that occluded the mask dots with diameters of 0.2–2.5°. Mask luminance contrast (Fig. 2f) was varied in the range 10–80% and the number of mask dots (Fig. 2g) in the range 0–180. Mask speed and motion type were tested (Fig. 2h) by changing the angular speed (expressed as maximum visual angle displacement per s) for three types of motion: 3D sphere (standard); 2D rotation of an array of crosses (approximately the same size as the sphere); and 1D left-to-right linear motion of a similar array.

Gestalt effects

To test the contour smoothness effect (Fig. 3a), we varied the tangential deviation of line segments arranged in a 2.1° (diameter) circle, between smooth (0° deviation) and sun-shaped (90° deviation). Observers reported disappearance of any part of the circle.

To test the effect of proximity, we varied the spacing in two groups of three dots each, from 0.3° (Fig. 3b, left) to 0.75° (Fig. 3b, right). The groups were separated by 2.1° along the *x* axis. Observers reported the disappearance of any dot ('parts' condition) and the disappearance of whole groups ('wholes' condition).

To test object competition, we presented two elliptical line configurations (1.8°×2.2°, with 0.6° displacement) of different colours (Fig. 3c, top right), with observers reporting the complete disappearance of each configuration and partial disappearance of any part.

To test the co-operation and rivalry of non-overlapping stimuli under MIB conditions (Fig. 4), we used two adjacent Gabor patches. These had 8 cycles per degree and were three wavelengths apart, 2° from fixation in the upper left quadrant, on a grey background with a 'rotating sphere' made of black dots. They were shown to five observers.

To test the distribution of MIB across space, we presented a single yellow dot at 30 different locations within 5° from fixation, with a 5.5° radius sphere, three times for 45 s periods. These were shown to seven observers.

Received 12 February; accepted 17 April 2001.

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or the authors' web site (http://www.weizmann.ac.il/~masagi/MIB/mib.html).

Acknowledgements

We thank J. D. Mollon for recently drawing our attention to the work of Grindley and Townsend[13]. We thank E. Freeman, B. Zenger, S. Gepshtein, H. Reed, J. Pettigrew and M. Merzenich for their helpful comments.

Correspondence and requests for materials should be addressed to Y.S.B. (e-mail: yoram@ski.org).

Interaction with the NMDA receptor locks CaMKII in an active conformation

K.-Ulrich Bayer*, Paul De Koninck*†, A. Soren Leonard‡§, Johannes W. Hell‡§ & Howard Schulman*

** Department of Neurobiology, Stanford University School of Medicine, Stanford, California 94305-5125, USA*
‡ Department of Pharmacology, University of Wisconsin, Madison, Wisconsin 53706-1532, USA

Calcium- and calmodulin-dependent protein kinase II (CaMKII) and glutamate receptors are integrally involved in forms of synaptic plasticity that may underlie learning and memory. In the simplest model for long-term potentiation[1], CaMKII is activated by Ca^{2+} influx through NMDA (*N*-methyl-D-aspartate) receptors and then potentiates synaptic efficacy by inducing synaptic insertion[2,3] and increased single-channel conductance[4] of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors. Here we show that regulated CaMKII interaction with two sites on the NMDA receptor subunit NR2B provides a mechanism for the glutamate-induced translocation of the kinase to the synapse in hippocampal neurons. This interaction can lead to additional forms of potentiation by: facilitated CaMKII response to synaptoc Ca^{2+}; suppression of inhibitory autophosphorylation of CaMKII; and, most notably, direct generation of sustained Ca^{2+}/calmodulin (CaM)-independent (autonomous) kinase activity by a mechanism that is independent of the phosphorylation state. Furthermore, the interaction leads to trapping of CaM that may reduce down-regulation of NMDA receptor activity[5]. CaMKII–NR2B interaction may be prototypical for direct activation of a kinase by its targeting protein.

