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Caldesmon and a 20-kDa actin-binding fragment of caldesmon inhibit tension development in skinned gizzard muscle fiber bundles

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ABSTRACT **Caldesmon is known to inhibit actin-activated myosin ATPase activity in solution, to inhibit force production when added to skeletal muscle fibers, and to alter actin movement in the *in vitro* cell motility assay. It is less clear that caldesmon can inhibit contraction in smooth muscle cells in which caldesmon is abundant. We now show that caldesmon and its 20-kDa actin-binding fragment are able to inhibit force in chemically skinned gizzard fiber bundles, which are activated by a constitutively active myosin light-chain kinase in the presence and absence of okadaic acid. This inhibitory effect is reversed by high concentrations of Ca^{2+} and calmodulin. Therefore, caldesmon may act by increasing the level of myosin phosphorylation required to obtain full activation. Our results also suggest that caldesmon does not act to maintain force in smooth muscle by cross-linking myosin with actin since competition of binding of caldesmon with myosin does not cause a reduction in tension.**

Regulation of smooth muscle contraction is dependent on the level of myosin phosphorylation (1) but there is evidence that other factors, such as the mode of stimulation (2), may alter the response to phosphorylation. Thus, a relaxed state may be associated with a high level of phosphorylation of the 20-kDa regulatory light chain of myosin (LC_{20} phosphorylation) (3, 4, 23), while the rate of relaxation may be regulated at apparently basal levels of LC_{20} phosphorylation (5). Interest in a second regulatory mechanism has been stimulated by the discovery of two actin-binding proteins, caldesmon (6) and calponin (7), which inhibit actin activation of myosin ATPase activity in solution (8–10). Caldesmon is particularly interesting since it is localized in the contractile actomyosin domain of smooth muscle (11), and it has been shown to be able to inhibit the binding of myosin-ATP cross-bridges to actin when added to single skeletal muscle fibers with a resulting inhibition of force production (12). This is an artificial situation, however, since caldesmon is not a component of striated muscle. Our own preliminary work indicated that exogenous caldesmon can inhibit force production.§ Furthermore, Katsuyama *et al.* (14) showed that caldesmon peptides, which presumably displace caldesmon from actin in muscle cells, can activate these cells to produce force. Thus, there is indirect evidence that caldesmon can inhibit contraction in smooth muscle.

We have now attempted to show the feasibility that caldesmon is a modulator of force production in smooth muscle. This was done by using chemically skinned chicken gizzard fibers, which were activated by adding a constitutively active fragment of myosin light-chain kinase (I-MLCK) (15). In this way, we were able to phosphorylate the regulatory chains of myosin necessary for activation of contraction without inactivating the Ca^{2+}/calmodulin-dependent activation pathway by binding caldesmon to calmodulin. This is preferential to thiophosphorylation of myosin, which disrupts the normal phosphorylation–dephosphorylation cycle of myosin. To facilitate diffusion of caldesmon into the fibers, we used a 20-kDa, actin-binding fragment of caldesmon in most of the experiments. This C-terminal fragment (16) inhibits ATP hydrolysis (17) and has been shown to function in the striated muscle system (12). Our results show that caldesmon and the actin-binding fragment of caldesmon shift the relation between force and LC_{20} phosphorylation toward higher levels of phosphorylation. In contrast, the N-terminal myosin-binding fragment of caldesmon and a maleylated derivative of the 20-kDa actin-binding fragment of caldesmon had no effect on force production.

MATERIALS AND METHODS

Chicken gizzards were collected at a local farm immediately after sacrifice of the animals and brought to the laboratory in oxygenated, ice-cold physiological saline solution containing 150 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM $CaCl_2$, 24 mM Hepes (pH 7.4 at 37°C), and 10 mM glucose. Small fiber bundles ≈15 mm long and 0.2–0.3 mm in diameter were gently teased out of the superficial circumferential muscle layer, skinned with Triton X-100, and stored as described (18). Fibers were used within 4 days.

Calmodulin was purified from bovine testicles according to ref. 19. Gizzard MLCK was isolated as described by Ngai *et al.* (20) and hydrolyzed with trypsin (1:40, wt/wt; Sigma) for 15 min at 25°C according to ref. 15. Caldesmon was purified by a modification of the method of Bretscher (21, 22). The actin-binding (16) and myosin-binding (17) fragments of caldesmon were prepared from chymotryptic digestion of caldesmon. As a control, lysine residues of the 20-kDa fragment were maleylated by treatment with a 10-fold excess of maleic anhydride at pH 8.0 for 5 min (24). Protein concentrations were determined by the Lowry method (25) with bovine serum albumin as a standard.

