Functional identification of Gd^{3+} binding site of metabotropic glutamate receptor 1α

Hideki Abe[a,b,1], Michihiro Tateyama[a,b], Yoshihiro Kubo[a,b,*]

[a] *Department of Physiology and Cell Biology, Tokyo Medical and Dental University, Graduate School and Faculty of Medicine, D-566, 1-5-45 Yushima, Bunkyo, Tokyo 113-8519, Japan*
[b] *CREST, Japan Science and Technology Corporation, Kawaguchi, Saitama 332-0012, Japan*

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Abstract **We previously reported that the metabotropic glutamate receptor1α (mGluR1α) has a sensitivity to extracellular polyvalent cations such as Ca^{2+} and Gd^{3+} as well as glutamate. Gd^{3+} binding site was recently identified by crystal structure analysis at the interface of two subunits including Glu238, but it remains unknown whether this site is functionally involved in the activation of mGluR1α by Gd^{3+} or not. We analyzed the ligand sensitivity of the Glu238Gln mutant, and observed that the sensitivity to extracellular Gd^{3+} was completely lost, while the sensitivity to glutamate and Ca^{2+} was not affected. We also observed that the presence of Gd^{3+} increased the sensitivity of mGluR1α to glutamate, and that this effect was again lost by Glu238Gln mutation. These results suggest that the binding of Gd^{3+} or a related endogenous substance to this site, alone or in cooperation with glutamate binding at a distant site, leads mGluR1α to activation.**

Key words: Metabotropic glutamate receptor; Ca^{2+}; Gd^{3+}; Structure function; Polyamine

1. Introduction

The G protein-coupled metabotropic glutamate receptor1α (mGluR1α) [1,2] is one of eight mGluR subtypes (mGluR1 ~ mGluR8) that have been cloned and classified into three categories. Group I mGluRs (mGluR1 and mGluR5) couple to phospholipase C to activate protein kinase C and promote the release of Ca^{2+} from intracellular stores. There is also substantial evidence that group I mGluRs play critical roles in some forms of synaptic plasticity, including long-term potentiation and long-term depression [3–7].

We previously reported that mGluR1α is activated not only by glutamate but also by extracellular Ca^{2+} ($[Ca^{2+}]_o$) and Gd^{3+} ($[Gd^{3+}]_o$) in *Xenopus* oocytes [8], and in transiently transfected Human embryonic kidney cells (HEK293) [9], although we could not observe responses to $[Gd^{3+}]_o$ in a stably transfected Chinese Hamster Ovary (CHO) cell line to which we applied a high dose (more than 1 mM) of $[Gd^{3+}]_o$ [10,11]. Based on the difference in the sensitivity to $[Ca^{2+}]_o$ between mGluR1 and mGluR2, we identified S166 in the extracellular domain as a site critical for the sensitivity to $[Ca^{2+}]_o$ [8].

The crystal structure of the extracellular domain of mGluR1α was solved by X-ray diffraction analysis, and two glutamate binding pockets were identified in the dimer structure [12] (Fig. 1, blue). It was also shown that mGluR1α has two conformational states, open–open/Resting and closed–open/Active, and the binding of glutamate shifts the equilibrium between the two states to the closed–open/Active state [12]. Here 'open' and 'closed' mean the relative position of the upper and lower lobes of each clam-like subunit. In Fig. 1, the subunit on the left is in the closed state, while that on the right is in the open state [12]. The discrimination of 'Resting' and 'Active' is based on the manner of the contact between the dimer interface at the upper lobe [12].

Binding of Ca^{2+} to mGluR1α was not clearly observed in a crystal at S166 (Fig. 1, red) adjacent to the glutamate binding site (Fig. 1, blue) [12]. A high-affinity binding site for Mg^{2+} and possibly for Ca^{2+} was identified at N118 by crystal structure analysis [12]. However, being located too far from S166, this site was speculated not to be part of the reported site critical for the sensitivity to $[Ca^{2+}]_o$. These results show the possibility that the agonistic effect of $[Ca^{2+}]_o$, with a K_d value larger than 2 mM, is not due to a high-affinity binding to S166, but due to a sort of loose interaction here.

On the other hand, the binding of $[Gd^{3+}]_o$ to mGluR1α was clearly observed in a crystal grown in the presence of 0.5 mM Gd^{3+} [13]. It located at the dimer interface where negatively charged amino acids including E238 are clustered (Fig. 1, green, E238 in cyan), and the site was distant from the binding pocket for glutamate (Fig. 1, blue). In the presence of both glutamate and Gd^{3+}, mGluR1α was shown to take the third conformational state, closed–closed/Active [13], a state different from that caused by glutamate alone. In spite of these structural observations, it remains unknown whether the $[Gd^{3+}]_o$ binding site actually plays a functional role in the activation of mGluR1α by $[Gd^{3+}]_o$.

