Ca^{2+}/Calmodulin-Dependent Activation and Inactivation Mechanisms of αCaMKII and Phospho-Thr_{286}-αCaMKII†

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ABSTRACT: Thr_{286} autophosphorylation is important for the role of αCaMKII in learning and memory. Phospho-Thr_{286}-αCaMKII has been described to have two types of activity: Ca^{2+}-independent partial activity and Ca^{2+}/calmodulin-activated full activity. We investigated the mechanism of switching between the two activities in order to relate them to the physiological functioning of αCaMKII. Using a fluorometric coupled enzyme assay and smooth muscle myosin light chain (MLC) as substrate, we found that (1) Ca^{2+}-independent activity of phospho-Thr_{286}-αCaMKII represents 5.0 (±3.7)% of the activity measured in the presence of optimal concentrations of Ca^{2+} and calmodulin and (2) Ca^{2+} in the presence of calmodulin activates the enzyme with a K_m of 137 (±56) nM and a Hill coefficient $n = 1.8$ (±0.3). In contrast, unphosphorylated αCaMKII has a K_m for Ca^{2+} in the presence of calmodulin of 425 (±119) nM and a Hill coefficient $n = 5.4$ (±0.4). Thus, the activity of phospho-Thr_{286}-αCaMKII is essentially Ca^{2+}/calmodulin dependent with MLC as substrate. In physiological terms, our data suggest that αCaMKII is only activated in stimulated neurones whereas Ca^{2+}/calmodulin activation of phospho-Thr_{286}-αCaMKII can occur in resting cells (∼100 nM [Ca^{2+}]). Stopped-flow experiments using Ca^{2+}/TA-cal [Ca^{2+}/2-chloro-(ϵ-amino-Lys_{75})-[6-[4-(*N*,*N*-diethylamino)phenyl]-1,3,5-triazin-4-yl]calmodulin] showed that at 100 nM [Ca^{2+}] partially Ca^{2+}-saturated Ca^{2+}/cal·phospho-Thr_{286}-αCaMKII complexes existed. These are likely to account for the activity of the phospho-Thr_{286}-αCaMKII enzyme at resting [Ca^{2+}]. Ca^{2+} dissociation measurements by a fluorescent Ca^{2+} chelator revealed that the limiting Ca^{2+} dissociation rate constants were 1.5 s^{-1} from the Ca^{2+}/cal·αCaMKII and 0.023 s^{-1} from the Ca^{2+}/cal·phospho-Thr_{286}-αCaMKII complex, accounting for the differences in the Ca^{2+} sensitivities of the Ca^{2+}/cal·αCaMKII and Ca^{2+}/cal·phospho-Thr_{286}-αCaMKII enzymes.

Calmodulin regulates a wide range of cellular processes via its Ca^{2+}-dependent activation of target proteins. Calmodulin binds many target proteins with similar high affinities because the linker between the binding lobes is flexible and can adapt to binding different target sequences (*1*, *2*). α-Ca^{2+}/calmodulin-dependent protein kinase II (αCaMKII)[1] is a major target for Ca^{2+}/calmodulin activation in neurones. αCaMKII is an oligomeric kinase of 660 kDa molecular mass that consists of 12 identical α subunits arranged as two stacked hexameric rings (*3*, *4*). This unique oligomeric structure of αCaMKII allows the enzyme to sense cellular Ca^{2+} oscillations and give a prolonged response between elevations of [Ca^{2+}] within the cells (*5*). The α isoform of CaMKII is the predominant isoform in the hippocampus, and its function is associated with the onset of long-term potentiation (*6*) and spatial learning (*7*, *8*). αCaMKII is able to phosphorylate itself and also a wide range of substrates ranging from transcriptional activators to ion channels (*9*−*11*). Ca^{2+}/calmodulin-dependent autophosphorylation of αCaMKII at residue Thr_{286} has a role in spatial learning as demonstrated by experiments with transgenic mice expressing only the nonphosphorylatable (T286A) mutant analogue of αCaMKII (*12*). Thr_{286} autophosphorylation is a special feature of αCaMKII which has two important consequences: (a) it increases the affinity of the enzyme for Ca^{2+}/calmodulin 10^4-fold (*13*, *14*) and (b) it results in a Ca^{2+}-independent partial kinase activity (*15*).

In this study, the mechanism of activation and inactivation of αCaMKII and phospho-Thr_{286}-αCaMKII by Ca^{2+} was investigated. First, Ca^{2+} dependence of the activation of αCaMKII was determined and compared with that of the phospho-Thr_{286}-αCaMKII enzyme to dissect the effect of Thr_{286} autophosphorylation on the activation mechanism. Second, Lys_{75}-labeled TA-cal (*16*) whose fluorescence is affected by Ca^{2+} and target protein binding was used to study the Ca^{2+}-dependent interaction of Ca^{2+}/calmodulin with αCaMKII and phospho-Thr_{286}-αCaMKII as well as the kinetics of Ca^{2+}/calmodulin·αCaMKII and Ca^{2+}/calmodulin·

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; αCaMKII, α-Ca^{2+}/calmodulin-dependent protein kinase II; phospho-Thr_{286}-αCaMKII, Thr_{286}-phosphorylated αCaMKII; DTT, 1,4-dithiothreitol; EGTA, 1,2-bis(2-aminoethoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; LDH, lactate dehydrogenase; MLC, chicken gizzard smooth muscle myosin light chain; MLCK, smooth muscle myosin light chain kinase; NADH, reduced nicotinamide adenine dinucleotide; PDE, phosphodiesterase; PEP, phosphoenolpyruvate; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PK, pyruvate kinase; TA-cal, 2-chloro-(ϵ-amino-Lys75)-[6-[4-(*N*,*N*-diethylamino)phenyl]-1,3,5-triazin-4-yl]calmodulin.

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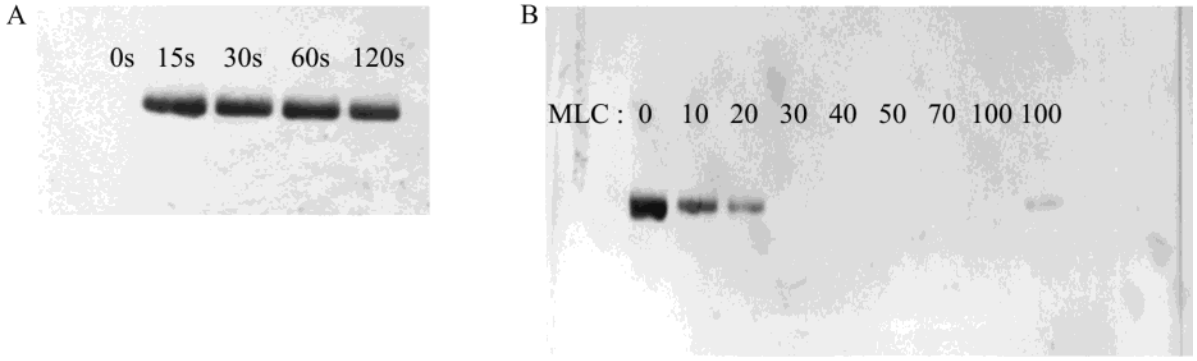

FIGURE 1: Western blot analysis of Thr_{286} autophosphorylation of αCaMKII. (A) Activation of Thr_{286} autophosphorylation of αCaMKII by Ca^{2+}/calmodulin. 6 μM αCaMKII was mixed with 8 μM calmodulin and 1 mM ATP at 21 °C as described in Materials and Methods. Reaction samples were taken at time intervals 0, 15, 30, 60, and 120 s and were quenched with SDS sample buffer. 3.3 μg of αCaMKII was loaded in each lane. (B) Competition between MLC target phosphorylation and Ca^{2+}/calmodulin-induced Thr_{286} autophosphorylation of αCaMKII. αCaMKII (1 μM) was incubated in the presence of 5 μM calmodulin, 1 mM ATP, and increasing concentrations of MLC (0, 10, 20, 30, 40, 50, 70 and 100 μM) for 15 s at 21 °C as described in Materials and Methods. 1 μg of αCaMKII was loaded in each lane. The last lane represents Thr_{286}-autophosphorylated αCaMKII after incubation of the enzyme in the presence of 100 μM MLC for 2 h at 21 °C. Western blotting was performed as previously described (*14*).

phospho-Thr_{286}-αCaMKII dissociation induced by Ca^{2+} sequestration.

