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Brain Myosin-V, a Calmodulin-carrying Myosin, Binds to Calmodulin-dependent Protein Kinase II and Activates Its Kinase Activity*

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Myosin-V, an unconventional myosin, has two notable structural features: (i) a regulatory neck domain having six IQ motifs that bind calmodulin and light chains, and (ii) a structurally distinct tail domain likely responsible for its specific intracellular interactions. Myosin-V copurifies with synaptic vesicles via its tail domain, which also is a substrate for calmodulin-dependent protein kinase II. We demonstrate here that myosin-V coimmunoprecipitates with CaM-kinase II from a Triton X-100-solubilized fraction of isolated nerve terminals. The purified proteins also coimmunoprecipitate from dilute solutions and bind in overlay experiments on Western blots. The binding region on myosin-V was mapped to its proximal and medial tail domains. Autophosphorylated CaM-kinase II binds to the tail domain of myosin-V with an apparent K_d of 7.7 nM. Surprisingly, myosin-V activates CaM-kinase II activity in a Ca^{2+}-dependent manner, without the need for additional CaM. The apparent activation constants for the autophosphorylation of CaM-kinase II were 10 and 26 nM, respectively, for myosin-V *versus* CaM. The maximum incorporation of ^{32}P into CaM-kinase II activated by myosin-V was twice that for CaM, suggesting that myosin-V binding to CaM-kinase II entails alterations in kinetic and/or phosphorylation site parameters. These data suggest that myosin-V, a calmodulin-carrying myosin, binds to and delivers CaM to CaM-kinase II, a calmodulin-dependent enzyme.

The primary intracellular receptor for Ca^{2+} in neuronal cells is calmodulin, which mediates the calcium signal by reversible, Ca^{2+}-regulated binding to many target enzymes, which include the calmodulin-dependent protein kinases (reviewed in Ref. 1). Recently, unconventional myosins that carry calmodulins as light chains have been identified in nervous tissue and implicated in neuronal processes such as phototransduction in retina (2, 3), growth cone dynamics (4, 5), and synaptic function (6, 7). One of these, a class V myosin from brain (BM-V),[1] is an oligomeric molecule composed of two identical heavy chains of 212 kDa, and 12–14 light chains in the 10–20-kDa range including multiple calmodulins (reviewed in Refs. 8–11). The native molecule thus has two mechanochemical head domains each bearing a well conserved ATP-binding site, an actin-binding site, and sequence homology with other known myosins.

There are two notable structural features of this myosin. 1) A regulatory neck domain is composed of six tandem IQ motifs, which are consensus sequences for light chain binding (12). Calmodulin is the major light chain (13) and copurifies with BM-V at a stoichiometry of about 4 mol/mol of heavy chain (14). The actin-activated, MgATPase activity of BM-V is tightly and cooperatively regulated by Ca^{2+}, presumably via its binding to calmodulin (15). Recently, light chains, homologous to the essential light chains of conventional myosins, have also been shown associated with BM-V.[2] 2) A tail domain, having intercalated coiled-coil and globular segments, is structurally distinct from that of other classes of myosins and thought to be responsible for the specific intracellular interactions and/or locations of this myosin. Very little is known about its structure and properties, although we have shown it to be phosphorylated by the multifunctional, calmodulin-dependent protein kinase II (CaMKII) (17). No functional significance for this fact has yet been assigned.

CaMKII is an abundant protein in brain with a broad substrate specificity. It associates with the actin cytoskeleton (18), as well as with synaptic vesicles (19) and postsynaptic densities (20), and is involved in neuronal processes such as neurotransmitter synthesis and release, ion channel regulation, and long term potentiation (reviewed in Refs. 1 and 21). Its kinase activity is regulated by Ca^{2+}/calmodulin and autophosphorylation in a complex manner (see Ref. 1). In a cellular milieu, how CaMKII is regulated, what it phosphorylates, and when may be determined in part by the subcellular colocalization of the participating components, including calmodulin as well as specific substrates. A general role for the calmodulin-carrying, unconventional myosins in the subcellular localization of calmodulin has been suggested (22).

In this paper we present biochemical evidence, which suggests that BM-V can bind to and deliver calmodulin to a calmodulin-requiring enzyme, CaMKII. We show that CaMKII co-

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[1] The abbreviations used are: BM-V, brain myosin-V; aa, amino acid(s); DTT, dithiothreitol; TFP, trifluoperazine; IPTG, isopropyl-1-thio-β- D-galactopyranoside; MBP, maltose-binding protein; GST, glutathione *S*-transferase; CaMKII, calmodulin-dependent protein kinase II; PAGE, polyacrylamide gel electrophoresis.

[2] F. S. Espindola, D. M. Suter, R. E. Cheney, S. M. King, and M. S. Mooseker, manuscript in preparation.

immunoprecipitates with BM-V from a Triton X-100-soluble extract of synaptosomes. Furthermore, the purified proteins bind to each other in both immunoprecipitation and overlay experiments. We use the overlay technique to determine the binding affinity and to map the binding site(s) on the BM-V molecule. Finally, we show that both the autophosphorylating activity of CaMKII and substrate phosphorylation of BM-V are activated in this complex.

EXPERIMENTAL PROCEDURES

Materials—[γ-^{32}P]ATP (5000 Ci/mmol) was purchased from Amersham Pharmacia Biotech and NEN Life Science Products. Electrophoresis chemicals, molecular mass standards, imidazole, ATP (grade II), EDTA, EGTA, phenol, glycose, maltose, IPTG, dithiothreitol, ampicillin, tetracycline, chloramphenicol, aprotinin, benzamidine, and lysozyme were purchased from Sigma. Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were obtained from Sigma or Promega. Anti-rabbit and anti-mouse IgGs conjugated to alkaline phosphatase were from Promega. Pefabloc was from Roche Molecular Biochemicals. DNA purification paper (NA4S) was from Schleicher & Schuell. Klenow, alkaline phosphatase and T4 DNA ligase were from Life Technologies, Inc. Restriction enzymes were purchased from Life Technologies, Inc. and New England Biolabs. *Taq* polymerase was from Perkin Elmer. Pansorbin cells were from Calbiochem. Chromatography media were from Amersham Pharmacia Biotech. Grade I water, prepared using the Milli-Q or Toraypure systems, was used in all solutions.

Antibodies—The following monoclonal antibodies were obtained commercially: anti-α-subunit of CaMKII and anti-synaptophysin (Roche Molecular Biochemicals), and anti-syntaxin and anti-200-kDa neurofilament protein (Sigma). Polyclonal antibodies against brain myosin II purified from chick brains as described previously (23) and against chicken brain myosin-V (tail domain expressed in bacteria, Ref. 13) were generated in rabbits and purified by affinity to the respective antigen.

Purification of Proteins—Myosin-V was purified from chick brain essentially as described by Cheney (24). Approximately 8 calmodulins copurify per BM-V with this procedure (14), which includes several precipitation steps and two cromatography columns, indicating their tight association with BM-V. Final fractions containing pure BM-V were pooled, dialyzed against 20 mM imidazole-HCl, pH 7.4 (containing 75 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EGTA, 1 mM DTT), and stored up to 2 weeks at 4 °C or with 30% sucrose at −20 °C.

Calmodulin was purified from bovine brain, either fresh or frozen, by the method of Gopalakrishna and Anderson (25), followed by purification on HPLC to remove low molecular weight contaminants, as follows: 0.5 ml of calmodulin (0.2 mM in 26 mM HEPES, pH 7.2, containing 0.5 mM EGTA) from the Gopalakrishna and Anderson preparation was loaded onto a MonoQ HR 5/5 column (Amersham Pharmacia Biotech) and eluted with a gradient of NaCl (0–487 mM in 8 min; 487–503 mM in 4 min; 503–750 in 3 min). Calmodulin elutes at about 495 mM NaCl and is separated from a closely pre-eluting contaminant by judicial fraction collection and reapplication to the column. The final preparation of calmodulin is highly purified as judged by Coomassie Blue and silver staining on low molecular weight gels.