In this study we aimed to functionally identify the Gd^{3+} binding site (E238) and compared the sensitivities of mGluR1α wild type (w.t.) and E238GlnQ as well as S166D mutants to glutamate, $[Ca^{2+}]_o$ and $[Gd^{3+}]_o$. We also analyzed

*Corresponding author. Fax: (81)-3-5803 5156.
E-mail address: ykubo.phy2@tmd.ac.jp (Y. Kubo).

[1] Present address: Wisconsin Regional Primate Research Center, University of Wisconsin, 1223 Capitol Court #222, Madison, WI 53715, USA.

Abbreviations: mGluR1α, metabotropic glutamate receptor1α; $[Ca^{2+}]_o$, extracellular Ca^{2+}; $[Gd^{3+}]_o$, extracellular Gd^{3+}; $[Ca^{2+}]_i$, intracellular Ca^{2+}; w.t., wild type; CSF, cerebro spinal fluid

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the effect of the constant presence of $[Gd^{3+}]_o$ on the sensitivity of mGluR1α to glutamate.

2. Materials and methods

2.1. Molecular biology

Point mutants were made using mutated primers and a QuickChange site-directed mutagenesis kit (Stratagene), and were subcloned into $pCXN_2$ [14] expression vector. The FLAG epitope (DYKDDDDK) was inserted into the N-terminus of mGluR1α between amino acids 57 and 58 by the two-step PCR (polymerase chain reaction)-based mutagenesis method as described previously [15], and then subcloned into $pCXN_2$. The sequence of the constructs was confirmed by DNA sequencing (ABI Prism 310).

HEK293 were transfected with the appropriate expression plasmids using LipofectAMINE PLUS (Invitrogen) according to the manufacturer's instructions. Also cotransfected was pDsRed-N1 (10% of the total DNA) (Clonetech) encoding a fluorescent reporter protein, DsRed. The cells were used between 24 and 48 h after transfection.

2.2. Measurement of intracellular Ca^{2+} ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was measured essentially as described previously [11] with some modifications. Culture medium was mixed with 5 μM Fura-2 AM (Molecular Probe) and 0.01% Cremophor EL (Sigma) by vortexing and by ultrasonic vibration for 15 s, after which transiently transfected cells were incubated in this mixture for 20–40 min at 37°C under 5% CO_2. The cells were then rinsed and incubated at room temperature for up to 1.5 h in basal solution (140 mM NaCl, 4 mM KOH, 10 mM HEPES (pH 7.4)) supplemented with 1 mM $MgCl_2$, 1.5 mM $CaCl_2$ and 10 mM glucose. In some experiments, the coverslip was rinsed with basal solution supplemented with 0.3 mM Mg^{2+} just before the experiments. This rinse was done only for a few minutes to avoid depletion of $[Ca^{2+}]_i$ stores, which would hamper responses. The coverslip was then placed in a recording chamber filled with 200 μl of basal solution supplemented with 0.3 mM Mg^{2+} and 0 mM (Figs. 3B,C and 4) or 1.5 mM $[Ca^{2+}]_o$ (Fig. 3A). To achieve abrupt changes in ligand concentration, 400 μl of 150% concentrated stock solution was applied by pipet. $[Ca^{2+}]_i$ was monitored as a function of the ratio of the 510 nm Fura-2 emission excited by illumination at 340 and 380 nm applied using a Lambda DG-4 light source and filter exchanger (Sutter, Novato, CA, USA). Fura-2 fluorescence was recorded using a MicroMAX 512BFT cooled CCD camera (Roper Scientific, Tucson, AZ, USA) and Metafluor imaging software (Universal Imaging, Downingtown, PA, USA).

2.3. Immunocytochemistry

To analyze surface expression of mGluR1α, live cells expressing FLAG-tagged mGluR1α were first blocked in 5% skim milk in phosphate-buffered saline (PBS) without detergent on ice for 1 h and then incubated with an anti-FLAG monoclonal antibody (clone M2, Sigma; 4 μg ml^{-1}) on ice for 1 h. They were then fixed in 4% paraformaldehyde in PBS on ice for 10 min, rinsed with PBS three times, and blocked in 5% skim milk in PBS without detergent at 37°C for 1 h. They were incubated with Alexa488-conjugated anti-mouse IgG (1:500; Molecular Probes) in 5% skim milk without detergent at 37°C for 1 h. Labeled cells were rinsed and mounted in PermaFluor (Shandon, Pittsburgh, PA, USA). Fluorescent images were captured with a BX-50 microscope (Olympus) equipped with an AxioCam CCD camera unit (Zeiss) and were analyzed with Scion Image software (Scion Corp., Frederick, MD, USA). To compare the expression level of mGluR1α quantitatively, we always carried out staining of three samples in parallel, and used a constant exposure time of 10 s in all cases.

