In Vitro Reconstitution of a Memory Switch of CaMKII by NMDA Receptor-Derived Peptide

Running title: Reconstitution of a CaMKII Memory Switch

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Abstract

Ca^{2+}/Calmodulin (Ca^{2+}/CaM)-dependent protein kinase II (CaMKII) has been shown to play a major role in establishing memories through complex molecular interactions including phosphorylation of multiple synaptic targets by CaMKII. However, it is still controversial whether CaMKII itself serves as a molecular memory or not because of a lack of the direct evidence. Here we show that a single holoenzyme of CaMKII *per se* serves as an erasable molecular memory switch. We reconstituted Ca^{2+}/CaM-dependent CaMKII autophosphorylation in the presence of protein phosphatase 1 (PP1) *in vitro*, and found that CaMKII phosphorylation shows a switch-like response with history dependence (hysteresis) only in the presence of an *N*-methyl-*D*-aspartate (NMDA) receptor-derived peptide, but not in its absence. This hysteresis is Ca^{2+}- and protein phosphatase 1 (PP1)-concentration dependent, indicating that the CaMKII memory switch is not simply caused by NMDA receptor-derived peptide lock of CaMKII in an active conformation. Mutation of a phosphorylation site of the peptide shifted the Ca^{2+} range of hysteresis. These functions may be crucial for induction and maintenance of long-term synaptic plasticity at hippocampal synapses.

Introduction

In the brain, Ca^{2+}/Calmodulin (Ca^{2+}/CaM)-dependent protein kinase II (CaMKII) has been shown to play a critical role in our learning and memory including synaptic long-term potentiation (LTP) (1-3). CaMKII has been theoretically hypothesized as a 'memory switch', which transforms a transient signal into persistent LTP. CaMKII forms a large symmetrical holoenzyme in which 12 kinase domains are tightly packed around a central ring-shaped scaffold (4, 5), and the cooperative autophosphorylation by neighboring subunits forms two stable steady states: a dephosphorylated 'OFF' state and a phosphorylated 'ON' state at threonine 286 (T286) (6, 7). By LTP inducing stimulation, a transient Ca^{2+} signal switches CaMKII from a dephosphorylated 'OFF' state to a phosphorylated 'ON' state for establishing memories.

To realize such a memory switch at synapses, the interaction between CaMKII and an *N*-methyl-*D*-aspartate (NMDA) receptor subunit GluN2B has been shown to be critically important. The interaction of CaMKII and GluN2B is required for persistent activation and translocation of CaMKII (8, 9), persistent T286 phosphorylation (10), and LTP maintenance (11, 12). Thus, the interaction between CaMKII and GluN2B is a key factor of the memory formation. However, the interaction between CaMKII and GluN2B as a memory switch has not been well described.

Here, in an *in vitro* reconstitution system (13), we report that CaMKII operates as a memory switch only in the presence of a GluN2B-derived peptide (residues 1,289-1,310; N2Bs), but not in its absence. An essential property of a memory switch, hysteresis, shows that the Ca^{2+} range of the memory switch is regulated by the phosphorylation of N2Bs at serine 1303 (S1303). Our results demonstrate that this CaMKII system can be a minimal component of an erasable memory switch, and no

complex molecular interaction with multiple synaptic targets is needed for enabling this function.

Materials and Methods

CaMKII expression and purification

Wild-type rat alpha-CaMKII cDNA and baculovirus containing alpha-CaMKII cDNA were kindly provided by Dr. Yasunori Hayashi (RIKEN Brain Science Institute) (14) and Haruhiko Bito (University of Tokyo). The virus containing alpha-CaMKII was added to 175-cm^2 monolayer cultures of Sf21 cells for 60–72 h, and the cells were then harvested and stored at −80 °C until protein purification (13, 15-18).

