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Short communication

Calcium- and calmodulin-dependent phosphorylation of AMPA type glutamate receptor subunits by endogenous protein kinases in the post-synaptic density

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Abstract

We have detected immunoreactivities of AMPA receptor subunits GluR1–4 in post-synaptic density (PSD) fraction and tested whether they can be phosphorylated by endogenous kinases. Incubation of PSD with Ca^{2+} and calmodulin increased phosphorylation of GluR1 and GluR2/3. The phosphorylation of GluR1 was largely blocked by a Ca^{2+}/calmodulin-dependent protein kinase type II inhibitor. Thus Ca^{2+}/calmodulin-dependent phosphorylation of glutamate receptor may be a mechanism underlying enhanced post-synaptic receptor responsiveness in LTP.

Keywords: α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid type glutamate receptor; Phosphorylation; Post-synaptic density; Excitatory amino acid; Calcium/calmodulin-dependent protein kinase; Synaptic plasticity

Long-term potentiation (LTP) of synaptic efficacy is a cellular model for learning and memory [3,4]. An increase in the responsiveness of post-synaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid type glutamate receptor (AMPA receptor) subsequent to Ca^{2+} influx has been suggested to underlie LTP at hippocampal CA1 synapses [7,10,16,22,23,34,35]. It has been proposed that AMPA receptor phosphorylation by Ca^{2+}-activated protein kinases plays a pivotal role in LTP expression [19]. Evidence for this hypothesis includes: (1) phosphorylation of the endogenous or recombinantly expressed AMPA receptor GluR1 subunit by Ca^{2+}/calmodulin-dependent protein kinase type II (CaMKII) or protein kinase C (PKC) [24,31]; (2) blockade of the *N*-methyl-D-aspartate (NMDA) receptor-mediated phosphorylation of AMPA receptor GluR1 subunit by a CaMKII inhibitor in cultured hippocampal cells [31]; (3) enhancement of AMPA receptor responses [24,36] and excitatory post-synaptic currents (EPSCs) [17] by intracellular injection of activated CaMKII; and (4) occlusion of the CaMKII-induced enhancement of EPSCs with LTP [17].

In order to elucidate further the phosphorylation of AMPA receptor, we utilized a biochemically isolated fraction of post-synaptic density (PSD), an electron-dense structure beneath post-synaptic membrane [13]. The PSD fraction contains various regulatory proteins, including CaMKII [12], PKC [33] adenylate cyclase, protein kinase A (PKA) and its anchoring protein AKAP-79 [6]; they are expected to be preserved with respect to their native integrity, such as protein conformation and mutual interaction. Using this preparation, we studied the Ca^{2+}- and calmodulin-dependent phosphorylation of AMPA receptor subunits.

PSD was purified as described by Sahyoun et al. [28] from rat cerebral cortex. In brief, the cerebral cortex was homogenized in 0.32 M sucrose, 1 mM $NaHCO_3$, 1 mM $MgCl_2$ and 0.5 mM $CaCl_2$ (solution A) and then centrifuged at $1000 \times g$ for 10 min. A part of the supernatant was centrifuged at $17\,000 \times g$ for 20 min to obtain a crude synaptosomal fraction. The remaining supernatant was ad-

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justed to 0.8 M sucrose by adding 2.0 M sucrose and 1 mM $NaHCO_3$ and then centrifuged at 48 000 × *g* for 1 h. The resultant pellet was homogenized in 1% Triton X-100, 10 mM Tris–HCl (pH 7.5), 0.16 M sucrose and respun at 48 000 × *g* for 1 h. The insoluble pellet was resuspended in solution A, overlaid on a discontinuous sucrose density gradient of 1.0, 1.5 and 2.0 M, respectively, and centrifuged at 230 000 × *g* for 2 h. The interface between the 1.5 and 2.0 M layers was recovered as a PSD fraction. All the above procedures were performed at 4°C.