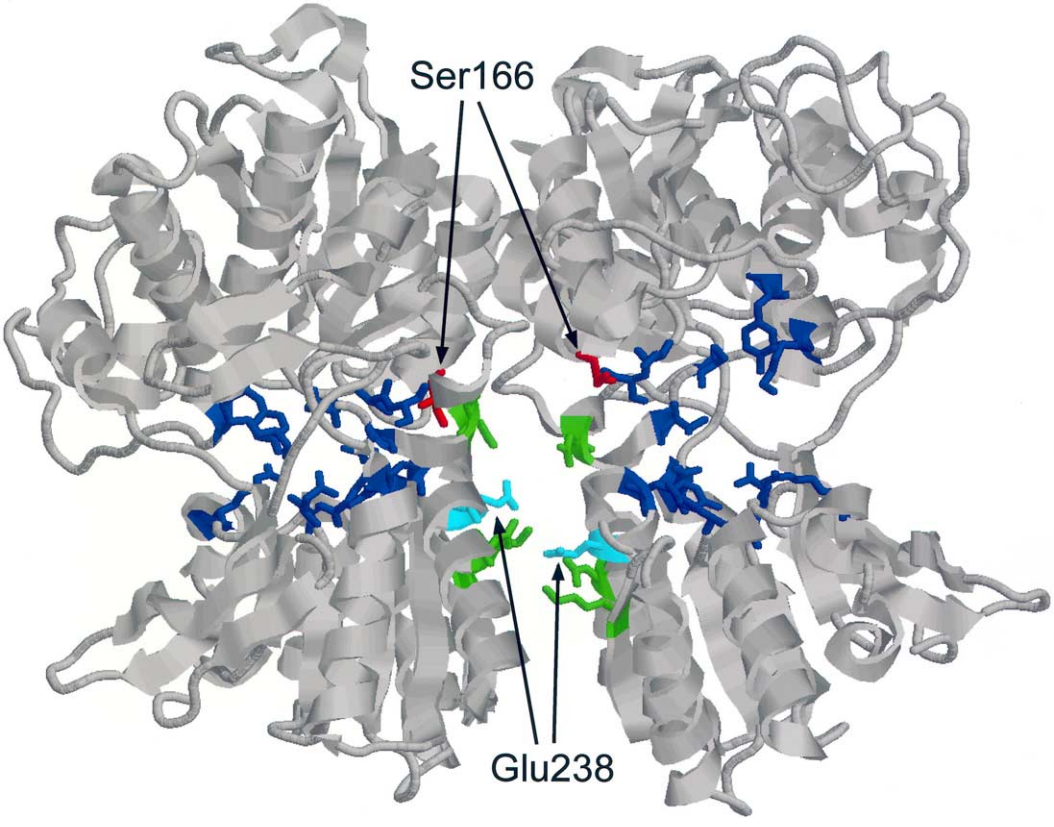

Fig. 1. Structure of the ligand binding domain of mGluR1α. A graphic presentation was created by Rasmol software based on the data #1EWK in the Protein Data Bank [12]. It represents a structure in a closed–open/Active state [12]. The two subunits show a dimeric structure. Amino acid residues reported to be involved in the glutamate binding (Y74, S164, S165, S186, T188, D208, Y236, E292, G293, D318, R323, K409) [12] are marked in blue, and those in $[Gd^{3+}]_o$ binding (D191, D242, K260) [13] are marked in green, except for E238, which is marked in cyan. S166 we identified to have an influence to sensitivity to $[Ca^{2+}]_o$ [8] was marked in red. E238 and S166 which were mutated in this study are marked by arrows.