Alpha-CaMKII was purified essentially as described (13, 15-18). Briefly, cell pellets were resuspended in lysis buffer (50 mM PIPES at pH 7.0, 1 mM EGTA, 1 mM EDTA, 2.5% Betaine, and 1× complete protease inhibitor cocktail (Roche, Basel, Switzerland)) (15), lysed by brief sonication, and clarified by centrifugation at 100,000 × g, 4°C, for 0.5 hour. The supernatant was loaded onto a 2-ml phosphocellulose column (P11 cation exchange resin; GE Healthcare, Buckinghamshire, UK) equilibrated with wash buffer (50 mM PIPES at pH 7.0, 200 μM EDTA, 100 mM NaCl, and 1× complete protease inhibitor cocktail) (18). The column was washed in the wash buffer, and CaMKII was eluted with Wash buffer plus 500 mM NaCl. The eluted fractions were then applied to a Sephacryl S300 Sepharose gel filtration column (GE healthcare) that had been equilibrated in 30 mM MOPS at pH 7.2, 200 mM KCl, 10 μM EDTA, and 1 mM 2-mercaptoethanol (Gel filtration buffer) (18). The CaMKII-containing fractions were collected, and concentrated by a centrifugal filter unit (NMWL: 100 kDa, Amicon Ultra-4; Millipore, Bedford, MA, USA) with gel filtration buffer plus 50% glycerol. Thirty-50 μM CaMKII (subunit concentration) was subdivided in 1.5 ml tubes, and stored at −80°C until use.

Protein phosphatase 1 (PP1) expression and purification

The His-tagged PP1a catalytic subunit cDNA in the vector pDR540 was kindly provided by Dr Angus Clark Nairn (Yale University) (13, 19, 20). *Escherichia coli* BL21 cells harboring the vector were grown at room temperature in 1 liters of LB medium with 50 mg/ml ampicillin and 100 mM $CoCl_2$ until OD_{600} = 0.4, and the expression of PP1 was induced overnight with 0.5 mM isopropyl β-D-thiogalactoside. The cells were harvested, and resuspended in 10 mM Tris-HCl at pH 8.0, 30 mM imidazole, 10% glycerol, 300 mM NaCl, 1 mM $CoCl_2$, and 1 mM phenylmethylsulfonyl fluoride (resuspension buffer). The cell suspension was lysed by 30-s sonication, and centrifuged at 30,000 × *g*, 4°C for 30 min. The supernatant was loaded onto a 2-ml Ni-NTA agarose column (Qiagen, Hilden, Germany) equilibrated with resuspension buffer. The column was washed with wash buffer containing 30 mM MOPS, pH 7.2, 200 mM KCl, and 10% glycerol, and PP1 was eluted with wash buffer plus 400 mM imidazole. The PP1 containing fractions were pooled, applied to a HiTrap desalting column (GE healthcare) equilibrated with wash buffer to remove imidazole, and concentrated using a centrifugal filter unit (NMWL: 10 kDa, Amicon Ultra-4; Millipore). Approximately 5 μM PP1 was subdivided in 1.5 ml-tubes, and stored at −80 °C until use. Our PP1 had K_m = 10 μM and k_{cat} = 0.043 s^{-1} for dephosphorylation of CaMKII at T286.

Preparation of CaMKII phosphorylated at T286

After incubation with 19 μM free Ca^{2+} for 10 min at 4°C, almost all CaMKII showed an upward band shift in Phos-tag western blotting (21), indicating phosphorylation. The shifted band reacted to a mouse monoclonal antibody 22B1, which specifically

recognizes CaMKII phosphorylation at T286. We used this preparation of CaMKII phosphorylated at T286 as a phosphorylated 'ON' state for 'coming down' experiments. We used a 2-fold concentrated system of Ca^{2+}-EGTA buffer for the preparation of indicated free Ca^{2+} solutions (Figs. 1-3; Figs. S1 and S2) (13), and calculated the free Ca^{2+} concentrations based on a fluorescence titration with Fluo-4 (the dissociation constant of Ca^{2+} for EGTA, 110 nM; Invitrogen, Carlsbad, CA, USA).