The generation of autonomous CaMKII activity has an important role in various forms of synaptic plasticity, and has been regarded as 'molecular memory', as the kinase remains active after the initial Ca^{2+} stimulus has subsided (see refs 1, 6, 7 for review). Autonomous CaMKII activity depends on autophosphorylation of T286 in its auto-inhibitory domain. Autophosphorylation requires coincident binding of at least two Ca^{2+}/CaM molecules to a dodecameric CaMKII holoenzyme[8,9] and enables Ca^{2+}-spike-frequency decoding by the kinase[10]. T286 autophosphorylation also results in greatly enhanced CaM binding (CaM trapping)[11] by the highly abundant α-CaMKII, which may sequester CaM and limit its availability for the NMDA receptor and other synaptic proteins. The initial Ca^{2+} stimulus for CaMKII activation can be provided by the NMDA receptor, the only known activity-dependent binding partner for CaMKII at the synapse[12–14]. Binding of α-CaMKII to the NMDA receptor was reported to require autophosphorylation[14], whereas NMDA-stimulated translocation of the kinase to neuronal synapses does not[15,16]; thus the two events did not seem to be linked. Our binding studies, however, now demonstrate that stimulation by Ca^{2+}/CaM is sufficient to induce binding of α-CaMKII to the cytoplasmic carboxy terminus of NR2B (residues 839–1,482) and that autophosphorylation is not required. In fact, this NR2B domain contains two sites with different modes of regulated CaMKII binding (Fig. 1), a Ca^{2+}/CaM-regulated site within residues 1,120–1,482 of NR2B (NR2B-C) and a phosphorylation-regulated site within residues 839–1,120 (NR2B-P). CaMKII binds to NR2B-

† Present address: Centre de Recherche Université Laval Robert-Giffard, Beauport, Québec G1J 2G3, Canada.
§ Present address: Department of Physiology (A.S.L.) and Department of Pharmacology (J.W.H.), University of Iowa, Iowa City, Iowa 52246-1109, USA.

P only if it is autophosphorylated at T286. Neither unphosphorylated wild-type kinase nor mock autophosphorylated T286A mutant bound this site even in the presence of Ca^{2+}/CaM. Autophosphorylated wild-type kinase binds to both sites even after removal of Ca^{2+}/CaM, and a T286D mutant mimics the binding characteristics of autophosphorylated kinase (Fig. 1). Most importantly, activation by Ca^{2+}/CaM alone, in the absence of ATP, is sufficient to induce interaction of wild type and T286A CaMKII with NR2B-C (Fig. 1).

We investigated whether the autophosphorylation-independent translocation of CaMKII observed in hippocampal neurons[15,16] could be reconstituted in a heterologous system by coexpression with NR2B. In unstimulated HEK cells, green fluorescent protein (GFP)-labelled CaMKII is evenly distributed throughout the cytoplasm. After generating a Ca^{2+} stimulus, both wild type and T286A mutant CaMKII translocate, but only when coexpressed with NR2B (Fig. 2a). However, a CaMKII mutant (I205K) that does not bind to NR2B *in vitro* (see Supplementary Information) does not show this movement even in the presence of NR2B (Fig. 2a). The I205K mutation does not reduce CaM affinity and allows normal kinase activation by Ca^{2+}/CaM[17]. Kinase and receptor are localized together after stimulation (Fig. 2b), further implicating a direct interaction. The autophosphorylation-deficient T286A mutant also co-localizes with the receptor, but the NR2B-binding-deficient I205K mutant does not (Fig. 2b). In hippocampal neurons, this I205K mutant also fails to translocate after stimulation with glutamate (Fig. 2c), a treatment that readily induced translocation of both wild type and T286A mutant kinase to postsynaptic sites (Fig. 2c; see also Supplementary Information). These data demonstrate that binding to NR2B can provide a mechanistic basis for the stimulation-induced translocation of CaMKII in neurons.

Although Ca^{2+}/CaM is necessary to initiate binding of CaMKII to NR2B-C, binding persists after washes with EGTA that effectively strip CaM from the kinase–receptor complex (Fig. 3a). During washes with Ca^{2+} but without CaM, however, CaM remained bound to the complex. Thus, interaction with NR2B-C seems to increase the CaM affinity of CaMKII, similar to CaM trapping by the autophosphorylated kinase, as CaM dissociates from unphosphorylated kinase that is not complexed with NR2B-C with an off rate of approximately 2 s^{-1} (ref. 11). To test this hypothesis and to narrow the region of NR2B-C involved, we examined the effects of various NR2B-derived peptides on the dissociation of an IAEDANS-labelled CaM (CaM^I) from CaMKII during a 'chase' with an excess of unlabelled CaM[11,18] (Fig. 3b). The short NR2B-C-derived peptide N2B-s (residues 1,289–1,310) reduced the dissociation rate of CaM^I from the kinase by about 20-fold. By contrast, the control peptide N2B-con (residues 1,095–1,119), which was derived from NR2B-P and has an amino-acid content similar to N2B-s, had little effect. The ability of the short N2B-s peptide to simulate the effect of the NR2B-C segment on CaM trapping suggests that it contains some or all of the residues within NR2B-C that anchor CaMKII to the NMDA receptor.

