Chemoattractant-mediated Increases in cGMP Induce Changes in *Dictyostelium* Myosin II Heavy Chain–specific Protein Kinase C Activities

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Abstract. Myosin II heavy chain (MHC)–specific protein kinase C (MHC-PKC) isolated from the ameba, *Dictyostelium discoideum*, regulates myosin II assembly and localization in response to the chemoattractant cAMP (Abu-Elneel et al. 1996. *J. Biol. Chem.* 271:977–984). Recent studies have indicated that cAMP-induced cGMP accumulation plays a role in the regulation of myosin II phosphorylation and localization (Liu, G., and P. Newell. 1991. *J. Cell. Sci.* 98: 483–490). This report describes the roles of cAMP and cGMP in the regulation of MHC-PKC membrane association, phosphorylation, and activity (hereafter termed MHC-PKC activities). cAMP stimulation of *Dictyostelium* cells resulted in translocation of MHC-PKC from the cytosol to the membrane fraction, as well as increasing in MHC-PKC phosphorylation and in its kinase activity. We present evidence that MHC is phosphorylated by MHC-PKC in the cell cortex which leads to myosin II dissociation from the cytoskeleton. Use of *Dictyostelium* mutants that exhibit aberrant cAMP-induced increases in cGMP accumulation revealed that MHC-PKC activities are regulated by cGMP. *Dictyostelium* streamer F mutant (*stmF*), which produces a prolonged peak of cGMP accumulation upon cAMP stimulation, exhibits prolonged increases in MHC-PKC activities. In contrast, *Dictyostelium* KI-10 mutant that lacks the normal cAMP-induced cGMP response, or KI-4 mutant that shows nearly normal cAMP-induced cGMP response but has aberrant cGMP binding activity, show no changes in MHC-PKC activities. We provide evidence that cGMP may affect MHC-PKC activities via the activation of cGMP-dependent protein kinase which, in turn, phosphorylates MHC-PKC. The results presented here indicate that cAMP-induced cGMP accumulation regulates myosin II phosphorylation and localization via the regulation of MHC-PKC.

CAMP stimulation of the ameba *Dictyostelium* generates a number of responses such as increase in cGMP accumulation (26, 46), influx of Ca^{2+} (1, 6), production of inositol phosphates (13), changes in the amount of filamentous actin (15), changes in the phosphorylation rates of myosin II heavy chain (MHC)[1] and light chains (MLC) (4), and changes in cell movement and spreading (38, 43).

Studies on mutants lacking normal myosin II have indicated that it is not required for cell motility. It is, however, needed for efficient chemotaxis, and myosin II is thought to be involved in the regulation of cell polarity (45). Several lines of evidence have shown a correlation between myosin II reorganization, phosphorylation, and *Dictyostelium* chemotaxis (4, 24, 29, 47). In response to cAMP, the myosin II that exists as thick filaments translocates to the cortex (47). This translocation is correlated with a transient increase in the phosphorylation rates of both MHC and MLC (3, 4). It has therefore been suggested that cAMP-induced myosin II phosphorylation responses are part of the chemotactic sensing mechanism (3, 29).

In *Dictyostelium*, at least four different MHC kinase (MHCK) activities have been purified partially or almost to homogeneity (for review see 40). In vitro phosphorylation of MHC by some of these kinases inhibits myosin II thick filament formation (8, 18, 33, 34). We have isolated a novel protein kinase C (MHC-PKC) that phosphorylates *Dictyostelium* MHC specifically (34, 35). In vitro phosphorylation of MHC by this kinase inhibits myosin II thick filament formation by inducing the formation of a bent monomer of myosin II whose assembly domain is tied up in an intramolecular interaction that precludes the intermolecular interaction necessary for thick filament formation (33, 34). The MHC-PKC that is expressed during *Dictyostelium* development has been implicated in the observed increase in MHC phosphorylation in response to cAMP

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1. *Abbreviations used in this paper:* MHC, myosin II heavy chain; MHCK, myosin II heavy chain kinase; MHC-PKC, a protein kinase C that phosphorylates *Dictyostelium* MHC specifically; MLC, myosin II light chain; MLCK, myosin II light chain kinase.

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(34). Recently, we found that elimination of MHC-PKC results in the abolishment of this cAMP-induced MHC phosphorylation, indicating that MHC-PKC is the enzyme that phosphorylates MHC in response to cAMP (2). MHC-PKC null cells exhibit substantial myosin II overassembly in vivo, as well as aberrant cell polarization, chemotaxis, and morphological differentiation. Cells that overexpress MHC-PKC contain highly phosphorylated MHC, exhibit impaired myosin II localization, and have no apparent cell polarization and chemotaxis (2). These findings establish that, in *Dictyostelium*, the MHC-PKC plays a critical role in regulating the cAMP-induced myosin II localization required for cell polarization and, consequently, for efficient chemotaxis.

Recent studies have indicated that cAMP-induced accumulation of cGMP contributes to regulating myosin II phosphorylation and localization (21, 22, 23). The *Dictyostelium* streamer F mutant (*stmF*) is defective in the structural gene for cGMP-specific phosphodiesterase and shows prolonged cAMP-induced cGMP accumulation that correlates with a prolonged period of cell elongation during cell chemotaxis (9, 36, 42). A study of the cytoskeleton in this mutant revealed that association of myosin II with the Triton X-100–insoluble cytoskeleton and phosphorylation of MHC were also prolonged (22, 23). Liu et al. (21) further investigated the effect of cGMP on MHC phosphorylation and association with the Triton-insoluble cytoskeleton in a *Dictyostelium* KI-10 mutant that lacks the normal cAMP-induced cGMP accumulation, and in which chemotaxis towards cAMP does not occur (19). It was shown that in this mutant the cAMP-induced localization and phosphorylation of myosin II are absent (21).

In the present study we investigated the possibility that cGMP regulates myosin II via the regulation of MHC-PKC. For this purpose we characterized the behavior of MHC-PKC in the cGMP-aberrant mutant cell lines *stmF*, KI-10, and KI-4. A comparison of the kinetics of the cAMP-induced changes in MHC-PKC membrane association, phosphorylation, and in activity of control and mutant cells revealed that these changes are prolonged in the *stmF* mutant and fail to occur in KI-10 and KI-4 mutants. We show further that MHC-PKC is phosphorylated by a cGMP-dependent protein kinase, which may regulate its activities.

Materials and Methods

Cell Culture and Development

Growth and development in suspensions of *Dictyostelium discoideum* strain Ax2 cells were as described (4). *Dictyostelium discoideum* parental strain XP55, streamer mutants NP368 and NP377 derived from XP55 (36), and the nonchemotactic mutants KI-10 and KI-4 (19) were grown in shaken suspension in association with *Escherichia coli* B\r in 17 mM Na^+/K^+ phosphate buffer, pH 6.0. Ameba were harvested from the bacterial growth flasks at a density of less than 5×10^6 cells/ml, washed free of bacteria in MES buffer (20 mM MES [pH 6.8], 0.2 mM $CaCl_2$, 2 mM $MgSO_4$), and resuspended at a density of 2×10^7 cells/ml to initiate development. Cells were shaken at 100 rpm at 22°C for 3.5 hr and treated with caffeine (4) before use.

Expression of MHC-PKCΔST

All DNA manipulations were carried out using standard methods (37). Brief description of the plasmid construct is given below, full details will be given elsewhere (Dembinsky, A., H. Rubin, and S. Ravid, manuscript submitted for publication). The vector pDXA-HY contains the actin-15 promoter and allows the expression of proteins carrying a NH_2-terminal His-tag (25). pDXA-MHC-PKCΔST was constructed as follows: the vector pBS-MHCK (35) contains a 2.6-kb MHC-PKC cDNA clone was digested with SmaI and SwaI that deleted a coding region at the 3′ end of MHC-PKC resulting in the deletion of a cluster of 21 serine and threonine residues which are the MHC-PKC autophosphorylation sites (Dembinsky A., H. Rubin, and S. Ravid, manuscript submitted for publication), the resulting MHC-PKC fragment was named MHC-PKCΔST. The MHC-PKCΔST was cloned into pDXA-HY digested with SmaI. pDXA-MHC-PKCΔST was used for the transformation of MHC-PKC null cells (2) using calcium phosphate precipitate (12) and the clones were selected on the basis of their resistance to G418 (Boehringer-Manneim Biochemicals, Indianapolis, IN).

Purification of His-tagged MHC-PKCΔST

50 ml of 2×10^6 cells/ml expressing MHC-PKCΔST were washed twice in 20 mM phosphate buffer (pH 6.5) and the cells were lysed in 1 ml lysis buffer containing 20 mM Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.5), 1% Triton, 0.2% NP-40, 200 mM KCl, 5 mM β-mercaptoethanol, 2 mM PMSF, 200 μM leupeptin, and 200 μM pepstatin. The extracts were centrifuged in a microfuge for 15 min at 4°C and the supernatant was incubated with 50 μl of a slurry of Ni^+-agarose beads (Qiagen Inc. Chatworth, CA) in 20 mM phosphate buffer (pH 6.5) and 200 mM KCl for 1 h at 4°C. The bead–protein complex was washed three times with lysis buffer, twice with lysis buffer containing 20 mM imidazole, and twice with lysis buffer containing 50 mM imidazole. The MHC-PKCΔST was eluted with 100 μl of lysis buffer containing 150 mM imidazole and then eluted with 100 μl of lysis buffer containing 250 mM imidazole.