CaMKII autophosphorylation assay

CaMKII autophosphorylation experiments were performed based on a previous study (13) at 4° C in 20 μL of 2 μM CaM, 0.025 μM PP1, 2 μM CaMKII (subunit concentration), 50 μM N2Bs or Ctl, 5 mM Ca^{2+}-EGTA, 4 mM $MgCl_2$, 2 mM ATP, 200 mM KCl, 30 mM MOPS at pH 7.2, and 0.1% w/v BSA (standard condition). All reactions in this study were performed under the standard condition, unless specified. N2Bs and Ctl were peptides derived from residues 1,289-1,310 (KAQKK NRNKL PRQHS YDTFV DL) and 1,095-1,119 (SAKSR REFDE IELAY RRRPP RSPDH) of GluN2B, respectively (GL Biochem, Shanghai, China) (8), and CaM was purchased from Wako Pure Chemicals (Osaka, Japan). For 'coming down' experiments from a phosphorylated 'ON' state of CaMKII, 8.4 μL of solution A (2.38× solution of CaM, PP1, CaMKII, N2Bs/Ctl, and BSA in 400 mM KCl and 30 mM MOPS at pH 7.2) was first added to 5 μL of solution B (2× solution of Ca^{2+}-EGTA in 30 mM MOPS at pH 7.2) (Calcium Calibration Buffer Kit, Invitrogen). Then, 1.6 μL of solution C (12.5× solution of $MgCl_2$ and ATP in 200 mM KCl and 30 mM MOPS at pH 7.2) was added to initiate a reaction for preparation of a phosphorylated 'ON' state of CaMKII. The mixture of solutions A, B and C gave 19 μM free Ca^{2+} in 15 μL of 2.7 μM CaM, 0.33

μM PP1, 2.7 μM CaMKII, 67 μM N2Bs or Ctl, 3.3 mM Ca^{2+}-EGTA, 5.3 mM $MgCl_2$, 2.7 mM ATP, 267 mM KCl, 30 mM MOPS at pH 7.2, 0.13% w/v BSA, and CaMKII was phosphorylated at T286 for 10 min incubation (21). Then, 5 μL of solution D (2× solution of K^+-EGTA and Ca^{2+}-EGTA mixture in 30 mM MOPS at pH 7.2) (Calcium Calibration Buffer Kit, Invitrogen) was added to obtain the final reaction condition with indicated free Ca^{2+} concentration. For 'going up' experiments from a dephosphorylated 'OFF' state of CaMKII, the order of addition of solutions C and D was reversed, and solutions A, B, D and C were sequentially added.

Autophosphorylation reactions were stopped by applying the reaction buffer to Laemmli's sodium dodecyl sulfate (SDS) sample buffer, and the samples were subjected to SDS polyacrylamide gel electrophoresis, followed by immunoblotting with a mouse monoclonal antibody 22B1, which specifically recognizes CaMKII phosphorylation at T286. T286 phosphorylation were quantified by the software Phoretix 1D (TotalLab, Newcastle upon Tyne, UK). The Ca^{2+} dose-responses of CaMKII phosphorylation were fitted by the Hill equation:

$$f_{\mathrm{Hill}}(\mathrm{Ca}^{2+}) = \frac{A_{\min} \cdot [\mathrm{EC}_{50}]^{n_h} + A_{\max} \cdot [\mathrm{Ca}^{2+}]^{n_h}}{[\mathrm{EC}_{50}]^{n_h} + [\mathrm{Ca}^{2+}]^{n_h}},$$

where $A_{\max}$ and $A_{\min}$ are the maximum and minimum of T286 phosphorylation, respectively, n_h is the Hill coefficient, and EC_{50} is the half maximal effective concentration.

For the kinase inhibitor (K252a) experiment, 4 μl of 50 uM K252a in 25% v/v DMSO was added to 40 μl of the solution mixture at 3h after the initiation of reaction (Fig. 1*B*). A control experiment was also performed with the same solution without K252a.

