Calcium/Calmodulin-dependent Protein Kinase II Phosphorylation Drives Synapse-associated Protein 97 into Spines*

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Synapse-associated protein 97 (SAP97) has been involved in the correct delivery and clustering of glutamate ionotropic receptors to the postsynaptic compartment. Here we demonstrate that synaptic trafficking of SAP97 itself was modulated by calcium/calmodulin-dependent protein kinase II (CaMKII) in cultured hippocampal neurons. CaMKII activation led to increased targeting of SAP97 into dendritic spines, whereas CaMKII inhibition was responsible for SAP97 high colocalization in the cell soma with the endoplasmic reticulum protein disulfide-isomerase. No effect was detected for other members of the membrane-associated guanylate kinase protein family, such as SAP102 and PSD-95. Transfection of activated αCaMKII T286D dramatically increased concentration of both endogenous and transfected SAP97 at postsynaptic terminals. In vitro CaMKII phosphorylation of the SAP97 N-terminal fusion protein and metabolic labeling of transfected COS7 cells indicated SAP97-Ser-39 as a CaMKII phosphosite in the SAP97 protein sequence. Moreover, transfection in hippocampal neurons of SAP97 mutants that blocked or mimicked Ser-39 phosphorylation had effects similar to those observed upon inhibiting or constitutively activating CaMKII. Further, CaMKII-dependent SAP97-Ser-39 phosphorylation determined a redistribution of the glutamate receptor subunit (GluR1) of the AMPA receptor. In conclusion, our data show that CaMKII-dependent SAP97-Ser-39 phosphorylation regulates the association of SAP97 with the postsynaptic complex, thus providing a fine molecular mechanism responsible for the synaptic delivery of SAP97 interacting proteins, i.e. ionotropic glutamate receptor subunits.

The correct recruitment of ionotopic glutamate receptor (iGluR) subunits into the postsynaptic compartment is a highly regulated process that requires the concerted action of diverse intracellular elements modulating association/dissociation of key protein complexes within multiple intracellular compartments. Among molecular associations regulating subcellular targeting of iGluRs subunits, interaction with members of the membrane-associated guanylate kinase (MAGUK) protein family has been proposed (1). In fact, MAGUK protein family members have been addressed as organizing elements in excitatory neurons (2). MAGUKs are characterized by a common multimodular structure including three PDZ domains, a Src homology domain 3, and a guanylate kinase-like domain. Members of this family act as molecular scaffolds for iGluRs mainly by direct interaction with an SH3 motif on the cytoplasmic termini of their binding proteins. Recently, members of this family have been proposed to be involved in iGluRs trafficking (3).

Among MAGUKs, SAP97 is the rat homologue of the Droso-philin (Dlg) and human (hDlg) discs large tumor suppressor protein. In the mammalian central nervous system, SAP97 has been described as enriched both at pre- and postsynaptic compartments, where it has been implicated in the processing and surface expression of ion channels in neurons. In contrast, few synaptic AMPA receptors associate with SAP97 (8), suggesting that SAP97 dissociates from the receptor complex at the plasma membrane. These data imply the existence of a fine-tuning of iGluRs interactions with SAP97 mediated by a still unknown partner.

Recently, different members of MAGUK have been identified as possible new targets for CaMKII (6,9–10), raising the possibility of a new mechanism regulating MAGUK function in the postsynaptic neuron. In fact, although several CaMKII substrates have been identified in the last few years (10), little is known about the functional role of specific CaMKII-dependent phosphorylation processes that take place in vivo in the postsynaptic compartment. Recently, studies from our group (6) showed that CaMKII-dependent phosphorylation of SAP97-Ser-232 within the PDZ1 domain disrupts SAP97 interaction with NR2-type NMDA subunits and revealed NR2A, thereby regulating synaptic targeting of SAP97 in the postsynaptic compartment. Recently, studies from our group (6) showed that CaMKII-dependent phosphorylation of SAP97-Ser-232 within the PDZ1 domain disrupts SAP97 interaction with NR2A, thereby regulating synaptic targeting of this NMDA receptor subunit.

