Calponin–Calmodulin Interaction: Properties and Effects on Smooth and Skeletal Muscle Actin Binding and Actomyosin ATPases†

Steven J. Winder,‡,§ Michael P. Walsh,‡,∥ Charles Vasulka,⊥ and J. David Johnson*,⊥

MRC Signal Transduction Group and Department of Medical Biochemistry, The University of Calgary, 3330 Hospital Drive Northwest, Calgary, Alberta, Canada T2N 4N1, and Department of Medical Biochemistry, The Ohio State University Medical Center, Columbus, Ohio 43210

Received June 16, 1993; Revised Manuscript Received September 28, 1993®

ABSTRACT: Smooth muscle calponin bound to the biologically active fluorescent calmodulin [2-(4′-maleimidoanilino)naphthalene-6-sulfonic acid·calmodulin] (MIANS·CaM) with a K_d of 80 nM and produced a 3.4-fold fluorescence enhancement. PKC-phosphorylated calponin (1.3 mol of P_i/mol) bound to CaM with ~15-fold lower affinity. Calponin inhibited CaM (10 nM) activation of the Ca^{2+}-/CaM-activated cyclic nucleotide phosphodiesterase (PDE) with an IC_{50} of 138 nM. The calponin–CaM interaction was Ca^{2+}-dependent: half-maximal binding of calponin to MIANS·CaM occurred at pCa 6.6 with a Hill coefficient of 2.4. Stopped-flow fluorescence kinetic analysis demonstrated that EGTA chelation of Ca^{2+} from CaM disrupted the MIANS·CaM–calponin complex at a rate of 1 s^{-1}. Calponin bound MIANS·CaM at a rate of $(6.0 \pm 1.8) \times 10^6$ M^{-1} s^{-1}, and melittin and unlabeled brain CaM both disrupted the MIANS·CaM–calponin complex at a rate of 0.3 ± 0.1 s^{-1}. These studies suggest that calponin binds CaM with 80-fold lower affinity than myosin light-chain kinase and that calponin associates with CaM much slower than it associates with caldesmon or myosin light-chain kinase. The physiological relevance of the CaM–calponin interaction was evaluated by analysis of the effects of Ca^{2+}-CaM on (i) the interaction of calponin with actin and (ii) calponin-mediated inhibition of actin-activated myosin MgATPase activity. Ca^{2+}-CaM half-maximally inhibited calponin (2 μM) binding to smooth and skeletal muscle actins (9 μM) at 5.4 and 11 μM CaM, respectively. Ca^{2+}-CaM failed to reverse calponin inhibition of smooth or skeletal muscle actin-activated myosin MgATPases, even at molar ratios of CaM that were supraphysiological relative to actin and calponin. Consistent with these findings, Ca^{2+}-CaM, under ATPase reaction conditions, failed to dissociate calponin from actin. We conclude that calponin's physiological function (inhibition of myosin cross-bridge cycling) is probably not modulated by its interaction with CaM.

Calponin is a thin filament associated protein that has been implicated in the regulation of smooth muscle contraction since the isolated protein inhibits the actin-activated MgATPase activity of phosphorylated smooth muscle myosin (Winder & Walsh, 1990). Calponin was originally isolated as an actin-binding protein that interacted with CaM[1] in a Ca^{2+}-dependent manner (Takahashi et al., 1986). The purified protein was also found to bind to tropomyosin (Takahashi et al., 1988). The inhibitory effect of calponin on the actomyosin MgATPase is due to its interaction with actin, since it is independent of tropomyosin and Ca^{2+} (Winder & Walsh, 1990). Several mechanisms have been considered that may regulate calponin-mediated inhibition of the actomyosin MgATPase and thereby the contractile state of smooth muscle: (i) direct binding of Ca^{2+} to calponin (Takahashi et al., 1987); (ii) binding of GTP to calponin (Takahashi & Nadal-Ginard, 1991); (iii) phosphorylation and dephosphorylation of calponin (Winder & Walsh, 1990; Winder et al., 1992); and (iv) binding of Ca^{2+}-CaM or another Ca^{2+}-binding protein such as S100 or SMCaBP-11 (an 11-kDa smooth muscle Ca^{2+}-binding protein referred to as caltropin) to calponin (Wills et al., 1993a). As part of a series of biochemical studies designed to evaluate the significance of these potential regulatory mechanisms, we report here a complete kinetic analysis of the interaction between Ca^{2+}-CaM and calponin, the effects of Ca^{2+}-CaM on the binding of calponin to actin, and the effects of Ca^{2+}-CaM on calponin-mediated inhibition of the actin-activated myosin MgATPase.

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP (>5000 Ci/mmol) was purchased from Amersham (Oakville, ON, Canada). 2-(4′-Maleimidoanilino)naphthalene-6-sulfonic acid (MIANS) and 2′-(*N*-methylanthraniloyl)-cGMP were obtained from Molecular Probes (Eugene, OR), and melittin was from Boehringer-Mannheim (Indianapolis, IN). Electrophoresis reagents were purchased from Bio-Rad Laboratories (Mississauga, ON, Canada). RS-20, the synthetic peptide corresponding to the CaM-binding domain of smooth muscle MLCK (residues 796–815 with the sequence: ARRKWQKTGHAVRAIGRLSS; Olson et al., 1990), was generously provided by Dr. Fred Fay (University of Massachusetts Medical Center). All other chemicals were reagent grade or better and were purchased from CanLab (Edmonton, AB, Canada).

† This work was supported by grants from the Medical Research Council of Canada (to M.P.W.) and from the National Institutes of Health (R01 DK33727 to J.D.J.).

* Author to whom correspondence should be addressed. The Department of Medical Biochemistry, The Ohio State University Medical Center, 333 Hamilton Hall, 1645 Neil Ave., Columbus, OH 43210. Telephone: (614) 292-0104. Fax: (614) 292-4118.

‡ The University of Calgary.

§ Recipient of a fellowship from the Alberta Heritage Foundation for Medical Research. Current address: MRC Laboratory of Molecular Biology, Hills Rd., Cambridge, U.K. CB2 2QH.

∥ Recipient of an Alberta Heritage Foundation for Medical Research Medical Scientist Award.

⊥ The Ohio State University Medical Center.

® Abstract published in *Advance ACS Abstracts,* November 15, 1993.

[1] Abbreviations: CaD, caldesmon; CaM, calmodulin; CaM kinase II, Ca^{2+}- and calmodulin-dependent protein kinase II; MIANS, 2-(4′-maleimidoanilino)-naphthalene-6-sulfonic acid; MLCK, myosin light-chain kinase; PDE, Ca^{2+}- and calmodulin-activated cyclic nucleotide phosphodiesterase; PKC, Ca^{2+}- and phospholipid-dependent protein kinase C.

