0026-895X/08/7404-1163–1169$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 74:1163–1169, 2008

Vol. 74, No. 4
48819/3392263
Printed in U.S.A.

Mutations to the Kainate Receptor Subunit GluR6 Binding Pocket That Selectively Affect Domoate Binding[S]

Yihong Zhang,[1] Naushaba Nayeem, and Tim Green

Department of Pharmacology, School of Biomedical Sciences, University of Liverpool, Ashton Street, Liverpool, United Kingdom

Received May 16, 2008; accepted July 28, 2008

ABSTRACT

Kainate receptor responses to domoate are characterized by large steady-state currents and slow deactivation kinetics. To improve our understanding of these responses, we mutated residues at the mouth of the agonist binding pocket of GluR6 using whole-cell electrophysiology to characterize the effects of the mutants. We identified two residues where mutations had significant ligand-specific effects. One, Met691, forms a hydrogen bond that seems to facilitate domoate binding by affecting the main-chain conformation. We found that mutation of Met691 to alanine significantly attenuated responses to domoate but had no effect on responses to glutamate, confirming the importance of this main-chain interaction in GluR6. The second residue, Val685, is located at the mouth of the binding pocket, adjacent to the domoate side-arm. Mutation of Val685 to glutamine increased the rate of decay from steady-state responses to domoate by more than 50-fold but had no effect on the rate or extent of desensitization or on the kinetics of responses to either glutamate or kainate. The V685Q mutant also significantly reduced the potencies of both glutamate (peak) and domoate (peak and steady-state). Empirical analysis using a basic kinetic model indicated that the V685Q phenotype could be fully explained by faster ligand dissociation. The V685Q mutant accelerated receptor deactivation without affecting either desensitization or gating, making it a potentially useful tool for further dissection of ligand binding and gating in kainate receptors.

Ionotropic glutamate receptors (iGluRs) selective for kainate (KA) and AMPA (collectively termed non-NMDA receptors) are activated by a wide range of ligands in addition to their endogenous agonist glutamate (Glu). These agonists share a similar binding mode but can vary greatly in their subunit selectivity, affinity, potency, and efficacy. They bind a conserved domain in iGluRs formed from two segments (S1 and S2) that flank the pore domain. This S1S2 domain can be recombinantly expressed as a soluble domain (Kuusinen et al., 1995), which has enabled the determination of structures for both AMPA (Armstrong et al., 1998) and KA subunits (Mayer, 2005; Nanao et al., 2005; Naur et al., 2005). In both subtypes, the S1S2 domain has a bilobed clamshell structure, with the ligand binding site located in the cleft between lobes (Fig. 1). Extensive studies, particularly in the AMPA-selective subunit GluR2, have shown that ligands activate the receptor by inducing the closure of the S1S2 domain clamshell, with the degree of domain closure related to agonist efficacy (Armstrong and Gouaux, 2000; Jin et al., 2003).

Domoate (Dom), a neurotoxin associated with amnesiac shellfish poisoning, is a high-potency partial agonist at both native (Lerma et al., 1993) and recombinant (Sommer et al., 1992; Köhler et al., 1993) KA-selective iGluRs. In radioligand binding assays, it exhibits an affinity constant of 2 nM at the GluR5 subunit (Lomeli et al., 1992), and 9 to 11 nM at the GluR6 subunit (Lomeli et al., 1992; Swanson et al., 1997). At both subunits, Dom elicits desensitizing responses with significant steady-state currents (Sommer et al., 1992; Köhler et al., 1993). This contrasts with responses to either Glu or KA, where steady-state currents are much smaller (GluR5) or virtually absent (GluR6) (e.g., see Swanson et al., 1997). Dom is structurally similar to KA but is distinguished by a longer side-arm with a terminal carboxyl group, projecting from position 4 of the pyrrole ring. Dom binds to GluR6 in an orientation essentially equivalent to that of Glu and KA, with its side-arm pointing out of the binding cleft (Mayer, 2005; Nanao et al., 2005). The degree of cleft closure is less than for the Glu complex, consistent with the action of Dom as a partial agonist.

This work was supported by the UK Medical Research Council.

[1] Current affiliation: Department of Physiology, University of Bristol, University Walk, Bristol, United Kingdom.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.108.048819.

[S] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: iGluR, ionotropic glutamate receptor; Dom, domoate; KA, kainate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA, *N*-methyl-D-aspartate; HEK, human embryonic kidney; WT, wild type.