Here we show that CaMKII-mediated phosphorylation of an additional SAP97 site, Ser-39, within the L27 domain is necessary and sufficient to drive SAP97 to the postsynaptic compartment in cultured hippocampal neurons. In addition, SAP97-Ser-39 phosphorylation represents a key step governing GluR1-containing AMPA receptor delivery to the postsynaptic complex, thus suggesting SAP97 as a multimodular element where distinct domains play differential roles in organizing the glutamatergic synapse.
**EXPERIMENTAL PROCEDURES**

**Neuronal Cultures, Transfection, and Immunofluorescence Labeling**—Low density or high density hippocampal neuronal cultures were prepared from E18-E19 rat hippocampi as previously described (11). Neurons were transfected using the calcium phosphate precipitation method at 10 days in vitro. Mutated products were obtained by using the QuikChangeTM site-directed mutagenesis kit (Stratagene, La Jolla, CA). Hippocampal neurons were fixed in 100% methanol at −20 °C for 15 min. Transfected cells were used 4 days after transfection. Primary and secondary antibodies were applied in GDB previously described (13). Antibodies were purchased from Molecular Probes (Eugene, Oregon). Clonal protein disulfide-isomerase (DSI) antibody were purchased from Calbiochem, 10 μM ryanodine (Sigma), 100 μM thapsigargin (Tocris, Bristol, UK), 10 μM Ant-AIP-2 (Calbiochem), 10 μM KN-93 (Sigma), 10 μM H89 (Sigma), 1 μM PP2 (Calbiochem), 0.1 μM GF109203X (Calbiochem).

**TIF Preparation**—Triton-insoluble fractions (TIF) were isolated from neurons harvested at 10–14 DIV as previously described (6).

**Metabolic Labeling**—COS-7 cells were transfected with empty vector, GFP-SAP97wt, aCcAim-1T286D, GFP-SAP97/S39A,S232A, αCaMKII-T286D, or GFP-SAP97/S39A, αCaMKII-T286D. 48 h after transfection, cells were preincubated for 2 h in phosphate-free minimal essential medium. The medium was aspirated and replaced with fresh phosphate-free minimal essential medium containing [32P]orthophosphate (500 μCi/ml). After 2 h, okadaic acid 0.2 μM was added to the medium and cells were incubated for an additional hour. Cells were washed with ice-cold washing buffer containing complete set of protease inhibitors (Complete™ Roche Diagnostics). Each solubilized sample was then incubated overnight at 4 °C with anti-SAP97 polyclonal antibody. Protein A-Sepharose beads washed in the same buffer were added and incubation continued for 2 h. Western blots were performed on all immunoprecipitated samples to verify that equal amounts of SAP97 protein were being precipitated under all conditions.

**Image Acquisition and Quantification**—Confocal images were obtained using a Nikon ×60 objective with sequential acquisition setting at 1024 × 1024 pixels resolution. Each image was a z series projection of ~8–12 images taken at 0.5–1.0 μm depth intervals. Pharmacologically treated and transfected neurons were chosen randomly for quantification from two to five coverslips from three to five independent experiments for each construct. Quantification of confocal experiments was performed using Bio-Rad Laserpix software. Both image acquisition and quantification of the fluorescence signal were performed by investigators who were “blind” to the experimental condition. Quantification of Western blot analysis and autoradiography was performed by means of computer-assisted imaging (Quantity-One™ System; Bio-Rad), and statistical evaluations were performed according to one-way analysis of variance followed by Bonferroni as a post hoc comparison test.

**Cloning, Expression, and Purification of GST Fusion Protein—**SAP97 fragments were subcloned downstream of glutathione S-transferase (GST) in the BmMHI and HindIII sites of the expression plasmid pGEX-KG by PCR using plaque-forming unit polymerase (Promega). The inserts were fully sequenced with the ABI Prism 310 genetic analyzer (ABI Prisma). SAP97-GST fusion proteins were expressed in *Escherichia coli*, purified on glutathione-agarose beads (Sigma), and eluted as previously described (12).

