Multifunctional Ca^{2+}/Calmodulin-dependent Protein Kinase Is Necessary for Nuclear Envelope Breakdown

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Abstract. The role of multifunctional Ca^{2+}/calmodulin–dependent protein kinase (CaM kinase) in nuclear envelope breakdown (NEB) was investigated in sea urchin eggs. The eggs contain a 56-kD polypeptide which appears to be a homologue of neuronal CaM kinase. For example, it undergoes Ca^{2+}/calmodulin–dependent autophosphorylation that converts it to a Ca^{2+}-independent species, a hallmark of multifunctional CaM kinase. It is homologous to the α subunit of rat brain CaM kinase. Autophosphorylation and substrate phosphorylation by the sea urchin egg kinase are inhibited in vitro by CaMK(273–302), a synthetic peptide corresponding to the autoinhibitory domain of the neuronal CaM kinase. This peptide inhibited NEB when microinjected into sea urchin eggs. Only one mAb to the neuronal enzyme immunoprecipitated the 56-kD polypeptide. Only this antibody blocked or significantly delayed NEB when microinjected into sea urchin eggs. These results suggest that sea urchin eggs contain multifunctional CaM kinase, and that this enzyme is involved in the control of NEB during mitotic division.

Cytosolic free calcium has been implicated in the regulation of mitosis in plant and animal cells (reviewed in Poenie and Steinhardt, 1987; Hepler, 1989). Recently, evidence has been presented which specifically implicates transient increases in intracellular free calcium ($[Ca^{2+}]_i$) in nuclear envelope breakdown (NEB)[1] (Steinhardt and Alderton, 1988; Twigg et al., 1988; Silver, 1989). It has been demonstrated that a Ca^{2+} transient immediately precedes NEB in sea urchin embryos and that buffering of Ca^{2+} with Ca^{2+} chelators will prevent NEB (Steinhardt and Alderton, 1988). Additionally, premature Ca^{2+} rises can trigger early NEB, provided there has been an adequate period of protein synthesis (Steinhardt and Alderton, 1988; Twigg et al., 1988). These results have been usefully extended in similar experiments on early division cycles in the sand dollar embryo (Silver, 1989).

One possible target of the increase in intracellular free Ca^{2+} which precedes NEB is multifunctional Ca^{2+}/calmodulin–dependent protein kinase (CaM kinase). This enzyme has been characterized from a number of species and tissues, including sea urchin egg (reviewed in Schulman, 1988). It exhibits a broad substrate specificity; its substrates include microtubule-associated protein 2 (MAP-2), tau, vimentin, synapsin I, and tyrosine hydroxylase. It may function to orchestrate diverse effects of elevated Ca^{2+}. The enzyme from rat brain has been most extensively characterized and shown to consist of isozymes composed of various ratios of subunits of 54 (α), 58 (β'), 60 (β), 59 (γ), and 60 kD (δ) in a holoenzyme containing 10–12 subunits. Each of the subunits can undergo a Ca^{2+}/calmodulin-dependent autophosphorylation. The amino acid sequences of all subunits, deduced from cDNA clones, shows them to be highly homologous (Bennett and Kennedy, 1987; Hanley et al., 1987; Lin et al., 1987; Bulleit et al., 1988; Tobimatsu et al., 1988; Tobimatsu and Fujisawa, 1989). The NH_2-terminal half of each protein contains a catalytic domain, followed by a regulatory domain that contains autophosphorylation sites near the calmodulin-binding domain and, finally, an association domain which maintains the multimeric structure (Schulman and Lou, 1989). Peptides corresponding to portions of the regulatory domain have been shown to selectively inhibit the activity of the kinase in vitro and thus constitute the autoinhibitory domain of the kinase (Kelly et al., 1988; Payne et al., 1988; Malinow et al., 1989).

An enzyme that shows several similarities to the rat brain kinase has been partially purified from sea urchin (*Arbacia punctulata*) egg actomyosin (Chou and Rebhun, 1986). It has a major 56-kD subunit that undergoes apparent autophosphorylation, a native molecular weight of 405,000, and has been shown to phosphorylate MAP-2, myosin light chains, and casein. It has been suggested that the sea urchin egg kinase may represent a homologue of multifunctional CaM ki-

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1. *Abbreviations used in this paper*: CaM kinase, Ca^{2+}/calmodulin–dependent protein kinase; MAP-2, microtubule-associated protein 2; MPF, maturation promoting factor; NEB, nuclear envelope breakdown.

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nase that is involved in regulating Ca^{2+}-dependent events during early development (Chou and Rebhun, 1986; Poenie and Steinhardt, 1987).

Here we describe the effects of microinjecting a selective peptide inhibitor of multifunctional CaM kinase and mAbs to this kinase on NEB in sea urchin eggs. We also show that sea urchin egg extracts contain a polypeptide that resembles the α subunit of the rat brain kinase. Our results support the hypothesis that a sea urchin egg kinase similar to the neuronal multifunctional CaM kinase is involved in the control of NEB during mitotic division.

Materials and Methods

Materials

L. pictus sea urchins were obtained from Marinus, Inc. (Long Beach, CA) and maintained in laboratory aquaria in natural sea water collected off shore Bodega Bay, CA. Sea urchins were fed *Macrocystis* sp. kelp. Shedding of eggs and sperm was induced by injection of 0.55 M KCl into the interperitoneal cavity. Bovine brain calmodulin and cytosolic rat brain multifunctional CaM kinase were purified as described (Schulman and Greengard, 1978; Schulman, 1984). Immunoblotting kit was purchased from Promega Biotic (Madison, WI). Nitrocellulose was from Schleicher & Schuell, Inc. (Keene, NH). Pansorbin was from Calbiochem-Behring Corp. (La Jolla, CA). [γ-^{32}P]ATP (7,000 Ci/mmol) was purchased from ICN K & K Laboratories Inc. (Irvine, CA). SDS electrophoresis reagents other than acrylamide were from Bio-Rad Laboratories (Richmond, CA). Fura-2 was obtained from Molecular Probes (Eugene, OR). All other chemicals were from Sigma Chemical Co. (St. Louis, MO). CaM kinase substrate, autocamtide-2, and peptides CaMK(273-302) and CaMK(284-302), generously provided by Dr. R. W. Tsien, were synthesized using an Applied Biosystems, Inc. (Foster City, CA) automated synthesizer, purified by HPLC, and assessed by sequencing.

Microinjection

For microinjection, eggs were allowed to settle onto a poly-DL-lysine (P-9011; Sigma Chemical Co.) coated glass coverslip cemented with silicone sealant (Dow Corning Corp., Midland, MI) into a circular hole machined into the bottom of a Corning 35-mm diameter polystyrene petri dish. All microinjection experiments were in natural sea water at 18°C. Microinjection was performed using a laboratory-made air pressure modulated apparatus consisting of a 20 cc B-D Yale ground glass hypodermic syringe connected via Leur Lok fittings and polyethylene tubing to a modified Leitz microinstrument collar. Omega dot glass capillary tubing was used to make injection pipettes with a tip diameter of ~2 μM. Injection and subsequent observations were performed on the stage of a Zeiss IM 35 inverted microscope using a Nikon UV-F 40× lens. Peptide injection solutions consisted of: 3.8 mM CaMK(273-302) or 5.4 mM CaMK(284-302) in 130 mM KCl, 10 mM NaCl, 10 mM MOPS, pH 7.0. Antibody injection solutions contained CB-β-1, CB-α-1, or CB-α-3 IgG (15-66 mM) in PBS or CB-β-1 IgG (30 mM) in 200 mM Tris-HCl, 130 mM Na citrate, pH 8.0, containing 0.02% sodium azide.

Injection volume and free Ca^{2+} were determined by fluorescence. Fura-2 (5.4 mM) was microinjected along with the peptide and antibody solution. The volume of solution injected was estimated by measurement of total cell fluorescence increase at 500-530 nm (excitation 350 nm) due to the inclusion of fura-2 in the injection solution, and the resulting concentration of antibody or peptide within the cell calculated by assuming the egg to be a sphere with a diameter of 100 μM.

