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The neurotoxin domoate causes long-lasting inhibition of the kainate receptor GluK5 subunit

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Abstract

Iontropic glutamate receptors (iGluRs) are responsible for fast excitatory neurotransmission in the mammalian brain, and are critical regulators of neuronal activity and synaptic plasticity. The three main types of iGluRs (AMPA, NMDA, and kainate receptors) are composed of distinct subunit populations. The tetrameric kainate receptors can be assembled from a combination of five different types of subunits (GluK1-GluK5). GluK1-3 subunits are able to produce functional homomeric receptors, while GluK4-5 are obligate heteromers, and must assemble with a GluK1-3 subunit. The neurotoxin domoate is widely used as an agonist at kainate-type receptors because it produces a less desensitizing response compared to glutamate. We have identified an additional, subunit-dependent action of domoate at recombinant kainate receptors. When applied to heteromeric GluK2/K5 receptors, domoate generates a small, long-lasting, tonic current. In addition, brief exposure to domoate inhibits the GluK5 subunit, preventing its activation by other agonists for several minutes. These characteristics are not associated with the GluK1, K2, or K4 subunits and can be prevented by a mutation in GluK5 that reduces agonist binding affinity. The results also show that the domoate-bound, GluK2/K5 heteromeric receptors can be fully activated by agonists acting through the GluK2 subunit, suggesting that the subunits within the tetramer can function independently to open the ion channel, and that the domoate-bound state is not a desensitized or blocked conformation. This study describes new properties associated with domoate action at kainate receptors, and further characterizes the distinct roles played by different subunits in heteromeric receptors.

1. Introduction

The ionotropic glutamate receptors responsible for fast excitatory neurotransmission are classified into three types: AMPA, NMDA and kainate (Traynelis et al., 2010). AMPA receptors mediate most of the post-synaptic response to glutamate, while NMDA receptors are critical for activity-dependent synaptic plasticity. Kainate receptors (KARs) have

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important roles in the regulation of neuron excitability and neurotransmitter release throughout the brain (Traynelis et al., 2010; Contractor et al. 2011). Dysregulation of their activity has been suggested to contribute to a variety of neurological disorders including epilepsy, neurodegeneration, and pain (Vincent and Mulle, 2009; Bhangoo and Swanson, 2013; Lerma and Marques, 2013). KARs have a particularly significant role in temporal lobe epilepsy, where the abnormal recurrent excitatory circuit that develops in the hippocampus is mediated primarily by KARs (Epsztein et al., 2005). Genetic linkages have been reported between mutations in KAR subunits and a variety of neuropsychiatric disorders, including schizophrenia, autism, and obsessive-compulsive disorder (Contractor et al., 2011).

The tetrameric KARs can assemble from a combination of five different subunits (GluK1-GluK5). The GluK1-3 subunits are able to form functional homomers, while the GluK4 and GluK5 subunits (formerly known as KA1 and KA2) are obligate heteromers. Homomeric kainate receptors are characterized by low kainate affinity and rapid desensitization kinetics (Contractor et al., 2011). The GluK2 subunit is the most widely expressed of the KAR subunits, while the GluK1 subunit is highly expressed primarily in the developing brain, with more restricted expression in the adult (Bahn et al., 1994). GluK3 subunits produce homomeric receptors with exceptionally low sensitivity to glutamate, and are important contributors to presynaptic kainate receptors in the hippocampus, where they may co-assemble with GluK2 subunits and regulate neurotransmitter release (Bettler et al., 1992; Bahn et al., 1994; Schiffer et al., 1997; Pinheiro et al., 2007). The GluK4 and GluK5 subunits form functional receptors only in combination with the GluK1-3 subunits. When incorporated into KARs, they increase glutamate sensitivity, slow desensitization kinetics at low agonist concentrations, and alter pharmacological profiles (Jane et al., 2009; Contractor et al., 2011). It is likely that most post-synaptic KARs are heteromeric. The GluK5 subunit is widely expressed throughout the brain, while GluK4 is found almost exclusively in the hippocampus (Herb et al., 1992; Bahn et al., 1994). With the exception of some GluK1-specific agonists and antagonists, few subunit-selective ligands for KARs have been identified (Jane et al., 2009). This lack of pharmacological tools has limited progress towards determination of the functional roles of distinct kainate receptor isoforms.

Domoate is a naturally-produced neurotoxin structurally related to kainate (Costa et al., 2010). It is an agonist at both kainate- and AMPA-type glutamate receptors, but at low concentrations can selectively activate kainate receptors (Bureau et al., 1999; Jane et al., 2009). Domoate has been particularly useful for experimental purposes because it produces a relatively non-desensitizing response, compared to the endogenous agonist glutamate. It activates GluK1- and GluK2-containing receptors with similar EC_{50} 's (Alt et al., 2004), but does not act as an agonist at GluK3 homomeric receptors, despite binding with high affinity (Bettler et al., 1992; Schiffer et al., 1997). Thus, domoate has been considered a relatively non-specific, partial agonist of most homomeric and heteromeric kainate receptors.

We used patch-clamp recordings from transfected HEK-293T cells to examine the activity of domoate at heteromeric kainate receptors, focusing on those containing the GluK5 subunit. Addition of the GluK5 subunit had little effect on the domoate EC_{50} , compared to GluK2 homomers. However, application of domoate to GluK2/K5 receptors produced a

prolonged tonic current and a long-lasting inhibition of the GluK5 subunit, reducing the response of the receptor to other agonists.

2. Materials and Methods

2.1. Cell culture and transfection of mammalian cells

Full-length cDNAs for rat kainate receptor subunits (obtained from Dr. S. Heinemann, Salk Institute, San Diego CA) in mammalian expression vectors were transfected into the human embryonic kidney cell line HEK-293T (GenHunter, Nashville, TN). Point mutations were generated using the QuikChange procedure and products (Agilent Technologies, Santa Clara, CA). Oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA) and mutations were verified by DNA sequencing (University of South Carolina Environmental Genomics core, Columbia, SC). Cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were passaged by a 2 min. incubation with 0.025% trypsin/0.01% EDTA solution in phosphate buffered saline (10 mM Na₂HPO₄, 150 mM NaCl, pH=7.3).

