A Monoclonal Antibody against Brain Calmodulin-Dependent Protein Kinase Type II Detects Putative Conformational Changes Induced by Ca^{2+}-Calmodulin

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ABSTRACT: A mouse monoclonal IgG1 antibody has been generated against the soluble form of the calmodulin-dependent protein kinase type II. This antibody recognizes both the soluble and cytoskeletal forms of the enzyme, requiring Ca^{2+} (EC_{50} = 20 μM) for the interaction. Other divalent cations such as Zn^{2+}, Mn^{2+}, Cd^{2+}, Co^{2+}, and Ni^{2+} will substitute for Ca^{2+}, while Mg^{2+} and Ba^{2+} will not. The antibody reacts with both the α- and β-subunits on Western blots in a similar Ca^{2+}-dependent fashion but with a lower sensitivity. The affinity of the antibody for the kinase is 0.13 nM determined by displacement of ^{125}I Bolton-Hunter-labeled kinase with unlabeled enzyme. A variety of other proteins including tubulin do not compete for antibody binding. The M_r 30000 catalytic fragment obtained by proteolysis of either the soluble or the cytoskeletal form of the kinase fails to react with the antibody. Calmodulin and antibody reciprocally potentiate each other's interaction with the enzyme. This is illustrated both by direct binding studies and by a decrease of the $K_{m_{app}}$ for calmodulin and an increase in the V_{max} for the autophosphorylation reaction of the enzyme. The antibody thus appears to recognize and stabilize a conformation of the kinase which favors calmodulin binding although it does not itself activate the kinase in the absence of calmodulin. Since the M_r 30000 catalytic fragment of the kinase is not immunoreactive, either the antibody combining site of the kinase must be present in the noncatalytic portion of the protein along with the calmodulin binding site or proteolysis interferes with the putative Ca^{2+}-dependent conformational change. Thus, monoclonal antibodies can be useful tools in elucidating the mechanism by which Ca^{2+} and calmodulin act on the kinase molecule.

Antibodies have proven useful in quantitating calmodulin-dependent protein kinase type II (Miller & Kennedy, 1985; Erondu & Kennedy, 1985), in the identification of enzyme on Western blots (Kennedy et al., 1983; McGuinness et al., 1983, 1984; Woodgett et al., 1984; Kelly et al., 1984), and in its histochemical localization (Erondu & Kennedy, 1985; McGuinness et al., 1984; Ouimet et al., 1984; Hendry & Kennedy, 1986; Vallano et al., 1986). In general, both subunits of the kinase (α and β) react with antibody although some clones have been reported to be subunit specific (Miller & Kennedy, 1985). This cross-reactivity of the subunits is not surprising given the functional similarity and the relatedness apparent in iodopeptide maps (Kennedy et al., 1983; Kelly et al., 1984; McGuinness et al., 1984). The mouse monoclonal IgG1 antibody described in this paper reacts with a Ca^{2+} and calmodulin-dependent epitope in the kinase subunits at a site distinct from the calmodulin binding site and from the catalytic site. Such an observation suggests that calmodulin binding generates a distinct conformational change in the protein kinase which can be detected by the monoclonal antibody.

Materials and Methods

Sigma was the source for poly(ethylene glycol) (M_r 20000), bovine brain calmodulin, tetramethylbenzidine, *p*-nitroblue tetrazolium chloride, and 5-bromo-4-chloro-3-indolyl phosphate (toluidine salt). Bio-Rad provided electrophoresis supplies. Ultrogel AcA 34 was from LKB. Pharmacia supplied CNBr-activated Sepharose. Enzyme-labeled antibodies were obtained from Litton Bionetics. GVWP filters and 0.45-μm nitrocellulose sheets were purchased from Millipore. DEAE-cellulose came from Whatman. Ultrapure ammonium sulfate was from Schwarz/Mann. EM Labs provided the DEAE-Fractogel. ^{125}I-Labeled Bolton-Hunter reagent (2200 Ci/mmol) was obtained from ICN. New England Nuclear supplied the [γ-^{32}P]ATP (30-50 Ci/mmol).