N2B-s is homologous to the segment surrounding T286 in the auto-inhibitory domain of the kinase (Fig. 3c) and may therefore mimic some of its interactions[17]. The T286 segment interacts with two sites on the kinase, and thus N2B-s could bind to either one of these sites (Fig. 3d). In the unstimulated or basal state, interaction of the T286 segment with a groove on the surface of the kinase (T site) serves to keep the kinase inactive by positioning an adjacent pseudo-substrate sequence in the substrate-binding site (S site)[17]. In the activated state of the kinase, Ca^{2+}/CaM displaces the T286 segment from the T site and enables it to interact with a vacant S site on a neighbouring subunit. NR2B-C (or N2B-s) might preferentially occupy either the vacated T or S site. Binding by means of the S site would result in inhibition of the kinase.

We tested the effect of CaMKII interaction with the NR2B-C fragment on kinase activity in a phosphorylation assay (Fig. 4a). NR2B-C-immobilized kinase retained about 70% of maximal Ca^{2+}/CaM-stimulated activity. Notably, the immobilized CaMKII remained active even after removal of Ca^{2+}/CaM with EGTA. This autonomous activity was 19% of the maximal Ca^{2+}/CaM-stimulated activity seen with NR2B-C-bound kinase. Such autonomous activity has previously been observed only after autophosphoryla-

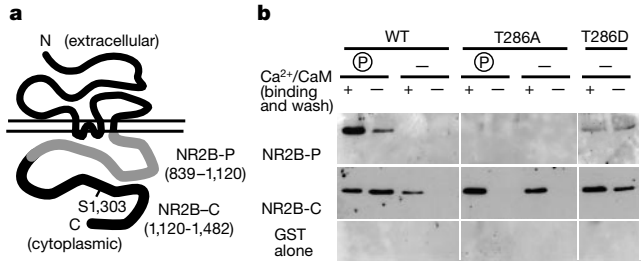

Figure 1 Differential regulation of CaMKII binding to two regions on NR2B. **a**, Schematic representation of the NR2B topology indicating the fragments used and the S1,301 phosphorylation site. **b**, Binding of α-CaMKII wild type (WT), T286A or T286D to immobilized fusion proteins of GST with NR2B-P or NR2B-C under various conditions. Kinase was either previously autophosphorylated or not (P or –), and the binding reactions and washes were done either in the presence or absence of Ca^{2+}/CaM as indicated. Bound CaMKII is visualized by immunoblotting the eluted protein using a specific antibody.

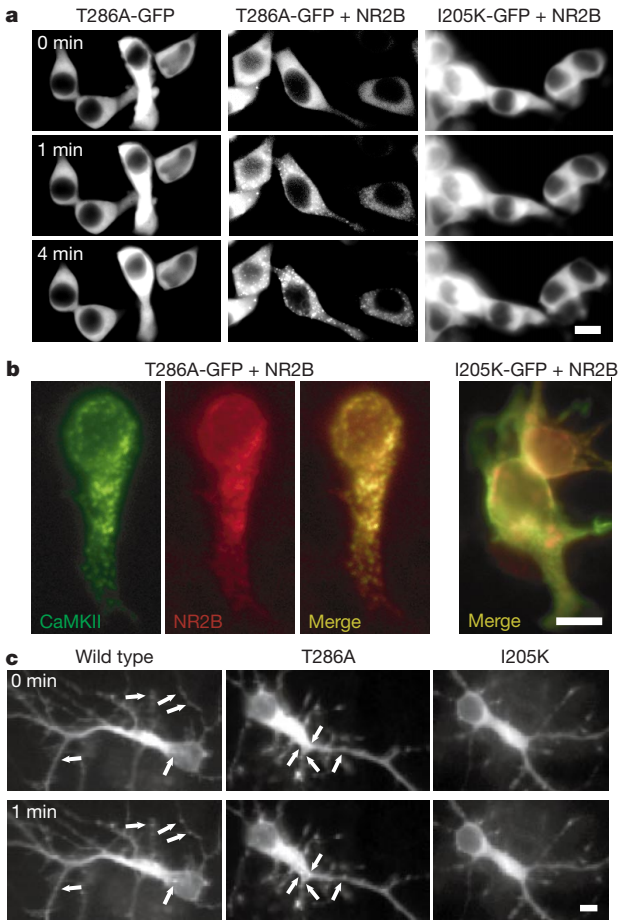

Figure 2 CaMKII translocation in HEK cells and in neurons. **a**, α-CaMKII-GFP T286A or I205K mutants were expressed in HEK cells, either alone or together with NR2B. The localization of the labelled kinase before and after a Ca^{2+} stimulus is monitored. **b**, Co-localization of α-CaMKII-GFP (green, GFP) and NR2B (red, immunostain) in stimulated HEK cells. **c**, Translocation of α-CaMKII-GFP in hippocampal neurons after stimulation with glutamate is monitored. Wild type (n = 8 of 12) and T286A mutant kinase (n = 2 of 4) rapidly translocates to postsynaptic sites (arrows; see Supplementary Information); the NR2B-binding deficient I205K mutant does not (n = 0 of 7). Scale bars, 10 μM.