2.4. Statistical analysis

Data are presented as means ± S.E. Means were compared using unpaired *t*-tests unless specified otherwise. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Cell surface expression of mGluR1α

The difference in responses between cells transfected with mGluR1α w.t. and those with mutants could be due to the difference in either the expression level or the ligand sensitivity. We first analyzed the surface distribution of FLAG-tagged mGluR1α w.t., S166D and E238Q by performing primary antibody reaction at 4°C without permeabilizing the cells (Fig. 2A–C). In cells expressing mGluR1α w.t., the cell surface receptors appeared as bright punctate spots of fluorescence suggestive of receptor clustering at the plasma membrane (Fig. 2A). S166D and E238Q mutants showed highly similar distributions (Fig. 2B,C). These surface expression levels did not show statistically significant difference (Fig. 2D, 107.3 ± 6.1 A.U. ($n = 23$, w.t.), 93.6 ± 3.8 A.U. ($n = 75$, S166D), 94.7 ± 5.3 A.U. ($n = 44$, E238Q)). Thus, the changes of the ligand-evoked responses of the mutants observed in the following experiments were believed not to be due to the changes of the surface expression level but to the ligand sensing function.

3.2. Comparison of the ligand sensitivities of mGluR1α w.t., S166D and E238Q

The responses of mGluR1α were monitored optically as increases in $[Ca^{2+}]_i$ in HEK293 transfectants loaded with the fluorescent Ca^{2+} indicator Fura-2. Examples of the responses of mGluR1α w.t., S166D and E238Q to 10 μM glutamate in the presence of 1.5 mM $[Ca^{2+}]_o$ are shown (Fig. 3A), and their dose–response relationships were compared (Fig. 3B). The sensitivity to glutamate was not at all changed by E238Q mutation, while it was reduced by S166D mutation as reported previously [9,11]. The effect of S166D mutation to glutamate sensitivity was less clear in the absence of $[Ca^{2+}]_o$ [8,11].

An abrupt increase in $[Ca^{2+}]_o$ from 0 to 10 mM also evoked transient increase in $[Ca^{2+}]_i$ in HEK293 cells expressing mGluR1α w.t. (Fig. 3C). These responses reached their peaks with a time course which was similar to that of the responses to 100 μM glutamate but significantly faster than that of the $[Ca^{2+}]_i$ transients in mock transfected cells probably due to influx of Ca^{2+}, as we reported previously [9]. Thus, $[Ca^{2+}]_o$-evoked changes in $[Ca^{2+}]_i$ were not initiated by the Ca^{2+} influx but by metabotropic reactions. We assumed $[Ca^{2+}]_i$ transients that reached their peak within 50 s after changing $[Ca^{2+}]_o$ to be $[Ca^{2+}]_o$-evoked responses mediated by mGluR1α. S166D mutant did not show clear evoked-responses to 10 mM $[Ca^{2+}]_o$ (Fig. 3C), suggesting much reduced sensitivity, while E238Q mutant showed clear responses (Fig. 3C). The sensitivity of E238Q to $[Ca^{2+}]_o$ was at a similar level to w.t., as shown in the plots of the dose–response relationship (Fig. 3D).

Recordings of the responses to 0.1 mM $[Gd^{3+}]_o$ are shown in Fig. 3E. The dose–response relationship of w.t. for $[Gd^{3+}]_o$ was different from those for glutamate and $[Ca^{2+}]_o$, in that it