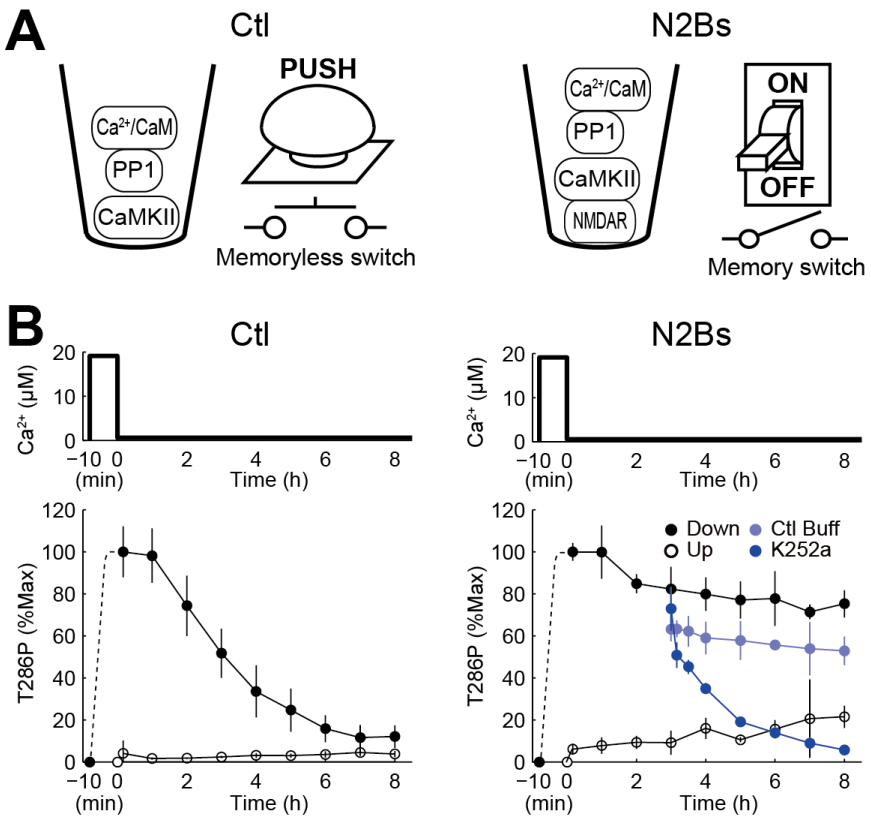

FIGURE 1 *In vitro* reconstitution of a memory switch comprising CaMKII and N2Bs. (***A***) *In vitro* reconstitution system with a control peptide (Ctl) (GluN2B 1,095-1,119), where CaMKII functions as a memoryless switch (left), and with N2Bs (GluN2B 1,289-1,310), where CaMKII functions as a memory switch (right). (***B***) Ca^{2+} pulse stimulation (black); 20 μM Ca^{2+} for 10 min and 0.56 μM (basal Ca^{2+} concentration) afterwards (top). T286 phosphorylation of CaMKII (T286P) with Ctl (bottom, left) or with N2Bs (bottom, right) in response to the pulse stimulation (filled circles) and to the basal Ca^{2+} concentration (open circles). The CaMKII kinase inhibitor (K252a) (dark blue circles) or control buffer (light blue circles) was added at 3 h. Error bars indicate mean ± SD ($n \geq 3$).

Results

Memory switch devices usually have two stable states (‘ON’ and ‘OFF’ states), and store information by switching between them. A push button is a typical memoryless switch because it responds just during stimulation (Fig. 1*A*, left), whereas a toggle switch is a typical memory switch because it keeps its response persistently even after stimulation (Fig. 1*A*, right). To examine whether CaMKII can actually serve as a memory switch, we reconstituted CaMKII phosphorylation *in vitro* based on an earlier study (13). Purified CaMKII together with CaM, PP1, adenosine triphosphate (ATP), and $MgCl_2$ was incubated in a Ca^{2+}-EGTA buffer system at 4° C where free Ca^{2+} concentration could be controlled (Materials and Methods, Fig. 1*A* and *B*). We examined CaMKII phosphorylation in the presence of a control peptide (residues 1,095-1,119 of GluN2B, Ctl) or the GluN2B-derived peptide that has been shown to interact with CaMKII (residues 1,289-1,310, N2Bs) (8). Consistent with the previous finding (13), in the presence of Ctl, the addition of a Ca^{2+} pulse transiently increased phosphorylation of CaMKII at T286, which subsequently decreased to a basal level (Fig. 1*B*, left black line). By contrast, in the presence of N2Bs, phosphorylation of CaMKII at T286 increased and was persistently retained (Fig. 1*B*, right black line). The persistent phosphorylation was blocked by the addition of K252a, a CaMKII inhibitor, indicating that the persistent phosphorylation requires the kinase activity of CaMKII, and is not due to irreversibility or slow dephosphorylation. The persistent phosphorylation is consistent with the previous finding that N2Bs locks CaMKII in an active conformation *in vitro* (8, 9). However, unlike the previous finding, the persistent phosphorylation requires basal Ca^{2+} concentration (0.56 μM). If the basal Ca^{2+} concentration was set below 0.2 μM, the phosphorylation of CaMKII at T286 decreased to a basal level within

6 hours even in the presence of N2Bs (Fig. S1*B*, T286P at 0.13 μM Ca^{2+}) .