CaMKII was purified from rat forebrains by calmodulin affinity chromatography as described by Goldenring *et al.* (26) with modifications. Rat forebrains in lots of 5 were rapidly removed and homogenized with an Omni high speed tissue grinder in 10 ml of ice-cold 50 mM imidazole-HCl, pH 6.9, containing 10 mM EDTA, 10 mM EGTA, 2 mM DTT, 1 mM Pefabloc, 2.0 μg/ml aprotinin, and 1 mM benzamidine. All further steps were done at 4 °C unless otherwise stated. The homogenate was centrifuged at 40,000 × *g* for 20 min and the supernatant recentrifuged at 100,000 × *g* for 1 h. The resulting supernatant was applied at a flow rate of 30 ml/h to a phosphocellulose column (60-ml bed volume) equilibrated in 50 mM imidazole-HCl, pH 6.9, containing 1 mM EDTA and 2.0 mM DTT. The column was washed with 5 volumes of equilibration buffer and eluted with the same buffer adjusted to pH 8. Eluting fractions were assayed for Ca^{2+}/calmodulin-dependent autophosphorylation of 50–60-kDa polypeptides characteristic of CaMKII, or otherwise assayed by probing dot blots of the column fractions with a monoclonal antibody against the α-subunit of CaMKII. Fractions enriched in CaMKII were pooled, supplemented to 1 mM free Ca^{2+}, and incubated for 1 h at room temperature with 3–5 ml of calmodulin-Sepharose 4B equilibrated in 50 mM imidazole-HCl, pH 8.0, containing 1 mM $CaCl_2$ and 1 mM DTT. The resin was loaded into a column and washed with 5 volumes of equilibration buffer alone and 2 volumes of equilibration buffer containing 500 mM NaCl, followed by elution with 50 mM imidazole-HCl, pH 7.4, containing 1.5 mM EGTA. Fractions of 0.5 ml were collected and assayed for Ca^{2+}/calmodulin-dependent autophosphorylation activity, or analyzed by dot blots for the α-subunit of CaMKII. Fractions enriched in CaMKII were pooled, assayed for total protein and stored at -20 °C in 30% sucrose.

Cloning and Expression of Fusion Proteins—Molecular cloning techniques used were essentially as described by Sambrook *et al.* (27). The subcloning of the head, neck, and complete tail domains of chicken BM-V in fusion with maltose-binding protein (MBP; 42 kDa) in the pIH902 vector was described by Espreafico *et al.* (13). The medial tail and globular tail domains were subcloned in fusion with glutathione *S*-transferase (GST; 27.5 kDa) in the pGEX vector (Amersham Pharmacia Biotech). The sequence corresponding to the proximal tail domain (nt2811–3440) was amplified by polymerase chain reaction using primers containing restriction sites for *Eco*RI at their ends. The amplified fragment was digested with this enzyme and inserted into the *Eco*RI site of the pIH902 vector. The predicted molecular mass of this fusion protein is 65.2 kDa (aa 911–1122). The medial tail domain was derived from the 32a clone in pBS vector (13), digested with *Xmn*I. The fragment of 962 base pairs (nucleotides 3425–4387) was purified and inserted into the *Sma*I site of the pGEX3 vector. The predicted molecular mass of the expressed fusion protein is 62.5 kDa (aa 1117–1435). The sequence encoding the globular tail was also derived from the 32a clone, digested with *Hpa*I and *Eco*RV, resulting in a 1.2-kilobase pair fragment that was inserted into the *Sma*I site of the pGEX3 vector. The predicted molecular mass of this fusion protein is 70.4 kDa (aa 1440–1830). All of the fusion proteins mentioned above were expressed in *Escherichia coli* XL1Blue strain. The whole tail of BM-V was also subcloned and expressed using the pET vector, which resulted in its fusion at the N terminus with a 16-amino acid segment derived from the T7 vector. This construct was the same as that cloned in fusion with MBP in the pIH902 vector and was produced by cutting the 8c clone in pBS vector (13) with *Eco*RI and ligating this fragment (nucleotides 2778–6599) into the *Eco*RI site of pET5a. This whole tail fragment encodes 102.4 kDa of BM-V (aa 899–1830) and was expressed using the pLysS strain of *E. coli*. The proteins mentioned above were expressed after induction by IPTG and were identified in SDS-PAGE by comparison of non-induced and induced bacterial lysate samples and by immunoblotting when appropriate.

Immunoprecipitation—Antibodies were purified by affinity column chromatography as described by Espreafico *et al.* (13). The flow-through fraction from the affinity column did not recognize BM-V in Western blots and was therefore used as the "preadsorbed" serum control. Pansorbin cells were washed in 10 volumes of TBS/Tween (40 mM Tris buffer, pH 8.0, containing 150 mM NaCl and 0.5% Tween 20) by several cycles of centrifugation and resuspension. Aliquots of affinity-purified antibody or preadsorbed serum were incubated with the washed cells for 2 h at room temperature in the proportion of 2 mg of protein/ml of Pansorbin cells. The cells were then washed three times with 10 volumes of TBS/Tween. Protein samples in microcentrifuge tubes were incubated for 2 h at room temperature with 50 μl of the antibody-laden Pansorbin cells under gentle agitation. The cells were collected by a 60 s spin in a microcentrifuge (this first supernatant, referred to as "*s*" in the figures, was saved and analyzed together with the final immunoprecipitate, "*p*") and washed by three centrifugation/resuspension cycles in TBS/Tween. Finally, the wash supernatant was carefully removed and the Pansorbin pellet dissolved in SDS-PAGE sample buffer, boiled for 2 min, centrifuged for 2 min in a microcentrifuge, and this supernatant (referred to as "*p*" in the figures) analyzed by SDS-PAGE and immunoblotting using appropriate antibodies.

"Overlay" Technique—Western blots of protein samples on reinforced nitrocellulose membranes (0.22 μm) were blocked by incubation with 5% nonfat dried milk in TBS/Tween for at least 2 h. The protein fractions to be used as probes were incubated with the Western blot under gentle agitation for 2–2.5 h. The filters were then washed with TBS/Tween with three quick changes, followed by four washes of 5 min each. Binding of the probing protein to polypeptides on the Western blot was detected in two ways: 1) by incubating the filter with probe-specific antibodies for 1 h and revelation with alkaline phosphatase-conjugated secondary antibodies, or 2) if the protein probe was radioactive, by autoradiography of the dried blot.

