1998). We therefore searched for EAAs in marine organisms and isolated a novel di-amino, di-acid, dysiherbaine (DH, Fig. 1), from the sponge Dysidea herbacea (Sakai et al., 1997).

In the present study, we have explored the pharmacological specificity of DH in radioligand binding and electrophysiological experiments. We have also carried out a more detailed characterization of the behavioral responses to DH injection in mice. These studies demonstrate that DH is an agonist for both ionotropic and metabotropic GluRs and has strikingly potent seizurogenic activity.

Materials and Methods

Convulsant Action in Mice

Male ddY mice (16–25 g, 4–6 weeks old; Owada experimental animals, Iwate, Japan) were kept in cages with free access to their standard diet until use. Five or more animals were tested for each group. All efforts were made to minimize both suffering and the number of animals used. Animals received i.p. or i.c.v. injections of drug solution or vehicle. i.c.v. administrations were performed according to a method described previously (Laursen and Belknap, 1986) with some modifications. The sample solution (20 μl) was injected slowly over 5 s at 2 mm lateral to the midline of the skull, 3 mm rostral to a line down through the anterior base of the ears, at a depth of 3.5 mm. Normal saline was used as a control vehicle and did not induce any notable change in animal behaviors.

The behavior of animals was observed following drug administration for 3 or 6 h continuously. Severity of seizures was classified based on a behavioral scale developed to assess toxicity of DOM (Tasker et al., 1991), with some modifications into seven grades as follows: grade 0, normal; grade 1, hypoactivity (20–40 s of immobility); grade 2, occasional scratching and/or 40 to 60 s of immobility without rigidity; grade 3, frequent scratching, vigilant staring, hyperactivity, and/or head bobbing; grade 4, loss of balance, and/or sudden running or jumping accompanied by frequent scratching; grade 5, forepaw or neck clonus and/or rearing with complete loss of balance; grade 6, rigid and splayed hind limbs with forepaw clonus and neck clonus plus rearing; grade 7, tonic convulsion followed by death. The highest score observed in each 10-min interval was averaged for 0 to 1, 2 to 3, and 5 to 6 h, respectively. The score from 1-h observation was probit-transformed by setting a score of 7.0 to be 100%. Two distinct ED_{50} values (doses that cause 50% of maximum seizure score) were calculated by linear regression of probit-transformed data in this study. The ED_{50(max)} value was calculated using the highest seizure score observed within the initial 1-h observation period after injection. The ED_{50(mean)} values of DH and DOM were calculated using averaged (as opposed to the highest) seizure scores obtained in six observation periods (10 min each, total 1 h). Linear regression of the data was performed with StatView software (SAS Institute Inc., Cary, NC).

Receptor Binding Studies

Synaptic membranes were prepared as previously described (Murphy et al., 1987). Briefly, male Sprague-Dawley rats (200–220 g) were decapitated and forebrains were homogenized with a Teflon-glass homogenizer in 10-fold volumes of 0.32 M sucrose solution. The homogenate was centrifuged at 10000 g for 10 min. After centrifugation of the supernatant (17,000 g for 20 min), the resulting pellet was suspended in distilled water. The suspension was centrifuged at 8000 g for 20 min. The supernatants and soft,uffy uppercoat layers of the pellets were collected and centrifuged (48,000 g for 20 min). After washing with a suitable buffer for each assay, the membrane pellets were stocked at −78°C until use. On the day of the assay, the frozen pellet was thawed at room temperature and incubated with 0.04% Triton X-100 in the assay buffer at 37°C for 30 min followed by centrifugation (48,000 g for 10 min). The detergent was removed by washing twice with the assay buffer. The conditions for each binding assay were as follows (ligand, ligand concentration, incubation temperature, incubation time, buffers): [3H]KA, 1 nM, 4°C, 1 h, 100 mM Tris-HCl (pH 7.1); [3H]AMP, 5 nM, 4°C, 1 h, 50 mM Tris-HCl (pH 7.4) and 100 mM KSCN; [3H]CGS-19755, 10 nM, 4°C, 1 h, 50 mM Tris-HCl (pH 8.0) (London and Coyle, 1979; Murphy et al., 1987, 1988).