0006-2960/93/0432-13327$04.00/0

Protein Purification. The following proteins were purified by previously described methods: plant CaM from wheat germ (Strasburg et al., 1988) with modifications described by Mills et al. (1988), bovine brain CaM (Walsh et al., 1984), rabbit skeletal muscle myosin (Persechini & Rowe, 1984) and actin (Zot & Potter, 1981), and chicken gizzard smooth muscle myosin (Persechini & Hartshorne, 1981), actin (Ngai et al., 1986), tropomyosin (Smillie, 1982), MLCK (Adelstein & Klee, 1981), and calponin (Winder & Walsh, 1990). Bovine brain PDE was purified as described by Sharma et al. (1983) up to and including the CaM–Sepharose step.

Electrophoresis. SDS–PAGE was performed on 7.5–20% polyacrylamide gradient slab gels (1.5 mm thick) with a 5% acrylamide stacking gel, in the presence of 0.1% (w/v) SDS, at 36 mA in the discontinuous buffer system of Laemmli (1970). Gels were stained in 45% (v/v) ethanol and 10% (v/v) acetic acid containing 0.14% Coomassie Brilliant Blue R-250 and were diffusion-destained in 10% (v/v) acetic acid.

Protein Concentrations. Protein concentrations were determined by the Coomassie Blue dye binding assay (Spector, 1978) using dye reagent purchased from Pierce Chemical Co. (Rockford, IL) and γ-globulin as the standard. The concentrations of the following protein solutions were determined using the indicated values for the absorbance of a 1% solution with a path length of 1 cm: smooth muscle myosin, 4.5 at 280 nm (Okamoto & Sekine, 1978); skeletal muscle myosin, 5.3 at 280 nm (Margossian & Lowey, 1982); actin, 6.3 at 290 nm (Lehrer & Kerwar, 1972); calmodulin, 1.95 at 277 nm (Klee, 1977); tropomyosin, 2.9 at 278 nm (Eisenberg & Kielley, 1974). Calponin and phosphorylated calponin concentrations were determined by quantitative amino acid analysis using norleucine as an internal standard.

Phosphorylation of Calponin. Calponin (0.2 mg/mL) was incubated at 30 °C for 60 min with PKC (0.5 μg/mL) in 25 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 48.4 μM L-α-phosphatidyl-L-serine, 12.3 μM diolein, and 1 mM [γ-^{32}P]ATP ($\sim$100 cpm/pmol). Reaction volumes ranged from 8 to 25 mL. Reactions were stopped by the addition of excess EDTA (1.2 mM excess). Samples (50 μL) were withdrawn for the quantification of protein-bound phosphate (Walsh et al., 1983). The stoichiometry of calponin phosphorylation was 1.3 ± 0.3 mol of P_i/mol of calponin (mean ± SD, n = 11). The remainder of the reaction mixture was applied to a column (2 × 10 cm) of CM-Sephadex previously equilibrated with 20 mM Tris-HCl (pH 7.5), 50 mM KCl, and 1 mM dithiothreitol. Phosphorylated calponin bound to the column and was eluted with 20 mM Tris-HCl (pH 7.5), 1 M KCl, and 1 mM dithiothreitol. Fractions containing phosphorylated calponin (identified by SDS–PAGE and autoradiography) were pooled and dialyzed *vs* 2 × 10 L of 20 mM Tris-HCl (pH 7.5), 0.1 M KCl, and 1 mM dithiothreitol.

Fluorescent Labeling of CaM. Wheat CaM was labeled with MIANS as described by Kasturi et al. (1993). Incorporation was determined to be 1.0 mol of MIANS/mol of CaM, assuming E_{320} = 20 000 M^{-1} cm^{-1} for MIANS (Prendergast et al., 1983).

Fluorescence Measurements. MIANS·CaM was excited at 320 nm, and fluorescence emission was monitored at 440 nm in a Perkin-Elmer LS5 spectrofluorometer at 22 °C. pCa curves were calibrated by determining that Fura 2 fluorescence (excitation at 340 nm, emission at 510 nm) increased half-maximally at pCa = 6.9. pCa was varied as described in Kasturi et al. (1993).

Assay of Cyclic Nucleotide Phosphodiesterase. PDE was assayed by monitoring the fluorescence decrease that occurs upon hydrolysis of 2′-(*N*-methylanthraniloyl)-cGMP, as described by Johnson et al. (1987). CaM stimulation of the PDE was 25-fold over the basal value (the activity measured in the absence of CaM).

Rapid-Mixing/Stopped-Flow Experiments. Stopped-flow experiments were carried out in an SF.17MV fluorescence stopped-flow instrument (Applied Photophysics Ltd., Leatherhead, U.K.) with a dead time of 1.6 ms at 22 °C. Excitation was at 320 nm, and MIANS·CaM fluorescence emission was monitored through a 440-nm narrow bandpass (10 nm) interference filter. The curve-fitting program (software by P. J. King, Applied Photophysics) uses the nonlinear Levenberg–Marquardt algorithm.

Effect of CaM on Calponin Binding to Actin. Smooth or skeletal muscle actin (9 μM) was incubated with calponin (2 μM) and various concentrations of CaM in 20 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM dithiothreitol, and 1 mM ATP (and 0.1 M KCl in the case of smooth muscle actin) at 20 °C in a reaction volume of 0.2 mL for 20 min, prior to centrifugation at 109000g for 45 min. An equal volume (0.2 mL) of SDS gel sample buffer was added to each supernatant and boiled. The pellets were resuspended in SDS gel sample buffer diluted 1:1 with H_2O (0.4 mL) and boiled. Samples (150 μL) of supernatants and pellets were subjected to SDS–PAGE. The proportion of calponin bound to F-actin was determined by laser densitometry of the Coomassie Blue stained gels with an LKB Model 2202 Ultroscan laser densitometer equipped with a Hewlett-Packard Model 3390A integrator.

Effect of CaM on Actin-Activated Myosin MgATPase Activities. The effect of CaM on smooth muscle actin-activated myosin MgATPase activity was investigated as follows. Smooth muscle myosin (1 μM) was incubated at 30 °C with smooth muscle actin (6 μM), tropomyosin (2 μM), CaM (0.6 or 30 μM), and MLCK (74 nM) in the absence or presence of calponin (2 μM) in 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 60 mM KCl, and 1 mM [γ-^{32}P]-ATP (19.7 cpm/pmol) in a reaction volume of 1.1 mL. Reactions were started by the addition of ATP. Samples (0.2 mL) were withdrawn at 1, 2, 3, 4, and 5 min for measurement of ATP hydrolysis (Ikebe & Hartshorne, 1985) and myosin phosphorylation (Walsh et al., 1983). The effect of CaM on skeletal muscle actin-activated myosin MgATPase activity was investigated as follows. Skeletal muscle myosin (0.57 μM) was incubated at 30 °C with skeletal muscle actin (6 μM) and CaM (0–50 μM) in the absence or presence of calponin (2 μM) in 25 mM Tris-HCl (pH 7.5), 50 mM KCl, 3.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM dithiothreitol, and 1 mM [γ-^{32}P]ATP (17.0 cpm/pmol) in a reaction volume of 0.3 mL. Reactions were started by the addition of ATP. Samples (50 μL) were withdrawn at 0.5, 1, 1.5, 2, and 2.5 min for quantification of ATP hydrolysis (Ikebe & Hartshorne, 1985). Calponin binding to actin under ATPase conditions was determined by centrifuging the ATPase reaction mixtures at 109 000g for 45 min and quantification of calponin in the supernatant and pellet following SDS–PAGE, as described above for actin binding. In the absence of actin, calponin was recovered exclusively in the supernatant.

