Ca\textsuperscript{2+}/calmodulin-kinase II enhances channel conductance of \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors

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ABSTRACT The ability of central glutamatergic synapses to change their strength in response to the intensity of synaptic input, which occurs, for example, in long-term potentiation (LTP), is thought to provide a cellular basis for memory formation and learning. LTP in the CA1 field of the hippocampus requires activation of Ca\textsuperscript{2+}/calmodulin-kinase II (CaM-KII), which phosphorylates Ser-831 in the GluR1 subunit of the \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionate glutamate receptor (AMPA-R), and this activation/phosphorylation is thought to be a postsynaptic mechanism in LTP. In this study, we have identified a molecular mechanism by which CaM-KII potentiates AMPA-Rs. Coexpression in HEK-293 cells of activated CaM-KII with GluR1 did not affect the glutamate affinity of the receptor, the kinetics of desensitization and recovery, channel rectification, open probability, or gating. Single-channel recordings identified multiple conductance states for GluR1, and coexpression with CaM-KII or a mutation of Ser-831 to Asp increased the contribution of the higher conductance states. These results indicate that CaM-KII can mediate plasticity at glutamatergic synapses by increasing single-channel conductance of existing functional AMPA-Rs or by recruiting new high-conductance-state AMPA-Rs.

Long-term potentiation (LTP) is a prolonged enhancement in synaptic efficacy thought to underlie certain forms of learning and memory, and recent studies have begun to unravel mechanisms that may be involved in this phenomenon (1, 2). It has been shown that LTP in the CA1 field of the hippocampus requires elevation of calcium in the postsynaptic spine and is accompanied by activation of Ca\textsuperscript{2+}/calmodulin-kinase II (CaM-KII; refs. 3–5), which phosphorylates \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole-propionate glutamate receptors (AMPA-Rs; ref. 6). CaM-KII has unusual biochemical properties that make it a unique transducer of postsynaptic signaling (7–9). AMPA-Rs, which are comprised of the GluR1 subunit, potentiates receptor current (13, 14). In this paper, we have investigated the molecular mechanism by which CaM-KII phosphorylation of Ser-831 in GluR1 potentiates its current, and our data show that CaM-KII significantly increases the occurrence of high-conductance states in GluR1 channel activity. Because induction of LTP often results in an increase in single-channel conductance of postsynaptic AMPA-Rs (15), our results indicate one molecular mechanism by which CaM-KII mediates synaptic plasticity.

EXPERIMENTAL METHODS

Experiments were performed on HEK-293 cells expressing GluR1 receptor subunit either alone or with a constitutively active mutant of CaM-KII (His-282 to Arg) as described (6, 13). In vitro mutagenesis of Ser-831 to Asp and Glu in GluR1 was performed by using the Quick change site-directed mutagenesis kit from Stratagene and was checked by sequencing (13). Patch pipettes were filled with (in mM) 160 CsCl, 2 MgCl\textsubscript{2}, 4 Na\textsubscript{2}ATP, 1 EGTA, and 10 Hepes. Extracellular solution contained (in mM) 165 NaCl, 2.5 KCl, 1 MgCl\textsubscript{2}, 2 CaCl\textsubscript{2}, and 5 Hepes. Constitutively active and heat-inactivated (HI) CaM-KII at 0.4 \(\mu\)M was added to the patch pipette where indicated. Series resistance was below 11 M\(\Omega\) and 60–80% compensated. Glutamate was applied to the lifted cells or outside-out patches by a piezo-driven application system. The rate of solution exchange was 1–3 ms (n = 5) and 0.2–0.5 ms (n = 6) for whole-cell and outside-out patches, respectively, determined as the time for a 10–90% change in the current amplitude during application of 30% diluted external solution to the cell or patch. Macroscopic currents were collected at a 0- to 2-kHz bandwidth and digitized at 10 kHz. Kinetic parameters of glutamate-evoked currents were obtained by simultaneously fitting their rising and decaying phases by the sum of two exponentials (pclamps software).

Nonstationary fluctuation analyses (16) were performed for the currents evoked by 70- to 100-ms applications of 10 mM glutamate at time intervals of 4 s. From 36 to 93 currents were collected for each analysis, with allowed run-down below 10%. Single-channel currents were recorded in the cell-attached mode at 0- to 2-kHz bandwidth and were digitized at 20 kHz. GluR1 channel activity was evoked by the inclusion of 10 \(\mu\M\) AMPA in the patch pipette and was recorded at pipette holding potentials of 70 mV or 80 mV. Membrane potential (−35 mV to −51 mV) was measured immediately after cell-attached observations within the first 3 s of the whole-cell configuration. GluR1-channel activity was identified as (i) currents that were present uniquely in the patches with AMPA as the agonist from only those cells having whole-cell current on application of 10 mM glutamate (18 patches from 18 cells) and never observed in cells without the glutamate-evoked

This paper was submitted directly (Track II) to the Proceedings office. Abbreviations: AMPA-R, \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionate glutamate receptor; CaM-KII, Ca\textsuperscript{2+}/calmodulin-kinase II; HI, heat-inactivated; LTP, long-term potentiation.