Binding studies with recombinant GluR5 and GluR6 KA receptor subunits in HEK293 cells were carried out as described previously (Swanson et al., 1997). [3H]KA displacement assays were performed as described previously (Swanson et al., 1997). The [3H]KA concentrations used in this study were 20 to 27 and 13 to 20 nM for GluR5

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**Fig. 1.** Structures of dysiherbaine and other KA receptor agonists.
and GluR6 assays, respectively. Nonspecific binding was defined in the presence of 1 nM glutamate. The GluR5 and GluR6 cDNAs were generously donated by Dr. Peter Seeburg (Max-Planck-Institute, Heidelberg, Germany) and Dr. Stephen Heinemann (The Salk Institute, La Jolla, CA).

Electrophysiological Studies

**Culture Preparation.** Hippocampal neurons were prepared from newborn rat pups and grown in microdot cultures as described previously (Bekkers and Stevens, 1991). Cultures were maintained in Dulbecco's modified minimal medium supplemented with 10% heat-inactivated horse serum, 20 mM glucose, 1% N2 supplement, and penicillin/streptomycin. After 4 to 5 days in vitro, non-neuronal cell division was inhibited by exposure to 35 μM fluorodeoxyuridine and 75 μM uridine for 1 to 3 days. Recordings were obtained from cells that had been cultured for 2 to 3 weeks.

**Electrophysiology.** Whole-cell voltage-clamp recordings were made from neurons using standard patch-clamp techniques. Glass fragments of coverslips with adherent cells were placed in a perfusion chamber and rinsed with a buffer of composition (in mM): NaCl, 150; KCl, 2.8; CaCl2, 1.8; MgCl2, 1; HEPES, 10; tetrodotoxin, 0.001 (pH was adjusted to 7.3 with NaOH). Experiments were performed at room temperature (22–25°C) and recorded on an Axopatch 200B amplifier using pClamp8 software (Axon Instruments, Foster City, CA). Patch pipettes had initial resistances of 4 to 5 MΩ when filled with an internal solution composed of (in mM): CsF, 95; CsCl, 25; Cs-HEPES, 10; Cs-EGTA, 10; NaCl, 2; Mg-ATP, 2; QX-314, 10; TEA-Cl, 5; 4-aminopyridine, 5 (pH adjusted to 7.3 with CsOH). Drugs were applied through three-barrel glass tubing attached to a piezo bimorph controlled by pClamp8 software, which allowed rapid exchange of solutions to the neurons. To test for activation of GABA receptors, DH (50 μM) was applied to cultured hippocampal interneurons in the presence of GYKI 53655 (100 μM), CNQX (50 μM), DL-2-amino-5-phosphonovaleric acid (50 μM), and CPPCOEt (100 μM) to block AMPA, kainate, NMDA, mGlu receptors, respectively. GYKI 53655 (100 μM) was added to perfusion solutions to isolate KA receptor currents. Dose-response curves were fitted to the Hill equation using Origin software (MicroCal Software, Inc., Northampton, MA).

**Ca2+ Imaging of Recombinant Group 1 Metabotropic Glutamate Receptors (mGluR1 and mGluR5)**

Standard calcium phosphate precipitation techniques were used to transiently transfet HEK293 cells with plasmids containing rat mGluR1 or mGluR5 cDNAs. Two to three days after transfection, cells were incubated in 3 μM Fura-2 AM (Molecular Probes, Eugene, OR) for 20 to 40 min at room temperature in the presence of 0.02% pluronic F-127. The cells were then transferred to a recording chamber and continuously perfused with HEPES-buffered saline containing (in mM): NaCl 135; KCl 5; CaCl2 2; sucrose 20; glucose 10; HEPES 5 (pH 7.4). Ratiometric Ca2+ imaging experiments were performed on an inverted microscope (Axiovert 100 TV; Zeiss, Thornwood, NY) equipped for epifluorescence microscopy. Samples were excited with a xenon lamp and the excitation wavelengths (350 or 380 nm) were selected using a polychromatic illumination system (TILL Photonics, Planegg, Germany). Images were acquired using a cooled charge-coupled device camera (MicroMAX, Roper Scientific, Trenton, NJ) controlled by Imaging Workbench software from Axon Instruments. 100 μM (S)-DHPG was used to elicit calcium signals in mGluR-expressing cells.