The essential property of a memory switch is hysteresis, which is dependence of a state not only on its current environment but also on its past environment (22). We therefore examined whether CaMKII phosphorylation exhibits hysteresis in our reconstitution system, which can be seen as a distinctive splitting of Ca^{2+} dose-response curves for 'coming down' from a phosphorylated 'ON' state and for 'going up' from a dephosphorylated 'OFF' state (13, 22). We measured the 'going up' Ca^{2+} dose-response from a dephosphorylated 'OFF' state to a phosphorylated 'ON' state by increasing Ca^{2+}, and the 'coming down' Ca^{2+} dose-response from a phosphorylated 'ON' state to a dephosphorylated 'OFF' state by decreasing Ca^{2+} (Fig. 2*A*). As previously described (13), in the presence of Ctl, the 'going up' and 'coming down' Ca^{2+} dose-response curves exhibited similar switch-like responses with the same EC_{50}s (Fig. 2*A*, left; Fig. S1*A*), which give 50% of the maximum response. By contrast, in the presence of N2Bs, although both Ca^{2+} dose-response curves exhibited similar switch-like responses, the 'coming down' Ca^{2+} dose-response curve shifted to the left of the 'going up' Ca^{2+} dose-response curve (Fig. 2*A*, center; Fig. S1*B*). This result indicates that CaMKII phosphorylation exhibits hysteresis, meaning that once phosphorylated, CaMKII phosphorylation can be maintained at a Ca^{2+} concentration between 0.2 μM and 1.0 μM at 6 hour after the initiation of a reaction, which is lower than the EC_{50} of the 'going up' Ca^{2+} dose-response curve, and can remember the previous Ca^{2+} concentration (23). The hysteresis is apparently a Ca^{2+} concentration-dependent process. Furthermore, by increasing PP1 concentration, the hysteresis completely disappeared even with N2Bs (Fig. 2*A*, right; Fig. S1*C*), indicating that the hysteresis is a PP1-dependent process. Thus, hysteresis of CaMKII phosphorylation is a Ca^{2+}- and PP1-dependent reversible

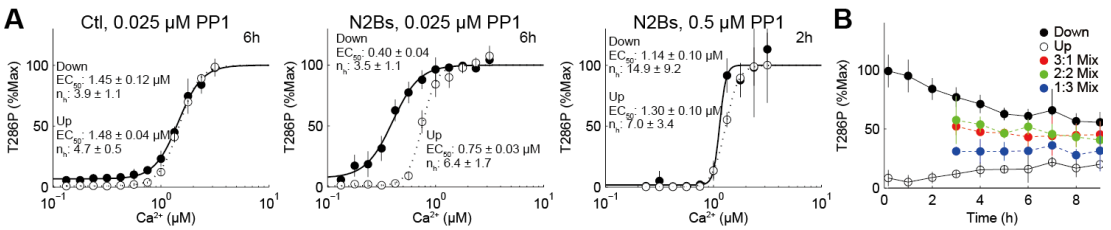

FIGURE 2 Hysteresis of T286 phosphorylation in the presence of N2Bs. (***A***) 'Coming down' Ca^{2+} dose-response curve of T286 phosphorylation (T286P) from a phosphorylated 'ON' state (filled circles) and that of 'going up' from a dephosphorylated 'OFF' state (open circles) in the absence (left) or the presence of N2Bs (middle and right). The concentrations of PP1 were 0.025 μM (left and middle) and 0.5μM (right), and the incubation time was 6 h. Solid and dotted lines denote the best fits to the Hill equation. The EC_{50}s and Hill coefficients (n_h) are indicated with 95% confidence intervals. (***B***) T286 phosphorylated (black) and dephosphorylated (white) CaMKII at 3 h was mixed with ratios of 3:1 (red), 2:2 (green), or 1:3 (blue).