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RESULTS

Several receptor parameters could be affected by phosphorylation and contribute to the potentiating effect of CaM-KII on AMPA-R current. We examined those parameters in HEK-293 cells expressing GluR1 alone or coexpressed with activated CaM-KII, which greatly enhances phosphorylation of GluR1 at Ser-831 (13, 14). Expressed GluR1 in HEK-293 cells seems to be a valid model, because potentiation of its current by CaM-KII closely mimics potentiation of AMPA-R/synaptic current in cultured hippocampal neurons or hippocampal slices by infused or expressed CaM-KII (6–10). Fig. 1A shows that dose–response curves for glutamate in these two situations were remarkably similar with EC50 and Hill coefficients of about 0.7 mM and 1.1, respectively, either with or without CaM-KII coexpression (Table 1). This result indicated no significant effect of CaM-KII on GluR1 affinity for glutamate and was not a likely mechanism for GluR1 current potentiation in our previous experiments (6, 13) as we used a saturating concentration of glutamate (10 mM).

However, the number of functional receptors (N), channel conductance (γ), channel open probability (Po), and the electrochemical potential through the channel (V − Vrev, where V is the membrane potential and Vrev is the reversal potential for the current) could theoretically contribute to the CaM-KII potentiation, because they determine the AMPA-R channel open probability (19) through their product: I(γNPo(V − Vrev)). Fig. 1B and C shows I–V curves for GluR1 in whole-cell and outside-out patch recording configurations. There were no significant shifts in Vrev in either configuration with or without CaM-KII coexpression (Table 1). Under whole-cell conditions, I–V curves exhibited a strong inward rectification (Fig. 1B Lower) that was reduced dramatically on excision of patches (Fig. 1C Lower), consistent with a voltage-dependent inhibition of AMPA-Rs by endogenous polyamines (18). Release from polyamine block on CaM-KII phosphorylation could be a mechanism for the potentiation of GluR1 currents. Because the rectification index (18) was not affected by coexpression with CaM-KII (Fig. 1D; Table 1), an effect of CaM-KII on polyamine block was unlikely. Ser-831 in GluR1 is the regulatory CaM-KII phosphorylation site (13, 14); therefore, we attempted to mimic the kinase effect by introducing a negative charge through mutation of Ser-831 to glutamate and aspartate (S831E and S831D, respectively). The mutants were functional receptors, and their analyses confirmed the negative result on polyamine block, as both had rectification properties indistinguishable from those of the wild-type GluR1 receptor (Table 1).

Desensitization of GluR1, which occurs within milliseconds (19, 20), could attenuate peak current by decreasing Po. This attenuation was particularly true for whole-cell current, where the rate of agonist delivery was limited by cell geometry. Indeed, the rate of AMPA-R desensitization by glutamate was about 15–30% slower for whole-cell than for outside-out patch recordings (Fig. 2A and B), reflecting perhaps an asynchrony in channel activation and thus in desensitization. We, however, did not observe an acceleration of this rate on saturation of the agonist-binding step at glutamate concentrations above 1 mM (Fig. 2C), indicating the recording of an intrinsic transition to a desensitized state. We used 10 mM glutamate to assess the rate of desensitization. Desensitization, tested either in whole-cell or in outside-out patches, was not affected detectably by CaM-KII coexpression or by the mutations of Ser-831 (Fig. 2 A, B, and D; Table 1). In addition, no changes were found in the rate of recovery from desensitization (Fig. 2 E and F; Table 1). Together, these observations...
indicate that CaM-KII did not modulate receptor desensitization or recovery.