The phosphorylation reaction by endogenous kinases in PSD fraction was carried out in following mixture: 20 μg PSD protein, 40 mM HEPES-NaOH (pH 7.5), 5 mM magnesium acetate, 0.1 mM EGTA, 0.4 mM $CaCl_2$, 1 μM calmodulin, 50 μM [γ-^{32}P]ATP (Amersham), 0.5 μM microcystin LR (in final volume 200 μl). To measure Ca^{2+}- and calmodulin-independent phosphorylation, $CaCl_2$ and calmodulin were omitted and 10 mM EGTA was added. The reaction was carried out at 30°C for 3 min unless otherwise stated and terminated by adding a final concentration of 0.5% SDS and 10 mM EDTA. In order to dissociate each subunit of the AMPA receptor, the mixture was denaturated at 100°C for 5 min [29]. Then, it was diluted by adding 4 vols. of 1.25% Nonidet P-40, 12.5 mM Tris–HCl (pH 7.4), 187.5 mM NaCl, 6.25 mM EDTA and 1.25 mM EGTA. In some experiments, the association of subunit was retained by omitting the denaturation step and solubilized by directly adding 4 vols. of 0.125% SDS, 1.25% Nonidet P-40, 12.5 mM Tris–HCl (pH 7.4), 187.5 mM NaCl, 6.25 mM EDTA and 1.25 mM EGTA followed by rotation at 4°C for 1 h. The solution from both sets of conditions was centrifuged at 18 500 × *g* for 20 min at 4°C to precipitate insoluble debris. Protein A Sepharose CL-4B (20 μl of 1 : 1 slurry, Pharmacia) was added to the supernatant to pre-absorb non-specific resin binding and the solution was again centrifuged at 18 500 × *g* for 5 min at 4°C. After reaction with anti-AMPA receptor antibodies (1 μg/sample, Chemicon International) at 4°C for 2 h to overnight with continuous rotation, the immunocomplex was absorbed onto Protein A Sepharose CL-4B resin (20 μl of 1 : 1 slurry) by further rotating at 4°C for 2 h. Finally, the resin was spun down and washed 3 × with 50 mM Tris–HCl (pH 7.4), 600 mM NaCl and 0.5% Triton X-100 with brief vortexing at each time. The resultant resin was subjected to SDS-polyacrylamide gel electrophoresis and subjected to autoradiography. For the quantification of phosphorylation, the radioactivity function of BAS-2000 (Fujifilm) was used. Autoradiographies were exposed for different durations and quantification was performed within linear range. The background value obtained from an appropriate portion of the same lane was subtracted. PKC-inhibitor peptide (Gibco-BRL/Life Technologies) was used at 2 μM; PKA-inhibitor peptide (Sigma), 5 μM; genistein (Gibco-BRL/Life Technologies), 10 μM; and autocamtide-2-related inhibitory peptide (AIP; custom-synthesized by Accord) [11], 10 μM. All the experiments were performed at least twice and one representative result is shown.

Coomassie brilliant blue staining patterns of the crude synaptosomal fraction and the PSD fraction revealed several protein bands (Fig. 1A) reported to be enriched in the PSD fraction [5,28]. Western blots of these preparations using antibodies against the AMPA receptor subunits clearly detected comparable immunoreactivities of GluR1, GluR2/3 and GluR4 in both crude synaptosomal and PSD fractions, although immunoreactivity against GluR4 was rather weak (Fig. 1B) (see also [1,27]).

We next carried out an immunoprecipitation of the AMPA receptor subunits under a non-denaturing condition such that the subunits keep their association. Immunoprecipitation under a denaturing condition, where the subunits