RESULTS

To determine the affinity of calponin and PKC-phosphorylated calponin (P-calponin) for CaM, we conducted titration of MIANS-labeled wheat CaM (MIANS·CaM). MIANS·CaM retains its Ca^{2+}-dependent biological activity and undergoes a fluorescence increase which is specific for CaM target protein binding (Mills et al., 1988; Kasturi et al., 1993).

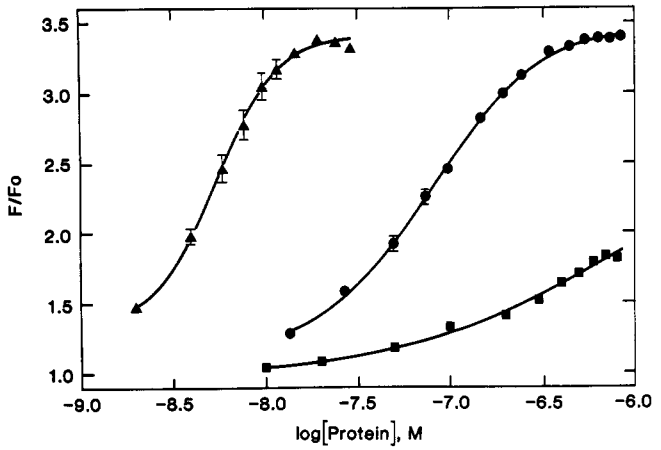

FIGURE 1: Enhancement of MIANS·CaM fluorescence by binding of MLCK, calponin, and phosphorylated calponin. Increasing concentrations of MLCK (▲), calponin (●), or P-calponin (■) were added to 10 nM MIANS·CaM in 10 mM MOPS (pH 7.0), 90 mM KCl, 0.1 mM $CaCl_2$, and 1 mM dithiothreitol (total volume = 1 mL). F represents the fluorescence intensity of MIANS·CaM in the presence of target protein; F_o represents the fluorescence intensity of MIANS·CaM in the absence of added protein. Each point represents the mean ± SEM of three independent determinations. Error bars are included only when they are larger than the symbols.

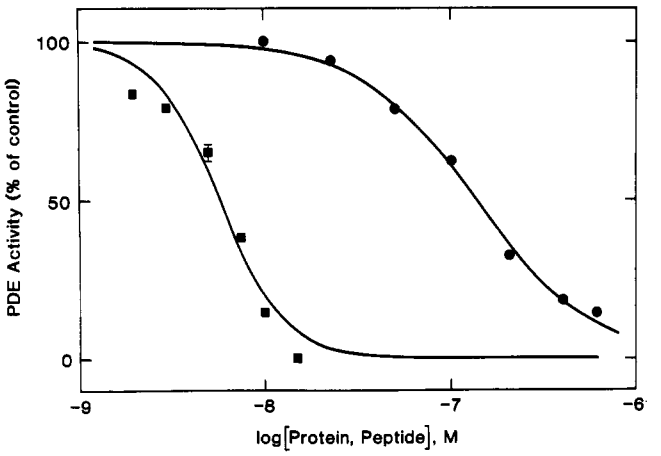

FIGURE 2: Inhibition of calmodulin-activated cyclic nucleotide phosphodiesterase by RS-20 and calponin. The indicated concentrations of RS-20 peptide (■) or calponin (●) were added to a cuvette containing 10 nM brain CaM in 10 mM MOPS (pH 7.0), 0.73 mM $CaCl_2$, 5 mM $MgCl_2$, and 8 μM 2′-(*N*-methylanthraniloyl)-cGMP, and the reaction was started by the addition of PDE (2.25 μg/mL). CaM produced a 25-fold enhancement of PDE activity; 100% activity represents a hydrolysis rate of 1.6 nmol of cGMP hydrolyzed/min.

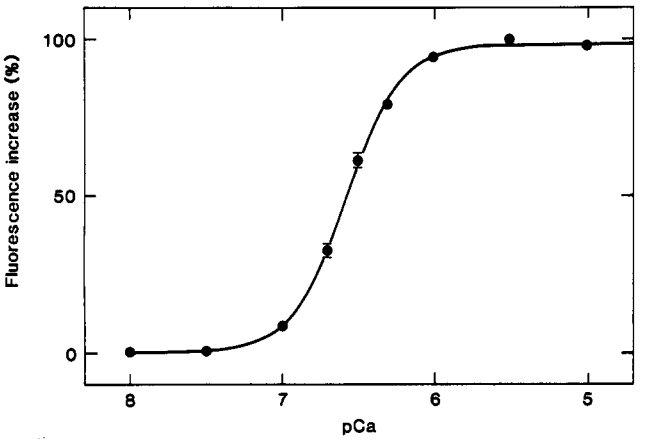

FIGURE 3: Ca^{2+} dependence of calponin binding to MIANS·CaM. MIANS·CaM (50 nM) and calponin (200 nM) were mixed in 0.2 M MOPS (pH 7.0), 90 mM KCl, and 2 mM EGTA. $CaCl_2$ was added to achieve the desired Ca^{2+} concentration as described by Kasturi et al. (1993). A 100% fluorescence increase corresponds to a smaller (2.25-fold) fluorescence increase than that shown in Figure 1 because 200 nM calponin does not fully saturate 50 nM CaM. Each point represents the mean ± SEM of three independent measurements. Error bars are included only when they are larger than the symbols.

Figure 1 compares the effects of MLCK, calponin, and P-calponin on the fluorescence emission intensity of 10 nM MIANS·CaM in the presence of Ca^{2+}. MLCK induced a large increase in MIANS·CaM fluorescence (F/F_o = 3.4) which was half-maximal at 5.5 nM MLCK, consistent with its high affinity for CaM (K_d = 1 nM; Stull et al., 1986). Calponin induced a similar 3.4-fold increase in fluorescence intensity which was half-maximal at ~80 nM. P-calponin induced a much smaller increase in MIANS·CaM fluorescence: at 0.7 μM (the highest concentration used), P-calponin induced less than one-half the fluorescence increase observed upon binding of MLCK or calponin to MIANS·CaM. If we assume that saturating amounts of P-calponin produce the same enhancement in MIANS·CaM fluorescence as native calponin, the half-maximal fluorescence increase occurs at ~1.2 μM P-calponin. Thus, calponin binds to CaM with a K_d of ~80 nM, compared to a K_d of 1 nM for MLCK. Phosphorylation of calponin produces a 15-fold reduction in the affinity of calponin for CaM (K_d ~1.2 μM).