The cells were transiently transfected using calcium phosphate precipitation. A total of 5 µg of cDNA was transfected into the cells. For homomeric receptors, 4 µg of GluK1 or GluK2 was transfected. To produce heteromeric receptors, plasmids were added to the cells in a 1:3 ratio (GluK2:GluK4 or 5), previously shown to optimize formation of a homogeneous population (Barberis et al., 2008). To allow identification of positively transfected cells, 1 µg of the plasmid pHookTM-1 (Invitrogen Life Technologies, Grand Island NY) which encodes a surface antibody, was also included (Chesnut et al., 1996). The cells were incubated at 3% CO₂ for 4–6 hrs with the DNA/calcium phosphate mixture at 3% CO₂ and then treated with a 15% glycerol solution in BES-buffered saline (25 mM BES(N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid), 140 mM NaCl, 0.75 mM Na₂HPO₄) for 30 sec. The selection procedure for pHook expression was performed 18–28 hrs later. The cells were trypsinized and mixed for 30–60 min. with approximately 6×10^5 magnetic beads coated with antigen for the pHook antibody (Chesnut et al., 1996). Although the trypsin treatment is relatively mild, potential effects of trypsin on kainate receptor subunits have not been investigated. Bead-coated cells were isolated using a magnetic stand, resuspended into DMEM, and plated onto collagen-coated glass coverslips and incubated overnight prior to electrophysiological recordings.

2.2. Electrophysiological recordings

Cells were used for voltage-clamp recordings 44–52 hours after transfection. For all recordings the external solution consisted of (in mM): 150 NaCl, 3 KCl, 10 HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 1 CaCl₂, 0.4 MgCl₂ at pH 7.4 and osmolarity 295–305 mOsm. Recording electrodes were filled with an internal solution of (in mM): 130 CsGluconate, 5 CsCl, 0.5 CaCl₂, 10 HEPES, 5 CsBapta, 2 MgCl₂, 2 MgATP, 0.3 NaGTP with pH 7.4 and osmolarity 290–300 mOsm. All agonists were diluted into external solution from freshly made or frozen stocks in water. Patch pipettes were pulled from borosilicate glass with an internal filament (World Precision Instruments, Sarasota, FL) on a

two-stage puller (Narishige, Japan) to a resistance of 5–10 MΩ. Drugs were applied to cells using a stepper solution exchanger with a complete exchange time of <50 msec (open tip, SF-77B, Harvard Apparatus, Holliston, MA). This whole-cell recording mode will not allow capture of fast desensitization and thus the peak current to high concentrations of agonists will likely be underestimated. There was a continuous flow of external solution through the chamber. Currents were recorded with an Axon 200B (Foster City, CA) patch clamp amplifier, filtered at 2 KHz (low-pass Bessel filter) and digitized at 20 KHz.

2.3. Data Analysis and statistical tests

Whole-cell currents were analyzed using the programs Clampfit (pClamp10.3 suite, Axon Instruments, Foster City, CA) and Prism (Graphpad, San Diego, CA). Concentration-response data was fit with a four-parameter logistic equation

$$(\text{Current} = [\text{Minimum current} + (\text{Maximum current} - \text{Minimum Current})] / 1 + (10^{(\log \text{EC}_{50} - \log[\text{agonist}]) * n}))$$

where n represents the Hill number. Fits were made to normalized data with the measured peak current expressed as a percentage of the maximum response for each cell. In cases where a saturating agonist concentration could not be reached due to low affinity of a mutated subunit, the maximum current was estimated by fitting the current amplitudes while fixing the Hill number to that obtained from the fit to data from wild-type receptors. ANOVA and Tukey-Kramer multiple comparisons tests were performed using the Instat program (Graphpad) with a significance level of $p < 0.05$.

3. Results

3.1. Domoate inhibits the response of GluK2/K5 receptors to glutamate

Addition of the GluK5 subunit to produce heteromeric GluK2/K5 kainate receptors causes several changes in receptor properties compared to GluK2 homomers, including sensitivity to a variety of agonists such as glutamate and AMPA (Herb et al, 1992, Jane et al., 2009). The neurotoxin domoate has been reported to behave as an agonist at most kainate receptors (Sommer et al., 1992; Swanson et al., 1997; Shiffer et al., 1997; Alt et al., 2004). When applied to GluK2/K5 receptors, 10 μM domoate produced a weakly desensitizing and slowly deactivating current compared to that generated by 10 μM glutamate. The response to domoate did not decay completely, instead resulting in a tonic inward current that required several minutes to return to the original baseline (Fig 1A, 1B). The amplitude of this current 1 min. after domoate application averaged 56.7 ± 12.4 pA ($n=7$) which was $15.0 \pm 1.2\%$ of the peak response to 10 μM glutamate in the same cells.

In addition, activation of GluK2/K5 receptors by domoate inhibited the response to subsequent applications of glutamate (Fig 1A, 1C). Glutamate (10 μM) was applied for 2 sec at 1 min intervals to establish a stable response prior to a 2 sec application of 10 μM domoate. Glutamate was then re-applied at 1 min intervals to determine the time course of recovery of responsiveness to glutamate. 10 μM glutamate was selected for use as a reference response as it produced little rundown and therefore was amenable to repeated applications. Because of the shift in baseline produced by domoate, the peak amplitude of the response to glutamate was measured using the original baseline prior to domoate application. Full recovery of the initial response to glutamate required 15–20 minutes, and

was accompanied by a loss of the tonic inward current and a return to the original baseline. In contrast, repeated applications of either 10 μ M glutamate or domoate alone produced stable responses (Fig 1B). Domoate applications caused an inward shift in the baseline, but did not reduce the subsequent response to domoate. This indicated that the receptor itself could still respond fully to the agonist action of domoate, possibly through activation of the GluK2 subunits.