Free Ca^{2+} was calculated from the fluorescence ratio (R) obtained by dividing the fluorescence (500-530 nm) values from excitation at 350 nm by those at 385 nm. This ratio changes in the same direction as $[Ca^{2+}]_i$ and can be used to calculate $[Ca^{2+}]_i$ from the equation $[Ca^{2+}]_i = K(R-R_o)/(R_s-R)$, where R_o is the ratio at 0 Ca^{2+} and R_s is the ratio at saturating Ca^{2+} (Grynkiewicz et al., 1985). K represents $K_d(F_o/F_s)$, where K_d is the effective dissociation constant for fura-2, previously determined to be 7.74×10^{-7} (Poenie et al., 1985), F_o is the fluorescence at 385 nm in 0 Ca^{2+}, and F_s is the fluorescence at 385 nm in saturating Ca^{2+} solutions. In these experiments K was modified by an average viscosity correction factor of 0.85 (Poenie et al., 1986). R_o and R_s were obtained from droplets of fura-2 in reference solutions containing 50 μM fura-2, 155 mM KCl, 25 mM NaCl, 100 mM MOPS, pH 7.02, and either 10 mM K_2H_2-EGTA or 1 mM $CaCl_2$. Excitation light was provided by 5 nm bandwidth interference filters at 350 and 385 nm rotated by a stepping motor at 0.5-4 Hz. The emitted light was collected through a 500-530-nm interference filter and photon counted in synchrony with the stepping motor so that signals from the two excitation wavelengths were stored in separate memories of an IBM AT computer.

Preparation of Sea Urchin Egg Subcellular Fractions

Eggs to be used in biochemical experiments were washed three times in natural sea water and twice in Ca^{2+}-free artificial sea water consisting of 484 mM NaCl, 27 mM $MgCl_2$, 10 mM KCl, 29 mM $MgSO_4$, and 2 mM $NaHCO_3$, pH 8.0. Eggs were sedimented (800 *g* for 60 s) for sea water changes. All procedures for the isolation of subcellular fractions were carried out at 4°C. 5 ml packed fertilized or unfertilized eggs were homogenized in 3 vol of buffer (25 mM PIPES, pH 7.0, 5 mM EGTA, 1 mM β-mercaptoethanol, 0.15 M Na perchlorate, 0.28 mg/ml soybean trypsin inhibitor, 30 μg/ml aprotinin, 1.5 mM PMSF) using a motor-driven Teflon-on-glass homogenizer. The homogenate was centrifuged at 10,000 *g* for 15 min. The supernatant of this homogenization was collected and centrifuged at 150,000 *g* for 30 min. The supernatant of the second centrifugation (designated "cytosol") was applied to a Sephadex G-50 column (2.5 × 50 cm) equilibrated in 25 mM PIPES, pH 7.0, 5 mM EGTA, 0.9 M glycerol, and 0.15 M NaCl. Material that eluted in the void volume of this column was collected and applied to a DE-52 column (Whatman Inc., Clifton, NJ) (1.5 × 15 cm) equilibrated in 25 mM PIPES, pH 7.0, 0.2 M NaCl, 2 mM EGTA, 10% glycerol, 2 μg/ml aprotinin, and 1 mM PMSF. Material that did not bind to the column (calmodulin-depleted cytosol) was collected and stored frozen at −70°C. The pellets of the first and second centrifugations (designated crude mitochondrial and microsomal fractions, respectively) were resuspended in homogenizing buffer containing 10% glycerol and stored frozen at −70°C. Protein was determined by the method of Bradford (1976).

Autocamtide-2 Phosphorylation

Autocamtide-2 phosphorylation was assayed by a modification of the method of Hanson et al. (1989). Assays contained 50 mM PIPES, pH 7.0, 15 mM $MgCl_2$, 1 mg/ml BSA, 10 μM autocamtide-2, 20 μM [γ-^{32}P]ATP (0.4 Ci/mmol), and either 1.0 mM EGTA (minus Ca^{2+}/calmodulin) or 1.0 mM EGTA, 1.3 mM $CaCl_2$, and 20 μg/ml calmodulin (plus Ca^{2+}/calmodulin), in a total volume of 50 μl. Reactions were initiated by addition of extract and terminated after 20 s by addition of TCA to a final concentration of 5%. ^{32}P incorporated into peptide was assayed by applying two-thirds of the supernatant volume to phosphocellulose paper (P81; Whatman Inc.) according to Roskoski (1985) and Cerenkov radiation determined in a scintillation spectrophotometer (LS 3801; Beckman Instruments, Inc., Palo Alto, CA).

Endogenous Phosphorylation and Autophosphorylation

Sea urchin egg subcellular fractions (100 μg) or purified rat brain CaM kinase (25-180 ng) were incubated for 3 min at 30°C in a solution containing 50 mM PIPES, pH 7.0, 15 mM $MgCl_2$, 5 μM [γ-^{32}P]ATP (5 Ci/mmol), and either 1.0 mM EGTA (minus Ca^{2+}/calmodulin) or 1.0 mM EGTA plus 1.2 mM $CaCl_2$ and 20 μg/ml calmodulin (plus Ca^{2+}/calmodulin) in a total volume of 100 μl. The reaction was terminated by the addition of 50 μl of SDS-stop solution, electrophoresed on 9% SDS polyacrylamide gels, and autoradiographed as described (Schulman, 1984).

Antibody Preparation

Monoclonal hybridomas secreting antibodies against rat brain CaM kinase were obtained as described (Scholz et al., 1988). Specificity of the mAbs for the α and β subunits of CaM kinase was determined by immunoblotting against samples of purified, soluble rat brain kinase. IgG class and subclass were determined using a mouse immunoglobulin subtype identification kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). Immunoglobulins from individual hybridomas were purified from ascites fluid by affinity chromatography on protein A-Sepharose according to the method of Ey et al. (1978). Purified antibody was dialyzed against 40 mM $NaPO_4$, 0.15 M NaCl, pH 7.4, 0.05% Na azide and stored frozen at −70°C. Purity of IgG

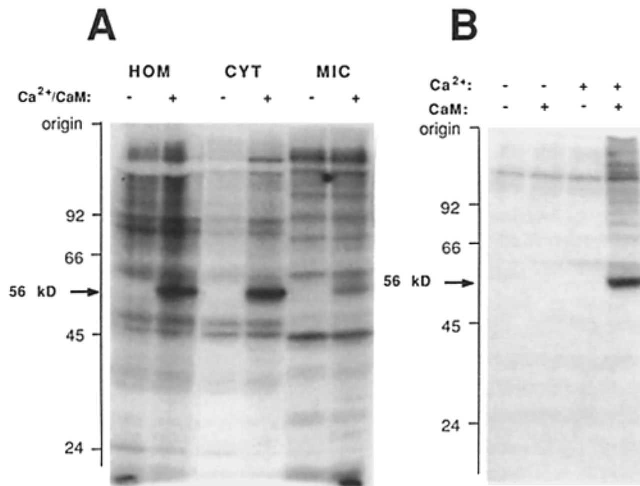


Figure 1. Ca^{2+}- and calmodulin-dependent phosphorylation of polypeptides in sea urchin egg extracts. (*A*) Subcellular fractions (*HOM*, homogenate; *CYT*, cytosol; *MIC*, microsomal fraction) prepared from unfertilized *L. pictus* eggs were incubated for 3 min at 30°C in a solution containing 50 mM PIPES, pH 7.0, 15 mM $MgCl_2$, 1 mM EGTA, 5 μM [γ-^{32}P]ATP (5 Ci/mmol), with (+) or without (−) 1.2 mM $CaCl_2$ and 20 μg/ml calmodulin. The reaction was terminated by the addition of SDS sample buffer and the samples were electrophoresed on 9% SDS polyacrylamide gels dried and subjected to autoradiography. The positions of molecular mass markers in kilodaltons are indicated. The position of the major polypeptide (calculated M_r = 56 kD) whose phosphorylation is increased in the presence of Ca^{2+} and calmodulin is indicated by an arrow. (*B*) Calmodulin-depleted cytosol prepared from *L. pictus* eggs was incubated. As in *A*, except that Ca^{2+} and calmodulin were deleted individually from the reaction. The positions of the molecular mass markers are indicated in kilodaltons and the position of the major phosphopeptide is indicated by an arrow.

preparations, as assessed by SDS-PAGE, was >95%. Affinities of individual mAbs for the kinase were determined from double-reciprocal plots of the binding of ^{125}I mAb to purified rat brain CaM kinase absorbed to microtiter plate wells (Kennel, 1982).

