

Regulation of Actin Microfilament Integrity in Living Nonmuscle Cells by the cAMP-dependent Protein Kinase and the Myosin Light Chain Kinase

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Abstract. Microinjection of the catalytic subunit of cAMP-dependent protein kinase (A-kinase) into living fibroblasts or the treatment of these cells with agents that elevate the intracellular cAMP level caused marked alterations in cell morphology including a rounded phenotype and a complete loss of actin microfilament bundles. These effects were transient and fully reversible. Two-dimensional gel electrophoresis was used to analyze the changes in phosphoproteins from cells injected with A-kinase. These experiments showed that accompanying the disassembly of actin microfilaments,

phosphorylation of myosin light chain kinase (MLCK) increased and concomitantly, the phosphorylation of myosin P-light chain decreased. Moreover, inhibiting MLCK activity via microinjection of affinity-purified antibodies specific to native MLCK caused a complete loss of microfilament bundle integrity and a decrease in myosin P-light chain phosphorylation, similar to that seen after injection of A-kinase. These data support the idea that A-kinase may regulate microfilament integrity through the phosphorylation and inhibition of MLCK activity in nonmuscle cells.

CYCLIC AMP is a key second messenger which mediates the biological effects of B-adrenergic hormones (for reviews see references 19, 20). These hormones activate adenylate cyclase, catalyzing the production of intracellular cAMP. The mode of action of cAMP is thought to be solely through the activation of the cAMP-dependent protein kinase (A-kinase)¹ by promoting the dissociation of inactive holozyme into regulatory subunits and active catalytic subunits (reviewed in reference 34). The catalytic subunits catalyze the phosphorylation of certain protein substrates which serve, in many cases, to regulate their subsequent biological activity (for reviews see references 19, 20, 34, 40, 59). Glycogen metabolism is the best-characterized process regulated by B-adrenergic agents and A-kinase. For example, the activation of A-kinase by the hormone epinephrine results in a cascade of phosphorylation of enzymes which leads to the breakdown of glycogen to glucose 1-phos-

phate. In addition to its regulatory role in glycogen metabolism, A-kinase also has been implicated in mediating a number of other intracellular events including the regulation of cell shape (43, 44, 53, 72), cell motility (5), and the control of cell growth and proliferation (42, 49, 74).

Several studies have indicated a role for A-kinase in the regulation of cytoskeletal structure (5, 53). The control of smooth muscle and nonmuscle microfilament (MF) contraction via phosphorylation of myosin light chain kinase (MLCK) by A-kinase has also been suggested (2, 21, 39, 55, 57, 66). In addition, changes in intermediate filament organization (such as those that occur during mitosis) may be mediated by the phosphorylation of vimentin catalyzed by A-kinase (12, 14, 17, 30–33, 41, 64). Likewise, there have been reports suggesting that changes in cell shape may be influenced by phosphorylation of microtubule-associated proteins by A-kinase (13, 46, 62).

In the present studies, we have examined the role of A-kinase in the regulation of actin MF structure and function in nonmuscle cells. In contrast to skeletal muscle, actin-myosin interaction in smooth and nonmuscle cells appears to be controlled by the phosphorylation of the myosin P-light chain (MLC) (23, 50, 51, 58; for reviews see references 22, 35, 39, 57). The phosphorylation of myosin light chain is catalyzed by the enzyme MLCK, the activity of which is dependent upon calcium and calmodulin, which serve as cofactors (21,

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1. *Abbreviations used in this paper:* A-kinase, cAMP-dependent protein kinase; db-cAMP, N⁶-dibutyryl-cAMP; MIX, 3-isobutyl-1-methylxanthine; MF, microfilament; MLC, myosin P-light chain; MLCK, myosin light chain kinase; rh-phalloidin, rhodamine-conjugated phalloidin.

25, 36, 39). The enzymatic activity of MLCK can be inhibited *in vitro* and *in vivo* by phosphorylation of the enzyme catalyzed by A-kinase (21, 26, 60). This phosphorylation interferes with Ca^{++} /calmodulin binding to MLCK and thereby results in the inhibition of MLCK activity *in vitro* (reviewed in reference 1).

In an effort to examine the possible role of A-kinase in the regulation of MLCK and actin MF structure/function in living nonmuscle cells, we examined the effects of activating A-kinase in cells by raising intracellular cAMP levels using defined drugs or by directly microinjecting the purified A-kinase catalytic subunit into living fibroblasts. We show that within 30 min after elevation of A-kinase activity, virtually all of the MF bundles are disrupted. Moreover, we provide evidence that these changes in actin MF integrity after A-kinase elevation are temporally accompanied by both an increase in MLCK phosphorylation and a concomitant decrease in level of myosin light chain phosphorylation. These results support the model suggested previously for the regulation of smooth muscle actomyosin structure/function in which phosphorylation and subsequent inhibition of MLCK activity is catalyzed by A-kinase in nonmuscle cells.

Materials and Methods

Cell Culture

Rat embryo fibroblast (REF-52) cells were cultured in a humidified atmosphere containing 5% CO_2 , 95% air as described previously (45). Cells were plated 2–3 d before use for microinjection (69).

Isolation of A-Kinase and Purification of the Catalytic Subunit

The free catalytic subunit of the A-kinase was purified from bovine heart (9). Purified catalytic subunit was stored as stock solution at 4°C in 5.0 mM 2-[*N*-morpholino]ethanesulphonic acid (pH 6.5), 100 mM NaCl, 0.2 mM EDTA, 15 mM beta-mercaptoethanol.

Drug Studies

REF-52 cells growing on glass coverslips were incubated with a mixture of dibutyryl cAMP (db-cAMP) and 3-isobutyl-1-methylxanthine (MIX) (both from Sigma Chemical Co., St. Louis, MO). Drugs were used at a concentration of 1 mM final, diluted from 1.0-M DMSO stock solutions. Solutions containing the same amount of DMSO without drugs were used as controls. After the appropriate incubation period, the cells were analyzed by phase-contrast microscopy. Alternatively, the cells were fixed and the distribution of the actin microfilaments determined by incubation with rhodamine-conjugated phalloidin (rh-phalloidin) (generously provided by T. Wheiland, Max-Planck-Institut, Heidelberg, Federal Republic of Germany) as described below.

Microinjection and Immunofluorescence

Cells were microinjected as described by Feramisco (27) using glass capillary needles. The purified A-kinase catalytic subunit was diluted to 1.0 mg/ml into an injection buffer (100 mM potassium glutamate, 40 mM potassium citrate, 1.0 mM MgCl_2 , pH 7.2). As it is estimated that the level of A-kinase in cells is ~ 0.2 – 2.0 μM (8), and we are injecting a 25- μM solution of the catalytic subunit which is diluted 20-fold by injection, we estimate that the microinjection procedure elevates the endogenous level of A-kinase by three-to-fivefold. Active MLCK was purified from bovine trachea as described elsewhere (2), before conjugation to Affigel (20 mg MLCK/ml Affigel). A 42% ammonium sulfate fraction of whole serum (~ 5 mg) directed against smooth muscle MLCK was affinity purified against native MLCK before being dialyzed against PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 , 8.0 mM Na_2HPO_4). Affinity-purified anti-MLCK and other monospecific antibodies were concentrated by sucrose dialysis (to 1–5 mg/ml) before being dialyzed into $0.5 \times$ PBS for microinjection. For im-

munofluorescence, cells were fixed for 5 min in 3.7% formalin (in PBS) followed by a 30-s extraction in -20°C acetone before staining for actin. Actin MFs were visualized with rh-phalloidin a specific stain for F-actin (73). Cells injected with primary antibodies directed against MLCK or control proteins were incubated for 60 min before fixation and extraction as described above. Injected antibodies were subsequently visualized by a 30-min incubation with affinity-purified fluorescein-conjugated goat anti-rabbit antibody (CooperBiomedical, Inc., Malvern, PA) diluted 1:100. Cells were simultaneously stained for actin MFs with rh-phalloidin. The cells were analyzed on a photomicroscope (model III, Carl Zeiss, Inc., Thornwood, New York) using a $63\times$ (1.3 NA) lens. Fluorescent images were recorded onto Kodak Tri-X pan (developed in Diafine; Acufine, Inc., Chicago, IL) and phase images on Kodak 2415 technical pan (developed in Rodinal; Agfa-Gevaert, Teterboro, NJ).

Metabolic Pulse Labeling

Microinjection studies involving metabolic pulse labeling used small (1-mm²) glass coverslips ("chips") onto which cells were plated and allowed to grow for 2–3 d. Coverslips having approximately the same number of cells were selected and microinjected with either purified A-kinase, antibodies specific to MLCK, or control solutions. Precisely the same number of cells on each chip were injected and any residual uninjected cells removed by scraping with the micropipette. After injection, the chips were transferred to humidified chambers for varying periods of time. For labeling, the chips were washed once in phosphate-free DME and then incubated for 30-min periods in 5 μl of phosphate-free DME (supplemented with dialyzed serum to 10%) containing 250–500 μCi [³²P]H₃PO₄ (carrier free; Amersham International, Amersham, United Kingdom). Gard and Lazarides (31) have shown that the specific activity of [³²P]ATP achieved in cells after incubation with [³²P]H₃PO₄ does not change appreciably after activation of A-kinase. Because of the prohibitively small sample size of microinjected cells, it was not feasible to determine the specific activity of the radiolabeled ATP in these samples directly. However, the phosphoprotein changes in REF-52 cells induced by either injection of A-kinase or by the addition of cell-permeable cAMP analogues to the culture medium were similar to those reported by Gard and Lazarides (31), suggesting that the procedures used in the present study are valid. After the labeling period, the cells were briefly washed twice in DME lacking phosphate and transferred to Eppendorf tubes containing $\sim 5 \times 10^6$ unlabeled cells in 20 μl of sample buffer (62.5 mM Tris-Cl [pH 6.8], 5 mM dithiothreitol [DTT], 0.5% [wt/vol] SDS, 7.5% [wt/vol] glycerol, and 2% [wt/vol] NP-40). After boiling, samples were lyophilized and resuspended in 10 μl of 1.0 mg/ml DNase and 1.0 mg/ml RNase in 50 mM Tris-Cl (pH 7.4), 10 mM MgCl_2 , and incubated for 5 min at 37°C to digest nucleic acids. Samples were then diluted with IEF sample buffer (9.5 M urea, 2% [wt/vol] NP-40, 5% [wt/vol] beta-mercaptoethanol, 2% [wt/vol] 6–8 ampholines) such that the final concentration of SDS was $<0.1\%$ (wt/vol).

