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Isolation of the bile canalicular actin–myosin II motor

(myosin light chain/motility/liver cell)

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ABSTRACT **Cytoskeleton-rich canalicular membranes (CCMs) with preserved cytoskeleton and demembranated CCMs, consisting only of cytoskeletal elements, were used to examine the relationship of pericanalicular microfilaments, myosin II phosphorylation, and canalicular contraction. The components of CCMs were visualized by fluorescence microscopy using the filamentous actin probe rhodamine-phalloidin and by electron microscopy, before and after incubation in 1 μM Ca^{2+}/1 mM ATP (contraction solution). Canalicular contraction (luminal closure) was evaluated by morphometric analysis. Myosin II was extracted from CCMs, purified by immunoprecipitation, and analyzed on Western blots. In sequential experiments, autoradiographs of gels from [γ-^{32}P]-ATP-treated CCMs in the presence or absence of Ca^{2+} were examined after 0.25, 0.50, 1, 2, 3, 5, and 10 min, and the effects of W7 (a calmodulin antagonist) and ML9 (a myosin light chain kinase inhibitor) were evaluated. The results showed that phosphorylation of the 20-kDa protein was low in controls but enhanced beginning 0.25–0.50 min after addition of contraction solution. Both W7 and ML9 significantly inhibited this reaction and inhibited canalicular contraction. The results indicate that phosphorylation of the regulatory 20-kDa myosin light chain of canaliculus-associated myosin II coincides with or precedes contraction of the canaliculus. We conclude that the canalicular contractile apparatus is composed of actin filaments and a myosin II motor.**

Bile canaliculi are specialized areas of the apical cell membrane of adjacent liver cells. Cytoskeletal filaments associated with the canalicular membrane consist predominantly of a circumferential pericanalicular actin–myosin band (1, 2) and an outer sheath of intermediate filaments (1, 3). Although disputed (4), there is mounting evidence that actin-filament-mediated canalicular contractions play an important physiological role in intrahepatic bile flow (3, 5, 6). The present investigation utilized isolated segments of the canalicular system prepared by a method known to yield cytoskeleton-rich canalicular membranes (CCMs) (1). It has been shown (1) by immunoelectron microscopy that CCMs were surrounded by actin filaments associated with myosin II and that canalicular contraction, defined as partial or complete luminal closure, elicited by exposure to a contraction solution was coincident with a change in distribution of pericanalicular actin and myosin. Here, the effects of myosin light chain (MLC) phosphorylation are examined on CCMs and on the isolated canalicular cytoskeleton.

MATERIALS AND METHODS

Preparation of CCMs. CCMs were isolated (1) by a modification of the conventional methods for canalicular membrane preparation (7) by using protease inhibitors [0.1 mM phenylmethylsulfonyl fluoride (PMSF)/aprotonin (1 mg/liter; Sigma)] to preserve more of the canaliculus-associated cytoskeleton. Isolated CCMs were suspended in 75 mM KCl/5 mM $MgCl_2$/1 mM EGTA/aprotonin (1 mg/liter)/0.1 mM PMSF/10 mM imidazole hydrochloride, pH 6.9 (solution A). For demembranation, CCMs were incubated in 1% Triton X-100 by the method of Owaribe *et al.* (8).

Treatment with Contraction Solutions. CCMs and demembranated CCMs (DCCMs) were incubated at 37°C in solution A, solution A with ATP added to 1 mM, or solution A with 1 μM free Ca^{2+} ($CaCl_2$) with or without ATP. Free Ca^{2+} in solution was calculated by the formula of Fabiato *et al.* (9). The effects of the calmodulin (CaM) antagonist, W7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride] (10), and of the protein kinase inhibitor with high affinity for MLC kinase, ML9 [1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride] (11), on contraction and on phosphorylation were determined by preincubating CCMs with 50 μM W7 or with 10 μM ML9 for 3 min before stimulation with Ca^{2+} plus ATP.

