Trafficking of α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid Receptor (AMPA) Receptor Subunit GluA2 from the Endoplasmic Reticulum Is Stimulated by a Complex Containing Ca\(^{2+}\)/Calmodulin-activated Kinase II (CaMKII) and PICK1 Protein and by Release of Ca\(^{2+}\) from Internal Stores

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Background: ER exit is a rate-limiting step for GluA2 synaptic delivery, which regulates AMPAR trafficking and synaptic calcium dynamics.

Results: ER calcium release, CaMKII activity and PICK1 are important for GluA2 ER exit.

Conclusion: ER-mediated intracellular calcium dynamics regulate GluA2 trafficking out of the ER.

Significance: Novel Ca\(^{2+}\)-dependent signaling pathways underlie the regulation of GluA2 trafficking from the ER.

The GluA2 subunit of the AMPA receptor (AMPAR) dominantly blocks AMPAR Ca\(^{2+}\) permeability, and its trafficking to the synapse regulates AMPAR-dependent synapse Ca\(^{2+}\) permeability. Here we show that GluA2 trafficking from the endoplasmic reticulum (ER) to the plasma membrane of cultured hippocampal neurons requires Ca\(^{2+}\) release from internal stores, the activity of Ca\(^{2+}\)/calmodulin activated kinase II (CaMKII), and GluA2 interaction with the PDZ protein, PICK1. We show that upon Ca\(^{2+}\) release from the ER via the IP3 and ryonodine receptors, CaMKII that is activated enters a complex that contains PICK1, dependent upon the PICK1 BAR (Bin-amphiphysin-Rvs) domain, and that interacts with the GluA2 C-terminal domain and stimulates GluA2 ER exit and surface trafficking. This study reveals a novel mechanism of regulation of trafficking of GluA2-containing receptors to the surface under the control of intracellular Ca\(^{2+}\) dynamics and CaMKII activity.

Membrane proteins are synthesized in the ER,\(^4\) and the vast majority of them are eventually delivered to the plasma mem-

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\(^{4}\) The abbreviations used are: ER, endoplasmic reticulum; CaMKII, Ca\(^{2+}\)/calmodulin-activated kinase II; AMPAR, AMPA receptor; PDZ, postynaptic density (PSD)-95/Discs large/zO-1; BAR, Bin-amphiphysin-Rvs; PICK1, protein interacting with C-kinase 1; GRIP, glutamate receptor interacting protein; ABP, AMPA receptor-binding protein; KN93, N-[2-[[3-(4-chlorophenyl)-2-propenyl]methylamino[methyl]phenyl]-N-(2-hydroxyethyl)-4-methoxybenzene sulfonamide; KN92, 2-[N-(4’-methylbenzene-sulfonyl)]amino-N-(4’-chlorophenyl)-2-propenyl-N-methylbenzylamine phosphate; Endo H, endoglycosidase H; CNQX, 6-cyano-7-nitro-

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domain, whereupon GluA2 binds the PICK1 protein (2, 3, 15, 16).

Synaptic GluA2-lacking AMPA receptors may be replaced by ones containing GluA2. Such replacement has been reported during the early phase of long term potentiation (Refs. 17 and 18, but see Ref. 19), in cerebellar stellate neurons (20–23), and at synapses of dopaminergic ventral tegmental area neurons of cocaine-sensitized rats (24), in the latter case involving the activation of group I metabotropic glutamate receptors. Where characterized in ventral tegmental area dopamine neurons, GluA2 insertion depends upon synaptic activity and thus differs from the reported GluA2 synapse trafficking mechanism in hippocampal neurons, which is activity-independent (25, 26).

Here we have investigated the mechanism of GluA2 surface trafficking. We found that release of Ca$^{2+}$ from the ER via the IP3 and ryanodine receptors activates CaMKII, which enters a complex that contains PICK1. Association of this complex with GluA2 stimulates GluA2 exit from the ER leading to trafficking to the plasma membrane. We suggest that this pathway acts as a feedback mechanism to limit Ca$^{2+}$ influx through Ca$^{2+}$-permeable AMPARs.

**EXPERIMENTAL PROCEDURES**

**Expression Vectors**—Plasmids and viral vectors expressing PICK1, GluA1, and GluA2 have been described before (27–29). Expression plasmids of CaMKIIα and the CaMKIIα (H282R) mutant were a gift from Professor Thomas R. Soderling at the Oregon Health Sciences University. Expression plasmids of GFP-CaMKIIα mutants were a gift from Professor Tobias Meyer at Stanford University. Expression plasmids of CaMKIIα and its mutants were created by PCR amplification and inserted into the pEGFP-N2 vector. Sindbis viral vectors of GFP-CaMKIIα mutants were created by PCR amplification and inserted into the pSinRep5 viral expression vector. The Sindbis viral vector expressing a truncation mutant of CaMKIIα encoding the catalytic domain was a gift from Professor Roberto Malinow of the University of California, San Diego.