**Fusion Protein Phosphorylation—**GST-SAP97 purified fusion proteins were incubated with αCaMKII (1:50) (New England Biolabs, Beverly, MA) at 37 °C in the presence of 20 μM HEPES (pH 7.4), 10 mM MgCl2, 1 mM dithiothreitol, 2.4 μM calmodulin, 2 μM CaCl2 with 100 μM ATP (100 Ci/tube, 3000 Ci/mmol; Amershams BioSciences). The reaction was initiated by the addition of kinase solution to the reaction mixture. The reaction was carried out for 5 min and stopped by the addition of SDS sample buffer.

**Surface Expression Assays—**Chymotrypsin (Sigma) treatments and cross-linking experiments by means of BS3 (Pierce) were performed as previously described (13).

**Antibodies**—The following antibodies were used: mouse αCaMKII antibody and polyclonal GluR1 antibody were purchased from Chemicon International, Inc. (Temecula, CA); polyclonal SAP97 antibody, polyclonal SAP102 antibody, monoclonal PS97-95 antibody, and monoclonal protein disulfide-isomerase (DSI) antibody were purchased from Affinity BioReagents Inc. (Golden, CO); monoclonal SAP97 antibody was purchased from StressGen (Victoria, British Columbia, Canada); polyclonal anti-GFP and AlexaFluor 488, 555, 568, and 633 secondary antibodies were purchased from Molecular Probes (Eugene, Oregon).

## RESULTS

### Differential Distribution of MAGUK Family Members in Hippocampal Neurons—

We examined by confocal labeling the distribution pattern of SAP97 and SAP102 in cultured hippocampal neurons (Fig. 1, A–J). PSD-95 was used as a marker of postsynaptic structures. SAP97 displayed a diffuse labeling in the somatic cytoplasm; immunoreactivity was also present in dendrites where both diffuse and punctate staining are present. A moderate colocalization pattern with PSD-95 was observed, indicating the presence of endogenous SAP97 at the postsynaptic side of excitatory synapses (Fig. 1, A–D). SAP102 labeling revealed a more intense staining in the soma than in the dendrites (Fig. 1E). No punctate SAP102 labeling and colocalization with PSD-95 were detectable, suggesting the absence of SAP102 in dendritic spines.

Neuronal activation by means of 15 min of treatment with NMDA (50 μM) led to an increased punctate staining of SAP97 in the dendritic compartment, leading to a higher colocalization degree with PSD-95 (Fig. 1, F–J). Quantification of SAP97 punctate staining revealed a quantitative increase of SAP97 immunoreactivity in PSD-95-positive dendritic spines (Fig. 1K; p < 0.01, NMDA versus control). On the other hand, no modification of SAP102 immunostaining was induced by NMDA treatment (Fig. 1J).

To confirm that endogenous SAP97 was expressed in the postsynaptic compartment of cultured neurons and to confirm its trafficking after NMDA treatment, a biochemical approach was also used. TIF was obtained from control and NMDA-treated neurons (see Ref. 6) and protein levels measured in the homogenate and TIF. The same amount of proteins from homogenate and TIF was loaded on the SDS-PAGE for Western blot analysis. As shown in Fig. 1L, SAP97 is barely detectable in TIF in untreated neurons; NMDA treatment, confirming confocal experiments, significantly increases SAP97 immunostaining in TIF without affecting the total SAP97 protein level in the homogenate (p < 0.01; +57.2 ± 8.1%, NMDA versus control expressed as SAP97 ratio TIF/homogenate). No alteration of PSD-95 immunostaining in both homogenate and TIF was observed after NMDA treatment. No SAP102 signal was present in TIF. As expected, treatment of hippocampal cultures with NMDA leads to a higher staining of αCaMKII in TIF (14).

### CaMKII Inhibition Affects SAP97 Distribution in Hippocampal Neurons—

Previous data from our laboratory demonstrated that NMDA exposure, leading to a maximal CaMKII activation, produced an in vivo SAP97 phosphorylation in cultured hippocampal neurons; CaMKII inhibitor KN-93 reduced SAP97 phosphorylation to basal levels, indicating a key role for this kinase in SAP97 phosphorylation (6). These data and other recent evidence (10) identified SAP97 as substrate for CaMKII.

