During prolonged application of glutamate (20 min), patterns of increase in intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]i) were studied in HEK-293 cells expressing metabotropic glutamate receptor, mGluR1α or mGluR5α. Stimulation of mGluR1α induced an increase in [Ca$^{2+}$]i that consisted of an initial transient peak with a subsequent steady plateau or an oscillatory increase in [Ca$^{2+}$]i. The transient phase was largely attributed to Ca$^{2+}$ mobilization from the intracellular Ca$^{2+}$ stores, but the sustained phase was solely due to Ca$^{2+}$ influx through the mGluR1α receptor-operated Ca$^{2+}$ channel. Prolonged stimulation of mGluR5α continuously induced [Ca$^{2+}$]i oscillations through mobilization of Ca$^{2+}$ from the intracellular Ca$^{2+}$ stores. Studies on mutant receptors of mGluR1α and mGluR5α revealed that the coupling mechanism in the sustained phase of Ca$^{2+}$ response is determined by oscillatory/non-oscillatory patterns of the initial Ca$^{2+}$ response but not by the receptor identity. In mGluR1α-expressing cells, activation of protein kinase C selectively desensitized the pathway for intracellular Ca$^{2+}$ mobilization, but the mGluR1α-operated Ca$^{2+}$ channel remained active. In mGluR5α-expressing cells, phosphorylation of mGluR5α by protein kinase C, which accounts for the mechanism of mGluR5α-controlled [Ca$^{2+}$]i oscillations, might prevent desensitization and result in constant oscillatory mobilization of Ca$^{2+}$ from intracellular Ca$^{2+}$ stores. Our results provide a novel concept in which oscillatory/non-oscillatory mobilizations of Ca$^{2+}$ induce different coupling mechanisms during prolonged stimulation of mGluRs.

Ca$^{2+}$ can transduce many diverse cellular processes. Such diversity may be achieved by different amplitude and distinct spatial and temporal patterns of Ca$^{2+}$ response (1). In B lymphocytes, the amplitude and duration of Ca$^{2+}$ signaling controls differential activation of pro-inflammatory transcriptional regulators (2). In differentiating neurons, the frequency of [Ca$^{2+}$]i oscillations affects expression of specific neuronal phenotypes such as channel maturation and neurotransmitter expression (3). Compartmentalization of Ca$^{2+}$ signaling is also important in different cellular processes. For example, cytosolic Ca$^{2+}$ signals activate c-fos gene transcription through the serum response element, but nuclear Ca$^{2+}$ signals activate it through cyclic AMP response element (4).

Stimulation of two metabotropic glutamate receptor subtypes, mGluR1α and mGluR5α, triggers the release of Ca$^{2+}$ from the intracellular stores through inositol 1,4,5-trisphosphate (InsP3)$^3$ formation (InsP3/Ca$^{2+}$ pathway) (5–8). We recently reported that transient application (1–60 s) of glutamate induces single-peaked intracellular Ca$^{2+}$ mobilization in mGluR1α-transfected cells but elicits [Ca$^{2+}$]i oscillations in mGluR5α-transfected cells (9). The response patterns of the [Ca$^{2+}$]i increase depend upon the identity of a single amino acid, aspartate (at position 854) or threonine (at position 840), located within the G-protein-interacting domains of mGluR1α and mGluR5α, respectively. Phosphorylation of threonine (840) of mGluR5α by PKC interferes with the signal transduction through mGluR5α. We hypothesized that repetitive phosphorylation and dephosphorylation of mGluR5α could induce [Ca$^{2+}$]i oscillations by signaling on and off. In mGluR1α, non-phosphorylation at aspartate (854) produces a non-oscillatory and PKC activator-resistant Ca$^{2+}$ response (9). This previous study provides the first evidence that an agonist can produce oscillatory/non-oscillatory patterns of Ca$^{2+}$ response by stimulating different receptor subtypes. However, it remained uncertain whether and how these two mGluRs control different cellular processes depending on their oscillatory/non-oscillatory Ca$^{2+}$ responses.