For each of the above experimental groups, five animals were used to prepare the CCM and DCCM samples. Fifty CCM samples were examined from each animal.

Assessment of Contraction by Fluorescence Microscopy. CCMs and DCCMs were fixed 0.25, 0.5, 1, 2, 3, 5, or 10 min after incubation in 1% paraformaldehyde for 15 min at 4°C, then stained with 0.15 μM rhodamine-phalloidin (Molecular Probes) for 30 min, and examined in a Nikon fluorescence microscope (Diaphot TMD-EF). The fluorescent images were evaluated for changes in caliber of the canalicular lumen and the number of canaliculi showing no change, partial closure, or complete closure. An IBAS II image analysis system (Zeiss) was used to measure the change of canalicular caliber. Changes in canalicular diameter were measured at five points along each canaliculus. Statistical significance was determined using the Student *t* test.

Electron Microscopy. For transmission electron microscopy, CCMs and DCCMs from each experimental group were fixed with 1.2% (vol/vol) glutaraldehyde, treated with 0.2% tannic acid, and postfixed with 0.25% osmium tetroxide (OsO_4). After *en bloc* staining with 2% (wt/vol) uranyl acetate, samples were dehydrated in a graded ethanol series and embedded in Epon/Araldite. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Philips EM400 transmission electron microscope.

Purification of Myosin by Gel Filtration. Hepatocytes were isolated from rat liver as described (12). Myosin was purified by a modification of the method of Pollard *et al.* (13). In brief, hepatocyte pellets or CCM pellets were suspended in extraction buffer [0.6 M KCl/15 mM pyrophosphate/30 mM imidazole/5 mM $MgCl_2$/3 mM dithiothreitol (DTT), pH 7.0] for 30 min at 4°C. After centrifugation, 2 mM $MgCl_2$ was added to

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Abbreviations: CCM, cytoskeleton-rich canalicular membrane; DCCM, demembranated CCM; MLC, myosin light chain; MHC, myosin heavy chain; DTT, dithiothreitol; CaM, calmodulin.

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the supernatant (pH 6.4). Pellets of crude actomyosin obtained were dissolved in 0.6 M KI/5 mM ATP/5 mM DTT/1 mM $MgCl_2$/20 mM imidazole, pH 7.0, by gentle homogenization, and the solution was clarified by centrifugation at 100,000 × *g* for 20 min. The supernatant was fractionated on a 2.5 × 100 cm column of Bio-Gel A-15m equilibrated with 0.6 M KCl/1 mM DTT/10 mM imidazole, pH 7.0. SDS/PAGE (5–15% linear gradient) was according to the method of Laemmli (14). Purified myosin separated by SDS/PAGE was visualized by staining the gel for 2 h with Coomassie blue.

Western Blot Analysis. Purified myosin resolved by SDS/PAGE (14) was transferred to nitrocellulose by electroelution. The blots were incubated with a monoclonal antibody to MLCs (20 kDa) (Sigma) for 2 h at room temperature in Tris-buffered saline containing 1% gelatin and then stained using immunoperoxidase. Molecular mass standards used were *Escherichia coli* galactosidase, 116 kDa; rabbit muscle phosphorylase, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase B, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.3 kDa.

Immunoprecipitation of Myosin. CCMs were incubated in Ca^{2+} and [γ-^{32}P]ATP. [γ-^{32}P]ATP stock was prepared by adding [γ-^{32}P]ATP to a solution of unlabeled ATP to 0.1 mM ATP (0.16 Ci/mmol; 1 Ci = 37 GBq). To stop the reaction, CCM samples were mixed with an equal volume of 1.8 M KCl/30 mM pyrophosphate/60 mM imidazole/10 mM $MgCl_2$/0.2 mM DTT, pH 7.0, cooled to 0°C, homogenized, and centrifuged at 100,000 × *g* for 60 min. The supernatant was incubated for 30 min with monoclonal antibodies against nonmuscle myosin II (Biomed Instruments, Fullerton, CA) at 37°C and then for 30 min with protein A-Sepharose CL-4B beads (Pharmacia) at 4 mg/ml. Immunoprecipitation with the nonmuscle myosin antibody was performed in 0.5% SDS. The absorbed material was eluted from the Sepharose beads with 2% (wt/vol) SDS and analyzed by SDS/PAGE (14). The gels were dried and subjected to autoradiography. As standards, the following ^{14}C-methylated protein molecular mass markers (Amersham) were used: rabbit muscle myosin, 200 kDa; phosphorylase *b*, 92.5 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; lysozyme, 14.3 kDa.