Experimental Protocol and Solution Composition. For isometric force recording, paired, thin fiber bundles (diameter, 100–150 μm) were attached with a cellulose-based glue between an AME 801 force transducer (SensoNor, Horton, Norway) and a glass rod extending from a micrometer drive. We chose to use paired fibers as controls rather than using each fiber as its own control because I-MLCK was difficult to wash out and therefore each fiber was incubated with I-MLCK only once. After mounting, the fiber bundles were

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Abbreviations: LC_{20}, 20-kDa regulatory light chain of myosin; MLCK, myosin light-chain kinase; I-MLCK, constitutively active fragment of MLCK.

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§Preliminary reports of this work were presented at the Biophysical Society Meeting, February 9–13, 1992, Houston (13).

incubated in rigor solution containing 150 mM KCl, 25 mM imidazole (pH 7.0 at 21°C), 4 mM EGTA, 3 mM $MgCl_2$, 2 mM dithioerythritol, to which hexokinase (400 units/ml; Boehringer Mannheim) and glucose (10 mM) were added for 15 min to deplete endogenous ATP (Fig. 1). The fibers were then transferred to rigor solution containing I-MLCK (0.18 mg/ml) and soybean trypsin inhibitor (0.5 mg/ml; Sigma) in the absence (control) or presence of caldesmon fragments or intact caldesmon for 30 min to allow for diffusion of the proteins into the fibers. Tension development was initiated by switching to the MgATP-containing relaxing solution in the continued presence of the proteins until a plateau was reached. The proteins were finally washed out in contraction solution. Relaxing solution consisted of 50 mM KCl, 25 mM imidazole (pH 7.0 at 21°C), 1 mM ATP, 4 mM $MgCl_2$, 4 mM EGTA, 2 mM dithioerythritol, 4 mM creatine phosphate, phosphocreatine kinase (140 units/ml; Boehringer Mannheim). Contraction solution had the same composition as relaxing solution but with 4 mM $CaCl_2$ and 1 μM calmodulin. Free Ca^{2+} was calculated as described (26). To all solutions, which did not contain caldesmon or fragments of caldesmon, the appropriate amount of buffers in which the proteins were dissolved was added. Okadaic acid was dissolved in Me_2SO and diluted with the incubation solutions; the final concentration of Me_2SO was <0.05% and did not affect tension development.

Determination of LC_{20} Phosphorylation. LC_{20} phosphorylation was determined in the same fiber bundles that were used for the tension measurements by two-dimensional polyacrylamide gel electrophoresis as described (5) with minor modifications.

Statistics. The data were expressed as the mean ± SEM. Student's *t* test was used to determine significant differences between two population means, and a *P* value of <0.05 was considered statistically significant.

RESULTS

Under the conditions of our experiments, incubation of skinned gizzard fibers with I-MLCK caused a submaximal contraction of 60–70% of the maximal force elicited by 30 μM Ca^{2+} and 1 μM calmodulin. Original traces of tension development are shown in Fig. 1. In the presence of caldesmon at 0.4 mg/ml (Fig. 1*B*), active tension induced by I-MLCK is nearly completely inhibited. Washing out caldesmon with Ca^{2+} (30 μM) and calmodulin (1 μM) reversed the inhibitory effect. In Fig. 1*C*, addition of the 0.4-mg/ml fragment to I-MLCK-activated fibers produced a plateau of force that was only 40% of the maximal force; the rate of tension development appears to be depressed somewhat. Another fragment of caldesmon, the N-terminal myosin-binding fragment, had no effect on force production (Fig. 1*D*), showing that the effect of caldesmon and its actin-binding fragment is specific.