When glutamate was applied following domoate exposure, the inward current produced by glutamate was followed by an outward current that approached the original baseline (Fig 1D). This apparent reduction in the tonic current was likely a result of glutamate-induced desensitization which closed the tonically open channels. The tonic current recovered within 1–2 seconds following the removal of glutamate, consistent with the time course of recovery from desensitization (Heckmann et al., 1996; Fisher and Mott, 2013).

To determine whether recovery from domoate inhibition required activation of the channel by glutamate, four applications of external solution without agonist were applied at 1 min intervals following the response to 10 μ M domoate (Fig 1E). The recovery was then tested with an application of glutamate. An average of $49.2 \pm 7.1\%$ ($n=4$) of the peak current response to 10 μ M glutamate was recovered after 5 minutes, comparable to the $49.9 \pm 3.9\%$ ($n=7$) recovered at this time point when 10 μ M glutamate was applied every minute (Fig 1C). These results suggest that the recovery process is strictly time-dependent, and is not influenced by stimulation of the receptor.

3.2. Prolonged inhibition by domoate is associated only with the GluK5 subunit

GluK1 and GluK2 subunits are expressed in the mammalian brain in region-specific and developmentally regulated patterns (Bahn et al., 1994). These subunits are capable of forming homomeric receptors which can be activated by a variety of kainate receptor agonists including domoate. While 300 μ M glutamate ($\sim EC_{50-70}$) causes rapid and complete desensitization of these receptors, domoate produces a slowly desensitizing response (Figure 2A).

The activation of GluK1 homomeric receptors by 10 μ M domoate did not result in a tonic current and had no effect on subsequent responses to 300 μ M glutamate (Figure 2). At homomeric GluK2 receptors, 10 μ M domoate generated a slowly decaying response, but the current returned to baseline within 1 min. (Fig 2A). The response of these receptors to 300 μ M glutamate was reduced by about 50% when applied 1 minute after domoate, with the full amplitude response recovering by 3 min. (Figure 2A, 2C). In addition to the reduced amplitude, the extent of desensitization produced by glutamate in these receptors was briefly affected by domoate exposure, with an increased steady state current apparent at the 1 min. time point after domoate (Fig 2B).

The GluK4 subunit shares many characteristics with the GluK5 subunit, including most pharmacological properties and the requirement for co-assembly with GluK1-3 subunits (Partin et al., 1993; Jane et al., 2009; Contractor et al., 2011). GluK2/K4 heteromers responded to 10 μ M domoate with a weakly desensitizing and slowly decaying current similar to that produced by GluK2 homomers. In addition, a small tonic current averaging

8.9±3.7 pA (3.5±1.1% of the response to 30 μ M glutamate, n=4) was observed 1 min after domoate. However, the baseline returned to its original level by 2–3 minutes (Figure 2A). Although rapid desensitization characteristics cannot be examined given the relatively slow solution exchange rate associated with whole cell recordings, the current response to glutamate at 1 minute after domoate exposure showed little difference in the extent of desensitization, in contrast to the GluK2 homomers (Figure 2B). Domoate also inhibited subsequent responses to glutamate of GluK2/K4 receptors, although the time course of recovery was much more rapid than for GluK2/K5, with full recovery within 5 mins (Figure 2C). A substantial and long-lasting tonic current as well as a prolonged inhibition of subsequent responses to agonist produced by domoate therefore appears to be a distinct property of the GluK5 subunit. Interestingly, it has been reported that GluK3 homomeric receptors are not activated by domoate, and that domoate exposure causes a long-lasting inhibition of these receptors (Shiffer et al., 1997), suggesting some shared characteristics in the responses of the GluK3 and GluK5 subunits.

3.3. Inhibition of the GluK5 subunit by domoate does not prevent full activation of the heteromeric receptor by GluK2 agonists

The results shown in Fig. 1 demonstrated that domoate application reduced subsequent responses of GluK2/K5 receptors to 10 μ M glutamate, but not to domoate. This suggested that agonists acting through the GluK2 subunit could fully activate the receptor channel, even when the GluK5 subunits within the heteromer were inhibited by domoate. To further test this hypothesis, we examined the effect of domoate on subsequent responses to other KAR agonists. At μ M concentrations, AMPA is an agonist at GluK2/K5 receptors, but not GluK2 homomers (Herb et al., 1992), suggesting that it activates the receptor solely through binding to the GluK5 subunit. Domoate effectively inhibited responses of GluK2/K5 receptors to 30 μ M AMPA, with a recovery time course similar to that observed with 10 μ M glutamate (Fig 3). In contrast to AMPA, both homomeric and heteromeric receptors are activated by kainate, with similar EC₅₀s (1–5 μ M) for GluK2 and GluK2/K5 (Alt et al., 2004). The response to 1 μ M kainate was only modestly inhibited by domoate, with a rapid recovery similar to that of GluK2 homomers (Fig 3), consistent with kainate activation of the receptor primarily through binding to the GluK2 subunits. Finally, we examined whether higher concentrations of glutamate might also be able to fully activate the domoate-bound receptors. GluK2 homomeric receptors are much less sensitive to glutamate compared to GluK2/K5 receptors (Alt et al., 2004; Barberis et al., 2008; Fisher and Mott, 2011). Thus, 10 μ M glutamate represents an EC₇₅₋₈₀ concentration for GluK2/K5 receptors, but only an EC₅₋₁₀ concentration for GluK2 homomers. When a glutamate concentration sufficient to fully saturate the GluK2 binding sites (10 mM) was applied, the current amplitude recovered with a time course similar to that of GluK2 homomers (Fig 3). The presence of the GluK5 subunit in these receptors was apparent from the long-lasting change in the baseline produced by domoate application (Fig 3A). Together, these results demonstrate that inhibition of the GluK5 subunits by domoate does not prevent a full response of the receptor through activation of the GluK2 subunits within the heteromer. This suggests that domoate inhibits only the bound subunits (i.e. GluK5), and does not hold the receptor in a prolonged desensitized state or block conductance through the ion channel.