Two-dimensional Gel Electrophoresis

Samples were analyzed by two-dimensional electrophoresis using the method of O'Farrell (48). First dimension electrofocusing gels contained 9.5 M urea 2% (wt/vol) ampholines, 2% (wt/vol) NP-40, and 5% (wt/vol) DTT. The ratio of ampholines was varied in the electrofocusing gel mixture to facilitate better resolution of different cellular protein components. The mixtures used were 60% (wt/vol) ampholine pH 3–10, 40% (wt/vol) ampholine pH 5–7 for resolving the whole cellular protein mixture, 50% (wt/vol) ampholine pH 4–6, 50% (wt/vol) ampholine pH 5–7, to resolve myosin light chain and 50% (wt/vol) ampholine pH 5–7, 50% (wt/vol) ampholine pH 6–8 to resolve MLCK. Second dimension gels contained either 12.5 or 15% (wt/vol) acrylamide and 0.1% (wt/vol) bis-acrylamide (11). Immediately after electrophoresis the gels were either processed for electrophoretic transfer (see below) or fixed in 50% (wt/vol) TCA at 70°C to hydrolyze residual nucleic acid and then stained to visualize the molecular mass markers. After drying, the phosphoproteins were visualized by autoradiography at -70°C on Kodak XAR-5 film, using Cronex Lightening Plus intensifying screens.

Electrophoretic Transfer and Immunoblotting

Immediately after electrophoresis in the second dimension, proteins were electrophoretically transferred to nitrocellulose as described previously (15). For MLCK, gels were transferred for 24 h at 50 V; whereas for myosin light chain, transfer was complete within 90 min at 80 V. (This difference reflects the slower rate at which MLCK transfers.) On completion of the transfer, nitrocellulose sheets were incubated with 1% (wt/vol) BSA to block

free protein binding sites before being incubated with either monospecific rabbit anti-myosin light chain antibodies (50) (kindly provided by J. Stull, University of Texas, Dallas, Texas) diluted 1:100 in PBS/BSA (PBS supplemented with 0.05% [wt/vol] BSA) or rabbit MLCK antibodies, diluted 1:100 into PBS/BSA. Primary antibodies were visualized by incubation with an affinity-purified horseradish peroxidase-conjugated goat anti-rabbit antibody (Cooper Biomedical, Inc.) diluted 1:1,000, before subsequent color development with 4-chloronaphthol (Sigma Chemical Corp.) (0.3% [wt/vol] in methanol) diluted 1:15 in PBS containing 20 μ l H₂O₂. In all cases, after electrophoretic transfer the gels were stained and processed for autoradiography as described above to ensure that protein transfer had been fully effective. In no case did any phosphoproteins other than residual vimentin remain in the gel.

Electron Microscopy

Cells were grown on UV-sterilized formvar-coated glass coverslips. After injection of A-kinase, the cells were fixed for 20 min with 2% (wt/vol) glutaraldehyde in PBS with further fixation in 75 mM cacodylate (pH 7.4) supplemented with 4.5% (wt/vol) sucrose. Cells were washed with PBS and fixed afterward with 1% (wt/vol) OsO₄ in 75 mM cacodylate (pH 7.4) for 10 min. After a water wash, the cells were stained with saturated aqueous uranyl acetate for 5 min. After staining and washing in water, the formvar was stripped from the coverslip, inverted, and raised to the water surface. Copper grids were placed over the injected cells and after serial ethanol dehydration, cells were critical point dried in a Bomar SPC-900 using CO₂ as the transitional fluid. Cells were analyzed on an electron microscope (model 200ZX; Philips Electronic Instruments, Inc., Mahwah, NJ) at 100 kV.

Results

Morphological Changes in Cells Treated with Drugs that Activate Endogenous A-Kinase

Initial experiments were performed to assess the morphological consequences of elevating endogenous A-kinase levels in REF-52 cells. For these studies we used, simultaneously, two drugs that freely pass into cells and elevate intracellular cAMP levels: (a) db-cAMP, an analog of cAMP, and (b) MIX, an inhibitor of the cyclic nucleotide phosphodiesterases. REF-52 cells, growing on 35-mm dishes (Falcon Labware, Oxnard, CA), were incubated in the presence of both 1 mM db-cAMP and 1 mM MIX. After a 60-min incubation period at 37°C, marked alterations in cellular morphology were observed (Fig. 1). The most obvious changes entailed a rapid "rounding" of the cells involving a pronounced thickening around the nucleus, a reduction in cytoplasmic phase detail, and a corresponding appearance of phase-dense regions in the cell periphery. The rounding effect observed in these experiments appears only to involve a change in cell morphology, with the cells showing no apparent loss of contact with the substratum and therefore remaining well spread. (In this work, the term "rounding" refers solely to this perinuclear thickening and loss of phase detail.) These effects on cell morphology were fully reversible. If the drugs were removed after a 60-min incubation period and the cells further incubated in normal medium, they regained their well-spread and flattened morphology within ~3–6 h (data not shown, but see below). In other experiments we have determined that these drug-induced changes in cell morphology can be maintained for up to 20 h provided that the cells are maintained in the presence of the drugs. Again, the effects can be reversed by simple removal of the drugs. In addition, little or no cell death was observed as a result of the drug treatment. Finally, both the extent and kinetics of such morphological changes appeared dependent upon the con-

centrations of the drugs used. At higher concentrations of the drugs (e.g., >1 mM), there occurred a more rapid rounding of the cells; however, the overall extent of the changes appeared similar to that observed when using lower concentrations (e.g., 1 mM) of each drug. Conversely, lower concentrations of the drugs (<1 mM) induced progressively less dramatic changes in cellular morphology with the kinetics of such changes being somewhat slower. Similar morphological changes were observed when another cAMP analog, 8-bromo-cAMP, was used to treat the cells.

Because of the intimate relationship between cell shape and the organization of the cytoskeleton, we examined whether alterations in the integrity of the cytoskeleton might accompany the morphological changes observed after drug treatment. Particular attention was paid to the actin MFs since previous studies had demonstrated similar changes in cell morphology after treatment with agents (e.g., cytochalasins) that perturb MF organization (10, 52, 54, 70; reviewed in references 65, 71) or treatment with hormones that increase cAMP (5). REF-52 cells, growing on glass coverslips, were incubated with 1 mM db-cAMP and 1 mM MIX for 60 min and then fixed; alternatively, the drugs were removed and the cells were incubated in drug-free medium for an additional 90 min before fixation. The distribution of the actin-containing MFs was analyzed by incubation with rh-phalloidin, an agent that binds specifically to F-actin (73). Shown in Fig. 2 A are the control cells (no drug treatment) stained for the distribution of the actin MFs. A rather typical distribution of the actin MFs and the stress fibers was observed. In contrast, the majority of those cells incubated in the presence of the drugs ($\geq 70\%$) no longer displayed any fibrillar actin MF bundles. Instead only residual punctate actin staining could be observed near the cell periphery and in some cases very near the nucleus (Fig. 2 D). Some of the cells (such as the cells seen in the upper right of Fig. 2 D) displayed residual stress fiber bundles for reasons as yet unknown. Removal of the drugs and further incubation of the cells in complete medium resulted in a gradual restoration of their normal cell morphology and an accompanying return of normal MF organization. For example, in cells allowed to recover from the drugs for 90 min a partial return of normal actin MF staining was observed throughout the cytoplasm (Fig. 2 F). By 6 h of recovery, the distribution of actin filaments appeared identical to that of the cells exposed to the drugs (data not shown).

Morphological Changes in Living Fibroblasts after Microinjection of the Catalytic Subunit of A-Kinase

Although db-cAMP and MIX have the overall effect of elevating intracellular cAMP levels and activating endogenous A-kinase, we were concerned that they may have other effects as well. Therefore, to ascertain in a direct fashion whether A-kinase might be involved in these changes in cell morphology and actin filament integrity, we examined the consequences of elevating intracellular A-kinase activity directly by microinjection of the purified catalytic subunit into the cytoplasm of living cells. Different amounts of the enzyme were used in initial experiments. Concentrations of <5 mg/ml were tested, and we decided to routinely use a concentration of 1 mg/ml purified catalytic subunit. As discussed in Materials and Methods, we estimate that injection of such a

