Sensitivity of CaM Kinase II to the Frequency of Ca^{2+} Oscillations

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The transduction of many cellular stimuli results in oscillations in the intracellular concentration of calcium ions (Ca^{2+}). Although information is thought to be encoded in the frequency of such oscillations, no frequency decoder has been identified. Rapid superfusion of immobilized Ca^{2+}- and calmodulin-dependent protein kinase II (CaM kinase II) in vitro showed that the enzyme can decode the frequency of Ca^{2+} spikes into distinct amounts of kinase activity. The frequency response of CaM kinase II was modulated by several factors, including the amplitude and duration of individual spikes as well as the subunit composition and previous state of activation of the kinase. These features should provide specificity in the activation of this multifunctional enzyme by distinct cellular stimuli and may underlie its pivotal role in activity-dependent forms of synaptic plasticity.

Many types of cells exhibit spikes or oscillations in the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) during endogenous rhythmic activity or in response to stimuli such as repetitive action potentials or a constant exposure to agonists that act on the phosphoinositide cascade. Information may be encoded by the frequency, amplitude, duration, and number of these oscillations (*1*). However, the molecular mechanisms that underlie the decoding of such information are unknown. In the brain, stimulus frequency–dependent changes in synaptic transmission, such as long-term potentiation (LTP) and long-term depression (LTD), involve the action of CaM kinase II (*2–4*), suggesting that this enzyme plays a role in decoding the frequencies of synaptic inputs. CaM kinase II phosphorylates and regulates multiple cellular targets that contribute to neurotransmission, neuronal plasticity, cell excitability, gene expression, secretion, and cell shape (*5*), and it would therefore appear ideally suited to a role in translating changes in $[Ca^{2+}]_i$ into changes in cell function. The regulatory features of the enzyme, especially its Ca^{2+}- and calmodulin-dependent autophosphorylation at Thr^{286} (*6*), prolong the effect of brief Ca^{2+} transients. This autophosphorylation disrupts the kinase regulatory domain, thereby converting the enzyme to a Ca^{2+}-independent (autonomous) form (*5*), and results in the trapping of bound calmodulin (*7*). Computer simulations based on the physical, catalytic, and regulatory properties of the kinase predict that it may be capable of decoding the frequency of repetitive Ca^{2+} spikes (*8*, *9*).

We investigated whether CaM kinase II can decode the frequency of Ca^{2+} oscillations in vitro. We immobilized the kinase on a solid support to be able to superfuse it rapidly with a pulsatile flow of solutions containing either high or low Ca^{2+} concentrations (Fig. 1). To allow immobilization of the multimeric kinase, we inserted a hemagglutinin (HA) tag at the COOH-terminus (association domain) of α– and β–CaM kinase II at the cDNA level (*10*) and expressed the constructs in COS-7 cells (*11*). The HA-tagged kinase present in extracts of transfected cells was then adsorbed onto polyvinylchloride (PVC) plastic coated with a monoclonal antibody to HA (*12*). The tag allowed immobilization of CaM kinase II from cell extracts (*13*) in antibody-coated PVC microtiter plates, as judged by assays of activity with the synthetic peptide autocamtide-3 (AC-3) as substrate (Fig. 2A) (*14*). Immobilized CaM kinase II retained its ability to undergo autophosphorylation at Thr^{286} in the presence of Ca^{2+}, calmodulin, and adenosine triphosphate (ATP), typically acquiring 60 to 80% autonomous activity (*5*) (Fig. 2A). The concentration of calmodulin that produced half-maximal activation (CaM_{50}) of immobilized α–CaM kinase II was ~80 nM, which was about three times the value for immobilized β–CaM kinase II (Fig. 2B), similar to results obtained with the soluble kinases (*5*).

