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Purification and Characterization of Calmodulin-Dependent Multifunctional Protein Kinase from Smooth Muscle: Isolation of Caldesmon Kinase†

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ABSTRACT: Previously, it was reported that smooth muscle caldesmon is a protein kinase and is autophosphorylated [Scott-Woo, G. C., & Walsh, M. P. (1988) *Biochem. J. 252*, 463-472]. We separated a Ca^{2+}/calmodulin-dependent protein kinase from caldesmon in the presence of 15 mM $MgCl_2$. The Ca^{2+}/calmodulin-dependent caldesmon kinase was purified by using a series of liquid chromatography steps and was characterized. The subunit molecular weight (MW) of the kinase was 56K by SDS gel electrophoresis and was autophosphorylated. After the autophosphorylation, the kinase became active even in the absence of Ca^{2+}/calmodulin. The substrate specificity of caldesmon kinase was similar to the rat brain calmodulin-dependent multifunctional protein kinase II (CaM PK-II) and phosphorylated brain synapsin and smooth muscle 20-kDa myosin light chain. The purified kinase bound to caldesmon, and the binding was abolished in the presence of high $MgCl_2$. Enzymological parameters were measured for smooth muscle caldesmon kinase, and these were K_{CaM} = 32 nM, K_{ATP} = 12 μM, $K_{caldesmon}$ = 4.9 μM, and $K_{Mg^{2+}}$ = 1.1 mM. Optimum pH was 7.5-9.5. The observed properties were similar to brain CaM PK-II, and, therefore, it was concluded that smooth muscle caldesmon kinase is the isozyme of CaM PK-II in smooth muscle.

It is well-known that the change in the intracellular Ca^{2+} concentration affects many physiological processes including muscle contraction. One of the major systems which transmits this signal to a change in the cell function is the major intracellular Ca^{2+} binding protein calmodulin and calmodulin-dependent enzymes.

In smooth muscle, the increase in the intracellular Ca^{2+} concentration causes the activation of a calmodulin-dependent protein kinase, myosin light chain kinase (MLC kinase). MLC kinase phosphorylates 20-kDa myosin light chain and activates the actomyosin ATPase activity. This is followed by the initiation of contraction (Hartshorne, 1987; Adelstein & Eisenberg, 1980). Although phosphorylation of myosin is thought to be a key event in the initiation of smooth muscle contraction, several recent results using intact smooth muscle strips have suggested that other control mechanisms may exist which modulate smooth muscle contraction. Murphy and his

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colleagues (Dillon et al., 1981; Aksoy et al., 1982, 1983) showed that force development of intact smooth muscle strips was maintained even after the level of myosin phosphorylation decreased. On the other hand, free load shortening velocity decreased in parallel with decreasing myosin phosphorylation. During this phase, chemical energy usage was also decreased (Siegman et al., 1980; Butler et al., 1984). These noncycling or slow cycling cross-bridges were called "latch bridges".

It has been suggested by several investigators that caldesmon may be involved in the "latch" mechanism because it inhibits the actin-activated ATPase activity of myosin (Marston et al., 1985; Kamm & Stull, 1985; Walsh, 1987). Caldesmon was originally isolated as a calmodulin and actin binding protein in chicken gizzard smooth muscle (Sobue et al., 1981) and later shown to be present also in other smooth muscle tissues (Clark et al., 1986) and various nonmuscle tissues (Kakiuchi et al., 1983; Ngai & Walsh, 1985). Ngai and Walsh (1984) have reported that the inhibition of actomyosin ATPase activity by caldesmon is abolished by the phosphorylation of caldesmon which is catalyzed by an endogenous Ca^{2+}/calmodulin-dependent kinase. Subsequently, it was suggested (Scott-Woo & Walsh, 1988a) that caldesmon itself is the calmodulin-dependent protein kinase responsible for its autophosphorylation. However, the amino acid sequence of chicken gizzard caldesmon did not contain a consensus sequence for the ATP binding region which should be present in protein kinases (Bryan et al., 1989).

In this paper, we purified the Ca^{2+}/calmodulin-dependent protein kinase from chicken gizzard caldesmon fraction. The purified kinase was characterized, and we concluded that the kinase is an isoenzyme of calmodulin-dependent multifunctional protein kinase II.

Materials and Methods

Smooth muscle myosin was isolated from frozen turkey gizzards (Ikebe & Hartshorne, 1985a). Myosin subfragment 1 (S-1)[1] was obtained by the proteolysis of myosin with *Staphylococcus aureus* protease as described previously (Ikebe & Hartshorne, 1985b). Smooth muscle 20-kDa light chain was prepared from smooth muscle myosin as described previously (Ikebe et al., 1988; Hathaway & Haeberle, 1983). Calmodulin was prepared from frozen bull testes (Walsh et al., 1983). Heat-treated caldesmon was purified from frozen turkey gizzard by a method of Bretscher (1984), and non-heat-treated caldesmon was purified from frozen chicken gizzard according to Ngai and Walsh (1984). Rat brain calmodulin-dependent protein kinase II was prepared according to Hashimoto et al. (1987). Calmodulin affinity column was prepared by coupling 50 mg of calmodulin with 15 g of cyanogen bromide activated agarose (Sigma). Brain synapsin I was a gift from Dr. Degenero, (University of Massachusetts). Phosphorylase *b*, histones IIS and IIIS, casein, and phosvitin were obtained from Sigma Chemical Co. (St. Louis, MO).