The only binding-site contact specific to Dom is a hydrogen bond to the main-chain NH of Tyr488. However, there is also a hydrogen bond from the Met691 sulfur to the main-chain NH of Asp687, which seems to bend the main-chain conformation compared with GluR2. This moves it away from the Dom side-arm (Nanao et al., 2005). Other GluR5 and GluR6 structures have a similar conformation (Supplementary Fig. 1). In addition to these direct and indirect interactions, there are two further side chains at the mouth of the binding pocket, Ala490 and Val685, that are close to the side-arm of Dom. Alanine 490 is located at the top of the binding cleft in lobe I (Fig. 1) and is conserved in all non-NMDA subunits with the exception of KA1 (where it is a valine). It has not been the subject of previous mutagenesis studies in iGluR subunits. Valine 685 is located at the lower front rim of the binding cleft in lobe II and is conserved in iGluR subunits as either a valine, isoleucine, or leucine. In GluR6, mutation of this residue to leucine, the amino acid found in AMPA subunits, slows desensitization of Glu and KA responses (Fleck et al., 2003) while switching the homologous residues in GluR5 and KA2 affects agonist affinity (Sanders et al., 2006). In AMPA-selective subunits, mutations to this site variously affect KA affinity in GluR1 (Mano et al., 1996), and agonist efficacy in GluR2 and GluR4 (Armstrong et al., 2003; Madden et al., 2004), whereas in NMDA subunits, mutations to the homologous residues in NR1, NR2A, and NR2B reduce agonist potency (Kuryatov et al., 1994; Laube et al., 1997; Anson et al., 1998; Lummis et al., 2002).

In this study we have investigated the binding of Dom to GluR6 by introducing mutations to Ala490, Val685, and Met691. Mutations to the first two sites were introduced to test whether receptor activation by Dom could be selectively blocked by increasing the size of these side chains. Met691 was mutated to determine whether the displaced main-chain conformation observed in the GluR6 S1S2-Dom structure (Nanao et al., 2005) was required for activation of GluR6 by Dom. In all cases, electrophysiological characterization of Glu, KA, and Dom current response kinetics was used to assess the effects of GluR6 mutants expressed in HEK cells.

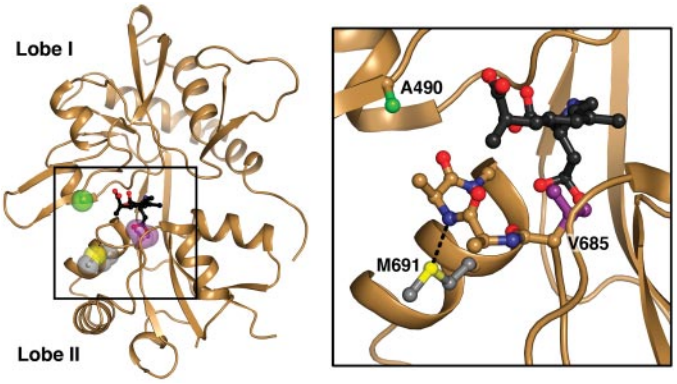

Fig. 1. Location of mutated residues within the GluR6 ligand binding cleft. The GluR6 S1S2 domain is shown in cartoon representation (left, main chain in gold; Dom in black). Side chains are shown for the three residues mutated in this study: Ala490 (green), Val685 (magenta), and Met691 (gray; sulfur in yellow). Right, the boxed region around the ligand is shown enlarged. The main chain and β carbons of residues Glu686 to Gly688 are included to show the hydrogen bond between the Met691 sulfur and the Asp687 main chain nitrogen (dashed line). Atomic coordinates are from the GluR6 S1S2-Dom complex, protomer B (protein data bank accession no. 1YAE; Nanao et al., 2005).

Materials and Methods

Mutagenesis. Mutagenesis was carried out on a rat GluR6 cDNA clone carrying a glutamine codon at the Gln/Arg editing site. Residue numbering is for the full-length subunit. Mutants were generated using the QuikChange protocol and *Pfu* turbo polymerase (Stratagene, La Jolla, CA) (Zhang et al., 2006). All mutants were confirmed either by sequencing of the entire open reading frame, or by sequencing after subcloning of the 1.65-kb XagI-Eco47III fragment. Structural hypotheses and figures were generated from the coordinates of the GluR6 S1S2-Dom structure (Protein Data Bank accession no. 1YAE) (Nanao et al., 2005) using MacPyMOL (http://pymol.sourceforge.net/).

Cell Culture and Electrophysiology. HEK-293 cells (European Collection of Cell Cultures, Porton Down, UK) were maintained at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum and penicillin/streptomycin (Sigma-Aldrich, Poole, UK). Cells were transiently transfected with cDNA plasmids encoding GluR6 and mutants thereof, using either calcium phosphate (for biochemical assays) or Lipofectamine 2000 (for electrophysiology; Invitrogen, Paisley, UK). Whole-cell recordings were carried out essentially as described previously (Zhang et al., 2006). In brief, recordings were made 12 to 72 h after transfection at a holding potential of −70 mV using a HEKA Elektronic EPC 10 amplifier. The electrode solution contained 110 mM CsF, 30 mM CsCl, 4 mM NaCl, 0.5 mM $CaCl_2$, 10 mM HEPES, and 5 mM EGTA (adjusted to pH 7.3 with CsOH). The external bath solution contained 150 mM NaCl, 2.8 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 10 mM HEPES (adjusted to pH 7.3 with NaOH). Rapid agonist application was achieved by using a Burleigh LSS-3200 piezo, driving movement of a theta tube pulled to an outside diameter of 300 μm. The rate of solution exchange determined by open-tip junction currents was <250 μs. Recordings were performed on small-diameter cells (20 μm), lifted into the perfusion stream. Both KA and Dom were purchased from Tocris Bioscience (Bristol, UK).