These observations led us to test whether CaMKII-dependent SAP97 phosphorylation is involved in SAP97 trafficking from intracellular compartments into dendritic shafts and spines. To this purpose, hippocampal cultures were exposed to NMDA in the absence or presence of specific CaMKII inhibitors. Two different strategies have been used to inhibit CaMKII: i) incubation with the competitive inhibitor KN-93, and ii) with the autoinhibitory peptide (AIP-2) fused to the antennapedia peptide (Ant). Both KN-93 (Fig. 2, D–F) and Ant-AIP-2 (Fig. 2, G–I) were able to modify SAP97 distribution, leading to a higher immunoreactivity of SAP97 within the soma and to a parallel decrease of SAP97 staining in the proximal and distal dendrites when compared with untreated neurons (Fig. 2, A–C). Quantification of the dendritic versus soma SAP97 immunostaining by measuring the relative fluorescence intensity revealed a significant decrease of SAP97 fluorescent signal in dendritic structures as a consequence of CaMKII inhibition.
Modulation of SAP97 Trafficking by CaMKII

**Fig. 1.** NMDA treatment influences SAP97 subcellular localization. Hippocampal neurons were either left untreated (A–E, control) or treated for 15 min with NMDA (F–J, 50 μM, fixed, and immunolabeled for SAP97 (green; A, B, F, G) or SAP102 (green; E, J) and PSD-95 (red). Scale bar, 10 μM. Higher resolution views shown in panels B–D and G–I. Areas of overlap appear yellow. K, quantification of the confocal experiment. NMDA treatment causes a significant increase in the percentage of SAP97 clusters on the total number of PSD-95-positive spines compared with control values (*, p < 0.01, NMDA versus control). L, Western blot analysis of the homogenate or TIF obtained from control or NMDA-treated high density hippocampal cultures. The same amount of proteins was loaded in each lane. NMDA treatment leads to a higher SAP97 localization in the TIF, leaving the total amount of SAP97 unaltered. CaMKII, but not PSD-95, immunostaining was more intense in the TIF of NMDA-treated cultures. Notably, SAP102 levels were undetectable in the TIF.

Because in control untreated neurons a pronounced SAP97 intracellular staining in the cell soma was observed, we performed colocalization experiments with an endoplasmic reticulum-specific marker, protein disulfide-isomerase; SAP97 showed a good colocalization with DSI in untreated cultures (Fig. 3, A–C; see Ref. 4). NMDA treatment led to a decreased colocalization between SAP97 and the ER marker in the cell soma (Fig. 3, D–F). Of relevance, coincubation with CaMKII inhibitor Ant-AIP-2 determined a high colocalization degree between SAP97 and the ER marker, DSI (Fig. 3, G–I).

It has been shown that calcium-induced calcium release from ER, usually associated to activation of ryanodine receptors (RyRs), is triggered by entry of calcium through NMDA receptor channels (15). Because it is known that CaMKII can be associated to RyRs (16, 17), we tested whether CaMKII-dependent modulation of SAP97 trafficking was correlated to activation of RyRs by examining the distribution pattern of SAP97 in neurons exposed to caffeine (Fig. 4). Caffeine treatment induced SAP97 trafficking to “spine-like” structures (as confirmed by double labeling of neurons with the postsynaptic protein marker PSD-95; data not shown), both in the absence and the presence of D-2-amino-5-phosphonopentanoic acid (Fig. 4A). In addition, RyRs blocking with high concentrations of ryanodine antagonized the effects of NMDA on SAP97 distribution (Fig. 4A), suggesting that calcium-induced calcium release from RyRs is necessary to trigger SAP97 trafficking. No...
Fig. 2. CaMKII inhibition leads to SAP97 redistribution toward the cell soma. Hippocampal neurons were either left untreated (A–C, Control) or exposed to KN-93 (D–F, 10 μM), Ant-AIP-2 (G–I, 10 μM), Ant-AIP-2 (10 μM) + NMDA (J–L, 50 μM), fixed, and immunolabeled for endogenous SAP97 (green) and PSD-95 (red). Merge data are shown on the right. Blocking CaMKII activity either by KN-93 or Ant-AIP-2 results in a dramatic loss of SAP97 staining from spines or distal dendrites paralleled by a redistribution of SAP97 itself toward the cell soma. PSD-95 localization is not influenced by CaMKII inhibition. Scale bar, 10 μM. M, CaMKII inhibition does not influence SAP102 subcellular localization; Ant-AIP-2-treated cultures were fixed and immunostained for SAP102. N, quantification of experiments shown in panels A–L. To this aim, the ratio of dendrites to cell soma average fluorescence was computed and averaged. *, p < 0.001 versus controls, **, p < 0.0005 versus control, analysis of variance. O, Western blot analysis of the TIF obtained from control or NMDA-treated cultures in the absence or presence of CaMKII inhibitor Ant-AIP-2. The same amount of proteins was loaded in each lane. NMDA treatment leads to a higher SAP97 localization in the TIF, leaving PSD-95 distribution unaltered. SAP97, but not PSD-95, immunostaining was decreased in the TIF of Ant-AIP-2-treated cultures.