We used immobilized CaM kinase II in PVC tubing for all subsequent experiments in a pulsatile-flow setup (Fig. 1). This device allows rapid flow and complete exchange of the solution in the reaction tubing within 20 ms and can deliver single or multiple pulses of solution with precise kinetics (*15*). The kinase remained attached inside the tubing during rapid flow (*16*), and its maximal activity did not decay during exposure to activators (Ca^{2+}, calmodulin, and ATP) at 30°C and autophosphorylation for 6 s (*17*).

Autophosphorylation at Thr^{286} is an intersubunit reaction that requires the coincident binding of two calmodulin molecules on two proximal subunits in the holoenzyme (*8*, *18*). Thus, submaximal activation of the kinase by a single Ca^{2+} spike may fail to result in autophosphorylation, whereas repeated spikes may lead to accumulation of calmodulin on the enzyme and to autophosphorylation. To investigate the frequency dependence of stimulation of autophosphorylation by Ca^{2+} spikes, we first determined the calmodulin concentrations that produced submaximal kinase autophosphorylation in a 6-s reaction. We exposed either α– or β–CaM kinase II to various concentrations of calmodulin in the presence of saturating Ca^{2+} and assayed generation of autonomous CaM kinase II activity, a measure of CaM kinase II autophosphorylation (*5*). The

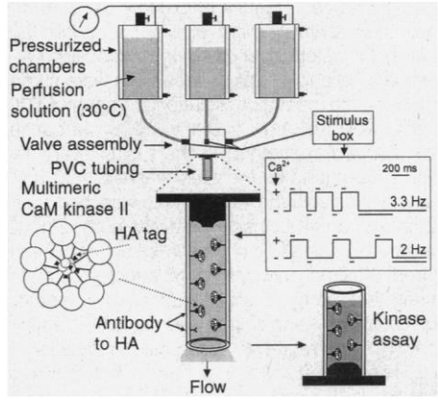

Fig. 1. Immobilization and superfusion of CaM kinase II. Multimeric α– or β–CaM kinase II containing HA tags in the central core (*10*, *28*) was immobilized in PVC tubing that was connected to a pulse-flow device. Perfusion solutions [50 mM Pipes (pH 7.0), 10 mM $MgCl_2$, 0.01% BSA] containing either Ca^{2+}, calmodulin, and ATP or EGTA were contained in air-pressurized (350 kPa) chambers (200 ml) maintained at 30°C by circulating water in an outer cylinder. Solutions flowed in Teflon tubing to solenoid poppet valves (General Valves, Fairfield, New Jersey) arranged in a manifold assembly. Opening and closing of the valves were controlled by a ValveBank stimulus box (AutoMate Scientific, Oakland, California). Brief pulses of Ca^{2+} were generated by opening the Ca^{2+} valve for 50 ms (bars above pulse traces), allowing solution flow in the PVC tubing (*15*), and stopping flow for the remaining duration of the pulse, after which the EGTA valve was opened for 50 ms (bars below pulse traces). Frequencies were varied by stopping flow between Ca^{2+} pulses for different periods. At the end of each stimulus sequence, a 400-ms opening of the EGTA valve provided a wash. Tubing was subsequently removed and cut into two equal pieces that were then plugged at one end. Deactivation solution in the tubes was gently aspirated, and 30 μl of phosphorylation assay mixture was added (*14*).

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slopes of the dose-dependent increase in autonomy induced by Ca^{2+} and calmodulin (Fig. 3A) were steeper than those obtained for substrate phosphorylation (Fig. 2B), with Hill coefficients of 1.6 to 1.8 and CaM_{50} values that were two to three times those for substrate phosphorylation (Fig. 2B), consistent with the requirement for two calmodulin binding events for autophosphorylation. The CaM_{50} for autophosphorylation of β–CaM kinase II was about one-fourth that of α–CaM kinase II.