MATERIALS AND METHODS

Proteins. αCaMKII was overexpressed in baculovirus-transfected Sf9 insect cells and purified as previously described in ref *14*. Pig brain calmodulin was purified as previously described (*16*). The purification procedures were modified and extended to remove EGTA from all protein samples used for Ca^{2+} dissociation kinetic measurements by quin-2 (see below) as follows: Mono Q FPLC was carried out using 20 mM Tris·HCl, pH 7.5, 1 mM DTT and protease inhibitors as previously described; however, 50 μM $CaCl_2$ was added instead of 2 mM EGTA. EGTA used in the preceding calmodulin−sepharose chromatography for αCaMKII was reduced to approximately 20 μM by this treatment. To reduce EGTA concentration below the detectable level (<0.01 μM), αCaMKII was dialyzed for 24 h at 4 °C, with 6 × 2 L of standard buffer solution. αCaMKII activity of the dialyzed and Thr_{286}-autophosphorylated samples was checked prior to stopped-flow kinetic experiments by the NADH-coupled assay described below. MLC was obtained by overexpression in BL21 *Escherichia coli* cells and purified from inclusion bodies (*17*). Mouse monoclonal anti-phospho-Thr_{286}-αCaMKII antibody was purchased from Upstate Biotechnology.

Protein Concentration Measurements. Protein concentrations were determined spectrophotometrically. The concentration of αCaMKII (subunits) was measured using ϵ_0 = 64805 M^{-1} cm^{-1} (280 nm) calculated from the amino acid composition (*14*). For MLC, the value ϵ_0 = 4400 M^{-1} cm^{-1} (280 nm) was calculated from the amino acid composition, and for pig brain calmodulin, ϵ_0 = 1800 M^{-1} cm^{-1} (280 nm) was used (*14*). The concentration of TA-calmodulin was determined as described in ref *16*.

Labeling of Calmodulin. Pig brain calmodulin was labeled with 2,4-dichloro-6-[4-(*N*,*N*-diethylamino)phenyl]-1,3,5-triazine as described in ref *16*.

Time Course of Ca^{2+}/Calmodulin-Dependent Thr_{286} Autophosphorylation of αCaMKII. αCaMKII (6 μM), 8 μM calmodulin, and 1 mM ATP were incubated in 50 mM K^+-PIPES, pH 7.0, 100 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, and 0.5 mM $CaCl_2$ at 21 °C for various times from 0 to 120 s. The reaction was terminated using SDS sample buffer. The phospho-Thr_{286}-αCaMKII was detected by western blotting using a specific mouse monoclonal anti-phospho-Thr_{286}-αCaMKII antibody as described in ref *14*. The time course was assessed by densitometry using an Alpha Innotech Corp. densitometer with Fluorochem version 2.00 software. The densities of the bands (Figure 1A, lanes 2−5) were identical within 3% and were below maximal saturation as previously shown (*14*).

Inhibition of Thr_{286} Autophosphorylation of αCaMKII by MLC Substrate. αCaMKII (1 μM), 5 μM calmodulin, 1 mM ATP, and varying concentrations of MLC in the range of 0−100 μM were incubated in 50 mM K^+-PIPES, pH 7.0, 100 mM KCl, 2 mM $MgCl_2$, 5 mM DTT, and 0.5 mM $CaCl_2$ at 21 °C for 15 s. The reaction samples were precipitated with 10% TCA, centrifuged at 16000*g* for 10 min at 4 °C, washed twice with acetone, and resuspended in SDS sample buffer. Phospho-Thr_{286}-αCaMKII was detected by western blotting using a specific mouse monoclonal anti-phospho-Thr_{286}-αCaMKII antibody and quantified by densitometry (*14*).

Steady-State Phosphorylation Assays of Enzyme Activity. The activities of αCaMKII and phospho-Thr_{286}-αCaMKII enzymes were assayed by measuring the steady-state rate of phosphorylation of the regulatory light chain of chicken gizzard myosin (MLC). Phospho-Thr_{286}-αCaMKII was generated by preincubating the enzyme with equimolar calmodulin and 1 mM ATP in the same solution as defined above for 15 s at 21 °C. We have observed that significant inhibition (90%) of the specific activity of αCaMKII to phosphorylate MLC occurs when the enzyme is incubated in the presence of Ca^{2+}/calmodulin and ATP at 30 °C (C. Fraser and K. Török, unpublished data); this does however not occur using our experimental protocol as described above. Thus there is no evidence of inhibitory autophosphorylation in our system.

The reactants were 100-fold diluted to final concentrations of 0.2 μM phospho-Thr_{286}-αCaMKII, 0.2 μM calmodulin, and 10 μM ATP. The assay mix contained 5 μM calmodulin and 1 mM ATP; thus the contribution from the additional reactants was at sufficiently low concentration not to

influence the measurements. The maximum effect by the carryover of 5 μM Ca^{2+} from the prephosphorylation mixture to the assay mix was to increase [Ca^{2+}] from 145 to 147 nM in the solution with the lowest buffering capacity of 0.5 mM Ca^{2+} and 2 mM EGTA. The 2 nM difference was within the experimental error. In all of the other Ca^{2+}/EGTA solutions, either the $CaCl_2$ or the EGTA concentrations were higher, and thus the change in [Ca^{2+}] concentration was $\ll$2 nM.

A continuous enzyme-linked spectrofluorometric assay was used to determine ADP production by monitoring the decrease in NADH fluorescence due to its oxidation to NAD^+ (*14*, *18*). Fluorescence excitation was at 340 nm, and emission was set to 460 nm. The experiments were carried out at 21 °C. Typically, to 0.5 mL of assay solution were added 5 mM DTT, 4.5 units of LDH, 2 units of PK, 2 mM PEP, and 22 μM NADH. The concentrations of calmodulin, ATP, MLC, and enzyme were 5 μM, 1 mM, 100 μM and 0.2 μM, respectively, unless otherwise specified. The enzyme was added last to the reaction mixture. Basal enzyme activity in the absence of calmodulin was <2% of that measured in the presence of calmodulin.

Free Ca^{2+} concentrations of Ca^{2+}/EGTA mixtures were calculated using a K_d value of 4.35×10^{-7} M, which was determined in buffer conditions and ionic strength similar to those used in this study (*19*). A Fortran program was written to solve the quadratic equation $[Ca^{2+}] = \{b \pm (b^2 - 4[Ca^{2+}]_0[EGTA]_0)^{1/2}\}/2$, where $b = K_d + [Ca^{2+}]_0 + [EGTA]_0$ from total Ca^{2+}, $[Ca^{2+}]_0$, total EGTA, $[EGTA]_0$ and the K_d. To verify the calculated [Ca^{2+}] concentrations, the fluorescent Ca^{2+} indicator fluo3 was titrated in Ca^{2+}/EGTA mixtures. The best fit to the titration curve gave a K_d value of 433 ($\pm$55) nM for fluo3, in good agreement with the K_d value of 390 nM previously described for fluo3 (Molecular Probes), thus verifying our calculated [Ca^{2+}] values.

Calmodulin when purified contained 2 mM EGTA; it was then 2$\times$ desalted by gel filtration with H_2O and lyophilized. Our calmodulin preparation was thus essentially free of Ca^{2+}. Calmodulin was also free of EGTA; this was confirmed by quin-2 experiments.

Stopped-Flow Kinetic Experiments. Pre-steady-state measurements of changes in TA-cal fluorescence observed at 90° to the excitation beam were performed using a double-mixing stopped-flow spectrofluorometer, Hi-Tech SF-53, with a dead time of 1.6 ms. Fluorescence excitation was set to 365 nm with a 1 nm slit width, and fluorescence emission from TA-cal was collected using a 400 nm cutoff filter. Samples were excited with a 100 W mercury arc lamp. For records <200 s photobleaching of TA-cal was negligible. The standard buffer solution contained 50 mM K^+-PIPES, pH 7.0, 100 mM KCl, 2 mM $MgCl_2$, and 1 mM DTT. For EGTA-induced dissociation of Ca^{2+}/TA-cal·αCaMKII and Ca^{2+}/TA-cal·phospho-Thr_{286}-αCaMKII complexes, 25 nM TA-cal saturated with 0.25 mM $CaCl_2$ and 200 nM enzyme, unless otherwise specified, was rapidly mixed. Syringe B contained various concentrations of EGTA (0.5−20 mM) in the same buffer solution. Concentrations of reactants in the mixing chamber are always given. All experiments were carried out at 25 °C.

Ca^{2+} dissociation was measured by quin-2 (Molecular Probes) and stopped flow as follows: fluorescence excitation was set to 320 nm with a 1 nm slit width, and fluorescence emission from quin-2 was collected using a 530 nm cutoff filter. Quin-2 (92.5 μM) in standard buffer solution with no added Ca^{2+} was mixed with 1 μM calmodulin, TA-cal, or calmodulin·αCaMKII and calmodulin·phospho-Thr_{286}-αCaMKII complexes in 20 μM Ca^{2+}-containing buffer solution (mixing chamber concentrations). Care was taken that all protein components were free of EGTA.