**Uptake Assay on Glutamate Transporters**

Madin-Darby canine kidney (MDCK) cells stably expressing subtypes of human excitatory amino acid transporters (EAAT1, EAAT2, or EAAT3) were seeded onto 96-well plates and cultured in Dulbecco's modified minimal essential medium containing 10% dialyzed fetal bovine serum and antibiotics (G418) for 2 days before the uptake assay. The uptake assay was performed as previously described (Shimamoto et al., 1998). The relative specific uptake of [3H]glutamate was determined from three different experiments.

**Drugs**

Dysiherbaine was isolated and purified according to a previously described procedure (Sakai et al., 1997). GYKI 53655 was custom synthesized by Research Biochemicals International (Natick, MA). All other chemicals were commercially available at highest purity from Nakarai Tesque (Kyoto, Japan), Sigma Chemical Co. (St. Louis, MO), Tocris Cookson (Bristol, UK), Research Biochemicals International, and Diagnostic Chemicals Ltd. (West Royalty, Canada). Radiolabeled compounds were purchased from PerkinElmer Life Sciences (Boston, MA).

**Results**

**DH Is a Potent Convulsant.** We first compared the behavioral responses to i.c.v. injection of DH and other representative excitatory amino acids. The rank order of potency for the tested EAAs was determined from the ED50(max) values (see Materials and Methods), which were estimated as the doses that gave 50% of the maximum seizure score in the initial 1-h observation period after injection of the drugs. The entire 1-h period was used for comparative purposes, because some compounds induced only short-lasting behavioral changes. As reported previously, behavioral changes induced after injection of NMDA- and non-NMDA-type EAAs were clearly different in terms of duration of convulsant behavior (Chiamulera et al., 1992).

DH was the most potent epileptogenic compound tested (Table 1). All the non-NMDA-type EAAs induced status epilepticus-like recurrence of seizures that lasted for hours, in contrast to NMDA or trans-ACPD, which induced violent convulsive behavior lasting 10 to 30 min without recurrence. At low concentrations, DH and DOM elicited a different set of seizure behaviors compared with KA. For example, stereotypic scratching, circling, or running were characteristic of lower doses of DH or DOM; in contrast, lower doses of KA caused immobility or rigidity with only occasional stereotyped behaviors. At higher doses however, DH, DOM, and KA elicited similar myoclonic or whole body convulsions. This similarity between the reactions to DH, DOM, and KA suggested that DH might elicit convulsive action via the activation of non-NMDA receptors. Because DOM is the most potent seizurogenic EAA characterized to date (Stewart et al., 1990), and its behavioral effects resembled those of DH, we used DOM for comparison in the subsequent behavioral tests.

DH produced seizures in a dose-dependent fashion after i.c.v. injection (Fig. 2A). Higher doses of DH (>40 pmol/mouse) produced recurrent status epilepticus-like seizures in all mice. At 100 and 80 pmol/mouse, 5 of 5 and 2 of 5 animals died, respectively. At 40 pmol/mouse, none of the mice died within 3 h, but 2 of 10 died in the 12 h following the administration. At lower doses (5–20 pmol/mouse), all animals showed behavioral abnormalities, including scratching, loss of balance, and occasional myoclonus convolution. All animals injected with 5 to 20 pmol of DH survived for at least a week. The behavioral changes caused by DH injection resembled those elicited by DOM. However, DH was about 7 times more potent than DOM: ED50(mean) values (see Materials and Methods) for DH and DOM were 13 pmol/mouse (7.6–26, 95%...
TABLE 1
Comparison of epileptogenic activity of DH with DOM and several representative EAAs in mice
Linear regression of probit-transformed dose-response data was used to estimate ED_{50} values. Note that DH shows the most potent epileptogenic activity among the EAAs tested.