We exposed α–CaM kinase II to short (200 ms) repetitive pulses of activation solution containing 100 nM calmodulin, which resulted in ~20% autonomy after continuous exposure for 6 s (Fig. 3A). Between pulses, we perfused a deactivation solution containing 500 μM EGTA. Stimulation at 1 Hz for up to 100 pulses produced little autonomy of α–CaM kinase II (6 to 7%), even though the enzyme had been exposed to activators for a total period of 20 s (Fig. 3B). In contrast, stimulation at 4 Hz generated >60% autonomy after 100 pulses. At an intermediate frequency of 2.5 Hz, ~30% autonomy was obtained after 100 pulses. These results indicate that the generation of CaM kinase II autonomy in response to pulsatile exposure to activators is frequency-dependent—possibly because, at high frequencies, when spike intervals are shorter than the time required for complete dissociation of calmodulin (*7*), each successive spike recruits calmodulin onto holoenzymes that still retain subunit-bound calmodulin. Such recruitment would increase the probability of autophosphorylation, which itself reduces the dissociation rate of calmodulin between Ca^{2+} spikes (*7*). Thus, autophosphorylation and calmodulin trapping during the early set of Ca^{2+} spikes may facilitate autophosphorylation during subsequent spikes in a feed-forward or functionally cooperative (*19*) manner. Such behavior should give rise to an early exponential increase in autonomy with successive Ca^{2+} spikes. In fact, the increase in autonomy during the first 30 pulses at 4 Hz is best described by a power relation (Fig. 3B).

If partial autophosphorylation potentiates the response of the enzyme to additional stimulation, then the response of a partially phosphorylated CaM kinase II to a subthreshold stimulation frequency should be increased. To test this hypothesis, we first induced phosphorylation of α–CaM kinase II to yield ~13% autonomy (*20*) and then delivered 0 to 16 pulses at 1 Hz (Fig. 3B). The rate of increase in autonomy for partially phosphorylated kinase was almost four times that for the unphosphorylated enzyme. Thus, the initial state of CaM kinase II activity affects its response to subsequent Ca^{2+} stimulation, with autophosphorylation shifting sensitivity to lower stimulation frequencies.

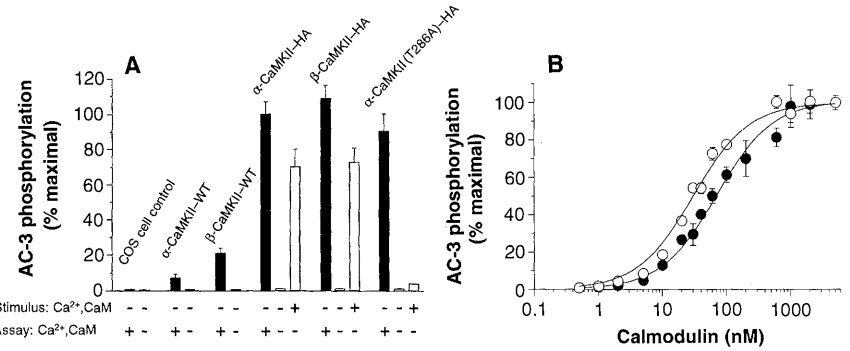

Fig. 2. Properties of immobilized CaM kinase II. (**A**) Immobilization of recombinant CaM kinase II and kinase activity assays. COS-7 cell extracts (*11, 13*) were added to PVC microtiter plates coated with antibodies to HA. Adsorption of kinase was determined by assaying AC-3 phosphorylation (*14*), which is expressed as a percentage of the maximal value measured with HA-tagged α–CaM kinase II (α-CaMKII–HA). Control extracts were obtained from COS cells transfected with vector DNA. Wild-type kinase without tag (WT) bound poorly to the plates. Ca^{2+}-independent activity after stimulation (*14*) in the wells (open bars) was a consequence of Thr^{286} autophosphorylation, because an α–CaM kinase II mutant in which Thr^{286} was replaced by alanine [α-CaMKII(T286A)–HA] (*5*) showed little Ca^{2+}-independent activity. Data are means ± SEM (n = 5). (**B**) Calmodulin dependence of activation of immobilized CaM kinase II. Activity of immobilized α–CaM kinase II (●) and β–CaM kinase II (○) was measured (*14*) in the presence of various concentrations of calmodulin at saturating Ca^{2+} (500 μM) and is expressed (means ± SEM, n = 4) as a percentage of maximal AC-3 phosphorylation obtained with 5 μM calmodulin. Lines represent fits to the Hill equation [$A = A_{max}/(1+10^{n\{\log(CaM_{50})-\log[CaM]\}})$], with the Hill coefficient (n) set to 1 and maximal activity (A_{max}) set to 100%. The concentration of calmodulin producing half-maximal activation (CaM_{50}) was 79 ± 8 nM for α–CaM kinase II and 30 ± 2 nM for β–CaM kinase II.