We report here that prolonged stimulation of mGluR1α induced an increase in [Ca$^{2+}$]i that consisted of an initial transient peak and a subsequent steady plateau or an oscillatory increase in [Ca$^{2+}$]i. The transient phase was largely attributed to Ca$^{2+}$ release from intracellular Ca$^{2+}$ stores, but the sustained phase was solely due to Ca$^{2+}$ influx through a mGluR1α receptor-operated Ca$^{2+}$ channel. On the other hand, prolonged stimulation of mGluR5α continuously induced [Ca$^{2+}$]i oscillations through mobilization of Ca$^{2+}$ from the intracellular Ca$^{2+}$ stores. The coupling mechanism in the sustained phase of Ca$^{2+}$ response is determined by oscillatory/non-oscillatory patterns of the initial Ca$^{2+}$ response but not by the receptor identity. Thus, during prolonged stimulation of mGluRs, oscillatory/non-oscillatory patterns of Ca$^{2+}$ response lead to different coupling mechanisms in Ca$^{2+}$ signaling.

**MATERIALS AND METHODS**

Fura-2 acetoxyethyl ester (Fura-2/AM) and phorbol-12-myristate-13-acetate (PMA) were from Wako Pure Chemical Industries. SK&F96365 and nimodipine were from Funakoshi. The mGluR1α antagonist, 1α-(4-phenyl)carbamoyl-1α,7α-dihydroxy-7H-hydroxymimo- cyclopentaprol[b]chomeren (10) was synthesized in our laboratory.

For construction of the mutant receptors, mGluR1α(T) and mGluR5α(D), aspartate (854) of mGluR1α and threonine (840) of mGluR5α were changed into threonine and aspartate, respectively, as described previously (9). The cDNA encoding rat mGluR1α, mGluR5α,

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1 The abbreviations used are: InsP3, inositol 1,4,5-trisphosphate; PKC, protein kinase C; Fura-2/AM, Fura-2 acetoxyethyl ester; PMA, phorbol 12-myristate 13-acetate; mGluR, metabotropic glutamate receptor.
or a mutant receptor was inserted into the eukaryotic expression vector, pEF-BOS. After transfection of the above plasmids, HEK-293 cells expressing mGluR1α, mGluR5α, or a mutant receptor were selected with 400 μg/ml geneticin and isolated by a single cloning step (9). These cells were loaded for 45 min with Fura-2/AM (6 μM) dissolved in balanced salt solution containing 135 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.9 mM MgCl₂, and 10 mM HEPES (pH 7.4). After incubation, the coverslips were mounted in a laminar flow chamber with a flow rate of 2 ml/min at 32 °C. The chamber was mounted on a Nikon inverted stage microscope. [Ca²⁺], measurement was started at 15 min after superfusion of balanced salt solution. Light from a Xenon lamp was filtered through either of two different band-pass filters (340 nm or 380 nm) in the excitation path and conducted to the specimen on the microscope stage through a diachronic mirror. The excitation wavelength was constantly switched between 340 nm and 380 nm. The fluorescence emitted from the cells was passed through a band-pass filter (510 nm). The video images were obtained using an intensified charge-coupled camera. Output from the camera was digitized and stored by a computerized imaging system (Hamamatsu, Argus 50). Ratios of sequential 340/380-nm excitation image pairs were compared with a standard curve for free Ca²⁺ constructed from shallow solutions of known Ca²⁺ and Fura-2 concentration. EGTA (1 mM) was included instead of CaCl₂ in all experiments using a Ca²⁺-free extracellular buffer. The application of reagent or change of external medium is indicated in each graph by a bar or set of bars.