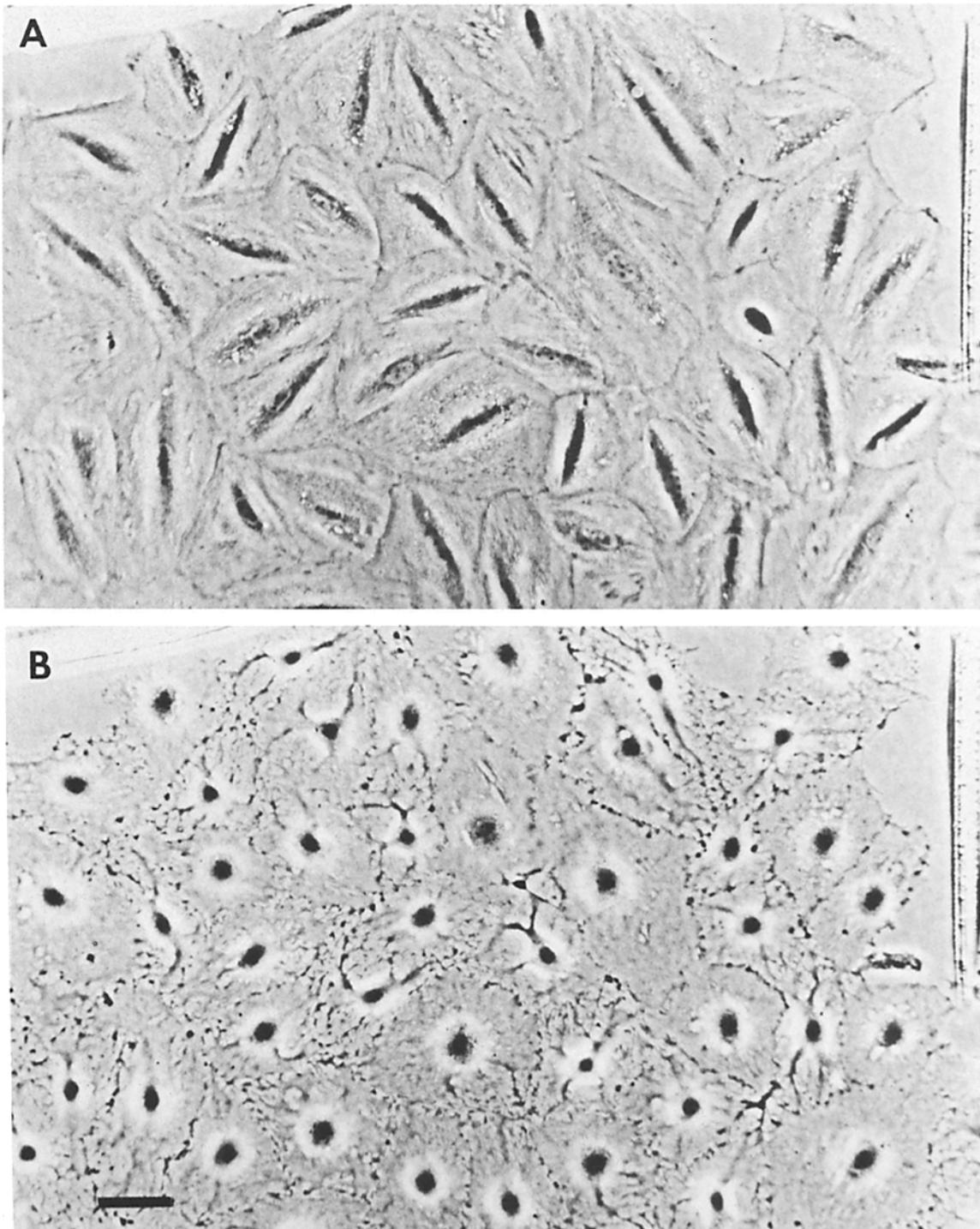


Figure 1. Changes in cellular morphology after treatment of cells with db-cAMP and MIX. Endogenous A-kinase activity levels were elevated by treating REF-42 cells with 1 mM db-cAMP (a freely cell-soluble analogue of cAMP) and 1 mM MIX (an inhibitor of the cAMP phosphodiesterase). After a 60-min exposure to the drugs, changes in cell morphology were recorded by phase-contrast microscopy. (A) Cells before the addition of the drugs. (B) Cells after a 60-min exposure to the drugs. Bar, 20 μ m.

solution of catalytic subunit results in an elevation of the A-kinase level to three to five times the maximal level of active catalytic subunits that could be obtained by stimulation of the endogenous A-kinase in cells.

Within 30 min after injection of A-kinase, REF-52 cells began to display an exaggerated thickening (rounding) near

the cell nucleus (Fig. 3 *b*). In addition, regions near the cell periphery appeared more pronounced or phase dense. These morphological changes after microinjection of the purified A-kinase appeared very similar to those observed in cells treated with db-cAMP and MIX (e.g., compare this to Fig. 1, but note that the cells were photographed at higher

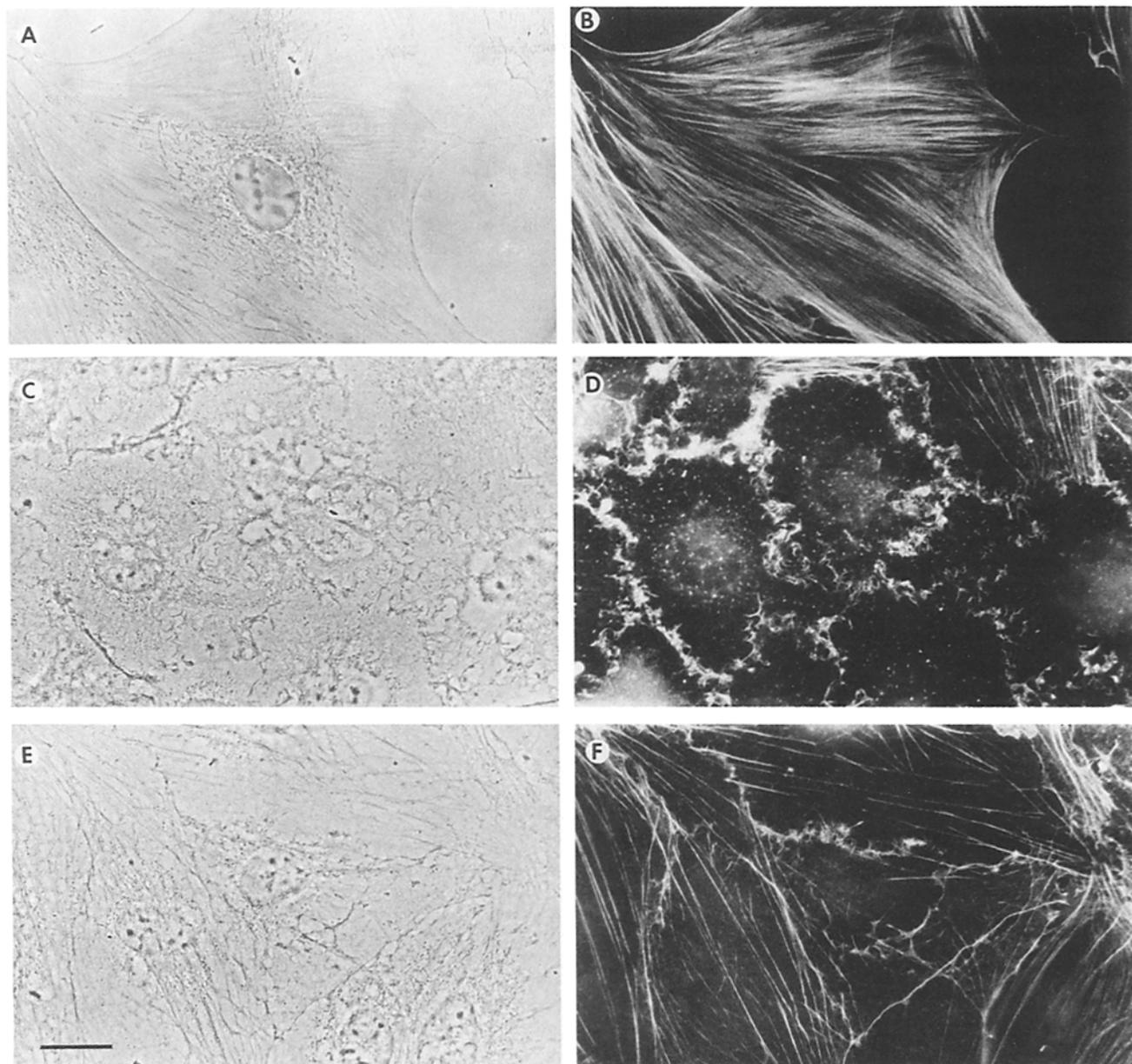


Figure 2. Treatment of cells with db-cAMP and MIX results in reversible changes in the integrity of the actin microfilaments. REF-52 cells, growing on glass coverslips, were treated with 1 mM db-cAMP and 1 mM MIX. After a 60-min exposure, one coverslip was removed and fixed with formalin as described in Materials and Methods. For the remaining cells, the medium containing the drugs was removed, the cells were washed three times with fresh culture medium, and then further incubated in the same for 90 min. These cells were then fixed with formalin and analyzed for the distribution of actin. Staining of the actin MFs was determined using rh-phalloidin as described in Materials and Methods. *A*, *C*, and *E* are the phase-contrast micrographs and *B*, *D*, and *F* are the corresponding fluorescent micrographs. (*A* and *B*) Untreated cells. (*C* and *D*) Cells treated with drugs for 60 min. (*E* and *F*) Cells after treatment with drugs for 60 min, removal of drugs, and further incubation for 90 min. Bar, 10 μ m.

magnification in Fig. 1). Moreover, the changes induced by microinjection of the catalytic subunit were also transient, by 24 h after injection the cells had regained their normal and well-spread character (Fig. 3 c). Microinjection of comparable amounts of BSA or A-kinase inactivated by multiple rounds of freezing and thawing had no effect on the morphology of the cells. Finally, injection of lower concentrations of the enzyme resulted in both a slower rate of change in cell morphology as well as a faster recovery of the cells to their normal morphology (data not shown).

Having demonstrated that microinjection of exogenous

A-kinase into living cells resulted in morphological changes similar to that observed after treatment of the cells with drugs that activate endogenous A-kinase, we next examined whether similar changes would occur with respect to the structure of the actin MFs (Fig. 4). Within 30 min after injection of A-kinase, the MF bundles (visualized again by rh-phalloidin) were reduced in both their number and apparent thickness (Fig. 4, *C* and *D*). By 60 min after injection the cells had become maximally rounded and displayed virtually no MF bundles (Fig. 4, *E* and *F*).