Data Analysis and Modeling. Dom dose-response curves were determined using concentrations up to 1 mM for GluR6 wild-type (WT) and 3 mM for GluR6 V685Q. For Glu dose-response curves, concentrations up to 10 mM were used for both mutants. Analysis of channel kinetics was carried out using PulseFit and AxoGraph (Molecular Devices, Sunnyvale, CA). Kinetic modeling to investigate the effects of the V685Q mutant was carried out using Monte Carlo simulations and numerical integration in ChannelLab software (Synaptosoft, Decatur, GA). Kinetic models were based initially on that described in Bowie et al. (1998) (incorporated in the ChannelLab software). Further details of the modeling are given in Supplemental information.

Protein Characterization. Radioligand binding assays were performed using [vinylidene-^{3}H]kainic acid as described previously (Swanson et al., 1997; Zhang et al., 2006). [^{3}H]Kainate (specific activity, 47–58 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Beaconsfield, UK). In addition to separation by filtration, centrifugation, and precipitation (with polyethylene glycol 3000) were used to attempt to identify lower-affinity binding sites. Binding assays were performed on membranes prepared from both transiently and stably transfected HEK-293 cells, as well as soluble GluR6 S1S2 expressed in BL21(DE3) bacterial cells using a construct carrying the V685Q mutation.

Results

The partial agonist Dom activates recombinant GluR6 receptors with responses characterized by moderately fast desensitization, a high steady-state current, and slow deactivation kinetics (Swanson et al., 1997). There are, however, relatively few studies describing recombinant kainate receptor responses to Dom, so we began by measuring the currents

evoked by Dom in HEK cells transiently transfected with GluR6 WT. Recordings were made using whole-cell patch clamp and a rapid-perfusion system, with Dom (100 μM) applied for 1 or 4 s. As expected, we observed partially desensitizing currents with significant steady-state responses (16.2% relative to peak responses) and lower efficacy than either Glu or KA (response sizes were 0.39 relative to peak Glu responses) (Table 1). Desensitization rates (τ_{Des}) were best fitted with a double exponential and were around 5-fold slower than desensitization from responses to Glu (Table 1). The rate of current decay from the steady-state (τ_{Dec}SS), which was on the order of seconds, was also best fitted to a double exponential (Table 2).

We then determined the effects on GluR6 receptor currents of mutations to three sites (Met691, Ala490 and Val685), selected for their proximity to the side-arm of Dom (Fig. 1). The first of these, Met691, forms a hydrogen bond that seems to allow the adoption of a specific main-chain conformation to accommodate the long side-arm of Dom (Fig. 1, Supplemental Fig. 1) (Nanao et al., 2005). Current responses to Glu, KA, and Dom were determined for receptors in which this site was mutated to either alanine (M691A) or lysine (M691K), comparing these to GluR6 WT responses (Fig. 2A). For these and the other mutants described here, we measured four principal response characteristics: the rate and extent of desensitization (for all three ligands), the peak current size relative to Glu (relative efficacy; for KA and Dom), and the rate of deactivation from steady-state (Dom only).

By these criteria, the currents for both Met691 mutants in response to Glu and KA were similar to those of GluR6 WT (Fig. 2A and Table 1). The only significant effect was a reduction in the relative efficacy of KA at M691A ($p < 0.05$ unpaired t test). For responses to Dom, in contrast, both mutations had significant effects. Steady-state responses were significantly smaller than GluR6 WT, while the relative efficacy of Dom at the M691A mutant was also reduced (Fig. 2A and Table 1). These mutants, and particularly M691A, therefore attenuated responses to Dom relative to responses to Glu and KA. The final parameter tested, the deactivation rate, was accelerated for both M691A and M691K, with the bi-exponential decay observed for GluR6 WT becoming essentially a single exponential decay with a rate equivalent to (for M691A) or faster than (for M691K) the WT τ_1 (Table 2).

The second residue investigated, Ala490, is located ~5Å from the terminal carboxylate group of the Dom side-arm. The Cα-Cβ bond is oriented such that extension of the side chain might affect either the binding of Dom or its ability to activate the receptor (Fig. 1). We therefore introduced two amino acids with extended side chains at this site, methionine (A490M) and arginine (A490R). Neither mutant had any effect on receptor desensitization in response to the three agonists (Fig. 2B and Table 1). Relative ligand efficacies, however, were markedly increased for both mutants (Fig. 2B and Table 1). For the A490R mutant, responses to Dom were the same size as responses to Glu, whereas responses to KA were 50% larger. These changes in relative efficacy could be the result of either increased efficacy of KA and Dom at these mutants or reduced Glu efficacy and/or potency. The decay rate from steady-state Dom currents was also affected by the A490R mutation, being slower than for GluR6 WT (Table 2). The size of the change was not large, and the double exponential fit made direct statistical comparison impractical.

The final site within the S1S2 domain investigated for its potential to affect Dom responses was Val685. This residue has been extensively studied in other iGluR subunits; mutations have a variety of effects on ligand-specific responses. In the GluR6 S1S2-Dom complex, the two Cγ atoms of Val685 are within 3.5 to 4 Å of the side-arm carbon atoms and pyrrole ring methyl-carboxylate group (Nanao et al., 2005). We tested the effects of three mutations to this residue,

TABLE 1
Desensitization rates for mutant GluR6 subunits
Values are given as mean ± S.E.M. for n determinations. For double exponential fits, the contribution of the fast component is indicated as a percentage in brackets.