tion at T286, with maximal autonomy ranging between 20% and 80% (refs 7, 19). Immunoblotting with an antibody sensitive to phosphorylated T286 showed that the autonomously active NR2B-C-bound enzyme is not phosphorylated at T286 (data not shown, but see Fig. 5a). Indeed, even a CaMKII T286A mutant shows autonomous activity (33%) when bound to NR2B-C (Fig. 4a). Residual CaM cannot account for the autonomous activity as it was below the detectable level (see Fig. 3a), and is therefore at less than 1:50 stoichiometry to the kinase. The observed autonomous activity after NR2B-C binding is thus generated by a T286 phosphorylation-independent mechanism and is not due to trapping of CaM.

The most likely interpretation of the data above is that once NR2B-C occupies the T site of the kinase, it blocks interaction with the inhibitory T286 segment of that subunit so that the enzyme remains active even after dissociation of Ca^{2+}/CaM (Fig. 3d). As only some of the 12 subunits in each CaMKII holoenzyme[20] are likely to interact with NR2B, we tested the effect of soluble NR2B-derived peptides on CaMKII activity at concentrations that would saturate binding to all subunits (Fig. 4b, c). Peptides N2B-l (NR2B residues 1,259–1,310) and the shorter N2B-s are sufficient to generate autonomous activity of soluble CaMKII (Fig. 4b). The effect requires an initial exposure of the kinase to Ca^{2+}/CaM, as seen for NR2B-C binding. With increasing concentration of N2B-l, autonomous activity attains about 60% of initial maximal Ca^{2+}/CaM-stimulated activity (Fig. 4c), suggesting that N2B-l binds to most if not all kinase subunits with a direct activation of each one[8]. There is also some reduction in maximal stimulated activity at high concentration of peptide, which may result from secondary binding at the S site at such concentrations. This inhibitory effect is less prominent with the longer N2B-l peptide, which may have a greater preference for the T site owing to additional interactions. Phosphorylation of these peptides is not essential for this effect, as autonomy is also generated with peptide N2B-a in which S1,303 is replaced with alanine.

Can CaMKII that has been made partially autonomous by binding of some of its subunits to NR2B be further potentiated by T286 phosphorylation? Indeed, CaMKII bound to immobilized NR2B-C does phosphorylate T286, as detected by an antibody selective for phosphorylated T286, but only when stimulated by Ca^{2+}/CaM. Without Ca^{2+}/CaM, no T286 phosphorylation was observed even though phosphorylation of other residues does occur, as detected by ^{32}P incorporation (Fig. 5a). Phosphorylation of soluble CaMKII at T286 requires that CaM be bound to the subunit being phosphorylated to expose T286 for phosphorylation by a neighbouring subunit, a substrate-directed effect of CaM[8,9]. As