3.4. The long-lasting tonic current produced by domoate is not affected by CNQX

Many of the properties of domoate activity at GluK2/K5 receptors are comparable to that of another neurotoxin, dysiherbaine, which produces a prolonged inhibition of GluK1 or GluK2 homomeric receptors, and generates a long-lasting tonic current when applied to heteromeric GluK1/K5, but not GluK1/K4 receptors (Swanson et al., 2002). Interestingly, application of the competitive antagonist CNQX to heteromeric receptors bound to dysiherbaine caused an increase in current amplitude, suggesting that CNQX was able to act as a partial agonist at these bound receptors. To determine if the tonically activated state produced by domoate shared this characteristic, 100 μ M CNQX was applied to GluK2/K5 receptors 1 minute following an application of 10 μ M domoate (Fig 4). CNQX had little impact on the tonic current, causing an average $3.4 \pm 0.8\%$ ($n=5$) decrease in current amplitude. This slight inhibition was readily reversible, suggesting that CNQX did not displace the bound domoate, but instead transiently reduced channel activity. This could represent block of the channel pore, or an inverse agonist action mediated by binding to the GluK2 subunits. The partial agonist activity of CNQX at dysiherbaine-treated heteromeric receptors was ascribed to binding to the GluK5 subunit (Swanson et al., 2002) and our results are also consistent with this conclusion.

3.5. Mutation of the GluK5 agonist binding site prevents the inhibitory action of domoate

To determine whether the long-lasting inhibitory activity of domoate occurred through the agonist binding site of the GluK5 subunit, we created mutated GluK2 and GluK5 subunits with reduced agonist affinity. Exchanging a conserved glutamate residue within the S2 domain of the agonist binding site for aspartate dramatically reduces sensitivity of the GluK2 subunit to glutamate (Mah et al., 2005; Fisher and Mott, 2011). We generated this mutation in either the GluK2 (E738D) or GluK5 (E722D) subunit, and examined the concentration dependence of the peak amplitude response of the receptors to agonists. The maximum peak response is likely underestimated due to the effects of rapid desensitization. As expected, homomeric GluK2_(E738D) receptors were much less sensitive to domoate, with an average EC_{50} of 131.3 ± 43.0 μ M ($n=3$) for GluK2_(E738D) compared to 1.41 ± 0.25 μ M ($n=4$) for wild-type receptors (Figure 5B). Thus, this mutation has similar effects on the sensitivity of homomeric GluK2 receptors to both glutamate and domoate.

The heteromeric GluK2/K5 receptor has greater sensitivity for glutamate compared to homomeric GluK2 receptors, due to the higher binding affinity of the GluK5 subunit. In agreement with previous studies (Fisher and Mott, 2011), assembly of GluK2_(E738D) with wild-type GluK5 subunits did not alter glutamate sensitivity of the heteromeric receptors (average $EC_{50} = 5.70 \pm 0.45$ μ M, $n=3$, compared to 6.35 ± 0.96 μ M, $n=4$ for wild-type GluK2/K5 receptors) (Fig 5C). Although these receptors activated normally, higher concentrations of glutamate were required to produce desensitization, which apparently required occupation of the GluK2 subunits (Fisher and Mott, 2011). When the GluK5_(E722D) subunit was incorporated into heteromeric receptors, the glutamate sensitivity was reduced compared to wild-type GluK2/K5 receptors, with an average EC_{50} of 217.6 ± 35.1 μ M ($n=3$) (Fig 5C). This glutamate EC_{50} is similar to that of GluK2 homomers (Fisher and Mott, 2011), suggesting that these receptors are activated by glutamate binding to the wild-type GluK2 subunits within the heteromer. Incorporation of the mutated GluK5 subunit into these

receptors was apparent by the difference in desensitization properties compared to GluK2 homomers (see Figure 6A). The combined effects of these mutations suggest that the GluK2 and GluK5 subunits maintain their distinct glutamate sensitivities when assembled into the heteromeric tetramer and that binding of the higher affinity subunit drives activation of the channel by glutamate.

In contrast, the effect of these mutations on the response of heteromeric receptors to domoate showed a very different pattern. The average domoate EC₅₀ for wild-type GluK2/K5 receptors averaged $0.85 \pm 0.18 \mu\text{M}$ (n=3), similar to that of GluK2 homomers (Fig 5B). This suggested that, unlike glutamate, the GluK5 subunits may not have greater sensitivity to domoate than the GluK2 subunits. This is consistent with the higher domoate affinity for the GluK2 subunit compared to GluK5 found in radioligand binding assays (Herb et al., 1992). The effects of the binding site mutations provide further evidence for this suggestion (Fig 5B), as the GluK2/K5_(E722D) receptors had an average domoate EC₅₀ ($1.59 \pm 0.55 \mu\text{M}$, n=4) comparable to that of the GluK2 homomers. In contrast, the GluK2_(E738D)/K5 receptors had greatly reduced sensitivity to domoate, with an average EC₅₀ of $124.6 \pm 29.5 \mu\text{M}$ (n=4), similar to that of the homomeric GluK2_(E738D) receptors. This suggests that GluK5 subunits have relatively low sensitivity to domoate and that it acts as a weak partial agonist through these subunits. As a result, activation of heteromeric receptors occurs primarily through domoate binding to the GluK2 subunit. This is opposite to the pattern for glutamate activation of these receptors.