effect on SAP102 immunostaining was observed as a consequence of the treatments indicated above acting on ER stores (data not shown). Also under these experimental conditions, coinubation of CaMKII inhibitor Ant-AIP-2 was able to block any effect of caffeine on SAP97 trafficking as tested both by confocal analysis (Fig. 4C; *, p < 0.0005 caffeine versus control, **, p < 0.001 caffeine + Ant-AIP-2 versus control expressed as the ratio of dendrites to cell soma average fluorescence) and by Western blotting in the TIF (Fig 4D; *, p < 0.01, +67.0 ± 8.3%, caffeine versus control; **, p < 0.005, −69.5 ± 4.3%, caffeine + Ant-AIP-2 versus control), confirming the central role of CaMKII in these events.

CaMKII-dependent Phosphorylation of SAP97-Ser-39 Affects SAP97 Trafficking into Spines—Modulation of SAP97 trafficking by CaMKII was further studied in hippocampal neurons cotransfected with GFP-SAP97wt and αCaMKIIwt or active-T286D αCaMKII (Fig. 5A). Four days after transfection, neurons were fixed and confocal analysis was performed. Cotransfection of GFP-SAP97 with αCaMKIIwt did not alter the characteristic GFP-SAP97 somatodendritic distribution (see Figs. 5A and 7A). However, cotransfection with active-T286D αCaMKII led to a GFP-SAP97 punctate staining with a concomitant reduction of immunoreactivity in the cell body and dendrites (Fig. 5A). In addition, single αCaMKIIwt or active-T286D αCaMKII transfections had similar effects on endogenous SAP97 (Fig. 5B).

Several CaMKII phosphorylation consensus domains are distributed along the SAP97 sequence (6, 9). Previous data from our laboratory addressed Ser-232 within the PDZ1 domain as a CaMKII in vivo phosphorylation site in SAP97. CaMKII-dependent phosphorylation of this site is responsible for the dynamic modulation of SAP97-NR2A complex (6). Inspection of the SAP97 sequence reveals the presence of another domain containing a serine residue (Ser-39 in the L27 N-terminal domain) that could represent a putative phosphate acceptor site for CaMKII (Fig. 6A). A fusion protein of GST with the N-terminal SAP97 (7–223) domain was obtained and incubated with recombinant αCaMKII (1–325) in the presence of [γ-32P]ATP as phosphate donor. Fig. 6B is a representative autoradiograph of an in vitro CaMKII-dependent phosphorylation of the SAP97 (7–223) GST fusion protein; a radioactive band at about 50 kDa corresponding to the fusion protein (upper arrow) is clearly visible, confirming the presence of the CaMKII phosphosites in the N-terminal domain of SAP97 (right lane). The phosphorylation signal is specific because GST alone did not show any phosphoband (left lane). The 37-kDa
phospho-Ser-39. SPAP97(S39D)-transfected neurons compared with all other constructs (Fig. 7B). Coimmunoprecipitation analysis of the TIF fractions purified from high density hippocampal cultures left untreated (control) or treated with caffeine (10 mM, 15 min) with or without CaMKII inhibition mediated by Ant-AIP-2. SAP97 subcellular distribution into this fraction, following these pharmacological treatments, confirms the subcellular localization observed by confocal microscopy.