The results of several experiments done by the protocol of Fig. 1 are shown in Fig. 2. Each histogram shows a control (c) paired with an experiment in the presence of a polypeptide (p). Intact caldesmon inhibited the active tension by 50% under the conditions shown, but the effects on both the time course of force development and the steady-state force were quite variable. Of a total of 17 fibers, 8 were inhibited by >80% and 5 of those 8 reached a clear plateau of tension

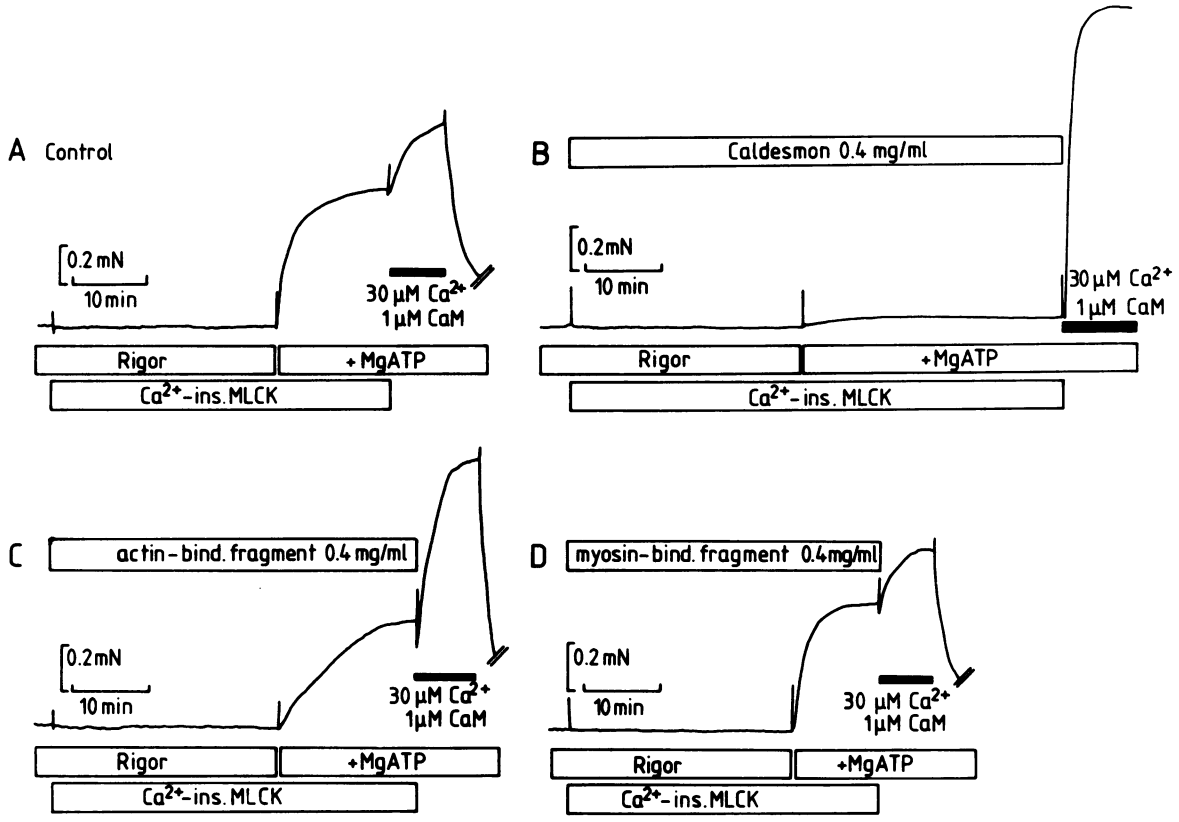

FIG. 1. Original tracings of force responses in Triton-skinned fiber bundles from chicken gizzard. Isometric force was measured in thin fiber bundles (100–150 μm), which were first incubated in rigor solution containing 10 mM glucose and hexokinase (400 units/ml) to deplete endogenous ATP. This was followed by incubation in rigor solution containing I-MLCK (0.18 mg/ml) with or without caldesmon or caldesmon fragments (myosin or actin binding). Thereafter, force was induced by switching to relaxing solution in the continued presence of I-MLCK and caldesmon or fragments until a plateau was reached. Finally, fibers were stimulated in contraction solution (30 μM Ca^{2+}) and 1 μM added calmodulin. The level of force produced in relaxing solution was depressed in the presence of caldesmon (*B*) and the actin-binding fragment of caldesmon (*C*).