**Antibodies**—The following antibodies were used: polyclonal and monoclonal anti-Myc, polyclonal and monoclonal anti-HA antibodies, monoclonal anti-tubulin, goat polyclonal anti-GRIP, and polyclonal anti-PICK1 N18 antibodies (Santa Cruz); polyclonal and monoclonal anti-FLAG antibodies, polyclonal anti-CaMKIIα antibody (Sigma); polyclonal anti-PICK1 antibody and monoclonal anti-pCaMKIIα antibody (Affinity BioReagents); monoclonal anti-CaMKII α antibody (Roche Applied Science); monoclonal anti-GluA2 and polyclonal anti-GluA2/3 (Chemicon) and polyclonal anti-GluA1 (Oncogene) antibodies; rabbit anti-GFP antibody.

**HeLa Cell Culture, Transfection, and Immunocytochemistry**—HeLa cells culture and immunostaining were performed as described before (36). Briefly, HeLa cells were seeded on glass coverslips and maintained in Dulbecco’s minimum Eagle’s medium (DMEM) culture medium, 10% fetal bovine serum, and antibiotics under 37 °C and 5% CO$_2$ in a cell culture incubator. After overnight incubation, cells were ∼30% confluent, and plasmids were introduced into cells using Effectene reagent (Qiagen) following the instructions of the manufacturer. 15–18 h after transfection, cells were washed with PBS and fixed with 4% paraformaldehyde, 4% sucrose for 15 min at room temperature (RT). After washing with PBS three times, cells were permeabilized with 0.2% Triton X-100 for 10 min at RT. Cells were then washed with PBS and blocked with 10% BSA for 1 h at RT. Primary antibody diluted in 3% BSA was incubated with cells for 1 h at RT. Cells were then washed 3 times with PBS for 5 min and incubated with secondary antibody conjugated with the appropriate fluorophore diluted in 3% BSA for 1 h at RT. After washing 3 times with PBS for 5 min, coverslips with cells were mounted with mounting oil, Citifluor (Ted Pella), and stored at 4 °C until examination under the confocal microscope.

**293T Cell Culture, Transfection, and Cell Lysate Preparation**—293T cells were seeded onto 6-cm Petri dishes and maintained in DMEM culture medium under 37 °C plus 5% CO$_2$ in a cell culture incubator. After overnight incubation, plasmids were introduced into cells using Effectene reagent (Qiagen) following the instructions of the manufacturer. 36–48 h after transfection, cells were washed once with PBS, and 0.5 ml 1% Triton X-100 buffer (150 mM NaCl, 2 mM EDTA, 50 mM Tris–HCl, pH 7.4, and 1% Triton X-100) plus protease inhibitor mixture tablet (Roche Applied Science) was added to each dish. Cells were collected into 1.5-ml Eppendorf tubes with Cell Lifter and agitated for 30 min to 1 h at 4 °C. Lysates were then clarified by microcentrifugation at 4 °C for 15 min at maximal speed. Supernatants were immediately used for experiments or stored in −80 °C until needed.

**Virus Production and Neuronal Infection**—Sindbis viruses encoding proteins of interest were produced based on instructions from the manufacturer (Invitrogen). Briefly, viral vectors encoding the proteins of interest were digested with the restriction enzyme, Not1, to linearize vectors, which were transcribed for 2 h at 37 °C using *in vitro* transcription/RNA capping kits (Ambion). RNAs were then electroporated into BHK cells (6 × 10$^6$ cells/electroporation) along with RNAs transcribed from the DH-26S helper plasmid (Invitrogen), which contains genes necessary for pseudovirus production. Electroporated BHK cells were then plated onto a 10-cm Petri dish in α-minimum Eagle’s medium (Invitrogen) and incubated at 37 °C and 5% CO$_2$ for about 30–40 h to allow virus production. The growth medium containing the viruses was subsequently collected, aliquoted, and frozen at −80 °C until needed.

Neurons seeded on coverslips were infected at days *in vitro* (DIV) 17–21, and neurons seeded on 6-cm Petri dishes were infected at DIV 10–14. For infections, viral stocks were thawed and diluted (generally 1:25) in 500 μl of conditioned Neurobasal medium (for neurons plated on coverslips) or in 1 ml of conditioned Neurobasal medium (for neurons plated on 6 cm Petri dishes) that was then placed over neurons with occasional rocking for 1 h. Neurons were then supplemented with additional conditioned Neurobasal medium for about 15–18 h until experimental manipulation.

**Primary Hippocampal Neuron Culture**—The day before dissection, coverslips or 6-cm Petri dishes were coated with poly-L-lysine in boracic acid buffer at 37 °C overnight. Before dissec-
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Pharmacological Manipulation of Hippocampal Cultures—To study the role of protein kinases and intracellular calcium dynamics in GluA2 surface expression and GluA2 ER exit, antagonists that inhibit protein kinases, ion channels, or receptors indicated in corresponding experiments were added to growth medium immediately after virus infection and maintained until immunostaining or metabolic labeling. Anti-glia growth drug was usually added into growth media for 1 h after Sindbis virus infection. The cells were incubated with the peptides for an additional 16 h before immunostaining or metabolic labeling.