Immunoprecipitation

Calmodulin-depleted cytosol from sea urchin eggs was incubated for 3 min at 30°C as described for endogenous phosphorylation, except that the reaction volume was 200 μl and the reaction was terminated by the addition of EDTA to a final concentration of 27 mM instead of by addition of SDS stop solution; the samples were then immediately placed on ice. All subsequent immunoprecipitation steps except for the final solubilization of the pellet were carried out at 4°C. Aliquots (80 μl containing 40 μg of sea urchin egg protein) of phosphorylated samples were added to 170 μl of concentrated immunoprecipitation buffer (30 mM Tris-HCl, pH 7.4, 7.5 mM EDTA, 1.5% Triton X-100, 0.225 M NaCl, 0.3 mg/ml BSA, 0.05% sodium azide, 1.5 mM o-phenanthroline, 3 mM PMSF). The samples were mixed and monoclonal (0.5–2.0 μl) or control (1.5–6 μl) ascites were added; the samples were incubated overnight at 4°C. Immune complexes were collected by adsorption to pansorbin and centrifugation. Each pellet was washed four times, with vigorous resuspension, in 1 ml added PBS-0.25% NP-40 containing 0.05% sodium azide. After boiling for 3 min in 150 μl added SDS-stop solution, the samples were centrifuged at room temperature for 2 min and solubilized proteins were analyzed by SDS-PAGE and autoradiography.

Immunoblot Analyses

Protein samples (100 μg/lane) were separated on 9% SDS polyacrylamide gels and transferred to nitrocellulose according to the method of Towbin et al. (1979). After preincubation for 1 h at 37°C in Tris-saline buffer (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl) containing 3% BSA, 0.1% Triton X-100, nitrocellulose sheets were incubated overnight at 4°C with monoclonal ascites fluids diluted 1:1,000. Immunoreactive bands were visualized by incubation with alkaline phosphatase–conjugated secondary antibodies (0.2 μg/ml, 1 h at room temperature), followed by visualization of antigen–antibody complexes with a mixture of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Results

Sea Urchin Eggs Contain a Homologue of Neuronal CaM Kinase

We tested for the presence of a homologue of multifunctional CaM kinase in *L. pictus* eggs since such an activity has been found associated with actomyosin from sea urchin (*A. punctulata*) egg (Chou and Rebhun, 1986). We incubated subcellular fractions of *L. pictus* eggs under phosphorylation conditions in the presence of [γ-^{32}P]ATP, either with or without added Ca^{2+} and calmodulin, and analyzed the phosphoproteins by SDS-gel electrophoresis and autoradiography (Fig. 1). In crude fractions from fertilized or unfertilized eggs the phosphorylation of a predominant 56-kD polypeptide was increased in a Ca^{2+}-dependent manner (Fig. 1 *A*). In calmodulin-depleted cytosol, the phosphorylation of the prominent 56-kD polypeptide, as well as that of other polypeptides, was dependent on the addition of both Ca^{2+} and calmodulin to the assay (Fig. 1 *B*).

A unique feature of multifunctional CaM kinase is its conversion to a Ca^{2+}-independent enzyme by a brief period of Ca^{2+}-dependent autophosphorylation. If EGTA is added af-

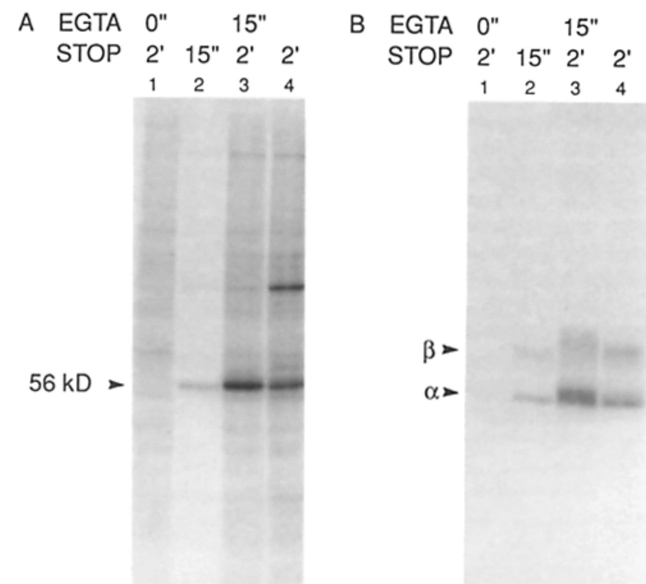


Figure 2. The sea urchin kinase exhibits both Ca^{2+}-dependent and Ca^{2+}-independent autophosphorylation. (*A*) Phosphorylation of the 56-kD protein in calmodulin-depleted cytosol was assayed for 2 min in the absence of Ca^{2+} (lane *1*), in the presence of Ca^{2+} for either 15 s (lane *2*) or 2 min (lane *4*), and after 15 s in the presence of Ca^{2+} followed by an additional 105 s in the absence of Ca^{2+} (EGTA added at 15 sec) (lane *3*) as described (Hanson et al., 1989; Lou and Schulman, 1989). The time at which EGTA is added and the time at which the reaction is terminated with SDS "stop" solution is indicated. (*B*) Autophosphorylation of purified rat brain CaM kinase (40 ng) was examined as above for comparison.

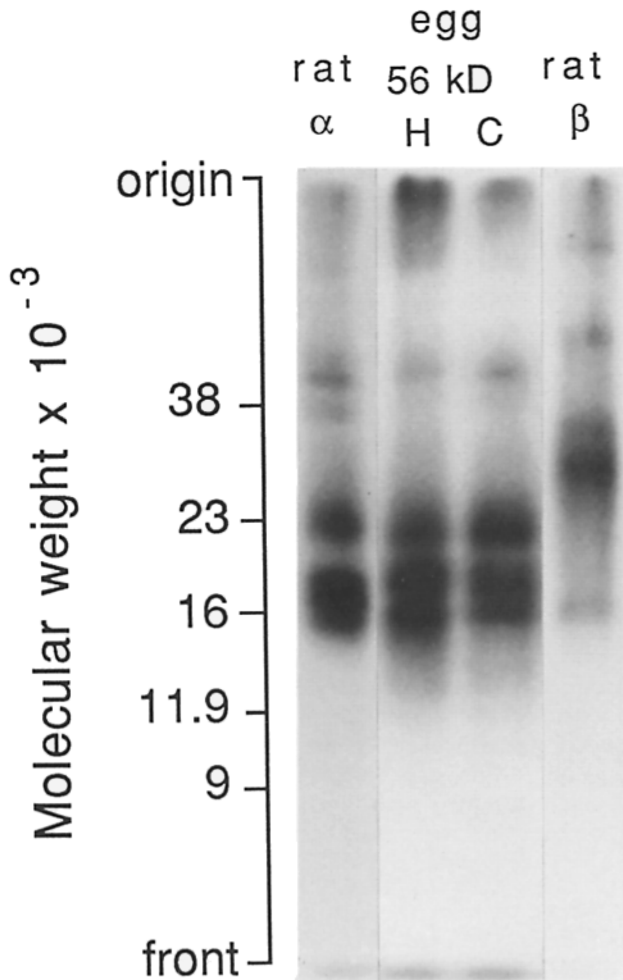


Figure 3. Comparison of phosphopeptides generated from autophosphorylated rat brain kinase subunits and endogenously phosphorylated 56-kD polypeptide from sea urchin egg. Rat brain kinase (0.4 μg) or sea urchin egg subcellular fractions (120 μg) were incubated for 3 min at 30°C as described in the legend to Fig. 2. SDS-PAGE was used to resolve autophosphorylated rat brain kinase subunits and a phosphorylated 56-kD polypeptide from *L. pictus* eggs. ^{32}P-labeled kinase subunits and 56-kD polypeptide were excised and subjected to partial proteolysis using 3 μg *S. aureus* V8 protease as described (Cleveland et al., 1977). The digestion products were separated on a 15% SDS polyacrylamide gel. The positions of migration of molecular mass markers (in kilodaltons) are indicated. (*rat α*) Rat brain kinase α (54 kD) subunit. (*egg 56 kD H*) 56 kD endogenous substrate from *L. pictus* egg homogenate phosphorylated in the presence of Ca^{2+}/calmodulin. (*egg 56 kD C*) 56 kD endogenous substrate from *L. pictus* egg cytosol phosphorylated in the presence of Ca^{2+}/calmodulin. (*rat β*) Rat brain kinase β, β', γ, δ (58–60 kD) subunits.