These mutations also demonstrate that both generation of the tonic current and prolonged inhibition of responses to glutamate are dependent upon activation of the GluK5 subunit by domoate. The tonic current was clearly evident in receptors containing wild-type GluK5 subunits, whether combined with wild-type or mutated GluK2 subunits (Fig 5A). However, heteromeric receptors containing the GluK5_(E722D) subunit did not generate this current in response to 10 μM domoate (Figs 5A, 6A). The same pattern was seen with the inhibition of responses to glutamate, which had a similar extent and time-course of recovery as wild-type receptors for GluK2_(E738D)/K5, but was completely absent for GluK2/K5_(E722D). Notably, the short-lived inhibition and alteration of the extent of desensitization produced by domoate in GluK2 homomeric receptors (Fig. 2) was also not produced in these GluK2/K5_(E722D) heteromeric receptors. This may suggest that occupancy of more than two GluK2 subunits by domoate is required to generate these effects.

4. Discussion

The neurotoxin domoate is produced by marine organisms such as red algae, and concentrated in shellfish and small fish that feed on this algae. In humans, consumption of shellfish contaminated with domoate was associated with brain damage and a variety of neurological symptoms termed ‘amnesic shellfish poisoning’ (Perl et al., 1990). Domoate exposure commonly causes seizures and death in marine mammals and birds (Pulido et al., 2008) and in sea lions, domoate intoxication produces status epilepticus and hippocampal damage analogous to that seen in humans with temporal lobe epilepsy (Buckmaster et al., 2014). These effects are consistent with excitotoxic activity of domoate mediated through kainate- and AMPA-type glutamate receptors.

Domoate has long been characterized as a high-affinity, weakly-desensitizing agonist with preferential activity at kainate receptors, and is widely used as an experimental tool to study these receptors. This study shows that domoate has additional effects at receptors containing the GluK5 subunit, producing a long-lived open state and preventing other agonists from activating the receptor through the GluK5 subunits. Both the tonic current and the inhibitory action of domoate required binding of domoate to the GluK5 subunit, and were prevented by mutations in the agonist binding site. The tonic current could be reduced by glutamate, suggesting that the open channel could be closed by transition into a desensitized state. However, the competitive antagonist CNQX did not effectively block the tonic current. Together, this suggests that domoate binding to GluK5 causes a conformational change to a very high affinity, but low efficacy open state. The long duration of the domoate-bound state indicates a high affinity binding, but μM levels are required to produce this state, suggesting that a transition to a high affinity state occurs after binding. This might be analogous to the agonist-induced conformational changes that lead to long-lived, high-affinity desensitized states. In contrast to desensitized states, however, the domoate-produced conformational change appears limited to the bound (GluK5) subunit, and does not alter the functional responses of unbound (GluK2) subunits within the tetramer. As a result, agonists acting through the GluK2 subunits generate nearly complete activation of the receptor, and apparently normal onset of desensitization.

These findings suggest that activation of only the two GluK2 subunits in the tetramer may be sufficient to generate a full response of heteromeric receptors. These observations are consistent with the emerging picture for independent functional roles of each subunit (or each dimer) within the tetrameric kainate receptor (Swanson et al., 2002; Mott et al., 2010; Fisher and Mott, 2011; Reiner and Isacoff, 2014). The poor agonist activity of domoate at GluK5 subunits suggests that domoate activates GluK2/K5 receptors only through the GluK2 subunits. Single channel recordings have shown that channel openings with similar conductance are produced by domoate at both GluK2 homomers and GluK2/K5 heteromers (Swanson et al., 1996). Although AMPA receptors have been shown to have different conductance states in response to different levels of binding site occupancy (Rosenmund et al., 1998), studies with kainate receptors indicated that they did not share this property (Smith and Howe, 2000). However, those experiments were performed using domoate as an agonist, and therefore may have been limited to only two full agonist sites within a heteromeric receptor.

These characteristics described for domoate have some striking similarities to the action of the neurotoxin dysiherbaine (Swanson et al., 2002). Dysiherbaine is a naturally-occurring compound produced by marine sponges, and has agonist activity at kainate receptors (Sakai et al., 1997; 2001). Application of dysiherbaine to homomeric GluK1 or GluK2 receptors initially activated the receptors, but then produced a long-lasting inhibition of these receptors, with less than 20% of the initial response to glutamate recovery at 20 minutes after dysiherbaine exposure. Thus, the duration of the inhibitory action is much greater than we observed with domoate. The subunit-dependence of the effects of these neurotoxins is also different. While domoate acts primarily through the GluK5 subunit, dysiherbaine was less effective at GluK2/K5 receptors, compared to GluK2 homomers, with full recovery of the response to glutamate within 5 minutes after dysiherbaine treatment. As observed with

domoate, a prolonged tonic current was also generated by GluK2/K5 receptors in response to dysiherbaine. This current could be reduced by desensitizing agonists, but unlike the domoate-associated current, could be increased by the competitive antagonist CNQX. In addition, the domoate-induced tonic current was produced immediately upon domoate application, and slowly decayed. In contrast, the dysiherbaine-activated current slowly developed following removal of dysiherbaine. The similarities between the effects of domoate and dysiherbaine are intriguing, and may represent a shared mechanism of action, while the differences between these two toxins might arise from unique characteristics associated with the different subunits responsible for mediating their effects.

The structural basis for the unique properties associated with the GluK5 subunit could be due to differences in the agonist binding site or the signal transduction and gating pathways. A great deal of information is available regarding the residues that contribute to domoate binding to the GluK2 subunit, from crystallographic analysis of the domoate-bound structure (Nanao et al., 2005) as well as extensive mutagenesis studies (Zhang et al., 2008). However, whether the domoate binding site in the GluK5 subunit might substantially differ from that in the GluK2 is not known. In general, the residues identified as critical for domoate binding to the GluK2 subunit are conserved among KAR subunits, and those that are variable are not unique to the GluK5 subunit. Variation at one site (N721 in GluK2) within the S2 binding domain has been identified to play an important role in many pharmacological differences between KAR subunits. GluK2 and GluK3 share an asparagine at this location, while GluK4 and GluK5 have a threonine residue and GluK1 has a serine. Mutations at this site showed that it controls the differing deactivation rates observed with GluK1 and GluK2 homomers in response to domoate, as well as the sensitivity to AMPA shared by GluK1, GluK4 and GluK5 (Swanson et al., 1997). This residue is also critical for some subunit-dependent responses to dysiherbaine (Swanson et al., 2002, Unno et al., 2011). Since the GluK4 and GluK5 subunits share homology at this site, however, it is less likely that this residue alone determines the distinct characteristics of GluK5-containing receptors.