Assay for Phosphorylation—Reaction mixtures containing 25 mM imidazole-HCl, pH 7.4, 1 mM EGTA, 4 mM $MgCl_2$, and 1 mM DTT were prepared in microcentrifuge tubes. CaMKII, BM-V, calmodulin, 100 μM free Ca^{2+}, and 50 μM trifluoperazine (TFP) were added as indicated in the figure legends. To obtain 100 μM free Ca^{2+}, $CaCl_2$ was added based on the calculation of the buffering capacity of 1 mM EGTA by Fabiato

(28). The reaction, at 35 °C, was initiated and stopped in two ways depending on the experiment: 1) reactions containing all other ingredients were initiated by addition of ~2 μCi of [γ-^{32}P]ATP to a final concentration of 10 μM and stopped by addition of SDS-PAGE sample buffer containing 1 mM EDTA and boiling, or 2) in experiments where the protein content was dilute, the reaction was initiated by addition of 100 μM free Ca^{2+} immediately after the addition of [γ-^{32}P]ATP and stopped by addition of EGTA to 2 mM, followed immediately by precipitation of total protein by addition of 0.10 volume of sodium deoxycholate, 0.15% w/v, and of 0.15 volumes of ice-cold 50% trichloroacetic acid. The precipitate was collected by centrifugation at 12,000 × *g* for 15 min at 4 °C, dissolved in SDS-PAGE sample buffer, and neutralized by ammonium vapor. Incorporation of ^{32}P into polypeptides was visualized by autoradiography of dried gels after SDS-PAGE and quantified by either of two methods. (*a*) Appropriate bands corresponding to the α and β subunits of CaMKII were cut from the gel, solubilized in 0.5 ml of 30 volume H_2O_2 by heating at 80 °C for 2 h, and quantified by Cerenkov scintillation counting in 5 ml of H_2O. (b) The dried gels were exposed on a storage phosphor screen, which was subsequently scanned using the STORM 840 PhosphorImager (Molecular Dynamics). Quantitative analysis on appropriate bands was performed using the ImageQuant software. In parallel determinations, both methods gave equivalent results, the STORM system being much quicker and easier.

Other Methods—Synaptosomes were prepared from rat cerebral cortex and purified over a Percoll gradient as described by Nagy and Delgado-Escueta (29). SDS-PAGE was performed using 6–15% linear gradient minigels (30). Immunoblotting was done as described by Towbin *et al.* (31), using alkaline phosphatase-conjugated secondary antibodies developed by the nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate. The free Ca^{2+} concentration indicated in the experiments was based on the calculation of 1 mM EGTA buffer capacity as described by Fabiato (28). Concentrations of BM-V and calmodulin were based on the following extinction coefficients: BM-V, 1.04 at 280 nm for 1.0 mg/ml solutions (14); calmodulin (in 1 mM EGTA), 2.0 at 276 nm for 1% solution (32). Otherwise, protein concentration was determined by the method of Bradford (33) using bovine serum albumin, fraction V, as a standard. The molecular weight of native BM-V was taken as 640,000 (14) and CaMKII holoenzyme as 600,000 (26).

RESULTS

BM-V Coimmunoprecipitates with CaMKII and Syntaxin from a Triton X-100-solubilized Extract of Synaptosomes—Synaptosomes from rat cerebral cortex were isolated on a Percoll gradient and examined by Western blotting for the presence of myosins and other proteins (Fig. 1). Myosins II and V were most concentrated in the 20% Percoll interphase, which corresponded to the highly enriched synaptosome fraction as verified by electron microscopy (Ref. 28; data not shown). The exclusion of neurofilament protein (NF200) and enrichment of synaptophysin, an integral membrane protein of synaptic vesicles, served as biochemical markers in this fraction and corroborated the integrity of the preparation. CaMKII was distributed throughout the gradient.

In a previous study (6), we showed that BM-V was partially solubilized by Triton X-100 extraction of lysed synaptosomes in the presence of millimolar concentrations of ATP. Since Triton extraction disrupts membranes, while many protein-protein interactions are maintained, we attempted to identify potential protein-ligands of BM-V in the Triton-solubilized fraction of synaptosomes by immunoprecipitation with antibodies against the BM-V tail domain. By immunodetection on Western blots, we were able to show coimmunoprecipitation of CaMKII and syntaxin, a synaptic plasma-membrane protein, with BM-V under these conditions (Fig. 2). The portion of the total CaMKII and syntaxin in the soluble fraction that cosedimented with BM-V was small; however, the fact that synaptophysin, also present in equivalent amounts in the synaptosome lysate, did not cosediment with these proteins argues against nonspecific aggregation. Additionally, none of these proteins were cosedimented with Pansorbin cells coated with pre-adsorbed antiserum. CaMKII and syntaxin participate in large, variably assembled protein and cytoskeleton complexes, formed during the cycle of events of synaptic transmission (reviewed in Ref. 34). Thus, these results suggest that BM-V may also be an eventual component of these supramolecular structures.

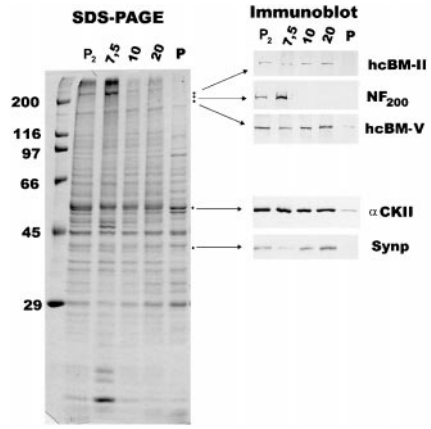

FIG. 1. **Myosins II and V are present in synaptosomes.** The crude "mitochondrial" pellet (*P2*) obtained from rat cerebral cortex was applied to a discontinuous Percoll gradient and the resulting visible bands which form at the interphases (*7.5*, *10*, and *20% Percoll*) and final pellet (*P*) were collected. Equivalent amounts of protein from aliquots of these fractions were subjected to SDS-PAGE and either stained with Coomassie Blue (*SDS-PAGE*) or blotted onto nitrocellulose and probed with specific antibodies, as indicated (*Immunoblot*). The positions of molecular mass standards are indicated to the *left* of the stained gel. Appropriate positions from the blot were cut and probed with the corresponding antibodies (see "Experimental Procedures"): *hcBMII*, heavy chain of brain myosin II; *hcBM-V*, heavy chain of brain myosin-V; *NF200*, neurofilament protein; *αCKII*, α-subunit of CaMKII; *Synp*, synaptophysin. The *arrows* indicate the relative positions of the probed proteins in the gel (note that the immunoblot is a composite of three blots from this overlapping region of the gel).

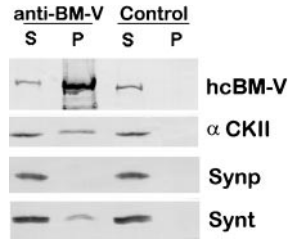

FIG. 2. **CaMKII and syntaxin coimmunoprecipitate with BM-V from Triton X-100-solubilized synaptosomes.** Synaptosomes were lysed in 9 volumes of 5 mM HEPES, pH 7.5, 1 mM EGTA, 2 mM DTT, 5 mM $MgCl_2$, 0.3 mM PMSF, 2 μg/ml aprotinin, and 1 mM benzamidine, containing 1% Triton X-100 and 5 mM ATP, and centrifuged at 10,000 × *g* for 20 min. The supernatant was incubated with Pansorbin cells laden with either BM-V tail antibodies (*anti-BM-V*) or pre-adsorbed antiserum (*control*), as described under "Experimental Procedures." Western blots were prepared from the first supernatant (*s*) and immunoprecipitated fraction (*p*), and appropriate positions from the blot were cut and probed with the corresponding antibodies: *hcBM-V*, myosin-V heavy chain; *αCKII*, α-subunit of CaMKII; *Synp*, synaptophysin; *Synt*, syntaxin.