RESULTS AND DISCUSSION

Ca²⁺ Responses in HEK-293 Cells Expressing mGluR1α—In HEK-293 cells expressing mGluR1α, prolonged application of 30 μM glutamate (20 min) induced an initial transient peak response followed by an oscillatory or a steady plateau Ca²⁺ response (Fig. 1, a and b). 44% of cells showed [Ca²⁺], oscillations, 39% showed steady plateau, [Ca²⁺], oscillations observed in the sustained phase of mGluR1α stimulation are characterized by base-line spikes of relatively constant amplitude. A minor population of cells showed three types of responses; a single transient peak response, an initial transient peak with a subsequent response best described as spike plateau, and an initial peak slowly descending to the base line followed by a base-line spiking type of [Ca²⁺], oscillations (data not shown). The transient peak of Ca²⁺ response in mGluR1α-expressing cells was largely attributed to Ca²⁺ mobilization from the intracellular Ca²⁺ stores, because Ca²⁺ response was only slightly reduced in the absence of external Ca²⁺ (Fig. 5a). In contrast, [Ca²⁺], oscillations during the sustained phase in mGluR1α-expressing cells were completely abolished in the absence of external Ca²⁺ (Fig. 1c). The oscillatory Ca²⁺ response in the sustained phase was blocked by 30 μM SK&F96365, a receptor-operated Ca²⁺ channel blocker (11) but not by 10 μM nifedipine, a voltage-gated Ca²⁺ channel blocker (12) (Fig. 2, a and b). Steady plateau Ca²⁺ response and other types of responses in the sustained phase of mGluR1α stimulation were also fully dependent on extracellular Ca²⁺, because Ca²⁺ response was only slightly reduced in the absence of external Ca²⁺ (Fig. 5a). In mGluR1α-expressing cells, in the absence of external Ca²⁺, 100 μM carbamol could still mobilize Ca²⁺ from intracellular Ca²⁺ stores during prolonged application of 30 μM glutamate (Fig. 3), indicating that during prolonged stimulation of mGluR1α, the InsP₃/Ca²⁺ pathway is turned off before Ca²⁺ stores are depleted. These results show that during prolonged stimulation of mGluR1α, the InsP₃/Ca²⁺-pathway is selectively desensitized, and Ca²⁺ entry from SK&F96365-sensitive Ca²⁺ channel solely contributes to the [Ca²⁺], increase in the sustained phase of mGluR1α stimulation.

[Ca²⁺], Oscillations in HEK-293 Cells Expressing mGluR1α

Fig. 1. Ca²⁺ responses elicited by prolonged application (20 min) of glutamate in HEK-293 cells expressing mGluR1α. Two patterns of Ca²⁺ response observed by prolonged application (20 min) of 30 μM glutamate (Glu) consisted of an initial transient peak followed by an oscillatory in 44% of cells (a) or a steady plateau increase in [Ca²⁺], in 39% of cells (b). [Ca²⁺], oscillations in the sustained phase of mGluR1α-stimulation were abolished in the absence of external Ca²⁺ (Ca²⁺-free, 1 mM EGTA) (c). Representative traces are presented.

or mGluR5α—Prolonged application of glutamate (20 min) constantly elicited sinusoidal [Ca²⁺], oscillations in cells expressing mGluR5α (Fig. 4a). In the late phase of stimulation, these [Ca²⁺], oscillations were not abolished in the absence of external Ca²⁺ (Fig. 4b). Thus, prolonged stimulation of mGluR5α continuously mobilizes Ca²⁺ from intracellular stores. In mGluR5α-expressing cells, the frequency of oscillations was lowered by the removal of external Ca²⁺. Thus, a source of extracellular Ca²⁺ is also involved in the generation of [Ca²⁺], oscillations, which is in good agreement with the notion that
the lack of Ca$^{2+}$ influx lengthens the time required for the refilling of intracellular Ca$^{2+}$ stores (13–15).