Mutant	Glu				KA				Dom			
	I_{peak}	τ_{Des}	des	n	$I_{KA/Glu}$	τ_{Des}	des	n	$I_{Dom/Glu}$	τ_{Des}	des	n
	nA	*ms*	*%*			*ms*	*%*			*ms*	*%*	
WT	6.2 ± 0.4	5.3 ± 0.2	99.4 ± 0.2	27	0.70 ± 0.05	4.1 ± 0.3	99.4 ± 0.1	13	0.39 ± 0.04	τ_1 = 26 ± 1.1 (97 ± 0.4%) τ_2 = 550 ± 95	83.8 ± 1.5	23
A490M	5.9 ± 1.8	3.6 ± 0.1	98.9 ± 0.4	5	0.94 ± 0.04*	5.3 ± 0.3	97.8 ± 0.9	5	0.68 ± 0.08*	τ_1 = 26 ± 0.5 (96 ± 1.4%) τ_2 = 250 ± 45	77.4 ± 4.2	5
A490R	3.2 ± 1.2	3.6 ± 0.6	99.7 ± 0.1	4	1.5 ± 0.2*	3.4 ± 0.4	99.6 ± 0.2	3	0.98 ± 0.07*	τ_1 = 21 ± 2.5 (97 ± 0.9%) τ_2 = 190 ± 30	87.8 ± 2.4	5
V685E	0.58 ± 0.25[a]	5.7 ± 0.6	99.9 ± 0.1	4	N.D.[b]	N.D.	98.5 ± 1.5	4	N.R.			4
V685K	1.9 ± 0.5[a]	4.2 ± 0.6	98.4 ± 0.9	3	N.D.[b]	5.3 ± 0.3	99.1 ± 0.7	12	N.R.			8
V685Q	6.0 ± 0.6	4.5 ± 0.2	99.4 ± 0.2	17	0.73 ± 0.03	4.7 ± 0.3	99.0 ± 0.2	13	0.24 ± 0.03*	τ_1 = 50 ± 3.0 (95 ± 0.7%) τ_2 = 1100 ± 300	82.4 ± 1.8	17
M691A	4.4 ± 1.9	4.4 ± 0.6	99.8 ± 0.03	4	0.45* ± 0.1	3.6 ± 0.5	99.9 ± 0.1	4	0.18 ± 0.04*	τ_1 = 15 ± 2.1 (95 ± 1.7%) τ_2 = 450 ± 170	95.4 ± 1.5*	4
M691K	7.2 ± 1.3	5.6 ± 0.6	99.6 ± 0.2	5	0.54 ± 0.07	4.8 ± 0.6	98.5 ± 1.0	4	0.24 ± 0.05	τ_1 = 13 ± 0.9 (95 ± 2.6%) τ_2 = 230 ± 55	94.3 ± 1.5*	4

N.D., not determined; N.R., no response.
* $P < 0.05$, unpaired t-test compared to GluR6 WT.
[a] Glu concentration was 10 mM.
[b] Mean peak responses to 100 μM KA were 0.06 ± 0.04 and 0.3 ± 0.09 nA, respectively.

changing it to glutamate (V685E), lysine (V685K), or glutamine (V685Q). The first two mutants significantly attenuated agonist responses (Fig. 3A and Table 1), with currents to 3 mM Glu averaging 39 ± 8 (n = 14) and 360 ± 80 pA (n = 8) for the V685E and V685K mutants, respectively. Larger responses were observed using 10 mM Glu (Table 1), indicating that this was to some extent the result of reduced Glu potency. Responses to 1 mM KA were also much smaller than those observed with GluR6 WT (4.0 ± 0.6 nA, n = 13), averaging just 58 ± 38 (n = 4) and 630 ± 130 pA (n = 14) for V685E and V685K, respectively. No currents were observed when 100 μM Dom was applied (Table 1). Where it was possible to determine the rate and extent of desensitization for Glu and KA responses, these were essentially unchanged compared with GluR6 WT (Table 1).

The third mutation tested at this site, V685Q, was somewhat more conservative, introducing a neutral glutamine compared with the charged groups in V685E and V685K. This mutation had a much more limited effect on receptor responses to Glu and KA; no changes were observed in the rate or extent of desensitization or the efficacy of KA relative to Glu (Fig. 3A and Table 1). Likewise, the desensitization properties of Dom responses for V685Q were indistinguishable from those of GluR6 WT. The relative efficacy of Dom seemed reduced (Table 1), but the most striking effect of this mutation was to the decay rate from steady-state Dom responses (Fig. 3). The decay rate of V685Q was accelerated more than 50-fold compared with the fast component of τ_{Dec}SS in GluR6 WT, with no slow component evident (Table 2). Comparison of traces for V685Q with GluR6 WT and selected other mutants where the decay rate was changed shows the dramatic nature of this effect (Fig. 3B), particularly given the otherwise relatively minor effects of this mutation.