To further investigate the molecular mechanism governing CaMKII-dependent trafficking of SAP97, we examined the spatial localization of transfected SAP97 constructs in hippocampal neurons, carrying point mutation into the previously identified CaMKII consensus sites (Fig. 7A). Single SAP97(S232D) mutation, mimicking phosphorylation, did not produce any significant effect on SAP97 localization in dendritic spines when compared with the wild type protein, suggesting that CaMKII phosphorylation of this site is not involved in SAP97 trafficking but only in the modulation of SAP97 interaction with NR2A as previously described (6). Similar results were obtained with the SAP97(S39A construct), whereas the SAP97(S39D) mutation led to an increased staining of SAP97 into “spine-like” structures (as confirmed by double labeling of neurons with the postsynaptic protein marker PSD-95; data not shown). Quantification analysis of the different SAP97 mutation constructs revealed a significative increase of spine-like clusters in SAP97(S39D)-transfected neurons compared with all other constructs (Fig. 7B). Colocalization experiments performed with synaptophysin indicated a specific postsynaptic immunoreactivity of SAP97(S39D) constructs in close opposition to the characteristic presynaptic synaptophysin staining (Fig. 7C). Moreover, double transfection of SAP97(S39A) + active-T286D αCaMKII showed that abrogation of the Ser-39 phosphosite is sufficient to block any effect of the active form of the kinase on SAP97 enrichment in punctate structures (Fig. 7D and see also Fig. 4A).

We then tested whether SAP97-Ser-39 was in vivo phosphorylated by CaMKII. GFP, GFP-SAP97wt, GFP-SAP97(S39A), or GFP-SAP97(S39A,S232A) were cotransfected in COS-7 cells together with αCaMKII-T286D. 48 h after transfection, we performed metabolic labeling with [32P]orthophosphate. Cell lysates were immunoprecipitated with anti-SAP97 antibody. Autoradiography revealed a significative reduction of 32P incorporation in SAP97(S39A)-transfected cells when compared with SAP97wt, indicating that Ser-39 represents a phosphorylation site in SAP97 under these experimental conditions (Fig. 6C, upper panel, −41.8% ± 6.3%, SAP97(S39A) versus SAP97wt, * p < 0.01). S39A,S232A mutation decreased CaMKII-dependent phosphorylation to barely detectable levels, indicating Ser-39 and Ser-232 as the two major in vivo CaMKII phosphosites in SAP97. Expression levels of all SAP97 constructs were comparable in all experimental conditions as tested by Western blotting using a polyclonal SAP97 antibody (Fig. 6C, lower panel).

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Modulation of SAP97 Trafficking by CaMKII

Abrogation of SAP97-Ser-39 Phosphosite Impairs AMPA Receptor Subunit GluR1 Spine Delivery—SAP97 has been shown to associate with the C-terminal PDZ binding domain of ionotropic glutamate receptor subunits in neurons (7). More recently, synaptic targeting of SAP97 has been demonstrated to lead to an increase in synaptic AMPA receptors, spine enlargement, and an increase in miniature excitatory post-synaptic current frequency (4), suggesting that SAP97 can affect the synaptic recruitment of AMPA receptors. We therefore asked whether the effect of CaMKII-dependent trafficking of endogenous and/or overexpressed SAP97 could affect synaptic targeting of endogenous GluR1-containing AMPA receptors.