Preparative and Analytical Procedures. Calmodulin-dependent protein kinase type II was purified by an abbreviated method based on the procedure of Bennett et al. (1983). Briefly, a 0-40% saturated ammonium sulfate fraction of rat brain cytosol was sequentially chromatographed in EGTA-containing buffer on an Ultrogel AcA 34 gel permeation matrix and then purified on calmodulin-Sepharose and finally on DEAE-Fractogel. Homogeneous kinase was obtained by sedimentation velocity centrifugation. The kinase (10-30 μg) was iodinated with 1 mCi of ^{125}I-labeled Bolton-Hunter reagent for 90 min on ice and unreacted reagent removed by Sephadex G-50 chromatography in 10 mM sodium phosphate, pH 7.5, 0.15 M NaCl (PBS), and 0.1% poly(ethylene glycol) (M_r 20000). Calmodulin was iodinated similarly (LeVine et al., 1985). Calmodulin-Sepharose was prepared with CNBr-activated Sepharose according to manufacturer's instructions. Metal ion-EGTA buffers in the presence and absence of magnesium were formulated with the aid of a BASIC computer program written by Dr. Steven Blanchard, Department of Chemotherapy, Glaxo, Inc.

^{125}I-Labeled calmodulin binding was determined for the cytoskeletal form of the kinase by filtration through Millipore GVWP 0.22-μm filters (LeVine et al., 1985) and for the soluble form of the kinase by gel permeation chromatography over Ultrogel AcA 34 equilibrated with PBS-0.1% poly(ethylene glycol)-0.1 mM $CaCl_2$. Initial rates of autophosphorylation were determined as described previously (LeVine et al., 1986). Duplicate determinations were within ±10%.

SDS-PAGE in 10% acrylamide-0.267% bis(acrylamide) gels was performed using the buffer system of Laemmli (1970),

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and the separated polypeptides were transferred to nitrocellulose (LeVine & Sahyoun, 1986). Transblots were blocked with 5% fetal calf serum in PBS for 1 h at room temperature. The primary antibody (culture–supernatant 1:20) and the second antibody (rabbit anti-mouse IgG–alkaline phosphatase 1:500) were each incubated with the transferred protein in PBS–5% fetal calf serum for 1 h at room temperature. 5-Bromo-4-chloro-3-indolyl phosphate and *p*-nitroblue tetrazolium were used to localize the immunoreactive product (Leary et al., 1983). Dot blots obtained with a Schleicher & Schuell apparatus were processed similarly.

Immunization and Production of Monoclonal Antibodies. Male SJL (4 weeks old) mice were injected with 10 μg of calmodulin–Sepharose eluate, containing >50% calmodulin-dependent protein kinase type II, emulsified in Freund's complete adjuvant. Three booster injections of 10 μg each of the affinity column eluate in Freund's incomplete adjuvant were given every 2 weeks. An intravenous tail vein injection with 10 μg of antigen in saline was administered 3 days before spleen removal. Primed lymphocytes were isolated from the spleen of an immunized animal and were fused with the mouse myeloma cell line P3X63.Ag8.653 by the poly(ethylene glycol)–dextran method (Kohler & Milstein, 1975, 1976) at a ratio of 2:1 lymphocytes to myeloma cells.

Hybrids were selected in HAT medium and the clones grown in 96-well plastic plates in 200 μL of RPMI 40 medium. Supernatants were analyzed for antibodies against the kinase by incubating 40 μL in 96-well Immunolon 2 plates (Dynatech) coated with 100 μL of 1 μg/mL pure calmodulin-dependent protein kinase type II in 10 mM sodium phosphate, pH 7.4. Nonspecific protein binding was blocked with 5% fetal calf serum in 10 mM sodium phosphate–0.15 M NaCl, pH 7.4. Bound mouse IgG was detected by an ELISA technique utilizing a 1:500 dilution of a rabbit anti-mouse IgG (H + L chain specific) conjugated to horseradish peroxidase and 100 μg/mL tetramethylbenzidine in 100 μL of 0.1 M sodium acetate, pH 5. OD_{450nm} was quantitated after the addition of 2 N H_2SO_4 in a Dynatech microtiter plate reader. Antibody-producing cells were cloned out by limiting dilution. Competition studies with ^{125}I-labeled kinase showed that all 22 clones tested recognized the same epitope.

Ascites fluid containing a high titer of one of the clones was produced by injecting 1×10^6 hybridoma cells into pristane-primed young adult male BALB mice. IgG1 was purified by DEAE-cellulose chromatography. At all stages of antibody production, the predominant subtype was IgG1 as determined by the Zymed kit. The presence of calmodulin itself in the monoclonal antibody preparations was ruled out.