Smooth muscle calmodulin-dependent protein kinase II was prepared from chicken gizzard as follows. The gizzards were trimmed of fat and connective tissue and minced. The minced muscle (250 g) was homogenized in 3 volumes of buffer A (4 mM EDTA, 1 mM EGTA, 50 mM NaCl, 2 mM DTT, 10 mM Tris-HCl, pH 7.5, and 5 mg/L leupeptin) for 20 s in a Waring blender and centrifuged at 10000*g* for 20 min. The muscle residue was homogenized in 2 volumes of buffer A and centrifuged at 10000*g* for 20 min. The residue was homogenized in 4 volumes of buffer B (1 mM EGTA, 1 mM $MgCl_2$, 40 mM KCl, 1 mM DTT, 20 mM Tris-HCl, pH 7.5, 0.2 mM PMSF, and 0.05% Triton X-100) and centrifuged at 10000*g* for 20 min. This washing step was repeated once more with 4 volumes of buffer B without Triton X-100. To the washed muscle residues were added 2 volumes of buffer C (80 mM KCl, 30 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.2 mM PMSF, and 40 mM Tris-HCl, pH 7.5) followed by homogenization and centrifugation at 12000*g* for 30 min. The supernatant was dialyzed against 10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 40 mM NaCl, and 20 mM Tris-HCl, pH 7.5. The dialyzed extract was applied to a DEAE-Sephacel column (2.5 cm × 35 cm) equilibrated with 1 mM EGTA, 40 mM NaCl, 1 mM DTT, and 20 mM Tris-HCl, pH 7.5. The calmodulin-dependent caldesmon kinase activity was recovered in the flow-through fraction. To the flow-through fraction were added 2 mM $CaCl_2$ and 3 mM $MgCl_2$, and the fraction was immediately applied to a calmodulin–Sepharose 4B column (1.5 cm × 20 cm) equilibrated with buffer D (1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.5 mM DTT, and 15 mM Tris-HCl, pH 7.5). The column was washed with buffer D and then with buffer D + 0.2 M NaCl. The kinase was eluted with buffer E (1 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, 0.5 M NaCl, and 15 mM Tris-HCl, pH 7.5). The kinase fraction was combined and dialyzed against 15 mM Tris-HCl, pH 7.5, and 0.5 mM DTT before being applied to a CM-5PW HPLC column (Toyo Soda Co. Ltd., Japan) attached to a Perkin-Elmer series 410 HPLC system. The column was equilibrated with 1 mM $MgCl_2$, 0.5 mM DTT, and 10 mM Tris-HCl, pH 7.5. The kinase was eluted with a linear NaCl gradient (0–0.4 M). The kinase fraction was combined and concentrated with sucrose and stored at −80 °C. Autophosphorylation experiments were done with the CM-5PW purified enzyme, and the characterization of enzyme activity was done both with calmodulin column EGTA eluate and with CM-5PW-purified enzyme. Electrophoresis was carried out on 7.5–20% polyacrylamide gradient slab gels by using the discontinuous buffer system of Laemmli (1970). Gels were stained in 0.06% Coomassie Brilliant Blue R-250 (Sigma). Molecular weight markers used were smooth muscle myosin heavy chain (200 000), β-galactosidase (116 000), phosphorylase *b* (97 400), bovine serum albumin (66 000), ovalbumin (45 000), carbonic anhydrase (29 000), smooth muscle myosin light chains (20 000 and 17 000), and α-lactalbumin (14 200). Phosphorylation of caldesmon was measured as follows: Heat-treated caldesmon, 0.1 mg/mL, and various amounts of kinase were incubated at 25 °C in a solution containing 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 30 mM Tris-HCl, pH 7.5, and 5 μg/mL calmodulin. The reaction was started by addition of 0.1 mM [γ-^{32}P]ATP (30 000 cpm/nmol). Protein concentrations were determined by the dye binding procedure of Spector (1978) or by spectrophotometric measurements for calmodulin ($A^{1\%}_{277}$ = 1.9) (Klee, 1977).

Results

Purification of CaM PK-II from Smooth Muscle. It was reported previously (Ngai & Walsh, 1984) that caldesmon which was prepared from gizzard smooth muscle without heat treatment showed a calmodulin-dependent kinase activity and was autophosphorylated. We confirmed that caldesmon purified from chicken gizzard without heat treatment contains Ca^{2+}/calmodulin-dependent caldesmon kinase activity. We attempted to dissociate the kinase activity from caldesmon by

[1] Abbreviations: SDS, sodium dodecyl sulfate; S-1, myosin subfragment 1; CaM PK-II, calmodulin-dependent multifunctional protein kinase II; HPLC, high-performance liquid chromatography; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; PMA, phorbol 12-myristate 13-acetate.