Software. Steady-state fluorescence data were analyzed using the GraFit software program, version 4.0. The steady-state activation data were fitted to the Hill equation $V = V_{max}[S]^n/(K_m + [S]^n)$. The steady-state TA-cal fluorescence data were fitted to the following equation, which describes cooperative binding, $F = F_0 + F_{max}[S]^n/(K_{1/2} + [S]^n)$, where F is the TA-cal fluorescence intensity that is an observation related to the amount bound, F_0 is the background fluorescence, F_{max} is the maximum TA-cal fluorescence intensity, n is the Hill coefficient, [S] is the free Ca^{2+} concentration and $K_{1/2}$ corresponds to the Ca^{2+} concentration required for half-maximal fluorescence. Stopped-flow kinetic data were fitted to exponentials using the RK-2 software program (Hi-Tech).

RESULTS

Ca^{2+}/Calmodulin-Dependent Thr_{286} Autophosphorylation of αCaMKII and Its Inhibition by MLC. To separately assess unphosphorylated and phospho-Thr_{286}-αCaMKII enzymes, it was checked that Thr_{286}-autophosphorylated enzyme was formed and, in turn, that αCaMKII enzyme remained unphosphorylated in our steady-state assay conditions when so required. Inhibition occurs when autophosphorylation of the enzyme is carried out at 30 °C (C. Fraser and K. Török, unpublished data). This is probably due to inhibitory autophosphorylation. This, however, is unlikely to occur in our experimental protocol of autophosphorylation as described in Materials and Methods as it does not lead to inhibition.

We assessed the state of Thr_{286} autophosphorylation by two means: first, we monitored the time course of Thr_{286} autophosphorylation by western blotting with a specific phospho-Thr_{286} antibody as described in ref *14*. Figure 1A shows a western blot of the Thr_{286} autophosphorylation reaction at time intervals from 0 to 120 s. Densitometric analysis of the blot revealed that the reaction was complete within the first 15 s (Figure 1A). Thus the phospho-Thr_{286}-αCaMKII enzyme was generated in our assay conditions. Second, Ca^{2+}/calmodulin dissociation kinetics are dramatically different from unphosphorylated αCaMKII compared to the Thr_{286} phosphoenzyme in saturating Ca^{2+}. As described in ref *14*, Ca^{2+}/TA-calmodulin dissociates at 0.34 s^{-1} from unphosphorylated αCaMKII whereas the rate of its dissociation from the Thr_{286} phosphoenzyme complex is 0.0027 s^{-1}. We took advantage of this difference to assess whether the αCaMKII preparation was Thr_{286} phosphorylated. The lack of any dissociation at 0.3 s^{-1} was taken as evidence for completion of Thr_{286} autophosphorylation.

The effects of various concentrations of MLC substrate on Thr_{286} autophosphorylation were tested. In the presence of 10 and 20 μM MLC, Thr_{286} autophosphorylation was inhibited by 50% and 75%, respectively. MLC concentrations greater than 30 μM were sufficient to essentially fully block Thr_{286} autophosphorylation of αCaMKII (Figure 1B).

Ca^{2+}/Calmodulin-Dependent Activation of αCaMKII and Phospho-Thr_{286}-αCaMKII Enzymes. Enzyme activation by

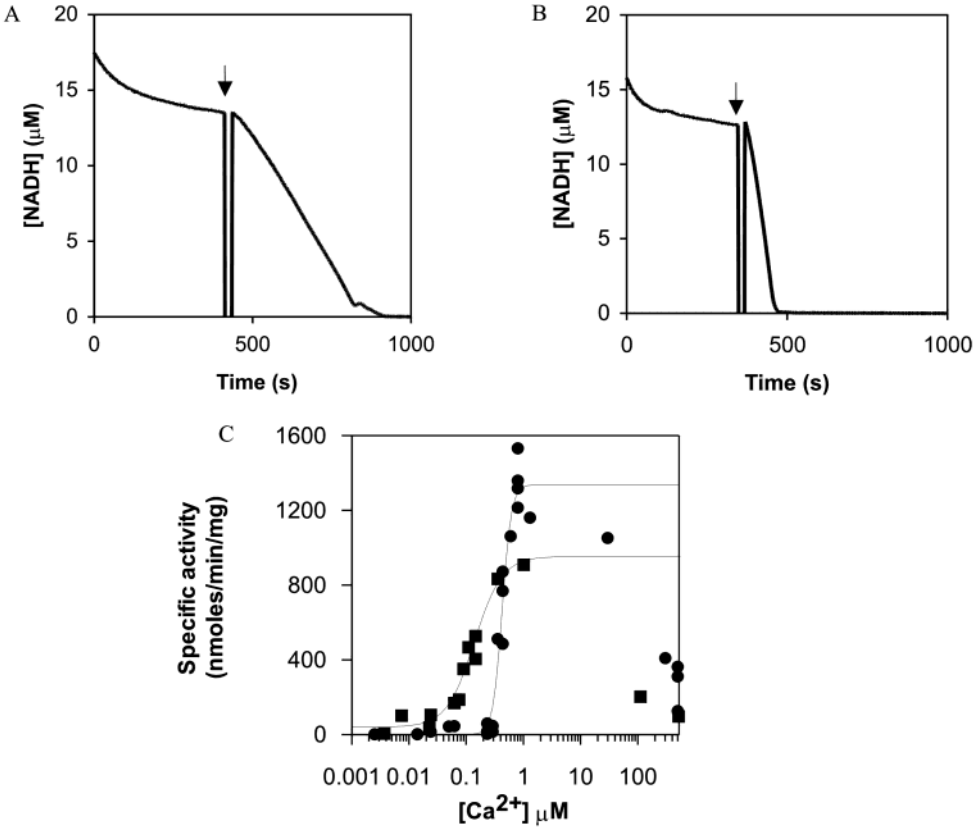

FIGURE 2: Measurement of steady-state αCaMKII and phospho-Thr_{286}-αCaMKII activation by Ca^{2+}. Prior to addition of αCaMKII (indicated by arrow), the assay components were allowed to equilibrate until a steady, slow NADH decay was obtained. The αCaMKII assay mix consisted of the following: 50 mM K^+-PIPES, pH 7.0, 100 mM KCl, 2 mM $MgCl_2$, $CaCl_2$ as indicated below, LDH (9 units/mL), PK (4 units/mL), 100 μM MLC, 2 mM PEP, 1 mM ATP, 22 μM NADH, 5 mM DTT and 5 μM calmodulin. The temperature was 21 °C. (A) Measurement of αCaMKII activity at 500 μM free Ca^{2+} concentration. The specific activity of the enzyme was 362 nmol of MLC phosphorylated min^{-1} (mg of αCaMKII)$^{-1}$. (B) Measurement of phospho-Thr_{286}-αCaMKII activity at 355 nM free Ca^{2+} concentration. αCaMKII (0.2 μM final, 19.2 μM stock concentration), preincubated with 20 μM calmodulin and 1 mM ATP, was added to initiate the reaction. The specific activity of the enzyme was 837 nmol of MLC phosphorylated min^{-1} (mg of phospho-Thr_{286}-αCaMKII)$^{-1}$. (C) Ca^{2+} dependence of steady-state activation of αCaMKII (●) and phospho-Thr_{286}-αCaMKII (■). The V_{max} for αCaMKII was 1337 (±54) nmol of MLC phosphorylated min^{-1} (mg of αCaMKII)$^{-1}$. Half-maximal activation (K_m) occurred at 425 (±119) nM [Ca^{2+}] with a best-fit value for the Hill coefficient of 5.4 (±0.4). The V_{max} for phospho-Thr_{286}-αCaMKII was 972 (±86) nmol of MLC phosphorylated min^{-1} (mg of phospho-Thr_{286}-αCaMKII)$^{-1}$ at 1 μM [Ca^{2+}]. Half-maximal activation (K_m) occurred at 137 (±56) nM [Ca^{2+}] with a best-fit value for the Hill coefficient of 1.8 (±0.3).