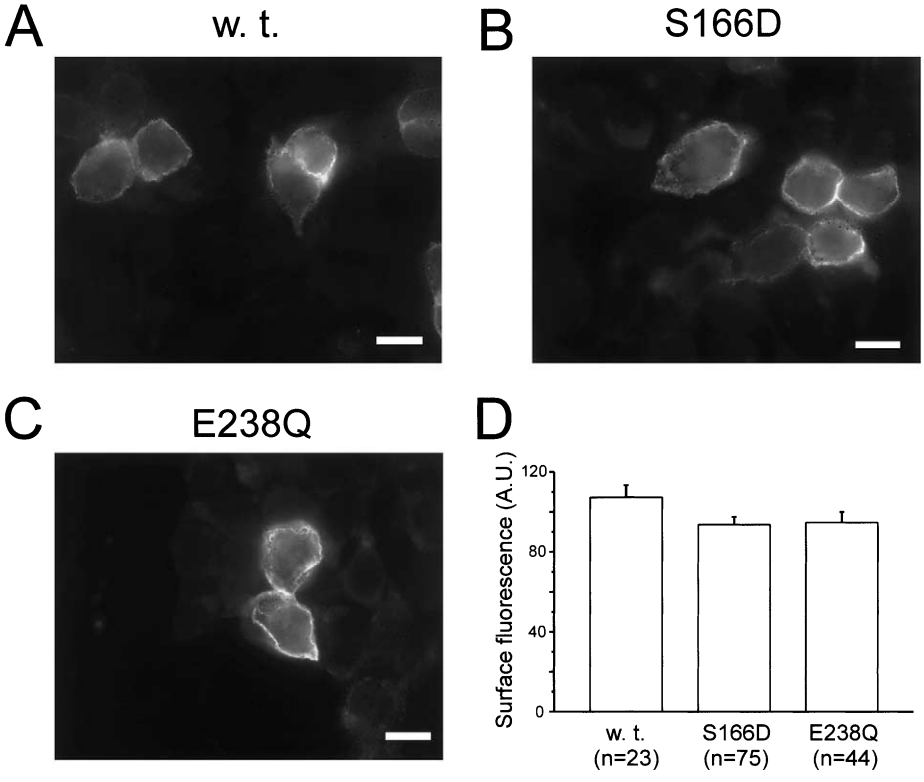

Fig. 2. Effects of S166D or E238Q mutation on the cell surface expression of mGluR1α. Fluorescence micrographs of HEK293 cells expressing FLAG-tagged mGluR1α w.t. (A), S166D (B), or E238Q (C). The scale bars indicate 10 μm. D: Quantitative comparison of the surface expression levels of mGluR1α w.t., S166D and E238Q. The average fluorescence intensities in 2×10 μm regions of cell surface were measured. Bars depict means ± S.E.