Preparation of MHC-PKC Antibody–Staphylococcus A Cell Mixture

50 μl of *Staphylococcus A* (Sigma Chemical Co., St. Louis, MO) that had been washed three times in 1× lysis buffer (see below) plus 1 mg/ml BSA were added to 50 ml MHC-PKC antibody (35) and incubated at 4°C on a rotator for at least 30 min before the addition of cell lysate.

Phosphorylation

^{32}P labeling and cAMP stimulation of *Dictyostelium* amebas and immunoprecipitation of ^{32}P-labeled MHC from cell lysates were carried out according to the method of Berlot et al. (4). To immunoprecipitate the ^{32}P-labeled MHC-PKC from cell lysates, developed cell suspension containing up to 5×10^6 cells/ml were added to an equal vol of ice-cold 2× lysis buffer (40 mM Tris-Cl [pH 7.5], 0.2% NP-40, 2 mM DTT, 10 mM EDTA, 2 mM PMSF, 200 μM leupeptin, 200 μM pepstatin, 50 mM sodium pyrophosphate, 200 mM NaF, 2 mM ATP, and 200 mM potassium phosphate [pH 7.5]) and centrifuged for 5 min in a microcentrifuge at 4°C. The supernatants were added to preadsorbed MHC-PKC antibody–*Staphylococcus A* cell mixture prepared as described above and incubated for at least 1 h at 4°C with rotation. The samples were centrifuged in a microcentrifuge and the pellets resuspended in 1 ml of 1× lysis buffer with 1 mg/ml BSA. The pellets were washed twice with 1× lysis buffer with 1 mg/ml BSA and then once with 1 ml 1× lysis − BSA, resuspended in SDS sample buffer and boiled for 5 min. The supernatants from a microcentrifuge spin were loaded on sodium dodecyl sulfate–polyacrylamide gels (SDS-PAGE). Densitometric scanning of the Coomassie blue–stained gels was used to determine the relative amounts of immunoprecipitated MHC and MHC-PKC and the amounts of ^{32}P incorporated into MHC and MHC-PKC were determined using autoradiography and Phosphorimaging with a bioimage analyzer (Bas2000; Fuji Co., Tokyo, Japan). Relative phosphorylation of MHC and MHC-PKC were determined by dividing the values obtained with the PhosphorImager by the values obtained by scanning of the Coomassie blue–stained gels.

Phosphorylation of MHC-PKCΔST in response to cGMP stimulation was carried out as follows: cells were developed in MES buffer as described above. 100-μl aliquots of developed cells were withdrawn and added to 100 μl of a reaction mixture containing 0.2% Triton X-100, 2 mM $MgCl_2$, 7.5 mM Tris-HCl (pH 7.5), 20 μM γ-[^{32}P]ATP, and cGMP (20 pmol per 10^7 cells), the mixture was incubated for 10 min at room temperature. Reactions were stopped by the addition of equal vol of 2× lysis

buffer and the phosphorylated MHC-PKCΔST was isolated using Ni^+-agarose beads as described above. The bead–protein complex was analyzed by SDS-PAGE, autoradiography, and Phosphorimaging as described above.

Triton-resistant Cytoskeleton Analysis

Triton-insoluble cytoskeleton analysis was performed as described previously (11). Supernatant and cytoskeletal pellet fractions were resuspended in SDS-PAGE sample buffer, boiled for 5 min, and electrophoresed on 7% SDS-PAGE gels. The relative amounts of myosin II were determined by SDS-PAGE gel analysis as described above.

Biochemical Analysis of MHC-PKC Distribution

After resuspension of 10^7 developed cells in 1 ml of sonication buffer (10 mM Tris-HCl, pH 7.5; 50 mM KCl, 2 mM PMSF, 200 μM leupeptin, 200 μM pepstatin), they were stimulated with 1 μM cAMP and lysed by sonication using an ultrasonic cell disruptor (XL; Misonix Inc. Framington, NY) XL with a small-sized tip at 50% output power, and the extract was spun in a microcentrifuge for 20 min at 4°C. The soluble fraction immunoprecipitated with MHC-PKC antibody as described above. MHC-PKC was extracted from the insoluble fraction using sonication buffer containing 0.5 M KCl, the extract was spun in a microcentrifuge for 10 min at 4°C, and the solubilized MHC-PKC was immunoprecipitated as described above. To quantify the amounts of MHC-PKC in the soluble and insoluble fractions the immunoprecipitated MHC-PKC from both fractions was electrophoresed on 7% SDS-PAGE gels and the Coomassie blue–stained gels were analyzed as described above.

MHC-PKC Activity

MHC-PKC activity was assayed by two methods. In the first method, MHC-PKC was immunoprecipitated from the soluble and insoluble fractions as described above and the immunoprecipitates were incubated with LMM58 (0.5–1 mg/ml), 6 mM $MgCl_2$, 0.2 mM [γ-^{32}P]ATP (500 cpm/pmol), 1 mM DTT for 10 min at 22°C on a rotator. Reaction was initiated by the addition of ATP and stopped by spinning out the *Staphylococcus A* cell mixture containing the MHC-PKC and adding of 5% trichloroacetic acid to the supernatant to precipitate the LMM58. The precipitated LMM58 were pelleted in a microcentrifuge after incubation for 10 min on ice, washed twice with 5% TCA, resuspended in 20 μl of SDS sample buffer, and electrophoresed on 7% SDS-PAGE gels. To determine incorporation of ^{32}P into LMM58, bands corresponding to LMM58 were cut out of the Coomassie blue–stained gels and counted in a scintillation counter in 5 ml of scintillation fluid. The amounts of MHC-PKC immunoprecipitated from the different cell fractions were determined using densitometric scanning of the Coomassie blue–stained gels and normalized to the total amount of protein determined as described (5).

In the second method, MHC-PKC–specific activity was assayed directly on the kinase extracted from the insoluble cell fraction. After resuspension of 10^7 developed cells in 1 ml of sonication buffer, cells were lysed by sonication as described above, and the extract was spun for 20 min at 4°C. MHC-PKC was extracted from the insoluble fraction using sonication buffer containing 0.5 M KCl, and the crude kinase was assayed for its activity as described above. Protein concentration was determined as described (5). The effect of cGMP on MHC-PKC activity was assayed after lysis of the cells using the Microson, as described above, in sonication buffer containing 20 pmol cGMP per 10^7 cells. MHC-PKC was extracted from the insoluble fraction and assayed for kinase activity as described above. The effect of the cGMP-dependent protein kinase inhibitor KT5823 on MHC-PKC activity was assayed as follows: Ax2 cells at 1×10^6 cells/ml were grown in HL5 containing 12.5 nM KT5823 (Calbiochem Corp., La Jolla, CA) for 24 h. Cells were developed in MES buffer as described above in the presence of 12.5 nM KT5823, and MHC-PKC was extracted from the insoluble fraction of the cells and assayed for kinase activity as described above.

Phosphorylation by cGMP-dependent Protein Kinase

MHC-PKC was immunoprecipitated from 5×10^6 developed Ax2 cells, and the His-tagged MHC-PKCΔST was purified as described. To block the autophosphorylation sites of MHC-PKC, the protein was preincubated in 40 mM Tris-HCl [pH 7.5] containing 0.2 mM ATP and 6 mM $MgCl_2$ for 30 min at 30°C in a shaking water bath. To this was added [γ-^{32}P]ATP (500 cpm/pmol), 300 U cGMP-dependent protein kinase (Promega Corp., Madison, WI) and 2 μM cGMP, and the mixture was incubated for 10 min at 30°C in a shaking water bath. Samples were pelleted in a microcentrifuge and resuspended in 1 ml of sonication buffer followed by three washes with 1 ml of sonication buffer. The pellets were resuspended in SDS-PAGE sample buffer and boiled for 5 min. The supernatants from a microcentrifuge spin were then loaded on SDS-PAGE gels. Gels were analyzed using autoradiography and Phosphorimaging, as described above. Phosphorylation of MHC-PKCΔST by cGMP-dependent protein kinase was done with Ni^+-agarose purified MHC-PKCΔST (see above) and carried out as described for MHC-PKC except for the preincubation with ATP.

Results

MHC Is Phosphorylated at the Cell Cortex by MHC-PKC in Response to cAMP

In previously reported experiments (4), cAMP stimulation of *Dictyostelium* Ax3 cells led to increases in phosphorylation of MHC in vivo and in vitro. The observed rates of phosphorylation coincided with the association of myosin II with the Triton-insoluble cytoskeleton (3). We have recently shown that MHC-PKC is the kinase that phosphorylates MHC in response to cAMP stimulation (2). To determine whether the effects of cAMP on MHC phosphorylation and localization are exerted via its regulation of MHC-PKC, we studied the kinetics of MHC phosphorylation and Triton insolubility in response to cAMP stimulation, and compared them with the kinetics of cAMP-induced MHC-PKC membrane association, phosphorylation, and kinase activity. To examine the phosphorylation of MHC in response to cAMP stimulation, we immunoprecipitated myosin II from caffeine-treated cAMP-stimulated ^{32}P-labeled *Dictyostelium* Ax2 strain (see Materials and Methods). To examine the Triton-insolubility properties of myosin II, we isolated caffeine-treated, cAMP-stimulated, actin-enriched Triton-insoluble cytoskeletons (see Materials and Methods).