ter a threshold level of Ca^{2+}-dependent phosphorylation has been attained, the kinase continues to autophosphorylate in the absence of Ca^{2+} (Miller and Kennedy, 1986). We therefore tested whether phosphorylation of the 56-kD protein exhibits this hallmark of CaM kinase. Indeed, a 15-s prephosphorylation in the presence of Ca^{2+} stimulates phosphorylation of the 56-kD (Fig. 2 *A*, lane 2) which continues during the subsequent 105 s even if Ca^{2+} is chelated at 15 s (Fig. 2 *A*, lane *3*). Without this prephosphorylation, no phosphorylation of the 56-kD protein is seen during a 120-s reaction without Ca^{2+} (Fig. 2 *A*, lane *1*). Ca^{2+}-independent phosphorylation is comparable to or greater than that seen during a 120-s reaction in the continuous presence of Ca^{2+} (Fig. 2 *A*, lane 4). Purified rat brain CaM kinase shows the same properties (Fig. 2 *B*). Ca^{2+}-dependent phosphorylation of the 56-kD protein was not affected by dilution of the enzyme and is, therefore, an intramolecular reaction similar to the autophosphorylation of neuronal CaM kinase (data not shown). These characteristics are consistent with the 56-kD phosphoprotein being a subunit of a sea urchin egg homologue of multifunctional CaM kinase.

We performed phosphopeptide mapping experiments in order to determine whether the 56-kD polypeptide most closely resembles the α or β subunits of the rat brain kinase. We utilized *S. aureus* V8 protease digestion followed by one-dimensional phosphopeptide mapping since this method detects differences between autophosphorylated α and β subunits of the rat brain kinase which are 85% homologous (Tobimatsu and Fujisawa, 1989). The pattern of phosphopeptides generated from the 56-kD egg polypeptide showed considerable similarity to that from the α subunit of the rat brain kinase and clear differences from the β subunit (Fig. 3). We will therefore refer to the 56-kD polypeptide as the "α-like" subunit from sea urchin egg.

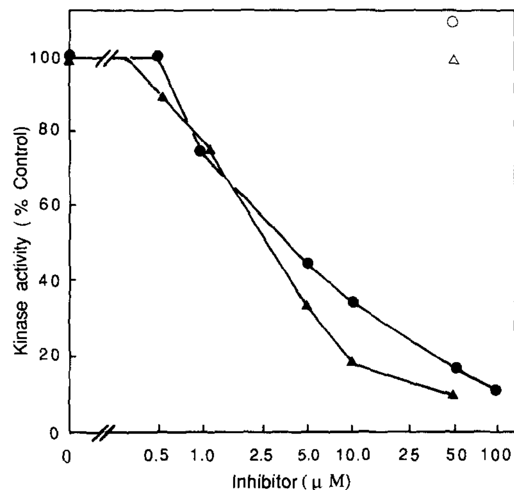


Figure 4. Effects of CaMK(273-302) and CaMK(284-302) on Ca^{2+}/calmodulin-dependent phosphorylation of autocamtide-2 by rat brain kinase and by sea urchin egg extracts. Ca^{2+}/calmodulin-dependent phosphorylation of autocamtide-2 was assayed in the presence or absence of various concentrations of CaM kinase inhibitor peptides for 20 s at 30°C. The enzyme reaction (50 μl) contained: 50 mM PIPES, pH 7.0, 15 mM $MgCl_2$, 1 mg/ml BSA, 1.0 mM EGTA, 1.3 μM $CaCl_2$, 20 μg/ml bovine brain calmodulin, 10 μM autocamtide-2, 20 μM [γ-^{32}P]ATP (0.3 Ci/mmol), and either 9.2 μg of egg calmodulin-depleted cytosol (●, ○), or 25 ng of purified brain kinase (▲, △). Inhibitory peptide CaMK(273-302) (●, ▲) or control peptide CaMK(284-302) (○, △) were added at the indicated concentrations. The level of Ca^{2+}/calmodulin-dependent phosphorylation obtained in the absence of inhibitory peptides were set at 100%. These levels were: egg calmodulin-depleted cytosol, 1.17 × 10^7 dpm/min/mg; brain CaM kinase, 4.25 × 10^9 dpm/min/mg. The level in egg homogenate is 1.32 × 10^6 dpm/min/mg.

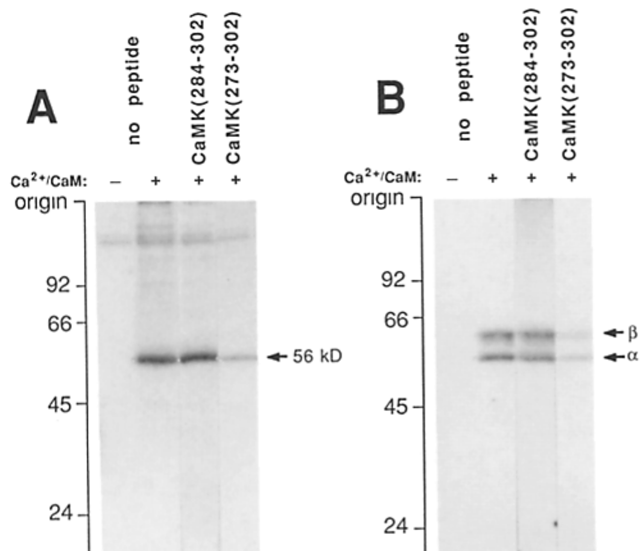


Figure 5. Inhibition of the endogenous phosphorylation of the 56-kD protein and of autophosphorylation of rat brain CaM kinase by CaMK(273-302). Endogenous phosphorylation of sea urchin egg calmodulin-depleted cytosol (9.2 μg, *A*) or of purified rat brain CaM kinase (25 ng, *B*) was measured in the presence or absence of CaMK(284-302) or CaMK(273-302). Assays contained 50 mM PIPES, pH 7.0, 15 mM $MgCl_2$, 20 μM ATP, 0.2 mg/ml BSA, and 1.0 mM EGTA, either without (−) or with (+) 1.3 mM $CaCl_2$ and 20 μg/ml calmodulin. Samples were incubated for 30 s at 30°C and the reaction terminated by the addition of SDS sample buffer. (*A*) Autoradiograph of the endogenously phosphorylated polypeptides in sea urchin egg calmodulin-depleted cytosol; (*B*) autoradiograph of the autophosphorylated rat brain kinase. Where indicated, reaction mixtures contained 50 μM CaMK(273-302) or 50 μM CaMK(284-302). The positions of molecular mass markers in kilodaltons are indicated. The positions of the α and β subunits of the rat brain kinase and of the 56-kD α-like egg polypeptide are indicated by arrows.

We reasoned that brain and egg CaM kinases might contain homologous catalytic sites and would therefore phosphorylate similar substrates and be sensitive to the same inhibitors. We therefore assayed CaM kinase activity utilizing the synthetic peptide autocamtide-2, which we have found to be an excellent substrate of neuronal CaM kinase. This substrate is selective for CaM kinase; it is not phosphorylated by protein kinase C or by cAMP-dependent protein kinase (Hanson et al., 1989).