Results

Characterization of the Monoclonal Antibody. Screening of culture supernatants from 96-well plates containing cells from the hybridoma fusion yielded a positive ELISA reaction for over 50% of the wells. Surviving clones were recloned twice. One clone was selected on the basis of antibody production. All the other subclones competed for the same epitope on antigen adsorbed to plastic.

Immunological probing of electrophoretically blotted cytoskeletal enzyme preparations and calmodulin–Sepharose eluates established that the antibody specifically recognized the α- and β-subunits of the calmodulin-dependent protein kinase type II (Figure 1A, lane 3), albeit at a sensitivity much lower than for native enzyme dot blotted onto nitrocellulose. Such an epitope-dependent discrepancy is often observed for monoclonal antibodies (Coding, 1986). Some immunoreactive high molecular weight material was also observed as a smear

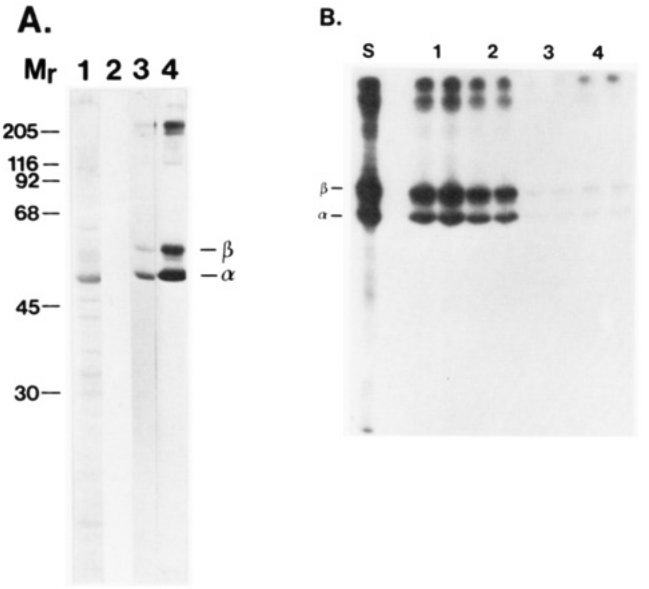

FIGURE 1: Polypeptide specificity of the monoclonal antibody. (A) Immunological probing of a Western blot. Thirty micrograms of postsynaptic density protein enriched in kinase (Sahyoun et al., 1985) was dissolved in SDS sample buffer, electrophoresed, transferred to nitrocellulose, and probed with a 1:20 dilution of culture supernatant containing the monoclonal antibody against the kinase followed by a second antibody conjugated to alkaline phosphatase as described under Materials and Methods. Lane 1, Ferridye (Janssen) stained polypeptide profile with the position of molecular weight ($\times 10^{-3}$) standards indicated; lane 2, immunoreactivity in the absence of calcium (1 mM EGTA); lane 3, immunoreactivity in the presence of 100 μM $CaCl_2$; lane 4, immunoreactivity in the presence of 100 μM $CaCl_2$ and 5 μg/mL calmodulin; calcium was present throughout all incubations while calmodulin was present only in combination with the primary (monoclonal) antibody. (b) Autoradiographic visualization of immunoprecipitated ^{125}I-labeled kinase; 40 000 cpm of ^{125}I-labeled kinase was incubated with 12.5 μL of culture supernatant in 20 mM Tris-HCl, pH 7.6, 0.15 M NaCl, 100 μM $CaCl_2$, and 0.1% poly(ethylene glycol) (M_r 20 000) (TBS–PEG) in a final volume of 100 μL in polystyrene tubes for 90 min at room temperature. Twenty microliters of a suspension of Pansorbin preincubated with goat anti-mouse antibody under the same conditions (5 μL of Pansorbin, 2 μg of secondary antibody, and 13 μL of TBS–PEG) was then added with vortexing, and the incubation was continued for an additional 30 min at room temperature. Four milliliters of 2 M urea, 0.1 M glycine, and 1% Triton X-100 was added to stop the reaction and to wash the Pansorbin. The Pansorbin was collected by centrifugation at 2000*g* for 10 min in a Sorvall HL-8 rotor at 4 °C. Under these conditions, 20–25% of the added radioligand was specifically bound, with nonspecific binding (1 mM EGTA or excess unlabeled kinase) accounting for 1–3% of the added radioactivity. Duplicate determinations were within ±5%. Immunoreactivity of specific polypeptides was determined by SDS–PAGE of the Pansorbin pellet suspended in 100 μL of SDS sample buffer heated at 60 °C for 5 min. Fifty microliters was separated on 10% acrylamide–0.267% bis(acrylamide gels), fixed, dried under vacuum, and autoradiographed at −90 °C on XAR-2 film with an intensifier screen. Duplicate lanes are shown. S, starting material; lane 1, 100 μM $CaCl_2$; lane 2, 100 μM $CaCl_2$ and 13 ng of unlabeled purified kinase; lane 3, 100 μM $CaCl_2$ and 2 μg of unlabeled purified kinase; lane 4, 1 mM EGTA.