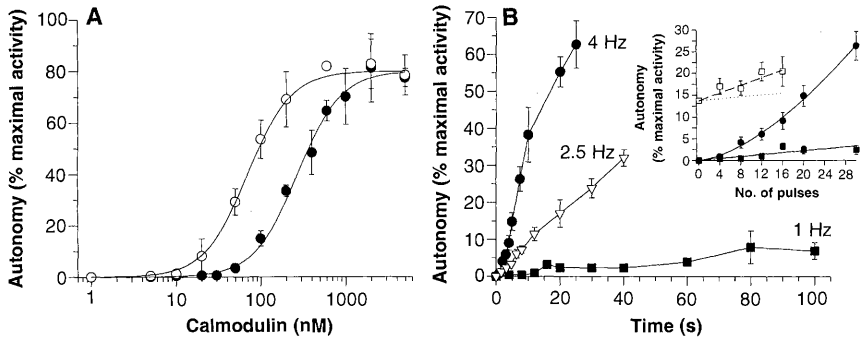

Fig. 3. Stimulation of CaM kinase II by pulse flow. (**A**) Calmodulin dependence of CaM kinase II autonomy, indicating a requirement of two calmodulin binding events for autophosphorylation. Immobilized α–CaM kinase II (●) and β–CaM kinase II (○) were stimulated for 6 s with perfusion solution containing 500 μM $CaCl_2$, 250 μM ATP, and 1 to 5000 nM calmodulin (solution was refreshed in the tubing every second with a 50-ms flow) and then washed (400 ms) with deactivation solution. Each tubing was then cut in two (2 cm), and CaM kinase II activity (autonomous and maximal Ca^{2+}- and calmodulin-stimulated) was measured. Maximal activity was not affected by any treatment. Autonomy is expressed as a percentage of maximal Ca^{2+}- and calmodulin-stimulated activity. Data are means ± SEM (n = 4). Lines represent fits to the Hill equation. For α– and β–CaM kinase II, Hill coefficient = 1.6 ± 0.1 and 1.8 ± 0.1; maximal autonomy = 80 ± 2 and 81 + 1%; and CaM_{50} = 271 ± 17 and 70 ± 3 nM, respectively. (**B**) Frequency dependence of α–CaM kinase II autophosphorylation induced by pulse stimulation with Ca^{2+} and calmodulin. Four to 100 stimulus pulses (200 ms) were delivered at 1, 2.5, or 4 Hz by flowing perfusion solution containing either Ca^{2+} (500 μM), calmodulin (100 nM), and ATP (250 μM) or EGTA (500 μM) in tubing as described in Fig. 1. Autonomy is expressed as in (A). Data are means ± SEM (n = 4). (**Inset**) Initial rise in autonomy at 4 Hz (●)or 1 Hz (■) plotted over the first 30 pulses. The response to stimulation at 4 Hz was best fitted by a power function ($y = ax^b$) [solid curved line; sum of square deviations (SSD) = 0.121], whereas the response to stimulation at 1 Hz did not differ from a linear relation (solid straight line). The response to 1-Hz stimulation was enhanced when 4 to 16 pulses were delivered to partially autophosphorylated enzyme (*20*) (□) (means ± SEM, n = 7). The linear fit (dashed line) indicates a slope of 0.47 ± 0.05, almost four times that for unphosphorylated kinase responding to 1-Hz stimulation (0.12 ± 0.02; dotted line, shifted up to same initial level of autonomy as that of the prephosphorylated enzyme for comparison).