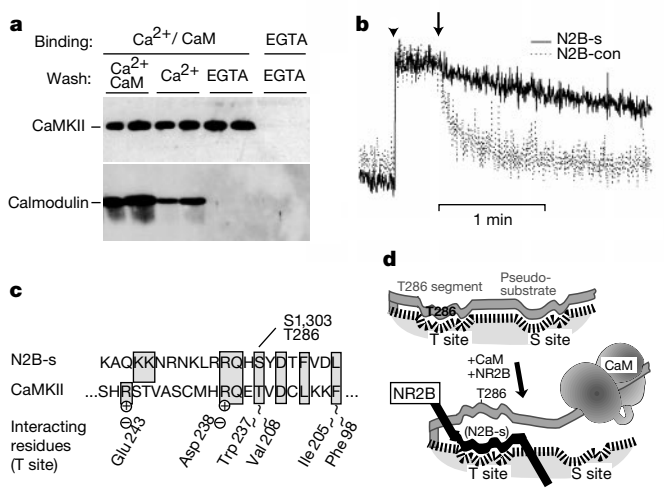

Figure 3 An NR2B fragment sufficient to induce CaM trapping by CaMKII. **a**, CaMKII incubated with immobilized NR2B-C in the presence of Ca^{2+}/CaM or EGTA, and washed with buffers containing EGTA, CaM and/or Ca^{2+}. Bound protein was eluted and tested for α-CaMKII and CaM by immunoblotting. **b**, CaM^{I} fluorescence increases on binding to CaMKII (ref. 18) (added with peptide present, arrowhead). Dissociation is initiated by the addition of excess unlabelled CaM (arrow), and is significantly decreased by 25 μM of N2B-s, but not by N2B-con. **c**, NR2B-derived CaMKII-binding peptide N2B-s and the auto-inhibitory region of CaMKII are homologous (boxed residues). CaMKII residues that interact with the auto-inhibitory region[17] are indicated. T286 of the kinase and S1303 of the receptor are marked. **d**, Model for CaMKII binding to NR2B. Binding of Ca^{2+}/CaM to individual CaMKII subunits displaces the auto-inhibitory region from the S and T sites (substrate binding and T286-segment-binding site) of the kinase. The vacated T site can then interact with the NR2B region around S1,303, preventing rebinding of the auto-inhibitory region and leaving the S site accessible for autonomous substrate phosphorylation.

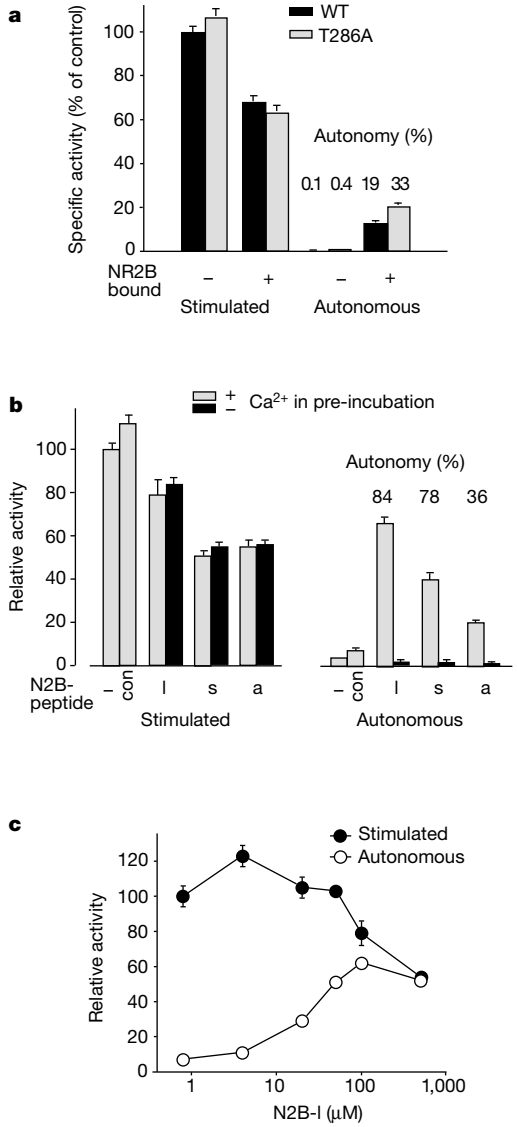

Figure 4 Interaction of CaMKII with NR2B-C generates autonomous kinase activity by a mechanism that does not require autophosphorylation or CaM trapping. **a**, α-CaMKII wild type (WT) or T286A mutant was bound to immobilized NR2B-C, and CaM was removed by EGTA washes (see Fig. 3a). Specific kinase activity of the NR2B-bound CaMKII was compared with soluble enzyme, either in the presence of Ca^{2+}/CaM (stimulated) or EGTA (autonomous). **b**, CaMKII was pre-incubated with NR2B-derived peptides (–, no peptide; or N2B-l, -s, -a or -con), CaM and either Ca^{2+} or EGTA (+ or – Ca^{2+}). EGTA was added to dissociate CaM from the kinase–peptide complexes, and after 8 min Ca^{2+}/CaM stimulated or autonomous activity was measured. **c**, Stimulated and autonomous kinase activity is dependent on the N2B-l concentration during pre-incubation (compare with **b**). For **a**–**c** The specific activity of Ca^{2+}/CaM-stimulated kinase (preincubation without peptide) was set as 100%. All data points are means ± s.e.m.