We found that in response to cAMP stimulation, MHC was transiently associated with the Triton-insoluble cytoskeleton and phosphorylated (Fig. 1 *A*). These results are similar to those obtained for *Dictyostelium* Ax3 strain (3). The phosphorylation of MHC occurred later than the association of the myosin II with the cytoskeleton, so that, when the association was half completed (Fig. 1 *A*, 20 s point) the phosphorylation had scarcely begun. The peaks of phosphorylation and association are however coincident (Fig. 1 *A*, 40 s point). These results may indicate that the MHC phosphorylation occurs in the cell cortex. Myosin II dissociation from the cytoskeleton is correlated with highly phosphorylated MHC; 50 s after cAMP stimulation, myosin II association with the cytoskeleton returned to its basal level, while the MHC contains 76% of the maximum amount of phosphate obtained 40 s after cAMP stimulation. These findings, along with our recent observation that the membrane-associated MHC-PKC phosphorylates MHC in response to cAMP (2), indicate that MHC is first associated with the cytoskeleton and is then phosphorylated at the cell cortex by MHC-PKC which results in myosin II dissociation from the cytoskeleton.

In mammalian cells, activation of PKC results in its translocation from the cytosol to the particulate fraction, apparently as part of the activation process (31). It was

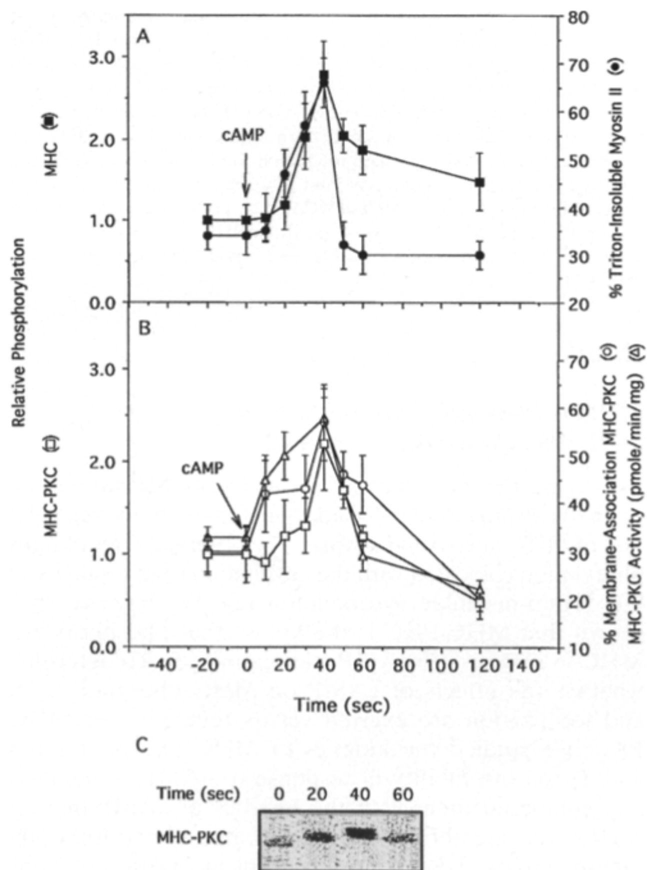

Figure 1. cAMP induces changes in myosin II and MHC-PKC behavior. In *A*, aliquots of a developed and caffeine-treated cell suspension were removed before and after stimulation with 1 μM cAMP, added to ice-cold Triton mixture, and centrifuged as described in Materials and Methods. Percent of insoluble MHC was determined by quantitating the amounts of MHC in the pellets and in the supernatants using densitometric scanning of the Coomassie blue–stained gels , and calculating the ratio of the amount of MHC in the pellet to the total amount of MHC for each time point. The rate of MHC phosphorylation was determined by immunoprecipitation of MHC from ^{32}P-labeled cells and its quantitation using the PhosphorImager as described in Materials and Methods. The relative phosphorylation rate is the ratio of the rate of MHC phosphorylation measured in vivo at a given time after cAMP stimulation to the mean rate of MHC phosphorylation measured in vivo before cAMP stimulation. In *B*, aliquots of developed cell suspensions were removed before and after stimulation with 1 μM cAMP, lysed by sonication, and centrifuged as described in Materials and Methods. Percent of membrane-associated MHC-PKC was determined by quantitating the amounts of MHC-PKC immunoprecipitated from the pellets and from the supernatants using densitometric scanning of the Coomassie blue–stained gels, and calculating the ratio of the amount of MHC-PKC in the pellets to the total amount of MHC-PKC for each time point. The rate of MHC-PKC phosphorylation was determined by immunoprecipitation of MHC-PKC from ^{32}P-labeled cells lysed in NP-40 mixture as described in Materials and Methods, and its quantitation by the PhosphorImager. The relative phosphorylation rate is the ratio of the rate of MHC-PKC phosphorylation measured in vivo at a given time after cAMP stimulation to the mean rate of MHC-PKC phosphorylation measured in vivo before cAMP stimulation. MHC-PKC–specific activity was determined by lysing cAMP-stimulated cells by sonication, extracting of the MHC-PKC from the pellet and subjecting to kinase assay as described (34). *C*, an image obtained by the PhosphorImager of MHC-PKC immunoprecipitated from cAMP-stimulated, ^{32}P-labeled cells. (error bars equal ±SEM; n = 5).

therefore of interest to determine whether cAMP stimulation affects the cell localization properties of MHC-PKC. For this purpose, Ax2 cells were developed, treated with caffeine, stimulated with cAMP, and lysed by sonication, and MHC-PKC was immunoprecipitated from the soluble and the insoluble fractions using specific MHC-PKC polyclonal antibody, as described in Materials and Methods. As shown in Fig. 1 *B*, before cAMP stimulation ~30% of the MHC-PKC was to be found in the insoluble fraction, whereas cAMP stimulation was followed by a rapid transient association of up to 58% of the MHC-PKC with the membrane fraction. These results indicate that MHC-PKC translocated to the membrane in response to cAMP stimulation. The association of MHC-PKC with the membrane preceded the association of myosin II with the cytoskeleton and its phosphorylation, such that, when the association of MHC-PKC was half completed (Fig. 1 *B*, 10 s point), the myosin II cytoskeletal association and phosphorylation had barely begun. Association of MHC-PKC with the membrane peaked ~40 s after cAMP stimulation, similar to myosin II cytoskeletal association and phosphorylation (Fig. 1 *A*).

To determine the effect of cAMP stimulation on MHC-PKC phosphorylation, MHC-PKC was immunoprecipitated from cAMP-stimulated ^{32}P-labeled Ax2 cells as described in Materials and Methods. MHC-PKC undergoes autophosphorylation as a result of an intramolecular event, with each mole of the kinase incorporating ~20 mole of phosphate (34). Autophosphorylation of mammalian PKC, as well as of the *Dictyostelium* 130-kD-MHCK, has been shown to increase their activity (27, 28). In addition to the autophosphorylation mechanism, it is apparent from the MHC-PKC sequence that it contains several potential phosphoryl ation sites for kinases such as cAMP- and cGMP-dependent protein kinases (35). The term MHC-PKC phosphorylation used here describes the sum of both types of phosphorylations. Fig. 1 *C* shows an image, obtained from the PhosphorImager, of immunoprecipitated ^{32}P-labeled MHC-PKC from a typical experiment. The immunoprecipitated MHC-PKC migrated on SDS-PAGE gel as a band with an apparent molecular mass of 84–90 kD. The differences in MHC-PKC migration rates reflect different extents of its phosphorylation, as shown in Fig. 1 *B*. In response to cAMP stimulation, MHC-PKC was transiently phosphorylated with a peak of phosphorylation at ~40 s. The membrane association of MHC-PKC (half-maximal, 10 s) preceded the phosphorylation of MHC-PKC (half-maximal, 30 s). Thus, MHC-PKC phosphorylation takes place in the cell membrane.

To determine whether MHC-PKC membrane association and/or phosphorylation are required for MHC-PKC activation, we examined the activity levels of MHC-PKC in soluble and insoluble fractions isolated from Ax2 cells stimulated with cAMP as described in Materials and Methods. Since *Dictyostelium* contains several soluble MHCKs (for review see 40), we first immunoprecipitated the MHC-PKC from the soluble and insoluble fractions, de-

termined its concentration, and then assayed it for kinase activity as described in Materials and Methods. The soluble MHC-PKC showed only 5% of the kinase activity present in the membrane-associated MHC-PKC (data not shown). These results suggest that the cytosolic MHC-PKC has greatly reduced activity, and that MHC phosphorylation by MHC-PKC takes place at the cell cortex. To determine the effect of cAMP on MHC-PKC specific activity, we solubilized the kinase from cell membranes and assayed it for kinase activity as described in Materials and Methods. As mentioned above, *Dictyostelium* contains several MHCKs; however, all except MHC-PKC reside in the cytosol (for review see 40). Another indication that MHC-PKC is the only MHCK activity present in the membrane fraction comes from the observations that cells in which the MHC-PKC gene was disrupted exhibit no MHCK activity in the membrane fraction (2). Accordingly, all subsequent kinase assays were performed on MHC-PKC that was solubilized from the cell membrane fraction. Fig. 1 *B* shows that cAMP stimulation of Ax2 cells resulted in a transient increase in membrane-associated MHC-PKC kinase activity. The half-maximal MHC-PKC activity was obtained 10 s after cAMP stimulation and peak activity occurred at 40 s. The cAMP-stimulated increases in MHC-PKC activity coincide with the cAMP-stimulated membrane-association and phosphorylation of MHC-PKC, suggesting that these processes may be required for activation of MHC-PKC.