in Western blots of cytoskeletal preparations containing the kinase. The origin of this reactivity is unknown; it could be cross-reactive polypeptides distinct from the kinase or possibly undissociated kinase complex. The monoclonal antibody could also immunoprecipitate radioiodinated, purified, soluble protein kinase in a Ca^{2+}-dependent and displaceable fashion (Figure 1B).

A radioimmunoassay was developed using ^{125}I-labeled Bolton–Hunter reagent to modify the kinase; oxidative iodination produced highly aggregated preparations. A species-specific goat anti-mouse IgG (H + L) secondary antibody immobilized on Pansorbin was used to separate free from

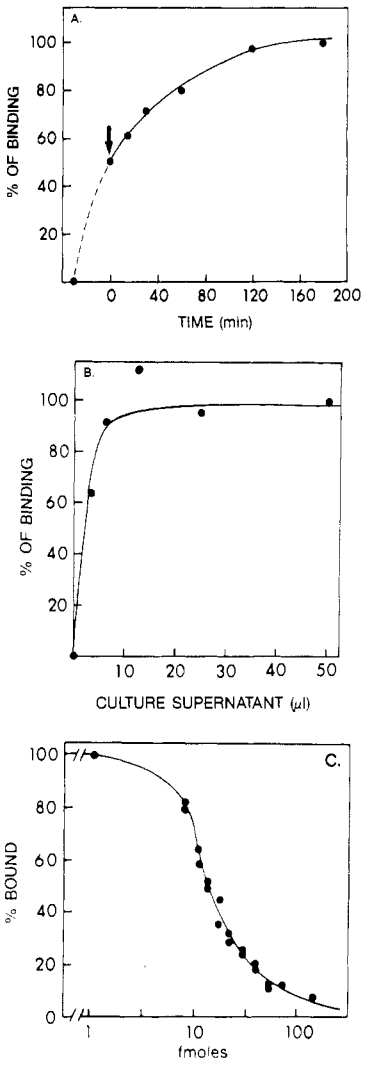

FIGURE 2: Radioimmunoassay of the monoclonal antibody. (A) Time course of association of ^{125}I-labeled kinase with antibody. Primary (monoclonal) antibody and ^{125}I-labeled kinase were incubated together for various periods of time under the conditions described in the legend to Figure 1B. Pansorbin–secondary antibody complex was added and incubated for a further 30 min before collection of the Pansorbin by centrifugation and the ^{125}I in the pellets determined. Nonspecific binding was assessed with 1 mM EGTA replacing calcium. The time of incubation is taken as the total time that antibody and antigen were together. Thus, the arrow indicates the point at which the primary antibody, the ^{125}I-labeled kinase, and the secondary antibody–Pansorbin complex were mixed all at one time. Longer times represent preincubation of monoclonal antibody with ^{125}I-labeled kinase before addition of the second antibody–Pansorbin complex. (B) Antibody concentration dependence of association with ^{125}I-labeled kinase. Different amounts of primary (monoclonal) antibody were incubated with a constant amount of ^{125}I-labeled kinase (40 000 cpm) for 90 min at room temperature before a saturating amount of secondary antibody–Pansorbin complex was added. (C) Determination of antigen affinity for antibody by displacement of ^{125}I-labeled kinase with pure kinase. Increasing amounts of purified unlabeled kinase (x axis) were mixed with a constant amount of ^{125}I-labeled kinase (40 000 cpm) and processed as indicated in the legend to Figure 1B. EC_{50} = 8.6 ng/assay = 0.13 nM kinase.

antibody-bound antigen. Control reactions omitted the primary monoclonal antibody. Figure 2A illustrates a time course of binding at 25 °C of primary antibody to 0.11 nM (1.32 nM subunits) labeled kinase (40 000 cpm) using a saturating amount of Pansorbin and secondary antibody. Figure 2B shows the antibody concentration dependence of the reaction. With a 90-min primary antibody incubation and a 30-min exposure to secondary antibody–Pansorbin complex, the apparent K_d of the antibody for the kinase was 0.13 nM (8.6