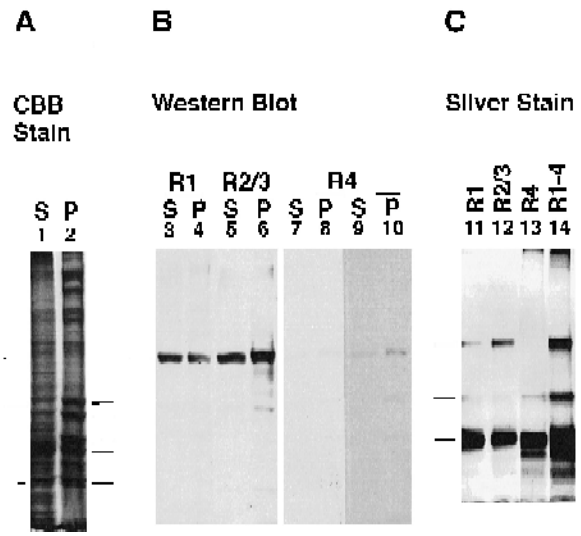

Fig. 1. Presence of AMPA-type glutamate receptors in the PSD fraction. (A) Coomassie brilliant blue staining patterns of the crude synaptosomal fraction (S, lane 1) and the PSD fraction (P, lane 2) each at 20 μg/lane. The bars on the left indicate the positions of the molecular-weight markers (97, 66 and 42 kd from top respectively). The bars on the right are major proteins found in PSD as identified by molecular weight and comparison with previous reports (protein 1a, protein 1b, tubulin, α-subunit of CaMKII, actin). (B) Western blotting of the crude synaptosomal (lanes 3, 5, 7 and 9) and PSD (lanes 4, 6, 8 and 10) fractions using antibodies for GluR1 (R1, lanes 3–4), GluR2/3 (R2/3, lanes 5–6) and GluR4 (R4, lanes 7–10), each used at 0.1 μg/ml. The same preparation as in (A) was used. Lanes 3–8: 2 min exposure. Lanes 9–10: 10 min exposure. The bar denotes a position of AMPA receptor subunit immunoreactivities. Note the enrichment of GluR2/3 and GluR4 in the PSD fraction compared with the crude synaptosomal fraction. (C) Silver staining of immunoprecipitated AMPA receptor subunits from the PSD fraction. For lanes 11–13, PSD fraction (20 μg) was denatured in the presence of 0.5% SDS at 100°C for 5 min and diluted five fold to give 0.1% SDS and 1% Nonidet P-40 (denaturing condition). AMPA receptor subunits were then immunoprecipitated using antibodies for GluR1 (lane 11), GluR2/3 (lane 12) and GluR4 (lane 13) (1 μg/sample each). For lane 14, the PSD fraction was solubilized with 0.1% SDS and 1% Triton X-100 at 4°C (non-denaturing condition). The AMPA receptor was immunoprecipited with the mixture of all three antibodies (R1–4). The bars on the left indicate from top AMPA receptor subunits, bovine serum albumin (a preservative of antibody) and the immunoglobulin heavy chain, respectively.

dissociate from each other, was also carried out (Fig. 1C). In agreement with the Western blotting, GluR1 and GluR2/3 were immunoprecipitated from PSD fraction whereas GluR4 was barely detectable (Fig. 1C, lanes 11–13). Since the antibody was used at the saturating concentration (1 μg for 20 μg PSD), the amount of immunoprecipitates is likely to reflect the amount of AMPA receptor subunit proteins. A mixture of three antibodies under the non-denaturing condition (Fig. 1C, lane 14) caused a marked immunoprecipitation more than the sum of the immunoprecipitates with each individual antibody in the denaturing condition. Since four subtypes of AMPA receptor are considered to form an oligomeric complex in PSD, it may behave as a multivalent antigen to facilitate the immunoprecipitation.