The affinity of calponin for Ca^{2+}-CaM was also investigated by examining its efficacy as an inhibitor of Ca^{2+}- and CaM-activated cyclic nucleotide phosphodiesterase (PDE). Half-maximal inhibition of PDE activated by 10 nM CaM occurred at 138 nM calponin (Figure 2). By contrast, the CaM antagonist peptide RS-20, which has a high affinity for Ca^{2+}-CaM (K_d = 1 nM; Lukas et al., 1986), caused half-maximal inhibition of PDE at 6 nM, and nearly total inhibition was observed at 1 mol of RS-20/mol of CaM. These results, therefore, support the conclusion that calponin has a relatively low affinity for Ca^{2+}-CaM. Calponin had no effect on basal PDE activity (activity in the absence of CaM), and calponin-mediated inhibition of CaM-activated PDE was overcome by the addition of excess CaM (data not shown).

The Ca^{2+} dependence of the calponin–CaM interaction is shown in Figure 3. Half-maximal binding of calponin to MIANS·CaM occurred at pCa 6.6 with a Hill coefficient of 2.4.

Since Ca^{2+} binding to MIANS·CaM produces only a small decrease in its fluorescence intensity in the absence of a CaM-binding protein (Mills et al., 1988; Kasturi et al., 1993), it was possible to determine the rate of EGTA-induced disruption of the MIANS·CaM–target protein complexes. Figure 4 shows that EGTA disrupts the MIANS·CaM–calponin complex at a rate of 1 ± 0.2 s^{-1}. For comparison, EGTA disrupts the MIANS·CaM–MLCK complex at a rate of 4 s^{-1} (Figure 4). The CaM–calponin complex, therefore, is dissociated much more slowly upon Ca^{2+} chelation than is the higher affinity CaM–MLCK complex.

CaM-binding peptides such as melittin and RS-20 do not affect MIANS·CaM fluorescence in the presence of Ca^{2+} (Kasturi et al., 1993). It was, therefore, feasible to use stopped-flow fluorescence to determine the rate of melittin- and CaM-induced dissociation of the MIANS·CaM–calponin complex. Figure 5 shows that both melittin (1 μM) and excess unlabeled brain CaM (8 μM) disrupt the MIANS·CaM–calponin complex at the same rate (0.31 ± 0.10 s^{-1}).

We attempted to determine the second-order rate constant for the association of calponin with increasing concentrations of MIANS·CaM by approximating pseudo-first-order reaction conditions. Figure 6 shows that MIANS·CaM (50 nM) associates with 150 nM calponin at 3.3 s^{-1}, 350 nM calponin

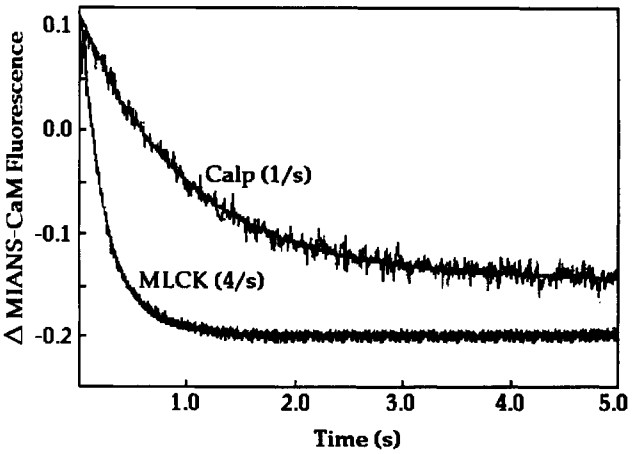

FIGURE 4: Kinetic analysis of EGTA-induced disruption of MIANS·CaM–calponin and MIANS·CaM–MLCK complexes. MIANS·CaM (100 nM) and calponin (400 nM) or MIANS·CaM (250 nM) and MLCK (250 nM) in 10 mM MOPS (pH 7.0), 90 mM KCl, 1 mM dithiothreitol, and 0.1 mM $CaCl_2$ were rapidly mixed with 5 mM EGTA in the same buffer without Ca^{2+}. Dissociation of the complexes was monitored by the decrease in fluorescence intensity measured in a stopped-flow apparatus, as described in Materials and Methods. Each curve (a signal average of 5–7 traces) was fit to a single exponential with a normalized variance of $<1 \times 10^{-4}$. The rate constants were determined to be 1 s^{-1} for the MIANS·CaM–calponin complex and 4 s^{-1} for the MIANS·CaM–MLCK complex. In four additional experiments, the rate of EGTA-induced complex disruption of the MIANS·CaM–calponin complex was 1.0 ● 0.2 s^{-1}. Control experiments in which MIANS·CaM, target protein, and Ca^{2+} were rapidly mixed with buffer containing Ca^{2+} indicated that >90% of the EGTA-induced fluorescence decrease was observed kinetically.

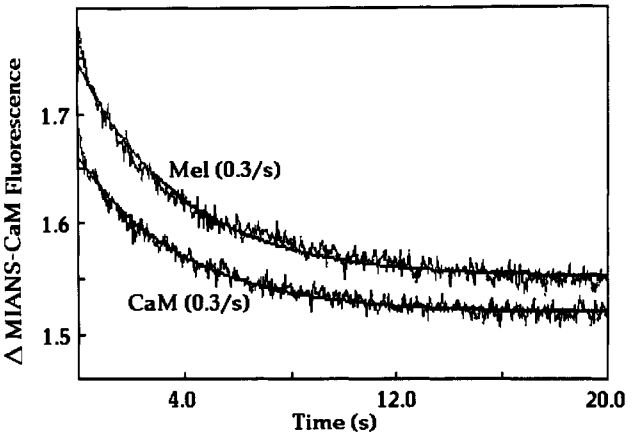

FIGURE 5: Kinetic analysis of melittin- and CaM-induced disruption of the MIANS·CaM–calponin complex. MIANS·CaM (100 nM) and calponin (400 nM) in 10 mM MOPS (pH 7.0), 90 mM KCl, 1 mM dithiothreitol, and 0.5 mM $CaCl_2$ were rapidly mixed with an equal volume of melittin (1 μM) or unlabeled brain CaM (8 μM) in the same buffer at 22 °C. Dissociation of the MIANS·CaM–calponin complex was monitored as described in the Materials and Methods section. Each curve (a signal average of six traces) was fit to a single exponential with a normalized variance of $<6 \times 10^{-5}$. The rate constant for dissociation of the MIANS·CaM–calponin complex by either melittin or unlabeled brain CaM was 0.31 s^{-1}. In four additional experiments, the rate constant for the dissociation of this complex was 0.31 ± 0.10 s^{-1}.

at 3.9 s^{-1}, and 650 nM calponin at 4.4 s^{-1}. Clearly, there is very little increase in association rate with increasing [calponin]. Similar experiments measuring the rates of MLCK and caldesmon binding to MIANS·CaM showed large linear increases in association rate with increasing concentrations of protein (Kasturi et al., 1993). This suggests that the association of CaM with calponin may be rate-limited by a slower isomerization process on calponin. At the highest [calponin] used in Figure 6, calponin is present at a 13-fold molar excess over CaM. Under these conditions, the reaction