To characterize the frequency response of CaM kinase II, we exposed the enzyme to only 30 pulses of 200 ms (equal to 6 s of total exposure to Ca^{2+}, calmodulin, and ATP). This protocol, at 100 nM calmodulin, assured that autonomy did not approach saturation even at high frequencies. Under these conditions, α–CaM kinase II autophosphorylation was markedly dependent on the frequency of stimulation (Fig. 4A).

We next examined the effect of pulse duration (80, 200, 500, or 1000 ms) on the frequency response of α–CaM kinase II. We fixed total exposure to Ca^{2+}, calmodulin, and ATP at 6 s for all frequencies by adjusting the number of pulses (Fig. 4A). Varying the pulse duration shifted the frequency response and changed its steepness. Increasing the pulse duration presumably increases the number of calmodulin binding events per pulse, which enhances the probability of coincident binding of calmodulin on neighboring subunits and of Thr^{286} autophosphorylation within a given pulse. Therefore, the frequency response to 500-ms pulses (resembling the time course of cardiac spikes) is shifted to lower frequencies compared with that for shorter pulses. At 1 Hz, for instance, the response of the enzyme to a 6-s exposure to activators was approximately seven times greater when delivered as 500-ms pulses rather than as 200-ms pulses. Similarly, α–CaM kinase II was poorly activated even at 3.3 Hz with 80-ms pulses (simulating brief neuronal spikes), compared with the activation obtained with 200-ms pulses at this frequency. As the pulse duration was reduced, the threshold for activation became sharper, whereas longer pulses evoked responses over a broader range of frequencies (Fig. 4A).

We examined the effect of the amplitude of Ca^{2+} and calmodulin spikes on the frequency response of α–CaM kinase II. Increasing the concentration of calmodulin (at saturating Ca^{2+}) in the stimulus pulses broadened the frequency response; a sharper threshold and increased signal-to-noise ratio were obtained at lower concentrations of calmodulin (Fig. 4B).

The affinity of β–CaM kinase II for calmodulin is greater than that of α–CaM kinase II (Figs. 2B and 3A) (*5*). Generation of autonomy for this isoform was also frequency-dependent (Fig. 4C), and, at all stimulus frequencies, the autonomous activity of β–CaM kinase II was greater and its response range was broader than for α–CaM kinase II. Thus, differential incorporation of α and β subunits may modulate the frequency response of the enzyme. In fact, the frequency-response curve of α– and β–CaM kinase II heteromers (*21*) was intermediate between those of homomers (Fig. 4C), suggesting that differential expression of α and β subunits at a given site might modulate the frequency response to Ca^{2+} oscillations.

Our data demonstrate that CaM kinase II can act as a frequency decoder of Ca^{2+} oscillations in vitro. This property is critically dependent on the effectiveness of the individual Ca^{2+} spikes in stimulating autophosphorylation of the enzyme (Fig. 4), such that, in a given cellular compartment, the variables that contribute to this effectiveness, including calmodulin availability (*22*), will determine the frequency response of the enzyme. CaM kinase II phosphatases would be expected to act in concert with the conditions described above to suppress the response of the enzyme to low frequencies and produce a sharper threshold for activation (*8*). After the threshold for kinase activation is reached and some subunits of the enzyme become autophosphorylated, the response of the kinase to low-frequency stimuli is increased (Fig. 3B), presumably because CaM kinase II autophosphorylation is functionally cooperative (*19*). This critical property may enable the kinase (i) to maintain an autophosphorylated state at subthreshold activation after stimulation by agonists or induction of LTP (*23*), which may underlie the large extent of basal CaM kinase II autonomy (8 to 20%) observed in the hippocampus (*24*); (ii) to act as molecular "switch" (*4*, *25*); and (iii) to serve as a tag of the history of activity at a synapse (*26*). Autonomy of CaM kinase II does, in fact, set the stimulus frequency threshold at which the enzyme responds (Fig. 3B) and determines the stimulus frequency threshold required to induce LTP or LTD (*3*).