These results, along with the finding that MHC-PKC phosphorylates MHC in response to cAMP (2), may indicate that MHC-PKC is the mediator between the extracellular cAMP signal and MHC, and that the regulation of MHC by cAMP occurs via regulation of MHC-PKC.

Mutants with Abnormal cAMP-induced cGMP Accumulation Exhibit Aberrant Myosin II and MHC-PKC Behavior

As mentioned above, cAMP stimulation of *Dictyostelium* ameba generates a number of responses including increases in cGMP. cGMP has been implicated in the regulation of MHC phosphorylation and cytoskeleton association (21, 22, 23). We have postulated that one way for cGMP to exert its effect on myosin II is via the regulation of MHC-PKC activities. To investigate this possibility, we first examined the kinetics of MHC phosphorylation and cytoskeletal association both in *Dictyostelium* mutants that exhibit aberrant cAMP-induced increases in cGMP accumulation or have aberrant cGMP binding activity and in the parental strain XP55 (hereafter termed control cells), and compared them to the kinetics of the kinase activities of MHC-PKC in these cells (Fig. 2). The mutants used were the streamer mutants NP377 or NP368 (hereafter termed *stmF* mutants) which exhibit a prolonged increase in cGMP in response to cAMP stimulation (30), the KI-10 mutant which lacks the normal cAMP-induced cGMP response (19), and the KI-4 mutant which has nearly normal cAMP-induced cGMP response but has aberrant cGMP-binding activity (20). Newell and colleagues (21, 22, 23), in characterizing the MHC phosphorylation and cytoskeleton association in cGMP mutants, presented their results as percent changes in these parameters in response to cAMP stimulation. Here we further characterized and quantified these changes. As demonstrated below such quantification is important for an understanding of the mechanism of MHC regulation by cGMP.

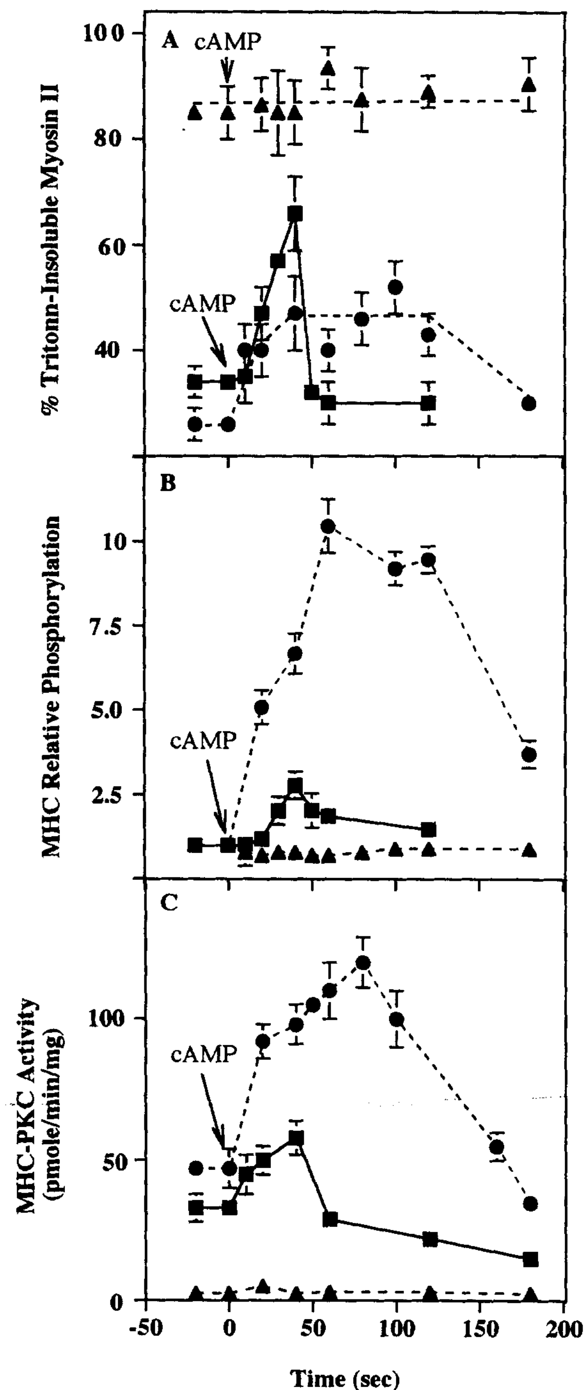

Figure 2. cAMP-induced changes in MHC membrane association and phosphorylation and in MHC-PKC activity in cGMP mutants. Conditions were as described in Fig. 1. (error bars equal ±SEM; $n = 4$). -■-, control; --●--, *stmF*; -▲-, KI = 10.

cAMP stimulation of the control cells resulted in a transient increase in myosin II association with the cytoskeleton (Fig. 2 *A*), with a pattern similar to that exhibited by Ax2 cells (Fig. 1 *A*). cAMP stimulation of *stmF* mutants

also resulted in an increase in cytoskeletal myosin II, but the response was more prolonged than that of the control cells (Fig. 2 *A*), as reported earlier (22, 23). The percent of cytoskeleton-associated myosin II 40 s after cAMP stimulation was lower in *stmF* mutants than in control cells (45 vs. 66% ; see Fig. 2 *A*). Also, the basal level of cytoskeletal myosin II was lower in *stmF* mutants than in control cells (26 vs. 35%). cAMP stimulation of KI-10 mutants did not affect the association of myosin II with the cytoskeleton, as also reported previously (21). Surprisingly, ~85% of the myosin II in these mutants was found to lie in the cytoskeleton regardless of cAMP stimulation (Fig. 2 *A*).

cAMP stimulation of control cells resulted in a transient increase in MHC phosphorylation (Fig. 2 *B*) similar to that in Ax2 cells (Fig. 1 *A*). cAMP stimulation of *stmF* mutants resulted in prolonged phosphorylation of MHC (Fig. 2 *B*), as found previously (23). The extent of cAMP-induced MHC phosphorylation was higher in *stmF* mutants than in control cells, with the peak of phosphorylation being about fourfold higher than that in controls. This is higher than reported by Liu and Newell (23) probably due to low basal levels of phosphorylation resulting from pretreatment of the cells with caffeine. Similar to control cells, the membrane association of myosin II occurred before MHC phosphorylation, indicating that MHC phosphorylation occurred at the cell cortex. cAMP stimulation did not affect MHC phosphorylation in KI-10 mutants (Fig. 2 *B*), as also reported by Liu et al. (21); only basal levels of MHC phosphorylation could be detected in these cells (Fig. 2 *B*).

We then examined whether the aberrant cAMP-induced cGMP increases in the cGMP mutants had a similar effect on the behavior of MHC-PKC to that on myosin II. After cAMP stimulation, the patterns of MHC-PKC-specific activity bore a striking resemblance to those of MHC phosphorylation (Fig. 2 *C*). Thus, in *stmF* mutants, stimulation by cAMP resulted in a prolonged increase in MHC-PKC–specific activity that was twice as high as in the control cells and preceded the phosphorylation of MHC. KI-10 mutants exhibited negligible amounts of MHC-PKC activity regardless of cAMP stimulation. Similar results were also obtained for KI-4 mutants (data not shown). Furthermore, in cAMP-stimulated *stmF* mutants, both the patterns of prolonged phosphorylation and cytoskeletal association of MHC and the prolonged specific activity of MHC-PKC correlated well with the prolonged cGMP increase in these mutants (data not shown) (30). These results suggest that cGMP regulates MHC via the regulation of MHC-PKC.

cAMP-induced Increases in cGMP Are Correlated with Increases in MHC-PKC Membrane Association and Phosphorylation

Membrane-associated MHC-PKC in mutant and control cells was assayed by immunoprecipitation of MHC-PKC from the soluble and insoluble fractions of caffeine-treated cAMP-stimulated cells as described in Materials and Methods. Phosphorylation levels of MHC-PKC in these cells were determined by immunoprecipitation of MHC-PKC from caffeine-treated, cAMP-stimulated ^{32}P-labeled cells using specific MHC-PKC polyclonal antibody as described in Materials and Methods. As shown in Fig. 3, MHC-PKC in the control cells underwent rapid membrane association and phosphorylation, with a peak at 40 s after cAMP stimulation, as described for Ax2 cells (Fig. 1 *A*). In the *stmF* mutants, the membrane association of the MHC-PKC was prolonged by cAMP stimulation compared with the control cells. The cAMP-dependent MHC-PKC phosphorylation lasted longer and was 20% higher than that in control cells. In the KI-10 mutants, the MHC-PKC membrane association and phosphorylation were unaffected by cAMP stimulation. The amount of membrane-associated MHC-PKC after cAMP stimulation of KI-10 cells was similar to basal levels in control cells (Fig. 3 *A*), while the level of MHC-PKC phosphorylation after cAMP stimulation was even lower than the basal phosphorylation in control cells (Fig. 3 *B*). Similar results were obtained for KI-4 mutants (data not shown). The patterns of MHC-PKC membrane association and phosphorylation exhibited by the *stmF* and KI-10 mutants resemble those of the cAMP-induced increases in cGMP in these mutants (30). These results support the idea that cGMP is involved in the regulation of MHC-PKC and suggest that cGMP is required for MHC-PKC activation and translocation to the membrane. The similar behavior of MHC-PKC in KI-10 and KI-4 mutants may indicate that cGMP regulates the MHC-PKC activities via a cGMP-binding protein.