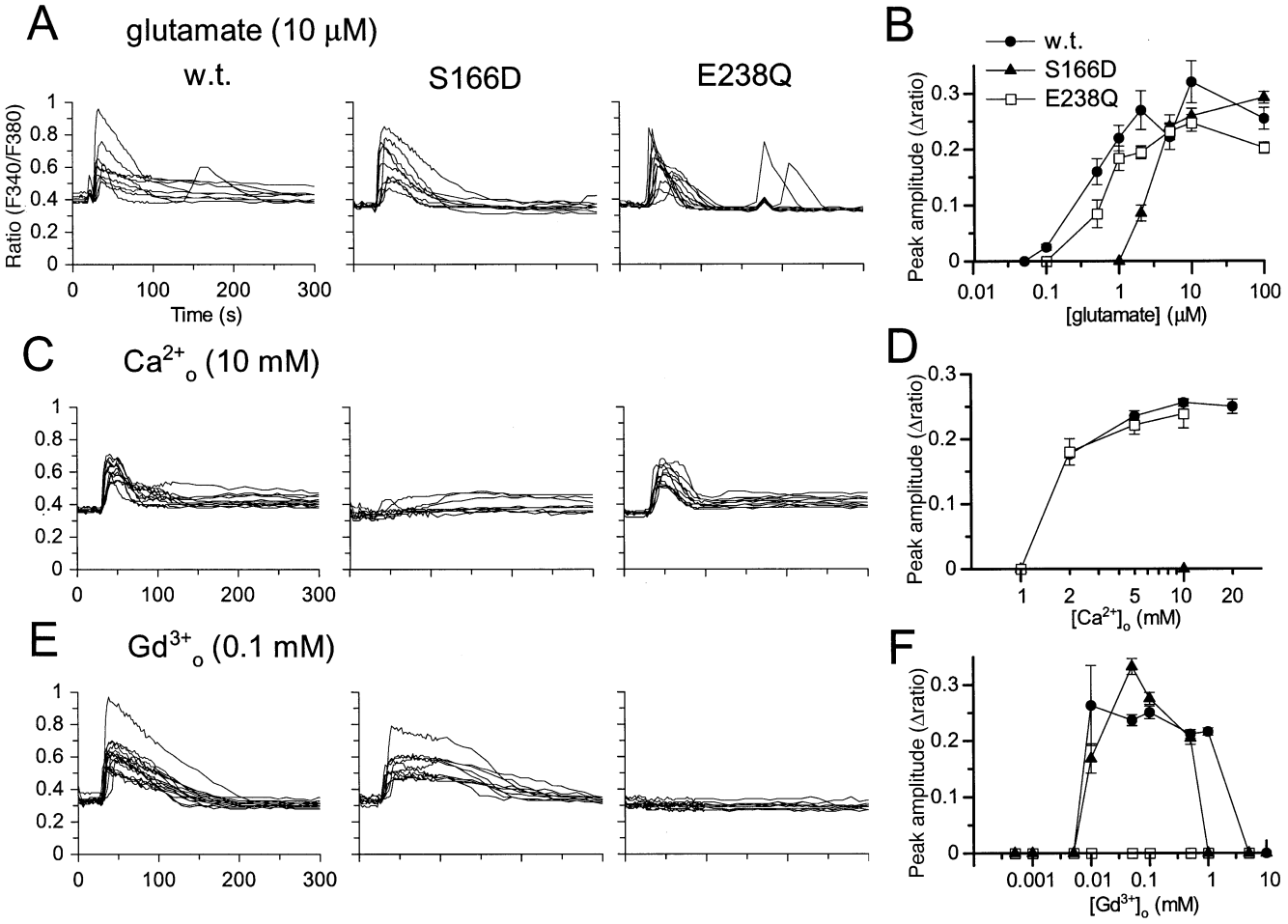

Fig. 3. Ligand-evoked $[Ca^{2+}]_i$ transients mediated by mGluR1α w.t., S166D and E238Q. A: Glutamate-evoked $[Ca^{2+}]_i$ transients. The traces show the transient increases in $[Ca^{2+}]_i$ evoked by 10 μM glutamate monitored as the changes in the ratio of 510 nm Fura-2 emission excited by illumination at 340 and 380 nm; $[Ca^{2+}]_o = 1.5$ mM. Cells expressed mGluR1α w.t. (left), S166D (middle) or E238Q (right). B: Plots of glutamate dose–response relationship for w.t. (filled circles), S166D (filled triangles) and E238Q (open squares). C,D: Same as A and B. Recordings of the response to abrupt increase in $[Ca^{2+}]_o$ from 0 to 10 mM (C) and $[Ca^{2+}]_o$ dose–response relationship (D) are shown. E,F: Same as A and B. Recordings of the responses to 0.1 mM $[Gd^{3+}]_o$ (E) and Gd^{3+} dose–response relationship (F) are shown. $[Ca^{2+}]_o = 0$ mM.

was bell-shaped, i.e. the amplitude of responses decreased at both low and high concentrations (Fig. 3F). As the rate of decay of the Gd^{3+} response was accelerated at a high concentration of the cation [9], we believe that this abrupt loss of effect at high concentration is due to an inactivating (or desensitizing) process taking place prior to activation. S166D mutant showed a bell-shaped dose–response relationship similar to that of w.t., but the sensitivity was completely lost in E238Q. As D191, D242 and K260 (Fig. 1 green) are also located at the $[Gd^{3+}]_o$ binding site [13], we examined the sensitivity of $[Gd^{3+}]_o$ of D191N, D242N and K260Q mutants. D191N and K260Q mutants showed a similar sensitivity to w.t., while that of D242N was slightly decreased (data not shown). These results demonstrate the primary significance of E238 together with D242 in the activation of mGluR1α by $[Gd^{3+}]_o$.

3.3. *Effect of the constant presence of $[Gd^{3+}]_o$ on the response of mGluR1α to glutamate*

In the previous section, it was shown $[Gd^{3+}]_o$ alone has an agonistic action and it is lost in E238Q mutant. If $[Gd^{3+}]_o$ acts as an agonist by changing the equilibrium of the two conformations by binding to negatively charged amino acid(s) at the interface of two subunits, $[Gd^{3+}]_o$ could potentiate the effect of glutamate on the activation which binds at a distant site (Fig. 1, blue). We, therefore, investigated the effect of $[Gd^{3+}]_o$ on glutamate-evoked responses. In Fig. 4A, responses to 100 μM glutamate in the presence of various $[Gd^{3+}]_o$ was analyzed. $[Gd^{3+}]_o$ was basally included in the bath in this experiment, and glutamate was applied without changing $[Gd^{3+}]_o$ after the responses triggered by $[Gd^{3+}]_o$ subsided. The responses of mGluR1α w.t. to glutamate were significantly increased by low concentrations (e.g. 10 μM) of $[Gd^{3+}]_o$, and this effect was not observed in E238Q mutant. In the presence of high concentrations of $[Gd^{3+}]_o$, glutamate responses disappeared completely. It is possible that the effect of high concentrations of $[Gd^{3+}]_o$ to eliminate responses to $[Gd^{3+}]_o$ described above also suppressed responses to glutamate. However, this suppressive effect of $[Gd^{3+}]_o$ was not affected by E238Q mutation (Fig. 4A), suggesting that the structural background for it is different from E238.