These results demonstrate a unique response to domoate associated with the presence of the GluK5 subunit and predict that neurons expressing this subunit would respond to domoate with a prolonged inhibition to activation by GluK5 agonists, as well as a substantial tonic current. These properties could allow domoate to be used as a pharmacological tool to identify functional surface expression of GluK5-containing kainate receptors. Further studies into the structural basis of this response may also provide insight into the subunit-specific differences in ligand binding and channel gating associated with the different KAR subunits.

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References

- Alt A, Weiss B, Ogden AM, Knauss JL, Oler J, Ho K, Large TH, Bleakman D. Pharmacological characterization of glutamatergic agonists and antagonists at recombinant human homomeric and heteromeric kainate receptors in vitro. *Neuropharmacology*. 2004; 46:793–806. [PubMed: 15033339]
- Bahn S, Volk B, Wisden W. Kainate receptor gene expression in the developing rat brain. *J Neurosci*. 1994; 14:5525–5547. [PubMed: 8083752]
- Barberis A, Sachidhanandam S, Mulle C. GluR6/KA2 kainate receptors mediate slow-deactivating currents. *J Neurosci*. 2008; 28:6402–6406. [PubMed: 18562611]
- Bettler B, Egebjerg J, Sharma G, Pecht G, Hermans-Borgmeyer I, Moll C, Stevens CF, Heinemann S. Cloning of a putative glutamate receptor: a low affinity kainate-binding subunit. *Neuron*. 1992; 8:257–265. [PubMed: 1371217]
- Bhangoo SK, Swanson GT. Kainate receptor signaling in pain pathways. *Mol Pharmacol*. 2013; 83:307–315. [PubMed: 23095167]
- Buckmaster PS, Wen X, Toyoda I, Gulland FMD, Van Bonn W. Hippocampal neuropathology of domoic acid-induced epilepsy in California sea lions (*Zalophus californianus*). *J Comp Neuro*. 2014; 522:1691–1706.
- Bureau I, Bischoff S, Heinemann SF, Mulle C. Kainate receptor-mediated responses in the CA1 field of wild-type and GluR6-deficient mice. *J Neurosci*. 1999; 19:653–663. [PubMed: 9880586]
- Chesnut JD, Baytan AR, Russell M, Chang MP, Bernard A, Maxwell IH, Hoeffler JP. Selective isolation of transiently transfected cells from a mammalian cell population with vectors expressing a membrane anchored single-chain antibody. *J Immunol Methods*. 1996; 193:17–27. [PubMed: 8690927]
- Contractor A, Mulle C, Swanson GT. Kainate receptors coming of age: milestones of two decades of research. *Trends Neurosci*. 2011; 34:154–163. [PubMed: 21256604]
- Costa LG, Giordano G, Faustman EM. Domoic acid as a developmental neurotoxin. *NeuroToxicology*. 2010; 31:409–423. [PubMed: 20471419]
- Epsztein J, Represa A, Jorquera I, Ben-Ari Y, Crepel V. Recurrent mossy fibers establish aberrant kainate receptor-operated synapses on granule cells from epileptic rats. *J Neurosci*. 2005; 25:8229–8239. [PubMed: 16148230]
- Fisher JL, Mott DD. Distinct functional roles of subunits within the heteromeric kainate receptor. *J Neurosci*. 2011; 31:17113–17122. [PubMed: 22114280]
- Fisher JL, Mott DD. Modulation of homomeric and heteromeric kainate receptors by the auxiliary subunit Neto1. *J Physiol*. 2013; 591:471–4724.
- Heckmann M, Bufler J, Franke C, Dudel J. Kinetics of homomeric GluR6 glutamate receptor channels. *Biophys J*. 1996; 71:1743–1750. [PubMed: 8889151]
- Herb A, Burnashev N, Werner P, Sakmann B, Wisden W, Seeburg PH. The KA-2 subunit of excitatory amino acid receptors shows widespread expression in brain and forms ion channels with distantly related subunits. *Neuron*. 1992; 8:775–785. [PubMed: 1373632]
- Jane DE, Lodge D, Collingridge GL. Kainate receptors: pharmacology, function and therapeutic potential. *Neuropharmacology*. 2009; 56:90–113. [PubMed: 18793656]
- Lerma J, Marques JM. Kainate receptors in health and disease. *Neuron*. 2013; 80:292–311. [PubMed: 24139035]
- Mah SJ, Cornell E, Mitchell NA, Fleck MW. Glutamate receptor trafficking: endoplasmic reticulum quality control involves ligand binding and receptor function. *J Neurosci*. 2005; 25:2215–2225. [PubMed: 15745947]
- Mott DD, Rojas A, Fisher JL, Dingledine RJ, Benveniste M. Subunit-specific desensitization of heteromeric kainate receptors. *J Physiol*. 2010; 588:683–700. [PubMed: 20026616]
- Nanao MH, Green T, Stern-Bach Y, Heinemann SF, Choe S. Structure of the kainate receptor subunit GluR6 agonist-binding domain complexed with domoic acid. *Proc Natl Acad Sci*. 2005; 102:1708–1713. [PubMed: 15677325]