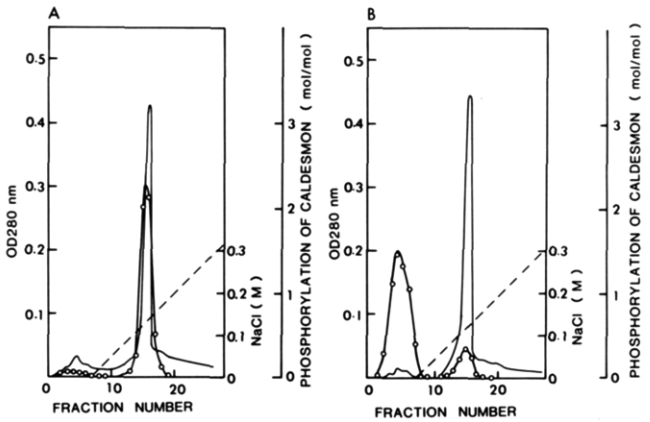

FIGURE 1: Separation of the caldesmon kinase activity from caldesmon. (A) The non-heat-treated caldesmon preparation was applied to a DEAE-5PW column equilibrated with 40 mM KCl, 1 mM DTT, and 20 mM Tris-HCl, pH 7.5, attached to the Perkin-Elmer series 410 HPLC system. (B) To the non-heat-treated caldesmon preparation was added 15 mM $MgCl_2$ and applied to a DEAE-5PW column equilibrated with 15 mM $MgCl_2$, 40 mM KCl, 1 mM DTT, and 20 mM Tris-HCl, pH 7.5, attached to the Perkin-Elmer series 410 HPLC system. After the column was washed with the equilibration buffer, a linear NaCl gradient was applied to elute the protein. Fractions (0.8 mL) were collected at a flow rate of 0.8 mL/min. An aliquot (50 μL) of each fraction was added to the caldesmon phosphorylation reaction mixture (final volume of 250 μL) to measure kinase activity. The reaction was stopped at 60 min by adding 5% TCA containing 1% sodium pyrophosphate. The extent of caldesmon phosphorylation was measured as described under Materials and Methods. (O—O) Caldesmon kinase activity; (- - -) NaCl concentration.