Ca^{2+} was determined in the presence of saturating 5 μM calmodulin and 100 μM MLC substrate under steady-state conditions. Figure 2A shows a representative steady-state assay of αCaMKII at 500 μM [Ca^{2+}]. Under these conditions, the specific activity of the unphosphorylated enzyme was 362 nmol min^{-1} (mg of αCaMKII)$^{-1}$. All steady-state assays were carried out in the presence of 100 μM MLC substrate. At this substrate concentration αCaMKII remained unphosphorylated upon addition to the reaction mixture (Figure 1B). Steady-state activation of the αCaMKII enzyme was studied over a range of free Ca^{2+} concentrations (2.5 nM to 500 μM). Figure 2C presents the specific activities of unphosphorylated αCaMKII as a function of [Ca^{2+}] concentration. Enzyme activity was increased by elevating [Ca^{2+}] to 1.0 μM and remained high up to 20 μM Ca^{2+}. A marked inhibition of the enzyme activity was observed as [Ca^{2+}] was further increased up to 500 μM. An increase of Mg^{2+} concentration from 2 to 10 mM in the assay did not abolish the inhibitory effect, indicating that inhibition was not due to any binding competition between Ca^{2+} and Mg^{2+} ions either to calmodulin or to the kinase (data not shown). Maximal activity (V_{max}) was 1337 (±54) nmol min^{-1} (mg of enzyme)$^{-1}$ for αCaMKII. Half-maximal activation (K_m) of the enzyme complex occurred at 425 (±119) nM [Ca^{2+}] (Figure 2C). Activation of αCaMKII was highly cooperative, with activity increasing from 10% to 90% of the maximum within a 2-fold raise (from 300 to 600 nM) of free Ca^{2+} concentration. The best-fit Hill coefficient (n) was 5.4 (±0.4) (Table 1).

Figure 2B shows a representative record of the phospho-Thr_{286}-αCaMKII enzyme activity with MLC substrate at 355 nM [Ca^{2+}]. In these conditions, the specific activity of the phospho-Thr_{286}-αCaMKII enzyme was 837 nmol min^{-1} (mg of phospho-Thr_{286}-αCaMKII)$^{-1}$. Steady-state activation of the phospho-Thr_{286}-αCaMKII enzyme was studied over the range of free Ca^{2+} concentrations of 2.5 nM to 500 μM (Figure 2B). As described in Materials and Methods, the carryover of 5 μM Ca^{2+} from the prephosphorylation mixture had negligible effect on the [Ca^{2+}] concentration in the assay mix. There was essentially no overlap between the activation curves of unphosphorylated and autophosphorylated αCaMKII (Figure 2C). Between 20 and 200 nM free Ca^{2+}, where the activity of unphosphorylated αCaMKII was <4% of its maximum, the activity of the Thr_{286}-autophosphorylated enzyme steadily increased up to 80% of its maximum in a Ca^{2+}-dependent manner. Maximal activity (V_{max}) measured

Table 1: Parameters of Ca^{2+} Binding and Activation of αCaMKII by Calmodulin and TA-cal As Determined by Steady-State and Equilibrium Binding Experiments

	steady state			equilibrium binding	
	K_m[a] (nM)	n	V_{max} (nmol min^{-1} mg^{-1})	$K_{1/2}$[b] (nM)	n[c]
TA-cal				458 (±99)	1.30 (±0.04)
αCaMKII	425 (±119)	5.4 (±0.4)	1337 (±54)	501 (±151)	1.50 (±0.06)
$αCaMKII_{T286-P}$	137 (±56)	1.8 (±0.3)	972 (±86)	89 (±28)	2.0 (±0.1)

[a] K_m denotes the Ca^{2+} requirement for half-maximal activation of the enzyme complexes by calmodulin. [b] $K_{1/2}$ denotes the Ca^{2+} requirement for half-maximal TA-cal fluorescence change in the absence and presence of αCaMKII. [c] n denotes the Hill coefficient.

at 1 μM [Ca^{2+}] was 972 (±86) nmol min^{-1} (mg of enzyme)$^{-1}$ for phospho-Thr_{286}-αCaMKII (Table 1). Half-maximal activation (K_m) occurred at 137 (±56) nM [Ca^{2+}] (Figure 2C and Table 1). Activation of phospho-Thr_{286}-αCaMKII by Ca^{2+} was less cooperative compared to unphosphorylated enzyme, displaying a Hill coefficient (n) of 1.8 (±0.3). At free Ca^{2+} concentrations of 20 nM, the activity of phospho-Thr_{286}-αCaMKII decreased to a mean specific activity of 50 (±37) nmol of MLC phosphorylated min^{-1} (mg of phospho-Thr_{286}-αCaMKII)$^{-1}$, corresponding to 5.0 (±3.7)% of its maximum activity. Ca^{2+}-independent activity was thus not thought to be significant with MLC as substrate.

Ca^{2+}-Dependent Interaction of TA-cal with αCaMKII and Phospho-Thr_{286}-αCaMKII. TA-cal fluorescence intensity undergoes a Ca^{2+}-dependent increase and is further enhanced by binding proteins (*16*, *20*). Fluorescence emission intensities of Ca^{2+}/TA-cal free in solution were compared with those of Ca^{2+}-free TA-cal and Ca^{2+}/TA-cal in complex with αCaMKII in the absence and presence of ATP. As seen in Figure 3A (record 1), the fluorescence intensity of TA-cal was the lowest in the presence of EGTA. Binding of Ca^{2+} to TA-cal resulted in a 4-fold increase in fluorescence intensity (record 2, Figure 3A). Further binding of αCaMKII to Ca^{2+}/TA-cal (relative fluorescence 1) enhanced the fluorescence intensity by 13% (relative fluorescence 1.13) (record 3, Figure 3A). The fluorescence difference between records 2 and 3 was significant; this fluorescence difference was used in stopped-flow kinetic experiments to measure the second-order rate constant of association of Ca^{2+}/TA-cal with αCaMKII in ref *14*. Addition of 1 mM ATP decreased fluorescence intensity by 40% (relative fluorescence 0.6) (record 4, Figure 3A). Addition of 4 mM EGTA (22 nM [Ca^{2+}]) reduced TA-cal fluorescence intensity to the basal levels but with a spectral red shift indicating TA-cal binding to phospho-Thr_{286}-αCaMKII at 22 nM [Ca^{2+}] (record 5, Figure 3A), suggesting incomplete dissociation of the Ca^{2+}/TA-cal·phospho-Thr_{286}-αCaMKII at 22 nM [Ca^{2+}]. This was consistent with the result of stopped-flow experiments presented below in Figure 4B. In contrast, addition of 4 mM EGTA (22 nM [Ca^{2+}]) to a solution of αCaMKII and Ca^{2+}/TA-cal in the absence of ATP reduced the fluorescence intensity to the basal levels, indicating no interaction between TA-cal and αCaMKII at 22 nM [Ca^{2+}] (record 6, Figure 3A).

[Ca^{2+}] dependence of the fluorescence increase of TA-cal alone and in the presence of αCaMKII or phospho-Thr_{286}-αCaMKII was studied as shown in panels B, C, and D of Figure 3, respectively. The [Ca^{2+}] requirement for half-maximal TA-cal fluorescence change, $K_{1/2}$, was 458 (±99) nM [Ca^{2+}] (Figure 3B) and in the presence of αCaMKII was 501 (±151) nM [Ca^{2+}] (Figure 3C), comparable to the [Ca^{2+}] requirement for half-maximal activation by calmodulin (Table 1). The best-fit Hill coefficient (n) for Ca^{2+} binding to TA-cal alone was 1.30 (±0.04) and to TA-cal in the presence of αCaMKII (in the absence of ATP) was 1.50 (±0.06). In comparison, n for activation by calmodulin (in the presence of ATP) was 5.4 (±0.4). In the interaction with phospho-Thr_{286}-αCaMKII, [Ca^{2+}] required for half-maximal TA-cal fluorescence change was 89 (±28) nM (Figure 3D and Table 1), while that for activation by calmodulin was 137 (±56) nM. The best-fit Hill coefficient (n) was 2.0 (±0.1), similar to that for activation (Figure 2C). These data suggest that ATP binding affects not only Ca^{2+}/calmodulin affinity for αCaMKII (*14*) but also the affinities of some of calmodulin's Ca^{2+} binding sites when in complex with αCaMKII.

Dissociation of Ca^{2+}/TA-cal and Its Complexes with αCaMKII and Phospho-Thr_{286}-αCaMKII by Ca^{2+} Sequestration. In the dissociation process of Ca^{2+}/TA-cal induced by EGTA at 20 mM final concentration (corresponding to a [Ca^{2+}] concentration of 5.5 nM) in the absence of any target, TA-cal fluorescence decreased from relative fluorescence 1 to 0.195 in a biphasic process (Figure 4A and Table 2). The dissociation kinetics of Ca^{2+}/TA-cal were independent of the final [Ca^{2+}] in the 5.5−433 nM range. Fluorescence decays upon Ca^{2+} sequestration from the Ca^{2+}/TA-cal·αCaMKII and Ca^{2+}/TA-cal·phospho-Thr_{286}-αCaMKII complexes were more complex (Figure 4A,B and Table 2). Both phases of TA-cal fluorescence decay were affected by αCaMKII and phospho-Thr_{286}-αCaMKII binding. In the phospho-Thr_{286}-αCaMKII complex, a slower, third phase of TA-cal fluorescence decay appeared (Figure 4B and Table 2).