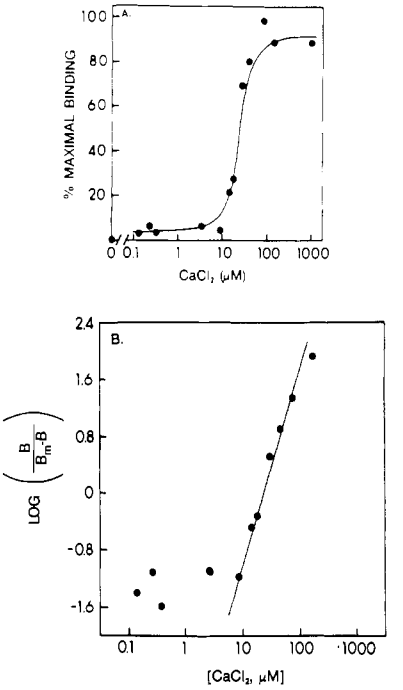

FIGURE 3: Calcium dependence of kinase association with the monoclonal antibody. (A) Binding of ^{125}I-labeled kinase to antibody as a function of calcium concentration. ^{125}I-Labeled kinase and primary (monoclonal) antibody were incubated and processed under the conditions described in the legend to Figure 1B with the free [Ca^{2+}] maintained by an EGTA buffer. (B) Hill plot representation of the data in part A. Slope = 2.8. Curvature of the plot is due to the mathematical form of the Hill relation (Greisow & Walker, 1986). Hill coefficients are only valid over the midrange of saturation.

ng/assay) as determined by displacement with unlabeled kinase (Figure 2C).

Ca^{2+}-Sensitive Conformational Epitope. In the presence of excess EGTA, the mouse monoclonal antibody fails to interact with the kinase in solution, on plastic microtiter plates, or on nitrocellulose Western blots. The EC_{50} for calcium required for antibody binding is 20 μM (Figure 3A) and is unaffected by the presence of 3 mM $MgCl_2$ (data not shown). The binding reaction is also markedly positively cooperative with respect to calcium concentration, yielding a Hill plot with a slope of 2.8 (Figure 3B). Once formed, the antibody–antigen complex is readily dissociable by the chelation of calcium (data not shown). Other divalent cations that substitute for Ca^{2+} in a variety of reactions including binding to calmodulin are similarly effective in supporting antibody binding to kinase (Table I). The specificity of the reaction is upheld by the fact that neither Mg^{2+} nor Ba^{2+} substitutes or competes for Ca^{2+}. Thus, this mouse monoclonal antibody appears to recognize a Ca^{2+}-dependent conformational epitope on the calmodulin-dependent protein kinase. Whether this is a physiologically relevant conformation remains to be determined.

Attempts To Identify the Epitope: Probable Localization of the Antibody Binding Site to the Noncatalytic Portion of the Kinase. Identification of the epitope was attempted by fragmentation of the native molecule with a variety of proteases: trypsin, chymotrypsin, *Staphylococcus aureus* V8 protease, and thermolysin. Such manipulations generate a proteolytically stable M_r 30 000 domain of the enzyme containing the catalytic activity of the M_r 50 000 subunit (LeVine & Sahyoun, 1987). All efforts resulted in total loss of immunoreactive material measured by dot blotting digests or transferring SDS–PAGE-separated polypeptides onto nitro-

Table I: Effect of Divalent Cations on Monoclonal Antibody Binding to Calmodulin-Dependent Kinase II[a]

	fraction of calcium-supported reactivity	
metal ion	immunoprecipitation	^{125}I-calmodulin binding
Mg^{2+}		
Ba^{2+}		0.27
Cd^{2+}	0.96	1.54
Zn^{2+}	0.94	1.51
Co^{2+}	0.93	1.18
Ni^{2+}	0.88	1.43
Mn^{2+}	0.90	0.93
Ca^{2+}	1.00	1.00

[a] Reactions were performed as indicated under Materials and Methods. 100 μM free divalent cation was used following sequestration of endogenous calcium with 50 μM EGTA. The ^{125}I-calmodulin binding assays also contained 3 mM $MgCl_2$. Results are expressed relative to the response induced by Ca^{2+}, taken as 1.00.

cellulose, or in direct and competitive formats of enzyme immunoassays on Immunolon 2 plates. Calcium–calmodulin fails to change the peptides produced or survival of immunoreactivity. Calmodulin binding and autophosphorylation sites are also lost under these conditions (LeVine & Sahyoun, 1987). The catalytic fragment is nonreactive with antibody even when microgram quantitites are assayed in competitive enzyme immunoassays.