Neuronal Immunocytochemistry—For fixed immunostaining, 15–18 h after infection, neurons were washed once with PBS and fixed with 4% paraformaldehyde, 4% sucrose for 10 min at RT. Neurons were then washed 3 times with PBS and permeabilized with 0.2% Triton X-100 for 8 min at RT. After permeabilization, neurons were blocked again with 10% BSA for 1 h at RT, and primary anti-Myc polyclonal antibody (A14, 0.5 μg/ml) or polyclonal anti-HA antibody (0.5 μg/ml) diluted in 3% BSA was incubated with neurons to detect total tagged receptors for 1 h at RT. Neurons were then washed 3 times with PBS and incubated with secondary antibody diluted in 3% BSA for 1 h at RT. Coverslips with neurons were then washed, mounted, and stored as described above.

Image Analysis and Quantitation—Immunofluorescence images were acquired on a Nikon PCM 2000 confocal microscope or a Zeiss Axioplan 2000 fluorescence microscope under 60× objective and, for the PCM2000, analyzed with Simple32 Imaging software (C-IMAGING Systems). All images were acquired at the same setting for one experiment. To analyze the effect of drugs treatments on Myc-GluA2 or HA-GluA1 trafficking, neuronal areas were defined as the extent of total Myc or HA staining, and total fluorescence was measured in this area. The ratio of surface Myc or HA signal to cell area was then calculated. However these ratios do not indicate actual receptor distribution but were used for relative differences across different treatment conditions. Each experimental manipulation was performed at least three times. Error bars are S.E., and t tests were carried out to determine the significance between two groups. Statistical differences for multiple groups were determined by one-way ANOVA.

Immunoprecipitation and Immunoblotting—For immunoprecipitation assays in 293T cells, appropriate amounts of 293T cell lysates were incubated with 1 μg of M2 monoclonal anti-FLAG antibody to immunoprecipitate FLAG-tagged species for 2 h at 4 °C. The immunocomplexes were precipitated with Protein G beads for 2 h at 4 °C. Immunoprecipitates were washed with lysis buffer, eluted with SDS-PAGE sample buffer, and heated at 100 °C for 5 min. The samples were then run on SDS-PAGE gels for electrophoresis. After electrophoresis, the protein samples in the SDS-PAGE gel were transferred overnight to nitrocellulose or PVDF membrane for Western blotting with the indicated antibodies.

Metabolic Labeling and Endo H Assay—DIV 10–14 neurons were washed once with warmed DMEM lacking Met/Cys (containing 0.5 mM glutamine and 1× B27; Invitrogen). Neurons were then Met-depleted in this medium for 20 min and pulsed with 160 μCi/ml of Expre35S (1175 Ci/mmol; PerkinElmer Life Sciences) for 15 min. Neurons were then washed 3 times with ice-cold PBS and lysed with Neuronal lysis buffer (400 μl/dish, 150 mM NaCl, 20 mM Hepes, 2 mM EDTA, 1% Triton, 0.1% SDS, pH 7.4, and 1× fresh protease inhibitor mixture tablets) for 1 h at 4 °C. Lysates were cleared for 20 min at 16,000 × g and used for immunoprecipitations. Lysates were incubated with 1.5 μg of Myc polyclonal antibody or 1.5 μg of GluA2 monoclonal antibody from Neuromab for 1 h at 4 °C. Twenty-

Coverslips or dishes were washed twice with PBS and stored in the incubator ready for plating neurons.

Primary hippocampal neuron cultures were obtained from E18–19 SD rat embryos. Pregnant rats were anesthetized with CO2, and embryos were then removed. All dissection work was carried out in ice-cold PHG buffer (1× PBS, 10 mM HEPES, and 0.6% glucose, pH 7.35). After decapitation of the embryos, hippocampi were isolated under a dissection microscope in the sterile hood. Hippocampi were collected and trypsinized in 1× trypsin for 15 min at 37 °C, washed 3 times in dissection buffer, and then resuspended in 5 ml of plating medium (minimal essential medium, 10% horse serum, 0.45% glucose, 1 mM pyruvate, 1× penicillin/streptomycin) warmed to 37 °C. Hippocampi were triturated with a 5 ml of sterile pipette until the cell suspension appeared homogeneous, and cells were then counted with a hemocytometer. Cells were plated at a density of 120,000 per coverslip or 1,000,000 per 6-cm Petri dish in plating medium. 2–4 h after plating, all media were removed and replaced with Neurobasal medium supplemented with B27 supplement (Invitrogen), glutamine (500 μM), and antibiotics. Every 4 days, half of the volume of medium remaining on the cells was removed and replaced with fresh Neurobasal medium. Anti-glia growth drug was usually added into growth media after 8 DIV.

Immunoprecipitation and immunoblotting assays in 293T and hippocampal cultures showed that the effect of CaMKII inhibition on GluA2 ER exit was concentration-dependent. The results suggest that CaMKII activity plays a role in regulating GluA2 ER exit in neurons.
five microliters of Protein A or G-agarose beads (Santa Cruz) were then added for 50 min. Immunoprecipitates were washed three times with lysis buffer.