CaM kinase II is present in many different tissues and cellular compartments and is thus exposed to a vast array of Ca^{2+} transients with distinct properties (*1*). Its ability to decode the frequency of Ca^{2+} spikes should provide specificity in the activation of this multifunctional enzyme.

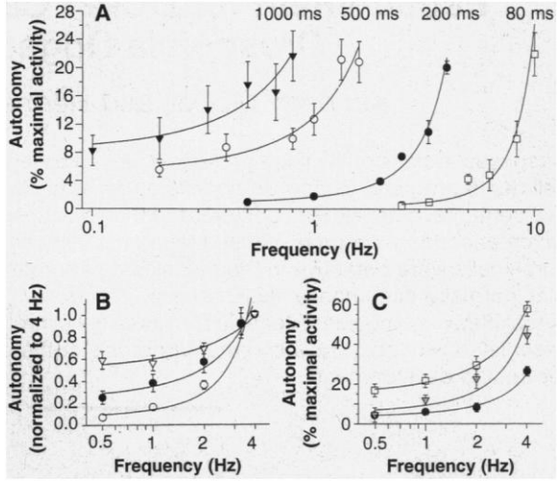

Fig. 4. Modulation of the frequency response of CaM kinase II. (**A**) Effects of Ca^{2+} pulse duration. α–CaM kinase II was exposed at various frequencies to different durations [80 ms (75 pulses, n = 3), 200 ms (30 pulses, n = 8), 500 ms (12 pulses, n = 5), or 1000 ms (6 pulses, n = 4)] of pulses of Ca^{2+}, calmodulin, and ATP, with a total exposure to activators of 6 s for each protocol. For the 80-ms pulse protocol, 2-cm tubing was used with a 20-ms solution flow (*15*). For each test frequency, maximal Ca^{2+}- and calmodulin-stimulated activity was unchanged. Autonomy (means ± SEM) is expressed as in Fig. 3. The lines represent fits to a single-exponential function ($y = ae^{bx}$) (*29*). The steepness of the curves increased as the pulse duration was reduced; at 80 ms, it was ~1.5, 3.2, and 4.9 times that at 200, 500, and 1000 ms, respectively. (**B**) Effect of Ca^{2+} and calmodulin pulse amplitude. The effective amplitude of Ca^{2+} pulses (200 ms) was varied by changing the calmodulin concentration: 30 nM (○), 100 nM (●), or 400 nM (▽). A different number of pulses was applied for each calmodulin concentration (60 at 30 nM, 30 at 100 nM, and 10 at 400 nM), so that maximal autonomy generated at 4 Hz would be approximately similar (20 to 25%). The slope of each frequency response was visualized by normalizing the mean autonomy values (± SEM, n = 4) relative to that at 4 Hz. Lines represent fits as in (A). The slope of the curves increased as the calmodulin concentration was reduced; at 30 nM, it was ~1.9 and 4.7 times that at 100 and 400 nM, respectively. (**C**) Effect of kinase subunit composition. The frequency response was determined as in (A) (30 pulses of 200 ms, 100 nM calmodulin) for either α–CaM kinase II (●), β–CaM kinase II (□), or α– and β–CaM kinase II heteromers (gray triangles). The slope of the curve for α–CaM kinase II was ~1.5 times that of β–CaM kinase II. The curves for α– and β–CaM kinase II homomers differed significantly from that of the pooled data ($P < 0.01$), whereas that for the heteromers did not ($P > 0.1$, F test).

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6. Autophosphorylation occurs at Thr^{286} in the autoinhibitory domain of the α isoform of CaM kinase II and at a comparable site in the other isoforms (*5*).