Addition of cGMP to Extract of Wild Type Cells Mimics the MHC-PKC Activity in stmF Mutants

To examine whether the activity of MHC-PKC is affected by cAMP-induced increases in cGMP, we mimicked the *stmF* mutant phenotype by adding cGMP to a lysed extract of control cells and assayed their membrane-associated MHC-PKC activity as described in Materials and Methods. Newell and Liu (30) have reported that in response to cAMP stimulation the maximum amounts of cGMP were ~8 pmol per 10^7 cells in control cells and ~16 pmol per 10^7 cells in *stmF* cells. We therefore used 20 pmol per 10^7 cells in our experiments. Addition of cGMP to extracts of control cells resulted in MHC-PKC activities two to three fold higher than in control cell extracts without cGMP (Fig. 4). The MHC-PKC activity detected in the cGMP-treated extracts was similar to that observed in *stmF* mutants, and the enzyme remained active as long as cGMP was present in the extract (Fig. 4). These results suggest that cGMP is a regulator of MHC-PKC activation.

cGMP-dependent Protein Kinase Inhibitor (KT5823) Inhibits MHC-PKC Activity

We have previously shown that the effect of cGMP on MHC-PKC activity is not exerted directly (34). Furthermore, our results with the KI-4 mutants indicate that cGMP-binding protein is involved in the regulation of MHC-PKC. We therefore investigated whether cGMP affects MHC-PKC via activation of a cGMP-dependent protein kinase that phosphorylates MHC-PKC and thereby activates it. Control cells were incubated with the cGMP-dependent protein kinase selective inhibitor KT5823 (Ki, 2.4 nM) (16) as described in Materials and Methods. The cells were then stimulated with cAMP and their membrane-associated MHC-PKC activity was determined. As shown in Fig. 4, no increase in MHC-PKC activity was observed in these cells. Moreover, the basal activity of MHC-

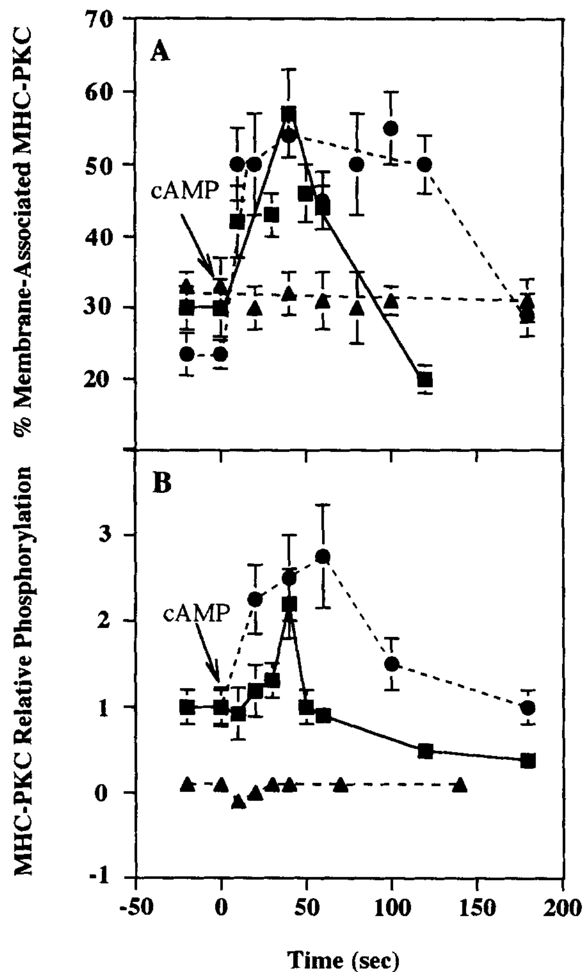

Figure 3. cAMP-induced changes in MHC-PKC membrane association and phosphorylation in cGMP mutants. Conditions were as described in Fig. 1. (error bars equal ±SEM; n = 5). -■-, control; --●--, *stmF*; -▲-, KI = 10.

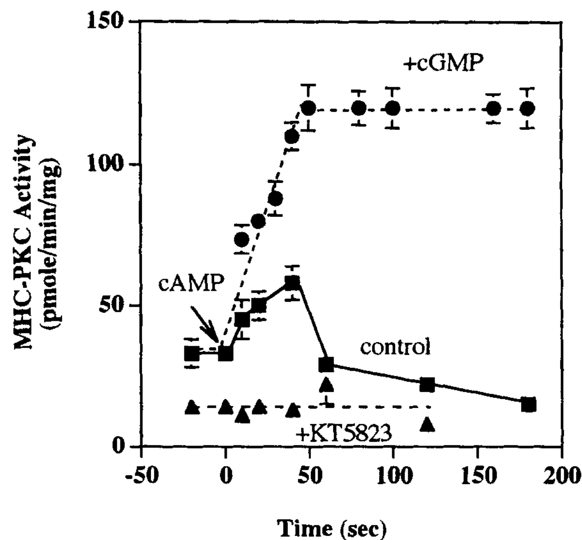

Figure 4. Effect of cGMP on MHC-PKC activity. Membrane-associated MHC-PKC activity was assayed as described in Fig. 1 and Materials and Methods. MHC-PKC activity in the presence of cGMP was assayed after lysis of the cells by sonication in the presence of 20 pmol cGMP per 10^7 cells. MHC-PKC activity in the presence of the cGMP-dependent protein kinase inhibitor, KT5823 was assayed in cells grown for 24 h and developed in the presence of KT5823. (error bars equal ±SEM; n = 6).

PKC in KT5823-treated cells was 2.5-fold lower than in untreated control cells (14 vs. 30 pmol/min/mg). Addition of cGMP to membrane isolated from KT5823-treated cells did not result in the activation of MHC-PKC (data not shown). To test whether the inhibition of MHC-PKC by KT5823 is exerted directly, we performed MHC-PKC kinase assay in the presence of KT5823, as described in Materials and Methods. Addition of the inhibitor did not affect MHC-PKC activity (data not shown), indicating that its effect is indirect. These results suggest that a cGMP-dependent protein kinase is involved in the phosphorylation and activation of MHC-PKC.

Addition of cGMP to an Extract of Cells Expressing MHC-PKCΔST Leads to its Phosphorylation

The detected phosphorylation levels of MHC-PKC described above were achieved mainly by autophosphorylation (since MHC-PKC is heavily autophosphorylated 34); this cannot be distinguished from its phosphorylation by other kinases, such as cGMP-dependent protein kinase. To determine whether MHC-PKC is phosphorylated in a cGMP-dependent manner on sites that are different from the autophosphorylation sites, we expressed in MHC-PKC null cells (2), a truncated MHC-PKC (MHC-PKCΔST) in which the autophosphorylation sites were eliminated (see Materials and Methods). In in vitro studies using the MHC-PKCΔST protein, we found that addition of ATP did not result in autophosphorylation of MHC-PKCΔST (Dembinsky A., H. Rubin, and S. Ravid, manuscript submitted for publication) in contrast to MHC-PKC (34). Furthermore, cAMP stimulation of cells expressing the MHC-PKCΔST resulted in very low level of phosphorylation compared with wild-type cells (Dembinsky, A., H. Rubin, and S. Ravid, manuscript submitted for publication). We used the MHC-PKCΔST cell line to study the cGMP-dependent MHC-PKC phosphorylation. For this purpose, developed MHC-PKCΔST cells were lysed in the presence of cGMP and [γ-^{32}P]ATP and the MHC-PKCΔST was immunoprecipitated as described in Materials and Methods. The addition of cGMP to an extract of MHC-PKCΔST cells resulted in its phosphorylation (Fig. 5). Similar results were obtained for MHC-PKCΔST isolated from cAMP-stimulated ^{32}P-labeled cells (data not shown). The results indicate that MHC-PKCΔST is phosphorylated in a cGMP-dependent manner on sites different from the autophosphorylation sites.

cGMP-dependent Protein Kinase Phosphorylates MHC-PKC and MHC-PKCΔST

We then addressed the question whether cGMP-dependent protein kinase phosphorylates MHC-PKC and MHC-PKCΔST directly. To investigate this possibility, MHC-PKC and MHC-PKCΔST were phosphorylated using a cGMP-dependent protein kinase isolated from bovine lung (10). Addition of cGMP stimulates an increase of ap-

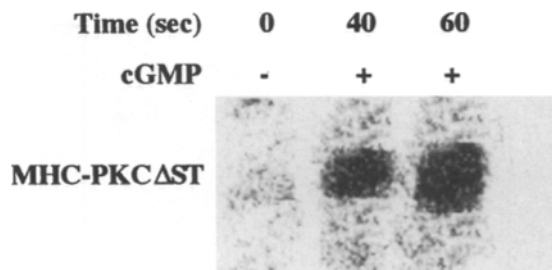

Figure 5. MHC-PKCΔST phosphorylation in response to cGMP. Cells expressing the MHC-PKCΔST protein were developed in MES buffer as described in Materials and Methods. 100-μl aliquots of developed cells were withdrawn and added to 100 μl of a reaction mixture containing ATP and cGMP and the mixture was incubated for 10 min at room temperature as described in Materials and Methods. The phosphorylated MHC-PKCΔST protein was purified with Ni^{+}-agarose beads and analysed using SDS-PAGE and autoradiography as described in Materials and Methods.

proximately three-fold in its protein kinase activity (10). To test whether the cGMP-dependent protein kinase phosphorylates the MHC-PKC directly, we first added unlabeled ATP and $MgCl_2$ to MHC-PKC immunoprecipitated from developed control cells, to avoid masking of cGMP-dependent protein kinase phosphorylation by MHC-PKC autophosphorylation. We then added [γ-^{32}P]ATP along with cGMP-dependent protein kinase and cGMP, as described in Materials and Methods. To test whether cGMP-dependent protein kinase phosphorylates MHC-PKC on sites different from the autophosphorylation sites, we used purified MHC-PKCΔST as a substrate for the cGMP-dependent protein kinase. Fig. 6 shows an autoradiogram of immunoprecipitated MHC-PKC or Ni^{+}-agarose–purified MHC-PKCΔST phosphorylated by cGMP-dependent protein kinase. The MHC-PKC or MHC-PKCΔST alone did not exhibit any phosphorylation. Some phosphorylation of MHC-PKC and MHC-PKCΔST was observed upon addition of cGMP-dependent protein kinase (Fig. 6). When cGMP was added together with the cGMP-dependent protein kinase, the phosphorylation increased by 5–10-fold (Fig. 6). These results suggest that cGMP-dependent protein kinase is capable of directly phosphorylating MHC-PKC on sites other than the autophosphorylation sites. The differences in the extent of MHC-PKC and MHC-PKCΔST phosphorylations are due to different amounts of protein used in these experiments. In vitro phosphorylation of MHC-PKC by cGMP-dependent protein kinase resulted in 20% increase in its specific activity (data not shown).