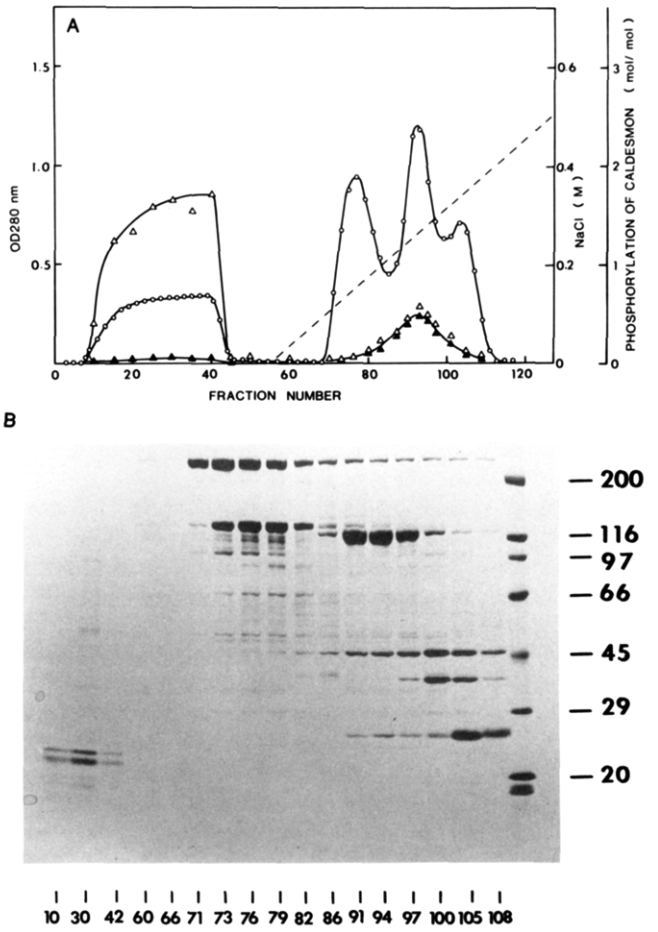

FIGURE 2: Separation of the caldesmon kinase from caldesmon and MLC kinase by DEAE-Sephacel chromatography. (A) Elution profile of DEAE-Sephacel chromatography. The high $MgCL_2$ extract of myofibrils was loaded on a DEAE-Sephacel column (2.5 cm × 35 cm) equilibrated as described under Materials and Methods. After the unbound proteins were completely washed out (monitored by the OD at 280 nm), a linear NaCl gradient from 0.04 to 0.5 M (250 mL + 250 mL) was applied to the column. The flow rate was 35 mL/h, and 6-mL fractions per each tube were collected. The activity of the kinase was measured as described in Figure 1. (O) OD at 280 nm; (Δ) phosphorylation of caldesmon in the presence of Ca^{2+}/calmodulin; (▲) phosphorylation of caldesmon in 1 mM EGTA; (- - -) NaCl concentration. (B) SDS–PAGE of the fractions from DEAE-Sephacel chromatography. Each fraction (75 μL) was mixed with 25 μL of SDS buffer solution containing 4% SDS, 0.04% bromphenol blue, 60% glycerol, and 100 mM Tris-HCl, pH 6.8, and boiled for 1 min, and 20 μL of the sample was subjected to SDS–PAGE on a 7.5–20% gradient gel. From left, fractions 10, 30, 42, 60, 66, 71, 73, 76, 79, 82, 86, 91, 94, 97, 100, 105, 108, and molecular weight standards ($\times 10^{-3}$).

liquid chromatography. Caldesmon with kinase activity was subjected to DEAE-5PW HPLC column, phosphocellulose column, and CM-cellulose column in the presence of 1 mM $MgCl_2$ in an attempt to separate caldesmon from the Ca^{2+}/calmodulin-dependent kinase activity; however, the kinase activity was always associated with caldesmon. As shown in Figure 1, the kinase activity was dissociated from caldesmon when non-heat-treated caldesmon which retains kinase activity was subjected to DEAE-5PW column chromatography in the presence of 15 mM $MgCl_2$. On the other hand, kinase activity coeluted with caldesmon from the column in the absence of $MgCl_2$ (Figure 1). Therefore, former attempts to purify the kinase from caldesmon were hampered by the lack of Mg^{2+} in the appropriate buffers which would diminish the tight association of the kinase with caldesmon. The $MgCl_2$-extracted protein from myofibrils (see Materials and Methods) was dialyzed against 10 mM $MgCl_2$ and applied to a DEAE-Sephacel column in order to separate the caldesmon and the calmodulin-dependent kinase. The calmodulin-dependent caldesmon kinase activity was recovered in the flow-through fraction (Figure 2A). Caldesmon (141 kDa in SDS–PAGE) was eluted in the first peak of the salt gradient fraction (Figure 2B), and no intact caldesmon was found in the flow-through fraction. Minor kinase activity was found at 0.27 M NaCl, but was not dependent on Ca^{2+}/calmodulin. The flow-through fraction was applied to a calmodulin affinity column (Figure 3). Most of the caldesmon kinase activity was recovered in the EGTA eluate although some activity was also found in the salt wash fraction. The activity in the salt wash fraction had a little activity even in EGTA. Therefore, the kinase in the salt wash fraction which expressed calmodulin-independent activity may be partially proteolyzed and may not bind strongly to calmodulin. It has been shown that proteolytic cleavage of the calmodulin-dependent kinase produces calmodulin-independent activity and diminishes the affinity of the enzyme for calmodulin (Levin & Sahyoun, 1987). The EGTA-eluted fraction was further purified by CM 5PW HPLC chromatography. As shown in Figure 4A, most of the kinase activity (fractions 40–60) was eluted at approximately 0.10 M NaCl and coincided with the appearance of a 56-kDa peptide. This peptide was a major component of the pooled kinase fractions and was autophosphorylated in the presence of Ca^{2+}/calmodulin (Figure 4B). The autophosphorylation was not found in the absence of Ca^{2+} (5 μg/mL calmodulin + 1 mM EGTA) or in the absence of calmodulin (O μg/mL calmodulin + 0.1 mM $CaCl_2$) (data not shown).

Characterization of Calmodulin-Dependent Caldesmon Kinase. Since the subunit molecular weight of smooth muscle calmodulin-dependent caldesmon kinase was similar to that of brain calmodulin-dependent protein kinase II and was autophosphorylated, as is the brain kinase, the properties of the

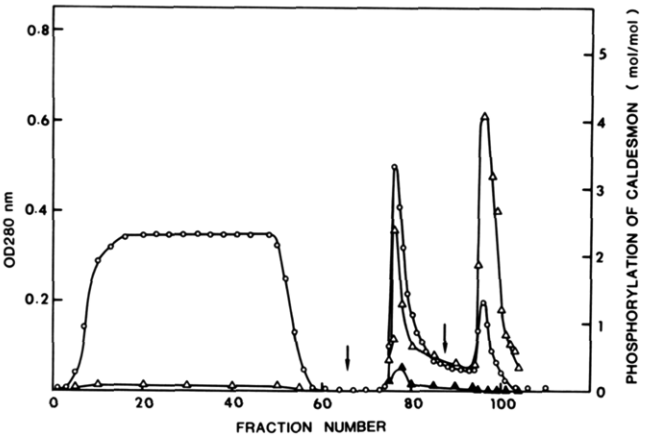

FIGURE 3: Purification of the caldesmon kinase by calmodulin–Sepharose 4B. The flow-through fraction of the DEAE-Sephacel column was applied to a calmodulin affinity column (1.5 cm × 20 cm). After the unbound proteins were washed out, 0.2 M NaCl was added to the column buffer (indicated by arrow), and the kinase was subsequently eluted by addition of 1 mM EGTA-containing buffer (indicated by arrow). The flow rate was 25 mL/h, and 3-mL fractions for each tube were collected. The kinase activity was measured as described in Figure 1. (○) OD at 280 nm; (△) phosphorylation of caldesmon in the presence of Ca^{2+}/calmodulin; (▲) phosphorylation of caldesmon in 1 mM EGTA.