To determine whether the decay rate from peak responses was also changed in V685Q, we used short applications of all three agonists (Glu and KA, 5 ms; Dom, 10 ms). For Glu and KA responses, the peak decay rates were fast for both GluR6 WT (3.8 ± 0.5 and 5.3 ± 1.1 ms, respectively, n = 5) and V685Q (2.9 ± 0.1 and 3.8 ± 0.3 ms, n = 8). For Dom re-

TABLE 2
Deactivation rates from steady-state for mutant GluR6 subunits in response to Dom.

Mutant	τ_1	τ_1	τ_2	n
	ms	*%*	*s*	
WT	630 ± 70	74 ± 5	2.5 ± 0.2	25
A490M	530 ± 90	54 ± 8	2.1 ± 0.2	5
A490R	980 ± 150	74 ± 4	4.2 ± 0.3	4
V685Q	12.5 ± 1.0	100		17
M691A	735 ± 30	100		2
M691K	190 ± 20	99.6 ± 0.3	1.5 ± 0.2	3

Fast (τ_1) and slow (τ_2) decay exponentials are shown in ms & s respectively, with the contribution of the fast component indicated (%τ_1).

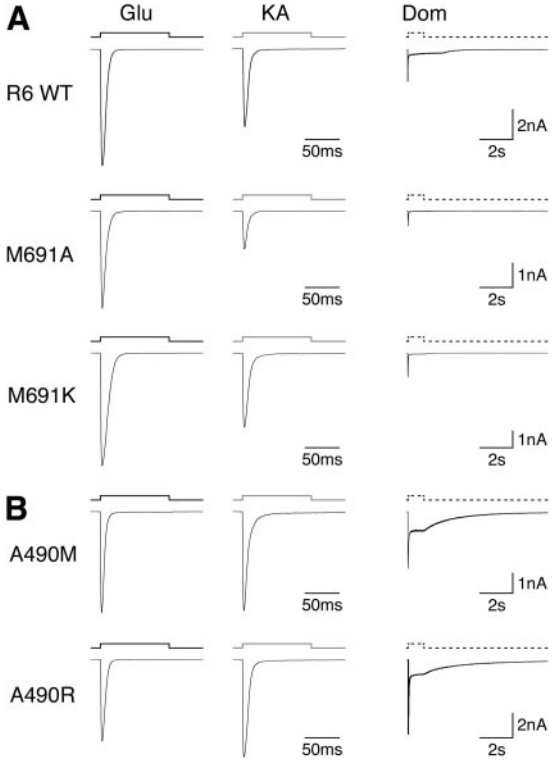

Fig. 2. Effects of mutations to Met691 and Ala490 on GluR6 agonist responses. A, representative responses to applications of Glu (3 mM), KA (1 mM), and Dom (100 μM), recorded from HEK cells expressing GluR6 WT and GluR6 mutants M691A and M691K. Glu and KA were applied for 100 ms (indicated by black and gray lines above traces, respectively), and Dom was applied for 1 s (dotted line above traces). B, representative responses to applications of Glu, KA, and Dom, recorded from cells expressing GluR6 mutants A490M and A490R, presented as in A.

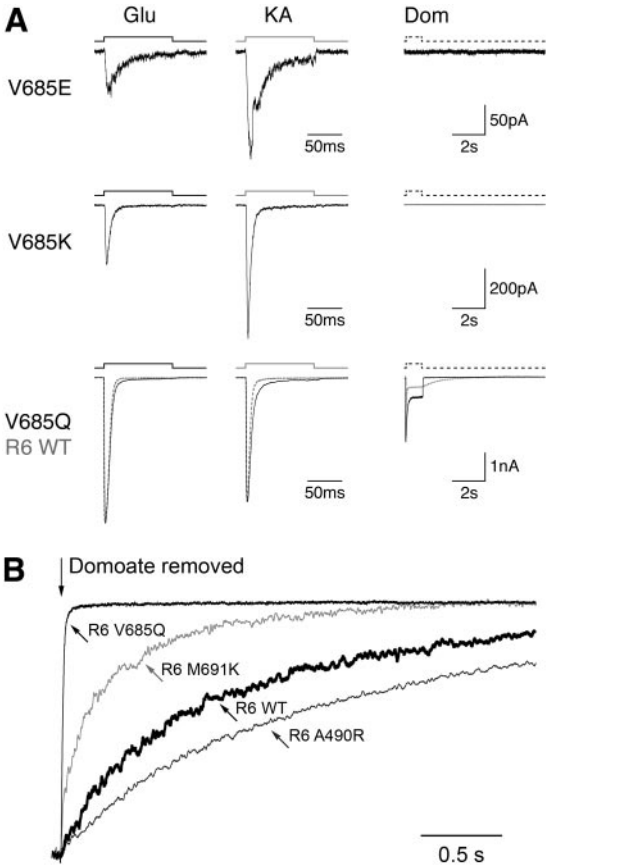

Fig. 3. Dom responses are selectively affected by mutations to Val685. A, representative responses to applications of Glu, KA, and Dom recorded from cells expressing GluR6 mutants V685E, V685K, and V685Q. For comparison, responses from GluR6 WT are superimposed on the traces for V685Q, normalized to the peak responses (gray, dotted). Agonist applications are indicated as in Fig. 2. B, decay from steady-state responses to Dom for GluR6 WT (thick trace) and GluR6 mutants A490R, V685Q, and M691K. Traces are the average of two to three responses, normalized from the point of ligand removal.