process. Together with the requirement of CaMKII kinase activity for persistent phosphorylation (Fig. 1*B*), this result suggests that the persistent phosphorylation is due to a regenerative cycle of phosphorylation and dephosphorylation, rather than direct CaMKII activation by N2Bs-binding. It has been reported that, once CaMKII becomes activated by Ca^{2+}/CaM in the absence of PP1, N2Bs keeps CaMKII persistently activated even after Ca^{2+} removal by the addition of EGTA (8). This irreversible activation of CaMKII by N2Bs-binding is a Ca^{2+}- or PP1-independent process. Therefore, the hysteresis in this study is likely to be a different process from the previous observation in terms of Ca^{2+}- and PP1-dependent reversibility (8). The hysteresis persisted up to 17 hours, although the difference in Ca^{2+} sensitivities (EC_{50}) gradually decreased (Fig. S1). In addition, we did not observe the PP1 dose-dependent shift of the EC_{50}, unlike the findings of a previous study (13). This PP1

dose-dependency of the EC_{50} may be due to the effect of imidazole in the PP1 solution, because imidazole has a large ionic strength, and chelates Ca^{2+}, resulting in lowering free Ca^{2+} concentration. Imidazole may shift the apparent EC_{50} under the conditions (13). We removed imidazole by a desalting column (Materials and Methods) and did not see the PP1-dependency of the EC_{50}.

We then analyzed whether persistent phosphorylation occurs in an intermolecular or intramolecular fashion. Phosphorylated 'ON' and dephosphorylated 'OFF' states of CaMKII were mixed with various ratios. The ratio between phosphorylated and non-phosphorylated CaMKII did not change for more than 6 hours after mixing (Fig. 2*B*), suggesting that cross-phosphorylation between holoenzymes did not occur, and that the persistent phosphorylation occurred only by autophosphorylation within a holoenzyme. This result is consistent with the previous observation that cross-phosphorylation between holoenzymes does not occur (24, but see 25).

N2Bs contains S1303 of GluN2B, a major phosphorylation site by CaMKII, and S1303 phosphorylation has been reported to interfere with CaMKII binding to GluN2B (26-28). Indeed, the phospho-mimetic (S1303D) and dephospho-mimetic (S1303A) mutants of N2Bs have been shown to have lower and higher binding affinities to CaMKII, respectively (26-28). We asked whether such N2Bs mutants affected the hysteresis of CaMKII phosphorylation by using two mutant N2Bs peptides, S1303A and S1303D. The 'going up' Ca^{2+} dose-response curve in the presence of N2Bs (S1303A) shifted left (Fig. 3*A*, dashed line; Fig. S2*A*), and in the presence of N2Bs (S1303D) shifted right (Fig. 3*C*, dashed line; Fig. S2*C*), compared with that in the presence of N2Bs (WT) (Fig. 3*B*, dashed line; Fig. S2*B*), suggesting that dephosphorylated N2Bs is more effective at triggering phosphorylation of CaMKII than phosphorylated N2Bs.

This result is consistent with the previous observation of the lower and higher binding affinities of dephospho- and phospho-mimetic N2Bs to CaMKII, respectively (26-28). By contrast, the 'coming down' Ca^{2+} dose-response curves appeared the same in the presence of any type of N2Bs (Fig. 3, solid lines; Fig. S2), indicating that the mutation of N2Bs affects only the 'going up' process but not the 'coming down' process. Importantly, the hysteresis appeared regardless of the amino acid mutation of S1303 (Fig. 3; Fig. S2). As a result, in the presence of N2Bs (S1303A), CaMKII had a narrower Ca^{2+} range of hysteresis, whereas, in the presence of N2Bs (S1303D), CaMKII had a broader Ca^{2+} range of hysteresis. This heterogeneity in Ca^{2+} range of hysteresis may contribute to a modifiable Ca^{2+} threshold for memory induction and persistent memory maintenance such as metaplasticity (29) in concert with other signaling mechanisms (30).