Sea urchin egg homogenate, cytosol, crude particulate fraction, and calmodulin-depleted cytosol all contained substantial Ca^{2+}/calmodulin-dependent autocamtide-2 kinase activity. The activity present in egg calmodulin-depleted cytosol corresponded to a specific activity of 17 nmol/min/mg, nearly comparable to the high activity seen in brain. The K_m for autocamtide-2 phosphorylation by calmodulin-depleted cytosol was 5 μM.

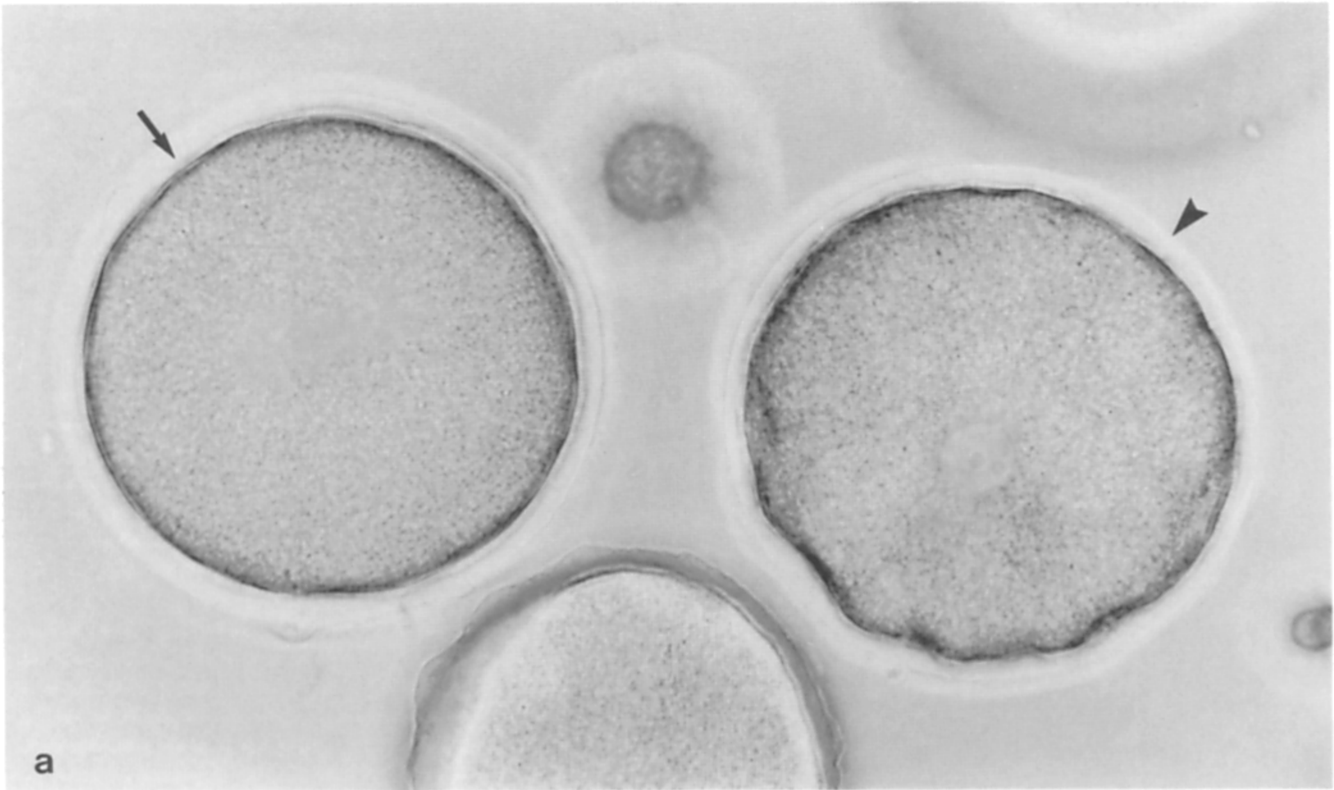
We tested whether the autoinhibitory peptide recently described as a selective inhibitor of neuronal CaM kinase (Malinow et al., 1989) might inhibit the sea urchin enzyme. The autoinhibitory peptide, CaMK(273–302), consists of amino acids 273–302, a segment of the regulatory domain of the α subunit of the neuronal CaM kinase (Lin et al., 1987), and is defective in calmodulin binding. CaMK(273–302) inhibits CaM kinase with an IC_{50} of 1–2 μM, but does not inhibit either cAMP-dependent kinase or protein kinase C at concentrations up to 50 μM (Malinow et al., 1989). As a control, we used a related peptide, CaMK(284–302), which lacks a critical region of the autoinhibitory domain and therefore does not inhibit CaM kinase.

The phosphorylation of autocamtide-2 by calmodulin-depleted cytosol fractions was strongly inhibited by CaMK (273–302) and was not affected by CaMK(284–302) (Fig. 4). The IC_{50}s for inhibition of the phosphorylation of autocamtide-2 by CaMK(273–302) were: 20 μM (homogenate), 7–8 μM (cytosol and particulate fractions), and 2–5 μM (calmodulin-depleted cytosol). These values compared favorably with the extent of inhibition of the rat brain kinase by CaMK(273–302) in a parallel assay (IC_{50} of 2.5 μM) (Fig. 4). CaMK(284–302), which does not inhibit rat brain kinase because it lacks a critical NH_2-terminal portion of the autoinhibitory domain, had no apparent inhibitory effects on the phosphorylation of autocamtide-2 by sea urchin egg extracts at concentrations up to 100 μM (Fig. 4).

If the 56-kD is a subunit of the sea urchin egg CaM kinase, its Ca^{2+}-dependent autophosphorylation should be inhibited by CaMK(273–302). We therefore tested the effects of the inhibitory peptide on endogenous phosphorylation in sea urchin egg extracts (Fig. 5 *A*). The endogenous phosphorylation of the α-like subunit in calmodulin-depleted cytosol was strongly inhibited by CaMK(273–302) (IC_{50}, ∼5 μM), but was only marginally affected by CaMK(284–302). The Ca^{2+}/calmodulin-dependent phosphorylation of other polypeptides in calmodulin-depleted cytosol was also strongly inhibited by CaMK(273–302) (Fig. 5 *A*). In comparison, the autophosphorylation of both α and β subunits of the rat brain kinase was similarly inhibited by CaMK(273–302) (apparent IC_{50} 2 μM for α subunit and 5–8 μM for β subunit) (Fig. 5 *B*). The autophosphorylation of the rat brain kinase was only slightly inhibited by CaMK(284–302).

Autoinhibitory Peptide CaMK(273–302) Delays the Onset of NEB

We microinjected CaMK(273–302) and its control peptide into sea urchin eggs because we reasoned that its potency and selectivity would allow us to test CaM kinase function during mitotic division. The effects of microinjecting CaMK (273–302) into *L. pictus* eggs on the subsequent occurrence of nuclear mitotic events are shown in Fig. 6. At 55 min after fertilization an uninjected control egg (Fig. 6 A, *arrow*) has just undergone NEB. Cells microinjected with the control peptide CaMK(284–302) similarly undergo normal NEB. By contrast, the egg that had been injected after pronuclear fusion to 40 μM CaMK(273–302) (Fig. 6 *A*, *arrowhead*) has a visible nuclear envelope. When examined 124 min after fertilization, the uninjected control is seen to progress to the four cell stage. The injected cell, however, has yet to undergo NEB. The nucleus is slightly larger, but the nuclear envelope is still intact. During the delay, the nuclear envelope remains as a distinct solid line enclosing the nuclear material. Cytoplasm is normal in appearance in cells injected with <3.5% of cell volume. Spindle asters are intact and normal in appearance in >95% of zygotes injected with the inhibitory peptide. All zygotes delayed by inhibitory peptide (or