The catalytic fragment is N-terminally blocked to Edman degradation, suggesting an N-terminal peptide (data not shown). This is supported by the recently published sequence information for both subunits of the holoenzyme derived from the cDNA sequence (Bennett & Kennedy, 1987; Lin et al., 1987) and the identification of the N-terminal amino acid of the α-subunit as *N*-acetylalanine by tandem mass spectroscopy (LeVine et al., 1987). Consensus catalytic site sequences are found within the first 250 residues of the protein separated from a putative calmodulin binding domain containing basic residues by an exposed "hinge" region susceptible to many proteases (Lin et al., 1987). Thus, by exclusion, the antibody combining site is inferred to be in the C-terminal M_r 20 000 portion of the protein. An alternative explanation which must also be entertained is that a conformational epitope rather than a colinear sequence of amino acids is recognized by the antibody.

Interaction between Calmodulin and Monoclonal Antibody Binding Sites. Calmodulin binding to both subunits of the kinase is also destroyed by the protease treatment that generates the catalytic fragment, suggesting that the calmodulin-interacting domain is also in the C-terminal segment. The calmodulin binding domain on the enzyme has been elucidated by the use of synthetic peptides corresponding to a sequence within a domain unique to Ca^{2+}–calmodulin-dependent protein kinases. The sequence of 25 amino acids corresponding to residues 290–314 binds calmodulin (Hanley et al., 1987) and is predicted to form an amphipathic helix containing multiple basic residues found in several other calmodulin-dependent protein kinases and calmodulin binding proteins (Nunnally et al., 1987). Further evidence for the binding of calmodulin to this region is found in a molecular genetic mapping study of a calmodulin binding protein containing a similar binding region (Sikela & Hahn, 1987).

The binding of calmodulin to the kinase appears to potentiate the interaction of the antibody with the enzyme. The EC_{50} for this effect is between 3 and 5 nM calmodulin in the presence of 100 μM $CaCl_2$ and 3 mM $MgCl_2$ (Figure 4A), consistent with the apparent K_d of calmodulin for the enzyme (LeVine et al., 1985). Conversely, antibody prebound to the