Immunoprecipitates were first denatured with 100 °C heating for 5 min in 0.5% SDS solution. When immunoprecipitates were cooled to RT, Endo H (Roche Applied Science) digestion was carried out. Enzyme reactions on immunoprecipitates were in 50 mM sodium citrate, pH 5.9, with 15 milliunits of enzyme per reaction. Digestions were at 30 °C for 16–19 h. Samples were separated on 6% SDS-PAGE gel, fixed with 45% methanol and 5% acetic acid buffer for 20 min, and amplified with amplification solution (Amersham Biosciences) for 30 min. Gels were then dried and autoradiographed.

RESULTS

CaMKII Is Required for GluA2 Trafficking to the Surface in Hippocampal Cultures—To test the roles of protein kinases in GluA2 surface trafficking, we measured the effects of various protein kinase inhibitors on GluA2 levels in the plasma membrane of cultured embryonic hippocampal neurons (17–21 DIV). We expressed Myc-GluA2 (Myc epitope tag fusion to GluA2 N terminus) from a Sindbis virus vector and treated the cultures with different kinase inhibitors. At 15–18 h after infection, surface and total Myc were measured by live and permeabilized cell immunofluorescent staining, respectively, and the ratio of surface Myc–GluA2 to total Myc–GluA2 was calculated.

Neither a PKC inhibitor (bisindolylmaleimide (2 μM)) nor a PKA inhibitor (KT5720; 2 μM) affected GluA2 plasma membrane levels. In contrast, a CaMKII inhibitor, KN93 (10 μM), significantly reduced the levels of surface GluA2 (Fig. 1A). As a control, the inactive analog KN92 did not significantly inhibit surface levels of GluA2 (Fig. 1B). This suggests a role for CaMKII in establishing the level of GluA2 in the neuronal plasma membrane.

To corroborate the role of CaMKII in GluA2 trafficking, we employed a different approach to inhibit neuronal CaMKII activity. We found that trafficking of MycGluA2 to the neuronal surface was sensitive to a peptide (myristoylated autocamtide-3 pseudosubstrate, 50 μM) that inhibits CaMKII activity (42), but not to a control peptide (Fig. 1C). Taken together these data demonstrate an important role of CaMKII in the regulation of GluA2 trafficking.

We previously showed that a large fraction of intracellular GluA2 resides in the endoplasmic (ER), where its glycosylation is sensitive to Endo H (7). We employed an Endo H assay to determine whether CaMKII is required for export of GluA2 from the ER, an early step in surface trafficking. CaMKII inhibition by KN93 significantly suppressed maturation of Myc-GluA2 as assayed by pulse-chase analysis of Endo H sensitivity. In contrast, KN92 did not suppress (Fig. 1D). Thus, CaMKII activity was specifically required for GluA2 exit from the ER.

Ca2+ Release from Internal Stores Stimulates GluA2 Surface Trafficking—Because CaMKII is a Ca2+/calmodulin-activated kinase, we next examined the roles of extracellular and intracellular Ca2+ in GluA2 surface expression. We found that blockage of Ca2+ influx through plasma membrane Ca2+ channels, including L-type voltage-gated Ca2+ channels (nimodipine 10 μM, data not shown) and ionotropic glutamate receptors (100 μM d-APV, N-methyl-D-aspartate receptor antagonist; CNQX 100 μM, AMPAR antagonist), did not impair GluA2 surface expression, nor did blockage of voltage-gated sodium channels with tetrodotoxin (2 μM) (Fig. 2A). This suggested that in cultured hippocampal neurons, GluA2 trafficking to the surface is not dependent on Ca2+ influx through channels in the plasma membrane or on neuronal activity. In agreement, a reagent (BAPTA, 2 mM) that chelates extracellular Ca2+ also failed to reduce GluA2 surface expression (Fig. 2A). Thus, extracellular Ca2+ influx is not required to maintain surface levels of GluA2.

Because Ca2+ release from the ER via IP3 and ryanodine receptors contribute to intracellular Ca2+ dynamics (30, 31), we examined the role of this release in GluA2 surface expression. Interestingly, simultaneous inhibition of the IP3 receptor by 2-APB (50 μM) and the ryanodine receptor by dantrolene (25 μM), but not inhibition of either receptor alone, significantly reduced surface GluA2 but did not inhibit GluA1, a control (Fig. 2, A and B). This suggests that release of Ca2+ from internal stores through either IP3 or ryanodine receptors is sufficient for the maintenance of GluA2 surface levels.

Regulation of Trafficking of Endogenous GluA2 by CaMKII Activity and Ca2+ Release from Internal Stores—Previous studies have shown that maturation of GluA2 at the ER is a rate-limiting step for surface expression of the receptor (7). To test the role of CaMKII activity and Ca2+ release from internal stores in native GluA2 maturation and forward trafficking, we treated neuronal cultures with KN93 or 2-APB and dantrolene and assayed GluA2 maturation through an Endo H assay. As shown in Fig. 2C, blockade of CaMKII activity or inhibition of Ca2+ release from internal stores suppressed maturation of endogenous GluA2 at the ER.