The kinetics of fluorescence changes upon Ca^{2+} sequestration from the Ca^{2+}/TA-cal·αCaMKII and Ca^{2+}/TA-cal·phospho-Thr_{286}-αCaMKII complexes were further analyzed in experiments in which the final [Ca^{2+}] was varied. The fluorescence of TA-cal returned to the basal level (0.195), suggesting that the complex Ca^{2+}/TA-cal·αCaMKII fully dissociated when the final [Ca^{2+}] was in the range of 5.5−293 nM (data not shown). The lack of protein dependence in the 50 nM to 1 μM αCaMKII concentration range (data not shown) suggests that the dissociation pathway is via Ca^{2+} dissociation. In contrast, in the same range of final [Ca^{2+}] concentrations, the fluorescence of the Ca^{2+}/TA-cal·phospho-Thr_{286}-αCaMKII complex did not reach the end point, and the rate of decay gradually decreased with increasing final [Ca^{2+}] (Figure 4B). Complete dissociation of the Ca^{2+}/TA-cal·phospho-Thr_{286}-αCaMKII complex only occurred when the final [Ca^{2+}] was 5.5 nM.

Measurement of Ca^{2+} Dissociation Kinetics by Stopped Flow and Quin-2. To understand the role of Ca^{2+} dissociation in the dissociation of calmodulin from αCaMKII and

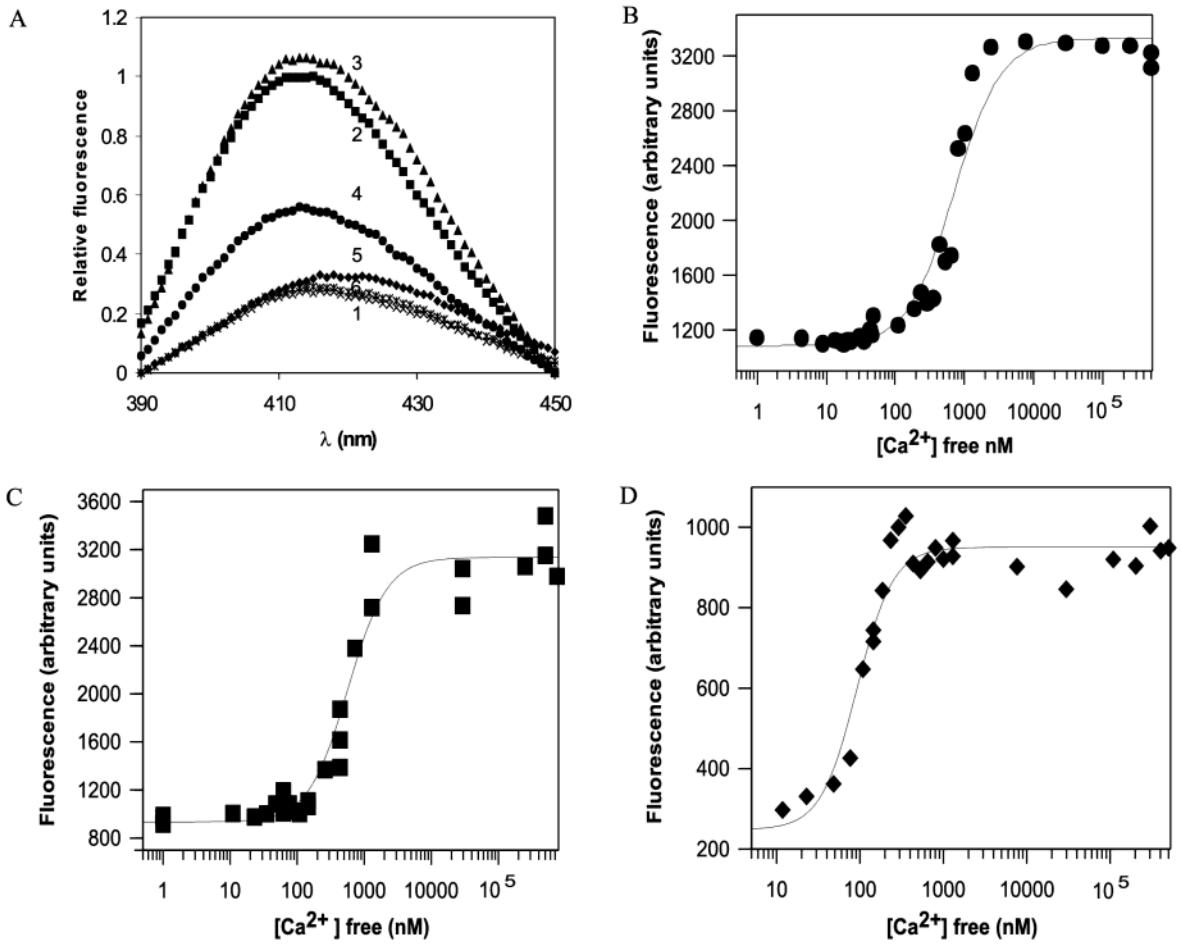

FIGURE 3: Steady-state fluorescence measurements of complexes of Ca^{2+}/TA-cal and αCaMKII. (A) Fluorescence emission spectra of TA-cal (12 nM) in the presence of 2 mM EGTA (1 nM [Ca^{2+}]) (record 1, ×), after addition of 2.1 mM $CaCl_2$ (0.1 mM [Ca^{2+}]) (record 2, ■), in the presence of αCaMKII (1 μM), 0.1 mM [Ca^{2+}], and 12 nM TA-cal (record 3, ▲), after addition of 1 mM ATP (record 4, ●), after addition of 4 mM EGTA (22 nM [Ca^{2+}]) (record 5, ◆), and finally in the presence of 100 nM αCaMKII, 12 nM TA-cal, and 4 mM EGTA (22 nM [Ca^{2+}]) (record 6, ∗). (B) Ca^{2+} dependence of the TA-cal fluorescence increase in the absence of αCaMKII. The fluorescence intensity of 12.5 nM TA-cal was measured at the indicated concentrations of free Ca^{2+}. Half-maximal TA-cal fluorescence was measured at a [Ca^{2+}] of 458 (±99) nM ($K_{1/2}$). (C) Ca^{2+} dependence of the interaction of TA-cal with αCaMKII. The fluorescence intensity of 12.5 nM TA-cal was measured in the presence of 200 nM αCaMKII at the indicated concentrations of free Ca^{2+}. Half-maximal TA-cal fluorescence was measured at a [Ca^{2+}] of 501 (±151) nM ($K_{1/2}$). (D) Ca^{2+} dependence of the interaction of TA-cal with phospho-Thr_{286}-αCaMKII. The fluorescence intensity of 12.5 nM TA-cal was measured in the presence of 12.5 nM phospho-Thr_{286}-αCaMKII at the indicated [Ca^{2+}]. Phospho-Thr_{286}-αCaMKII was generated by preincubating 1.25 μM αCaMKII in the presence of 1.25 μM TA-cal, 50 μM [Ca^{2+}], 2 mM $MgCl_2$ and 1 mM ATP for 15 s to allow Thr_{286} autophosphorylation. The autophosphorylated complex was then added to the assay mixture (0.5 mL), giving final concentrations of 11.8 nM [Ca^{2+}] (2 mM EGTA in the assay mixture), 12.5 nM phospho-Thr_{286}-αCaMKII, and 12.5 nM TA-cal. Half-maximal TA-cal fluorescence in the presence of phospho-Thr_{286}-αCaMKII was measured at a [Ca^{2+}] of 89 (±28) nM ($K_{1/2}$).

phospho-Thr_{286}-αCaMKII complexes, measurements were carried out using the fluorescent Ca^{2+} chelator, quin-2. As shown in Figure 4C, Ca^{2+} dissociation from calmodulin was seen as a single exponential process at 10 s^{-1}. From Ca^{2+}/calmodulin·αCaMKII and Ca^{2+}/calmodulin·phospho-Thr_{286}-αCaMKII complexes, however, two and three phases of Ca^{2+} dissociation were observed, respectively (Table 3). In addition to an initial Ca^{2+} release at 8−12 s^{-1}, an intermediate phase at 0.3−1.6 s^{-1} occurs. Most striking is an additional slow phase of Ca^{2+} dissociation from the calmodulin·phospho-Thr_{286}-αCaMKII complex at 0.023 s^{-1}. We considered that the amplitude of quin-2 fluorescence change represents dissociation from the two high-affinity sites of calmodulin (*21*−*24*). The amplitude obtained for the Ca^{2+} dissociation from the Ca^{2+}/calmodulin·αCaMKII complex was similar to that of Ca^{2+}/calmodulin, and it thus also corresponds to two sites. In contrast, the amplitudes of Ca^{2+} dissociation from the Ca^{2+}/calmodulin·phospho-Thr_{286}-αCaMKII complex correspond to one site at 8 s^{-1}, two sites at 0.3 s^{-1}, and one site at 0.023 s^{-1}, showing stabilization of every Ca^{2+} binding site.