Indirect immunofluorescence analyses of the A-kinase in-

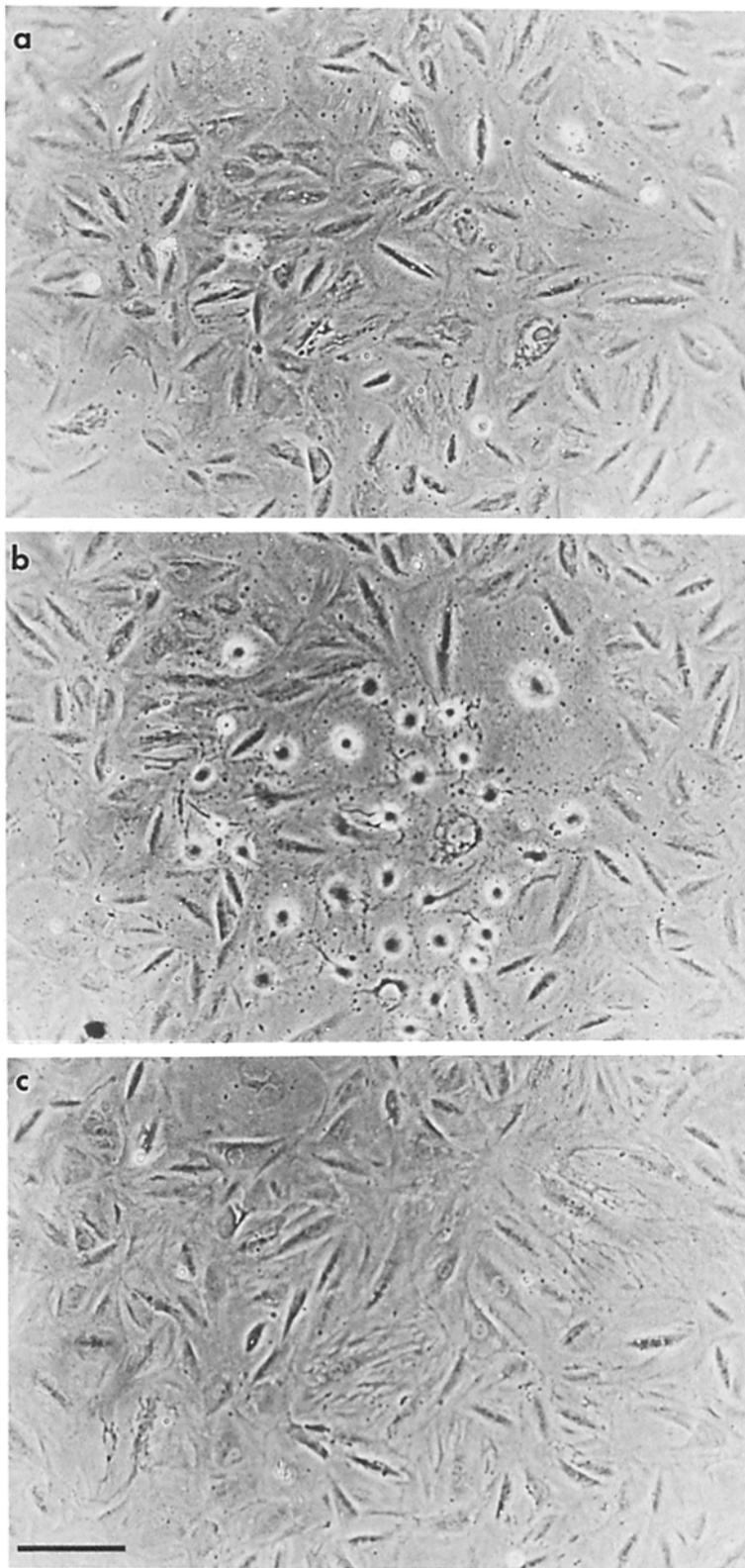


Figure 3. Morphological changes induced by microinjection of purified A-kinase. Approximately 25 REF-52 cells, growing on 35-mm Falcon dishes, were injected with a 1.0-mg/ml solution of the purified catalytic subunit of A-kinase. (The injected cells were marked by first placing an ink circle on the bottom of the culture dish.) Changes in the morphology of the living cells after microinjection were monitored by phase-contrast microscopy. (a) Cells before injection. (b) Cells 60 min after injection of the purified A-kinase. (c) Cells 24 h after injection of the purified A-kinase. Bar, 30 μ m.

jected cells using a variety of different antibodies specific to other MF-associated proteins (e.g., α -actinin, MLCK, myosin heavy chain, and tropomyosin) demonstrated the loss of intact MFs (data not shown). Further confirmation of the dissolution of the actin MFs was obtained by analyzing microin-

jected cells via whole mount EM (Fig. 5). While cells injected with the microinjection buffer alone displayed a typical pattern of MF bundles traversing the cytoplasm (Fig. 5 A), those cells injected with the purified kinase showed virtually no discernible 6-nm filaments (Fig. 5 B). Since the

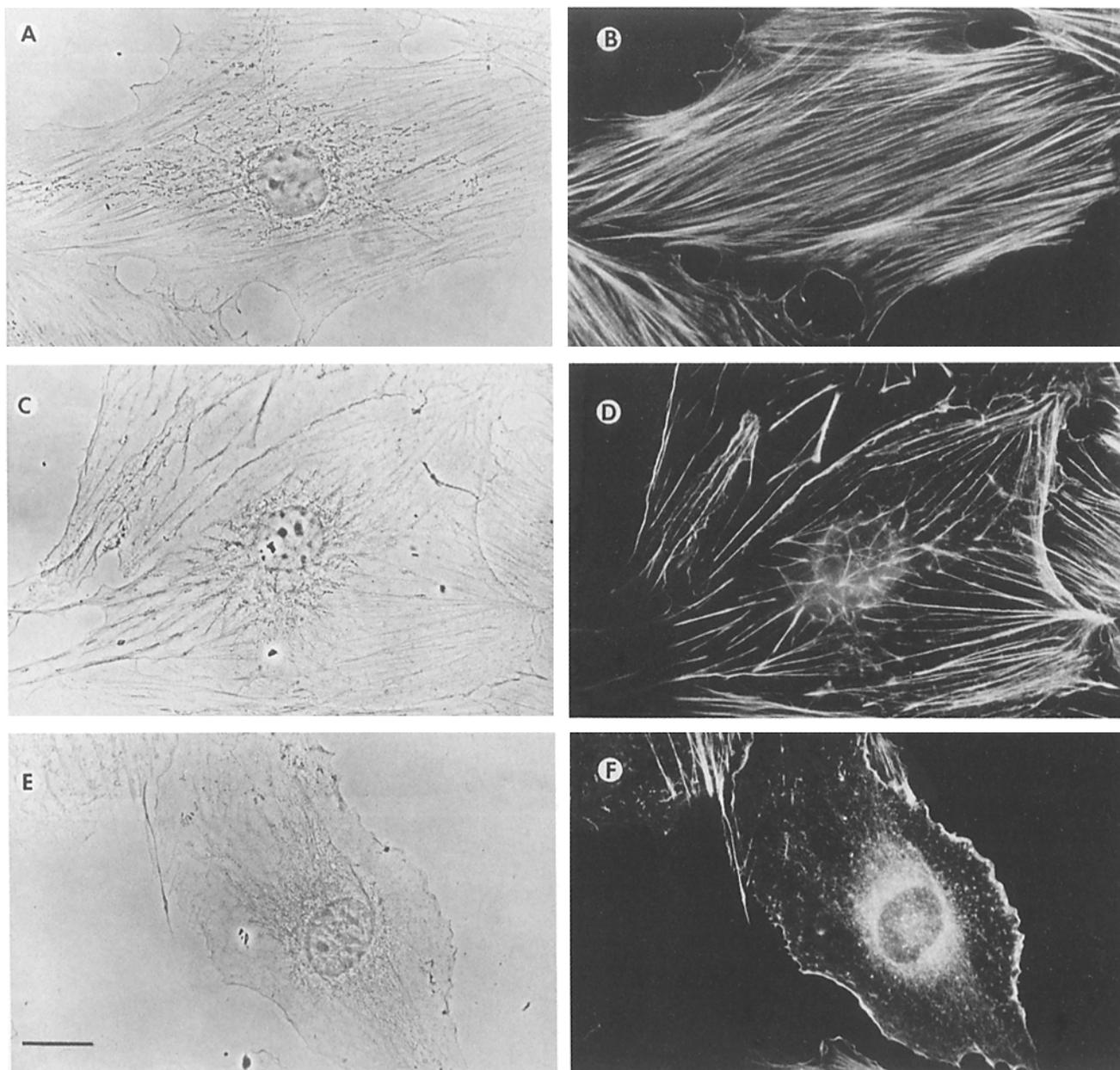


Figure 4. Changes in the integrity of MFs after microinjection of A-kinase. Subconfluent REF-52 cells, growing on glass coverslips, were injected with a 1.0-mg/ml solution of the purified catalytic subunit of A-kinase. At various times afterward, the cells were fixed and the distribution of the actin MFs was analyzed by incubation with rh-phalloidin. Shown are phase-contrast (*A*, *C*, and *E*) and the corresponding fluorescence (*B*, *D*, and *F*) micrographs of cells analyzed for the distribution of actin. (*A* and *B*) Control cells injected with microinjection buffer alone. (*C* and *D*) Cells injected with purified A-kinase and further incubated for 30 min. (*E* and *F*) Cells injected with purified A-kinase and further incubated for 60 min. Bar, 10 μ m.

fixation conditions for EM are different (2% glutaraldehyde) from those used for fluorescence, these findings support the conclusion that the injection of A-kinase results in the disruption of the MF.

To determine whether these alterations in the integrity of the MFs were reversible, we performed a time course experiment analyzing the cells at various times after injection of the purified kinase. REF-52 cells were injected with a 1.0-mg/ml solution of the kinase and at various times thereafter

the cells examined by phase-contrast and fluorescence microscopy for the distribution of the actin MFs (Fig. 6). 90 min after injection, the cells were still rounded and devoid of most of their fibrillar actin (Fig. 6, *A* and *B*). 3 h after injection the cells had begun to respread and exhibited a partial return of their actin MF bundles (Fig. 6, *C* and *D*). By 30 h after injection, both cell morphology and the distribution of actin appeared indistinguishable from that of the control cells (Fig. 6, *E* and *F*).