PICK1-dependent GluA2 Trafficking to Neuronal Surface—We have previously shown that the exit of GluA2 from the ER depends on the PICK1 protein (7), a Ca2+-responsive, PDZ protein that specifically binds the GluA2 C-terminal domain and mobilizes trafficking of GluA2 (28, 32–35). To study the role of PICK1 in CaMKII-dependent GluA2 trafficking to the neuronal surface, we took advantage of well-characterized GluA2 mutants (GluA2-AVKI and GluA2-SVKE) (27). Although GluA2-SVKE cannot bind to either PICK1 or GRIP/ABP, GluA2-AVKI selectively loses its ability to bind to GRIP/ABP without disrupting the interaction with PICK1 (27). We determined which of these MycGluA2 mutants trafficked by a CaMKII-dependent and ryanodine or IP3 receptor-dependent mechanism by comparing trafficking in inhibitor-treated cultures with trafficking in control cultures lacking drugs. As shown in Fig. 3, KN93 or dantrolene/2-APB inhibits trafficking of GluA2 and GluA2-AVKI to the neuronal surface. In contrast, neither KN93 nor dantrolene/2-APB has an effect on GluA2-SVKE delivery to the plasma membrane. These data suggest that the ability of CaMKII activity or internal calcium release to stimulate GluA2 trafficking depends on an interaction of PICK1 with GluA2.

PICK1 Associates with CaMKII in Heterologous Cells—We next investigated how Ca2+ release from internal stores,
CaMKII, and PICK1 could cooperate to control GluA2 trafficking. Based on the precedent that PKCα regulates GluA2 through a PICK1-PKCα complex (28, 36–38), we hypothesized that CaMKII might act through a complex containing PICK1 and CaMKII to stimulate GluA2 trafficking. To determine whether PICK1 enters such a complex, we expressed wild type PICK1 or PICK1 truncation mutants with CaMKIIα in 293T cells. Taking into account that activation of PKCα is required to form the PICK1-PKCα complex, we assayed whether the CaMKIIα mutant, CaMKIIα H282R, would coimmunoprecipitate with PICK1 after 293 cell expression (Fig. 4). The H282R mutation releases the kinase regulatory domain from its catalytic domain, as does binding of Ca²⁺-calmodulin to CaMKII, constitutively acti-

FIGURE 1. CaMKII activity is required for surface expression of GluA2 in cultured hippocampal neurons. A and B, requirement of CaMKII activation for surface delivery of Myc-GluA2 in neurons. Cultured hippocampal neurons (17–21 DIV) were infected with Sindbis virus expressing Myc-GluA2. PKC inhibitor, bisindolylmaleimide (BIS, 2 μM), or PKA inhibitor, KT5720 (2 μM) or CaMKII inhibitor, KN93 (10 μM), KN92 (10 μM) or the vehicle, DMSO, were added to cultured media immediately after infection. Surface and total Myc-labeled receptors were quantitated by immunocytochemistry as described under “Experimental Procedures.” The bar graph in A shows the mean ± S.E.; n = 29 for DMSO; n = 23 for bisindolylmaleimide; n = 24 for KT5720; n = 30 for KN93. The bar graph in B shows the mean ± S.E.; n = 37 for DMSO, KN93, or KN92. Significance between individual drug treatment and DMSO in A and B was determined with two-tailed t test; *, p < 0.001. **, p < 0.05 with one-way ANOVA; ***, p < 0.05 post hoc Fisher’s test. C, cultured hippocampal neurons (18–21 DIV) were infected with Sindbis virus expressing Myc-GluA2 (R) in the presence of control peptide (Cnt Pep) or the CaMKII inhibitor, autocamtide (AC3-I). Surface and total Myc-labeled receptor was quantitated by immunocytochemistry as described under “Experimental Procedures.” The bar graph shows the mean ± S.E.; n = 51 for control peptide and n = 50 for autocamtide-3. Significance was determined with two-tailed t test. ***, p < 0.05. D, CaMKII activity is involved in GluA2 exit from the ER. Cultured hippocampal neurons (∼12 DIV) were infected with Sindbis virus expressing Myc-GluA2. 15 h after infection, neurons were pulsed with [35S]Met-Cys for 20 min and chased for 5 h. CaMKII inhibitor, KN93 (10 μM), or the inactive analog, KN92 (10 μM), or the vehicle, DMSO, was added to the medium throughout the pulse-chase experiment. Myc-GluA2 was immunoprecipitated with an anti-Myc antibody, Endo H-digested, and analyzed on 6% SDS-PAGE followed by fluorography. Maturity glycosylated Myc-GluA2 is denoted with an empty arrowhead, and immaturely glycosylated Myc-GluA2 with a filled arrowhead. The bar graph in the bottom shows the quantitation (n = 4 for DMSO and KN93 and n = 3 for KN92. *, p < 0.05 with one-way ANOVA. **, p < 0.05 with post hoc Fisher’s test.).
CaMKII Regulates GluA2 ER Exit