Next, we compared the glutamate dose–response relationship in the presence and absence of 0.01 mM $[Gd^{3+}]_o$ (Fig. 4B). It was clear that the presence of 0.01 mM $[Gd^{3+}]_o$ sig-

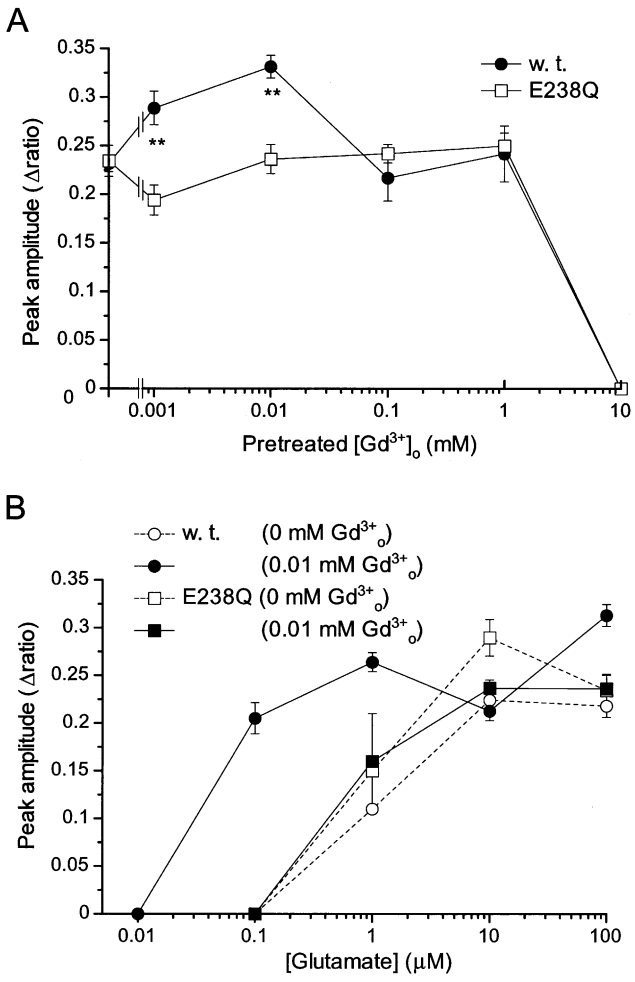

Fig. 4. Effect of the presence of $[Gd^{3+}]_o$ on the responses to glutamate. A: Amplitudes of the responses to 100 μM glutamate in the presence of various concentrations of $[Gd^{3+}]_o$. ** at 1 μM and 10 μM indicates significant difference ($P < 0.01$). B: Comparison of the glutamate dose–response relationship of mGluR1α w.t. (circles) and E238Q (squares) in the absence (open symbols) and presence (filled symbols) of 0.01 mM $[Gd^{3+}]_o$.

nificantly increased the sensitivity of mGluR1α to glutamate, while this effect was not observed in E238Q.

4. Discussion

We previously identified S166 of mGluR1α as a site critical for $[Ca^{2+}]_o$ sensing function [8], and a region including E238 was recently reported to be a $[Gd^{3+}]_o$ binding site by crystal structure analysis [13]. A $[Gd^{3+}]_o$ binding site different from that for glutamate is also reported on ionotropic kainate receptors [16]. In the present study we compared sensitivities of mGluR1α w.t., S166D and E238Q to glutamate, $[Ca^{2+}]_o$ and $[Gd^{3+}]_o$, and obtained the results as follows. (1) S166D mutation abolished responses to $[Ca^{2+}]_o$ (Fig. 3D) and decreased sensitivity to glutamate (Fig. 3B). (2) E238Q mutation abolished responses to $[Gd^{3+}]_o$ (Fig. 3F) without changing sensitivities to glutamate (Fig. 3B) and $[Ca^{2+}]_o$ (Fig. 3D). (3) $[Gd^{3+}]_o$ potentiated glutamate-evoked responses (Fig. 4), and this potentiating effect was lost completely by E238Q mutation (Fig. 4). (4) A high dose of $[Gd^{3+}]_o$ could not evoke responses and also suppressed responses to glutamate completely. This suppressive effect on glutamate-evoked responses was not affected by E238Q mutation.