Table I: Relative Rates of Phosphorylation (%)

substrates	smooth	brain
synapsin I[a]	950	1030
caldesmon	100[b]	100[b]
SM myosin 20-kDa light chain	80	75
histone IIIs	23	20
histone IIs	3.0	2.2
phosphorylase *b*	<0.1	<0.1
casein	62	42
phosvitin	<0.1	<0.1

[a] 0.1 mg/mL concentrations of substrates were used. Assay conditions are 5 μg/mL calmodulin, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 30 mM NaCl, and 30 mM Tris-HCl, pH 7.5 at 25 °C. [b] 100% activity was set for each enzyme.

smooth muscle caldesmon kinase were compared to the brain calmodulin-dependent multifunctional protein kinase II (CaM PK-II). The substrate specificity of the kinase was determined and compared to brain calmodulin-dependent protein kinase (Table I). Caldesmon kinase phosphorylated synapsin as well as smooth muscle 20-kDa light chain, casein, and histone IIIS but did not phosphorylate phosphorylase *b* or phosvitin. Phosphorylation of caldesmon and synapsin was only observed in the presence of Ca^{2+}/calmodulin but not in their absence (data not shown) and was not stimulated by phosphatidylserine + PMA or cAMP. A similar substrate specificity was also found with brain CaM PK-II (Table I). This is consistent with previous findings (Scott-Woo & Walsh, 1988a) that synapsin is phosphorylated by non-heat-treated caldesmon in a strictly Ca/calmodulin-dependent manner. It is known that the brain CaM PK-II is autophosphorylated and this produces a calmodulin-independent activity (Saitoh & Schwartz, 1985; Miller & Kennedy, 1986; Lai et al., 1986). Figure 5 shows the effect of autophosphorylation on the caldesmon kinase activity. The kinase was first incubated with 0.2 mM Ca^{2+}, 10 μg/mL calmodulin, and 0.1 mM ATP, at 25 °C for 10 min; then caldesmon was added to measure the kinase activity in the presence of 2 mM EGTA. During the preincubation, the caldesmon kinase (56 kDa) was autophosphorylated as judged from the autoradiogram (data not shown). By autophosphorylation, the kinase became active even in the presence of EGTA (Figure 5). The kinase activated by autophosphorylation also phosphorylated synapsin in the presence of EGTA (data not shown). These results are also similar to the results obtained with non-heat-treated caldesmon (Scott-Woo et al., 1990). The activation constant (K_{CaM}) of the kinase for calmodulin was 32 nM (Table II) as determined