sponses, the peak decay rate for V685Q was accelerated compared with GluR6 WT. Responses for the V685Q mutant were fitted to a single-exponential decay constant (τ_{Dec}-Peak = 7.8 ± 0.5 ms, n = 8), compared with the double-exponential (τ_1 = 20 ± 0.9 ms, 96%; τ_2 = 610 ± 100 ms, n = 13) necessary to fit GluR6 WT responses. To ensure that the use of whole-cell recording had not significantly affected our results, we measured the kinetics of GluR6 WT and V685Q responses in excised outside-out patches (Supplemental Fig. 2). Although the rise-times and desensitization rates for Glu and KA responses determined from patches were slightly faster, no difference was observed between desensitization rates of GluR6 WT compared with V685Q. We also observed no significant difference in the kinetic parameters of Dom responses determined by the two methods (see Supplemental information).

The lower relative efficacy of Dom observed for V685Q might be the result of either reduced intrinsic efficacy or reduced relative potency of Dom. We therefore carried out dose-response experiments to determine EC_{50} values for Glu (peak) and Dom (peak and steady state) at both GluR6 WT and V685Q (Fig. 4). For GluR6 WT, the Glu peak EC_{50} was 83 ± 22 μM (n = 8). This was increased 5-fold in the V685Q mutant (to 420 ± 40 μM, n = 10), making 3 mM Glu slightly submaximal. The Dom peak EC_{50} for GluR6 WT was 42 ± 7 μM (n = 4) and was more than 3-fold higher in V685Q (140 ± 30 μM, n = 3). Adjusting for differences in Glu potency, this change is sufficient to completely account for the apparent reduction in relative efficacy of Dom at V685Q. Our measured potency for peak responses to Glu at GluR6 WT was higher than some previous reports that quoted EC_{50} values in the range of 500 to 760 μM (e.g., Paternain et al., 1998; Bowie et al., 2003) but was closer to other published values (i.e., 200–300 mM; Traynelis and Wahl, 1997; Fleck et al., 2003; Li et al., 2003). One study reported EC_{50} values ranging from 60 to 690 μM depending on the ionic strength of the external solution (Bowie, 2002), suggesting that glutamate peak potency is particularly sensitive to variations in buffer conditions. Because our dose-response curves were all determined in parallel, this should not affect the relative difference in Glu peak EC_{50} between GluR6 WT and V685Q identified in this study (Fig. 4A).

For steady-state responses to Dom, the effect on EC_{50} values was even more marked (Fig. 4B), with a 380-fold change for V685Q (170 ± 50 μM, n = 3) compared with GluR6 WT (0.44 ± 0.23 μM, n = 3). As a result, the peak and steady-state EC_{50} values for V685Q responses to Dom were approximately equal. Given these large changes in ligand potencies, we were interested in the effects on ligand binding affinities, determined by saturation binding assay with radiolabeled kainate. However, no binding could be detected in the V685Q mutant using a range of methods and starting materials (see *Materials and Methods*), indicating that its affinity for kainate in this assay was significantly lower than GluR6 WT.

To identify which underlying receptor states were being affected by the V685Q mutation, we carried out empirical kinetic modeling to assess the effects of changing specific rate constants on macroscopic currents. We used a basic GluR6 model described by Bowie et al. (1998), which included a single open-state (biliganded) and two desensitized states (mono- and biliganded). The initial model parameters were modified to change three characteristics to approximate GluR6 WT responses to Dom: the steady-state current, the desensitization rate, and the decay rate from steady-state (see Supplemental information). A detectable steady-state current could be achieved either by increasing the rate of exit from the RA2d biliganded desensitized state (model A), or by assuming that in RA2d, the pore had a fractional conductance (model B). The desensitization rate was slowed by reducing the rate of entry into the RA2d desensitized state, whereas the rate of current decay was slowed by reducing the rate of exit from both biliganded closed states (RA2 and RA2d). These were the only rate constants found to significantly affect decay rates. These changes were all made empirically to generate two models that approximated the response characteristics of Dom responses at GluR6 (see Supplementary Table 1).

To mimic the effect of V685Q on τ_{Dec}SS in these two models, the rates of ligand dissociation from RA2 and RA2d were increased (Supplemental Table 1 and Supplemental Fig. 3). This had no effect on the peak response, the desensitization rate, or the size of the steady-state current. We also determined τ_{Dec}Peak and ligand potency for these models. The modeled peak decay rates were similar to those observed in cells, whereas for ligand potencies, model A provided the closest fit with the observed data (Supplemental Table 1). Although this modeling is subject to a number of significant