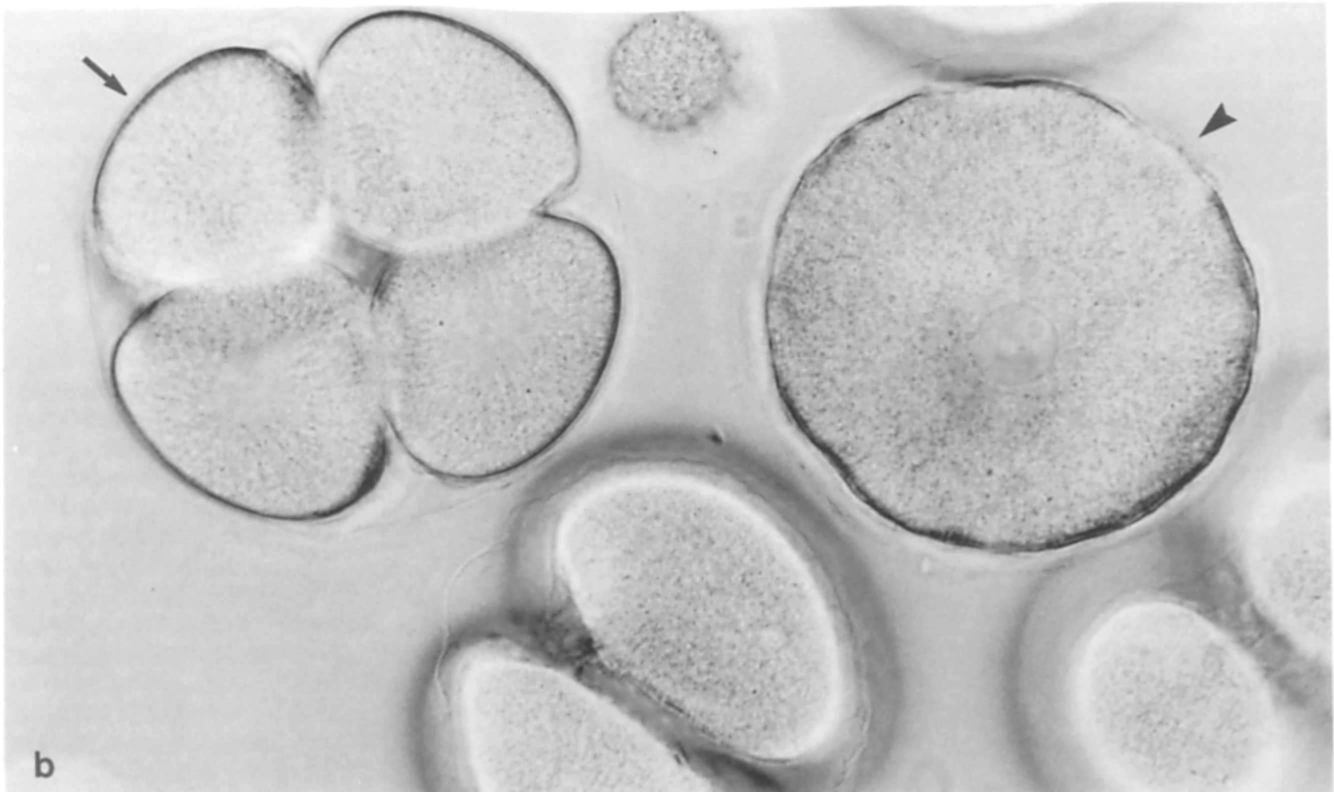


Figure 6. Effects of CaMK(273–302) on NEB in sea urchin eggs. *L. pictus* eggs were fertilized in synchrony and pressure microinjected after pronuclear fusion with CaMK(273–302) to 40 μM peptide (0.57% of cell volume). A microinjected cell is indicated with an arrowhead and uninjected control egg is indicated with an arrow at either 55 min (*a*) or 124 min (*b*) after fertilization.

Table I. Effects of CaMK(273–302) and CaMK(284–302) on NEB in Sea Urchin Eggs

Peptide	Time of injection	Egg volume	Final concentration	Normal	Delay	Duration of delay
		%	μm			min
CaMK(273-302)	before fertilization	0.5-1.1	20-40	0/17	17/17	26-88
CaMK(273-302)	after centrosome divides and asters form	1.1-1.8	40-70	0/4	4/4	19-62
CaMK(284-302)	before fertilization	1.7-3.5	60-190	8/9	1/9	9
CaMK(284-302)	before fertilization	4.1-4.8	220-260	1/4	3/4	11-27

Peptides were introduced into *L. pictus* eggs to the indicated estimated intracellular concentrations using pressure microinjection. Eggs that were microinjected before fertilization were fertilized 10–15 min after microinjection. The effects of peptide microinjection on NEB were monitored by comparison with uninjected cells fertilized in synchrony and maintained in the same dish. Delay in time to NEB in experimental eggs was expressed relative to the longest time to NEB among a population of at least six adjacent uninjected eggs. Within a dish of fertilized eggs, NEB was quite synchronous. The average time between the first and last control egg to undergo NEB was 6 min.

with antibody below) characteristically show a wrinkled cortex at the time of NEB in control zygotes. When NEB occurs after a delay, subsequent events (anaphase, cytokinesis) proceed on a normal time course. The delay from the first cell division is conserved during the second mitosis. A summary of our findings with CaMK(273-302) and CaMK(284-302) is given in Table I.

Microinjection of CaMK(273-302) to levels corresponding to estimated intracellular concentrations of 20–70 μM invariably delayed NEB (21/21 cells injected); delays ranged from 19-88 min in duration. Three of these delays could be characterized as blocks of NEB since these eggs were not monitored after delays of 88 min. In each case only a few minutes of the delay occurred before pronuclear fusion. To make sure that the bulk of the delay really occurred just before the stage of NEB, we injected four cells after centrosome division and aster formation had occurred (late prophase). In each case, a substantive delay occurred (Table I). In two additional experiments, one blastomere of a two cell embryo was injected with 22 or 31 μM inhibitory peptide. NEB in the injected blastomeres was delayed 27 and 39 min, respectively, while the uninjected blastomeres continued in synchrony with control embryos.

The duration of the delay in NEB was related to the amount of peptide injected, the highest concentrations usually giving the longest delays. A significant delay (~20 min) occurred at the lowest concentration of CaMK(273-302) injected, corresponding to an estimated intracellular concentration of 20 μM peptide. The pattern of fluctuations in intracellular free Ca^{2+} was checked in three of the peptide-injected eggs; these eggs showed a normal pattern of fluctuations in intracellular free Ca^{2+} at the appropriate times compared to control cells, up to and including the peak which normally oc-

Table II. Characteristics of mAbs to Rat Brain Multifunctional CaM Kinase

Name	Ig class, subclass	Subunit specificity	K_d
		KD	M
CB-β-1	IgG_{2b}	β (58-60)	4.8×10^{-10}
CB-α-1	IgG_1	α (54)	3.2×10^{-9}
CB-α-3	IgG_{2a}	α (54)	8.0×10^{-9}

Dissociation constants were determined from double-reciprocal plots of the binding data from solid phase immunosorbent assays measuring the binding of radioiodinated mAbs to purified rat forebrain kinase.