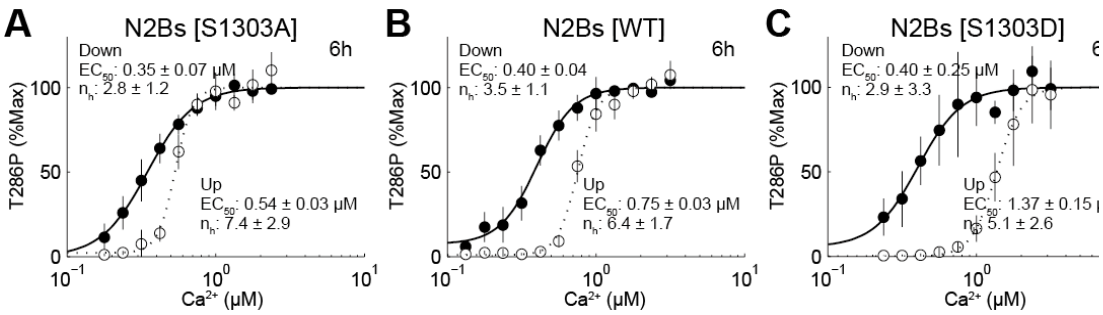

FIGURE 3 N2Bs phosphorylation state regulates the Ca^{2+} range of hysteresis. 'Going up' (filled circles) and 'coming down' (open circles) Ca^{2+} dose-response curves in the presence of the nonphospho-mimetic mutant peptide (S1303A) (***A***) or the wild-type peptide (WT) (***B***), or the phospho-mimetic peptide (S1303D) (***C***). Solid and dotted lines denote the best fits to the Hill equation. The EC_{50}s and Hill coefficients (n_h) are indicated with 95% confidence intervals.

Discussion

Accumulating experimental evidence has clearly shown the requirement of interaction between CaMKII and GluN2B in hippocampal LTP. The disruption of CaMKII and GluN2B interaction prevents synaptic translocation of CaMKII (9), persistent T286 phosphorylation (10), LTP induction (10, 31), LTP maintenance (11, 12), and extracellular signal-regulated kinase (ERK)-dependent synaptic structural plasticity (32). The CaMKII and GluN2B interaction also controls spatial learning in behaving mice (10, 33). Together with the function of the postsynaptic density (PSD) to protect CaMKII from dephosphorylation (34), the CaMKII memory switch can provide a theoretical basis of maintenance of modifiable and reversible LTP in hippocampus (11, 12). Maintenance of modifiable and reversible LTP cannot be attained by simple irreversible binding reactions between molecules. Such irreversible binding reactions easily fall in a single stable steady state by noisy molecular interactions, and the coupled molecules cannot function as a memory device. By contrast, the CaMKII memory switch accompanies Ca^{2+}- and PP1-concentration dependent hysteresis (Figs. 2 and 3). With this hysteresis, a dephosphorylated ‘OFF’ state of CaMKII is robust against temporal noise of basal Ca^{2+} concentration, and the dephosphorylated ‘OFF’ state switches to a phosphorylated ‘ON’ state only when a Ca^{2+} signal exceeds a modifiable threshold for LTP. Similarly, a phosphorylated ‘ON’ state of CaMKII is robust against temporal noise of basal Ca^{2+} concentration, and the phosphorylated ‘ON’ state switches to a dephosphorylated ‘OFF’ state only when PP1 concentration increases. Such phosphatase-dependent reversal of LTP (depotentiation) has been observed (35, 36), and described by a modeling study (37).

A FRET experiment has shown that CaMKII is not persistently but transiently

activated in spines by LTP inducing stimulation (38), supporting the idea that CaMKII does not serve as a memory switch. Similarly, a theoretical study has revealed that a CaMKII memory switch without GluN2B does not operate under physiological conditions (39). Our result indicates that the memory switch of CaMKII phosphorylation can be seen only in the presence of GluN2B-derived peptide (N2Bs), but not in its absence. This result supports the idea that GluN2B-associated CaMKII constitutes only a subpopulation of total spine population in LTP, and the signal of GluN2B-associated CaMKII activation is below the detectable level of multiphoton microscopy (3, 10, 38). Although this idea can account for the discrepancy among experiments (10, 11, 38), further studies in actual synapses are necessary to clarify the precise roles of CaMKII and GluN2B in LTP.