<table>
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<tr>
<th></th>
<th>i.c.v.</th>
<th>i.p., ED_{50}</th>
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<tr>
<td></td>
<td>ED_{50}/max*</td>
<td>ED_{50}/mean*</td>
</tr>
<tr>
<td>DH</td>
<td>0.006  (0.002–0.015, n = 25)</td>
<td>0.013 (0.0076–0.026, n = 30)</td>
</tr>
<tr>
<td>DOM</td>
<td>0.034  (0.001–0.12, n = 20)</td>
<td>0.034 (0.065–0.146, n = 25)</td>
</tr>
<tr>
<td>KA</td>
<td>0.28   (0.21–0.35, n = 20)</td>
<td>0.057 (3.8–8.6, n = 25)</td>
</tr>
<tr>
<td>AMPA</td>
<td>0.24   (0.10–0.59, n = 30)</td>
<td>0.43</td>
</tr>
<tr>
<td>NMDA</td>
<td>0.43   (0.28–0.64, n = 25)</td>
<td></td>
</tr>
<tr>
<td>trans-ACPD</td>
<td>9.7    (4.1–23, n = 30)</td>
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* The highest behavioral score obtained during 1 h after administration of drugs was assigned as the behavioral score for each dose.

** Averages of the six highest behavioral score obtained at each 10 min-observation for 1-h period was used to assign the behavioral score for each dose (see Results).

* 95% confidence limits, total number of animals used.

Confidence limit and 97 pmol/mouse (65–146, 95% confidence limit), respectively (Table 1).

In addition to a higher potency, we noted that DH-induced behavioral changes lasted much longer than those caused by DOM. Even at 3 h after administration of 20 pmol of DH [which is near the ED_{50(mean)}], behavioral changes were still clearly observed, whereas the effects of DOM (100 pmol) had largely diminished (Fig. 2A). In addition, the status epilepticus generated consistently by 40 pmol of DH lasted for at least 2 days, but was not lethal; in contrast, equivalent prolonged symptoms were not induced by DOM at any dosage tested.

The convulsant activity of systemically injected DH was also evaluated. Dose-dependent behavioral changes were observed after i.p. injections of DH and DOM, with ED_{50(mean)} values of 0.97 mg/kg (0.81–1.21 mg/kg, 95% confidence limit) for DH and 5.7 mg/kg (3.8–8.6 mg/kg, 95% confidence limit) for DOM based on the 1-h observations (Fig. 2B). The potency of DOM (estimated 1 h after injection) was comparable to a previously reported value (3.9 mg/kg, Tasker et al., 1991). The behavioral effects of DH also lasted longer than those of DOM in the i.p. administration. Mice exhibited recurrent spontaneous seizures for as long as 24 h following injection of at least 1.6 mg/kg DH. Convulsant behavior following i.p. injection of DOM, however, terminated within 6 h (Fig. 2B). These data underscore the potency and long duration of DH-induced status epilepticus.

**DH Displaces Radiolabeled Ligands from Non-NMDA Receptors.** The affinity of DH for ionotropic glutamate receptors was determined by radioligand binding techniques using rat brain synaptic membrane preparations. DH and other agonists were used to displace [3H]CGS-19755, [3H]AMPA, and [3H]KA from NMDA and non-NMDA glutamate receptors (Table 2 and Fig. 3). Table 2 summarizes K_i values and Hill coefficients estimated from the displacement curve for each drug. DH inhibited [3H]KA binding and [3H]AMPA binding with K_i values of 26 ± 4.0 and 153 ± 10 nM, respectively. These values were higher than those for unlabeled KA and AMPA displacement of their tritiated counterparts (1.8 and 3.9 nM, respectively). The Hill coefficient determined from DH displacement of [3H]KA binding was much smaller (0.52) than those of other ligands (Table 2), suggesting that DH was displacing [3H]KA from a heterogeneous population of binding sites. Additionally, some data points for DH displacement of [3H]KA both in low (<0.2 nM) and high (>100 nM) concentration range fitted rather poorly to the Hill-type model. A multiphasic model for [3H]KA displacement fitted better to the curve; however, such an analysis was not rigorously made, because we could not define the points of the inflection unambiguously. Therefore, the apparent K_i value for DH displacement of [3H]KA binding likely represents an average value from several binding sites. DH did not inhibit [3H]CGS-19755 binding at 10 μM, suggesting that the compound does not interact with NMDA receptors.