FIGURE 2. Calcium release from internal stores is required for surface delivery of GluA2. A, cultured hippocampal neurons (18–21 DIV) were infected with Sindbis virus expressing Myc-GluA2. Pharmacological reagents D-APV (100 μM), CNQX (100 μM), Triton X-100 (TTX; 2 μM), BAPTA (2 mM), 2-APB (50 μM), and dantrolene (1 μM) or the vehicle, DMSO, were added to the culture medium immediately after infection. Surface and total Myc-labeled receptor was quantitated by immunocytochemistry as described under “Experimental Procedures.” The bar graph shows the mean ± S.E.; n = 57 for DMSO, n = 29 for D-APV, n = 12 for CNQX, n = 18 for Triton X-100, n = 12 for BAPTA, n = 18 for 2-APB, n = 18 for dantrolene, and n = 18 for dantrolene/2-APB. Significance was determined with two-tailed t test. *p < 0.001. B, neurons were infected with Sindbis virus expressing HA-GluA1. Pharmacological treatment, immunostaining for surface and internal HA-GluA1, and quantitation of normalized surface levels were performed as described under “Experimental Procedures.” The bar graph shows the mean ± S.E.; n = 26 for DMSO and n = 30 for dantrolene/2-APB. Significance was determined with two-tailed t test. p > 0.05. ns, not significant. C, neurons were labeled with [35S]Met and [35S]Cys for 90 min and chased for 18 h in the presence of KN93 or dantrolene/2-APB or DMSO (control) as noted, and endogenous GluA2 was immunoprecipitated from whole cell extracts, treated with Endo H, and Endo H sensitivity was analyzed by polyacrylamide gel electrophoresis and autoradiography, all as described under “Experimental Procedures.” *, p < 0.05 with one-way ANOVA; **, p < 0.05 with post hoc Fisher’s test.

vating the kinase (39). CaMKIIα H282R coimmunoprecipitated with the WT PICK1 protein and the PICK1 mutant Δ121, which lacks the PICK1 PDZ domain. It also coimmunoprecipitated with a PICK1 mutant that contains the PICK1 coiled-coil domain (CC mutant), whose dimerization forms a BAR domain (33), and it also bound to PICK1 mutant 379Δ, which lacks sequences C-terminal to the coiled-coil domain. However, it did not coimmunoprecipitate with mutant 135Δ, which contains the PICK1 PDZ domain but lacks sequences C-terminal to it, including the coiled-coil
Thus, PICK1 coimmunoprecipitation with CaMKII requires the coiled-coil region, which can form a BAR domain, rather than the PDZ domain, which is the binding site for PKC. These results indicate that activated CaMKII can enter a complex containing PICK1, dependent upon the PICK1 BAR domain, although it did not determine if the interaction was direct or indirect.

To analyze further the roles of CaMKII domains in the interaction of CaMKII with PICK1 and to determine the effects of PICK1 interaction on CaMKII subcellular localization, we expressed wild type CaMKII or its truncation mutants in HeLa cells together with CaMKII, which contains the BAR domain and the site required for CaMKII interaction (Fig. 5, A and B). Δ121 by itself formed intracellular clusters, and CaMKII was diffuse on its own (Fig. 5A). A CaMKIIα mutant containing only the catalytic domain colocalized with Δ121 (Fig. 5B), but constructs containing the catalytic domain plus the regulatory domain or the catalytic, regulatory, and association domains (i.e., WT) did not colocalize and neither did the isolated CaMKII association domain or a mutant lacking the catalytic domain (Fig. 5B). In agreement with the coimmunoprecipitation studies, this indicated that the site on CaMKII necessary for entering a complex with PICK1 was the CaMKII catalytic domain. Because inclusion of the regulatory domain with the catalytic domain blocked colocalization, it appeared that only CaMKII with the unmasked form of the catalytic domain could enter into a complex with PICK1. The fact that the unmasked catalytic domain, which colocalizes with PICK1, corresponds functionally to the activated kinase, is consistent with the finding made through coimmunoprecipitation that PICK1 enters into a complex with the activated CaMKIIα (Fig. 4) via the catalytic domain of CaMKIIα (Fig. 5C).

The PICK1 BAR Domain Colocalizes with Activated Forms of CaMKII—CaMKII H282R colocalized with coexpressed CaMKII in clusters surrounding the nucleus (Fig. 5D), but on its own, H282R was diffusely distributed, further supporting that activated CaMKIIα binds directly or indirectly to the BAR domain and suggesting that Δ121 could recruit CaMKIIα to its subcellular location as does PICK1 for PKCα (28). Binding of Ca2+-calmodulin to CaMKIIα stimulates CaMKIIα Thr-286 autophosphorylation, which releases the regulatory domain from the catalytic domain and stably activates the kinase (40, 41). To evaluate the role of CaMKII autophosphorylation in kinase interaction with the PICK1 BAR domain, we coexpressed Δ121 with wild type CaMKIIα in HeLa cells (Fig. 5E). Although immunofluorescence revealed that the bulk of the CaMKII population was diffuse and did not colocalize with Δ121, Thr-286 autophosphorylation...
results demonstrated that the CaMKII neuron.