Purified CaMKII Coimmunoprecipitates with BM-V or Its Bacterially Expressed Tail Domain—To assay for direct binding between BM-V and CaMKII, we performed *in vitro* coimmunoprecipitation experiments from a dilute mixture of the two purified proteins using the BM-V tail antibodies (Fig. 3*A*). Coimmunoprecipitation of CaMKII and BM-V was observed in the absence of ATP (reaction 2, no phosphorylation condition) as well as under conditions where autophosphorylation was promoted (reaction 3). Both non-phosphorylated (seen in *lanes 2p* and *3p*) and phosphorylated forms of αCaMKII (the slower migrating band recognized by the αCaMKII antibody seen only in *lane 3p*) were detected. Controls demonstrated that the precipitation of CaMKII in this assay was dependent on the presence of both BM-V and the BM-V tail antibodies. Similarly,

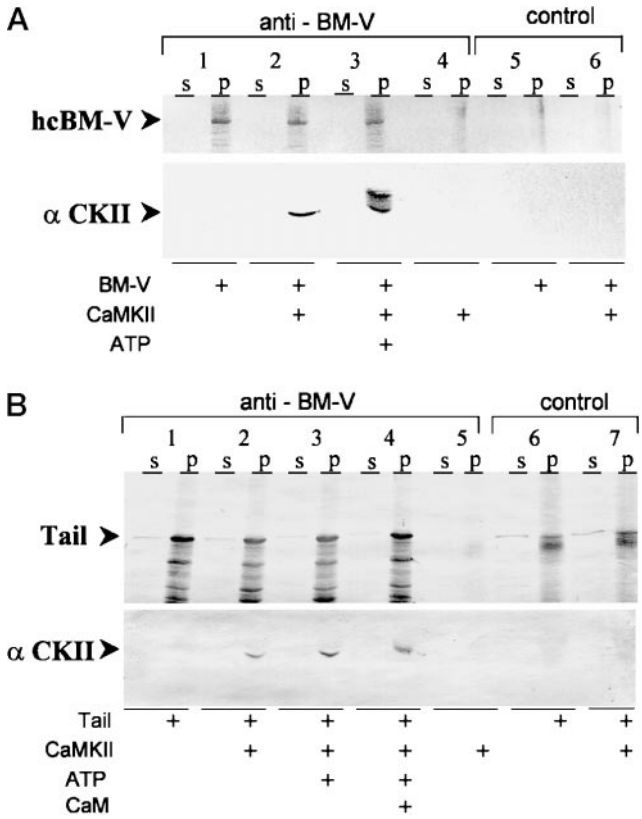

FIG. 3. **Purified CaMKII coimmunoprecipitates with purified BM-V or with bacterially expressed BM-V tail domain.** *A*, BM-V (4 μg) and/or CaMKII (1 μg) were incubated during 10 min at 37 °C in 170 μl of 25 mM imidazole-HCl, pH 7.4, containing 1 mM $MgCl_2$, 100 μM Ca^{2+} and, where indicated, 10 μM ATP. The reaction mix was then diluted to 1 ml with the same buffer, containing 1 mM ATP and 600 mM NaCl, and submitted to immunoprecipitation. Immunoblots were prepared from the first supernatant and immunoprecipitated fraction (*s* and *p*, see "Experimental Procedures") from reactions containing BM-V alone (*1* and *5*), BM-V + CaMKII (*2* and *6*), BM-V + CaMKII + ATP (*3*), or CaMKII alone (*4*). Pansorbin cells laden with BM-V tail antibodies were used in experiments 1–4, whereas in experiments 5 and 6 antiserum preadsorbed with excess BM-V was used as the control. The appropriate regions of the immunoblots were probed with tail antibodies (*hcBM-V*) and a monoclonal antibody against the α-subunit of CaMKII (*αCKII*), respectively. Note that the autophosphorylation of the α-subunit of CaMKII results in multiple phosphorylated species which migrate slower in SDS-PAGE (*lane 3*) (26). *B*, basically the same experiment as in *A* except that BM-V was replaced by 50 μl of bacterial extract of *E. coli* expressing the BM-V whole tail domain (clone 8c pET, see "Experimental Procedures"). Immunoblots were prepared from the first supernatant (*s*) and immunoprecipitated fraction (*p*), as above, from reactions containing tail alone (*1* and *6*), tail + CaMKII (*2* and *7*), tail + CaMKII + ATP (*3*), tail + CaMKII + 1.5 μM CaM + ATP (*4*), and CaMKII alone (*5*). Pansorbin cells laden with BM-V tail antibodies were used in experiments 1–5, whereas in experiments 6 and 7 anti-serum preadsorbed with excess BM-V was used as the control. The appropriate regions of the immunoblots were probed with tail antibodies (*Tail*) and a monoclonal antibody against the α-subunit of CaMKII (*αCKII*), respectively.

CaMKII coimmunoprecipitated with the bacterially expressed tail domain (Fig. 3*B*). Under conditions where autophosphorylation of CaMKII was promoted (reaction 4, note the requirement for calmodulin), it appeared that the phosphorylated α-subunit (based on electrophoretic mobility) was the principal component that coimmunoprecipitated with the tail domain (*lane 4p*).

To permit direct demonstration of the incorporation of ^{32}P into the phosphorylated species, reaction mixtures of BM-V and CaMKII, diluted 2- and 8-fold in relation to the CaMKII concentration of Fig. 3*A*, were incubated for 1 min at 37 °C upon addition of 10 μM [γ-^{32}P]ATP. The reaction was then

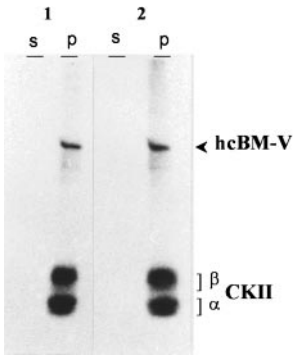

FIG. 4. **Phosphorylated BM-V and autophosphorylated α- and β-subunits of CaMKII coimmunoprecipitate.** Mixtures of 1 μg of BM-V and 1 μg of CaMKII in 345 μl of 25 mM imidazole-HCl, pH 7.4, containing 1 mM $MgCl_2$ were incubated for 10 min at room temperature and incubated as such (*1*) or diluted to 1380 μl (*2*), followed by 1 min at 37 °C with 10 μM [γ-^{32}P]ATP (1.5 μCi) and 100 μM Ca^{2+}. The reactions were then diluted to 3 ml with reaction buffer containing 1 mM cold ATP and incubated at room temperature for 50 min. Immunoprecipitation with tail antibody was performed as described in Fig. 3 in the presence of 600 mM NaCl, and the first supernatant (*s*) and immunoprecipitated pellet (*p*) were analyzed by autoradiography after SDS-PAGE. The positions of the heavy chain of BM-V (*hcBM-V*) and the α and β subunits of CaMKII (*CKII*) are indicated.

diluted to 3 ml containing 1 mM non-radioactive ATP and immunoprecipitation performed as described above. Autoradiography of the immunoprecipitated proteins separated on a SDS-PAGE gel (Fig. 4) demonstrated that (*a*) the α- and β-subunits of CaMKII were autophosphorylated and BM-V phosphorylated, and (*b*) the phosphorylated species were coimmunoprecipitated by tail antibodies. The relative intensity of the bands was not affected by 4-fold dilution of substrate and enzyme in the kinase reaction, suggesting that the phosphorylated species were effectively bound during the reaction and fully immunoprecipitated under these dilute conditions.

CaMKII Binds to the BM-V Heavy Chain and Its Bacterially Expressed Tail Domain; BM-V Binds to Both α and β Subunits of CaMKII—To further characterize the binding between BM-V and CaMKII, we used the "overlay" technique where Western blots of protein samples were incubated with protein ligand, followed by detection of binding by ligand-specific antibodies. When a Western blot of purified BM-V or of the MBP-tail fusion protein expressed in bacteria was incubated with purified CaMKII, its binding to the BM-V heavy chain and to the tail domain was detected by the CaMKII monoclonal antibody (Fig. 5, *lanes 2* and *5*). Reciprocally, when a blot of CaMKII was incubated with purified BM-V, both α and β subunits of CaMKII were detected by BM-V tail antibodies (Fig. 5, *lane 8*). These data strongly suggest that there is direct binding between these two proteins.