Phosphorylation Assay. The phosphorylation assay was carried out by the method of Keller and Mooseker (15). Phosphorylation of CCM and DCCM proteins was assayed in solution A with or without $CaCl_2$. [γ-^{32}P]ATP was added to unlabeled ATP to 0.1 mM ATP (0.16 Ci/mmol) and applied to CCMs and DCCMs. The reaction was started by adding the contraction solution and stopped at 0.25, 0.5, 1, 2, 3, 5, or 10 min by mixing an aliquot of the reaction mixture with an equal volume of 2× Laemmli SDS/sample buffer containing 2 mM EGTA and immediately boiling it for 1 min. Inclusion of EGTA in the sample buffer prevented calmodulin from migrating to a position that interfered with the identification of the 20-kDa MLC (16, 17). Samples were electrophoresed on a 5–15% linear gradient SDS/polyacrylamide gel. Proteins were stained with Coomassie brilliant blue R. Stained gels were dried and autoradiographs were prepared using Kodak XAR-5 x-ray film at −70°C with an intensifying screen. The ^{32}P radioactivity of the protein bands was examined by densitometric analysis (LKB Ultroscan XL enhanced laser densitometer, Stockholm) of the radioautographs.

RESULTS

Fluorescence and Electron Microscopy. Fluorescence microscopy (Fig. 1) showed that the CCM preparations consisted of segments of the bile canalicular network with cytoskeletal components attached to their membranes. Compared to other canalicular-enriched plasma membrane fractions from rat liver (7, 18), CCMs have two advantages: they consist of visible segments of the bile canalicular network and they have more 6-nm microfilaments and intermediate filaments. The filamentous actin probe, rhodamine-phalloidin, provided excellent definition of bile canaliculi. Overall, segments of bile canaliculi in CCM (Fig. 1*a*) and DCCM (Fig. 1*c*) samples appear to be similar and the canalicular lumen is clearly seen. In the presence of Ca^{2+} and ATP, the bile canalicular segments dramatically contracted (Fig. 1 *b* and *d*) and their lumens became narrower. Another frequent indication of contraction was the distorted or twisted appearance of the canaliculi in both membranated and demembranated Ca^{2+}/ATP-treated preparations.