Toward this aim, hippocampal neurons were transfected with GFP-SAP97 constructs and labeled with GluR1 antibody to identify subcellular distribution of GluR1-containing AMPA receptors (Fig. 8). Neurons transfected with GFP-SAP97(S39D) (Fig. 8B) showed a characteristic punctate GluR1 staining in spine-like structures with an apparent increase in the size of GluR1 clusters but with a very similar distribution to those of neighboring untransfected neurons (Fig. 8A). Similar data were obtained with GFP-SAP97wt (data not shown) in agreement with previous observations indicating an increase in the size of GluR1 clusters in GFP-SAP97-expressing neurons (4). On the other hand, transfection of GFP-SAP97(S39A) (Fig. 8C) resulted in a dramatic redistribution of GluR1 signal with an intense labeling in the cell soma and a diffuse dendritic staining, suggesting that CaMKII phosphorylation of SAP97-Ser-39 phosphosite can also be necessary for synaptic trafficking of SAP97-interacting proteins, i.e. GluR1. Quantification of spines versus dendritic shafts GluR1 immunostaining by measuring the relative fluorescence intensity revealed a significant decrease of GluR1 fluorescent signal in spine structures (p < 0.001, −48.7 ± 7.2%, GFP-SAP97(S39A) versus controls). In addition, experiments performed in untransfected neurons showed that inhibition of CaMKII by means of Ant-AIP-2 was responsible for a redistribution of endogenous GluR1 leading to a higher immunoreactivity within the soma and to a parallel decrease staining in the distal dendrites and spine-like structures (Fig. 8D). Quantification analysis revealed a significant decrease of GluR1 in spine structures as a consequence of CaMKII inhibition (p < 0.0005, −65.8 ± 6.8%, Ant-AIP-2 versus controls). We next examined the effects of CaMKII inhibition by treatment with Ant-AIP-2 on GluR1 surface expression. Control and Ant-AIP-2 hippocampal cultures were treated with the cross-linker BS3 or with chymotrypsin (Fig. 8E). A significant increase in the GluR1 intracellular pool was observed with both experimental devices (BS3, p < 0.01, +69.7 ± 10.3%, Ant-AIP-2 versus control; chymotrypsin, p < 0.01, +51.5 ± 9.8%, Ant-AIP-2 versus control).

DISCUSSION

The identification of molecular events governing the correct assembly of the different components of the glutamatergic synapse has emerged as a fundamental issue in the understanding of synaptic activity, plasticity, and neurodegenerative processes. In the last few years, several lines of evidence indicated
CaMKII activation as a key event in the regulation of glutamatergic synapses (18). In this report, we identify an additional role for CaMKII in modulating postsynaptic trafficking of SAP97, a member of the MAGUK protein family. In fact, our data show that SAP97-Ser-39 CaMKII-mediated phosphorylation is indeed necessary and sufficient to drive SAP97 to the postsynaptic compartment in cultured hippocampal neurons.

Recently, studies from our group demonstrated that CaMKII-dependent phosphorylation of SAP97-Ser-232 within the PDZ2 domain modulates the association/dissociation of the SAP97-NR2A complex (6), raising the possibility of novel strategies regulating SAP97 function in hippocampal neurons. Here we confirm and expand the concept that CaMKII activation or inhibition results in a direct regulation of SAP97 function; indeed, phosphorylation/dephosphorylation of Ser-39 entails changes in SAP97 distribution and, consequently, in SAP97 synaptic localization. Transfection in hippocampal neurons of SAP97 mutants that blocked or mimicked Ser-39 phosphorylation has effects similar to those observed upon inhibiting or constitutively activating CaMKII, thus clearly addressing SAP97-Ser-39 as necessary and sufficient for modulation of SAP97 trafficking by CaMKII. No effect is obtained when wild type enzyme is transfected, suggesting that it is not αCaMKII per se but rather kinase activation-autophosphorylation, the key molecular event in the modulation of SAP97 distribution. In addition, double S39A,S232A mutation abolished CaMKII-dependent phosphorylation of SAP97 in metabolic labeling experiments in transfected COS-7 cells. These data indicate the presence of two serine residues, in the L27 and PDZ1 domains, respectively, as major in vivo phosphosites in SAP97, supporting the idea that SAP97 acts as a multimodular element where distinct domains play differential roles for the correct delivery of excitatory glutamate receptors.