Multiple applications of glutamate to the same patches or cells showed a significant increase in amplitude of current fluctuations on coexpression or infusion of CaM-KII (Fig. 3A and B Left). This result suggested either an increase in single-channel conductance or change in channel $P_o$. We determined these parameters by applying nonstationary variance analysis (16), which confirmed a significant increase in single-channel conductance with perhaps a slight decrease in $P_o$ (Fig. 3A and B Right). It was possible in some outside-out patches (3 of 19 tested) to observe single-channel currents on the tails of macroscopic currents where channel $P_o$ was reduced dramatically by desensitization (Fig. 3C Left). Direct measurements of single-channel tail currents independently confirmed their estimation through variance analyses (Fig. 3C Right). The analyses identified an ≈70% increase in single-channel conductance on coexpression or infusion of activated CaM-KII (Fig. 3D Left; Table 1). In contrast, no significant change in $P_o$ was found (Fig. 3D Right; Table 1). The effect of CaM-KII on channel conductance was not observed by infusion of Hi CaM-KII but was mimicked by the Glu and Asp mutants (Fig. 3D; Table 1). These observations confirmed the critical role of Ser-831 as the regulatory CaM-KII phosphorylation site in GluR1.

We could not directly compare the number of functional GluR1 receptors with or without coexpression of CaM-KII, because independent sets of measurements were used. However, the channel conductance after CaM-KII action was 74 ± 6% greater ($n = 6$) than the conductance in untreated cells that closely matched the increase in whole-cell peak current on infusion of CaM-KII (53 ± 17%; $n = 11$; see refs. 6 and 13). Therefore, it was unlikely that a significant change in the number of receptors contributed to the potentiation of GluR1 current in HEK-293 cells. Previously, we have shown that conditions known to stimulate CaM-KII phosphorylation of AMPA-Rs in hippocampal neurons did not show a detectable increase in their surface expression (21).

To validate our estimation of CaM-KII phosphorylation of GluR1, we generated a series of recombinant GluR1 constructs with Ser-831 phosphorylated or unphosphorylated and coexpressed them with CaM-KII (Fig. 4). The GluR1 channel, similar to GluR4 and GluR2/GluR4 (22), can adopt multiple conductance states with values ranging from 9 pS to 28 pS (Fig. 4A; Table 2). High-conductance states could arise artifactually from the recruitment of new channels with high-conductance states. We attempted to distinguish these molecular mechanisms by directly analyzing single-channel activity. The cell-attached configuration was chosen over excised patches, because we wanted to maintain the cellular environment of the AMPA-Rs and because excised patches exhibited a rapid run-down of channel activity. Single-channel recordings indicated that the GluR1 channel, similar to GluR4 and GluR2/GluR4 (22), can adopt multiple conductance states with values ranging from 9 pS to 28 pS (Fig. 4A; Table 2). High-conductance states could arise artifactually from random superimpositions of single-channel openings from different receptors in the patch. However, chances of such superimpositions were below 0.1% of the total number of events in patches (the total $P_o$ of channel activity in individual patches was below 1%).

<table>
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<tr>
<th>Receptor</th>
<th>EC$_{50}$, µM</th>
<th>$n_H$</th>
<th>$V_{rev}$, mV</th>
<th>$G_{60}/G_{(100)}$, pS</th>
<th>$r_{des}$, ms</th>
<th>$r_{rec}$, ms</th>
<th>$\gamma$,† pS</th>
<th>$P_o$,†</th>
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<td>Cell</td>
<td>750 ± 50</td>
<td>1.1 ± 0.04</td>
<td>8.7 ± 2.2</td>
<td>0.045 ± 0.006</td>
<td>3.1 ± 0.12</td>
<td>98 ± 9</td>
<td>11.5 ± 1.3</td>
<td>0.79 ± 0.05</td>
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<td>9.2 ± 1.6</td>
<td>0.69 ± 0.12</td>
<td>2.5 ± 0.16</td>
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<tr>
<td>Cell</td>
<td>690 ± 140</td>
<td>1.1 ± 0.14</td>
<td>7.8 ± 1.2</td>
<td>0.043 ± 0.004</td>
<td>2.9 ± 0.12</td>
<td>112 ± 11</td>
<td>19.9 ± 1.2**</td>
<td>0.72 ± 0.06</td>
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<td>8.1 ± 2.0</td>
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<tr>
<td>Cell</td>
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<td>8.5 ± 1.5</td>
<td>0.052 ± 0.003</td>
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<td>13.2 ± 1.3</td>
<td>0.83 ± 0.06</td>
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<td>S831E R1</td>
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<tr>
<td>Cell</td>
<td>—</td>
<td>—</td>
<td>6.7 ± 2.7</td>
<td>0.051 ± 0.002</td>
<td>3.1 ± 0.22</td>
<td>127 ± 12</td>
<td>18.7 ± 1.3**</td>
<td>0.64 ± 0.05</td>
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<td>Cell</td>
<td>—</td>
<td>—</td>
<td>7.2 ± 1.6</td>
<td>0.041 ± 0.005</td>
<td>2.9 ± 0.14</td>
<td>122 ± 15</td>
<td>27.5 ± 2.9**</td>
<td>0.62 ± 0.07</td>
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Means ± SEM are shown, followed by the number of measurements (in parentheses). $n_H$, Hill coefficient; $V_{rev}$, reversal potential for the current; $G$, peak conductance at indicated membrane potential; $r_{des}$, time constants of the decays; $r_{rec}$, time constant; $\gamma$, channel conductance; $P_o$, channel open probability. ***, $P < 0.01$.
†Combined statistic of cells and patches for $\gamma$ and $P_o$.
‡Number of cells + number of patches.