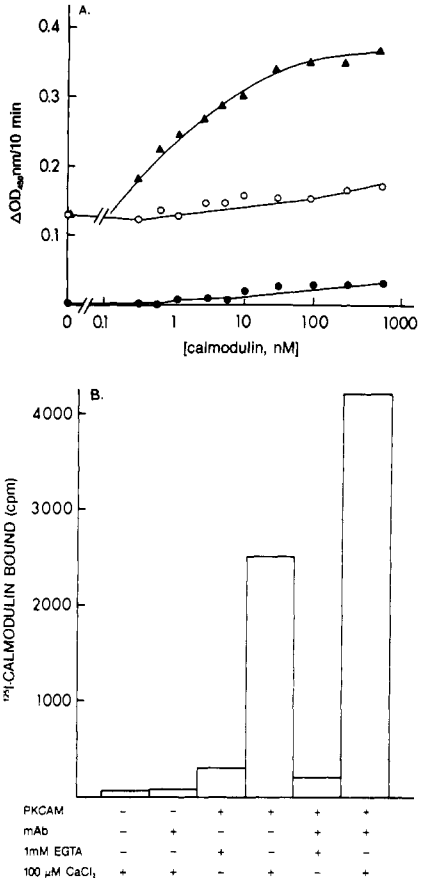

FIGURE 4: Simultaneous interactions of monoclonal antibody and calmodulin with the kinase. (A) Effect of increasing concentrations of prebound calmodulin on antibody binding to immobilized kinase. Purified kinase was coated onto Immunolon 2 96-well microtiter plates as described under Materials and Methods. After blocking with TBS–PEG, the enzyme was exposed to various concentrations of calmodulin in the presence (100 μM $CaCl_2$) and absence (1 mM EGTA) of calcium for 45 min at room temperature. After being washed with 0.15 M NaCl–0.05% Tween 20, primary (monoclonal) antibody, 10 μL of culture supernatant in 100 μL of TBS–PEG, was added to the pretreated enzyme in the absence or presence of calcium for 45 min at room temperature. Following washing, immunoreactivity was determined by incubating the wells with secondary antibody–horseradish peroxidase complex. (●) EGTA with the monoclonal antibody, preincubated with Ca^{2+} and calmodulin; (○) Ca^{2+} with the monoclonal antibody, preincubated with EGTA and calmodulin; (▲) Ca^{2+} with monoclonal antibody, preincubated with Ca^{2+} and calmodulin. (B) Effect of prebound antibody (mAb) on ^{125}I-labeled calmodulin binding to soluble kinase (PKCAM). Binding of ^{125}I-labeled calmodulin (30 000 cpm, 1 nM) to 250 ng of the soluble form of the kinase was performed in a total volume of 150 μL of TBS–PEG with either 100 μM $CaCl_2$ or 1 mM EGTA for 30 min at room temperature. When present, 20 μg of monoclonal antibody purified from ascites fluid was preincubated with 250 ng of the kinase before the addition of ^{125}I-labeled calmodulin. Kinase-associated ^{125}I-labeled calmodulin was isolated in the void volume of a 2-mL Ultrogel AcA 34 column equilibrated with TBS–PEG. Preincubation of the monoclonal antibody with calcium was without effect.

kinase enhances the subsequent interaction with ^{125}I-labeled calmodulin (Figure 4B). Calmodulin binding to kinase subunit polypeptides separated by SDS–PAGE and transblotted to nitrocellulose also enhances their interaction with antibody (compare lanes 3 and 4, Figure 1A).

Prebound antibody also dramatically increases the apparent affinity of the soluble form of the kinase for calmodulin as reflected in the initial rate of autophosphorylation of the enzyme subunits. Data are presented only for the α-subunit as the β-subunit gives similar results (data not shown). The 10-fold decrease in $K_{m_{app}}$ for Ca^{2+}-saturated calmodulin (Figure

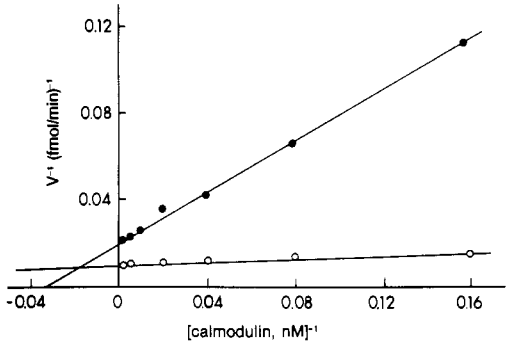

FIGURE 5: Effect of monoclonal antibody binding on kinase autophosphorylation. Effect of prebound antibody on calmodulin dependence of kinase α-subunit autophosphorylation. One hundred nanograms of kinase was incubated on ice with or without 10 μg of purified monoclonal antibody in 50 μL of 50 mM Tris-HCl, pH 7.6, 100 μM $CaCl_2$, 2 mM dithiothreitol, 3 mM $MgCl_2$, and 100 μg/mL bovine serum albumin in polystyrene tubes for 1 h. Fifty microliters of the same buffer containing an appropriate dilution of calmodulin was then added and incubated for a further 1 h on ice. Autophosphorylation was initiated by the addition of 20 μM ATP (4 μCi/assay) and [γ-^{32}P]ATP at 30 °C for 1 min. Kinase subunits were separated by SDS–PAGE, and ^{32}P incorporation was determined (LeVine et al., 1986). Results for the α-subunit (M_r 50000) are given in the figure. Similar data were obtained for the β-subunit (M_r 60000). Duplicate determinations were within ±10%. $K_{m_{app}}$ for calmodulin (−antibody) (●) = 28 nM; (+antibody) (○) = 2.7 nM. V_{max}(−antibody) (●) = 52 fmol of phosphate incorporated/min; (+antibody) (○) = 91 fmol of phosphate incorporated/min.

5) brings the concentration of calmodulin required for catalytic activation of the enzyme down to the binding constant for that ligand. The effect is antibody concentration dependent (data not shown). Antibody binding, therefore, appears to stabilize a conformational state of the enzyme, potentiating its interaction with calmodulin.