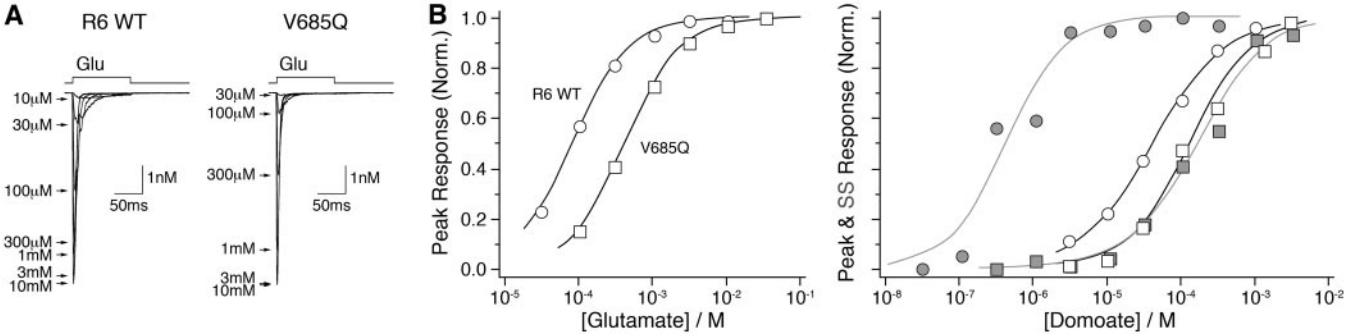

Fig. 4. Effects of V685Q mutation on Glu and Dom potency. A, overlaid traces from Glu dose-response experiment for GluR6 WT (left) and V685Q (right). Maximal peak heights at the different Glu concentrations are indicated by arrows to the left of the traces. For these examples the EC_{50} values were 88 and 382 μM for GluR6 WT and V685Q. B, normalized dose-response curves for GluR6 WT (circles) and V685Q (squares) in response to Glu (left) and Dom (right). White symbols are used for peak response values, and gray symbols for steady-state (SS) response values. Data were fitted to the Hill equation by nonlinear regression (solid black and gray lines for peak and SS, respectively). Data points are averages of multiple normalized determinations (Glu, n = 8 and 10 for R6 WT and V685Q, respectively; Dom, n = 4 and 3, respectively for peak and n = 3 for SS).

caveats (see *Discussion*), it does provide indirect support for the hypothesis that the phenotype of the V685Q mutation is principally the result of an increase in the rate of ligand dissociation.

Discussion

Residue Met691 Indirectly Facilitates Binding of Dom to GluR6. In this study, we sought to identify residues for which mutations would selectively affect GluR6 responses to Dom. We first tested whether the interaction between Met691 and the main-chain nitrogen of Asp687, which seems to result in an altered conformation relative to GluR2 in both GluR5 and GluR6 structures (Supplemental Fig. 1), was required for receptor activation by Dom. We found that this was indeed the case, with significant effects on Dom responses with two mutants (M691A and M691K) lacking hydrogen bonding potential (Fig. 2A and Table 1). The M691A mutation also affected responses to KA, with a reduction in relative efficacy. In both mutants, responses to Glu were indistinguishable from those of GluR6 WT (Table 1). Our data for M691K, where the site is mutated to the residue found in AMPA subunits, were consistent with previous observations that M691K had no effect on Glu or KA responses (Fleck et al., 2003).

Overall, these data support the hypothesis that the interaction between Met691 and Asp687 alters the main-chain conformation, facilitating the binding of Dom. Elimination of this interaction in M691A or M691K selectively attenuated Dom responses, having a more limited effect on responses to KA (which has a shorter side-arm) and no effect on responses to Glu. Assuming the Dom steady-state results from faster exit from the desensitized state (see below), the reduction in steady-state responses in these mutants is consistent with a destabilization of the open, or closed nondesensitized, states with Dom bound, increasing the relative stability of the desensitized state. Explaining the observed differences in ligand efficacy and deactivation rates between the M691A and M691K is more difficult. An indirect effect on the free energy and/or conformation of the Dom complex (for example through the additional positive charge in M691K) seems most likely.

Mutations to GluR6 Ala490 Affect Ligand Efficacy. Alanine 490 is located in lobe I at the mouth of the ligand binding cleft (Fig. 1). The effects of mutating this residue to either methionine or arginine were similar, although the phenotype of the A490R mutant was more pronounced. The relative efficacy of both KA and Dom were increased compared with Glu, whereas other response characteristics were unchanged (Fig. 2B and Table 1). The effect of these mutants was therefore relatively subtle and, if anything, the opposite of that predicted. The observed changes in relative efficacy might reflect real changes in the efficacy of KA and Dom but might also result from reduced Glu efficacy and/or potency. It is not possible to distinguish between these two possibilities from macroscopic recordings, but for A490M, at least, there is no evidence of reduced current amplitudes in response to Glu. Responses to Glu for A490R are smaller than WT, so an effect on Glu cannot be discounted. Some elements of the A490R phenotype (e.g., slower deactivation) might be explained by the positive charge favoring Dom binding through its carboxylate side-arm. This would not, however, help to explain either the effects on KA responses or the A490A phenotype. Overall, it is clear that these mutants did not selectively block Dom binding as intended, and further work will be required to explain their unexpected effects on receptor activation.