Table 1. Effect of CaM-KII (KII) and mutations of Ser831 (S831) to Glu (E) or Asp (D) on parameters of GluR1 (R1) tested as a population.
of two exponentials for the rise and decay shown by the thin line. (the conductance distributions was increased from states. Weighted single-channel conductance calculated from increased frequency of transitions to the higher conductance of increased open times. Rather, it was the result of the GluR1 phosphorylation was not an effect of better resolution increased contribution of the higher conductance states to GluR1 current mediated by CaM-KII (6, 13).

Table 1) and closely match the increase (53%) in macroscopic agreement with our nonstationary fluctuation analysis (Fig. 3; changes in weighted single-channel conductances are in a good about 22 pS for the Ser-831-to-Asp mutant (Table 2). These

FIG. 2. Effect of CaM-KII on the desensitization of GluR1. (A and B) Whole-cell (A) and outside-out patch (B) currents elicited by 10 mM glutamate at −80 mV in cells expressing GluR1 alone (Left) or the Ser-831-to-Glu mutant (Right). The currents were fitted by the sum of two exponentials for the rise and decay shown by the thin line. (C) \( \tau_{\text{des}} \) as a function of glutamate concentration for the whole-cell currents (\( n = 4 \)). (D) \( \tau_{\text{des}} \) for whole-cell (open bars) and outside-out (gray bars) currents for GluR1 expressed alone, for GluR1 coexpressed with CaM-KII, for GluR1 with HI CaM-KII in the patch pipette, and for Ser-831-to-Glu and Ser-831-to-Asp mutants. (E) Time course of recovery from desensitization measured as the amplitude ratio of the second current to the first in pairs of glutamate applications. Points were fitted by exponential (solid line) with the time constant \( \tau_{\text{rec}} \) (Inset) Currents elicited by paired applications of 10 mM glutamate (20-ms pulses) with time intervals 10–1,000 ms after the end of the first application. (F) \( \tau_{\text{rec}} \) for cells with GluR1 alone, for cells with GluR1 and CaM-KII, and for Ser-831-to-Glu and Ser-831-to-Asp mutants.

Fig. 4A Bottom). This gating of open states was not affected detectably by CaM-KII coexpression or the Ser-831-to-Asp mutation (Fig. 4 B and C Bottom; Table 2), suggesting that the increased contribution of the higher conductance states to GluR1 phosphorylation was not an effect of better resolution of increased open times. Rather, it was the result of the increased frequency of transitions to the higher conductance states. Weighted single-channel conductance calculated from the conductance distributions was increased from ϕ=13 pS for GluR1 alone to ϕ=19 pS on coexpression with CaM-KII and about 22 pS for the Ser-831-to-Asp mutant (Table 2). These changes in weighted single-channel conductances are in a good agreement with our nonstationary fluctuation analysis (Fig. 3; Table 1) and closely match the increase (53%) in macroscopic GluR1 current mediated by CaM-KII (6, 13).