The apparent multi-component displacement of [3H]KA might reflect affinity for both AMPA and KA receptors. To test more directly the affinity of DH for KA receptors, we displaced [3H]KA from recombinant KA receptor subunits expressed in HEK293 cells. DH inhibited binding of [3H]KA to GluR5 and GluR6 subunits with K_i values of 0.48 ± 0.10 and 1.28 ± 0.05 nM, respectively (Fig. 4). These data therefore support the interpretation that the higher affinity component in the rat brain displacement curves arose from DH interaction with neuronal KA receptors.

**DH Evokes Currents from Non-NMDA Receptors in Hippocampal Neurons.** Because DH selectively displaced non-NMDA ligands from synaptic membranes, we next tested whether DH had agonist or antagonist activity on neuronal non-NMDA receptor currents. DH evoked currents from glutamate receptors in cultured hippocampal neurons. Measurable inward current responses were observed at 0.1 μM DH, and the responses were saturating at 100 to 300 μM (Fig. 5A). Steady-state currents evoked by 100 μM DH had a mean amplitude of 4.1 ± 0.6 nA (n = 7), which was approximately 2-fold larger than the amplitude of steady-state currents evoked by 1 mM glutamate in the same neurons (1.9 ± 0.7 nA, n = 7). Indeed, the predominantly nondesensitizing DH currents were comparable in amplitude to peak glutamate currents (4.0 ± 0.9 nA). The majority of this current was mediated by AMPA receptors, as opposed to KA receptors, because the currents were inhibited to a large degree by the AMPA receptor antagonist GYKI 53655 (see below).
Materials and Methods

Concentration-response data for DH were obtained by measuring the steady-state currents evoked 0.03 to 300 μM DH (Fig. 5D, closed triangles). Hill curve fitting of the data yielded an EC50 value and Hill coefficient (nH) of 9.68 ± 0.03 μM and 1.24 ± 0.06, respectively (n = 7).

DH also activated KA receptors in cultured hippocampal neurons (Fig. 5B). KA receptor currents were isolated by applying DH in the presence of GYKI 53655 (100 μM). Steady-state currents elicited by application of low concentrations of DH activated slowly and saturated at approximately 3 μM. 1 μM DH evoked a mean steady-state current of 307 ± 110 pA (n = 4). At higher concentrations of DH (>10 μM), a desensitizing peak response was observed (data not shown). Hill curve fitting of dose-response data gave an EC50 value of 210 nM and an nH of 2.5 for activation of steady-state KA receptor currents by DH (n = 4). KA receptor currents evoked by 0.3 μM DH were blocked (91 ± 3%, n = 5) by high concentrations of CNQX (100 μM) (Fig. 5C).

In the presence of a cocktail of glutamate receptor blockers (100 μM GYKI 53655, 50 μM CNQX, 50 μM DL-2-amino-5-phosphonovaleric acid, and 100 μM CPCCOEt), we did not observe any currents evoked by 50 μM DH in cultured hippocampal interneurons (n = 4). In contrast, 100 μM GABA elicited robust currents from each of these cells. These data demonstrate that DH does not activate GABA_A receptors.