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10. We reasoned that positioning the HA tag in the association domain, at a site where an insert is normally found in some alternatively spliced δ isoforms of the enzyme [P. Mayer, M. Mohlig, H. Schatz, A. Pfeiffer, *Biochem. J.* **298**, 757 (1994)], should provide an exposed tag that would not interfere with assembly of the oligomer or with its catalytic and regulatory functions. Size fractionation chromatography revealed that the HA tag did not affect the oligomerization of the enzyme. Site-directed mutagenesis (with single-stranded M13–α–CaM kinase II cDNA and antisense oligonucleotide 5′-CCCTGGCCTGGTCCTTCACAGCTGAGCTCCAGGTCCGGCGTAGTCGGGGACGTCGTAAGGATAAGGAGCTCCATGGGGCAGGACGGAGGG-3′) was used to insert the HA tag (*8*) at the COOH-terminus of α–CaM kinase II as described (*27*). For β–CaM kinase II cDNA (in the SRα expression vector), we used a Transformer site-directed mutagenesis kit (Clontech, Palo Alto, CA) to insert a sense oligonucleotide (5′-CTCCAGTGGCCCCACTGCAGGGAGCTCCTTATCCGTACGAGTCCCCGACTACGCCGGACCTGGAGCTCAGCTGTGGAGCTGCGCCTGGTTTC-3′) encoding the HA tag.
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12. PVC tubing or microtiter plates were coated with antibodies to HA (BAbCO, Richmond, CA) [10 μg/ml in 20 mM $NaHCO_3$ (pH 9.6)] at 4°C overnight. Plates or tubing were washed with Pipes saline [50 mM Pipes (pH 7.0), 150 mM NaCl] and then incubated with 3% (w/v) bovine serum albumin (BSA) (ELISA grade, Sigma) in Pipes saline for 1 hour at room temperature.
13. COS-7 cell extracts were diluted 1:40 with Pipes saline containing 0.1% BSA, 10% (v/v) glycerol, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, leupeptin (10 μg/ml), and pepstatin (10 μg/ml) (Sigma) and then incubated in wells or tubing for 2 to 3 hours at 4°C. This procedure allowed approximately 10 to 15% of the kinase present in the extract to bind to the plastic, saturating the coated antibody. The plastic was washed three times with Pipes saline containing 0.1% BSA and 0.1% Tween-20 and once with the same solution without Tween-20 and was then maintained on ice in Pipes saline containing 0.1% BSA until experimental treatment.
14. Kinase activity was measured in 96-well microtiter plates in 50 mM Pipes (pH 7.0), 10 mM $MgCl_2$, 0.01% BSA, 15 μM AC-3, 25 μM [γ-^{32}P]ATP (1 Ci/mmol), and either 500 μM EGTA (Ca^{2+}-independent activity) or 500 μM $CaCl_2$ and 2 μM calmodulin (Ocean Biologicals, Edmonds, WA) (maximal Ca^{2+}-stimulated activity) in a total volume of 30 μl at 30°C. After 60 s, assays were terminated by pipetting and spotting assay mix on P81 phosphocellulose paper (*27*). The ability of the immobilized kinase to undergo autophosphorylation was examined by incubation for 15 s with stimulus buffer [50 mM Pipes (pH 7.0), 10 mM $MgCl_2$, 0.01% BSA, 250 μM ATP, 500 μM $CaCl_2$, 1 μM calmodulin], after which the solution was replaced with an assay mix containing 500 μM EGTA and the effect of autophosphorylation measured as Ca^{2+}-independent activity (autonomous activity), expressed as a percentage of the maximal Ca^{2+}-stimulated activity. Incubation of autophosphorylated α–CaM kinase II for up to 2 min did not result in a decrease in autonomous activity, suggesting that little or no phosphatase activity was present together with the immobilized enzyme.
15. The flow rate was 5 ml/s, assuring virtually complete (>95%) exchange of solutions inside a tubing (internal diameter, 1.58 mm) 2 cm in length with a 20-ms valve opening or, for most experiments, inside a tubing 4 cm in length with a 50-ms open time (>200 μl of flow through a 15-μl dead volume inside the manifold plus a volume of 80 μl inside the tubing).
16. After 100 pulses (50 ms each, 1 Hz) of perfusion solution (30°C), kinase activity in the tubing was 94 ± 5% (n = 6) of that in untreated tubing.
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19. The phosphorylation of Thr^{286} in one subunit facilitates that of Thr^{286} in a second subunit, resulting in functional cooperativity because a Thr^{286}-phosphorylated subunit can phosphorylate a neighbor without having to bind calmodulin (*7, 8*).
20. α–CaM kinase II was prephosphorylated by a 300-ms pulse of Ca^{2+}, calmodulin (1 μM), and ATP (from the third valve), which was followed by a 400-ms wash and 1-s delay prior to stimulation at 1 Hz. An initial control pulse, with Ca^{2+} and calmodulin but not ATP, generated no autonomy, and the subsequent response to 1-Hz stimulation did not differ from that obtained without a control pulse (Fig. 3B).
21. We confirmed co-assembly of both CaM kinase II isoforms by immunoprecipitation with an antibody to β–CaM kinase II (CB-β-1) [C. Baitinger, J. Alderton, M. Poenie, H. Schulman, R. A. Steinhardt, *J. Cell Biol.* **111**, 1763 (1990)] from a lysate of COS-7 cells transfected with equal amounts of cDNAs encoding HA-tagged α– and β–CaM kinase. Precipitated proteins were separated by SDS–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and subjected to calmodulin overlay blot analysis (*8*). Immunoprecipitated α– and β–CaM kinase II heteromers contained approximately equal amounts of each subunit.
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29. This exponential function is used to illustrate the relation between frequency of stimulation and CaM kinase II autonomy without any implied model. Data were fitted with PSI-PLOT software; the correlations for the fits in Fig. 4 were all ≥0.95, with SSD values of <0.35. The significance of differences between curves was determined with an F test comparing the variance of the pooled data set with the sum of the variance of the individual data sets.
30. We thank D. Profitt and R. Schneeveis for building the pulse-flow device; S. Sather for help with site-directed mutagenesis and early studies on immobilized kinase; A. Braun, P. Hanson, T. Meyer, A. Naini, and R. Y. Tsien for helpful discussions; and J. Ferrell and L. Stryer for their comments on the manuscript. Supported by NIH grants GM40600 and GM30179 and by a Human Frontier Science Program Organization fellowship to P.D.K.