NR2B-C should only bind one or a few of the subunits in each holoenzyme, it is likely that T286 is not exposed in the unbound subunits. We therefore activated every subunit by saturating the kinase with NR2B-derived peptides and tested for T286 autophosphorylation. Even full autonomy produced by the peptides did not lead to significant T286 phosphorylation in the absence of Ca^{2+}/CaM (Fig. 5b). However, once Ca^{2+}/CaM exposes T286, it is readily phosphorylated by peptide-bound subunits. Therefore, NR2B-C binding sustains kinase activity without CaM, but does not mimic the substrate-directed effect of CaM binding.

Binding of Ca^{2+}/CaM to CaMKII is inhibited by a 'burst' of autophosphorylation at T305 and T306, which is initiated when CaM dissociates from an autonomous enzyme[21,22]. We compared soluble CaMKII with NR2B-C-bound CaMKII under conditions known to produce the inhibitory burst (Fig. 5c). CaM binding to the soluble enzyme, as assessed by CaM overlay, is largely eliminated by the inhibitory burst of autophosphorylation. By contrast, the same conditions failed to reduce CaM binding for the NR2B-C-bound form of the kinase. Thus, binding to NR2B renders the kinase autonomous, as does T286 phosphorylation, but suppresses the inhibitory phosphorylation of T305/T306.

One possible substrate for CaMKII made autonomous by forming a kinase–receptor complex is the NMDA receptor itself[23]. CaMKII bound to NR2B-C phosphorylates the cytoplasmic domain of NR2B (Fig. 5a). In contrast to T286 autophosphorylation, NR2B-C phosphorylation occurs even without Ca^{2+}/CaM. However, Ca^{2+}/CaM further enhances the NR2B-C phosphorylation, probably owing to activation of non-autonomous unbound subunits. The phosphorylation of NR2B-C does not disrupt the interaction with the kinase, as no CaMKII was detected in the supernatant of the reactions (not shown). Ca^{2+} signals can stimulate both phosphorylation and dephosphorylation of NR2B[23,24], but the CaMKII–NR2B interaction generates a persistent kinase activity that outlasts the initial stimulus.

Stimulus-dependent binding to NR2B targets CaMKII to the signalling protein network of the postsynaptic density, where it is optimally positioned to decode local Ca^{2+} influx and mediate its proposed functions in learning and memory by modulating AMPA receptor-dependent neurotransmission[1–4], NMDA receptor inactivation[5,25], and the mitogen-activated protein kinase pathway[26]. In contrast to the previously identified CaMKII-anchoring proteins αKAP, F-actin and densin-180 (refs 27–30), NR2B affects not only the localization of the kinase but also directly regulates several of its physiologically important properties by mimicking a segment of its auto-inhibitory domain. In contrast to T286 phosphorylation, NR2B binding generates an autonomous and CaM-trapping state of CaMKII that cannot be reversed by phosphatases. It also suppresses inhibitory autophosphorylation at T305/306 that promote dissociation of the kinase from synaptic sites[16]. NR2B-induced CaMKII autonomy may preferentially occur with low-level stimuli as it does not require coincident binding of two CaM molecules[8,9], but may facilitate T286 autophosphorylation of neighbouring subunits on binding of a single CaM. In turn, autophosphorylation promotes CaMKII interaction with a second, phosphorylated T286-dependent binding site on NR2B. Thus, NR2B-binding and T286 autophosphorylation can function synergistically. Collectively, these mechanistic steps constitute a powerful feed-forward pathway for the regulation and potentiation of synaptic strength. □