DH Activates mGluR5 Receptors but Not mGluR1 Receptors. Because activation of mGluR1 metabotropic glutamate receptors can induce convulsant actions in vivo (Schoepp and Conn, 1993), we next examined the effect of DH on group I metabotropic glutamate receptors. We measured mGluR-mediated increases in intracellular calcium using the membrane-permeable Ca2+ indicator Fura-2 AM in ratiometric calcium imaging experiments. HEK293 cells expressing mGluR1 receptors gave a increased Ca2+ signal when 100 μM (S)-DHPG was applied, but no signal was observed upon application of 100 μM DH (Fig. 6A). In contrast, cells expressing mGluR5 receptors gave a robust signal with (S)-DHPG and also showed elevated Ca2+ levels when DH was applied (Fig. 6B). The calcium signal elicited by DH in mGluR5-expressing cells was comparable with that evoked by (S)-DHPG. These data demonstrate that DH is a subtype-selective group I mGluR agonist.

TABLE 2

<table>
<thead>
<tr>
<th></th>
<th>Ka (nM)</th>
<th>Hill Coefficient</th>
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<tbody>
<tr>
<td>KA binding</td>
<td></td>
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<tr>
<td>Glu</td>
<td>89 ± 4.3</td>
<td>1.04 ± 0.05</td>
</tr>
<tr>
<td>KA</td>
<td>1.8 ± 0.2</td>
<td>0.95 ± 0.15</td>
</tr>
<tr>
<td>DOM</td>
<td>1.5 ± 0.2</td>
<td>0.91 ± 0.07</td>
</tr>
<tr>
<td>DH</td>
<td>26 ± 4.0</td>
<td>0.52 ± 0.04</td>
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<tr>
<td>AMPA binding</td>
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<tr>
<td>Glu</td>
<td>62 ± 23</td>
<td>0.97 ± 0.06</td>
</tr>
<tr>
<td>AMPA</td>
<td>3.9 ± 0.1</td>
<td>1.01 ± 0.03</td>
</tr>
<tr>
<td>DOM</td>
<td>927 ± 80</td>
<td>0.71 ± 0.04</td>
</tr>
<tr>
<td>DH</td>
<td>153 ± 10</td>
<td>0.98 ± 0.05</td>
</tr>
<tr>
<td>CGS 19755 binding</td>
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<tr>
<td>Glu</td>
<td>62 ± 2.8</td>
<td>0.97 ± 0.04</td>
</tr>
<tr>
<td>NMDA</td>
<td>3,900 ± 370</td>
<td>0.89 ± 0.12</td>
</tr>
<tr>
<td>DH</td>
<td>&gt;100,000</td>
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*K values were calculated from the equation Ki = IC50/[radioligand]/Kd. The Ki values used here were 3.8 nM for [3H]KA, 10 nM for [3H]AMPA, and 24 nM for [3H]CGS 19755. Each IC50 value for DH, Glu, DOM, KA, and AMPA was determined based on the concentration-inhibition curve (Fig. 3) by the logistic equation: B = Bmax/[1 + IC50/[radioligand]]^n. Values represent the mean ± S.E.M. from at least three different experiments.

Fig. 2. Epileptogenic actions induced by DH and DOM in mice. Behavioral changes, induced by administration of DH and DOM i.c.v. (A) or i.p. (B), were graded using a seven-point scale (see Materials and Methods). Values are the mean scores ± S.E.M. Numbers in parentheses represent the observation period expressed by the time (h) after administration of drugs. More than five mice were used for each dose group. Note that behavioral changes induced by DH were observed for 3 h (i.c.v.) or 6 h (i.p.) after the administration.

These data therefore demonstrate that the activity of DH on hippocampal AMPA receptors is similar to that of KA, although of somewhat higher potency.
DH Does Not Interact with Glutamate Transporters.

Glutamate transporters play an important role in maintaining the extracellular concentration of glutamate below neurotoxic levels. To examine the activity of DH for transporters, inhibition of [14C]Glu uptake in MDCK cells permanently expressing EAAT1, EAAT2, or EAAT3 was measured. DH (1 mM) did not inhibit glutamate uptake, whereas DOM showed weak inhibition (IC50 = 550 μM) for EAAT2, a KA-sensitive subtype. The relative specific uptakes of [14C]Glu to control were 101 ± 3% (EAAT1), 101 ± 4% (EAAT2), and 106 ± 9% (EAAT3) in the presence of DH (1 mM).