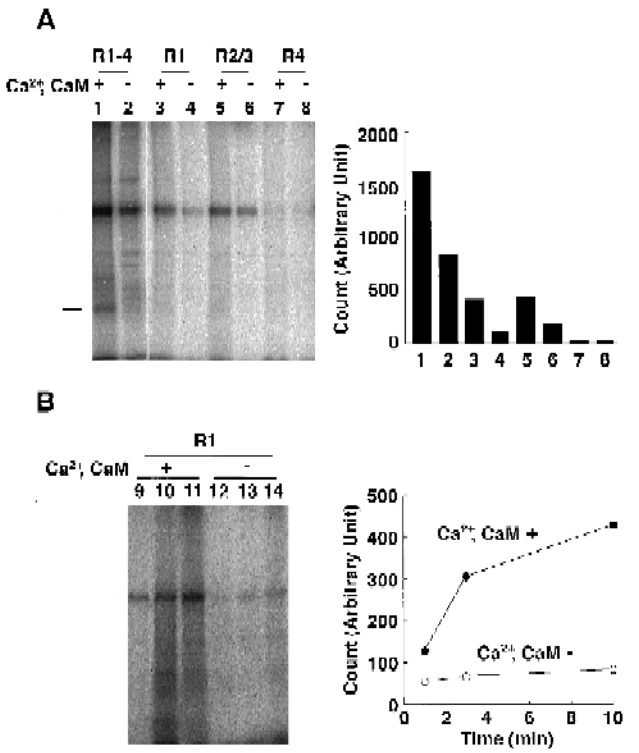

Fig. 2. Ca^{2+}- and calmodulin-dependent phosphorylation of AMPA receptor subunits by endogenous kinase(s) present in the PSD. (A) Phosphorylation of each subunit of AMPA receptor. Phosphorylation reaction was carried out in the presence of Ca^{2+} (0.4 mM) and calmodulin (CaM, 1 μM) (lanes 1, 3, 5 and 7) or in the presence of EGTA (10 mM) (lanes 2, 4, 6 and 8) without Ca^{2+} and calmodulin, for 3 min at 30°C. Then, the reaction mixture was immunoprecipitated with the mixture of antibodies for GluR1, GluR2/3 and GluR4 under non-denaturing conditions (lanes 1–2), anti-GluR1 (lanes 3–4), anti-GluR2/3 (lanes 5–6) and GluR4 (lanes 7–8) under denaturing condition. The bars on the left indicate AMPA receptor subunits (top) and autophosphorylated CaMKII α-subunit which was non-specifically precipitated (bottom). (B) The time course of phosphorylation of GluR1. The reaction was carried out in the presence of Ca^{2+} and calmodulin (lanes 9–11) or in the presence of EGTA without Ca^{2+} and calmodulin (lanes 12–14) for 1 min (lanes 9 and 12), 3 min (lanes 10 and 13) and 10 min (lanes 11 and 14). Each one representative autoradiography of experiments repeated at least twice is shown and graphical representations of the same data are shown in the right. Background was subtracted from an appropriate position of the same lane.

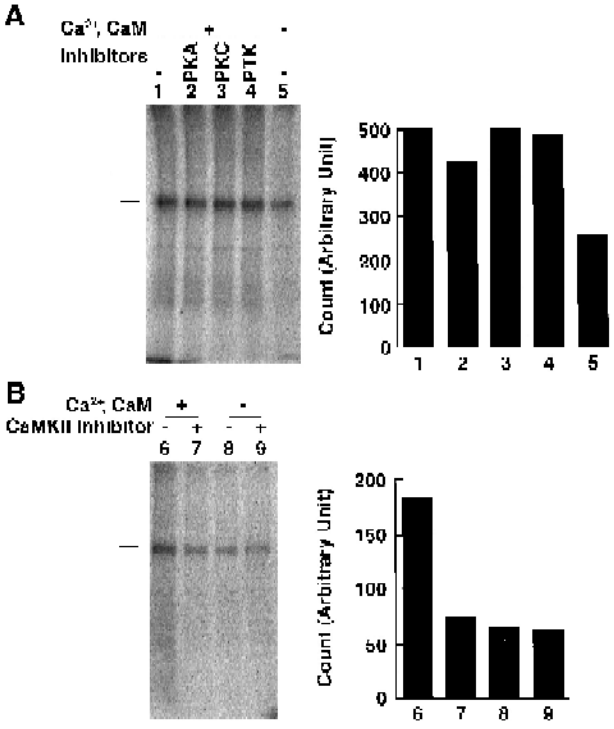

Fig. 3. Phosphorylation of GluR1 was inhibited by a CaMKII inhibitor. (A) Phosphorylation of GluR1 in the absence (lane 1) of inhibitors, or in the presence of a PKA inhibitor peptide (lane 2), a PKC inhibitor peptide (lane 3), genistein (a protein tyrosine kinase (PTK) inhibitor, lane 4), all with Ca^{2+} and calmodulin. Lane 5 is a control without Ca^{2+} and calmodulin but with 10 mM EGTA. The reactions were carried out for 3 min at 30°C. (B) Phosphorylation of GluR1 in the absence (lane 6) and in the presence (lane 7) of a CaMKII inhibitor (AIP, 10 μM). AIP did not affect background phosphorylation in the presence of EGTA without Ca^{2+} and calmodulin (lanes 8–9). The reactions were carried out in the presence of the PKA and PKC inhibitor peptides and genistein for 3 min at 30°C. The left bar, the AMPA receptor.