Discussion

Sequence information (Bennett & Kennedy, 1987; Hanley et al., 1987; Lin et al., 1987) and biochemical characterization (LeVine & Sahyoun, 1987) have identified several functional domains of the calmodulin-dependent protein kinase involved with catalytic activity, calmodulin binding, autophosphorylation, and subunit association. The lack of competition between calmodulin and antibody binding indicates that they bind at different sites or domains in the enzyme. Interestingly, these two sites appear to communicate because the binding of calmodulin or antibody reciprocally enhances the binding of the other ligand. The data suggest that the interaction of each ligand with the kinase induces or stabilizes a conformational change which favors the association of the other ligand. The properties of the monoclonal antibody described here, therefore, contrast the effects of other antibodies raised against calmodulin-regulated enzymes (Woodgett et al., 1984; Wang et al., 1983; Hansen & Beavo, 1986).

The additional requirement for Ca^{2+} or a substitute divalent cation for monoclonal antibody binding to the kinase may arise from several possibilities. Ca^{2+} may bind to the kinase directly, or serve as a "bridge" between the kinase and the antibody, bind to the antibody itself, or to both. The first possibility appears the most tenable in view of the ability of Ca^{2+} to induce conformational changes in Ca^{2+} binding proteins. These changes may manifest as alterations in antigenicity. Such a situation arises with antibodies raised against synthetic peptides derived from regions of the calmodulin molecule which adopt helical conformations as a consequence of calcium binding (Gariepy et al., 1986). The inability to determine the epitope or to isolate an immunoreactive peptide could arise from the multiple proteolysis sites predicted throughout the C-terminal M_r 20000 domain from sequence information, or from a conformational epitope possibly involving several regions of the kinase. Resolution of this question will require either a series of synthetic peptides or truncated molecules prepared from defined cDNA insert segments in expression systems.

While no E-F hand Ca^{2+} binding loop of the calmodulin type is present in either subunit of the calmodulin-dependent protein kinase, consensus sequences for other putative Ca^{2+} binding proteins remain to be defined. Conformation-specific antibodies directed against the Ca^{2+} complex of prothrombin, factor IX, and thrombospondin and the high molecular weight form of kininogen (Higashiyama et al., 1987) have been described although the sequence responsible for the epitope has not been elucidated.

A Ca^{2+}-dependent epitope has also been described in the acute-phase reactant protein C γ-carboxyglutamic acid containing region which is identical with domains found in factors VII, IX, and X of the clotting cascade, and protein Z (Ohlin & Stenflo, 1987). A very recent study on protein C documents clearly the presence of a Ca^{2+}-dependent antigenic site. A monoclonal antibody detects the Ca^{2+}-dependent conformational change and itself binds Ca^{2+}. Thus, Ca^{2+} is required for the antigen–antibody reaction by virtue of its binding both to protein C and to the monoclonal antibody (Stearns et al., 1988). The relatively low-affinity Ca^{2+} requirement and the use of the monoclonal antibody to probe the conformational changes required for protein C activation constitute a clear precedent for the conclusion arrived at in this paper concerning calmodulin-dependent protein kinase II.

The apparent positive cooperativity of antibody binding with respect to calcium concentration suggests the occurrence of multiple calcium binding sites on each enzyme subunit but can alternatively be attributed to subunit–subunit interactions similar to those invoked to explain the positive cooperativity of cytoskeletal enzyme activation by calmodulin (LeVine et al., 1986). The physiologic relevance of the relatively low-affinity ($EC_{50} \simeq 20$ μM) putative calcium binding to the kinase remains unclear. However, the kinase, which appears to exist in a cytoskeletal form itself [see Sahyoun et al. (1985) and references cited therein], may belong to the larger class of Ca^{2+} binding peripheral membrane or cytoskeletal polypeptides (Greisow & Walker, 1986; Schliewa, 1981). The high concentrations of calmodulin-dependent protein kinase type II in subcellular fractions such as postsynaptic densities may also allow it to function as a local, proteinaceous Ca^{2+} reservoir capable of regulating free Ca^{2+} concentrations in its microenvironment.

Thus, the use of a monoclonal antibody against calmodulin-dependent protein kinase II helps to define three communicating regions or domains in the enzyme which underlie calmodulin binding, antibody binding, and catalytic activity. The role of Ca^{2+} in antibody–enzyme interactions further raises the possibility of a direct Ca^{2+}–kinase interaction. Thus, monoclonal antibodies can be utilized effectively to study intramolecular events which may contribute to the mechanism of synaptic calcium signaling at the level of a major calmodulin-dependent protein kinase.

Registry No. Ca, 7440-70-2; protein kinase, 9026-43-1.

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