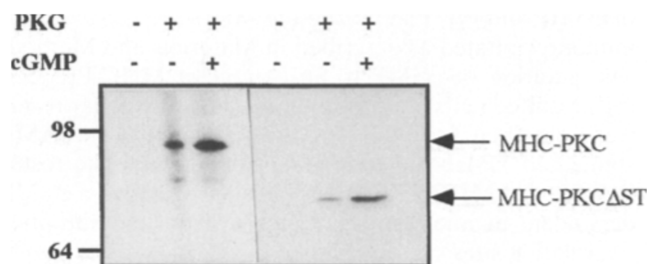

Figure 6. Phosphorylation of MHC-PKC and MHC-PKCΔST by cGMP-dependent protein kinase. MHC-PKC was immunoprecipitated from developed Ax2 cells and MHC-PKCΔST was purified using Ni^{+}-agarose beads in Materials and Methods. The proteins were added to a reaction mixture containing cGMP- and cGMP-dependent protein kinase, as described in Materials and Methods. The phosphorylated proteins were then subjected to SDS-PAGE and analyzed using autoradiography (see Materials and Methods).

Discussion

A central question for an understanding of the molecular basis of signal transduction mechanisms is how an external signal is transmitted to intracellular elements. In the case studied here, the question relates to how the extracellular cAMP signal is transmitted to myosin II which responds by translocating to the cortex, thereby initiating cell polarity and chemotaxis. In a previous report, we showed that MHC-PKC is the mediator of the cAMP signals (2); here we show that the extracellular cAMP regulates the MHC-PKC activities via cGMP-dependent protein kinase. The extracellular cAMP signal regulates myosin II assembly and localization by promoting intracellular cGMP accumulation which, in turn activates cGMP-dependent protein kinase that phosphorylates MHC-PKC thereby regulating its activities. MHC-PKC phosphorylates MHC and affects myosin II assembly properties, with resulting affects on myosin II localization.

cAMP stimulation results in translocation of MHC-PKC to the membrane (Fig. 1 *B*). Mammalian cell fractionation experiments have demonstrated that activation of PKC results in translocation of PKC activity from the cytosol to the particulate fraction (17, 48). Our present findings also indicate that cAMP-induced MHC-PKC translocation to the membrane is part of the enzyme activation: MHC-PKC translocation coincided with increase in MHC-PKC activity, whereas cytosolic MHC-PKC showed greatly reduced kinase activity. cAMP-induced increases in MHC cytoskeleton association were followed by its phosphorylation but were preceded by the observed increase in MHC-PKC membrane association and activation (Fig. 1). These findings, together with our recent finding that MHC-PKC phosphorylates MHC in response to cAMP (2), indicate that MHC is phosphorylated in the cell cortex by MHC-PKC in response to cAMP.

Several lines of evidence emerging from this study indicate that the extracellular cAMP affects the MHC-PKC via accumulation of cGMP and activation cGMP-dependent protein kinase: (*a*) the activity of MHC-PKC in *stmF* mutant is mimicked by the addition of cGMP to control cells (Fig. 4), yet the addition of cGMP to purified kinase does not alter its activity (34); (*b*) addition of the cGMP-dependent protein kinase inhibitor KT5823 to control cells inhibits the MHC-PKC activity but addition of the inhibitor to the kinase alone does not affect its activity (Fig. 4); (*c*) cGMP-dependent protein kinase isolated from lung is capable of phosphorylating MHC-PKC in vitro (Fig. 6); (*d*) MHC-PKC behavior is similar in the cGMP mutants, KI-10 and KI-4. The former mutant lacks the cAMP-induced cGMP accumulation while the latter shows nearly normal cAMP-induced cGMP accumulation but has an aberrant cGMP binding activity, indicating that cGMP per se does

not affect the MHC-PKC but rather a cGMP-binding protein which is possibly a cGMP-dependent protein kinase. The most direct evidence that cGMP-dependent protein kinase phosphorylates the MHC-PKC arises from our studies on the MHC-PKCΔST: MHC-PKCΔST is phosphorylated in cGMP-dependent manner (Fig. 5) and is phosphorylated in vitro by cGMP-dependent protein kinase (Fig. 6). As well cGMP-dependent protein kinase was recently identified in *Dictyostelium* (44), although its substrate is unknown. MHC-PKC phosphorylation by cGMP-dependent protein kinase may play a role in the activation and translocation of the kinase to the membrane since, in KI-10 and in KI-4 mutants, the MHC-PKC resides in the cytosol in inactive form. Furthermore, phosphorylation of MHC-PKC by cGMP-dependent protein kinase increases its activity in vitro. Analysis of the MHC-PKC sequence reveals that it contains at least two sites for cGMP-dependent protein kinase phosphorylation (35). We are currently attempting to express an MHC-PKC in which the putative cGMP-dependent protein kinase phosphorylation sites are converted to alanine residues. Experiments with this altered MHC-PKC protein will enable us to explore the in vivo role of MHC-PKC phosphorylation by cGMP-dependent protein kinase.

Phosphorylation of PKC by a heterologous kinase and its involvement in the regulation of PKC has been reported previously for PKCα (7). Cazaubon et al. (7) reported that PKC-α activation depends not only on lipid activators but also on phosphorylation by PKC kinase. These authors presented evidence that PKC must itself be phosphorylated before it can become catalytically active. Site-directed mutagenesis was used to identify phosphorylation of Thr497 as a critical event in PKC posttranslational modification and activation of PKC-α (7). Since this is not an autophosphorylation site, it appears that a PKC kinase is required in order to generate a primed PKC, which can then be activated by second messenger molecules (7). Thr497 is conserved among all conventional members of the PKC family including MHC-PKC (35). The identity of this kinase, however, from the results of this study suggest that a plausible candidate for a PKC kinase is a cGMP-dependent protein kinase.

The phosphorylation of MHC-PKC by cGMP-dependent kinase is the first of two stages of MHC-PKC phosphorylation that plays a role in its regulation. The second stage is a cAMP-dependent autophosphorylation which accounts for most of the MHC-PKC phosphorylation in our experiments and is responsible for the electrophoretic mobility shift of MHC-PKC (Dembinsky, A., H. Rubin, and S. Ravid, manuscript submitted for publication). Our results indicate that the autophosphorylation occurs in the membrane since the cAMP-induced increases in MHC-PKC membrane association coincides with the increases in its phosphorylation. Consistent with this hypothesis is our finding that, in KI-10 and KI-4 cells, the enzyme does not translocate to the membrane (Fig. 3 *A*) and contains a negligible amount of phosphates (Fig. 3 *B*). It is conceivable that the binding of MHC-PKC to the membrane provides the enzyme with the specific configuration that allows it to phosphorylate itself.

Further indications that cGMP plays a regulatory role in MHC-PKC activity are the findings that cAMP-induced increases in cGMP accumulation correlate well with increases in MHC-PKC membrane association and phosphorylation, and that these responses were also altered in cGMP mutants (Fig. 3). In the *stmF* mutant both the cAMP-induced MHC-PKC association with the membrane and phosphorylation were prolonged compared with control cells; the amount of MHC-PKC membrane association in the two cell lines was similar while the phosphorylation of MHC-PKC in *stmF* mutant was 20% higher and its activity was twice than that in control cells (Fig. 2 *C*, and Fig. 3 *B*). In contrast, the absence of cGMP accumulation in KI-10 mutant or the aberrant cGMP-binding activity in KI-4 mutant resulted in the absence of both MHC-PKC translocation to the membrane and its phosphorylation in response to cAMP. A simple explanation of these results is that MHC-PKC is phosphorylated in the cytosol by a cGMP-dependent protein kinase and that this phosphorylation is necessary for MHC-PKC activation and translocation to the membrane where autophosphorylation takes place. In *stmF* mutant, the prolonged cGMP accumulation prolongs the activation of cGMP-dependent protein kinase, leading to higher phosphorylated, more active MHC-PKC and its membrane association is prolonged. These results are consistent with the findings that phosphorylation of MHC-PKC by cGMP-dependent protein kinase increases its activity, and that addition of cGMP to an extract of control cells results in two to threefold more MHC-PKC activity as compared with untreated cells (Fig. 4). On the other hand, there is no cGMP-dependent MHC-PKC phosphorylation in KI-10 and KI-4 mutants. Therefore activation and translocation of MHC-PKC to the membrane do not occur, and the kinase resides in the cytosol in unphosphorylated, inactive form. In vitro phosphorylation of MHC-PKC by cGMP-dependent protein kinase resulted in only 20% increase in its acitivity compared with a two to threefold increase *in stmF* mutants (Fig. 2 *A*) and after the addition of cGMP to cell extracts (Fig. 4). Plausible explanations for these results are: the bovine cGMP-dependent protein kinase used in this study is not as efficient as the *Dictyostelium* cGMP-dependent protein kinase and/or the phosphorylation of MHC-PKC by cGMP-dependent protein kinase is not the only mechanism of its activation, for example cofactors such as calcium and diacylglycerol are also required for its activation.