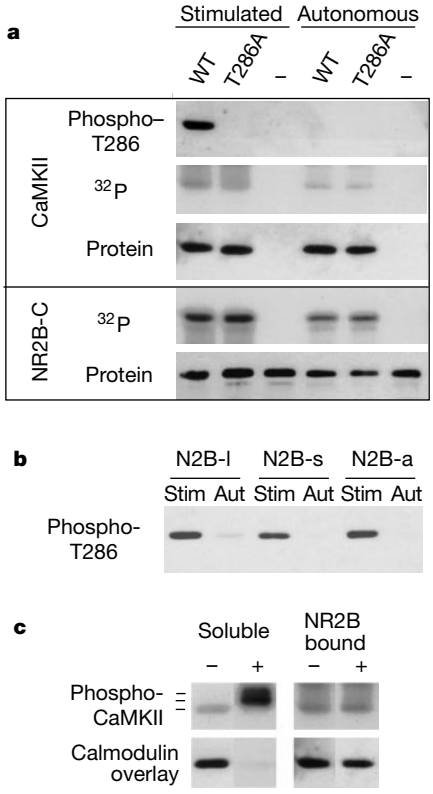

Figure 5 Phosphorylation within CaMKII–NR2B complexes. **a**, α-CaMKII wild type (WT), T286A or no kinase (–) bound to NR2B-C. Phosphorylation reactions contained either Ca^{2+}/CaM (stimulated) or EGTA (autonomous). Upper panels, phosphorylated CaMKII (^{32}P), T286-phosphorylated CaMKII (phospho-T286) and total CaMKII (protein), visualized by autoradiograph, immunoblotting with a phosphorylated T286-specific antibody, or CaM overlay. Lower panels, phosphorylated (^{32}P) and total NR2B-C (protein) visualized by autoradiography or immunoblotting. **b**, CaMKII was pre-incubated with peptides in the presence of Ca^{2+}/CaM. After addition of EGTA (8 min), samples were subjected to Ca^{2+}/CaM stimulated (stim) or autonomous (aut) auto-phosphorylation reactions. T286-phosphorylated CaMKII is visualized by immunoblotting with a phospho-specific antibody. **c**, Binding to NR2B can protect CaMKII from inhibitory burst autophosphorylation. CaMKII in solution and bound to NR2B-C are compared. As in **a**, T286 autophosphorylation was stimulated in the presence of [γ-^{32}P]ATP (–). EGTA was added and the reaction continued at 30 °C to induce burst phosphorylation (+). ^{32}P incorporation visualized by autoradiography (upper panel); burst phosphorylation leads to a bandshift of CaMKII. Burst phosphorylation at T305/306 inhibits CaM binding (CaM overlay, lower panel).

Methods

Fluorescence-based CaM dissociation assay

Baculovirus-based purification of CaMKII and the CaM dissociation assay will be described in detail elsewhere (S. I. Singla, A. Hudmon, J. M. Goldberg, J. L. Smith and H. Schulman, submitted manuscript). The assays contained 15 nM CaM^{I}, 300 nM CaMKII, 50 mM MOPS pH 7, 0.1 mg ml^{-1} BSA, 150 mM KCl, 2 mM Mg-ADP, 0.5 mM $CaCl_2$ and (in the chase) 4 μM unlabelled CaM.

Binding assays with immobilized GST fusion protein

Glutathione *S*-transferase (GST)–NR2B fusion proteins were expressed in bacteria and immobilized on anti-GST-antibody-coated 96-well plates (Gibco, BRL). Binding mix (50 μl) (100–200 nM kinase, 50 mM PIPES pH 7.0, 0.1% BSA, 150 mM NaCl, 0.1% Tween-20, and either 1 mM $CaCl_2$ and 3 μM CaM or 1 mM EGTA) was overlaid for 1 h at 4 °C after blocking with 5% BSA. Wells were washed 7–8 times over a period of 20 min with buffer containing EGTA (0.5–1 mM) or as indicated (1 mM $CaCl_2$ ± 300 nM CaM). Immobilized kinase activity was measured for 1 min at 30 °C (50 ml of 50 mM PIPES pH 7, 0.1 mg ml^{-1} BSA, 10 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP (1 Ci mmol^{-1}), 15 μM autocamtide-3 (AC3) peptide substrate, and either 0.5 mM $CaCl_2$ and 900 nM CaM or 0.5 mM EGTA) or the complexed proteins were phophorylated for 2 min on ice (similar buffers but without AC3). For immunoblot analysis using specific antibodies against α-CaMKII (Cbα2, Gibco), phosphorylated T286 (ABR) or CaM (Upstate Biotechnology), and for overlay with biotinylated CaM (STI), protein was eluted for 12 min in boiling SDS-loading buffer.

Translocation of CaMKII in HEK cells and neurons

Hippocampal cultures from neonatal rats were grown at high density (300–600 cells mm^{-2}) on 13 mm poly-D-lysine-coated aclar coverslips in neurobasal medium supplemented with B27, L-glutamine (500 μM), penicillin/streptomycin (50 U ml^{-1}/50 μg ml^{-1}) and cytosine arabinoside (5 μM, from day 2 to 5). Seven-day-old cultures were transfected in the presence of 10 μM CNQX (6-cyano-7-nitroquinoxaline-2,3-dione)/2-amino-5-phosphono pentanoate acid and 50 μM AP-5, using a custom-made microporator (platinum