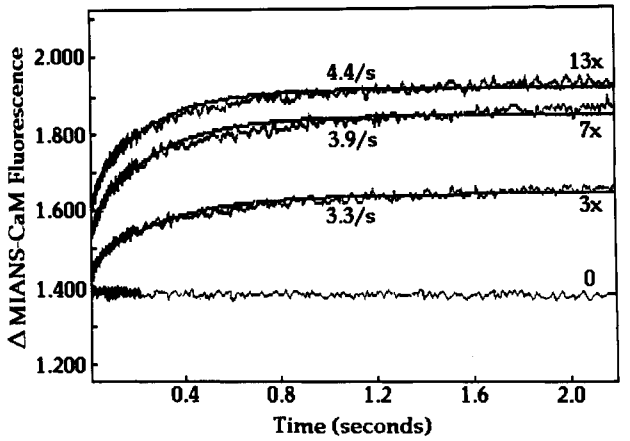

FIGURE 6: Kinetic traces of the rates of MIANS·CaM (50 nM) association with 150 (3×), 350 (7×), and 650 nM (13×) calponin. Calponin and MIANS·CaM in 10 mM MOPS (pH 7.0), 90 mM KCl, and 0.5 mM $CaCl_2$ were rapidly mixed at the indicated molar ratio using the stopped-flow apparatus described in the Materials and Methods section. MIANS·CaM fluorescence was excited at 320 nm and observed through a 440-nm narrow bandpass filter. The data (7–9 traces at each [calponin]) were well-fit as a single exponential process (variance $<1 \times 10^{-4}$). The rate constants for association of calponin with MIANS·CaM were determined to be 3.3 (3×), 3.9 (7×), and 4.4 s^{-1} (13×). The 7× and 13× data were displaced vertically along the ordinate to avoid superposition of the traces.

should follow first-order reaction kinetics, and the on-rate can be accurately calculated as k_{obsd}/[calponin]. An average k_{obsd} of 3.9 ± 1.2 was determined from three additional experiments of 13-fold excess calponin associating with MIANS·CaM. These data suggest an on-rate of ~(6.0 ± 1.8) × 10^6 M^{-1} s^{-1} for the association of CaM with calponin.

Since the K_d is equal to the off-rate/on-rate, this allows calculation of K_d from these kinetic parameters. Assuming an off-rate of 0.31 ± 0.10 (from Figure 5) and an on-rate of 6 × 10^6 M^{-1} s^{-1}, we calculate a K_d of ~52 nM, in good agreement with the K_d of 80 nM determined from direct binding.

The results described above indicate that calponin belongs to the class of low-affinity CaM-binding proteins, which includes caldesmon, rather than the class of high-affinity CaM-binding proteins such as MLCK and PDE. Calponin also binds to actin and tropomyosin, and its ability to inhibit the actin-activated myosin MgATPase is mediated by its interaction with actin (Winder & Walsh, 1990). We therefore investigated the effects of Ca^{2+}-CaM on (i) the binding of calponin to smooth and skeletal muscle actins and (ii) the calponin-mediated inhibition of smooth and skeletal muscle actin-activated myosin MgATPases.

Figure 7A shows the concentration-dependent dissociation of calponin from smooth muscle F-actin by Ca^{2+}-CaM. Results are shown for two different preparations of calponin. At 5.4 μM CaM, 50% dissociation was observed at actin and calponin concentrations of 9 and 2 μM, respectively. In the absence of Ca^{2+}, no dissociation of calponin from actin was observed at any concentration of CaM (data not shown). Ca^{2+}-CaM also induced dissociation of calponin from skeletal muscle actin (Figure 7B). In this case, half-maximal dissociation occurred at ~11 μM CaM. This is probably due to the lower ionic strength used in the experiments of Figure 7B compared with Figure 7A, since we have observed that the affinity of actin for calponin is increased at low ionic strength. Figure 7B also confirms that ~95% of actin is in the filamentous form under these conditions and that the equilibrium between G- and F-actin is unaffected by Ca^{2+}-CaM.

Table I shows that calponin inhibits the actin-activated MgATPase activity of smooth muscle myosin without affecting

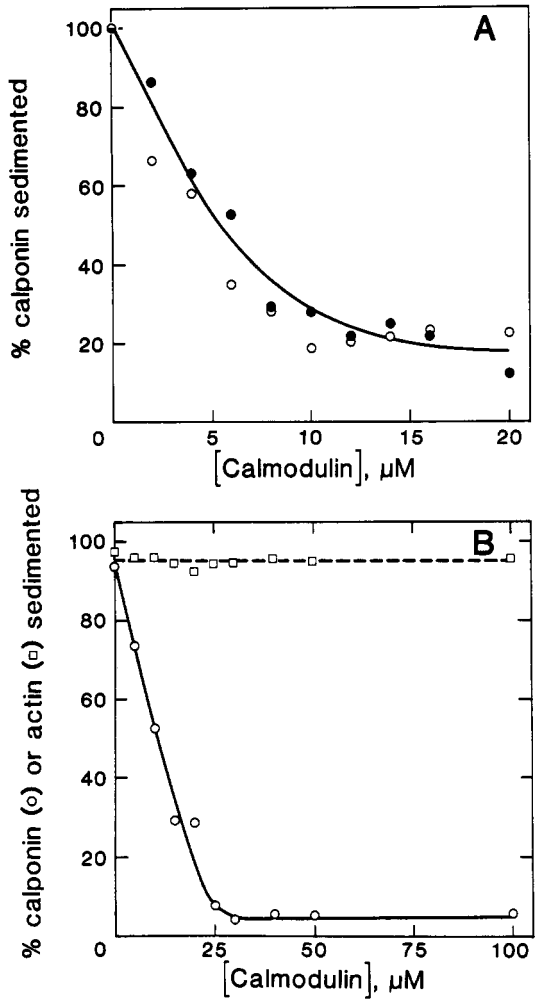

FIGURE 7: Effect of Ca^{2+}-CaM on calponin binding to F-actin. The binding of calponin (2 μM) to actin (9 μM) at the indicated CaM concentrations was quantified by the sedimentation method described in Materials and Methods. (A) Effect of CaM on calponin binding to smooth muscle actin. Symbols indicate data from two different calponin preparations. (B) Effect of CaM on calponin binding to skeletal muscle actin. Calponin (○) and actin (□) were quantified by scanning laser densitometry.