Together, these results demonstrate that the translocation of SAP97 to the postsynaptic compartment is regulated by CaMKII-dependent phosphorylation and consequently suggest a novel mechanism for the regulation of synaptic delivery of SAP97-interacting proteins, i.e. glutamate receptors. In fact, it is known that members of the MAGUK protein family have been implicated as major players in targeting and clustering of glutamate receptor subunits (1, 2). SAP97 interacts with GluR1 early in the biosynthetic pathway of GluR1-containing AMPA receptors; therefore, it may play a role in maturation of receptor complexes in the endoplasmic reticulum-cis-Golgi and delivery of receptors to synapses, but not in anchoring AMPA receptors at synapses (7, 8). In the last five years, several studies have been focused on understanding the multiple mechanisms by which AMPA receptor-mediated transmission is strengthened during long term potentiation, and the specific role of CaMKII in these mechanisms has been pointed out. Increased CaMKII activity, but not direct CaMKII phosphorylation of GluR1, was responsible for GluR1 delivery to the synapse (19, 20). This process requires interactions between GluR1 and PDZ domain-containing proteins. All these data suggest that some protein(s) other than GluR1 must be substrate(s) of CaMKII and participate in the regulated synaptic delivery of GluR1-containing AMPA receptors. Data presented here confirm and expand this hypothesis, identifying SAP97...
positive synaptic clusters. Biochemical fractionation experiments confirm confocal data showing a specific enrichment of SAP97 to a Triton-insoluble “PSD-like” fraction after NMDA activation when compared with the corresponding SAP97 level in TIF of untreated neurons. Co-treatment with different kinase inhibitors shows that SAP97 redistribution to spine-like structures is strictly dependent on CaMKII activation and specifically blocked by CaMKII inhibitors; in particular, treatment with CaMKII inhibitors leads to a strong colocalization pattern of SAP97 with the ER marker, DSI.

Recent observations indicate that activation of RyRs in the hippocampus can play a role in synaptic plasticity events through the elevation of CaMKII activity (16, 17), suggesting that CaMKII might represent a potential enzymatic target of the calcium-induced calcium release from ER ryanodine stores. Our data, showing that coinubcation with CaMKII inhibitor Ant-AIP-2 blocks any effect of caffeine on SAP97 trafficking, not only confirm a close relationship between calcium-induced calcium release and CaMKII function but also indicate the presence of different pools of CaMKII in hippocampal neurons activated upon different physiological stimuli.

Our data show that CaMKII affects SAP97 targeting by direct phosphorylation of SAP97-Ser-39. This phosphosite is located within the well described L27 N-terminal motif of SAP97 (22–24). It has been demonstrated that the L27 domain of SAP97 binds to Hrs, an endosomal ATPase that regulates protein sorting and has been implicated in vesicular endocytosis and exocytosis (22). In fact, the SAP97 (1–65) N-terminal domain, which is absent from PSD-95 and SAP102, has been shown to be responsible for subcellular membrane targeting of SAP97 in epithelial cells (24, 25), suggesting that the N terminus of SAP97 may also be involved in neuronal targeting. Our data strengthen the role of the SAP97 N-terminal domain in the modulation of SAP97 localization, identifying inside the L27 motif a specific CaMKII phosphosite that is not conserved in PSD-95 and SAP102.

However, our results do not exclude the possibility that other domains can affect subcellular targeting of SAP97 in neurons. Indeed, recent data showed that subcellular targeting of SAP97 to synaptic sites in primary dissociated hippocampal neurons is dependent on an alternatively spliced region between the SH3 and GK domains called the I3 region, a known protein 4.1 binding site (4). All these results suggest that SAP97 trafficking is a highly regulated mechanism of critical relevance for the correct delivery of excitatory glutamate receptors.

Together, our results confirm that CaMKII plays a central role in excitatory neurons and confirm that activation of CaMKII and consequent kinase autophosphorylation is crucial not only in the synaptic site but also in extrasynaptic compartments to initiate the biochemical cascade that potentiates synaptic transmission. In particular, our data show that CaMKII-dependent SAP97-Ser-39 phosphorylation regulates the association of SAP97 with the postsynaptic complex, thus providing a fine molecular mechanism responsible for the synaptic delivery of SAP97-interacting proteins, i.e. ionotropic glutamate receptor subunits.

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