We examined whether the glutamate receptor subunits in the PSD fraction might be phosphorylated by endogenous kinase(s) activated by Ca^{2+} and calmodulin. The PSD fraction was incubated with Ca^{2+} and calmodulin in the presence of [γ-^{32}P]ATP, solubilized under either non-denaturing or denaturing conditions and the AMPA receptor subunits were immunoprecipitated. An autoradiography of immunoprecipitates under the non-denaturing condition revealed efficient Ca^{2+}/calmodulin-dependent phosphorylation of the glutamate receptor complex (Fig. 2A, lanes 1–2). In order to identify the subunit which underwent phosphorylation, the immunoprecipitation was carried out under the denaturing condition. The phosphorylation was found both in GluR1 and GluR2/3 but not in GluR4 subtypes (Fig. 2A, lanes 3–8). Presumably, the quantity of GluR4 in our preparation was too small for its phosphorylation to be detected in our experimental condition. The Ca^{2+}/calmodulin-dependent phosphorylation of GluR1 was detectable as early as 1 min after the start of incuba-

tion and its intensity increased with time. These results suggest that the glutamate receptor in the PSD can be phosphorylated by endogenous kinase(s) activated by Ca^{2+} and calmodulin. Various kinases, such as CaMKII, PKC [25], PKA [6] and tyrosine kinase [15], can be potentially activated by Ca^{2+} in PSD. By using kinase type-specific inhibitors, we examined which of these kinases participate in the phosphorylation of GluR1 in the PSD fraction. The PKA inhibitor peptide reduced the phosphorylation of GluR1 by $\approx$30%, whereas the inhibitors for PKC or tyrosine kinase had no effect on GluR1 phosphorylation (Fig. 3A). In the presence of inhibitors for PKA, PKC and tyrosine kinase, the CaMKII inhibitor AIP [11] clearly inhibited the remaining GluR1 phosphorylation down to the background level (Fig. 3B). AIP had no effect on the background phosphorylation in the absence of Ca^{2+} and calmodulin. Thus, the Ca^{2+}- and calmodulin-stimulated phosphorylation of GluR1 in PSD can be accounted for mainly by CaMKII.

We utilized the PSD fraction to study the phosphorylation of AMPA receptors, firstly because it contains various regulatory factors [13] and also because it is known to preserve structural integrity as evidenced by electron microscopic analyses [5,28]. Thus, native interactions of proteins are expected to be intact. It also allows access of various reagent molecules, such as peptide inhibitors, to the target molecules.

Our PSD fraction retained a substantial amount of AMPA receptor subunit proteins, although most of the AMPA receptor proteins could be eluted in a soluble fraction under different conditions, such as temperature during treatment and detergent/protein ratio [32]. Since most lipid bilayer membrane would be disrupted in the PSD fraction by detergent treatment, AMPA receptor subunits might be anchored in PSD fraction by some structural proteins which are relatively resistant to detergent. In fact, the NMDA receptor has been reported to anchor with PSD-95 [14].

It has been controversial as to whether GluR1 is phosphorylated by CaMKII [26]. However, our study clearly demonstrated that GluR1 and GluR2/3 in the PSD fraction were phosphorylated in a Ca^{2+}/calmodulin-dependent manner. The phosphorylation of GluR1 was mostly inhibited by AIP, a specific inhibitor for CaMKII, but not by the inhibitors for PKC and protein tyrosine kinase. These results indicate that the AMPA receptor subunit GluR1 can be a substrate for CaMKII.

The phosphorylation of GluR1 was also inhibited by a PKA inhibitor peptide although to a lesser extent. This is consistent with what has been reported by Huganir and his colleagues in cultured cortical neurons and HEK293 cells [2,26]. Since our results were obtained in the condition favoring CaMKII activation, possible contributions of other kinases to AMPA receptor phosphorylation in the PSD remain to be seen.

Among various kinases present in the PSD, the regulatory role of CaMKII on the phosphorylation of glutamate receptor attracts much attention as a potential mechanism for expression of LTP. At hippocampal CA1 synapses, post-synaptic CaMKII activity is reported to be necessary [18,20,21,30] and sufficient [17] for the induction of LTP. Our present findings together with previous reports [24,26] support the hypothesis that the phosphorylation of AMPA receptor subunits by CaMKII is essential for LTP induction.

The mechanism by which AMPA receptor phosphorylation increases receptor responsiveness in LTP remains unclear. It can be mediated by a change in single channel properties of AMPA receptor [9] or by conversion of a non-conducting (or silent) receptor channel to a conducting (active) one. Alternatively, the subcellular distribution of AMPA receptor subunits may be modulated by phosphorylation, as reported for the NMDA receptor NR1 subunit [8], thereby up-regulating the functional AMPA receptor at the post-synaptic membrane. Whatever is the mechanism, phosphorylation of the AMPA receptor by CaMKII in PSD may be an essential mechanism underlying LTP.

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