We sought to determine the role of the pore apex residue, Arg-607, which imposes a barrier on assembly of channels with high GluA2 content that is not observed for GluA1, whose pore apex residue is Gln. Indeed, the hippocampal neuron ER contains a pool of dimeric and monomeric GluA2, whereas GluA1 is almost exclusively tetrameric and in the plasma membrane (6, 7). Changing the GluA2 pore apex from Arg to Gln overcomes the assembly barrier leading to rapid ER exit (6). To determine the role of the pore apex residue in the Ca\(^{2+}\)-dependent export of GluA2, we compared the sensitivities of GluA2 (R) and GluA2 (Q) to CaMKII or to ER Ca\(^{2+}\) release inhibitors. Significantly, trafficking of MycGluA2 (R), but not MycGluA2 (Q), was sensitive to KN93, and MycGluA2 (Q) trafficking exceeded the trafficking level of MycGluA2 (R) both in the presence and absence of KN93 (Fig. 7A). In addition, although MycGluA2 (R) is inhibited by the treatment with 2-APB and dantrolene, MycGluA2 (Q) is not sensitive to such treatment (Fig. 7B). These data suggested that the pore apex residue mutation to Gln, a change that promotes ER assembly of GluA2, may overcome the ER trafficking dependence on ER Ca\(^{2+}\) flux and CaMKII activity. Finally, to test if ER Ca\(^{2+}\) release from stores can contribute to CaMKII activation, we treated cultured neurons with DMSO or 2-APB/dantrolene. Five hours after treatment we performed a Western blot assay to measure total CaMKII\(\alpha\) and pCaMKII\(\alpha\). As shown in Fig. 7C, 2-APB/dantrolene treatment significantly reduced the levels of pCaMKII\(\alpha\), suggesting that Ca\(^{2+}\) release from ER stores contributes to CaMKII activation.

**DISCUSSION**

The AMPAR GluA2 subunit determines receptor biophysical properties, including single channel current, rectification, and Ca\(^{2+}\) conductance (4). Therefore, trafficking of GluA2-containing receptors to synapses plays a key role in shaping excitatory synaptic transmission. Trafficking of AMPARs to the synapse begins at the ER, where the AMPAR subunits are synthesized and assembled into a functional ion channel (43). In the present work we report a pathway for controlling trafficking of GluA2 from the endoplasmic reticulum to the plasma membrane. Ca\(^{2+}\) released from ER stores via the IP3 and ryanodine receptors activates CaMKII, which directly or indirectly associates with the BAR domain of PICK1 to form a complex that can bind to the C-terminal domain of GluA2. CaMKII activity then promotes the release of GluA2 from the ER whereupon the receptor traffics to the cell surface.

Previous studies from our laboratory have shown that RNA editing of the GluA2 pore, which changes the pore apex residue Gln-607 to Arg, resulted in a majority of intracellular GluA2 residing in the ER (6, 7). This raises a critical question for mechanisms underlying GluA2 trafficking: how is GluA2 trafficking out of the ER regulated? Here we show that GluA2 forward trafficking from the ER is stimulated by CaMKII activity. Pharmacological inhibition of CaMKII activity by either KN93 or a CaMKII inhibitory peptide prevented GluA2 trafficking out of the ER and to the plasma membrane. This suggests that CaMKII functions in the early secretory pathway of GluA2 trafficking. Surprisingly, inhibition of PKC activity failed to prevent GluA2 trafficking to the surface. PKC has been shown to phosphorylate GluA2 and regulates the interaction of GluA2 with

**FIGURE 4. PICK1 forms a complex with a constitutively active CaMKII\(\alpha\) mutant in 293T cells.** A, schematic drawings of PICK1 and PICK1 mutants tagged with FLAG in the C termini. B, CaMKII\(\alpha\) (H282R) was expressed alone or together with FLAG tagged PICK1 and PICK1 mutants in 293T cells. FLAG-tagged species in cell lysates were immunoprecipitated with an anti-FLAG antibody, and immunoprecipitates (IP) were subjected to Western blotting (W) assay with an anti-CaMKII antibody. 10% input used for immunoprecipitation was also probed with the indicated antibodies to confirm protein expression.

**CaMKII Regulates GluA2 ER Exit**
PDZ domain proteins (e.g., PICK1 and ABP/GRIP) (28, 37, 38). This suggests that PKC may primarily be involved in trafficking of GluA2 that has already exited the ER and arrived at the plasma membrane, whereas an earlier step in GluA2 trafficking, movement out of the ER, depends on CaMKII activity.