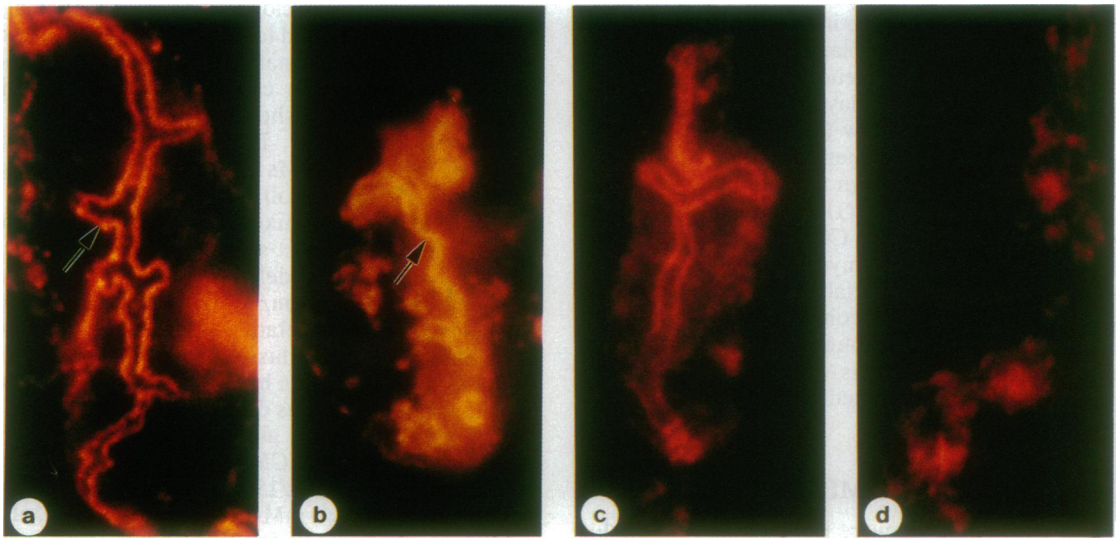

FIG. 1. Fluorescence micrographs of CCMs and DCCMs stained with rhodamine-phalloidin after incubation for 10 min at 37°C with or without contraction solution (1 μM Ca^{2+}/1 mM ATP). (×1400.) (*a*) Untreated CCMs. Rhodamine-phalloidin staining shows the distribution of actin filaments around the bile canaliculus. Note the lumen of the bile canaliculus (arrow). (*b*) Treated CCMs. Note the narrowed bile canalicular lumen (arrow). The canalicular segment is also markedly shortened and appears twisted. (*c*) Untreated DCCMs. Pericanalicular actin filaments remain after demembranation and canaliculi closely resemble those seen in membrane-intact preparations (compare with *a*). (*d*) Treated DCCMs. Overall structure resembles that seen in *b*; but the well defined CCM lumen is not as clear and this can be attributed to the absence of bile canalicular membranes.