The phosphorylation of MHC-PKC by cGMP-dependent protein kinase plays an important role in the regulation of MHC-PKC and, thereby, in the phosphorylation and localization of myosin II as shown by the cGMP mutants. In cGMP mutants the cAMP-induced association of myosin II with the Triton-insoluble cytoskeleton is abnormal (Fig. 2 *A*). cAMP stimulation of KI-10 mutants does not affect the localization of myosin II. Interestingly, ~85% of the myosin II in these mutants was to be found in the cytoskeleton regardless of cAMP stimulation (Fig. 2 *A*). On the other hand, in control cells, the basal level of Triton-insoluble myosin II was ~30% and this increased to 65% upon cAMP stimulation. Our interpretation of these results is that the absence of cGMP accumulation in response to cAMP stimulation in KI-10 mutant results from the absence of MHC-PKC translocation to the membrane (Fig. 3 *A*) and hence inactive MHC-PKC (Fig. 2 *C*). The absence of active MHC-PKC drives myosin II mole-

cules into filaments in vivo and these filaments have high affinity for the cortical cytoskeleton. Therefore, in the absence of cGMP accumulation, most of the myosin II is associated with the cytoskeleton. Similar results were obtained for MHC-PKC null cells (2).

In *stmF* mutant the association of myosin II with the Triton-insoluble cytoskeleton is more prolonged than in control cells. Yet only 50% of the myosin II was Triton-insoluble compared with 65% in control cells (Fig. 2 *A*). A simple explanation for this finding is that translocation of myosin II to the cortex and removal of myosin II from the cortex are two separate processes, both of which may be affected by cGMP. Silveira et al. (*Mol. Biol. Cell.* 5:152*a*) have shown that addition of cGMP to *Dictyostelium* lysates results in an increase of at least fivefold in MLCK activity. cGMP does not act directly on the kinase, as shown by the fact that addition of cGMP to purified kinase does not alter its activity (41). It is possible that cAMP-induced cGMP accumulation first activates MLCK, which phosphorylates MLC, thereby increasing the ATPase activity of myosin II (14), and thus enabling myosin II to move on actin filaments and to reach the cortex. In *stmF* mutant the process of cGMP accumulation is more prolonged than in control cells (36) and therefore more myosin II is activated by MLCK and the association of myosin II with the Triton-insoluble cytoskeleton is prolonged. On the other hand, the prolonged accumulation of cGMP also results in prolonged MHC-PKC activation. Consequently, more of the MHC is phosphorylated and the amount of myosin II associated with the Triton-insoluble cytoskeleton is less than in control cells. Indeed, the cAMP-stimulated MHC is two to five times more phosphorylated in *stmF* mutants than in control cells, while KI-10 mutant contains no cAMP-induced MHC phosphorylation (Fig. 2 *B*). It is possible that the amount of cytoskeletal myosin II in wild-type cells represents the balance between MLC and MHC phosphorylation.

Ostrow et al. (32) studied the role of the regulatory MLC (RMLC) phosphorylation in the function of myosin II in *Dictyostelium* cells. They found that RMLC is phosphorylated on serine 13 both in vivo and in vitro by MLCK (41). They further demonstrated that RMLC bearing a ser13ala substitution was not phosphorylated in vivo and in vitro by MLCK (41). Although this mutant RMLC had reduced actin activated ATPase activity, it rescued the defects of RMLC null cells (32). These results seems to indicate that RMLC phosphorylation does not play a role in the regulation of myosin II in vivo. However, it should be noted that the in vivo phosphorylation studies were done on vegetative cells (32). It is therefore possible that during *Dictyostelium* development, RMLC is phosphorylated on site(s) different than serine 13 and this phosphorylation is important for the regulation of myosin II during *Dictyostelum* chemotaxis. Consistent with this hypothesis are Silveira et al. (*Mol. Biol. Cell.* 5:152*a*) findings that *Dictyostelium* contains multiple MLCKs that respond differently to external stimuli. Furthermore, it has been proposed that RMLC can be phosphorylated by kinases other than the MLCK, including PKC and $p34^{cdc2}$ kinase (40). It is therefore possible that, during *Dictyostelium* chemotaxis, RMLC is phosphorylated by an MLCK different from that used by Ostrow et al. (32) or by another kinase and this phosphorylation affects myosin II localization during directed cell movement.

Based on the above results, we propose the following model for the role of cGMP in MHC-PKC activation and hence in myosin II localization, cell polarization, and chemotaxis (Fig. 7). An unstimulated cell is rounder because of a contractile shell formed by an actin–myosin II network in the cortex. This network presumably inhibits events necessary for pseudopodial projection. cAMP stimulation of one edge of the cell results in increased cGMP accumulation. cGMP activates MLCK (presumably via a cGMP-dependent protein kinase), which in turn phosphorylates MLC that drives myosin II movement on actin filaments to the cell cortex (Fig. 7). Concomitantly, cGMP

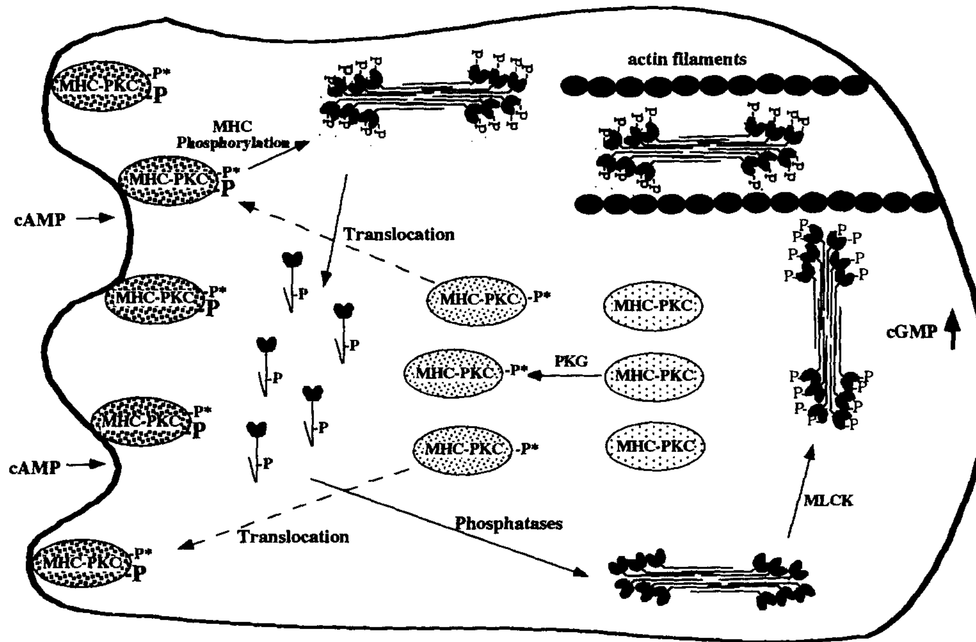

Figure 7. A model showing the role of cGMP in the interaction between myosin II and MHC-PKC, leading to chemotaxis. See Discussion for more details. *MHC-PKC*, MHC-specific PKC; *MLCK*, myosin II light chain kinase; *PKG*, cGMP-dependent protein kinase; *MHC*, myosin II heavy chain; *P**, MHC-PKC phosphorylation by PKG; *P*, MHC-PKC autophosphorylation.

activates a cGMP-dependent protein kinase, which phosphorylates the inactive MHC-PKC in the cytosol causing it to translocate to the membrane where it is localized at the site of cAMP stimulation in an active form. Active MHC-PKC phosphorylates the cortical MHC at the anterior part of the cell, causing disassembly of myosin II thick filament by inducing the formation of a bent monomer of myosin II. In this way, the cAMP- cGMP-dependent interaction between myosin II and MHC-PKC may play a major role in the generation of cell polarity for efficiently directed migration.