Table I: Effect of Ca^{2+}-CaM on Calponin-Mediated Inhibition of Smooth Muscle Actin-Activated Myosin MgATPase

calponin (μM)	calmodulin (μM)	ATPase rate[a] (nmol of P_i (mg of myosin)$^{-1}$ (min)$^{-1}$)	myosin phosphorylation[b] (mol of P_i (mol of myosin)$^{-1}$)	calponin bound to actin[a] (%)
0	0.6	126.5 ± 11.6	1.92 ± 0.15	
2	0.6	46.8 ± 8.0	1.96 ± 0.17	59.2 ± 5.3
2	30	57.2 ± 6.4	2.01 ± 0.16	55.1 ± 9.6

[a] Values are given as mean ± SD (n = 3). [b] Values are given as mean ± SD (n = 15).

myosin phosphorylation, as described previously (Winder & Walsh, 1990). An increase in the CaM concentration by 50-fold (to 30 μM) did not significantly reverse calponin inhibition of the ATPase, suggesting that Ca^{2+}-CaM, even at this very high concentration, does not induce dissociation of calponin from the actin filament. This was confirmed by direct examination of the effect of Ca^{2+}-CaM on the binding of calponin to actin under ATPase reaction conditions (Table I). Similar results were obtained with the skeletal muscle system (Figure 8). In the absence of CaM, 2 μM calponin inhibited the skeletal muscle actin-activated myosin MgATPase by 40%. An increase in the CaM concentration to 50 μM did not affect this inhibition. This concentration of Ca^{2+}-CaM also did not

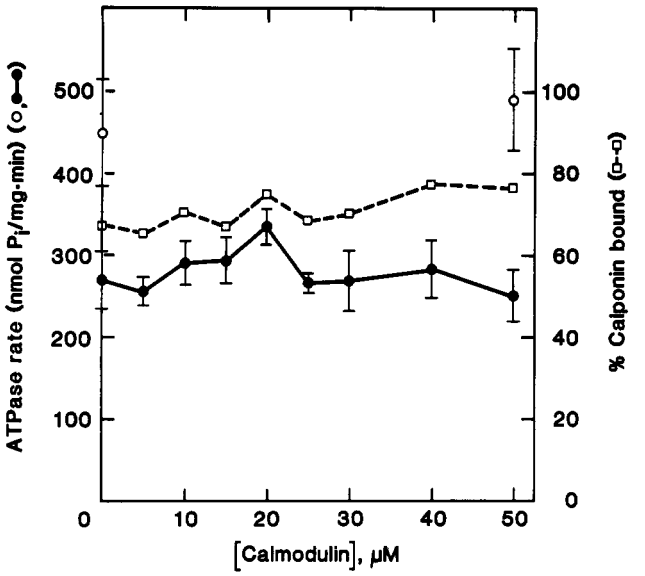

FIGURE 8: Effect of Ca^{2+}-CaM on calponin-mediated inhibition of skeletal muscle actin-activated myosin MgATPase. Skeletal muscle actin-activated myosin MgATPase activity was measured as described in Materials and Methods in the absence (○) or presence (●) of 2 μM calponin at the indicated concentrations of CaM. Values represent the mean of four determinations, and bars indicate SD. Calponin binding to actin under ATPase conditions is also shown (□). In this case, values represent the mean of two determinations.

affect the control ATPase activity in the absence of calponin, as shown in Figure 8. Again, Ca^{2+}-CaM did not induce dissociation of calponin from actin under ATPase reaction conditions (Figure 8).

DISCUSSION

CaM is involved in the regulation of a wide range of cellular functions, in most cases via its Ca^{2+}-dependent interaction with specific target proteins (Means et al., 1991). CaM-binding proteins can be divided into two groups: (i) high-affinity CaM-binding proteins having K_d values near 1 nM, e.g., enzymes such as MLCK (Kemp & Stull, 1990), PDE (Wang et al., 1991), calcineurin (type 2B protein phosphatase) (Klee & Cohen, 1988), and CaM kinase II (Colbran, 1992), which are activated by Ca^{2+}-CaM, and (ii) low-affinity CaM-binding proteins having K_d values of 80 nM or higher, which are generally nonenzyme proteins such as the actin- and myosin-binding protein, caldesmon (Sobue & Sellers, 1991). Calponin clearly belongs to the second group of CaM-binding proteins (Wills et al., 1993a; this study).

Wills et al. (1993a) have used acrylodan-labeled calponin to characterize calponin's interactions with CaM and other Ca^{2+}-binding proteins. CaM titrations of acrylodan–calponin (at 500 nM calponin) indicated a biphasic process consistent with CaM binding to higher affinity ($K_d \leq 220$ nM) and lower affinity (K_d = 2.5–3.4 μM) sites on calponin. Our studies with MIANS·CaM are consistent with their results, and we estimate a K_d of 80 nM for the CaM–calponin complex. Since our titrations were conducted at much lower concentrations of CaM (10 nM), where the K_{app} would more closely approximate the K_d, the K_d they report as ≤220 nM can be precisely defined as K_d = 80 nM in our studies. This K_d is also consistent with the analytical centrifugation results of Wills et al. (1993b), which suggest a K_{d1} of 100 nM and a second, much lower affinity complex of calponin with CaM. Our calponin titrations of MIANS·CaM saturate as a monophasic process with no evidence for the much lower affinity CaM–calponin complex, since we did not go beyond micromolar [calponin].

Table II: Affinity and Kinetic Parameters for the Interactions of Calmodulin with MLCK, Caldesmon, and Calponin

protein	K_d (nM)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1}) competition	k_{off} (s^{-1}) EGTA
MLCK[a]	1.1	2.8×10^7	0.031	3.5
caldesmon[a]	108	5.3×10^8	57.0	13.5
calponin[b]	52	$(6.0 \pm 1.8) \times 10^6$	0.31 ± 0.10	1.0

[a] These data are from Kasturi et al. (1993). [b] K_d was calculated from kinetic data: $K_d = k_{off}/k_{on}$.

While our binding data are similar to the results of Wills et al. (1993a), we find that MIANS·CaM binds calponin half-maximally at pCa 6.6, while they report that acrylodan–calponin binds CaM half-maximally at pCa 5.9. This difference could be due to differences in the calibration of the Ca^{2+} and EGTA stocks used in the Ca^{2+} titrations. Our pCa curves were calibrated by following the Ca^{2+}- induced increase in the fluorescence of Fura 2 acid. This fluorescence indicator binds Ca^{2+} with a K_d = 135 nM (Grynkiewicz et al., 1985), and we used its fluorescence to assure accurate calibration of our fluorescence *vs* pCa titrations (see Materials and Methods section). Without an independent method for pCa calibration, slight differences in either the Ca^{2+} or EGTA stock can result in a large difference in the Ca^{2+} dependence of any process. Our data indicate that calponin, like many other CaM target proteins [see Olwin et al. (1984) and Kasturi et al. (1993)], produces dramatic increases in Ca^{2+} binding to CaM and interacts with CaM half-maximally near pCa 6.5. In addition, EGTA disrupts the Ca^{2+}-CaM–calponin complex more slowly (1 s^{-1}) than it disrupts the Ca^{2+}-CaM–MLCK complex (4 s^{-1}), consistent with calponin producing large increases in CaM's affinity for Ca^{2+}.