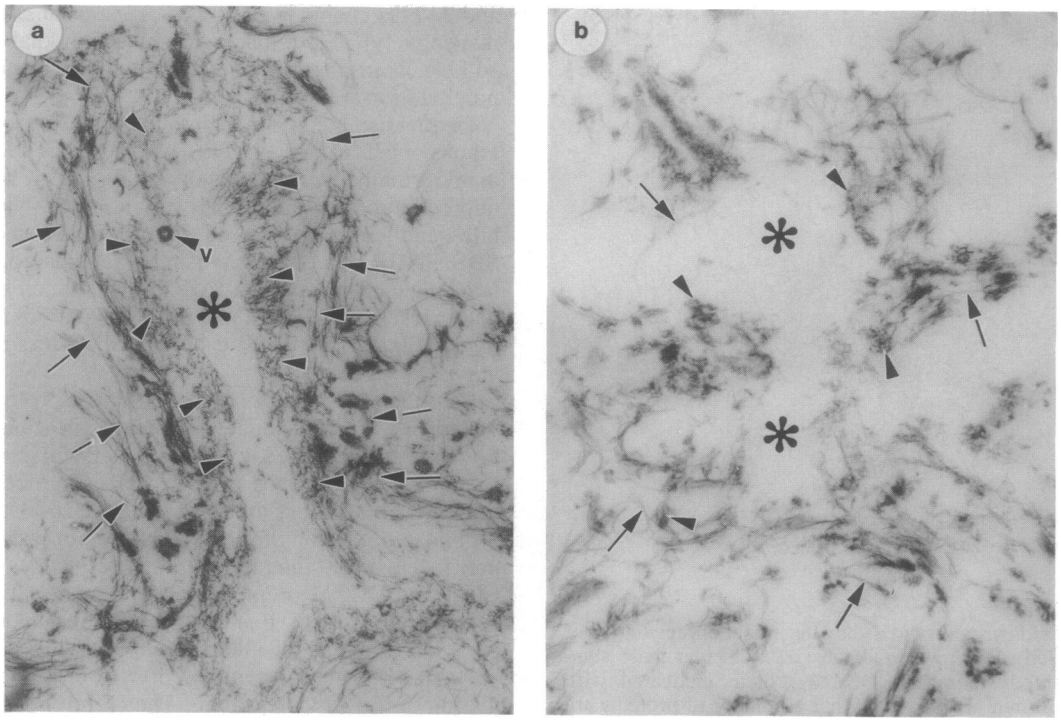

FIG. 2. Transmission electron microscopy. (*a*) Untreated DCCMs. The circumferential pericanalicular cytoskeleton is intact except for the microfilaments in the microvillous cores, which were lost during the demembranation procedure. Note that there are two distinct layers: an inner microfilamentous band (arrowheads) and an outer intermediate filament sheath (arrows). The canalicular lumen (*), normally filled with microvillous membranes, is empty. v, Coated vesicle. (×65,680.) (*b*) Treated DCCMs. Note the abundance of microfilaments and intermediate filaments (arrows) but the orderly structure seen in *a* is altered. Microfilament clumps are frequent (arrowheads). The lumen (*) is more irregular in outline than the control. (×79,200.)

By transmission electron microscopy, bile canaliculi and the associated microvilli, junctional complexes, microfilaments, and intermediate filaments were easily recognized. After the addition of contraction solution, CCMs became distorted, often appearing twisted. Microvillous microfilaments were frequently lost and pericanalicular microfilaments were clumped (data not shown). In DCCMs (Fig. 2), the overall configuration and appearance of the canaliculi were similar to those seen in CCMs except that the membranes were missing and only the cytoskeleton remained. Preparations consisted of a lumen surrounded by an inner band of microfilaments and an outer layer of cytokeratin-type intermediate filaments (1) (Fig. 2*a*). After treatment with contraction solution, the shape of the lumen was more irregular and much of the surrounding filamentous network appeared to have clumped (Fig. 2*b*). The intermediate filaments and microfilaments were easily discerned but the microfilament band was disrupted and its filaments appeared to be in clumps.

Assessment of Contraction by Fluorescence Microscopy. In the presence of Ca^{2+}/ATP, CCMs dramatically contracted. The narrowing of the bile canalicular lumen began between 30 s and 1 min after the addition of the contraction solution. After 3–5 min, the entire structure shortened and appeared twisted. As noted in a previous report (1), coincident with these changes, there were focal points of increased fluorescence in the pericanalicular region that represent microfilament aggregation or clumping. Canalicular closure with loss of a resolveable lumen accompanied by an increased intensity in membrane fluorescence occurred in 69.8 ± 2.1% (mean ± SEM) of CCMs after addition of Ca^{2+}/ATP, compared to 19.6 ± 2.8% in controls ($P < 0.001$). Ca^{2+} alone had no significant effect (18.2 ± 2.5%). Incubation in ATP alone caused significant changes (canalicular closure, 28.4 ± 3.6%; $P < 0.01$) compared to controls, but these changes were not as marked as those seen with Ca^{2+}/ATP. ATP alone in 5 mM EDTA and in the absence of Mg^{2+} gave results similar to those in controls (canalicular closure, 23.6 ± 2.9%). The results with ATP alone may be explained by proteolysis (19). W7 and ML9 inhibited the effects of Ca^{2+}/ATP on CCMs;