wires 7 mm apart) and a BioRad Gene Pulser (with Pulse Controller, 600 Ω; capacitance extender, 125 μF) set to deliver two 190 V pulses (30 s apart, at opposite polarity and with exponential decay τ-values of 30–40 ms). We injected 5 μl of expression vector (0.5 μg μl^{-1}) into the microporator before the pulses. Neurons were maintained for 16–24 h (in the presence of 25 μM AP-5) before imaging. HEK cells were transfected using lipofectamine Plus (BRL), passaged after 16–20 h onto coverslips and maintained for 6–24 h. Images were acquired on a Nikon inverted microscope with a ×40 (1.3 NA) oil immersion objective using a Pentamax cooled CCD camera controlled by Metamorph software (Universal Imaging). Cells were perfused with Hank's Balanced Salt Solution containing 1 mM $MgCl_2$ (for neurons), 1.2 mM $CaCl_2$ and 10–25 mM HEPES for 5–10 min at room temperature in a custom-made chamber. To induce a Ca^{2+} stimulus, HEK cells were treated with ionomycin/EGTA (10 μM/1 mM) for 2 min, and then perfused with 2 mM $CaCl_2$; neurons were stimulated with glutamate/glycine (100 μM/10 μM) (see ref. 15 and references therein). For immunostaining with NR2B antibody (Transduction Laboratories, 1:400; secondary antibody: GαM-Alexa594, Molecular Probes, 1:1000), cells were fixed after stimulation with 4% paraformaldehyde in 0.1 M phosphate buffer, and permeabilized with 0.1% Triton X-100 in PBS.

NR2B-derived peptides and CaMKII activity

The peptides N2B-l, -s, -a, and -con (NR2B 1,259–1,310, 1,289–1,310, 1,289–1,310/S1,303A and 1,095–1,119, respectively) were pre-incubated for 12 min with kinase (100 nM CaMKII, 100 μM peptide, 4.5 μM CaM, 10 mM PIPES pH 7.0, 0.1 mg ml^{-1} BSA, and 0.25 mM $CaCl_2$ or EGTA). EGTA was added (2.5 mM) and samples were diluted 1:5 into activity assays (see 'Binding assays', above, but with 100 μM AC3) or autophosphorylation reactions (without AC3).

Received 26 February; accepted 17 April 2001.

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Supplementary information is available on *Nature's* World-Wide web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

Acknowledgements

We thank A. Hudmon, S. Singla, M. Meffert, P. Nef and T. Bartfai for helpful discussions, and B. Barres, R. C. Malenka, T. Meyer and D. Mochly-Rosen for comments on the manuscript. Purified CaMKII and IAEDANS-CaM were provided by A. Hudmon and S. Singla. We also thank T. Meyer and colleagues for providing the α-CaMKII-GFP plasmid and help in designing the microporation technique. The research was supported by NIH grants (to H.S. and J.W.H.), a grant from Hoffmann-La Roche (H.S.), and an American Heart Award (J.W.H.). P.D.K. is a Career Awardee of the Burroughs Wellcome Fund and a Centennial Fellow of the Canadian Institutes of Health Research.

Correspondence and requests for materials should be addressed to K.-U.B. (e-mail: ulli.bayer@stanford.edu) or H.S. (e-mail: schulman@cmgm.stanford.edu).

Molecular mechanism of cAMP modulation of HCN pacemaker channels

Brian J. Wainger*, Matthew DeGennaro†, Bina Santoro*, Steven A. Siegelbaum*†‡ & Gareth R. Tibbs‡§

** Center for Neurobiology and Behavior; † Howard Hughes Medical Institute; ‡ Department of Pharmacology; and § Department of Anesthesiology, Columbia University, New York, NY 10032, USA*

Hyperpolarization-activated cation channels of the HCN gene family[1–6] contribute to spontaneous rhythmic activity in both heart[7] and brain[5,6,8]. All four family members contain both a core transmembrane segment domain, homologous to the S1–S6 regions of voltage-gated K^+ channels, and a carboxy-terminal 120 amino-acid cyclic nucleotide-binding domain (CNBD) motif. Homologous CNBDs are responsible for the direct activation of cyclic nucleotide-gated channels and for modulation of the HERG voltage-gated K^+ channel—important for visual and olfactory signalling[9] and for cardiac repolarization[10], respectively. The direct binding of cyclic AMP to the cytoplasmic site on HCN channels permits the channels to open more rapidly and completely after repolarization of the action potential[1,2,11], thereby accelerating rhythmogenesis[6–8]. However, the mechanism by which cAMP binding modulates HCN channel gating and the basis for functional differences between HCN isoforms remain unknown. Here we demonstrate by constructing truncation mutants that the CNBD inhibits activation of the core transmembrane domain. cAMP binding relieves this inhibition. Differences in activation gating and extent of cAMP modulation between the HCN1 and HCN2 isoforms result largely from differences in the efficacy of CNBD inhibition.