Discussion

In the present study, we demonstrate that dysiherbaine, a novel amino acid isolated from Micronesian sponge D. herba-cea, is a non-NMDA receptor agonist possessing the most potent convulsant activity among the known EAAs (Stewart et al., 1990; Chiamulera et al., 1992). Injection of higher concentrations of DH produced status epilepticus, which was previously characterized as a typical reaction to non-NMDA glutamate receptor agonists (Chiamulera et al., 1992), and in preliminary ligand binding studies DH displaced non-NMDA receptor ligands (Sakai et al., 1997); these observations suggested to us that DH could act as an agonist at non-NMDA receptors, which may in part underlie its epileptogenic action. This hypothesis was supported by subsequent radioligand binding and physiology experiments.

In radioligand binding studies using rat synaptic membranes, DH selectively interacted with non-NMDA receptors. Interestingly, despite the strong convulsant activity, DH displaced [3H]KA and [3H]AMPA with only moderate affinities (Ki values = 26 and 153 nM, respectively, Table 2) compared with the unlabeled counterparts of these radioligands. However, the Hill coefficient determined from DH displacement of [3H]KA binding (0.52) was particularly low compared with those of other ligands (or compared with DH displacement of [3H]AMPA). These results suggest that DH has discrete affinities for several KA-binding sites in rat brain, possibly comprised of both AMPA and KA receptor populations. If this is indeed the case, it is possible that the higher affinity DH binding sites correspond to those KA receptors involved in generation of seizure behavior (Mulle et al., 1998). This interpretation was supported by subsequent studies using recombinant kainate receptor subunits expressed in HEK293 cells. A high affinity of DH for KA receptors was clearly demonstrated by displacement of [3H]KA from recombinant KA receptors GluR5 and GluR6 (Ki = 0.48 and 1.28 nM for GluR5 and -6, respectively). The affinity of DH to GluR6 is 8 times higher than that of KA-selective ligand 4-methylglutamate (Zhou et al., 1997).

We next verified that DH is an agonist for non-NMDA ionotropic glutamate receptors with KA selectivity in electrophysiological studies using cultured hippocampal neurons. A large proportion of the DH-evoked current at higher concentrations was mediated by AMPA receptors, because GYKI 53655, a selective noncompetitive antagonist of AMPA receptors, substantially reduced mean current amplitudes. DH elicited currents in a concentration-dependent manner with an EC50 of 9.68 μM and a mean current amplitude of about 4 nA at a concentration of 100 μM DH. We also observed GYKI-resistant current when DH was applied to the neurons...
in the presence of the antagonist. The GYKI-resistant current was of relatively high affinity, with an EC$_{50}$ of 0.21 μM. DH appears to be approximately 50-fold more potent for KA receptors as compared with AMPA receptors in hippocampal neurons, a degree of selectivity similar to that described for SYM 2081 (Jones et al., 1997; Zhou et al., 1997). The steady-state KA receptor current saturated at approximately 3 μM and had maximum amplitude of about 300 pA. High concentrations of the non-NMDA receptor antagonist CNQX suppressed GYKI-resistant DH currents; the lower sensitivity of KA receptors to blockade by CNQX compared with AMPA receptors is consistent with a previous report (Paternain et al., 1996). Finally, our observation that KA receptors are present at much lower densities compared with AMPA receptors in cultured neurons is also consistent with previous data (Paternain et al., 1996; Wilding and Huettner, 1997).