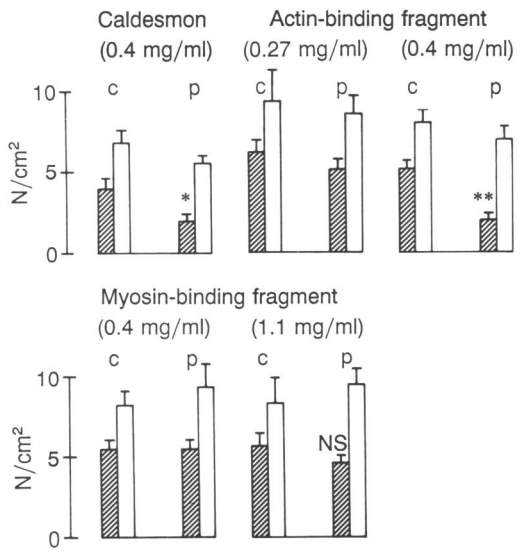

FIG. 2. Effect of caldesmon and fragments of caldesmon on force in relaxing solution and after washout in Ca^{2+}/calmodulin. Caldesmon and the 20-kDa actin-binding fragment of caldesmon inhibited force produced by muscle bundles activated without calcium in a concentration-dependent manner. The myosin-binding fragment was without significant effect. Experimental protocol is as described in Fig. 1. Only paired experiments were used for statistical analysis; values are means ± SEM, n = 7–17 fiber bundles from at least five different gizzards and three different preparations of caldesmon and the caldesmon-binding fragment. * and **, P values of <0.05 and <0.001; NS, not significantly different. Hatched bars, force in relaxing solution in the presence of I-MLCK; open bars, force in contraction solution (30 μM Ca^{2+}/1 μM calmodulin); c, control preparations; p, polypeptides.

within 45 min after activation. In 4 fibers (1 with a plateau) we observed 40% inhibition, and in 5 fibers (3 with a plateau) no inhibition was observed. We believe that this variability is largely due to the slow rate of diffusion of intact caldesmon into muscle fibers (12).

Much greater reproducibility was obtained by using the 20-kDa actin-binding fragment of caldesmon. This fragment inhibits ATP hydrolysis and also inhibits force production in skeletal muscle fibers (12). In the present case, little inhibition was observed by 0.27 μM actin-binding fragment. However, in 10 observations, the 0.4-mg/ml fragment reduced the active tension from 5.3 ± 0.4 N/cm² to 2.0 ± 0.4 N/cm². Because of the greater reproducibility of the 20-kDa fragment, this fragment was used for most of the subsequent experiments.

Several controls were run to ensure that the results observed with the 20-kDa fragment and intact caldesmon are specific and reversible. First, we noted that washing out the actin-binding fragment with contraction solution reversed its effect on force; the resulting force (6.9 ± 0.6 N/cm²) was not significantly different from the force in control fibers (8.1 ± 0.7 N/cm²; cf. Fig. 2). Washing the fibers in relaxing solution—that is, without Ca^{2+} and calmodulin—only partially reversed the inhibitory effect. Reversal of the inhibitory effect by Ca^{2+}/calmodulin was also observed in the continued presence of the fragment. In the absence of Ca^{2+}, calmodulin did not reverse the inhibitory effect of the 20-kDa fragment. In contrast, force was partially restored by the addition of Ca^{2+} in the absence of added caldmodulin. This partial reversal occurs since calmodulin is not totally extracted from the fibers during the skinning procedure. Thus, Ca^{2+} and calmodulin compete with the binding of the caldesmon fragment in the smooth muscle fiber just as they do in the skeletal muscle fiber (12) and in solution (L. Velaz, Y.-d. Chen, and J.M.C., unpublished data).

Two experiments were done to show that the inhibitory effect is due to specific binding of caldesmon to actin. First, the myosin-binding fragment of caldesmon, which does not inhibit ATPase activity in solution and does not bind to actin, has no effect on fiber tension. Figs. 1 and 2 show that the force elicited by I-MLCK was not altered even with the myosin-binding fragment at 1.1 mg/ml. This lack of effect could not be attributed to phosphorylation of the myosin-binding fragment. Second, we observed that treatment of the 20-kDa actin-binding fragment with maleic anhydride eliminated the ability of this fragment to inhibit actin-activated ATPase in solution. This modified actin-binding fragment had no effect on force when added to skinned gizzard fiber bundles.