- Partin KM, Patneau DK, Winters CA, Mayer ML, Buonanno A. Selective modulation of desensitization at AMPA versus kainate receptors by cyclothiazide and concanavalin A. *Neuron*. 1993; 11:1069–1082. [PubMed: 7506043]
- Perl TM, Bedard L, Kosatsky T, Gockin JC, Todd ECD, Remis RS. An outbreak of toxic encephalopathy caused by eating mussels contaminated with domoic acid. *N Engl J Med*. 1990; 322:1775–1780. [PubMed: 1971709]
- Pinheiro PS, Perrais D, Coussen F, Barhanin J, Bettler B, Mann JR, Malva JO, Heinemann SF, Mulle C. GluR7 is an essential subunit of presynaptic kainate autoreceptors at hippocampal mossy fiber synapses. *Proc Natl Acad Sci*. 2007; 104:12181–12186. [PubMed: 17620617]
- Pulido OM. Domoic acid toxicologic pathology: a review. *Mar Drugs*. 2008; 6:180–219. [PubMed: 18728725]
- Reiner A, Isacoff EY. Tethered ligands reveal glutamate receptor desensitization depends upon subunit occupancy. *Nature Chem Biol*. 2014; 10:273–280. [PubMed: 24561661]
- Rosenmund C, Stern-Bach Y, Stevens CF. The tetrameric structure of a glutamate receptor channel. *Science*. 1998; 280:1596–1599. [PubMed: 9616121]
- Sakai R, Kamiya H, Murata M, Shimamoto K. Dysiherbaine: a new neurotoxic amino acid from the Micronesian marine sponge *Dysidea herbacea*. *J Am Chem Soc*. 1997; 119:4112–4116.
- Sakai R, Swanson GT, Shimamoto K, Green T, Contractor A, Ghetti A, Tamura-Horikawa Y, Oiwa C, Kamiya H. Pharmacological properties of the potent epileptogenic amino acid dysiherbaine, a novel glutamate receptor agonist isolated from the marine sponge *Dysidea herbacea*. *J Pharm Exp Ther*. 2001; 296:650–658.
- Schiffer, Swanson GT, Heinemann SF. Rat GluR7 and a carboxy-terminal splice variant, GluR7b, are functional kainate receptor subunits with a low sensitivity to glutamate. *Neuron*. 1997; 19:1141–1146. [PubMed: 9390526]
- Smith TC, Howe JR. Concentration-dependent substate behavior of native AMPA receptors. *Nature Neurosci*. 2000; 3:992–997. [PubMed: 11017171]
- Sommer B, Burnashev N, Verdoorn TA, Keinänen K, Sakmann B, Seeburg PH. A glutamate receptor channel with high affinity for domoate and kainate. *EMBO J*. 1992; 11:1651–1656. [PubMed: 1373382]
- Swanson GT, Feldmeyer D, Kaneda M, Cull-Candy SG. Effect of RNA editing and subunit co-assembly on single-channel properties of recombinant kainate receptors. *J Physiol*. 1996; 492:129–142. [PubMed: 8730589]
- Swanson GT, Gereau RW, Green T, Heinemann SF. Identification of amino acid residues that control functional behavior in GluR5 and GluR6 kainate receptors. *Neuron*. 1997; 19:913–926. [PubMed: 9354337]
- Swanson GT, Green T, Sakai R, Contractor A, Che W, Kamiya H, Heinemann SF. Differential activation of individual subunits in heteromeric kainate receptors. *Neuron*. 2002; 34:589–598. [PubMed: 12062042]
- Traynelis SF, Wollmuth LP, McBain CJ, Menniti FS, Vance KM, Ogden KK, Hansen KB, Yuan H, Myers SJ, Dingledine R. Glutamate receptor ion channels: structure, regulation, and function. *Pharmacol Rev*. 2010; 62:405–496. [PubMed: 20716669]
- Unno M, Shinohara M, Takayama K, Tanaka H, Teruya K, Doh-ura K, Sakai R, Sasaki M, Ikeda-Saito M. Binding and selectivity of the marine toxin neodysiherbaine A and its synthetic analogues to GluK1 and GluK2 kainate receptors. *J Mol Biol*. 2011; 413:667–683. [PubMed: 21893069]
- Vincent P, Mulle C. Kainate receptors in epilepsy and excitotoxicity. *Neuroscience*. 2009; 158:309–323. [PubMed: 18400404]
- Zhang Y, Nayeem N, Green T. Mutations to the kainate receptor subunit GluR6 binding pocket that selectively affect domoate binding. *Mol Pharmacol*. 2008; 74:1163–1169. [PubMed: 18664604]

Highlights

- Domoate produces a prolonged inhibition of the GluK5 subunit of kainate receptors.
- Domoate induces a small, long-lasting tonic current through GluK5-containing receptors.
- GluK2 agonists activate GluK2/K5 heteromers despite block of the GluK5 by domoate.

GluK2/K5

Figure 1. Domoate activation of GluK2/K5 heteromers produces a long-lasting residual current and inhibition of subsequent responses to glutamate

A. Agonists were applied for 2 sec (solid line) to transfected HEK-293T cells voltage-clamped at -70 mV. Application of 10μ M domoate caused a prolonged inward shift from the original baseline, which is indicated by the dashed line. Following exposure to domoate, the amplitude of the next response to 10μ M glutamate was reduced. 10μ M glutamate was applied at 1 min intervals to measure recovery of the initial response.

B. Repeated applications of either glutamate (10μ M) or domoate (10μ M) do not influence subsequent responses to the same agonist. Agonists were applied for 2 sec at 1 min intervals. Time intervals (1, 5 and 10 mins) are in reference to the first trace shown.

C. The peak response to glutamate (10μ M) or domoate (10μ M) was normalized to the average amplitude of the first three agonist applications. To account for the prolonged tonic current, the peak amplitude was measured using the baseline from the first agonist treatment. 10μ M domoate was applied for 2 sec following three applications of agonist, as indicated

by the solid bar and arrow, except in the case of glutamate only. Open symbols represent mean $\pm$ SEM for responses to 10 μ M glutamate (n=7). Filled symbols represent the average amplitudes of responses to repeated applications of either glutamate (circles, n=6) or domoate (diamonds, n=3) only.