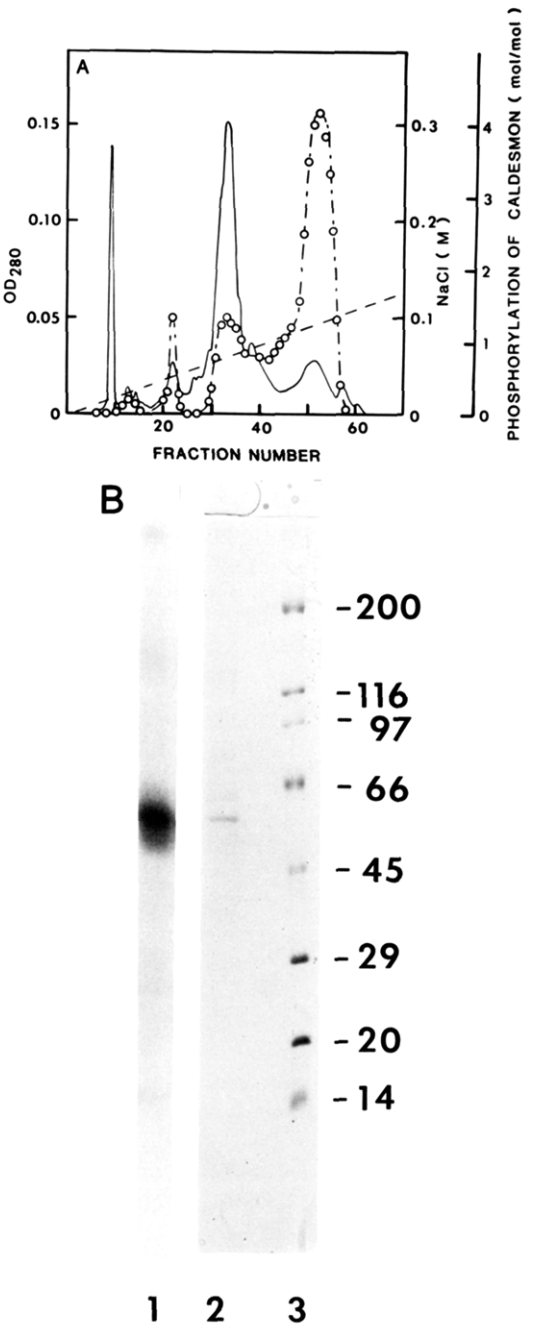

FIGURE 4: Purification of caldesmon kinase by CM-5PW HPLC chromatography. (A) Elution profile of CM-5PW chromatography. The EGTA fraction of the calmodulin affinity column was applied to a CM-5PW column attached to a Perkin-Elmer HPLC series 410 system. The kinase activity was measured as described in Figure 1. (—) OD at 280 nm; (---) NaCl concentration; (○) phosphorylation of caldesmon in the presence of Ca^{2+}/calmodulin. (B) Coomassie brilliant blue staining and autoradiograph of the purified caldesmon kinase. Fractions 49–55 were combined, concentrated by sucrose, and used as the purified kinase. Purified kinase (500 μL) was incubated with 0.05 mM [γ-^{32}P]ATP, 0.1 mM $CaCl_2$, 2 mM $MgCl_2$, and 30 mM Tris-HCl, pH 7.5, in the presence of 5 μg/mL calmodulin at 25 °C for 10 min. Twenty microliters was loaded onto a gel. Autoradiograph of gels using Kodak X-Omat AR film and Kodak intensifying screens: (1) autoradiogram of the autophosphorylated kinase; (2) Coomassie brilliant blue staining of the purified kinase; (3) molecular weight standards (×10^{-3}).

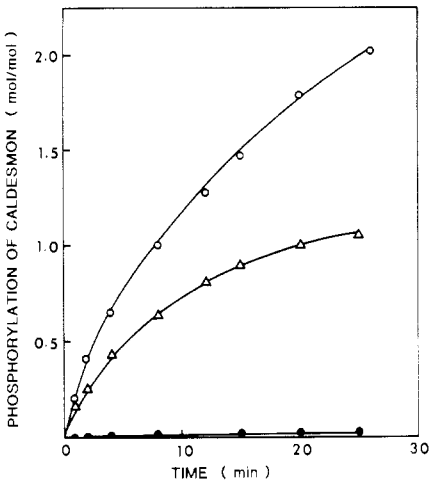

FIGURE 5: Effects of autophosphorylation of the caldesmon kinase on its activity. The caldesmon kinase (5 μg/mL) was preincubated for 5 min with 0.2 mM $CaCl_2$, 5 mM $MgCl_2$, and 30 mM Tris-HCl, pH 7.5, and 10 μg/mL calmodulin in the presence of 0.1 mM ATP (△) or its absence (○, ●). Heat-treated caldesmon (0.1 mg/mL) + 1 mM EGTA (△), or 0.1 mg/mL heat-treated caldesmon + 0.1 mM ATP (○), or 0.1 mg/mL heat-treated caldesmon + 0.1 mM ATP + 1 mM EGTA (●) were added to start the phosphorylation reaction.

Table II: Enzymatic Properties of Smooth Muscle Calmodulin-Dependent Protein Kinase II

	smooth muscle	rat brain
optimum pH[a]	7.5–9.0	7.5–9.0
$K_{i,NaCl}$ (mM)	85	85
K_{MgCl_2} (mM)	1.1	1.1
$K_{m,ATP}$ (μM)	12	
$K_{m,caldesmon}$ (mg/mL)	0.4 (4.9 μM)	
K_{CaM} (nM)	32	25

[a] Buffers used were acetate (pH 4–5.5), imidazole (pH 6–7), Tris-HCl (pH 7.5–9), and Tris–glycine (9.5–10). Assay conditions are the same as Table I.

by measuring the calmodulin dependence of the kinase activity. This value was lower than the previously reported half-maximal activation of the kinase activity in the non-heat-treated caldesmon (Scott-Woo & Walsh, 1988b). It was also found that brain calmodulin-dependent protein kinase II phosphorylated caldesmon (Table I) and the K_{CaM} was 25 nM which was similar to the reported value (Goldenring et al., 1983). The pH dependence of the caldesmon kinase activity was similar to the brain CaM PK-II (Table II). The activity increased above pH 5.5 and was maximum at pH 7.5–9.0. The dependence on ionic strength of the caldesmon kinase activity was also similar to the brain CaM PK-II (Table II). It is known that free Mg^{2+} is required to obtain maximum activity of brain CaM PK-II (Goldenring et al., 1983). Caldesmon kinase required free Mg^{2+} for its activity at a similar concentration required for the activation of brain CaM PK-II (Table II). The K_m of the kinase for ATP was 12 μM, and the K_m for caldesmon was 0.4 mg/mL (4.9 μM). The values for these parameters were similar to those reported for the kinase activity in non-heat-treated caldesmon (Scott-Woo & Walsh, 1988b). Therefore, it can be concluded that the association of the kinase with caldesmon does not alter its enzymatic properties. As mentioned earlier, the separation of the kinase activity from caldesmon using several liquid chromatography steps failed in the absence of high $MgCl_2$ concentration. This suggests that the kinase is tightly associated with caldesmon. To test whether or not the isolated kinase binds to caldesmon, the kinase was mixed with caldesmon and subjected to DEAE-5PW chromatography. When the purified kinase alone was applied to the column, all the activity was