What is the structural background for the potentiation effect of $[Gd^{3+}]_o$ on glutamate response, and its suppressive effect at high dose? We discuss three points in the following by combining our data with those of the crystal structure analysis [12,13]. (1) *Potentiation of glutamate response by $[Gd^{3+}]_o$*. The $[Gd^{3+}]_o$ binding site identified by crystal structure analysis is located at the dimer interface where negatively charged amino acids including E238 and D242 are clustered [13]. We speculate that binding of $[Gd^{3+}]_o$ neutralizes negative charges at the dimer interface, facilitating the approach of the interface at the lower lobes, and make the receptor enter a potentiated 'Active' state different from that caused by glutamate alone. The state could be closed–closed/Active proposed by Tsuchiya et al. in the presence of both glutamate and $[Gd^{3+}]_o$ [13]. (2) *Activation by $[Gd^{3+}]_o$ alone*. We demonstrated that $[Gd^{3+}]_o$ alone can activate mGluR1α, and E238 plays a critical role in this step. The results suggest a possibility that mGluR1α can take the conformation of open–open/Active. $[Gd^{3+}]_o$ might cause the approach of the dimer interface of the lower lobes, even if both of the two subunits are in the open state. Kunishima et al. predicted the existence of the open–open/Active state by model building [12]. (3) *Suppressive effect of $[Gd^{3+}]_o$*. $[Gd^{3+}]_o$ also leads mGluR1α to another unidentified inactivated (or desensitized) state, which was apparently observed by applying a high concentration of $[Gd^{3+}]_o$. There could be two possible explanations. (3a) mGluR1α might enter a kind of inactivated state, when firmly stabilized at the closed–closed/(potentiated) Active state by a high concentration of $[Gd^{3+}]_o$ and glutamate. Transitions between the Resting and the Active state might be necessary for the functional signal transduction. (3b) Tsuchiya et al. identified two additional $[Gd^{3+}]_o$ binding sites on the external surface of mGluR1α [13]. Although they discussed that they may not play critical roles for activation of mGluR1α, it is possible that the binding of $[Gd^{3+}]_o$ at these sites could cause suppression of the receptor function. Of these two possibilities, the latter might be more likely when the result is taken into account that E238Q mutation did not affect the suppressive effect.

There is one point with a discrepancy in the results. It is the difference between the effective range of the agonist effect of $[Gd^{3+}]_o$ alone (Fig. 3F) and that of the potentiating effect of $[Gd^{3+}]_o$ to glutamate responses in mGluR1α w.t. (Fig. 4A). This difference could be explained by the difference in the experimental conditions. In Fig. 3F, we abruptly applied Gd^{3+} to the bath and plotted the peak amplitude of $[Gd^{3+}]_o$-evoked responses. In Fig. 4A, Gd^{3+} at various concentrations was basally included in the bath, and we plotted the peak amplitude of glutamate-evoked responses. Thus, it is not surprising that the effective range of $[Gd^{3+}]_o$ differs in these two cases.

It goes without saying that Gd^{3+} does not exist physiologically in cerebro spinal fluid (CSF) at a concentration sufficient to activate mGluR1α. What then is the physiological role of the $[Gd^{3+}]_o$ binding site? One extreme and negative possibility is that the $[Gd^{3+}]_o$ binding site does not have any physiological significance at all, i.e. all positive results, the presence of the binding site in the crystal and the recorded responses, were observed just by chance. The second possibility is that the $[Gd^{3+}]_o$ binding of mGluR1α has a role to

detect toxic exogenous substances, i.e. heavy metals. The third possibility is that there is an endogenous extracellular substance X which mimics the action of $[Gd^{3+}]_o$. The substance X may regulate the sensitivity of mGluR1α to glutamate by a similar potentiating effect to that of $[Gd^{3+}]_o$.

An attractive and challenging question is what the substance X is. It would be positively charged, and small enough to fit in the dimer interface. One obvious candidate may be $[Ca^{2+}]_o$, as discussed by Tsuchiya et al. [13]. However, we showed that the agonistic effect of $[Ca^{2+}]_o$ alone was not affected by E238Q mutation (Fig. 3B), suggesting that E238 is not involved in the agonistic action of $[Ca^{2+}]_o$ alone. A possibility still remains that a potentiating effect of $[Ca^{2+}]_o$ on the glutamate-evoked response [11] is mediated by the binding of $[Ca^{2+}]_o$ to E238Q. To examine this possibility, we compared the dose–response relationships of glutamate in the presence and absence of $[Ca^{2+}]_o$. An enhancing effect by $[Ca^{2+}]_o$ on the sensitivity to glutamate was observed in mGluR1α w.t. and similarly in the E238Q mutant (data not shown). These results suggest that the enhancing effect of $[Ca^{2+}]_o$ on the glutamate-evoked response is not mediated by E238, either. As the second candidate for the substance X, we speculated polyamines such as spermine, spermidine and putrescine. These are long thin molecules with four, three or two positive charges, respectively. Although polyamines are more abundant in the cytoplasm, they is also present in CSF [17]. For these reasons, the shape, polyvalent positive charges of the molecules and existence in CSF, we decided to examine if polyamines have an agonistic effect and/or a potentiating effect on glutamate responses. However, none of them activated mGluR1α when applied alone, nor showed clear effects to enhance glutamate sensitivity (data not shown). Therefore, a physiological endogenous substance which interacts with mGluR1α at E238 still remains to be identified.

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