We also measured the effect of the actin-binding fragment on LC_{20} phosphorylation since dephosphorylation of the regulatory myosin light chain could cause a reduction in force. The 20-kDa fragment had no effect on the level of LC_{20} phosphorylation (data not shown). Thus, the 20-kDa fragment appeared to alter the coupling between force and LC_{20} phosphorylation. To investigate in more detail the relation between phosphorylation and force in the presence and absence of the actin-binding fragment, the level of phosphorylation was changed by addition of various concentrations of the phosphatase inhibitor okadaic acid to the relaxing solutions. In this series of experiments, the concentration of I-MLCK was adjusted so that force development occurred only in the presence of okadaic acid. Fig. 3 shows the noncumulative dose–response relation between force and okadaic acid with and without the actin-binding fragment. Fig. 3*A* shows the measured force, while the values in Fig. 3*B* are normalized to the maximum force produced by each fiber. Force was maximal in the presence of 0.3–1 μM okadaic acid and was not significantly enhanced by Ca^{2+} (30 μM) and calmodulin (1 μM). In the presence of the actin-binding fragment, the dose–response relation was shifted toward the right—i.e., toward higher concentrations of okadaic acid (Fig. 3). A similar shift in the dose–response relation was observed when okadaic acid was added cumulatively. In a second series of experiments, the fibers were fixed with trichloroacetic acid after incubation in I-MLCK at four concentrations of okadaic acid to determine LC_{20} phosphorylation. Fig. 4 shows that inhibition of force by the actin-binding fragment (0.03 and 0.1 μM okadaic acid) was not associated with a decrease in LC_{20} phosphorylation. At 0.3 and 1 μM okadaic acid, neither force nor phosphorylation was significantly different from that in controls. This resulted in an apparent shift in the force–phosphorylation relation. However, at high concentrations of okadaic acid, the interpretation of the data may have been complicated by the fact that under this condition, we also observed a certain degree of phosphorylation of caldesmon and the actin-binding fragment of caldesmon. As the proteins were only barely phosphorylated in the incubation medium alone, it is unlikely that phosphorylation of the proteins was due to a protein kinase contaminating purified caldesmon or MLCK. Therefore, the protein kinase is most likely contained in the skinned fibers.

DISCUSSION

By activating skinned smooth muscle fibers with the constitutively active fragment of MLCK in the absence of Ca^{2+}, we have shown that phosphorylation-dependent tension development is inhibited by caldesmon and a C-terminal, actin-binding fragment of caldesmon. The inhibitory effect is not associated with a decrease in LC_{20} phosphorylation and cannot be attributed to a calmodulin antagonistic action (27). The inhibitory activity of caldesmon can, however, be specifically reversed by the addition of Ca^{2+}/calmodulin to the fiber as predicted by solution studies (28). Neither the maleic

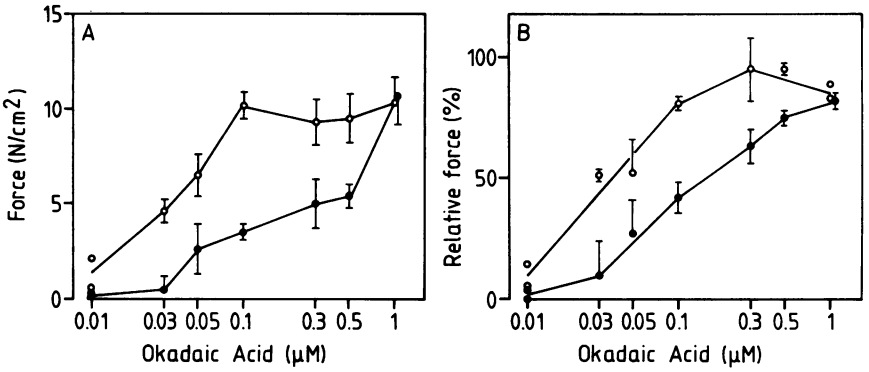

FIG. 3. Dose–response relation between force in relaxing solution with and without the actin-binding caldesmon fragment and okadaic acid. Experimental protocol is as described in Fig. 1 except the phosphatase inhibitor okadaic acid was added to the relaxing solution, which also contained I-MLCK. Note that each fiber was subjected to only one concentration of okadaic acid. (*A*) Absolute force values per cross-sectional area obtained in I-MLCK. (*B*) Force elicited by I-MLCK was expressed as percentage of the force obtained in contraction solution. Open circles, control fibers; solid circles, fibers incubated with actin-binding fragment of caldesmon (0.4 mg/ml). Values are means ± SEM, n = 3–4 fiber bundles.

anhydride-modified actin-binding fragment nor the myosin-binding fragment of caldesmon has any inhibitory activity. Thus, under conditions in which force is solely dependent on LC_{20} phosphorylation, the putative regulatory protein caldesmon modulates the coupling between force and LC_{20} phosphorylation (cf. Fig. 4).