Autophosphorylated CaMKII Binds to the BM-V Whole Tail Domain with High Affinity, and the Binding Site Is Centered around the Proximal Tail Region—In order to estimate the affinity of the CaMKII binding to BM-V, we developed a method of ligand detection on the immobilized tail domain with the overlay technique using autophosphorylated [^{32}P]CaMKII as the ligand probe. As shown in Fig. 6*A*, analysis of the overlaid blot by phosphor image scanning demonstrated that [^{32}P]CaMKII was bound to the immobilized tail at incubation concentrations in the nanomolar range. The data from a typical experiment, plotted in Fig. 6*B*, showed an apparently hyperbolic increase in [^{32}P]CaMKII binding with increased concentration. The reciprocal plot of the data (*inset*) gave an apparent K_d of 7.7 nM. Four equivalent experiments gave K_d values that ranged from 1.1 to 7.7 nM. In order to compare the binding of non-phosphorylated *versus* autophosphorylated CaMKII,

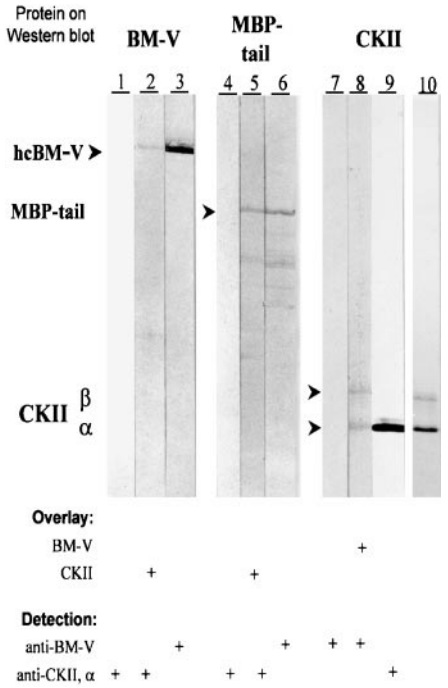

FIG. 5. **The reciprocal binding of BM-V and CaMKII is shown by an "overlay" assay.** Western blots of BM-V, MBP-Tail purified on an amylose column and CaMKII (*CKII*) were incubated either with CaMKII (lanes 2 and 5), with BM-V (*lane 8*) or with blocking solution only (*lanes 1*, *3*, *4*, *6*, *7*, and *9*) as described under "Experimental Procedures." *Lane 10* is a Coomassie Blue-stained gel of the purified CaMKII used in these studies. The blots were probed with a monoclonal antibody against the α-subunit of CaMKII (*lanes 1*, *2*, *4*, *5*, and *9*) or with BM-V tail antibodies (*lanes 3*, *6*, *7*, and *8*). The positions of BM-V heavy chain (*hcBM-V*), undegraded fusion protein (*MBP-Tail*) and α and β subunits of CaMKII (*CKII*) are indicated by *arrowheads* to the *right* of the figure.

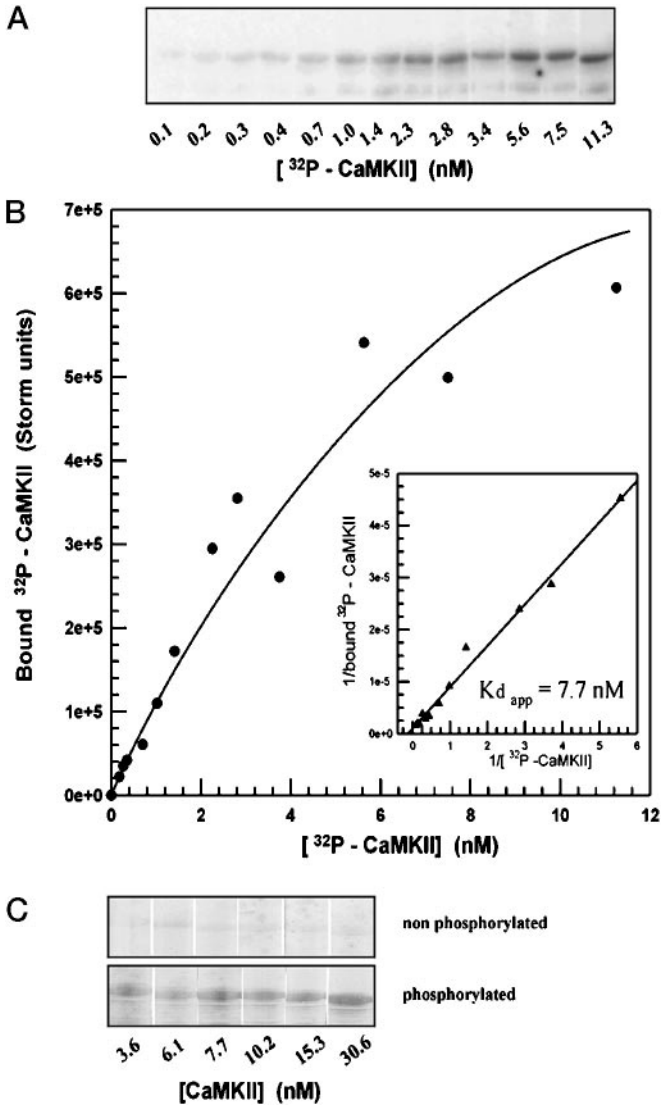

FIG. 6. **Determination of the apparent dissociation constant for CaMKII binding to BM-V.** *A*, pieces containing equal amounts of the BM-V whole tail domain, expressed in bacteria without fusion with MBP, were cut from Western blots and incubated with increasing concentrations of ^{32}P-autophosphorylated CaMKII, as indicated, in 380 μl of reaction mix containing 25 mM imidazole, pH 7.0, 2 mM EGTA, 12.5 μg/ml calmodulin, 4 mM $MgCl_2$, and 1 mM ATP. Blots were washed and air-dried, exposed on a storage phosphor screen, and imaged using the STORM 840 PhosphorImager. *B*, the data were quantitated using the ImageQuant software by defining a rectangle around the appropriate band and a second rectangle of equal area immediately above the band to subtract as nonspecific binding. The apparent dissociation constant was determined from a double-reciprocal plot (*inset*), since the amount of CaMKII bound was less than 1% of the total CaMKII at each concentration. Four equivalent experiments gave K_d values that ranged from 1.1 to 7.7 nM. *C*, direct comparison of the binding of non-phosphorylated *versus* phosphorylated CaMKII to the blotted tail is demonstrated in an equivalent experiment done by overlaying with CaMKII, followed by probing the blots with anti-αCaMKII and enzymatic detection as in Fig. 5.

equivalent overlay experiments were done except that they were probed with anti-αCaMKII, followed by enzymatic detection (Fig. 6*C*). The result demonstrated that autophosphorylated CaMKII bound much more strongly than non-phosphorylated CaMKII.

Based on these experiments, we attempted to map the binding site on the BM-V molecule by examining the binding of [^{32}P]CaMKII to recombinant proteins corresponding to several regions of BM-V (Fig. 7*A*). Strong binding was observed on the whole tail domain (*lane 8*) and the proximal tail segment (*lane 5*). Weaker, but clearly detectable binding was observed for the medial tail region (*lane 6*). No binding was detected over the head (*lane 4*) and globular tail (*lane 7*) segments, or over the GST or MBP proteins alone or proteins in the non-induced bacterial extract. We also have detected very weak binding to the expressed neck domain in separate experiments (data not shown), but due to difficulties in the bacterial expression of this domain, we have not obtained clearly comparative results. These composite data are illustrated on a linear map of BM-V (Fig. 7*B*) and suggest that the binding site for CaMKII is centered around the proximal tail region with some participation from adjacent regions.