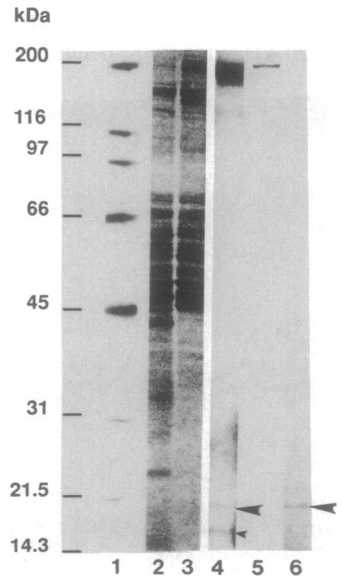

FIG. 3. Purification of myosin and Western blot analysis. Lanes: 1, molecular mass standards (kDa); 2, isolated liver cell homogenate (Coomassie blue-stained SDS gel); 3, CCMs (Coomassie blue-stained SDS gel); 4, purified myosin [Coomassie blue-stained SDS gel, note the 200-kDa, 20-kDa (large arrowhead), and 17-kDa (small arrowhead) bands]; 5, Western blot of purified myosin labeled with antibody to 200-kDa MHC; 6, Western blot of purified myosin labeled with antibody to 20-kDa MLC (large arrowhead).

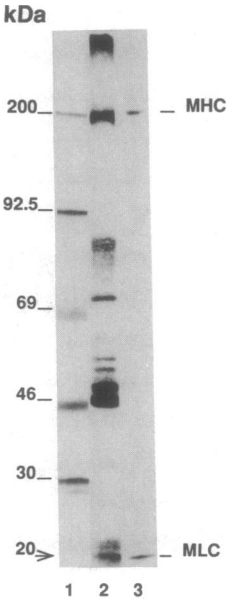

FIG. 4. Autoradiographs of gels showing immunoprecipitation of [γ-^{32}P]ATP-labeled CCM myosin. Lanes: 1, molecular mass standards; 2, extracted solubilized DCCM protein incubated with [γ-^{32}P]ATP for 10 min at 37°C (note that a number of proteins are labeled); 3, purified DCCM myosin shows two phosphorylated bands of 200 and 20 kDa, corresponding to the MHC and regulatory 20-kDa MLC.

(canalicular closure: with W7, 25.2 ± 3.3% of CCMs; with ML9, 26.8 ± 0.49% of CCMs; $P < 0.01$).

Myosin Purification by Gel Filtration and Western Blot Analysis. Analysis of the SDS/PAGE gels prepared after column chromatography of homogenates of whole rat liver showed that the 200-kDa myosin heavy chain (MHC) and the 20- and 17-kDa MLCs were eluted from the same lanes (data not shown). The proteins derived from homogenized isolated hepatocytes and from the CCMs are shown in Fig. 3, lanes 2 and 3, respectively. Lane 4 shows the 200-, 20-, and 17-kDa chains of purified CCM myosin II. Antibodies to MHC (200 kDa) and MLC (20 kDa) reacted specifically with 200-kDa and 20-kDa chains in the CCM preparations (lanes 5 and 6, respectively). To conclusively establish that the 200- and 20-kDa chains were derived from myosin, isotopic immunoprecipitation was performed.

Purification of Myosin by Immunoprecipitation. Autoradiographs of gels of [γ-^{32}P]ATP/Ca^{2+}-treated CCM showed that many proteins were phosphorylated (Fig. 4, lane 2). Immunoprecipitation with anti-myosin antibodies yielded purified bile-canaliculus-associated myosin. Autoradiographs of SDS/PAGE gels of this myosin revealed isotopic labeling of two single proteins of 200 and 20 kDa, which are the molecular masses of the MHC and of the regulatory MLC, respectively (Fig. 4, lane 3). The 17-kDa MLC was not phosphorylated.

Phosphorylation Assay. To identify the proteins phosphorylated in a Ca^{2+}-dependent manner, bile canalicular membranes were incubated with [γ-^{32}P]ATP with or without 1 μM Ca^{2+}. Autoradiographs showed that a number of polypeptides were phosphorylated including the 20-kDa protein (Fig. 5). Phosphorylation of the 20-kDa protein was greater in the presence of Ca^{2+} than in its absence (Figs. 5 and 6). In both CCM and DCCM preparations, phosphorylation of 20-kDa polypeptide increased immediately after addition of Ca^{2+} plus ATP and reached maximum levels after 1–3 min (Fig. 5). W7 and ML9 partially inhibited Ca^{2+}-dependent phosphorylation of the 20-kDa protein. The identity of the phosphorylated band immediately above the 20-kDa protein is unknown but is most likely calmodulin (15). The densitometric analysis of the radioautographs is summarized in Fig. 6. These results closely correlated with canalicular closure.