When caldesmon was first observed to bind to myosin, as well as to actin, the possibility was raised that caldesmon could contribute to force maintenance (29) such as in the latch state (30, 31). If caldesmon contributed to force maintenance, under our conditions, the myosin-binding fragment of caldesmon should have reduced that force by competing with caldesmon cross-linking. Yet high concentrations of the myosin-binding fragment had no effect on tension. A similar conclusion has been reached by Hemric and Haeberle (32, 33) using *in vitro* motility assays.

In the presence of caldesmon and the 20-kDa fragment, we observed an apparent decrease in the rate of force production. This does not necessarily mean that caldesmon alters a rate constant in the ATPase cycle of smooth muscle. It has already been shown that caldesmon has no effect on the rate constant of force redevelopment in skeletal psoas fibers (12).

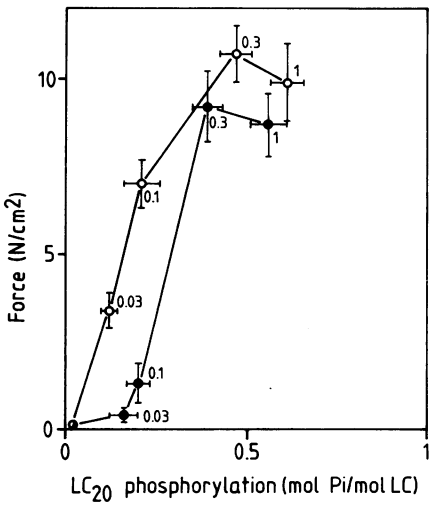

FIG. 4. Relationship between force and LC_{20} phosphorylation with and without the actin-binding fragment of caldesmon. Experimental protocol is as described in Fig. 3 but with a different preparation of fibers. Once force reached a plateau in relaxing solution containing I-MLCK and various concentrations of okadaic acid with or without the actin-binding fragment, the fibers were fixed in trichloroacetic acid, the proteins of the fibers were separated by two-dimensional gel electrophoresis, and the LC_{20} phosphorylation level was determined by densitometry of the silver- or Coomassie-stained gels. Open circles, control; solid circles, actin-binding fragment of caldesmon (0.4 mg/ml). Numbers indicate concentrations (μM) of okadaic acid.

A complication of the present work is that following our chemical skinning procedure the fibers contain residual caldesmon. We have not been able to quantitate the amount of lost caldesmon. Thus, it is likely that we are observing not the maximum effects of caldesmon but the difference between the level of inhibition due to endogenous caldesmon and that caused by exogenous caldesmon. Support for the idea that endogenous caldesmon is inhibitory comes from the recent experiments of Morgan and coworkers (14), who showed that a noninhibitory actin-binding peptide from caldesmon enhanced force. This enhancement was presumed to result from competitive inhibition of the binding of caldesmon to actin by the peptides. It is also possible that exogenous caldesmon would inhibit tension in muscle fibers that contain their full complement of endogenous caldesmon. This is so because, among smooth muscle cells, the caldesmon content is quite variable (34). The addition of excess caldesmon could therefore give a level of inhibition not normally seen in the cell. This is not an unusual situation since exogenous calmodulin is known to enhance the effect of endogenous calmodulin (35).

An interesting aspect of our data is that the inhibitory effects of caldesmon are reversed by high concentrations of okadaic acid. It is not clear at present whether this is due to the high level of myosin light-chain phosphorylation or to the simultaneous phosphorylation of the actin-binding fragment of caldesmon since both occur under our experimental conditions. If phosphorylation of caldesmon antagonizes its inhibitory effect, the effects of caldesmon and the 20-kDa actin-binding fragment are low estimates. The effect of phosphorylation of caldesmon on tension development in skinned fibers remains to be tested. Regardless, higher levels of myosin light-chain phosphorylation were required to maintain a given level of force. In this respect, caldesmon appears to act not as a switch but as a modulator of phosphorylation-dependent force. It would be interesting to know whether the differences in caldesmon content among smooth muscle types (34), such as the high content in phasic smooth muscles, are related to the differences in force–phosphorylation coupling observed in different types of smooth muscle (36).

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