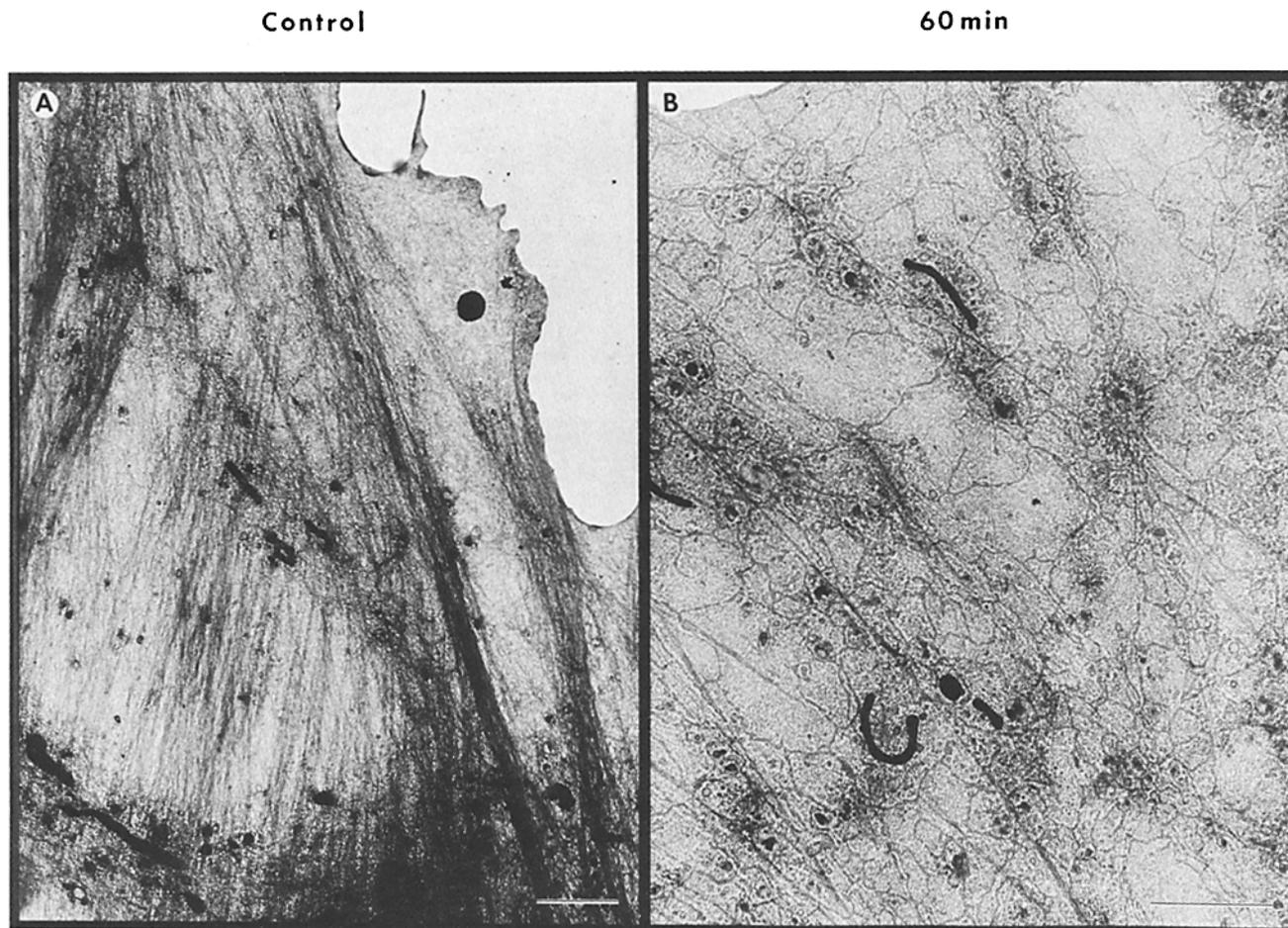


Figure 5. Analysis of cells microinjected with A-kinase by whole mount EM. REF-52 cells growing on formvar-coated glass coverslips were injected with the purified A-kinase catalytic subunit (1 mg/ml) or with microinjection buffer alone. 60 min after injection the cells were fixed and analyzed by whole mount EM as described in Materials and Methods. (A) A single cell injected with microinjection buffer alone. (B) A single cell injected with the purified catalytic subunit. Bar, 10 μ m.

Phosphoprotein Changes in Cells Injected with A-Kinase

As the mechanism of action of A-kinase is presumably via phosphorylation of substrate proteins, changes in the phosphorylation status of cellular proteins after microinjection of A-kinase were examined. For these studies, cells were plated on small 1-mm² glass coverslips. Each and every cell on a coverslip was injected either with the kinase or with the microinjection buffer alone, and at various times thereafter the cells were metabolically labeled with [³²P]H₃PO₄. After the labeling period the cells were solubilized and the labeled proteins analyzed by two-dimensional gel electrophoresis. The phosphoproteins were visualized by autoradiography of the gels (exposure times of 3–14 d with an enhancing screen). The phosphoprotein pattern of cells (~100 cells total) injected with buffer (i.e., control) and pulse labeled for the period 30–60 min after injection are shown in Fig. 7 A. This pattern appears similar to the typical phosphoprotein pattern obtained by labeling a whole dish of cells, indicating that the microinjection procedures alone have no effect on the phosphoprotein pattern. After injection of A-kinase, an overall increase in protein phosphorylation was observed within the first 30 min (Fig. 7 B). Although at least 20 protein species

showed changes in their phosphorylation status, the most prominent increase in phosphorylation occurred on vimentin (V) and two proteins that we have identified as the nuclear lamins A and C (L in Fig. 7). Similar changes in phosphorylation of both these proteins have been reported previously in cells treated with drugs or hormones that elevate cAMP (14, 30, 31, 33, 41). Our assignment of the positions of both vimentin and the nuclear lamins were confirmed by immunoblotting with the appropriate monospecific antibodies (data not shown). Cells labeled 30–60 min after injection (i.e., the period during which the major changes in cell morphology and actin organization occur) displayed both the maximal levels of protein phosphorylation and a number of new phosphoproteins (at least five) not apparent in the previous 30-min labeling period (Fig. 7 C). Labeling during the period 60–90 min after injection showed a gradual decrease in overall protein phosphorylation as compared to the levels observed during the previous time periods (Fig. 7 D).

Since a number of phosphoproteins including myosin light and heavy chain and MLCK have been identified as key proteins in the regulation of actomyosin contractility, we chose to further analyze whether changes in these phosphoproteins

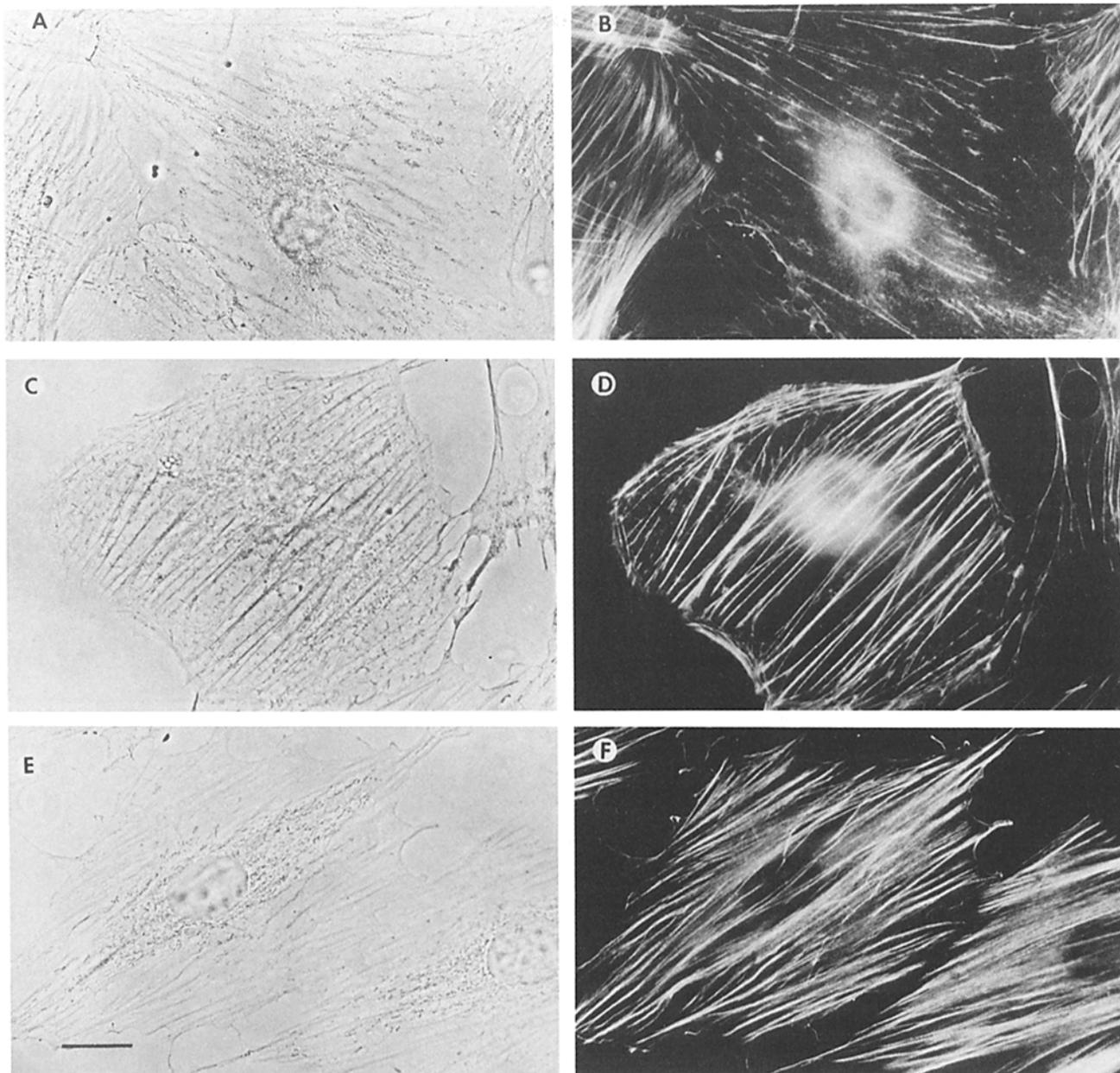


Figure 6. The effects on actin MF integrity after A-kinase injection are fully reversible. In an experiment similar to that described in Fig. 2, REF-52 cells were injected with purified A-kinase and the distribution of the actin MFs was analyzed at various periods after injection using rh-phalloidin. Phase-contrast micrographs (*A*, *C*, and *E*) and the corresponding fluorescence micrographs (*B*, *D*, and *F*) of the actin MFs are shown. (*A* and *B*) Cells 90 min after injection. (*C* and *D*) Cells 180 min after injection. (*E* and *F*) Cells 30 h after injection. Bar, 10 μ m.