It has been proposed that [Ca$^{2+}$], oscillations can be classified into two types, base-line spiking and sinusoidal oscillations, and can be driven by several different mechanisms (15–17). From this point of view, [Ca$^{2+}$] oscillations seen in mGluR1α-expressing and mGluR5α-expressing cells are clearly distinguished between base-line spiking and sinusoidal behavior. The mechanisms underlying these two [Ca$^{2+}$] oscillations are also distinct because [Ca$^{2+}$], oscillations in mGluR1α-expressing cells are fully dependent on external Ca$^{2+}$, but those in mGluR5α-expressing cells occur mainly through mobilization of Ca$^{2+}$ from the intracellular stores.

The Effect of PKC Activator on Ca$^{2+}$ Responses in HEK-293 Cells Expressing mGluR1α—In mGluR1α-expressing cells,
treatment with 100 nM PMA (18) reduced but did not abolish the Ca$^{2+}$ response induced by transient application (20 s) of 30 μM glutamate (Glu) was slightly reduced by removing external Ca$^{2+}$-free, 1 mM EGTA (a). 100 nM PMA reduced but not abolished Ca$^{2+}$ response elicited by transient application (20 s) of glutamate (b). This PMA-resistant Ca$^{2+}$ response was abolished in the absence of external Ca$^{2+}$ (c). The number of cells analyzed were 21 (a), 8 (b), and 16 (c). The data are the mean ± S.E. (vertical bars) at each time point.

FIG. 5. The effect of PKC activator on Ca$^{2+}$ responses elicited by transient application of glutamate (Glu) in the absence or presence of external Ca$^{2+}$ in HEK-293 cells expressing mGluR1α. Ca$^{2+}$ response elicited by transient application (20 s) of 30 μM glutamate (Glu) was slightly reduced by removing external Ca$^{2+}$-free, 1 mM EGTA (a). 100 nM PMA reduced but not abolished Ca$^{2+}$ response elicited by transient application (20 s) of glutamate (b). This PMA-resistant Ca$^{2+}$ response was abolished in the absence of external Ca$^{2+}$ (c). The number of cells analyzed were 21 (a), 8 (b), and 16 (c). The data are the mean ± S.E. (vertical bars) at each time point.

FIG. 6. Ca$^{2+}$ responses elicited by prolonged application (20 min) of glutamate in HEK-293 cells expressing mutant mGluRs. In cells expressing mGluR1α(T), prolonged application of 30 μM glutamate (Glu) constantly elicited [Ca$^{2+}$]i oscillations, which were not abolished in the absence of external Ca$^{2+}$-free, 1 mM EGTA (a). In cells expressing mGluR5a(D), prolonged application of 30 μM glutamate induced an initial transient peak response followed by a steady plateau in 20% of cells (b) or an oscillatory Ca$^{2+}$ response in 63% of cells (c). The sustained phase of Ca$^{2+}$ responses in mGluR5a(D)-expressing cells were abolished in the absence of external Ca$^{2+}$ (b and c). Representative traces are presented.
expressing mGluR5α(D), prolonged application of 30 μM glutamate (20 min) induced an initial transient peak response followed by a steady plateau or an oscillatory Ca²⁺ response (Fig. 6, b and c), both of which are identical with those in mGluR1α-expressing cells. 20% of cells showed [Ca²⁺]ᵢ oscillations, 63% showed steady plateau. The sustained phase of Ca²⁺ response in mGluR5α(D)-expressing cells was abolished in the absence of external Ca²⁺ (Fig. 6, b and c). These results indicate that the coupling mechanism in the sustained phase of Ca²⁺ response is determined by oscillatory/non-oscillatory patterns of the initial Ca²⁺ response but not by the receptor identity.