Mutation of Val685 to Glutamine Increases Rate of Dom Dissociation from GluR6. Valine 685 is the closest side chain to the carboxylate side-arm of Dom. For two of the mutations tested here, V685E and V685K, we observed a general reduction in response sizes. The potency and/or efficacy of Glu was reduced with both mutations, and responses to KA and Dom were small or absent (Fig. 3A and Table 1). These effects are perhaps unsurprising, given that both mutants introduce an additional charge in the vicinity of the binding pocket. The V685Q mutant, in contrast, which introduces a bulkier but uncharged side chain, had very specific effects. In terms of kinetics, only responses to Dom were affected, with peak and steady-state decay rates increased (Fig. 3A and Table 1). The V685Q mutation also affected ligand potency, reducing the peak EC_{50} of both Glu and Dom, and the steady-state EC_{50} of Dom (Fig. 4), as well as reducing KA affinity below the limit of detection by radioligand binding. Because glutamate is isosteric with glutamine, it might be expected that V685E would have an equivalent (or greater) effect on Dom dissociation rates than V685Q. In the absence of Dom responses with V685E this cannot be directly assessed. However, a larger effect on ligand dissociation by V685E (through destabilization of binding by the negative charge) would also be consistent with complete attenuation of Dom responses.

In general terms, the V685Q phenotype seemed consistent with our original hypothesis that mutating this residue would interfere with Dom binding. To investigate this further, we explored the effects of changing kinetic parameters in a basic KA receptor model. Kinetic modeling of KA and AMPA subunits has been described by a number of groups (e.g., Heckmann et al., 1996; Partin et al., 1996; Bowie et al., 1998; Bowie and Lange, 2002; Li et al., 2003), and the model we used is clearly a simplification of the true situation. At a minimum, there are additional desensitized/closed states (Bowie and Lange, 2002; Robert and Howe, 2003) and alternative open states corresponding to subconductance levels (Swanson et al., 1996), differential activation of which has been shown to underlie partial agonism in AMPA receptors (Jin et al., 2003). The question is therefore to what extent these additional elements might affect our interpretation, were they included in the model. In terms of the additional desensitized states, these underlie complex kinetics with multiple time-constants, but at least in the case of the V685Q mutant, single exponential decay rates indicate that a simpler model is sufficient. In addition, for our purposes, the size of the average open-state conductance for the partial agonist Dom relative to that of full-agonist responses should not affect the analysis. Overall, we therefore believe the basic model we have used serves as a useful approximation of the core events in KA receptor activation and was sufficient to explore potential underlying causes of the V685Q phenotype.

Two points of interest arose from our qualitative modeling: one general, and one related to the effects of V685Q. First, the steady-state currents observed with Dom are best explained by faster exit from the biliganded desensitized state (i.e., faster "resensitization"). We cannot, however, rule out other possibilities, including fractional currents in desensitized states. Faster resensitization has been proposed to underlie the steady-state responses observed in GluR2 Thr686

mutants (Robert et al., 2005). If this is also the case in GluR6, it implies that the desensitized state is less stable with Dom bound than with either Glu or KA bound. This is consistent with the observations of Fay and Bowie (2006), who found that the conformational difference between closed and desensitized states in GluR6 was less when Dom was bound compared with Glu.

The modeling also suggested a possible explanation for the phenotype of V685Q. The faster current decay observed in V685Q can be explained by faster ligand dissociation from closed and desensitized states. In our model, faster ligand dissociation increased the decay rate from the steady state and reduced ligand potency. We therefore propose that in both V685Q and GluR6 WT, the rate of receptor deactivation after removal of Dom is dominated by rates of ligand dissociation. This contrasts with GluR2 Thr686 mutants, where resensitization was found to dominate response kinetics (Robert et al., 2005). Whether this represents a real difference between AMPA and kainate receptor gating, relates only to Dom responses, or is simply a feature of these specific mutants will require further investigation.

This last question highlights the general difficulty in assessing whether mutants simply affect receptor kinetics and/or thermodynamics, as is often assumed, or actually alter the conformations of underlying receptor states. In this respect, we believe that the effect of V685Q is primarily restricted to changes in receptor kinetics. Although we initially hypothesized that V685Q would sterically block the Dom side-arm, reduced Glu potency indicates a more general effect on ligand binding and/or gating. This could be occurring through disruption of domain closure, consistent with the proposed role of the homologous site in GluR2 (Leu650). This has been described as a "wedge" in the binding pocket, preventing complete domain closure in the presence of the relatively bulky agonist KA (Holm et al., 2005). Reducing the size of Leu650 and its GluR4 homolog increases the efficacy of the partial agonist KA relative to Glu (Armstrong et al., 2003; Madden et al., 2004). By extension, increasing the size of Val685 in GluR6 may reduce domain closure. However, the reduction in ligand potency at V685Q, without any change in relative efficacy, points instead to an effect on binding, rather than gating. We therefore conclude that the V685Q mutant accelerates GluR6 deactivation through accelerated ligand dissociation, as a result of destabilization of Dom binding.

Acknowledgments

We thank Kate Davis for mutant generation, DNA purification, and HEK cell maintenance, and Max Nanao for initial discussions on mutations with the potential to affect domoate binding selectively. The rat GluR6 cDNA was a gift from Prof. Steve Heinemann (Salk Institute, La Jolla, CA).

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Address correspondence to: Tim Green, Department of Pharmacology, School of Biomedical Sciences, University of Liverpool, Ashton Street, Liverpool L69 3GE, UK. E-mail: tpgreen@liv.ac.uk