Hysteresis in Ca^{2+} sensitivity eventually disappeared under our experimental conditions (Figs. S1 and S2). This may raise the possibility that hysteresis is derived from slow convergence to a monostable state, rather than the bistable dynamics in the dynamical systems theory. However, considering that phosphorylation/dephosphorylation is stochastic event, stochastic phosphorylation/dephosphorylation may account for disappearance of hysteresis. Miller et al. computationally analyze the stochastic transition between the ON and OFF states of CaMKII phosphorylation, and estimate that the ON/OFF state of T286 phosphorylation is stable over a few years (40). They obtain this long lifetime where the PP1 activity is saturated. By contrast, in our experimental conditions, the PP1 activity is not saturated (2 μM CaMKII where K_m = 10 μM of PP1 for dephosphorylation). With this unsaturated PP1, the lifetime of the ON/OFF state can be much shorter (40). This short lifetime may explain the gradual decrease of hysteresis (Figs. S1 and S2).

Region of hysteresis in Ca^{2+} sensitivity ranged between 0.2 μM Ca^{2+} and 1.0 μM Ca^{2+} with 2 μM CaM (Fig. 2*A*). This Ca^{2+} range is above basal Ca^{2+} concentration in neurons (0.05-0.1 μM) (39, 41), and one may think that the CaMKII memory switch does not operate in neurons. However, when CaM concentration was increased to 10 μM, hysteresis appeared at the smaller Ca^{2+} concentration between 0.12 μM and 0.5 μM (data not shown), and the region of hysteresis is expected to cover 0.05-0.1 uM Ca^{2+} with 100 μM CaM in neurons . Thus, CaMKII can operate as a memory switch under physiological conditions.

The CaMKII memory switch was seen only in the presence of GluN2B-derived peptide (N2Bs), but not in its absence. Although the CaMKII memory switch can be explained by the bistable dynamics in the dynamical systems theory (6, 7, 40, 42), this bistable dynamics does not require GluN2B. Therefore, the CaMKII memory switch may be based not on the conventional bistable mechanism, but on other mechanism(s). Although the mechanism is unknown, the following two properties of CaMKII, such as slow dephosphoryation and decrease of the dissociation rate of Ca^{2+}/CaM from CaMKII, may not be direct cause of hysteresis. The slower T286 dephosphorylation caused by GluN2B-binding to CaMKII (43) may not be the direct cause. The CaMKII memory switch required not only the presence of a GluN2B-derived peptide (N2Bs), but also the larger than 0.2 μM Ca^{2+} for CaMKII activation (Fig. 1*B*, right; Fig. 2*A*). If the Ca^{2+} concentration was below 0.2 μM, the dephosphorylation rates of CaMKII in the presence and absence of N2Bs were almost similar (Fig. S1). This result suggests that hysteresis requires both N2Bs–binding and larger than 0.2 μM Ca^{2+}, rather than simply decreasing dephosphotylation. Decrease in the dissociation rate of Ca^{2+}/CaM from CaMKII by N2Bs may not be the cause of hysteresis (8). T286 phosphorylation itself

also leads to >1000-fold decrease in the dissociation rate of Ca^{2+}/CaM from CaMKII (44), whereas N2Bs leads to only 20-fold decrease (8). Despite the much stronger effect of T286 phosphorylation relative to N2Bs, the hysteresis appeared only in the presence of N2Bs, but not in its absence even when T286 phosphorylation occurred (Fig. 2*A* and *B*) (13). This suggests that hysteresis does not occur by simply decreasing the dissociation rate of Ca^{2+}/CaM from CaMKII.

Numbers of memory switches using artificial gene regulatory networks (45) and engineered molecules (46) have been proposed; however, few examples of memory switches in natural signaling systems have been clearly described (47, 48). To our knowledge, this is the first *in vitro* reconstitution and description of an erasable memory switch based on enzymatic reactions of a natural signaling system. The CaMKII memory switch occurred only within a holoenzyme, and no cross-phosphorylation between holoenzymes occurred. This indicates that the memory density of CaMKII can be extremely high, i.e., each CaMKII holoenzyme can remember different information in a solution. Thus, the CaMKII memory switch will provide us a design principle for energy efficient, high-speed and high-density memory devices, more than the memory switches using artificial gene regulatory networks.

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