occurred after injection of A-kinase. Because of the low molecular mass and expected isoelectric point of MLC, we were especially interested in examining proteins of low molecular mass that migrated near the acidic end of the gels. Consequently, the same gels analyzed in Fig. 7, *A-D*, were reexposed for a longer period of time and this region of the autoradiograms (*boxed area*) was magnified (*E-H*). In the case of cells injected only with the microinjection buffer, a prominent ≈ 20 kD protein near the acidic end of the gel was apparent. Owing to its similar size and isoelectric point to

that reported for the MLC, and its recognition by antibodies specific for MLC (see below), we have designated this protein as *M* in the figure. In cells injected with A-kinase and labeled 0–30 min after injection (*F*) a slight decrease in the phosphorylation of *M* was observed. Labeling of the cells 30–60 min (*G*) or 60–90 min after injection (*H*) revealed little or no continued phosphorylation of the *M* protein. While several other proteins exhibited a similar decreased phosphorylation with time after injection, the overall reduction in *M* protein phosphorylation appeared prominent.

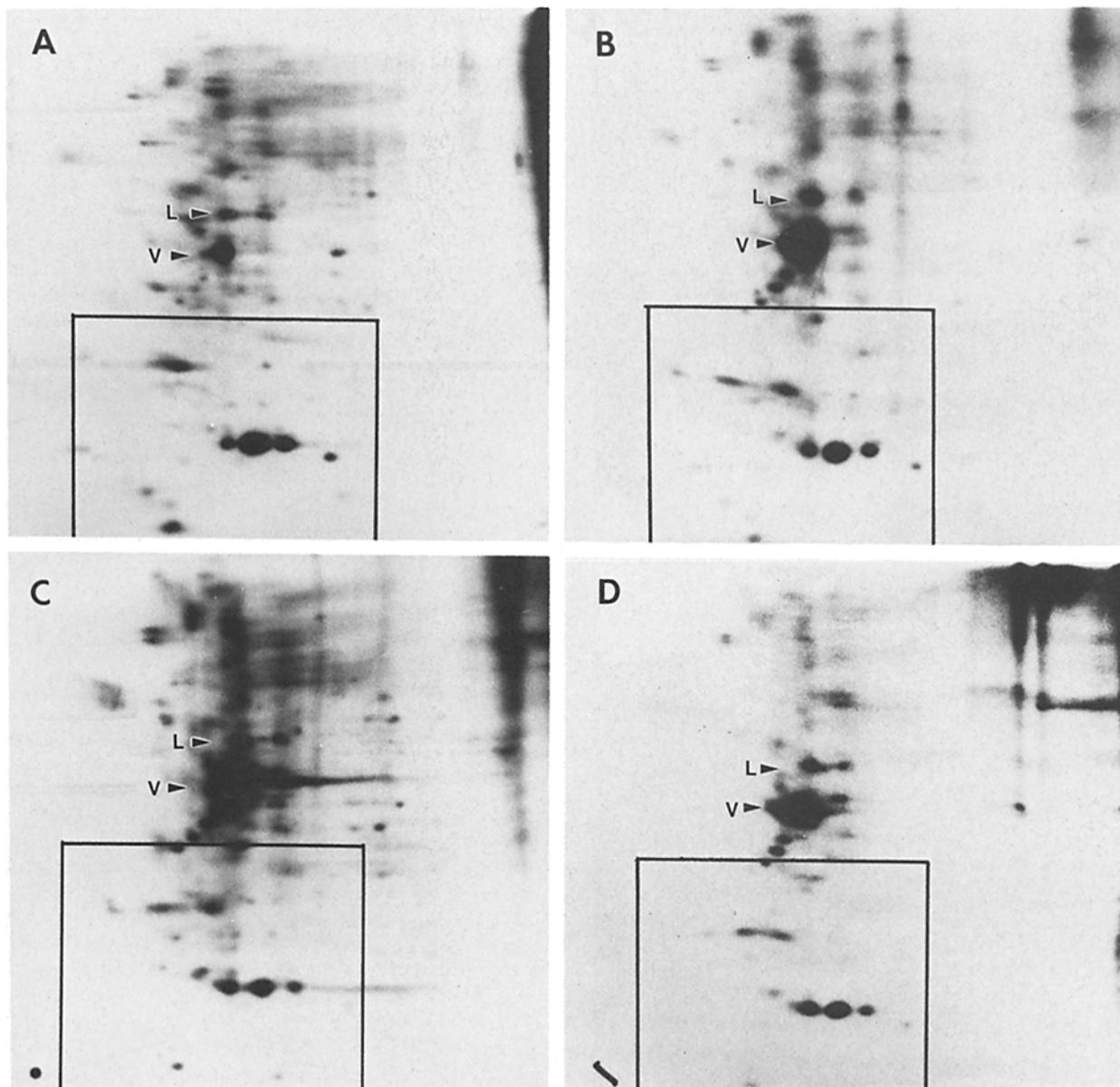
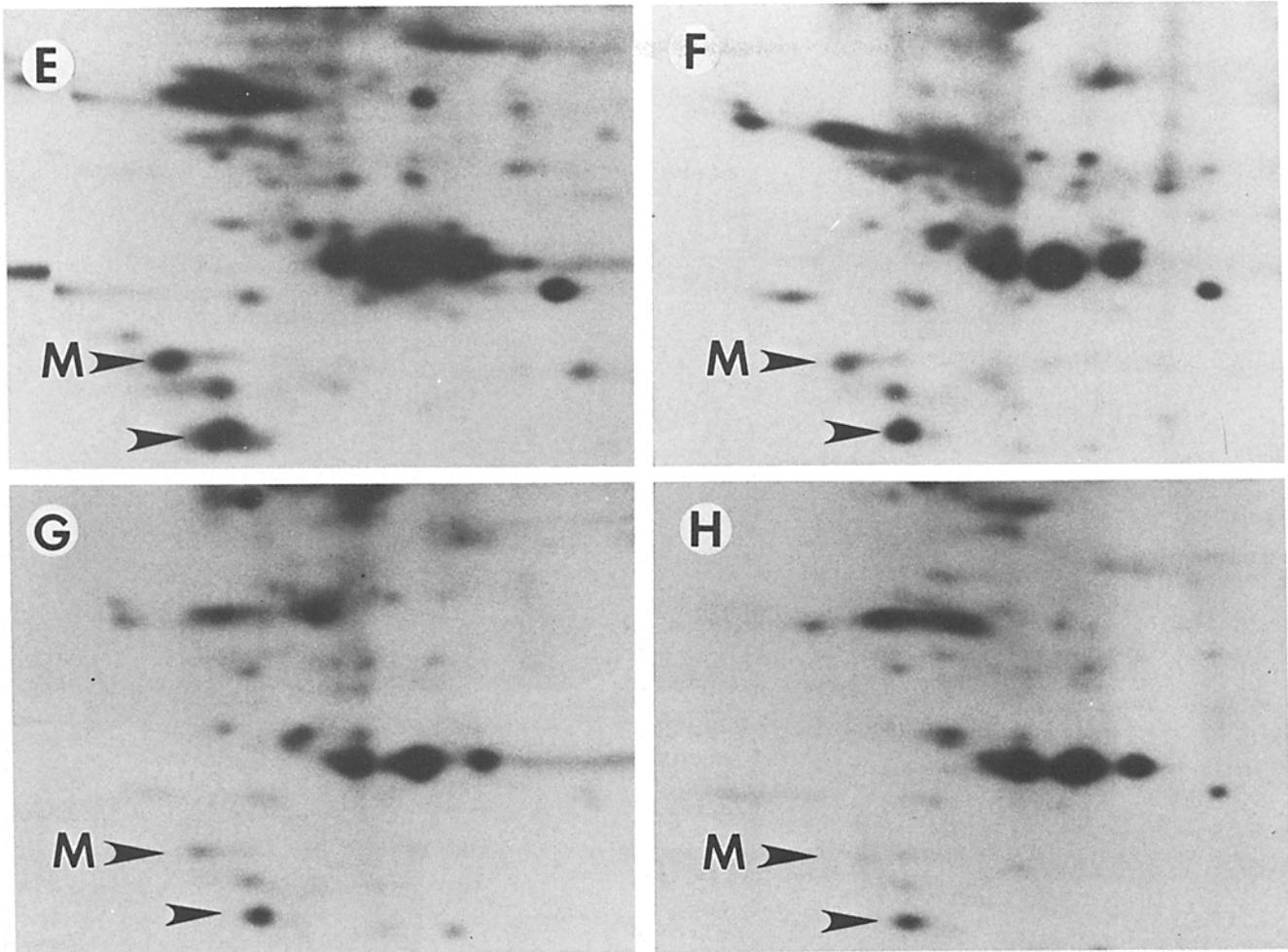


Figure 7. Analysis of phosphoprotein changes after microinjection of the catalytic subunit of A-kinase. REF-52 cells, growing on 1-mm² glass coverslips, were microinjected with either buffer alone or with a 1.0-mg/ml solution of the purified subunit of A-kinase. (Every cell on the coverslip was injected.) At various times after injection, the cells were rinsed with DME lacking phosphate and metabolically labeled with [³²P]H₃PO₄ for 30 min. After the labeling period, the cells were solubilized in lysis buffer and the labeled proteins analyzed by two-dimensional gel electrophoresis as described in Materials and Methods. Shown are autoradiograms of the gels (the acidic end is to the left) with the major phosphoproteins vimentin (*V*) and the nuclear lamins (*L*) marked (*A-D*). Because of the differential levels of phosphorylation among the various substrate proteins, we have included longer exposures (14 d instead of 3 d) of that region of the gel containing the putative myosin light chain (boxed areas of *A-D* which correspond to *E-H*, respectively). (*A*) Approximately 100 cells injected with microinjection buffer alone and labeled 30–60 min after injection. (*B*) Approximately 30 cells injected with A-kinase and labeled 0–30 min after injection. (*C*) Approximately 30 cells injected with A-kinase and labeled 30–60 min after injection. (*D*) Approximately 30 cells injected with A-kinase and labeled 60–90 min after injection. The regions of the gels containing the putative myosin light chain from *A*, *B*, *C*, and *D* are shown respectively in *E*, *F*, *G*, and *H*. (*E-H*) *M*, the position of the putative myosin light chain; arrowhead, the position of a different phosphoprotein in the same region as myosin light chain. (Note that three times as many cells were analyzed in panels *A* and *E* [control, microinjection buffer] owing to the higher incorporation of radiolabel in those cells injected with the A-kinase.)