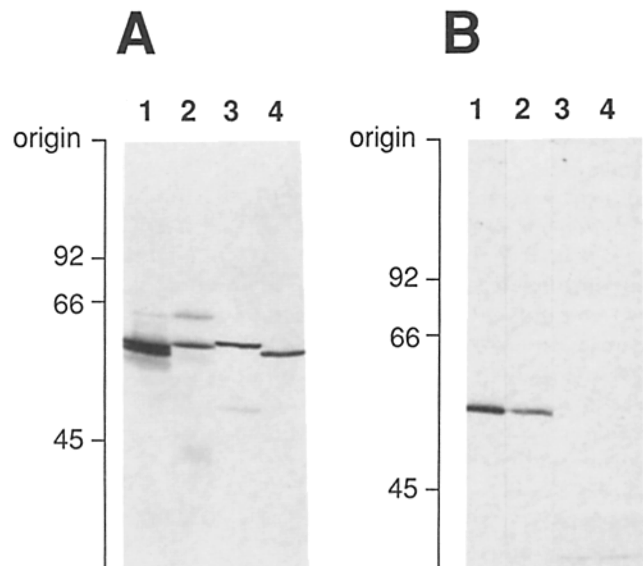


Figure 7. Immunoblot analysis of the cross-reactivity of mAbs CB-β-1 and CB-α-1 with polypeptides in *L. pictus* eggs and cell lines. Brain, eggs, or cells were homogenized in SDS sample buffer and centrifuged at 100,000 *g* for 30 min. Aliquots (100 μg) of protein from each extract were electrophoresed on adjacent lanes of 9% SDS polyacrylamide gels, transferred to nitrocellulose, and incubated overnight with CB-β-1 or CB-α-1 ascites (1:1,000 dilution). Bound antibodies were visualized using alkaline phosphatase-conjugated anti-mouse IgG (Promega Biotec). The positions of pre-stained molecular mass markers are indicated in kilodaltons. (*A*) CB-β-1; (*B*) CB-α-1. (lane *1*) Rat brain; (lane *2*) goldfish brain; (lane *3*) *L. pictus* egg; (lane *4*) REF-52 cells.

curs immediately before NEB (Poenie et al., 1985; Steinhardt and Alderton, 1988).

Injection of the control peptide CaMK(284–302) to estimated intracellular concentrations of 60–120 μM had, in six of seven cases, no effect on the timing of onset of NEB; NEB occurred in synchrony with uninjected controls. In one case, in which an estimated intracellular concentration of 80 μM was achieved, a 9-min delay occurred. At higher concentrations, corresponding to very large injection volumes (over 4% of the cell volume), NEB was delayed between 11 and 27 min (three of four cases) or proceeded normally (one case). At these higher volumes of injections, even control buffer solution (without peptide) resulted in delays of NEB in ~70% of injections. These high levels of injection disrupt the appearance of the cytoplasm, rendering it more granular, and the inhibition appears to reflect an artifactual effect of this disruption.

An mAb against Neuronal CaM Kinase Specifically Immunoprecipitates the α-like Subunit from L. pictus Egg

We have generated a panel of mAbs against the rat brain kinase, each of which can immunoprecipitate the phosphorylated holoenzyme. These include both α-selective and β-selective antibodies (Table II). We tested these mAbs to determine whether any cross-reacted with the 56-kD α-like subunit. When tested on immunoblots of denatured sea urchin egg polypeptides, mAb CB-β-1 cross-reacted strongly with a polypeptide of ~62 kD, and weakly with a 51-kD polypeptide (Fig. 7). The 62-kD polypeptide is of a similar but not identical M_r to the β and β′ subunits of the neuronal CaM kinase, which this antibody selectively recognizes (Scholz et al., 1988; Fig. 7). Antibody CB-β-1 also recognized a polypeptide of similar molecular weight to the β subunit in a variety of cells and tissues examined (Fig. 7). The antibody reacted with a 61-kD polypeptide in goldfish brain and a 60-kD polypeptide in REF-52 cells (Fig. 7), and a 60-kD polypeptide in PC12 cells and in primary mouse fibroblasts (data not shown). Although anti-α subunit antibodies recognized a polypeptide of similar molecular weight to the neuronal α subunit in brains of all species examined, including nonmammalian species, neither of these antibodies reacted strongly with immunoblotted polypeptides from any of the nonbrain tissues examined (Fig. 7). Weak cross-reactivity with a polypeptide of ~25,000 D was sometimes observed.

When endogenously phosphorylated polypeptides from sea urchin egg were incubated with control ascites or CB-β-1 ascites under "native" immunoprecipitation conditions (similar to those used to immunoprecipitate the phosphorylated holoenzyme of the brain kinase), 40–80% of the phosphorylated 56-kD polypeptide was specifically immunoprecipitated (Fig. 8, lane *4*). Control ascites immunoprecipitated <2% of the phosphorylated 56-kD protein (Fig. 8, lane *3*). In parallel experiments, CB-β-1 ascites precipitated 55–68% of the ^{32}P-labeled α subunit from a sample of purified, autophosphorylated rat brain kinase (Fig. 8, lane *6*). The α-like subunit was not immunoprecipitated by CB-α-1 or CB-α-3 ascites (data not shown). By analogy with its interaction with the brain enzyme, CB-β-1 is likely immunoprecipitating the α-like subunit in sea urchin cytosol that is complexed with a β-like subunit.

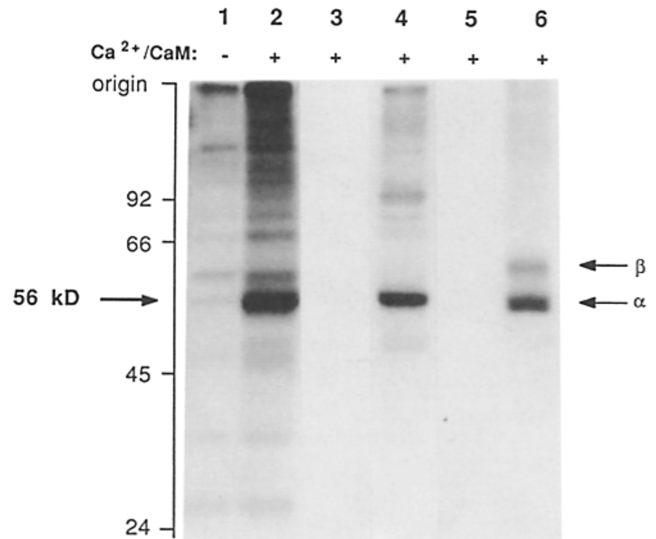


Figure 8. Immunoprecipitation of the α-like polypeptide from sea urchin egg calmodulin-depleted cytosol by mAb against rat brain CaM kinase. Sea urchin egg calmodulin-depleted cytosol (40 μg) was endogenously phosphorylated as described in the legend to Fig. 2 and incubated, without denaturing, with either 3 μl of control ascites or 1 μl of CB-β-1 ascites overnight at 4°C. Antibody-antigen complexes were collected and analyzed by 9% SDS polyacrylamide gels and autoradiography as described in "Experimental Procedures." (lanes *1* and *2*) Sea urchin egg calmodulin-depleted cytosol incubated in the absence and presence of Ca^{2+}/calmodulin, respectively; (lane *3*) control ascites pellet from sample phosphorylated in the presence of Ca^{2+}/calmodulin; (lane *4*) CB-β-1 ascites pellet from sample phosphorylated in the presence of Ca^{2+}/calmodulin. For comparison, lanes *5* and *6* show polypeptides immunoprecipitated by control or CB-β-1 ascites, respectively, from a sample (54 ng) of purified, autophosphorylated rat brain kinase. The positions of molecular mass markers in kilodaltons are indicated. Arrows indicate the positions of the 56-kD sea urchin egg polypeptide and of the α and β subunits of the rat brain kinase.

Microinjection of anti-CaM Kinase mAb Inhibits NEB

Since mAb CB-β-1 immunoprecipitates sea urchin egg CaM kinase, we reasoned that it may inhibit NEB. To address this question, we microinjected purified IgGs from each of three mAbs into sea urchin eggs before fertilization and monitored their effects on NEB (Table III). Neither CB-α-1 or CB-α-3 affected NEB at injection volumes estimated to correspond to intracellular concentrations of 45–580 and 45–660 nM IgG, respectively. In injected eggs, NEB occurred in synchrony with control, uninjected cells. Injection of ascites control also had no effect. In contrast, microinjection of CB-β-1 IgG to estimated intracellular levels of 16–238 nM blocked NEB (13 of 14 injections) and delayed NEB significantly, by 30 min, in one case. Injection of the citrate buffer in which the CB-β-1 IgG was prepared to a similar or greater percentage of the egg volume had no effect. Injection of a second preparation of CB-β-1 IgG (independently prepared in a different buffer) delayed NEB 25–30 min at injection volumes estimated to correspond to 51–57 nM intracellular concentration, and blocked NEB at higher levels (estimated 70–292 nM intracellular concentration).