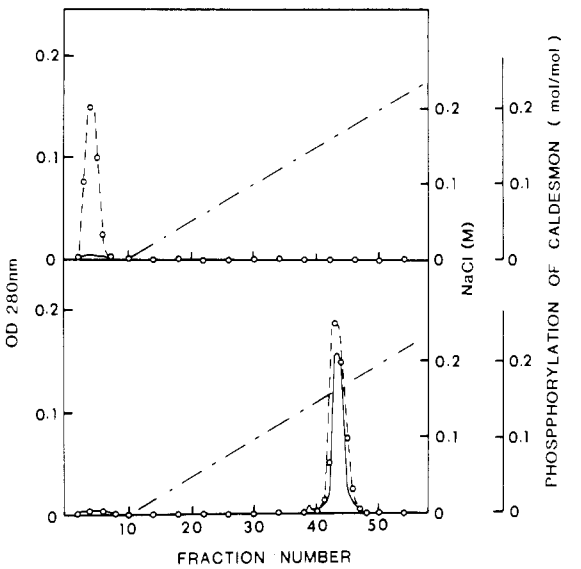

FIGURE 6: DEAE-5PW chromatography of purified CaM PK-II–caldesmon mixture. (Lower panel) Caldesmon (0.5 mg/mL) was mixed with purified CaM PK-II (1 μg/mL) in the solution containing 30 mM KCl, 30 mM Tris-HCl, pH 7.5, and 1 mM DTT and applied to the column. (Upper panel) The purified CaM PK-II alone was applied to the column. (—) OD at 280 nm; (○) caldesmon kinase activity; (---) NaCl concentration. Chromatography was carried out as described in Figure 1, and the kinase activity was assayed as described in Figure 1 except the reaction was stopped at 15 min.

eluted in the flow-through fraction. On the other hand, no kinase activity was found in the flow-through fraction, and all kinase activity coeluted with caldesmon when the kinase was mixed with caldesmon prior to loading on the column (Figure 6). These results supported the idea that smooth muscle CaM PK-II has a strong binding affinity for caldesmon.

Discussion

Ca^{2+}/calmodulin-dependent caldesmon kinase was purified from the myofibril fraction of chicken gizzard. During the several washing steps, all soluble proteins were removed. Therefore, the caldesmon kinase is likely to have an affinity to myofibrillar proteins. Attempts to separate the kinase activity from caldesmon by several types of liquid chromatography failed. In all cases, kinase activity was associated with the caldesmon fraction. These results suggest that the kinase has an affinity to caldesmon. This was directly shown by using the purified kinase and caldesmon (Figure 6). The dissociation of the kinase from caldesmon was achieved in the presence of high $MgCl_2$ concentration (Figure 1). Initially it was reported that Ca^{2+}/calmodulin-dependent kinase activity copurified with caldesmon (Ngai & Walsh, 1984), and subsequently it was reported (Scott-Woo & Walsh, 1988a) that caldesmon itself is a kinase which autophosphorylates. The present result shows that the Ca^{2+}/calmodulin-dependent caldesmon kinase activity present in the caldesmon preparation is due to a contaminating kinase. The salt dependence, pH dependence, Mg dependence, and ATP dependence for the autophosphorylation of caldesmon previously reported (Scott-Woo & Walsh, 1988b) are very similar to the values obtained for isolated smooth muscle caldesmon kinase. This suggests that the autophosphorylation of caldesmon previously observed is due to the phosphorylation by the contaminating kinase. The properties of purified Ca^{2+}/calmodulin-dependent caldesmon kinase were quite similar to rat brain CaM PK-II in many respects. (1) The subunit molecular weight of caldesmon kinase in SDS–PAGE was 56K. (2) The caldesmon