To confirm that this 20-kD protein designated M was indeed the MLC, Western blot analysis of two-dimensional gels was carried out using monospecific antibodies raised against the protein purified from smooth muscle (very kindly provided by J. R. Stull and colleagues). In addition, using an antibody to the MLCK, we determined its two-dimensional coordinates. In duplicate, cells were injected with purified A-kinase, labeled with [^{32}P]H $_3$ PO $_4$ for the period 30–60 min after injection (when the maximal changes in the actin MF organization occur), and then harvested. An aliquot of unlabeled “carrier” cells was added to the radiolabeled cells (to allow for Western blot analysis), the proteins were separated by two-dimensional gel electrophoresis and transferred to nitrocellulose, and the positions of MLC and MLCK were determined. After detection of the antibody reaction products, the nitrocellulose sheets were exposed to film to reveal the position of the [^{32}P]H $_3$ PO $_4$ -labeled proteins. Fig. 8 A shows the position of the myosin light chain as revealed after color development of the blot. Clearly, two spots are present which we believe correspond to the phosphorylated and dephosphorylated isoforms of the myosin light chain. Fig. 8 B shows the phosphoprotein pattern of the cells injected with buffer. The position of the heavily phosphate-labeled protein indicated corresponded exactly to that of the more abundant and acidic form of the two myosin light chain spots as determined by immunoblotting. In the cells

injected with A-kinase, this same protein was observed to contain significantly less radiolabel (Fig. 8 C). Hence, we conclude that the 20-kD protein whose overall incorporation of [^{32}P]H $_3$ PO $_4$ decreases after A-kinase injection is indeed the MLC. We have observed that the extent of MLC phosphorylation decreases with time after A-kinase injection and that by 75 min after injection little or no phosphorylation of the protein occurs. As was shown earlier, this is the same time after injection when the actin MF appeared maximally disassembled.

In the same studies, examining the phosphorylation status of MLCK, a result opposite to that obtained with myosin light chain was observed. In Fig. 8 D the position of MLCK, as determined by immunoblotting, is shown. The protein migrated with a relatively basic isoelectric point and appeared heterogeneous. In cells injected with microinjection buffer alone, little or no phosphorylated proteins could be observed in this region of the two-dimensional gels (Fig. 8 E, arrowhead). However, after injection of A-kinase, a series of phosphorylated proteins which migrated with the same coordinates as that determined for the MLCK were observed (Fig. 8 F, arrowhead). Note also the proteins directly under those designated by the arrowhead that appear, qualitatively, very similar to the intact MLCK. We suspect that these are proteolytic fragments of the intact protein as others have previously reported (2, 39). Hence, after

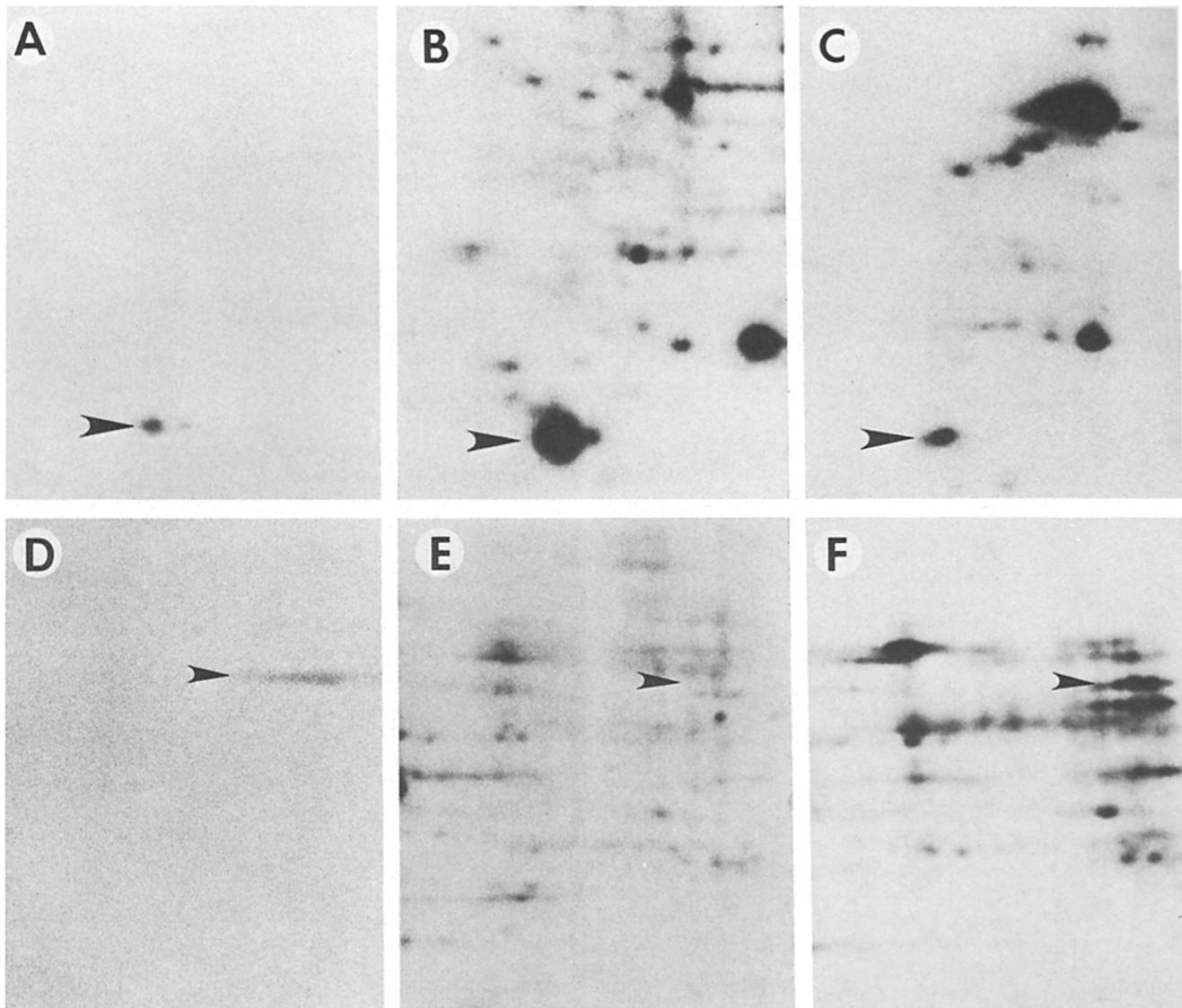


Figure 8. A-kinase-induced changes in MLCK and myosin light chain phosphorylation as determined by Western blot analysis. REF-52 cells growing on small (1-mm²) glass coverslips were microinjected with either the catalytic subunit of A-kinase or with a control protein. 30 min after injection the cells were labeled with [³²P]H₃PO₄ for 30 min, harvested, and then mixed with an excess of unlabeled REF-52 cells. The cell lysates were subjected to two-dimensional gel electrophoresis, the proteins transferred to nitrocellulose, and the position of either MLCK or myosin light chain determined by incubation with the appropriate antibody and subsequent visualization with a second horseradish peroxidase-conjugated antibody, and color development (A and D). The nitrocellulose was then exposed to film to reveal the phosphate-labeled proteins. Shown in B, C, E, and F are the autoradiogram. (A-C) Analysis of myosin light chain phosphorylation; A shows the position of the light chain as determined by immunoblotting, B the phosphate-labeled proteins in cells injected with control proteins, and C the phosphate-labeled proteins in cells injected with A-kinase. Note that only those portions of the gel analyzing myosin light chain are shown. (The position of the myosin light chain is indicated by an arrowhead.) (D-F) Analysis of MLCK phosphorylation; D shows the position of MLCK as determined by immunoblotting, E the phosphate-labeled proteins in cells injected with control protein, and F the phosphate-labeled proteins in cells injected with A-kinase. Note that only those regions of the gel analyzing MLCK are shown. (The position of MLCK is indicated by an arrowhead.)

A-kinase injection there occurred a transient increased phosphorylation of MLCK and a corresponding decreased level of new myosin light chain phosphorylation.