BM-V Activates the Protein Kinase Activity of CaMKII without Requirement for Exogenous Calmodulin—Previous work from our laboratory showing that BM-V is a substrate for CaMKII (17) was confirmed here in Fig. 4 using purified proteins. In order to better characterize this activity in terms of Ca^{2+} and calmodulin requirements, we performed phosphorylation reactions with the purified proteins under several conditions (Fig. 8). The surprising result was that a mixture of CaMKII and BM-V showed Ca^{2+}-dependent autophosphorylation of the kinase subunits and substrate phosphorylation of BM-V without requirement for exogenous calmodulin. Since this activity was abolished by micromolar concentrations of the calmodulin antagonist, trifluoperazine, these data suggest that BM-V, which has calmodulin light chains bound to its neck domain, was contributing one or more of its calmodulins to the regulatory domain of CaMKII. The immunoprecipitation of BM-V from aliquots used in kinase activation experiments resulted in the diminution of kinase activity (data not shown),

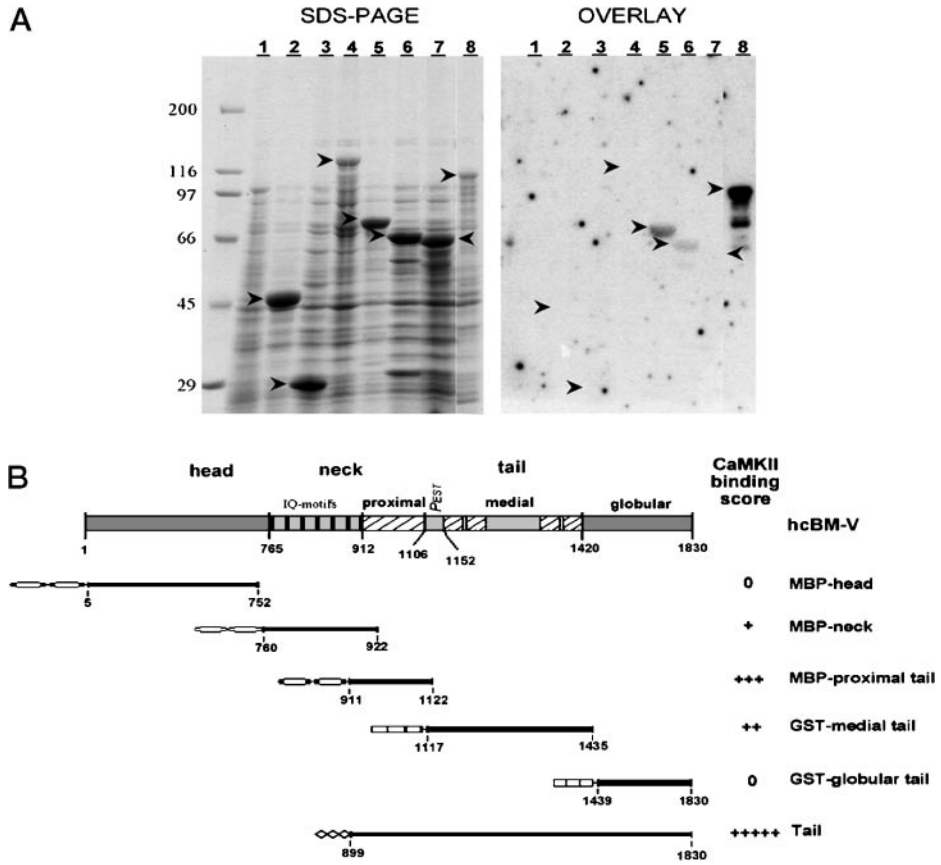

FIG. 7. **Mapping of the CaMKII binding site(s) on BM-V heavy chain by the overlay assay.** In *A*, recombinant proteins corresponding to defined segments of BM-V were expressed in bacteria, as described under "Experimental Procedures." Samples were analyzed by Coomassie Blue staining of SDS-PAGE gels, and Western blots of these bacterial extracts were incubated with autophosphorylated [^{32}P]CaMKII. CaMKII-binding regions were revealed by autoradiography (*overlay*). *Lane 1*, non-induced bacterial extract; *lane 2*, bacterial extract expressing MBP alone; *lane 3*, bacterial extract expressing GST alone. The other lanes contain the expressed recombinant proteins, as follows: *lane 4*, head domain, aa 5–752, in fusion with MBP; *lane 5*, proximal tail region, aa 911–1122, in fusion with MBP; *lane 6*, medial tail region, aa 1117–1435, in fusion with GST; *lane 7*, globular tail region, aa 1440–1830, in fusion with GST; *lane 8*, complete tail domain, aa 899–1830, in the pET5a vector. The positions of the recombinant proteins in the bacterial extracts after induction by IPTG are indicated by *arrowheads*. In *B* a linear model of the BM-V molecule and recombinant proteins is presented with relative scoring of CaMKII binding from the overlay data, as illustrated in *A*. The heavy chain of BM-V (*hcBM-V*) is divided into head (aa 1–765), neck (aa 766–912), and tail (aa 913–1830), which is further divided into proximal tail (aa 913–1106), medial tail (aa 1107–1420), which includes the PEST sequence, and globular tail (aa 1421–1830). The six IQ motifs are indicated by *stripes*, and the *cross-hatching* in the proximal and medial tail segments indicate coiled-coil structures. The recombinant proteins, represented by *bars* with N- and C-terminal amino acids indicated, are aligned below their positions on the linear structure. The MBP and GST fusion counterparts are represented at the N termini by *unconnected* and *fused rectangles*, respectively.

thus indicating that the presence of native BM-V was necessary for the full activation effect on CaMKII.

In order to quantitate this activation, the incorporation of ^{32}P into CaMKII induced by BM-V was determined. The time course of the incorporation was not linear under the conditions studied (Fig. 9) and, in fact, suggested the occurrence of a burst of autophosphorylating activity within 15 s of initiation of the reaction. We therefore used the shortest reaction time attainable in practice (15 s) to determine the effect of increasing BM-V concentration on ^{32}P incorporation. The activation of CaMKII by BM-V, together with that by exogenous calmodulin on the same preparation of CaMKII and under the same reaction conditions, is illustrated in Fig. 10*A*. Reciprocal plots of these data, shown in Fig. 10*B*, gave values of 10 and 26 nM for the apparent activation constants of BM-V and calmodulin, respectively, which is consistent with an exchange of about 2–3 calmodulins from BM-V to CaMKII under the conditions of the experiment. Notably, the maximum incorporation of ^{32}P into CaMKII activated by BM-V was about twice that by exogenous calmodulin. Although the protocol used here does not permit us to distinguish between the initial steady-state rate and a possible initial burst phase (Fig. 9), the data suggest that BM-V not only supplies calmodulin, but additionally the binding of BM-V to CaMKII entails alterations in kinetic and/or autophosphorylation site parameters.

DISCUSSION

An important target of Ca^{2+}/calmodulin in neuronal cells is CaMKII, whose kinase activity is stringently regulated by the Ca^{2+}-dependent binding of calmodulin. CaMKII is a multifunctional enzyme with a wide amplitude of potential cellular substrates (reviewed in Refs. 1, 21, and 35). Thus, its subcellular localization and specific associations within its immediate molecular vicinity are likely to be important factors in its cellular function and control. In the pre-synaptic region, for example, CaMKII is bound to synapsin I and synaptic vesicles where it regulates, via phosphorylation, the interactions between these components and the actin cytoskeleton (36, 37), an important regulatory process of neurotransmitter release (reviewed in Ref. 38). CaMKII is also associated with postsynaptic densities, submembranous actin-cytoskeleton structures believed to be involved in receptor regulation and synaptic plasticity (reviewed in Refs. 39 and 40). Autophosphorylation of CaMKII affects its reversible association with postsynaptic densities (41) and recent studies identified the polypeptides, p190 and p140, as major CaMKII-binding proteins (42).