DISCUSSION

Ca^{2+}/Calmodulin-Dependent Activation of α*CaMKII and Phospho-Thr_{286}-*α*CaMKII.* The activation curves of the two enzymes revealed that when 20% of the αCaMKII enzyme was active (at 300 nM [Ca^{2+}]), 90% of phospho-Thr_{286}-αCaMKII was in the active conformation. Phospho-Thr_{286}-αCaMKII was partially active in the 20−200 nM [Ca^{2+}] concentration range in saturating 5 μM calmodulin (Figure 2C). In the same range of [Ca^{2+}], the unphosphorylated αCaMKII was inactive (Figure 2C). Thr_{286} autophosphorylation increased the sensitivity of the autophosphorylated

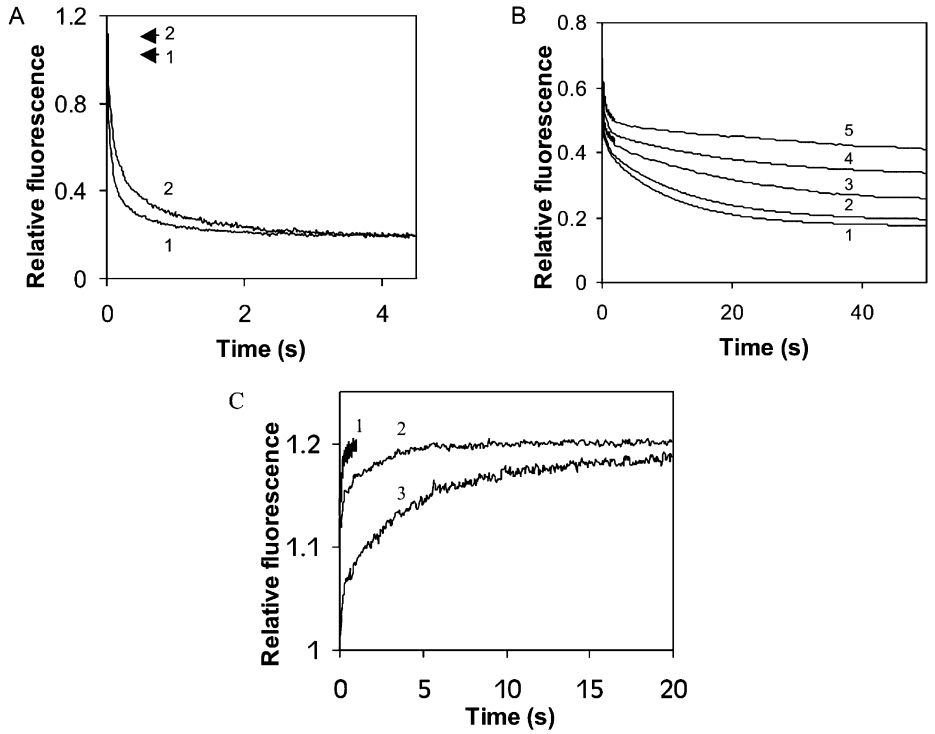

FIGURE 4: Dissociation kinetics of Ca^{2+}/TA-cal and its complexes with αCaMKII and phospho-Thr_{286}-αCaMKII upon Ca^{2+} sequestration. (A) TA-cal fluorescence changes in its complex with αCaMKII. A solution containing 25 nM TA-cal (record 1) or 25 nM TA-cal and 200 nM αCaMKII (record 2) and 0.25 mM Ca^{2+} was mixed with 20 mM EGTA (mixing chamber concentrations) in a stopped-flow apparatus. Initial fluorescence values were Ca^{2+}/TA-cal, 1 (arrow 1) and Ca^{2+}/TA-cal·αCaMKII, 1.13 (arrow 2). The final fluorescence was 0.195. The observed rates and amplitudes are shown in Table 2. (B) TA-cal fluorescence changes upon dissociation of the Ca^{2+}/TA-cal·phospho-Thr_{286}-αCaMKII complex by EGTA. A solution of 25 nM TA-cal, 0.2 μM αCaMKII and 0.5 mM ATP was mixed with EGTA to give 5.5, 27, 104, 184 and 293 nM final free $[Ca^{2+}]$ in records 1, 2, 3, 4 and 5, respectively (mixing chamber concentrations). The initial fluorescence of the Ca^{2+}/TA-cal·phospho-Thr_{286}-αCaMKII complex was 0.62. Kinetic parameters are shown in Table 2. (C) Ca^{2+} dissociation kinetics of calmodulin and its complexes with αCaMKII and phospho-Thr_{286}-αCaMKII measured by quin-2. 92.5 μM quin-2 was rapidly mixed with 1 μM calmodulin (record 1), 1 μM calmodulin and 1.1 μM αCaMKII (record 2), and 1 μM calmodulin, 1.1 μM αCaMKII and 0.5 mM ATP (record 3) in 20 μM $CaCl_2$ (reactant concentrations in mixing chamber). The excitation wavelength was 320 nM, and emitted light was collected using a 530 nm cutoff filter. Relative fluorescence 1 corresponds to the level of the 20 μM Ca^{2+}/quin-2 complex. Relative fluorescence in records 1 and 2 at time 0 is 1.1. The rates and amplitudes of quin-2 fluorescence changes are given in Table 3.

Table 2: Kinetic Parameters of the Dissociation of Ca^{2+}/TA-cal Complexes with αCaMKII and Phospho-Thr_{286}-αCaMKII upon Ca^{2+} Sequestration Monitored by TA-cal Fluorescence

	k_1 (s^{-1})	A_1	k_2 (s^{-1})	A_2	k_3 (s^{-1})	A_3	final $[Ca^{2+}]$ (M)
TA-cal	47 (±1.8)	0.67	5.4 (±0.3)	0.33			5.5×10^{-9}
αCaMKII[a]	14.5 (±0.2)	0.69	1.00 (±0.04)	0.31			5.5×10^{-9}
$\alpha CaMKII_{T286-P}$[a]	13.8 (±0.8)	0.35	1.00 (±0.05)	0.11	0.15 (±0.02)	0.54	5.5×10^{-9}

[a] αCaMKII and phospho-Thr_{286}-αCaMKII concentrations were 0.2 μM; the final $[Ca^{2+}]$ was 5.5 nM. Values of the mean and standard error (SE) of measurement are given.

Table 3: Kinetic Parameters of Ca^{2+} Dissociation of Ca^{2+}/cal Complexes with αCaMKII and Phospho-Thr_{286}-αCaMKII Measured by Quin-2 Fluorescence

	k_1 (s^{-1})	A_1 (V)[a]	k_2 (s^{-1})	A_2 (V)	k_3 (s^{-1})	A_3 (V)	final $[Ca^{2+}]$ (M)[b]
TA-cal	11.1 (±0.3)	0.180[c] (±0.003)					6.9×10^{-8}
calmodulin	10 (±1)	0.100 (±0.003)					6.9×10^{-8}
αCaMKII	11.5 (±1.4)	0.060 (±0.004)	1.6 (±0.3)	0.031 (±0.003)			6.9×10^{-8}
$\alpha CaMKII_{T286-P}$	8.0 (±1.4)	0.042 (±0.004)	0.31 (±0.03)	0.076 (±0.004)	0.023 (±0.016)	0.044 (±0.014)	6.9×10^{-8}

[a] The amplitude of quin-2 fluorescence increase is given as measured. Normalized amplitudes were obtained by considering that dissociation of two Ca^{2+} ions is measured from calmodulin, while dissociation of the other two Ca^{2+} ions is too fast to measure (*21*−*24*). On that basis the amplitude of 0.05 V corresponds to the dissociation of 1 mol of Ca^{2+}/mol of calmodulin. [b] Final $[Ca^{2+}]$ was calculated from mixing 92.5 μM quin-2 [K_d 25 nM (*24*)] and 20 μM $CaCl_2$. [c] The amplitude of quin-2 fluorescence changes when measuring Ca^{2+} dissociation from TA-cal was greater than that measured with calmodulin. This difference is most likely due to a contribution from TA-cal fluorescence in the conditions of measurement.

enzyme to Ca^{2+} compared to that of the unphosphorylated αCaMKII by about 3-fold. Half-maximal activation of αCaMKII occurred at ~0.5 μM $[Ca^{2+}]$, which corresponds to $[Ca^{2+}]$ concentrations within cells activated by an extracellular stimulus, whereas half-maximal activation of phospho-Thr_{286}-αCaMKII occurred at ~0.14 μM $[Ca^{2+}]$, which is in the range of resting intracellular $[Ca^{2+}]$ concentration (*25*).