DISCUSSION

Myosin is the mechanochemical transducer of contraction in smooth muscle and in nonmuscle cells (20, 21). Recent reports classify myosin motor molecules as a superfamily having seven classes in which class II myosins retain the overall properties of myosin II (22). Myosin II is involved in nonmuscle motility events such as locomotion, contraction of the cleavage ring in cytokinesis (20, 23, 24), and contraction of the terminal web of the intestinal brush border (16, 25, 26). Phosphorylation of the regulatory 20-kDa MLC by Ca/CaM-

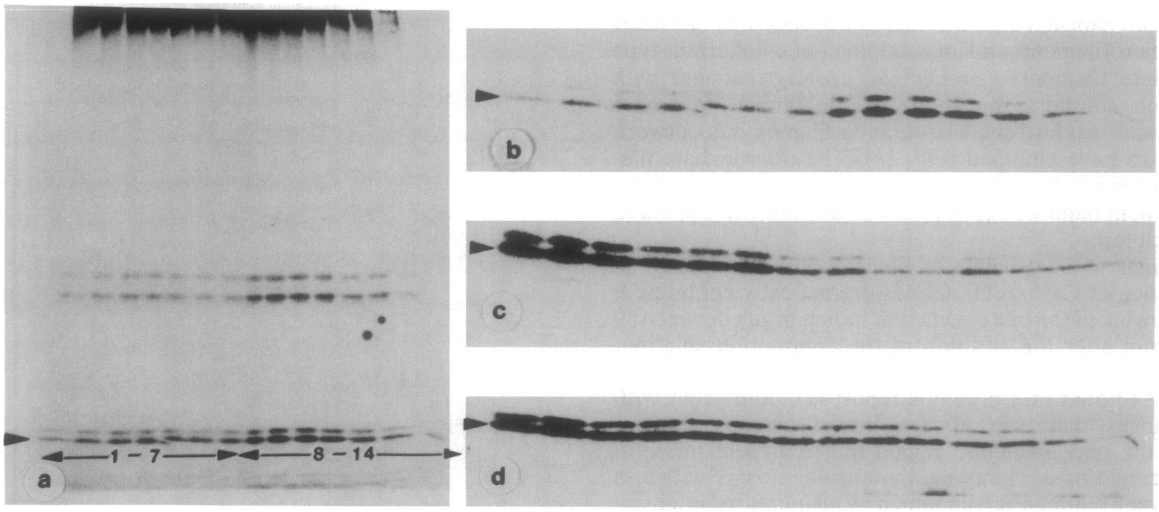

FIG. 5. Autoradiographs of DCCMs. Results shown are after incubation in 0.1 mM [γ-^{32}P]ATP after addition of isotope at 0.25, 0.50, 1, 2, 3, 5, or 10 min. Lanes: 1–7, in the absence of Ca^{2+}; 8–14, in the presence of Ca^{2+}. The 20-kDa protein is marked by an arrowhead. (*a*) Complete gel of the control in the absence and presence of calcium. Several proteins are phosphorylated. (*b*) Close-up view of the 20-kDa region shown in *a*. Note that with the addition of Ca^{2+}, there is enhanced phosphorylation of the 20-kDa protein. (*c* and *d*) Close-up views of the 20-kDa region after preincubation with W7 and ML9, respectively. (*c*) Enhancement of phosphorylation of the 20-kDa protein is impaired by W7 (lanes 8–14). (*d*) Enhancement of phosphorylation of the 20-kDa protein is impaired by ML9 (lanes 8–14). The slight fading in intensity in lanes 6, 7, 13, and 14 may be due to dephosphorylation and suggests that the phosphorylation may be transient.