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References

1. Abe, T., Y. Maeda, and T. Iijima. 1988. Transient increase of the intracellular calcium concentration during chemotactic signal transduction in Dictyostelium discoideum cells. *Differentiation.* 39:90–96.
2. Abu-Elneel, K., M. Karchi, and S. Ravid. 1996. Dictyostelium myosin II is regulated during chemotaxis by a novel protein kinase C. *J. Biol. Chem.* 271:977–984.
3. Berlot, C.H., P. Devreotes, and J.A. Spudich. 1987. Chemoattractant-elicited increases in Dictyostelium: myosin phosphorylation are due to changes in myosin localization and increases in kinase activity. *J. Biol. Chem.* 262:3918–3926.
4. Berlot, C.H., J.A. Spudich, and P.N. Devreotes. 1985. Chemoattractant-elicited increases in myosin phosphorylation in Dictyostelium. *Cell.* 43: 307–314.
5. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
6. Bumann, J., B. Wurster, and D. Malshow. 1984. Attractant-induced changes and oscillations of the extracellular calcium concentration in suspensions of differentiating Dictyostelium cells. *J. Cell Biol.* 98:173–178.
7. Cazaubon, S., F. Bornancin, and P. Parker. 1994. Threonine-497 is critical site for permissive activation of protein kinase Cα. *Biochem. J.* 301:443–448.
8. Cote, G., and S. McCrea. 1987. Selective removal of the carboxy-terminal tail end of the Dictyostelium myosin II heavy chain by chymotrypsin. *J. Biol. Chem.* 262:13033–13038.
9. Coukell, M.B., and A.M. Cameron. 1986. Characterization of revertants of stm F mutants of Dictyostelium discoideum: evidence that stm F is the structural gene of the stm cGMP-specific phosphodiesterase. *Dev. Genet.* 6:173–177.
10. Edelman, A.M., D.K. Blumenthal, and E.G. Krebs. 1987. Protein serine/threonine kinases. *Annu. Rev. Biochem.* 56:567–613.
11. Egelhoff, T.T., S.S. Brown, and J.A. Spudich. 1991. Spatial and temporal control of nonmuscle myosin localization: identification of a domain that is necessary for myosin filament disassembly in vivo. *J. Cell Biol.* 112:677-688.
12. Egelhoff, T.T., M.A. Titus, D.J. Manstein, K.M. Ruppel, and J.A. Spudich. 1991. Molecular genetic tools for study of the cytoskeleton in Dictyostelium. *Methods Enzymol.* 196:319–335.
13. Europe-Finner, G.N., and P.C. Newell. 1987. Cyclic AMP stimulates accumulation of inositol trisphosphate in Dictyostelium. *J. Cell Sci.* 87:158–169.
14. Griffith, L.M., S.M. Downs, and J.A. Spudich. 1987. Myosin light chain kinase and myosin light chain phosphatase: effects of reversible phosphorylation on myosin structure and function. *J. Cell Biol.* 104:1309–1323.
15. Hall, A.L., A. Schlein, and J. Condeelis. 1988. Relationship of pseudopod extension to chemotactic hormone-induced actin polymerization in amoeboid cells. *J. Cell Biochem.* 37:285–299.
16. Kase, H., K. Iwahashi, S. Nakanishi, Y. Matsuda, K. Yamada, M. Takahashi, C. Murakata, A. Sato, and M. Kaneko. 1987. K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. *Biochem. Biophys. Res. Comm.* 142:436–440.
17. Kraft, A.S., and W.B. Anderson. 1983. Phorbol esters increase the amount of Ca^{2+}, phospholipid-dependent protein kinase associated with plasma membrane. *Nature (Lond.).* 301:621–623.
18. Kuczmarski, E.R., and J.A. Spudich. 1980. Regulation of self-assembly: phosphorylation of Dictyostelium heavy chain inhibits formation of thick filaments. *Proc. Natl. Acad. Sci.* USA. 77:7292–7296.
19. Kuwayama, H., S. Ishida, and P.J.M. Van Haastert. 1993. Non-chemotactic Dictyostelium discoideum mutants with altered cGMP signal transduction. *J. Cell Biol.* 123:1453–1462.
20. Kuwayama, H., G.T. Viel, S. Ishida, and P.J.M. Van Haastert. 1995. Aberrant cGMP-binding activity in non-chemotactic Dictyostelium discoideum mutants. *Biochim. Biophys. Acta.* 1268:214–220.
21. Liu, G., H. Kuwayama, S. Ishida, and P.C. Newell. 1993. The role of cyclic GMP in regulating myosin during chemotaxis of Dictyostelium: evidence form a mutant lacking the normal cyclic GMP response to cyclic AMP. *J. Cell Sci.* 106:591–596.
22. Liu, G., and P.C. Newell. 1988. Evidence that cyclic GMP regulates myosin interaction with the cytoskeleton during chemotaxis of Dictyostelium. *J. Cell Sci.* 90:123–129.
23. Liu, G., and P.C. Newell. 1991. Evidence that cyclic GMP may regulate the association of myosin II heavy chain with the cytoskeleton by inhibiting its phosphorylation. *J. Cell Sci.* 98:483–490.
24. Malchow, D., R. Bohme, and H.J. Rahmsdorf. 1981. Regulation of phosphorylation of myosin heavy chain during the chemotactic response of Dictyostelium cells. *Eur. J. Biochem.* 17:213–218.
25. Manstein, D.J., H.P. Schuster, P. Morandini, and D.M. Hunt. 1995. Cloning vectors for the production of proteins in Dictyostelium discoideum. *Gene (Amst.).* 162:129–134.
26. Mato, J.M., P.J.M. Van Haastert, F.A. Krens, E.H. Rhijnsburger, F.C.P.M. Dobbe, and T.M. Konijn. 1977. Cyclic AMP and folic acid stimulated cyclic GMP accumulation in Dictyostelium discoideum. *FEBS* Lett. 79:331–336.
27. Medley, Q.G., J. Gariepy, and G.P. Cote. 1990. Dictyostelium myosin II heavy chain kinase A is activated by autophosphorylation: studies with Dictyostelium myosin II and synthetic peptides. *Biochemistry.* 29:8992–8997.
28. Mochly-Rosen, D., and D.E. Koshland. 1987. Domain structure and phosphorylation of protein kinase C. *J. Biol. Chem.* 262:2291–2297.
29. Nachmias, V.T., Y. Fukui, and J.A. Spudich. 1989. Chemoattractant-elicited translocation of myosin in motile Dictyostelium. *Cell Motil. Cytoskeleton.* 13:158–169.
30. Newell, P.C., and G. Liu. 1992. Streamer F mutants and chemotaxis of Dictyostelium. *Bioessays.* 14:473–479.
31. Nishizuka, Y. 1988. The molecular heterogeneity of protein kinase C and its implication for cellular regulation. *Nature (Lond.)* 334:661–665.
32. Ostrow, B.D., P. Chen, and R. Chisholm. 1994. Expression of myosin regulatory light chain phosphorylation site mutant complements the cytokinesis and developmental defects of *Dictyostelium* RMLC null cells. *J. Cell Biol.* 127:1945–1955.
33. Pasternak, C., P.F. Flicker, S. Ravid, and J.A. Spudich. 1989. Intermolecular versus intramolecular interactions of Dictyostelium myosin: possible regulation by heavy chain phosphorylation. *J. Cell Biol.* 109:203–210.
34. Ravid, S., and J.A. Spudich. 1989. Myosin heavy chain kinase from developed Dictyostelium cells: purification and characterization. *J. Biol. Chem.* 264:15144–15150.
35. Ravid, S., and J.A. Spudich. 1992. Membrane-bound Dictyostelium myosin heavy chain kinase: a developmentally regulated substrate-specific member of the protein kinase C family. *Proc. Natl. Acad. Sci.* USA 89:5877–5881.
36. Ross, F.M., and P.C. Newell. 1981. Streamers: chemotactic mutants of Dictyostelium discoideum with altered cGMP metabolism. *J. Gen. Microbiol. 127:339–350.*
37. Sambrook, J., E.F. Fritsch, and T. Maniatis (1989). Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 545 pp.
38. Segall, J.E. 1988. Analysis of responses of Dictyostelium discoideum amoebae to chemoattractant concentration changes. *J. Muscle Res. Cell Motil.* 9: 481–490.
39. Deleted in proof.
40. Tan, J.L., S. Ravid, and J.A. Spudich. 1992. Control of nonmuscle myosins by phosphorylation. *Annu. Rev. Biochem.* 61:721–759.
41. Tan, J.L., and J.A. Spudich. 1990. Dictyostelium myosin light chain kinase. Purification and characterization. *J. Biol. Chem.* 265:13818–13824.
42. Van Haastert, P.J.M., M. Van Campagne, and F.M. Ross. 1982. Altered cGMP-phosphodiesterase activity in chemotactic mutants of Dictyostelium discoideum. *FEBS Lett.* 147:149–152.
43. Varnum-Finney, B., N.A. Schroeder, and D.R. Soll. 1988. Adaptation in the motility response to cAMP in Dictyostelium discoideum. *Cell Motil. Cytoskeleton.* 9:9–16.
44. Wanner, R., and R. Wurster. 1990. Cyclic GMP-activated protein kinase from Dictyostelium discoideum. *Biochim. Biophys. Acta.* 1053:179–184.
45. Wessels, D., D.R. Soll, D. Knecht, W.F. Loomis, A. De Lozanne, and J. Spudich. 1988. Cell motility and chemotaxis in *Dictyostelium* amebae lacking myosin heavy chain. *Dev. Biol.* 128:164-177.
46. Wurster, B., K. Schubiger, U. Wick, and G. Gerisch. 1977. Cyclic GMP in Dictyosetlium discoideum: oscillations and pulses in response to folic acid and cyclic AMP signals. *FEBS Lett.* 76:141–144.
47. Yumura, S., and Y. Fukui. 1985. Reversible cyclic AMP-dependent change in distribution of myosin thick filaments in Dictyostelium. *Nature (Lond.)* 314:194–196.
48. Zalewski, P.D., I.J. Forbes, L. Valente, S. Apostolous, and N.P. Hurst. 1988. Translocation of protein kinase C to a Triton-insoluble sub-cellular compartment induced by the lipophilic gold compound auranofin. *Biochem. Pharmacol.* 37:1415–1417.