DISCUSSION

Our results show that among several GluR1 receptor parameters capable of potentiating peak current amplitude on phosphorylation by CaM-KII, only channel conductance was increased significantly (Tables 1 and 2). Because the magnitude of the change in channel conductance was sufficiently large to account for the CaM-KII-mediated enhancement of GluR1 peak current, it was unlikely that other parameters, such as a change in the number of functional GluR1 receptors with the same conductance properties, also occurred. If additional functional receptors were recruited, either they could preexist with a \( P_o \) of 0, or, if newly inserted, they would have to exhibit high conductance. Modulation of AMPA-R conductance by CaM-KII—and presumably by protein kinase C, because both kinases phosphorylate the same site (13, 14)—is unusual for glutamate-gated channels, because phosphorylation generally regulates other receptor parameters. For example, phosphorylation of N-methyl-d-aspartate receptors modulates their affinity for agonist, desensitization, channel gating, and \( P_o \), but not channel conductance (23, 24). Protein kinase A phosphorylates GluR1 on Ser-845 and GluR6 on Ser-666 and Ser-684 and increases AMPA/kainate receptor responsiveness through modulation of channel gating and \( P_o \) but without reported changes in channel conductance (25–30). Interestingly, 5-HT3 receptor channel conductance is potentiated similarly by protein kinase C through a redistribution of conductance states (31).
During LTP, CA1 hippocampal AMPA-Rs are phosphorylated by CaM-KII on the GluR1 subunit (6); thus, one might expect that LTP would increase AMPA-R channel conductance. Because direct measurement of single-channel currents of postsynaptic AMPA-Rs in CA1 pyramidal neurons is not technically possible, Benke et al. (15) used nonstationary fluctuation analyses to show that induction of LTP often results in an increase (184 ± 20% of control) in single-channel conductance. Our single-channel observations not only provide direct support for the possibility of such regulation of postsynaptic AMPA receptor function, but they indicate the likely molecular mechanism for CaM-KII in the mediation of LTP as well. Particularly, phosphorylation by CaM-KII of the GluR1 subunit of AMPA-Rs increases the occurrence of higher conductance states, which enhances the total current contributing to LTP. Indeed, knockouts enhance LTP (33), and it becomes increasingly important to determine whether knockouts of GluR1 or homologous replacements of GluR1 with Ser-831-to-Ala mutants obviate expression of LTP in the CA1 region of the hippocampus, as suggested by an antisense approach (34).

An increase in AMPA-R channel conductance by CaM-KII phosphorylation may be, however, one of several mechanisms contributing to LTP. Indeed, the phenomenon of “silent” AMPA-R synapses that become functional on LTP induction can be explained better by an increase in the number of functional synaptic AMPA-Rs (35, 36). Infusion of activated CaM-KII into CA1 hippocampal neurons potentiates kainate-evoked currents in a manner consistent with the modulation of $P_o$ or the number of channels (37). Furthermore, CaM-KII can increase exocytosis in dendrites of hippocampal neurons (38), and blocking membrane fusion in CA1 neurons reduces LTP (39). Although multiple mechanisms might contribute to LTP, our results, combined with the recent report by Benke et al. (15), indicate that one mechanism that enhances the responsiveness of postsynaptic AMPA-Rs during LTP is the CaM-

### Table 2. CaM-KII (KII) increases contribution of high-conductance states in single-channel activity of GluR1

<table>
<thead>
<tr>
<th>Receptor</th>
<th>$\gamma_1$ pS</th>
<th>$\gamma_2$ pS</th>
<th>$\gamma_3$ pS</th>
<th>$\gamma_4$ pS</th>
<th>$\gamma_1 + \gamma_4$</th>
<th>$%$</th>
<th>$\gamma_1^\dagger$ pS</th>
<th>$\tau_{o1}$ ms</th>
<th>$\tau_{o2}$ ms</th>
<th>$\tau_{o1}$§ %</th>
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<tr>
<td>R1</td>
<td>9.4 ± 0.3</td>
<td>13.6 ± 0.2</td>
<td>20.5 ± 0.8</td>
<td>28.3 ± 1.4</td>
<td>23 ± 2</td>
<td>12.6 ± 0.9</td>
<td>0.33 ± 0.07</td>
<td>2.1 ± 0.5</td>
<td>81 ± 4</td>
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<tr>
<td>R1 + KII</td>
<td>7.6 ± 0.9</td>
<td>12.4 ± 0.6</td>
<td>19.3 ± 1.2</td>
<td>29.5 ± 2.0</td>
<td>35 ± 6**</td>
<td>18.2 ± 1.2**</td>
<td>0.43 ± 0.07</td>
<td>3.0 ± 0.7</td>
<td>70 ± 4</td>
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<tr>
<td>S831D</td>
<td>8.4 ± 0.7</td>
<td>12.9 ± 1.0</td>
<td>22.2 ± 1.3</td>
<td>30.1 ± 1.8</td>
<td>83 ± 10**</td>
<td>21.5 ± 0.8**</td>
<td>0.41 ± 0.11</td>
<td>2.3 ± 0.7</td>
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Means ± SEM are shown, with the number of measurements (in parentheses) below. **, $P < 0.01$.

$^\dagger$ Combined contribution of $\gamma_1$ and $\gamma_4$ conductance states to the total distribution.

$^\ddagger$ Weighted value (see Experimental Methods).

§ Contribution of $\tau_{o1}$ to the total open times.
KII-mediated phosphorylation of AMPA-Rs, which increases channel conductance.

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