Table II compares the affinity and kinetic parameters for CaM's interaction with three of its smooth muscle target proteins: MLCK, caldesmon, and calponin. Calponin dissociates from CaM ~10 times more rapidly than the higher affinity MLCK, consistent with its lower affinity. However, calponin dissociates from CaM nearly 200 times slower than caldesmon (which has only slightly lower affinity). Calponin maintains its relatively low affinity for CaM in spite of its slow dissociation rate because calponin has a slow association rate with CaM. CaM binds caldesmon ~130 times more quickly and MLCK ~7 times more quickly than it binds calponin. This suggests that, during a [Ca^{2+}] transient in a smooth muscle cell, CaM could first associate with caldesmon (CaD) and then rapidly (57 s^{-1}) dissociate from CaD and bind MLCK. Because of the reduced on-rate of CaM for calponin and calponin's 80-fold lower affinity for CaM compared to MLCK, it is unlikely that calponin would interact with CaM during a [Ca^{2+}] transient. These kinetic studies suggest that CaM's interaction with calponin is much slower, and probably is not physiologically relevant, compared to the rates of CaM's association with CaD and MLCK.

The ability of calponin to inhibit the actin-activated myosin MgATPase is mediated by its interaction with actin (Winder & Walsh, 1990). Ca^{2+}-CaM is clearly capable of competing with smooth and skeletal muscle actins for binding calponin *in vitro*, suggesting that this may represent a mechanism for regulating the inhibitory effect of calponin on the actomyosin ATPase. However, this does not appear to be a physiologically relevant mechanism for the following reasons: (i) The concentration of Ca^{2+}-CaM required for half-maximal inhibition of calponin binding to actin is 5–11 μM at 2 μM calponin and 9 μM actin. The concentrations of CaM, calponin, and actin in nonarterial smooth muscles have been estimated to be 20–50, 150, and 870 μM, respectively (Grand et al., 1979; Takahashi et al., 1986; Hartshorne, 1987). Given that the affinity of calponin for smooth muscle actin is similar (K_d = 46 nM) (Winder et al., 1991) to that for CaM, at physiological molar ratios of the three proteins, therefore, calponin would be expected to bind exclusively to actin. (ii) In a reconstituted contractile system, prepared from purified smooth or skeletal muscle contractile and regulatory proteins, even very high molar ratios of Ca^{2+}-CaM to calponin and actin did not reverse calponin-mediated inhibition of the actin-activated myosin MgATPase. Consistent with these observations, Ca^{2+}-CaM failed to dissociate calponin from actin under ATPase reaction conditions. These results also suggest that the affinity of actin for calponin is significantly higher in the reconstituted system than with purified actin or, alternatively, the affinity of Ca^{2+}-CaM for calponin is significantly lower under the conditions used in the experiments involving calponin binding to the reconstituted contractile system than to actin alone.

Several other investigators have examined the effect of Ca^{2+}-CaM on the actomyosin ATPase using different *in vitro* contractile protein systems. Using smooth muscle actin (12 μM) and skeletal muscle myosin (0.25 μM), Marston (1991) observed little reversal of calponin-mediated inhibition (2.4 μM) of the actomyosin ATPase at Ca^{2+}-CaM concentrations up to 30 μM, i.e., similar to our observations. Makuch et al. (1991) observed complete reversal of skeletal muscle actomyosin ATPase at a molar ratio of Ca^{2+}-CaM:calponin of 10:1; this effect correlated with Ca^{2+}-CaM-induced dissociation of calponin from actin. However, the ATPase and binding experiments were carried out under different conditions, and as we have shown herein, binding is strongly dependent on the conditions. Abe et al. (1990) observed complete reversal of inhibition of smooth muscle actothiophosphorylated myosin MgATPase at 3 mol of Ca^{2+}-CaM/mol of calponin (20 μM actin, 3.4 μM calponin). However, the level of inhibition of the ATPase by calponin in these experiments was low (~30%). Calponin inhibited actin filament movement over immobilized smooth muscle phosphorylated myosin or skeletal muscle heavy meromyosin (Shirinsky et al., 1992); this effect was reversed by a high concentration (10 μM) of Ca^{2+}-CaM. Although the concentration of calponin used was not specified, it was presumably $\leq$2 μM. While there are quantitative differences in the effects of Ca^{2+}-CaM in these various studies, which may be due to variations in reaction conditions including the use of proteins from different sources, it is clear that reversal of the inhibitory effects of calponin on the actomyosin ATPase and motility, if it occurs at all, requires unphysiologically high concentrations of Ca^{2+}-CaM. All of these results, therefore, support the conclusion that calponin function is not regulated by the Ca^{2+}-dependent binding of CaM to calponin.

Of the other mechanisms that have been considered for the regulation of calponin function, phosphorylation/dephosphorylation and binding of another Ca^{2+}-binding protein such as SMCaBP-11 (Mani & Kay, 1990) appear to be the most attractive candidates. Phosphorylation by CaM kinase II or PKC abolishes the calponin–actin interaction and inhibition of the actomyosin ATPase (Winder & Walsh, 1990), and dephosphorylation by a type 2A protein serine/threonine phosphatase reverses these effects (Winder et al., 1992). As we have shown in this study, phosphorylation also significantly decreases the affinity of calponin for Ca^{2+}-CaM. Previously, phosphorylated calponin was shown to bind to a CaM–Sepharose affinity column in a Ca^{2+}-dependent manner (Winder & Walsh, 1990). Affinity chromatography, however, may not be sensitive to a 10–15-fold increase in K_d.

Wills et al. (1993a) demonstrated that the Ca^{2+}-binding proteins S-100b, isolated from bovine brain, and SMCaBP-11, isolated from smooth muscle, have significantly higher affinities for calponin than does CaM. It will be important to determine the effects of these proteins, particularly SMCaBP-11 (S-100 has not been identified in smooth muscle), on calponin-mediated inhibition of the actin-activated MgATPase activity of smooth muscle phosphorylated myosin.

The binding of Ca^{2+} or GTP to calponin is an unlikely physiological control mechanism. The affinity of calponin for Ca^{2+} is low (K_d = 7 μM; Takahashi et al., 1987). The more recent work of Wills et al. (1993a) has provided substantial evidence that calponin does not, in fact, bind Ca^{2+}. Furthermore, we have been unable to detect the binding of [^{35}S]-GTPγS to calponin under conditions in which binding to bovine brain G_i (the inhibitory GTP-binding protein coupled to adenylyl cyclase) was clearly detectable. We have also observed no effect of GTP on the interaction between calponin and actin as assessed by the sedimentation assay (S. J. Winder and M. P. Walsh, unpublished observations).

Therefore, we conclude the following: (i) Calponin is a low-affinity CaM-binding protein (K_d = 80 nM). (ii) The interaction of calponin with CaM is Ca^{2+}-dependent and occurs within the normal physiological range of intracellular free [Ca^{2+}]. In the presence of Ca^{2+}, CaM binds calponin very slowly ($t_{1/2}$ $\approx$0.2 s), perhaps due to a slow isomerization reaction that must occur in calponin to facilitate CaM binding. (iii) The affinity of calponin for Ca^{2+}-CaM is significantly reduced by phosphorylation. (iv) Actin competes with Ca^{2+}-CaM for calponin binding, such that at physiological molar ratios of the three proteins calponin should be bound exclusively to actin. However, as we have demonstrated earlier, calponin dissociates from actin upon phosphorylation (Winder & Walsh, 1990). (v) On the bases of the on- and off-rates for the association–dissociation of Ca^{2+}-CaM with the three major CaM-binding proteins of smooth muscle (MLCK, caldesmon, and calponin), it is unlikely that calponin would interact with CaM during a [Ca^{2+}] transient.