In this study, we found that mGluR1α and mGluR5α show distinct coupling mechanisms during prolonged stimulation by glutamate (20 min). In the initial phase of stimulation, both mGluR1α and mGluR5α trigger the release of Ca²⁺ from the intracellular stores through InsP₃/Ca²⁺ pathway. In the sustained phase of stimulation, in mGluR1α-expressing cells, InsP₃/Ca²⁺-pathway is desensitized, and Ca²⁺ entry solely contributes to [Ca²⁺]ᵢ increase. In contrast, in mGluR5α-expressing cells, InsP₃/Ca²⁺-pathway is not desensitized, and Ca²⁺ is mobilized continuously from the intracellular Ca²⁺ stores during the prolonged stimulation. In mGluR5α-expressing cells, Ca²⁺ entry is also involved in the generation of [Ca²⁺]ᵢ oscillations, because the frequency of oscillations is lowered by the removal of external Ca²⁺. At present, it is unclear whether or not the Ca²⁺-permeable channels, which are activated during stimulation of mGluR1α or mGluR5α, are the same. However, the coupling mechanisms during prolonged stimulation of these two mGluRs are clearly distinct in that mGluR5α continuously couples to InsP₃/Ca²⁺ pathway, but mGluR1α does not.

The studies on mutant receptors of mGluR1α and mGluR5α demonstrate that desensitization of the InsP₃/Ca²⁺ pathway occurs when the initial Ca²⁺ response is non-oscillatory. In contrast, the sinusoidal [Ca²⁺]ᵢ oscillations seen in mGluR5α- or mGluR1α(T)-expressing cells prevent the InsP₃/Ca²⁺ pathway from desensitization during prolonged stimulation of these receptors. The precise mechanism by which the sinusoidal [Ca²⁺]ᵢ oscillations avoid InsP₃/Ca²⁺ pathway desensitization is unclear; however, our earlier study of mGluR5α-controlled [Ca²⁺]ᵢ oscillations may give a hint (9). In that study, we showed that PKC inhibitors eliminate [Ca²⁺]ᵢ oscillations and convert Ca²⁺ response from an oscillatory to non-oscillatory pattern in mGluR5α-expressing cells. In contrast, the PKC activator, PMA, abolishes the Ca²⁺ response in mGluR5α-expressing cells (9). We suggested that phosphorylation of mGluR5α by PKC inactivates mGluR5α, thus resulting in the decrease of [Ca²⁺]ᵢ, whereas subsequent dephosphorylation of mGluR5α restores the signal transduction through mGluR5α, thus regenerating the [Ca²⁺]ᵢ increase. We had then proposed that repetitive cycles of phosphorylation and dephosphorylation of mGluR5α generate [Ca²⁺]ᵢ oscillations (9). Although not proven by experiments, continuous cycling of phosphorylation/dephosphorylation of mGluR5α may be provided by oscillations in the activity of PKC. During mGluR5α stimulation, not only [Ca²⁺]ᵢ, but also the cellular level of diacylglycerol, the other bifurcating limb of phosphoinositide pathway (19), would oscillate. It is conceivable that PKC activity that is known to be affected by Ca²⁺ and diacylglycerol would also oscillate (20). In the present study, we found that PKC is responsible for desensitization of InsP₃/Ca²⁺ pathway during mGluR1α stimulation. If PKC activity, which is first incremented by mGluR5α stimulation, decreases rapidly before the InsP₃/Ca²⁺ pathway is desensitized, such an oscillating PKC activity would prevent the InsP₃/Ca²⁺ pathway from desensitization in mGluR5α-expressing cells.

In cultured astrocytes, glutamate induces [Ca²⁺]ᵢ oscillations through mGluR5 (21, 22). Similar to the observations in mGluR5α-expressed HEK-293 cells (9), the PKC activator abolishes Ca²⁺ response in cultured astrocytes (21, 23). Moreover, both PKC inhibitor and PP1/PP2A phosphatase inhibitor convert Ca²⁺ response from an oscillatory to non-oscillatory pattern, suggesting that the same mechanism underlies the generation of [Ca²⁺]ᵢ oscillations in mGluR5-expressed heterologous and native cells (21). Thus, it may also occur in native cells that oscillatory/non-oscillatory mobilizations of Ca²⁺ result in distinct coupling mechanisms during prolonged stimulation of mGluRs. Although it remains to be elucidated whether these different coupling mechanisms would indeed establish diverging cellular processes, our results provide a new insight into Ca²⁺ signaling when long lasting stimuli are evoked in cells.

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