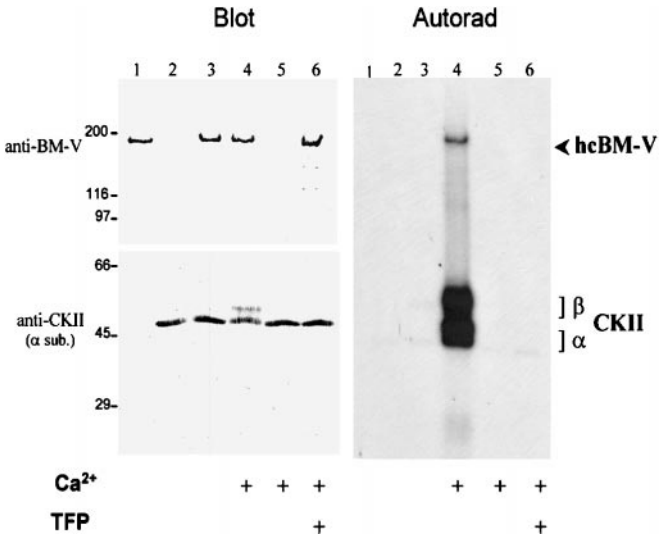

FIG. 8. **Protein kinase activity of CaMKII is activated by BM-V and Ca^{2+} without requirement for additional calmodulin.** Reaction mixtures (55 μl) contained 25 mM imidazole-HCl, pH 7.4, 4 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, 2.5 μg of BM-V, 0.25 μg of CaMKII, and, where indicated, 100 μM free Ca^{2+} and 50 μM TFP. The reactions were initiated by the addition of 2.4 μCi of [γ-^{32}P]ATP and stopped after 2 min at 35 °C with SDS-PAGE sample buffer. The experiments were: *1*, BM-V alone; *2*, CaMKII alone; *3*, BM-V + CaMKII, no Ca^{2+}; *4*, BM-V + CaMKII + Ca^{2+}; *5*, CaMKII alone + Ca^{2+}; *6*, BM-V + CaMKII + Ca^{2+} + TFP. Samples of the reactions were analyzed by SDS-PAGE and autoradiography of the dried gel (*Autorad.*). A Western blot of an equivalent gel was probed with BM-V tail antibodies and with monoclonal antibodies against the α subunit of CaMKII (*Blot*) to illustrate the protein content of the reactions. The heavy chain of BM-V (*hcBM-V*) and CaMKII (*CKII*, α- and β-subunits) are indicated to the *right* of the figure. Ca^{2+}-activated protein kinase activity was not detected in the BM-V fraction (data not shown).

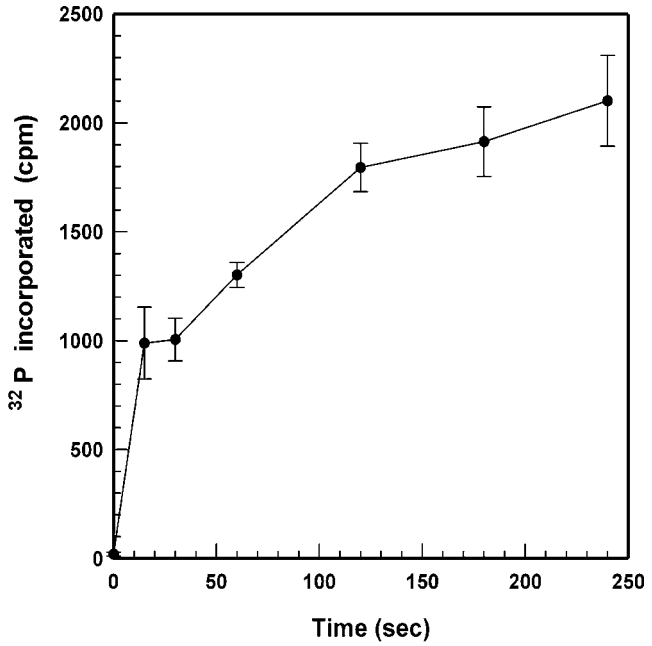

FIG. 9. **The time course of autophosphorylation of CaMKII activated by BM-V suggests an initial burst phase.** Reaction mixtures containing 15 nM BM-V + 2 nM CaMKII + Ca^{2+}, as described in Fig. 8, were initiated by the addition of 2.4 μCi of [γ-^{32}P]ATP, allowed to proceed for the various times indicated on the *abscissa*, and stopped by trichloroacetic acid/deoxycholate precipitation as described under "Experimental Procedures." Samples were applied to SDS-PAGE gels, bands corresponding to the α and β subunits of CaMKII were cut out and dissolved by H_2O_2, and the incorporation of ^{32}P was determined by Cerenkov counting.

Calmodulin would not be expected *a priori* to have limited access to target proteins, being small, highly soluble, and present in relatively high concentrations in brain (43). However, in the cell calmodulin is indeed discretely localized to several subcellular compartments, for example, postsynaptic densities (44), centrosomes and the mitotic apparatus (45), the contractile vacuole of *Dictyostelium* (46), and the growing bud and cytokinesis contractile ring in yeast (47). Furthermore, several proteins have been identified that bind calmodulin in the absence of Ca^{2+}, such as neuromodulin (48) and the unconventional myosins (reviewed in Ref. 12). The notion that calmodulin-carrying myosins may play a role in the determination of the subcellular localization of calmodulin has been suggested (22, 46, 49). Porter and collaborators (22) even suggested that calmodulin could translocate from myosin to other proteins as part of a mechanism of enzyme regulation.

In this paper, we present biochemical evidence that BM-V, an unconventional myosin which harbors at least 8 calmodulins on its neck domains, binds to CaMKII and activates its kinase activity in the presence of Ca^{2+}. Using a combination of immunoprecipitation and Western blot overlay techniques, we showed binding (i) between the native proteins for both phosphorylated and non-phosphorylated species; (ii) between denatured-renatured (Western blotted) polypeptides, corresponding to the heavy chain of BM-V and the α and β subunits of CaMKII, to the appropriate alternate native protein; and (iii) between autophosphorylated CaMKII and bacterially expressed proteins corresponding to the whole tail, medial tail, and proximal tail regions of the BM-V molecule, but not to the head domain nor to the C-terminal globular tail domain. The binding was resistant to 600 mM NaCl and 1% Triton X-100, and under the conditions measured (Western blots of the bacterially expressed tail domain overlaid with autophosphorylated CaMKII) was of relatively high affinity. The binding of non-phosphorylated CaMKII to the tail domain was also detected, although very much weaker and not quantifiable with our methods. We also demonstrated that BM-V was able to activate the kinase activities of CaMKII in a Ca^{2+}-dependent, trifluoperazine-inhibited manner without the need for additional calmodulin. Comparison between the apparent activation constants for BM-V *versus* calmodulin suggested that each BM-V provided 2 or more calmodulins. Interestingly, activation of the autophosphorylation of CaMKII by BM-V resulted in a higher maximum level of incorporation of ^{32}P than that by purified calmodulin. Our data suggested that this activation included an initial burst of autophosphorylation, but our experimental protocol did not allow us to distinguish this burst from the initial steady-state rate of autophosphorylation. However, this result did suggest that BM-V not only supplies calmodulin to CaMKII, but that its binding alters the kinetic properties or autophosphorylation sites of CaMKII. These biochemical results obtained *in vitro* are consistent with the possibility that these two proteins interact in the intracellular milieu.