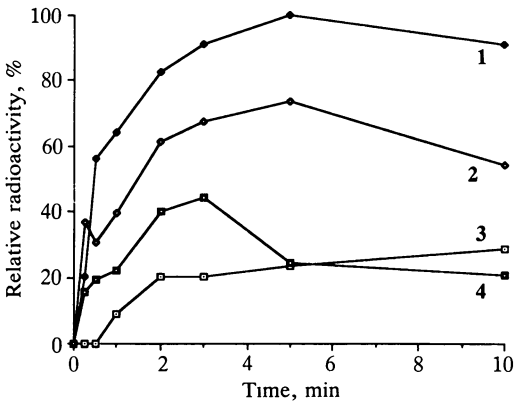

FIG. 6. Quantitation of phosphorylation of 20-kDa MLC in DCCMs after 0.25, 0.5, 1, 2, 3, 5, or 10 min of incubation from gels shown in Fig. 5. ^{32}P radioactivity of 20-kDa bands was measured by densitometry. The activity was defined as values relative to that of the 20-kDa polypeptide of the group treated with [γ-^{32}P]ATP plus Ca^{2+}. There is enhanced phosphorylation with Ca^{2+}/ATP (curve 1) compared with control values (curve 3); preincubation with W7 (curve 4) and ML9 (curve 2) shows partial inhibition.

activated MLC kinase is the accepted mechanism of contraction (20, 21, 27, 28). Myosin phosphorylation at other sites has different effects (29, 30). Contraction of skeletal and smooth muscle cells coincides with increased phosphorylation of the regulatory 20-kDa MLC (28) and the same is true of nonmuscle cells where phosphorylation is accompanied by motility and changes in actin organization (31). MLC phosphorylation stimulates Mg^{2+}ATPase activity and promotes the assembly of myosin into filaments (26). Myosin filaments have not yet been visualized *in situ* in nonmuscle cells and this is probably due to the preparatory methods used, their small size, and small number of filaments present (13, 26).

In the liver, there is accumulating evidence for canalicular contraction mediated by a similar mechanism involving actin filaments, myosin II, Ca^{2+}, ATP, CaM, and MLC kinase (3, 29, 32, 33). Canalicular contractions were first described in isolated hepatocyte couplets (12), they were subsequently shown to be Ca^{2+}/ATP-dependent (2, 6, 32), and they have recently been shown *in vivo* (5). The microinjection of the catalytic fragment of MLC kinase was shown to produce canalicular contraction in hepatocyte doublets (33). Inhibitors of filamentous actin polymerization such as cytochalasin and filamentous actin stabilizers such as phalloidin impaired canalicular contractions and bile flow (34). A review on the role of the cytoskeleton in canalicular physiology can be found in ref. 6.

In the present paper, we show that contraction of CCMs induced by ATP/Ca^{2+} coincides with phosphorylation of the regulatory 20-kDa MLC. These results and the inhibitory effects shown with W7 and ML9 are consistent with the model in which force generation requires phosphorylation of the 20-kDa MLC in a reaction that is regulated by Ca/CaM-dependent MLC kinase, as in other nonmuscle cells (20, 21, 24, 27). The findings suggest that the Ca^{2+}/ATP-induced contraction of microfilaments associated with bile canalicular membranes is a dynamic process resulting in narrowing or complete closure of the canalicular lumen. These results provide an important link in our understanding of actin filament interaction with myosin in bile canalicular contraction. In addition, the model describes how an apical junction (zonula adherens)-associated band of contractile microfilaments may regulate a vital organ function, in this instance, intrahepatic canalicular bile flow. It is also of special interest that the 20-kDa MLC phosphorylation and dynamic actin filament changes consistent with contraction are also observed with DCCMs since these preparations are composed solely of the isolated pericanalicular cytoskeletal components. Hence, this isolated pericanalicular cytoskeletal preparation, DCCMs, has all the constitutive components necessary for contraction. It contains a circumferential actin filament band and myosin II motor surrounded by a noncontractile sheath of intermediate filaments.

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