Steady-state activation of αCaMKII by Ca^{2+} showed positive cooperativity (Hill coefficient 5.4). Slight increases in Ca^{2+} concentration were sufficient to cause large increases in enzyme activity, suggesting a linkage between several Ca^{2+} binding sites involved in the activation process. Such highly cooperative Ca^{2+}-dependent activation has also been shown for calcineurin and may be consistent with a switch mechanism by which the unphosphorylated enzyme is inactivated upon Ca^{2+} sequestration within the cells (*26*). A similarly high Hill coefficient of $n \sim 5$ was described for the Ca^{2+} dependence of Thr_{286} autophosphorylation by Bradshaw et al. (*27*), published after this paper was submitted. It is important to note that, in our case, αCaMKII was activated by Ca^{2+}/calmodulin binding only, as Thr_{286} autophosphorylation was inhibited by the addition of a sufficiently high concentration of the substrate MLC (Figure 1B). The level of cooperativity in both this and in our work exceeds the number of Ca^{2+} binding sites in calmodulin. A Hill coefficient of >4 is reasonably attributed to the involvement of more than one calmodulin molecule in the reaction mechanism. Such behavior has been indicative of the involvement of two αCaMKII subunits and two calmodulin molecules, one bound to each subunit, in the intersubunit Thr_{286} autophosphorylation mechanism (*28*). The Hill coefficient of >4 found in our experiments may therefore indicate an intersubunit substrate phosphorylation mechanism in which the bound substrate is phosphorylated by an adjacent subunit. In contrast, activation of substrate phosphorylation by phospho-Thr_{286}-αCaMKII by Ca^{2+} was less cooperative with a Hill coefficient $n = 1.8$, suggesting a different mechanism and/or perhaps that fewer Ca^{2+} binding sites are involved in the activation of this complex.

The activities of both unphosphorylated and phospho-Thr_{286}-αCaMKII enzymes were inhibited as the free Ca^{2+} concentration increased from 10 to 500 μM. This effect was not due to any binding competition of Ca^{2+} and Mg^{2+} ions to calmodulin or αCaMKII, since an increase to 10 mM Mg^{2+} did not reduce the inhibitory effect. This inhibition of activity may be related to the mode of binding of calmodulin to the enzyme target at that range of Ca^{2+} concentration. Using our kinetic data, the bell-shaped Ca^{2+} dependence of phospho-Thr_{286}-αCaMKII may reflect activation by partially saturated calmodulin but inhibition by Ca^{2+}-saturated calmodulin. Unphosphorylated αCaMKII also has a bell-shaped Ca^{2+} activation curve, but the high cooperativity in its activition by Ca^{2+} suggests that the fully Ca^{2+}-saturated calmodulin·enzyme complex has full activity. Previous studies have observed inhibitory effects of calmodulin on αCaMKII activation and proposed a model in which αCaMKII possesses two calmodulin binding sites, one for activation and a second, low-affinity calmodulin binding site for inhibition (*29*). A number of additional studies have now reached the surprising conclusion that calmodulin mediates both inactivation and activation of target proteins (*29*−*32*). In anthrax adenylyl cyclase, the bell-shaped activation curve is explained by Ca^{2+} competition to displace Mg^{2+} in the catalytic site of edema factor (*33*).

In our study, using MLC as substrate and in saturating calmodulin, phospho-Thr_{286}-αCaMKII activity at <20 nM [Ca^{2+}] was 50 nmol min^{-1} mg^{-1}, corresponding to 5.0 (±3.7)% of its maximum activity. Partial activity of phospho-Thr_{286}-αCaMKII increased from 5% to 50% in the range of 20−100 nM [Ca^{2+}], suggesting that Ca^{2+}/calmodulin trapping was required for activation of phospho-Thr_{286}-αCaMKII. Autonomous or Ca^{2+}-independent partial activity of phospho-Thr_{286}-αCaMKII corresponding to 20−80% of the maximum activity has been described for substrate syntide 2 (*10*, *15*). Recent evidence also shows that in some cases autonomous activation of the kinase can be achieved by its substrate protein in a Thr_{286} autophosphorylation-independent manner (*11*). It has now become apparent that persistent kinase activity may be achieved by diverse mechanisms (*10*) involving direct activation of αCaMKII by the substrate (*11*), by Ca^{2+}/calmodulin-independent activation of phospho-Thr_{286}-αCaMKII (*10*, *15*), and by Ca^{2+}/calmodulin-dependent activation of phospho-Thr_{286}-αCaMKII, shown in this study.

Ca^{2+}-Dependent Interaction of TA-cal with αCaMKII and Phospho-Thr_{286}-αCaMKII. TA-cal is a highly fluorescent derivative of calmodulin which allows the mechanism of the interaction of calmodulin with Ca^{2+} and the target enzyme αCaMKII to be probed. Previous kinetic studies suggest that TA-cal binds to target proteins with comparable affinity to calmodulin but with different consequences for the target enzyme activity (*16*, *18*, *34*). Various derivatives of calmodulin can act on different calmodulin-dependent enzymes, and a particular derivative can act either as agonist or as antagonist (*35*). TA-cal has been reported to act as an agonist (activator) for PDE activation but as an antagonist for MLCK (*16*). Interestingly, TA-cal also activates plasma membrane Ca^{2+}-ATPase (*34*). For αCaMKII, TA-cal is shown to bind the enzyme with an affinity comparable to that previously published for wild-type calmodulin (*13*, *14*). TA-cal activates Thr_{286} autophosphorylation of αCaMKII and MLC phosphorylation by αCaMKII (C. Fraser and K. Török, unpublished data).

The Ca^{2+} dependence of the interaction of TA-cal with αCaMKII showed cooperativity (Hill coefficient 1.5) and required 501 nM [Ca^{2+}] for half-maximal complex formation (Table 1). This value is similar to that required for formation of Ca^{2+}/TA-cal and to that required for half-maximal activation of the enzyme (Table 1). Additionally, the cooperativity of $n = 1.3$ observed upon TA-cal binding (Figure 3C) is very little compared to that displayed upon activation of the enzyme ($n = 5.4$) (Figure 2C). These data indicate that the Ca^{2+} affinities of the low-affinity sites in TA-cal are not noticeably affected by the binding of αCaMKII alone, consistent with the data showing that the predominant binding conformation of calmodulin to αCaMKII in the absence of substrates is similar to that of free Ca^{2+}/calmodulin in solution (*14*). In contrast, in αCaMKII complexes containing substrates ATP and MLC as well, Ca^{2+} affinities of all four Ca^{2+} binding sites of calmodulin are similar, as suggested by the activity data in Figure 2C.

For the Thr_{286}-autophosphorylated enzyme, the requirements for Ca^{2+} for both complex formation and enzyme activation are greatly reduced. The interaction of TA-cal with phospho-Thr_{286}-αCaMKII as a function of [Ca^{2+}] showed positive cooperativity (Hill coefficient 2.0) and required 89 nM [Ca^{2+}] for half-maximal complex formation (Figure 3D and Table 1). These values are comparable to those obtained for phospho-Thr_{286}-αCaMKII activation by Ca^{2+} (Table 1). As seen by comparing the Ca^{2+} dependence of activity (Figure 2C) and TA-cal binding (Figure 3C,D), the presence of substrate MLC has no significant effect. These data

suggest that the partial Ca^{2+}/calmodulin-dependent activity observed at very low [Ca^{2+}] may be the result of formation of partially Ca^{2+}-saturated calmodulin complexes with phospho-Thr_{286}-αCaMKII. This finding is supported by the Ca^{2+} dissociation kinetic data presented for the phospho-Thr_{286}-αCaMKII enzyme.

Mechanisms of Ca^{2+} and Calmodulin Dissociation. Binding of targets to Ca^{2+}/calmodulin produces increases in calmodulin's affinity for Ca^{2+}. Several studies using targets such as MLCK (*37*, *38*), troponin I (*39*), and calmodulin-sensitive phosphodiesterase (*38*) have reported dramatic increases in Ca^{2+} binding to calmodulin. These increases in Ca^{2+} affinity are reflected by decreases in the rate of Ca^{2+} dissociation from the lower affinity N-terminal and higher affinity C-terminal EF-hands of calmodulin (*21*). Binding of the αCaMKII peptide to Ca^{2+}/calmodulin produces an increase in the Ca^{2+} affinity of the N-terminal regulatory domain from 14-fold to 350-fold and a decrease in the Ca^{2+} dissociation kinetics from 60-fold to 140-fold. Smaller effects are observed for the C-terminal domain of calmodulin, where the CaMKII peptide increases the apparent Ca^{2+} affinity 8−100-fold and slows the dissociation kinetics 13−32-fold (*22*).