D. Current trace from 1A shown on an expanded scale to demonstrate the response to 10 μ M glutamate (solid line) given 1 min after a 2 sec application of 10 μ M domoate. The dashed line indicates the original baseline prior to domoate exposure.

E. Recovery does not require glutamate application or channel activity. Four applications of external solution without glutamate were applied for 2 sec at 1 minute intervals following a 2 sec application of 10 μ M domoate. The extent of recovery was then determined with a 2 sec. application of 10 μ M glutamate. For the traces shown, the peak response 5 min after domoate(right trace) was 52% of the initial peak response to glutamate (left trace), prior to domoate exposure. The dashed line indicates the initial baseline, before domoate application.

Figure 2. Domoate does not produce prolonged inhibition or tonic current in GluK1- or GluK2-containing receptors that lack the GluK5 subunit

A. Homomeric GluK1 and GluK2 receptors and heteromeric GluK2/K4 receptors were transiently expressed in HEK-293T cells. Agonists were applied for 2 sec (solid line) at 1 min intervals to cells voltage-clamped at -70 mV. For all subunit combinations the traces shown were obtained from the same cell. The response to 10 μ M domoate for GluK1 and GluK2/K4 receptors was much larger than the response to glutamate. The domoate-activated current for these isoforms is therefore presented on a different scale than the glutamate-activated currents. The dashed line represents the baseline prior to domoate exposure. The glutamate concentration was sub-maximal ($\sim EC_{50-70}$) for each receptor isoform.

B. Current responses to glutamate at 1 minute (black trace) or 5 minutes (gray trace) after domoate exposure are shown on an expanded time scale with current amplitudes normalized to one another to clarify desensitization properties. As the GluK1 receptor currents were not inhibited by domoate, these traces are shown on the same amplitude scale. GluK2/K4 currents were also normalized to the same baseline, as a small tonic current was present at 1 minute post-domoate.

C. The amplitude of the response to glutamate was divided by the average response to the first three agonist applications and was measured using the baseline from the first agonist treatment. 10 μ M domoate was applied for 2 sec following three applications of glutamate, as indicated by the solid bar and arrow. Symbols represent mean $\pm$ SEM for responses to glutamate (n=4 for GluK1 and GluK2/K4; n=5 for GluK2). GluK2/K5 data is duplicated from Figure 1C.

Figure 3. Inhibition of the GluK5 subunit by domoate does not prevent channel activation by agonists acting through the GluK2 subunit

A. Heteromeric GluK2/K5 receptors were transiently expressed in HEK-293T cells and agonists were applied for 2 sec (solid line) at 1 min intervals to cells voltage-clamped at -70 mV. The dashed line represents the baseline prior to domoate exposure. Time points indicate time from domoate application.

B. The peak response to glutamate, AMPA or kainate was normalized to the average amplitude of the first three agonist applications (dashed line). The peak amplitude was measured using the baseline prior to exposure to domoate. 10 μ M domoate was applied for 2 sec following three applications of agonist, as indicated by the solid bar and arrow. 10 μ M glutamate data is duplicated from Figure 1C.

C. The peak response to agonist was measured at 5 min. after domoate exposure and normalized to the response before domoate as described in B. Bars represent mean $\pm$ SEM with the number of cells indicated by the number in parentheses. *** indicates a significant difference ($p < 0.001$) compared to the results from GluK2 using ANOVA and Tukey-Kramer multiple comparisons tests.

Figure 4. The tonic current produced by domoate exposure is not increased by CNQX
Representative current traces from heteromeric GluK2/K5 receptors expressed as previously described. 100 μ M CNQX was applied for 2 sec (solid line) to cells 1 minute after application of 10 μ M domoate. The dashed line indicates the original baseline prior to domoate. The current trace in response to CNQX is shown on the same scale as the response to domoate as well as at an expanded scale to clarify the effect on current amplitude.

Figure 5. Effect of binding site mutations on sensitivity to activation by domoate and glutamate

A. Representative current traces from HEK-293T cells transfected with the indicated subunits in response to 2 sec applications of 10 μ M domoate.

B. Concentration-response relationships for domoate were constructed by measuring the peak current amplitude and normalizing this to the maximal peak response to a saturating concentration of domoate for each cell. Symbols represent mean $\pm$ SEM and lines show the fit of a four-parameter logistic equation to the averaged data. For GluK2(E738D)-containing receptors, 1 mM domoate was not sufficient to produce a maximal response. Therefore, the peak response used to normalize the data was estimated for each cell by fitting the raw amplitude data and constraining the Hill number to that of the fit for GluK2 or GluK2/K5 wild-type receptors.

C. Concentration-response relationships for glutamate were constructed as described in B.

Figure 6. Mutation of the agonist binding site in GluK5, but not GluK2, prevents prolonged inhibition by domoate

A. Representative current traces from heteromeric receptors containing mutated subunits as indicated. Agonists were applied for 2 sec at 1 min intervals. Glutamate concentrations were submaximal for each isoform (EC_{60-70}). The dashed line represents the baseline prior to domoate exposure.

B. The peak response to glutamate was normalized to the average amplitude of the first three glutamate applications and was measured using the baseline from the first agonist treatment. 10 μM domoate was applied for 2 sec following three applications of agonist, as indicated by the solid bar and arrow. Symbols represent mean $\pm$ SEM for responses to 10 μM glutamate ($\text{GluK2}_{\text{E738D}}/\text{K5}$, n=5, filled squares) or 300 μM glutamate ($\text{GluK2}/\text{K5}_{\text{E722D}}$, n=5, open squares). Wild-type $\text{GluK2}/\text{K5}$ data is duplicated from Figure 1C.