A role of CaMKII in GluA2 trafficking suggests the involvement of Ca$^{2+}$ signaling in GluA2 forward transport. In hippocampus, CaMKII is highly expressed in pyramidal neurons (40, 41). Studies have shown that a substantial fraction of CaMKII is found at excitatory synapses where it may be strategically located to transduce signals generated by Ca$^{2+}$ influx through N-methyl-d-aspartate receptors to the regulation of intracellular pathways involved in synaptic plasticity (40, 41). However, in the current work, pharmacological manipulations preventing Ca$^{2+}$ influx from extracellular space failed to suppress GluA2 forward trafficking, suggesting that Ca$^{2+}$ influx through Ca$^{2+}$-permeable channels at the plasma membrane is unlikely to be necessary for GluA2 trafficking to the surface in hippocampal neurons. This is reminiscent of previous work that showed that blockade of neuronal activity did not prevent GluA2 trafficking to synapses (25). This is also a striking difference from the mechanism underlying trafficking of GluA1-con-
CaMKII regulates GluA2 trafficking out of the ER (46). Indeed, TARPs have recently been shown to be a phosphorylation substrate of CaMKII (47). Significantly, TARPs have also been proposed to contribute to AMPA receptor assembly in the ER (23, 48). In this scenario, we envision that calcium release from ER activates CaMKII, facilitating direct or indirect interaction of CaMKII with PICK1, which in turn brings complexes containing the PICK1 and CaMKII to GluA2 residing in the ER through the PICK1-GluA2 interaction. Activated CaMKII in the vicinity of GluA2 may stimulate GluA2 trafficking out of ER through TARP-dependent mechanisms.

It is worth noting that blockade of CaMKII activity and ER calcium release has a larger effect on virally expressed GluA2 than endogenous GluA2 (Figs. 1 and 2). Virally expressed GluA2 largely forms GluA2 homomers. In contrast, in hippocampal neurons native GluA2 mainly forms heteromers with GluA1 (10, 11). As many previous studies have shown that GluA1 trafficking out of ER and to the surface is different from endogenous GluA2 (Figs. 1 and 2). Virally expressed GluA2 exits the ER and hyperpolarization-activated AMPA receptors (HAMPARs) have recently been shown to be a phosphorylation substrate of CaMKII (47). Significantly, TARPs have also been proposed to contribute to AMPA receptor assembly in the ER (23, 48). In this scenario, we envision that calcium release from ER activates CaMKII, facilitating direct or indirect interaction of CaMKII with PICK1, which in turn brings complexes containing the PICK1 and CaMKII to GluA2 residing in the ER through the PICK1-GluA2 interaction. Activated CaMKII in the vicinity of GluA2 may stimulate GluA2 trafficking out of ER through TARP-dependent mechanisms.

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The finding that GluA2 exits the ER is under the regulation of ER calcium release suggests an unappreciated mechanism governing AMPAR maturation. Although the mechanism regulating internal calcium release that underlies GluA2 trafficking remains to be determined, it is possible that metabotropic glutamate receptors, which upon activation can mobilize intracellular calcium release from internal stores via IP3 receptors, may play a role in this. Indeed, a recent study shows that activation of metabotropic glutamate receptor 1 induces long term depression at excitatory glutamatergic synapses on dopamine neurons of the ventral tegmental area by removing synaptic GluA1-containing calcium-permeable receptors, which do not contain the GluA2 subunit, and by replacing them with GluA2-containing receptors (24). This suggests that metabotropic glutamate receptors function in promoting GluA2 trafficking to synapses in ventral tegmental area dopamine neurons.
Regulation of GluA2 trafficking by intracellular Ca\(^{2+}\) dynamics may have important implications in protecting neurons from ischemic insults. Substantial evidence shows that trafficking of GluA2-containing receptors to synapses in the hippocampus is impaired after ischemic insults, which may prime neuronal death after transient ischemia (49). Significantly, metabotropic glutamate receptors, which contribute to cognitive aging, stimulate the formation of IP3 (50), which activates the IP3 receptor, shown here to contribute to GluA2 trafficking. Therefore, promoting the delivery of GluA2-containing receptors to the surface through the mechanism discovered here may be utilized to reduce prevent neuronal death upon ischemia.

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FIGURE 7. Surface trafficking of GluA2 pore apex residue mutant R607Q displays reduced dependence on CaMKII and ER calcium. Cultured hippocampal neurons (18–21 DIV) were infected with Sindbis virus expressing Myc-GluA2 (R) or Myc-GluA2 (Q) in the presence of DMSO (control) or the KN93 (A1, A2) or 2-APB/dantrolene (B1, B2). Surface and total Myc-labeled receptor was quantitated by immunocytochemistry as described under "Experimental Procedures." The bar graph shows the mean ± S.E.; n = 32 for all conditions in A; n = 29 for DMSO and n = 30 for dantrolene/2-APB in B. *, p < 0.05 with one-way ANOVA. *, p < 0.05 with post hoc Fisher’s test. *, p < 0.05. C, 2-APB/dantrolene treatment reduced the levels of neuronal pCaMKII\(\alpha\). Significance was determined with two-tailed t test. *, p < 0.05.
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