In eggs injected with CB-β-1 IgG, pronuclear migration, pronuclear fusion, centration of pronuclei, centrosome divi-

Table III. Effects of mAbs against Rat Brain CaM Kinase on NEB in L. pictus Eggs

Sample microinjected	Intracellular concentration	Delay	NEB	
			Block	Normal
	nM			
CB-α-1	45–580			14/14
CB-α-3	42–660			10/10
CB-β-1	16–238	1/14 (30 min)	13/14	
Citrate buffer	1–3% vol of egg			no effect
Phosphate buffer	1–3% vol of egg			no effect
CB-β-1	51–57	3/3 (20–25 min)		
CB-β-1	70–292		19/19	
Ascites control	1–3% vol of egg			4/4

Eggs were fertilized 10–15 min after microinjection and the occurrence of NEB was monitored and compared to that of control cells fertilized at the same time in the same dish. Block is defined as the nonoccurrence of NEB by 60 min after fertilization.

sion, and spindle formation appeared to proceed normally. The pattern of fluctuations in intracellular free Ca^{2+} was checked in three of the eggs injected with CB-β-1 IgG. In the three cases monitored, while NEB was blocked, fluctuations in intracellular free Ca^{2+}, up to and including the peak which normally occurs immediately before NEB, were like uninjected controls.

Discussion

We have shown that a peptide corresponding to the autoinhibitory region of multifunctional CaM kinase, as well as an mAb directed against this enzyme, inhibit NEB when microinjected into sea urchin eggs. These results suggest that a sea urchin kinase similar to the neuronal multifunctional CaM kinase plays an important role in the control of NEB during mitotic division.

The effect of CaMK(273–302) on NEB is specific. In every case in which this peptide was injected, either before fertilization or after the formation of the spindle, it substantially delayed NEB. The control peptide, CaM(284–302), which is a very weak inhibitor of the brain and egg kinases, had minimal effects on NEB. The normal occurrence of Ca^{2+} transients in the cells injected with CaMK(273–302), as well as the nearly normal timetable of progression of pronuclear fusion, also argue against the possibility that the delays in NEB were due to disruption of the eggs due to the microinjection or to other side effects of the synthetic peptide. In the minority of cases in which microinjection of CaMK(284–302) was associated with delayed NEB, the effects may have been due to the large volume of the injection itself, which was used in order to achieve the highest possible intracellular concentration of the peptide, rather than to a direct inhibitory effect of the peptide.

An mAb against brain CaM kinase also inhibits NEB in sea urchin eggs. This antibody was effective at blocking NEB at estimated intracellular concentrations of ~20–70 μM and higher. The effect of this antibody is specific, since antibodies that did not cross-react with sea urchin egg polypeptides, and did not immunoprecipitate the α-like subunit, did not inhibit NEB in sea urchin eggs. The non-cross-reactive antibody did not affect NEB even at 2–30-fold higher concentrations than found effective for CB-β-1.

Chou and Rebhun (1986) previously reported that actomyosin from sea urchin eggs has an associated Ca^{2+}/calmodulin-dependent kinase with broad substrate specificity. We present here additional evidence that sea urchin eggs contain multifunctional CaM kinase. First, egg extracts exhibit an activity that phosphorylates autocamtide-2, a very specific peptide substrate for the multifunctional CaM kinase. The K_m for this peptide as a substrate of the sea urchin egg kinase is similar to that for the brain enzyme. In extracts from mammalian tissue, most of the Ca^{2+}/calmodulin-dependent phosphorylation of autocamtide-2 is due to the activity of multifunctional CaM kinase.

Second, the activity that phosphorylates autocamtide-2 is inhibited by the peptide CaMK(273–302), a selective neuronal CaM kinase inhibitor. Inhibition of NEB in eggs and inhibition of autocamtide-2 phosphorylation in egg extracts occurs at similar concentrations of the inhibitory peptide.

Third, eggs contain a 56-kD polypeptide that resembles the brain kinase in its display of Ca^{2+}-independent autophosphorylation after a brief prephosphorylation with Ca^{2+}. Conversion of CaM kinase activity from a Ca^{2+}-dependent to a Ca^{2+}-independent species is a distinguishing feature of neuronal CaM kinase (Schulman, 1988). This phenomenon is shared by the 56-kD polypeptide (Fig. 2, compare *A* and *B*). The similarity to the neuronal kinase extends to its phosphopeptide map, its sensitivity to CaMK(273–302), and its specific immunoprecipitation by an antibody to the rat brain kinase. These results strongly suggest that the 56-kD polypeptide represents a subunit of a homologous CaM kinase in sea urchin egg. The data are in agreement with previous findings which suggest that a 56-kD polypeptide which undergoes apparent autophosphorylation is a major subunit of the multifunctional CaM kinase in *A. punctulata* sea urchin eggs (Chou and Rebhun, 1986).

The results with the autoinhibitory peptide were further supported by experiments using antibodies directed against the brain CaM kinase. Of three mAbs directed against the brain kinase, only one appeared to cross-react with a sea urchin egg polypeptide, and this antibody was the only one of the three that blocked NEB when microinjected into sea urchin eggs. This antibody is the highest affinity of mAbs that we have generated against the rat brain kinase, and it recognizes the β subunit of the rat brain enzyme (Scholz et al., 1988). On immunoblots of sea urchin eggs, as well as in a variety of nonbrain cells and tissues, this antibody appears to recognize a 60–62-kD polypeptide that is similar in molecular mass to the β subunit of the rat brain kinase; it also

specifically immunoprecipitates the 56-kD α-like polypeptide from sea urchin egg cytosol.

Sea urchin mitotic apparatus contain a Ca^{2+}/calmodulin-dependent protein kinase that selectively phosphorylates an unidentified 62-kD polypeptide in the preparation (Dinsmore and Sloboda, 1988). Phosphorylation of this substrate enhances the rate of microtubule depolymerization and may function in vivo at the metaphase–anaphase transition (Dinsmore and Sloboda, 1988). We do not know whether the CaM kinase that participates in NEB is related to the kinase described above. The 62-kD polypeptide recognized by CB-β-1 does not appear to be heavily phosphorylated, but we cannot exclude the possibility that it is related to the 62-kD mitotic apparatus–associated phosphoprotein described by Dinsmore and Sloboda (1988).

Since the molecular masses of the 56-kD α-like polypeptide and the 62-kD polypeptide which this antibody recognizes on immunoblots are distinct, it appears unlikely that they represent the same protein. The 62-kD sea urchin egg polypeptide is likely related to the rat brain β subunit. Immunoprecipitation of the 56-kD α-like polypeptide by this anti-β subunit antibody only occurs with nondenatured enzyme and likely results from immunoprecipitation of a holoenzyme containing both types of subunits. Since we could not reliably detect phosphorylation of a 62-kD polypeptide in sea urchin egg extracts, we could not determine by phosphopeptide mapping whether the 62-kD polypeptide recognized on immunoblots is related to the β subunit. The available data, however, are consistent with the interpretation that in sea urchin egg as well as in brain, multifunctional CaM kinase contains distinct subunits in a multimeric enzyme. Determination of this will await further characterization of the sea urchin egg enzyme.

Recent studies suggest a central role of cyclins and their activation of protein kinase activity of maturation promoting factor (MPF) in the control of the early embryonic cell cycle (Murray and Kirschner, 1989; Draetta et al., 1989; Hunt, 1989; Eckberg, 1988). Multifunctional CaM kinase may participate in regulation of the cyclin/MPF complex or may be more distal to MPF action and participate selectively in NEB, one of the consequences of MPF activation (Whitaker and Patel, 1990). The specific delay or block of fertilization-induced NEB by antibody to CaM kinase and by the inhibitory peptide indicates that CaM kinase is on the normal physiological pathway for regulation of NEB and not part of a redundant or alternative pathway.

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