The question is whether an interaction between CaMKII and BM-V is indeed plausible based on what is known about the subcellular locations and functions of these two proteins. In a recent report, BM-V was shown to be associated with synaptic vesicles via the synaptobrevin-synaptophysin complex (7). These authors suggested that BM-V may be involved in the recruitment of synaptic vesicles to the pre-synaptic membrane. We also have detected BM-V in synaptic vesicle preparations,[3] although we demonstrated here its coimmunoprecipitation with syntaxin, an integral membrane protein of the synaptic

[3] F. Mani, E. M. Espreafico, and R. E. Larson, unpublished data.

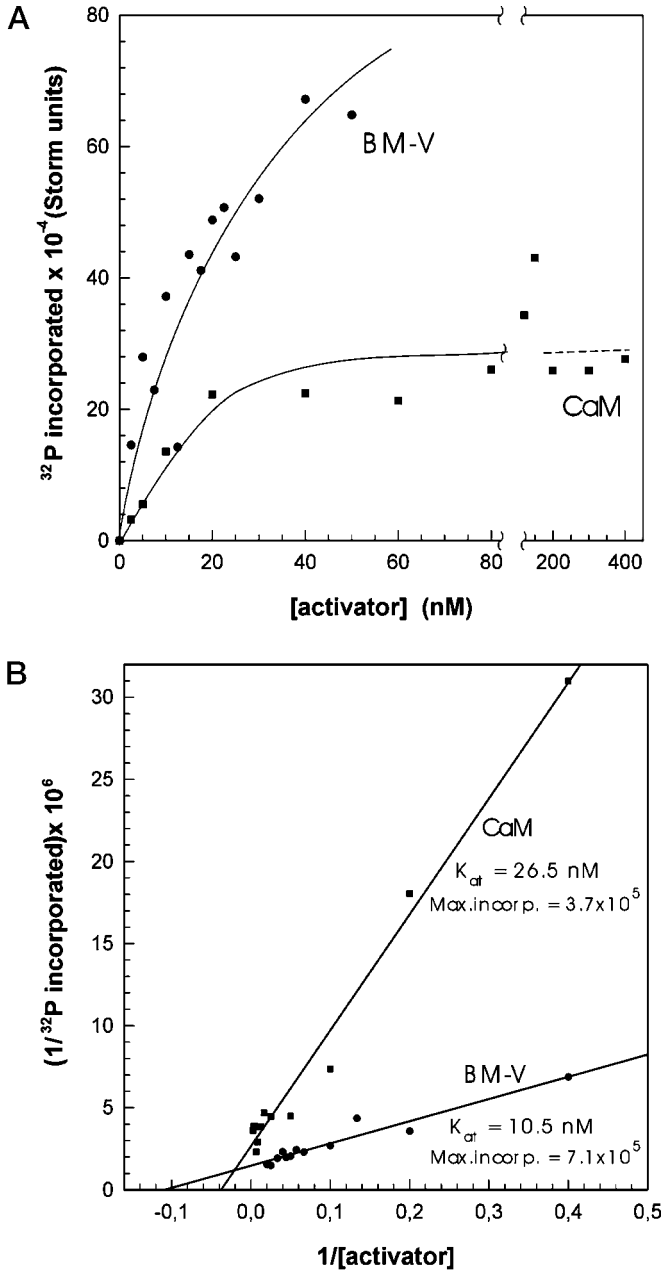

FIG. 10. **BM-V activates the autophosphorylation of CaMKII with high affinity.** The incorporation of ^{32}P into CaMKII after 15 s was determined with increasing concentrations of BM-V or calmodulin purified from bovine brain. The concentration of CaMKII was fixed at 3.3 nM in imidazole-HCl, pH 7.4, containing 4 mM $MgCl_2$, 1 mM EGTA, and 1 mM DTT. BM-V or calmodulin at the indicated concentrations was added to the reaction mix and incubated for 1 h at room temperature. 2.4 μCi of [γ-^{32}P]ATP followed by 100 μM free Ca^{2+} were added to initiate the reaction, which was stopped after 15 s by trichloroacetic acid precipitation, as described under "Experimental Procedures." The incorporation of ^{32}P into CaMKII was determined after SDS-PAGE by exposure of the dried gels to a storage phosphor screen and quantitated by the STORM system as described in Fig. 6. *A*, the data are plotted against the molar concentration of the respective activating proteins. *B*, double-reciprocal plots of the same data gave apparent affinity constants of 10.5 and 26.5 nM for BM-V and calmodulin, respectively. The range for four independent determinations was 10–19 nM and 12–52 nM for BM-V and calmodulin, respectively. The maximum incorporation of ^{32}P into CaMKII activated by BM-V was about twice that activated by calmodulin.

plasma membrane, from detergent extracts of synaptosomes. Integral membrane proteins of synaptic vesicles and the synaptic plasma membrane, together with associated and linking proteins, form multimeric complexes during different stages of the lifecycle of synaptic vesicles (reviewed in Refs. 34 and 38), which resist detergent extractions (50). Specifically, syntaxin and SNAP-25 tightly bind synaptobrevin as a priming event for vesicle fusion to the plasma membrane (51), although this complex seems to require the dissociation of synaptophysin (52). In accordance, we did not detect synaptophysin in the immunoprecipitate with BM-V and syntaxin (Fig. 2). Thus, the present data on BM-V associations are not necessarily in conflict. Clearly, further studies on the subfractionation of synaptosomes will have to be done to establish the precise interactions. In any case, the data do support the basic claim that BM-V is associated with components of the multimeric complexes involved in the synaptic vesicle exocytotic cycle.

Bi and collaborators (53) have provided evidence for essential roles of both motor proteins, kinesin and myosin, in the recruitment of vesicles to the release sites of Ca^{2+}-regulated exocytosis in living cells. Without identifying the myosin, these studies indicated that a myosin-mediated step affected Ca^{2+}-regulated exocytosis at a point upstream from the final fusion event, but downstream from the kinesin transport event. Importantly, the inhibition of CaMKII had similar effects at the same stage of exocytosis as did the inhibition of myosin. A crucial function of synaptic vesicle-bound CaMKII at this stage of exocytosis is the phosphorylation of synapsin I, which tethers synaptic vesicles to the actin cytoskeleton in its unphosphorylated form and releases this interaction when phosphorylated (37).

There is much evidence to suggest that BM-V has a role in vesicle transport (reviewed in Refs. 10 and 11), including small brain-derived vesicles containing the synaptic vesicle marker protein, SV2 (54). The IQ motifs in the neck domain of BM-V have high homology to the calmodulin-binding sequence of neuromodulin (GAP-43), which has been suggested to have a role in reversibly sequestering calmodulin at specific locations near calmodulin-requiring enzymes in the cell (48). Similarly, calmodulin binds to BM-V at low Ca^{2+} concentrations and is at least partially released at micromolar Ca^{2+} (15, 16).

A plausible hypothesis that unites the data we have presented here with that of others is that, upon elevation of intracellular free Ca^{2+}, calmodulin could translocate from BM-V to CaMKII, which in turn would autophosphorylate and then phosphorylate in its immediate environment synapsin I and BM-V. In the former case, the tethering effect of synapsin I on synaptic vesicles to the actin cytoskeleton would be released. In the latter case, we do not know what effect, if any, phosphorylation of the BM-V tail domain by CaMKII (17) would have, but we can speculate that new ties to the actin cytoskeleton via BM-V might occur that may participate in the mobilization of the synaptic vesicles from the "reserve pool" to the "releasable pool."

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