DH also stimulated calcium mobilization by heterologously expressed mGluR5 receptors, which belong to the group I family of receptors coupled to phospholipase C activity and phosphoinositide hydrolysis. Surprisingly, we found that this agonist activity was subtype-selective, because DH showed no agonist activity on mGluR1 receptors. This selective activity is relatively rare; only two agonists and three antagonists have been characterized with a similar preference for mGluR5 receptors (Doherty et al., 1997; Gasparini et al., 1999; Mannaioni et al., 1999; Varney et al., 1999). It is possible that mGluRs contribute to the epileptogenic activity of DH, although the different behavioral profiles of DH and trans-ACPD suggest that the selective action of DH on mGluR5 receptors does not account solely for the potent epileptogenic properties of DH. Further characterization of the pharmacological activity of DH on mGluRs, including group II and III receptors, is necessary to explore direct or indirect relationships of DH-induced seizure and mGluRs.
The striking potency that DH demonstrates for neuronal and recombinant KA receptors suggests that this activity predominantly underlies its efficacy for generation of seizures. KA receptors have been clearly implicated in the induction of seizures in the hippocampus (Ben-Ari, 1985; Sander et al., 1997; Mulle et al., 1998). Firing properties of CA3 pyramidal neurons are acutely sensitive to modulation by KA receptor agonists (Robinson and Deadwyler, 1981), and KA-induced seizures are commonly used as a model for the pathologies observed in human temporal lobe epilepsy (Nadler, 1981; Ben-Ari, 1985). In addition, susceptibility to KA-induced seizures was reduced in gene-targeted mice that lacked the GluR6 KA receptor subunit (Mulle et al., 1998).

The behavioral profile elicited by DH injection suggests that this compound may prove useful both as a new model for epileptogenesis studies and as a tool for characterizing the contribution of KA receptors to seizure behavior. Central or peripheral administration of DH in mice produced behavioral seizures that resembled those caused by DOM (Tryphonas et al., 1990a,b; Tasker et al., 1991) and to some extent with those by KA (Sperk, 1994). These similarities include preconvulsive behaviors and clonic convulsions followed by status epilepticus at the higher doses. However, the behavioral changes elicited by DH lasted much longer than those of DOM or other compounds. Furthermore, the ED50 values we calculated for DH, 13 pmol/mouse i.c.v., and 0.97 mg/kg, i.p. were approximately 7-fold lower than those calculated for DOM in parallel experiments. Two behaviors induced by DH—scratching at lower doses and long-lasting status epilepticus—were produced only by DOM among the other drugs we tested, suggesting that DOM and DH seizures may arise from activation of similar (or overlapping) receptor groups in the brain. Previously, it was proposed that DOM and KA activate different receptor populations to produce their seizureogenic behaviors, because in vivo excitotoxicity of DOM was significantly attenuated by NS 102, whereas that of KA was less sensitive to this drug (Tasker et al., 1996). Finally, it is worth noting that an intriguing difference emerged between the behaviors produced by DH and DOM: DH induced much longer lasting status epilepticus than other EAAs (including DOM). This difference could just be attributed to differences in the metabolic rate of the two drugs, or it could arise from a distinct spectrum of activities on ionotropic and metabotropic glutamate receptors.

Interestingly, in preliminary experiments DH was shown to activate homomeric recombinant GluR5 and GluR6 KA receptors at exceptionally low concentrations, whereas heteromeric KA receptors were significantly less sensitive to DH (G. T. Swanson, R. Sakai, A. Contractor, T. Green, H. Kamiya, and S. F. Heinemann, poster, 29th annual meeting, Society for Neuroscience, Miami Beach, FL, October 23–28, 1999). These results along with the potent binding affinity of DH to recombinant KA receptors suggest that DH exhibits a unique receptor subunit selectivity that may underlie its distinctive seizureogenic profile. Further characterization of the action and kinetics of DH on recombinant KA receptors is in progress.

In summary, we find that the novel amino acid DH is a non-NMDA agonist that possesses the most potent epileptogenic action among the known amino acids. Further investigations are necessary to fully characterize the mechanism of DH-evoked epileptogenic action. Nevertheless, unique binding, electrophysiological, and behavioral profiles, as well as the distinctive chemical structure of DH, warrant classification of this compound as a new type of EAA. We are now conducting a structure-activity relationship study of DH (Sasaki et al., 1999). With this information in hand, we anticipate that DH or its analogs may serve as useful pharmacological agents for investigating glutamate receptor function as well as lead compounds to develop therapeutic agents for central nervous system disorders.

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