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Requirement for DCP-1 Caspase During *Drosophila* Oogenesis

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Caspases, a class of cysteine proteases, are an essential component of the apoptotic cell death program. During *Drosophila* oogenesis, nurse cells transfer their cytoplasmic contents to developing oocytes and then die. Loss of function for the *dcp-1* gene, which encodes a caspase, caused female sterility by inhibiting this transfer. *dcp-1*$^-$ nurse cells were defective in the cytoskeletal reorganization and nuclear breakdown that normally accompany this process. The *dcp-1*$^-$ phenotype suggests that the cytoskeletal and nuclear events in the nurse cells make use of the machinery normally associated with apoptosis and that apoptosis of the nurse cells is a necessary event for oocyte development.

Apoptosis, a form of programmed cell death, is a mechanism used by organisms to remove cells that are superfluous, abnormal, or no longer needed (*1*, *2*). The failure of cells to undergo apoptosis at the appropriate time during development can lead to abnormal differentiation of tissues and the death of the organism (*3*). Large numbers of cells of the developing germ line of vertebrates and invertebrates are lost as a result of apoptosis (*4*); however, it is not clear if apoptosis is a required event in the germ line of some organisms.

The *Drosophila* ovary consists of individual egg chambers, each of which contains 16 sister germline cells that remain interconnected because of incomplete cytokinesis (*5*, *6*). One germ cell becomes an oocyte, and the rest develop into nurse cells that are linked to the oocyte by cytoplasmic bridges (ring canals). The nurse cells become polyploid and synthesize large amounts of RNA and protein, which are

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