kinase was autophosphorylated in the presence of Ca^{2+}/calmodulin and became a constitutively active enzyme. (3) The substrate specificity was similar to the brain CaM PK-II. (4) The activation constant for calmodulin (K_{CaM}) was 32 nM which was similar to the K_{CaM} of brain CaM PK-II (25 nM). (5) The pH dependence, salt dependence, and Mg^{2+} dependence of the caldesmon kinase were similar to those of brain CaM PK-II. (6) The K_m for ATP of the caldesmon kinase was 12 μM, and this value was similar to the reported value for rat brain CaM PK-II (Yamauchi & Fujisawa, 1983). Therefore, we concluded that Ca^{2+}/calmodulin-dependent caldesmon kinase is a smooth muscle isozyme of the CaM PK-II family. The similarities of the kinase activity in the non-heat-treated caldesmon to CaM PK-II have also been observed (Scott-Woo et al., 1990). While this paper was in preparation, Abougou et al. (1989) recently showed that the calmodulin-dependent kinase activity in the non-heat-treated caldesmon is due to the contaminating CaM PK-II. Our data support this conclusion and further characterize this enzyme by identifying its subunit molecular weight and its Mg^{2+}-dependent association with caldesmon. CaM PK-II has been purified or partially purified from several tissues. These are rat brain (Yamauchi & Fujisawa, 1983; Kennedy et al., 1983), rabbit skeletal muscle (Woodgett et al., 1983), bovine cardiac muscle (Kloepper & Lanat, 1984), rat lung (Schulman et al., 1985), rat spleen (Schulman et al., 1985), rat adrenal tumor (Vulliet et al., 1984), and rat pancreas (Cohn et al., 1987). CaM PK-II has not been isolated from smooth muscle, and this is the first report of the purification of CaM PK-II from smooth muscle.

Another important finding is the binding of the purified smooth muscle CaM PK-II to caldesmon. Since caldesmon is thought to be located on the thin filaments, it is likely that CaM PK-II is also located on the thin filaments.

Ngai and Walsh (1984) have shown that the inhibition of the smooth muscle actin-activated ATPase activity of myosin by caldesmon was abolished by the phosphorylation of caldesmon. Caldesmon is one of the most likely physiological substrates for the smooth muscle CaM PK-II purified in this study, since the kinase showed an affinity to caldesmon. Recently, Adam et al. (1988) reported that caldesmon was phosphorylated after the stimulation of intact arterial smooth muscle fiber by high $[K^+]$, an agonist which is known to increase the intracellular $[Ca^{2+}]$. Since the K_{CaM} (32 nM) for smooth muscle CaM PK-II (caldesmon kinase) was 30-100 times higher than the K_{CaM} of MLC kinase (0.3–1 nM), CaM PK-II requires higher free $[Ca^{2+}]$ than MLC kinase to supply enough Ca^{2+}/calmodulin for activation. Therefore, after the initial stimulation, the activation of MLC kinase probably precedes CaM PK-II activation. This is consistent with the results reported by Adam et al. (1988), who found the phosphorylation of caldesmon is slower than the phosphorylation of myosin. A more interesting feature of smooth muscle CaM PK-II is that the kinase was autophosphorylated (Figure 5) and became constitutively active. This suggests that the extent of caldesmon phosphorylation may be maintained even after the cytosolic free Ca^{2+} level is decreased. It has been found in intact smooth muscle that the level of caldesmon phosphorylation remains constant while the level of myosin phosphorylation decreases (Adam et al., 1983). Walsh and his colleagues have suggested (Ngai & Walsh, 1984; Ngai & Walsh, 1987) that caldesmon is dephosphorylated during the latch state since it is only inhibitory in the nonphosphorylated state. This is contrary to the results obtained by Adam et al. (1988), who found the extent of caldesmon phosphorylation remained high during the tension maintenance phase in intact smooth muscle. Whether or not the phosphorylation of caldesmon is related to the maintenance of tension development in the "latch" state is obscure and requires further study.

The physiological function of CaM PK-II has been suggested in several cellular events. Synapsin, the most effective in vitro substrate, was found to be a physiological substrate in brain, and it has been suggested that the phosphorylation of synapsin plays a role in the regulation of neurotransmitter release (Nestler & Greengard, 1984). It has also been suggested that CaM PK-II may affect the synthesis of neurotransmitters and hormones by phosphorylation of tyrosine hydroxlase, an enzyme which catalyzes the rate-limiting step in the biosynthesis of the catecholamines (Yamauchi & Fujisawa, 1982). Other possible physiological substrates are microtubule-associated protein II which regulates the assembly of microtubules (Yamauchi & Fujisawa, 1982), glycogen synthase which catalyzes the rate-limiting step in glycogen synthesis (Rylott et al., 1979; Kloepper & Lanat, 1984), and phospholamban which regulates the cardiac sarcoplasmic reticulum Ca^{2+}-pump ATPase (Lindemann & Watanabe, 1985; Iwasa et al., 1986). Recently we found (Ito et al., 1989) that the peptide which strongly binds to calmodulin so as to antagonize calmodulin and the peptide which strongly inhibits MLC kinase both markedly enhanced the transient Ca^{2+} increase in single smooth muscle cells. Preliminary work shows that these peptides also strongly inhibited the activity of smooth muscle CaM PK-II. This may indicate the possible role of smooth muscle CaM PK-II in the regulation of Ca^{2+} homeostasis in smooth muscle cells.

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