ACKNOWLEDGMENT

The authors are very grateful to Cindy Sutherland and Elaine Fraser for expert technical assistance and to Gerry Garnett for word processing.

REFERENCES

Abe, M., Takahashi, K., & Hiwada, K. (1990) *J. Biochem. (Tokyo) 108*, 835–838.

Adelstein, R. S., & Klee, C. B. (1981) *J. Biol. Chem. 256*, 7501–7509.

Colbran, R. J. (1992) *Neurochem. Int. 21*, 469–497.

Eisenberg, E., & Kielley, W. W. (1974) *J. Biol. Chem. 249*, 4742–4748.

Grand, R. J. A., Perry, S. V., & Weeks, R. A. (1979) *Biochem. J. 177*, 521–529.

Grynkiewicz, G., Poenie, M., & Tsien, R. Y. (1985) *J. Biol. Chem. 260*, 3440–3450.

Hartshorne, D. J. (1987) in *Physiology of the Gastrointestinal Tract*, 2nd ed. (Johnson, L. R., Ed.) pp 423–482, Raven Press, New York.

Ikebe, M., & Hartshorne, D. J. (1985) *Biochemistry 24*, 2380–2387.

Johnson, J. D., Walters, J. D., & Mills, J. S. (1987) *Anal. Biochem. 162*, 291–295.

Kasturi, R., Vasulka, C., & Johnson, J. D. (1993) *J. Biol. Chem. 268*, 7958–7964.

Kemp, B. E., & Stull, J. T. (1990) in *Peptides and Protein Phosphorylation* (Kemp, B. E., Ed.) pp 115–133, CRC Press, Boca Raton, FL.

Klee, C. B. (1977) *Biochemistry 16*, 1017–1024.

Klee, C. B., & Cohen, P. (1988) *Molecular Aspects of Cellular Regulation 5*, 225–248.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Lehrer, S. S., & Kerwar, C. (1972) *Biochemistry 11*, 1211–1217.

Lukas, T. J., Burgess, W. H., Prendergast, F. G., Lau, W., & Watterson, D. M. (1986) *Biochemistry 25*, 1458–1464.

Makuch, R., Birukov, K., Shirinsky, V., & Dabrowska, R. (1991) *Biochem. J. 280*, 33–38.

Mani, R. S., & Kay, C. M. (1990) *Biochemistry 29*, 1398–1404.

Margossian, S. S., & Lowey, S. (1982) *Methods Enzymol. 85*, 55–71.

Marston, S. B. (1991) *FEBS Lett. 292*, 179–182.

Means, A. R., VanBerkum, M. F. A., Bagchi, I., Lu, K. P., & Rasmussen, C. D. (1991) *Pharmacol. Ther. 50*, 255–270.

Mills, J. S., Walsh, M. P., Nemcek, K., & Johnson, J. D. (1988) *Biochemistry 27*, 991–996.

Ngai, P. K., Gröschel-Stewart, U., & Walsh, M. P. (1986) *Biochem. Int. 12*, 89–93.

Okamoto, Y., & Sekine, T. (1978) *J. Biochem. (Tokyo) 83*, 1375–1379.

Olson, N. J., Pearson, R. B., Needleman, D. S., Hurwitz, M. Y., Kemp, B. E., & Means, A. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2284–2288.

Olwin, B. B., Edelman, A. M., Krebs, E. G., & Storm, D. R. (1984) *J. Biol. Chem. 259*, 10949–10955.

Persechini, A., & Hartshorne, D. J. (1981) *Science 213*, 1383–1385.

Persechini, A., & Rowe, A. J. (1984) *J. Mol. Biol. 172*, 23–39.

Prendergast, F. G., Meyer, M., Carlson, G. L., Iida, S., & Potter, J. D. (1983) *J. Biol. Chem. 258*, 7541–7544.

Sharma, R. K., Taylor, W. A., & Wang, J. H. (1983) *Methods Enzymol. 102*, 210–219.

Shirinsky, V., Biryukov, K. G., Hettasch, J. M., & Sellers, J. R. (1992) *J. Biol. Chem. 267*, 15886–15892.

Smillie, L. B. (1982) *Methods Enzymol. 85*, 234–241.

Sobue, K., & Sellers, J. R. (1991) *J. Biol. Chem. 266*, 12115–12118.

Spector, T. (1978) *Anal. Biochem. 86*, 142–146.

Strasburg, G. M., Hogan, M., Birmachu, W., Thomas, D. D., & Louis, C. F. (1988) *J. Biol. Chem. 263*, 542–548.

Stull, J. T., Nunnally, M. H., & Michnoff, C. (1986) *The Enzymes 17*, 113–166.

Takahashi, K., & Nadal-Ginard, B. (1991) *J. Biol. Chem. 266*, 13284–13288.

Takahashi, K., Hiwada, K., & Kokubu, T. (1986) *Biochem. Biophys. Res. Commun. 141*, 20–26.

Takahashi, K., Hiwada, K., & Kokubu, T. (1987) *Hypertension 10*, 360a.

Takahashi, K., Abe, M., Hiwada, K., & Kokubu, T. (1988) *J. Hypertens. 6* (Suppl. 4), S40–S43.

Walsh, M. P., Hinkins, S., Dabrowska, R., & Hartshorne, D. J. (1983) *Methods Enzymol. 99*, 279–288.

Walsh, M. P., Valentine, K. A., Ngai, P. K, Carruthers, C. A., & Hollenberg, M. D. (1984) *Biochem. J. 224*, 117–127.

Wang, J. H., Sharma, R. K., & Mooibroek, M. J. (1991) in *Cyclic Nucleotide Phosphodiesterases: Structure, Regulation and Drug Action* (Beavo, J., & Houslay, M. D., Eds.) pp 3–18, John Wiley & Sons, Ltd., Chichester, U.K.

Wills, F. L., McCubbin, W. D., & Kay, C. M. (1993a) *Biochemistry 32*, 2321–2328.

Wills, F. L., McCubbin, W. D., & Kay, C. M. (1993b) *Biophys. J. 64*, A31.

Winder, S. J., & Walsh, M. P. (1990) *J. Biol. Chem 265*, 10148–10155.

Winder, S. J., Sutherland, C., & Walsh, M. P. (1991) *Adv. Exp. Med. Biol. 304*, 37–51.

Winder, S. J., Pato, M. D., & Walsh, M. P. (1992) *Biochem. J. 286*, 197–203.

Zot, H. G., & Potter, J. D. (1981) *Prep. Biochem. 11*, 381–395.