Rates for the Ca^{2+} chelator-induced dissociation of Ca^{2+}/calmodulin, Ca^{2+}/calmodulin·peptide, and Ca^{2+}/calmodulin·enzyme complexes have been reported. Peptide and MLCK binding to calmodulin has been shown to reduce the rate of Ca^{2+} dissociation from the N-terminal sites from 405 to 1.8−2.9 s^{-1} (a 140−225-fold decrease) and from the C-terminal Ca^{2+} binding sites from 2.4 to 0.1−0.4 s^{-1} (a 6−24-fold decrease) (*21*). Quin-2-induced dissociation of Ca^{2+} from a peptide derived from a calmodulin·CaMKII enzyme revealed biphasic dissociation with rates of 2.9 and 0.29 s^{-1} (*22*). Calmodulin labeled at Cys_{27} with the fluorescence probe MIANS has been used to study EGTA-induced dissociation of complexes of Ca^{2+}/calmodulin with the proteins caldesmon, calponin, and MLCK, reporting monophasic dissociations of 13.5, 1 and 3.5 s^{-1}, respectively (*23*).

Ca^{2+} dissociation from the C-terminal sites of Ca^{2+}/calmodulin bound to MLCK was reported at a rate of 0.18 s^{-1} (*21*). The rate of EGTA-induced disruption of the Ca^{2+}/cal·MLCK complex occurs at 3.5 s^{-1} (*21*). These data provide a mechanism of MLCK inactivation in which Ca^{2+} dissociation from the N-terminal sites of calmodulin, in the Ca^{2+}/cal·MLCK complex, appears to control the rate of inactivation as the [Ca^{2+}] falls. MLCK is thus inactivated before calmodulin is fully dissociated from the complex. For skeletal muscle MLCK, biphasic dissociation of Ca^{2+} at >1000 and 1.6 s^{-1} was measured (*24*), and the rate of inactivation was 1 s^{-1} (*24*). Inactivation of skeletal muscle MLCK is thus fit by a model in which full dissociation of calmodulin from the complex is coupled to both dissociation of Ca^{2+} and enzyme inactivation.

Ca^{2+} Dissociation Mechanism of Calmodulin As Detected by TA-cal and Quin-2. Half-maximal fluorescence of TA-cal was measured at [Ca^{2+}] of 460 nM, similar to that for Tyr_{99} fluorescence, which is attributed to reporting high-affinity Ca^{2+} binding to calmodulin (*40*). Remarkably, Tyr fluorescence is completely quenched in TA-cal (K. Török, unpublished data), suggesting proximity of the phenyl ring of Tyr_{99} and the anilino ring of the TA moiety. Ca^{2+} binding to the low-affinity sites ($K_d \sim 5\ \mu M$ at physiological ionic strengths) causes essentially no further fluorescence change in TA-cal. Ca^{2+} dissociation from calmodulin and TA-cal was observed at 11 s^{-1} at physiological ionic strength and 2 mM $MgCl_2$. This value is consistent with reported rates and with the view that Ca^{2+} dissociation is only measured from two high-affinity sites in these conditions, as dissociation from the two low-affinity sites is immeasurably rapid (*21*−*24*). TA-cal fluorescence changes upon Ca^{2+} sequestration occurred at 47 and 5.4 s^{-1}; 47 s^{-1} is too slow to represent Ca^{2+} dissociation from the low-affinity sites but is significantly faster than the measured Ca^{2+} dissociation from the high-affinity sites (*21*−*24*). Thus the first phase of free Ca^{2+}/TA-cal fluorescence change upon Ca^{2+} sequestration may represent a conformational change following rapid dissociation of Ca^{2+} from the low-affinity sites. The second phase of TA-cal fluorescence change is likely to correspond to Ca^{2+} dissociation from the high-affinity sites. The difference in the rates measured by the TA and the quin-2 probes may be due to reversibility of conformational change and Ca^{2+} binding, respectively.

Dissociation Mechanism of Ca^{2+}/cal·αCaMKII and Ca^{2+}/cal·Phospho-Thr_{286}-αCaMKII Complexes. The initial complexes of TA-cal with αCaMKII and phospho-Thr_{286}-αCaMKII in saturating Ca^{2+} are defined as complexes with $Ca^{2+}{}_4$/cal. To establish the preferable first step of dissociation, the kinetics of displacement by calmodulin and by Ca^{2+} sequestration are compared. Kinetic experiments studying displacement of $Ca^{2+}{}_4$/TA-cal from $Ca^{2+}{}_4$/TA-cal·αCaMKII have revealed a dissociation rate constant of 0.34 s^{-1} and from $Ca^{2+}{}_4$/TA-cal·phospho-Thr_{286}-αCaMKII of 0.003 s^{-1} (*14*). These rates are much slower than the observed rates of the fast phase of TA-cal fluorescence change at 14 s^{-1} upon Ca^{2+} sequestration from $Ca^{2+}{}_4$/cal·αCaMKII and $Ca^{2+}{}_4$/cal·phospho-Thr_{286}-αCaMKII or the 8−11 s^{-1} Ca^{2+} dissociation rates from these complexes measured by quin-2. Thus, the preferable first step of dissociation of the $Ca^{2+}{}_4$/TA-cal·αCaMKII or $Ca^{2+}{}_4$/TA-cal·phospho-Thr_{286}-αCaMKII complex involves Ca^{2+} dissociation from these complexes.

It is noteworthy that, in the process of Ca^{2+} dissociation from the Ca^{2+}/cal·αCaMKII complex measured by quin-2, only two sites are detected. This suggests that Ca^{2+} binding to the low-affinity sites is not sufficiently stabilized in the Ca^{2+}/cal·αCaMKII complex to be in the measurable range and that Ca^{2+} binding to calmodulin in complex with αCaMKII is similar to that of free calmodulin. This observation is consistent with observing little change in the dynamics of αCaMKII-bound calmodulin with a resonance energy transfer probe (*14*). While this paper was under review, the dissociation of all four Ca^{2+} ions at measurable rates was reported from the Ca^{2+}/cal·αCaMKII complex (*41*). This work was, however, carried out in the absence of Mg^{2+} and, therefore, cannot be directly compared with our result.

In contrast, Ca^{2+} dissociation from all four sites is measured in Ca^{2+}/cal·phospho-Thr_{286}-αCaMKII. Ca^{2+} dissociation rates of 10, 0.31, and 0.023 s^{-1} show significant stabilization of all four Ca^{2+} sites, consistent with high-affinity Ca^{2+}/cal binding by phospho-Thr_{286}-αCaMKII (*13*, *14*). The Ca^{2+} dissociation rates measured by quin-2 are close to the rates of TA-cal fluorescence decay upon Ca^{2+} sequestration by EGTA of 13.8, 1.0 and 0.15 s^{-1}. The differences may have to do with the different final [Ca^{2+}] concentrations and some Ca^{2+} rebinding as a result.

Our data with TA-cal show that resting [Ca^{2+}] levels prevent dissociation of the Ca^{2+}/cal·phospho-Thr_{286}-αCaMKII complex and suggest that, at a [Ca^{2+}] of 5−293 nM, calmodulin in complex with phospho-Thr_{286}-αCaMKII may be partially Ca^{2+} saturated (Figure 4B). The [Ca^{2+}] dependence of a partially Ca^{2+}-saturated complex of TA-cal with phospho-Thr_{286}-αCaMKII correlates well with the [Ca^{2+}] dependence of both TA-cal binding to (Figure 3D) and steady-state activity of (Figure 2C) phospho-Thr_{286}-αCaMKII, suggesting that a partially Ca^{2+}-saturated complex may represent the active Ca^{2+}/calmodulin-dependent phosphoenzyme. Inactivation of phospho-Thr_{286}-αCaMKII according to this model occurs by slow Ca^{2+} dissociation from the partially Ca^{2+}-saturated complex. In contrast, dissociation of the Ca^{2+}/TA-cal complex of unphosphorylated αCaMKII does not show a stable intermediate in the same Ca^{2+} concentration range and suggests that inactivation of αCaMKII occurs by Ca^{2+} dissociation at 1.6 s^{-1} from the Ca^{2+}/calmodulin-bound αCaMKII complex.

In conclusion, our findings relate to the function of αCaMKII in response to Ca^{2+} levels within the cells. In particular, the steady-state activation, equilibrium binding, and dissociation experiments suggest that unphosphorylated αCaMKII, in complex with Ca^{2+}/calmodulin, is highly responsive to Ca^{2+} stimulation in cells. Thr_{286} autophosphorylation transforms the molecule so that phospho-Thr_{286}-αCaMKII can be activated by calmodulin at resting [Ca^{2+}]. Thus, Thr_{286} autophosphorylation by a combination of increased sensitivity to [Ca^{2+}] and formation of stable and active partially Ca^{2+}-saturated complexes may provide a kinase with maintained activity at resting intracellular Ca^{2+} conditions albeit in a Ca^{2+}/calmodulin-dependent manner.

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