Effects of Microinjection of Antibodies Specific for MLCK on Actin MF Integrity and Myosin Light Chain Phosphorylation

The data described above are consistent with the idea that phosphorylation of MLCK catalyzed by A-kinase could re-

sult in the inactivation of MLCK, the subsequent dephosphorylation of MLC, and the dissolution of the actomyosin MFs. Therefore, we chose to test the effect of directly inhibiting MLCK activity *in vivo* by injecting antibodies specific for the kinase into living cells. For these studies rabbit polyclonal antibodies were affinity purified using native MLCK from smooth muscle. Before testing their efficacy *in vivo*, we tested the inhibitory activity of these antibodies on the Ca⁺⁺/calmodulin-dependent phosphorylation of purified myosin

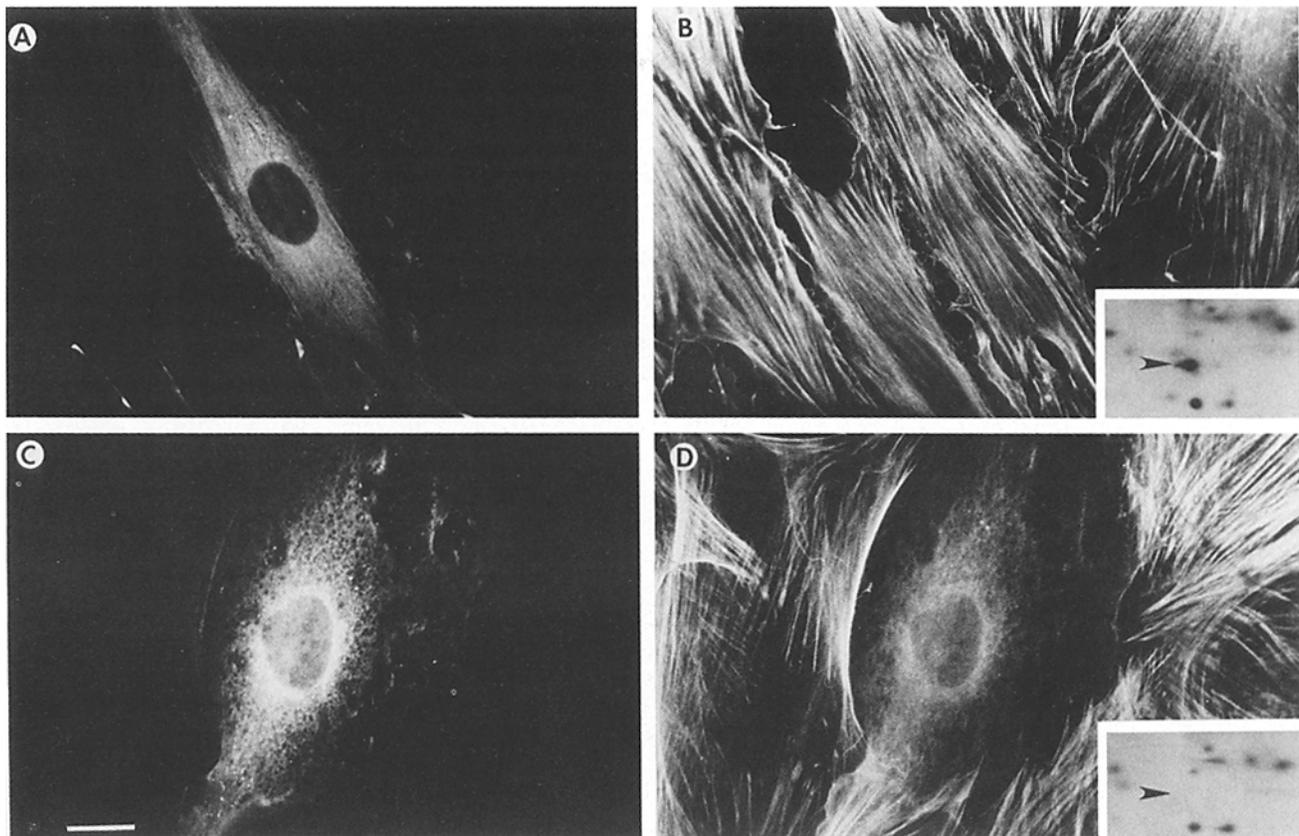


Figure 9. Microinjection of antibodies specific to native MLCK results in the dephosphorylation of myosin light chain and the disassembly of the actin MFs. REF-52 cells growing on glass coverslips were microinjected with either affinity-purified MLCK antibody or with a nonspecific control, fluorescently labeled antibody. 45 min after injection the cells were fixed and analyzed for the distribution of the actin MFs using rh-phalloidin. In parallel, cells growing on 1-mm² glass coverslips were injected with the same antibodies, and 45 min later labeled with [³²P]H₃PO₄ for a period of 1 h. The cells were then harvested and the labeled proteins analyzed by two-dimensional gel electrophoresis. Insets in *B* and *D* are the regions of the autoradiograms covering the area of migration of myosin light chain. The position of the myosin light chain is indicated by an arrowhead. (*A* and *B*) Cells microinjected with control antibodies and stained for either the injected antibody (*A*) or the actin MFs (*B*). Inset in *B* shows the position of the myosin light chain in the cells injected with the control antibody and labeled with [³²P]H₃PO₄. (*C* and *D*) Cells microinjected with anti-MLCK and stained for the injected antibody (*C*) or the actin MFs (*D*). Inset in *D* shows the position of the myosin light chain in the cells injected with the anti-MLCK antibody and labeled with [³²P]H₃PO₄.

light chain by purified MLCK *in vitro*. We found that a 0.5-mg/ml solution of the affinity-purified anti-MLCK antibody inhibited 90% of the Ca⁺⁺/calmodulin-dependent phosphorylation of MLC by purified MLCK. In contrast, up to 10-mg/ml solutions of control IgGs had little or no effect on the kinase activity under the same assay conditions. To assess the effects of the inhibitory antibodies *in vivo*, REF-52 cells, growing on glass coverslips, were injected with either control nonspecific antibodies (e.g., rabbit anti-rat IgG) or alternatively the affinity-purified rabbit anti-MLCK antibody. 45 min later, the cells were fixed and analyzed for the distribution of the actin MFs, using rh-phalloidin. To identify the injected cells, the coverslips were incubated with fluorescein-labeled goat anti-rabbit antibody. Fig. 9, *A* and *B*, shows a field of about six cells, one of which was injected with the control IgG as shown by the fluorescent staining of the injected antibody (Fig. 9 *A*). All of the cells, including the single cell injected with the control antibody, exhibited a normal distribution of actin MFs (*B*). Approximately 50 such injected cells were examined after injection of non-

specific IgGs, and all showed a normal distribution of actin MFs. Fig. 9, *C* and *D* shows a field of cells in which one cell was injected with the affinity-purified anti-MLCK antibody. Only that cell containing the MLCK antibody (*C*) showed a loss of filamentous actin staining (*D*). Of ~150 cells injected with affinity-purified anti-MLCK, at least 95% showed such a complete loss of actin MF structure by 45 min after injection. Dilution of this antibody to ~25 µg/ml abolished the effect on the microfilaments. Interestingly, cells injected with the affinity-purified MLCK antibody did not exhibit the same changes in cell morphology, such as the cell rounding observed after injection of A-kinase. Since in both cases the extent of actin reorganization appeared similar, apparently there must occur other changes in the cells after A-kinase injection that result in the rounded phenotype.

Finally, to ascertain whether the changes in actin MF integrity after injection of the MLCK antibody were correlated with changes in the phosphorylation status of myosin light chain, phosphorylation studies again were performed. As described before, ~100 cells on small coverslips were in-

jected with either a control, nonspecific antibody or with the affinity-purified anti-MLCK antibody; then the cells were labeled with [^{32}P]H $_3$ PO $_4$ for ~ 30 min, and the labeled proteins analyzed by two-dimensional gel electrophoresis. Cells injected with the control antibodies exhibited a typical pattern of myosin light chain phosphorylation (Fig. 9 B, *inset*). In contrast, those cells injected with the anti-MLCK antibody showed virtually no phosphorylated myosin light chain (Fig. 9 D, *inset*). Under these conditions, we observed no other major changes in protein phosphorylation such as that observed after microinjection of A-kinase. Namely, injection of the antibody did not result in any increase in MLCK, lamin, or vimentin phosphorylation like that seen in cells injected with A-kinase. Therefore, inhibition of MLC phosphorylation can occur either as a result of A-kinase-mediated phosphorylation of MLCK or alternatively by direct inhibition of MLCK activity via injection of anti-MLCK antibodies.

Discussion

Using the direct approach of microinjection, we have examined the role of A-kinase and MLCK in the regulation of non-muscle cell structure and the integrity of actin MFs. Elevation of A-kinase levels via microinjection of the purified catalytic subunit of A-kinase resulted in cell rounding and dissolution of the microfilaments. Using metabolic labeling of phosphoproteins after A-kinase injection, we provide data implicating changes in the phosphorylation status of myosin light chain and MLCK as a potential molecular basis for the changes observed in MF integrity.

Changes in Cellular Morphology after Elevation of A-Kinase Activity

A number of previous reports have detailed changes in cell morphology after activating endogenous A-kinase using either drugs or beta-adrenergic hormones (5). However, others (16, 44, 53, 72; reviewed in reference 43) have described opposite morphological effects of elevating A-kinase activity in transformed cells, which they termed "reverse transformation" (16, 43, 44, 53). In these latter studies, cells were observed to flatten by 24 h after the administration of the drugs that activated A-kinase.

We have studied the effects of elevating A-kinase levels in 20 different cell lines, several of which are transformed. In all cases, the predominant early effect of elevating endogenous A-kinase activity was to cause cells to round (with transformed cells appearing more rounded). These changes, which we observed during the first 6–12 h, were consistent whether using drugs, beta-adrenergic hormones, or after microinjection of A-kinase. We found, however, that cell rounding induced after inactivation of A-kinase via drug or hormone treatment was often times maintained only for <24 h, after which time the cells reverted to their original flattened morphology and become refractory to further exposure to the drugs. However, microinjection of these cells previously treated with drugs with A-kinase still resulted in cell rounding. Therefore, it would seem that the cell is capable of "down-regulating" the levels of A-kinase. Others have suggested that specific mechanisms exist by which cells can

modulate the level of A-kinase activity during different phases of the cell cycle (38, 74) and down-regulation mechanisms have been proposed for A-kinase in fibroblasts (7, 38). This down-regulation may be analogous to that described for C-kinase where there is an apparent loss of C-kinase activity in cells after chronic exposure to one of its activators, phorbol myristate acetate (18). Hence, we speculate that cell rounding was an early effect of the activation of A-kinase while cell flattening is a long-term effect associated with down-regulation of A-kinase.

Changes in MF Integrity and Protein Phosphorylation after Elevation of A-Kinase Activity through Microinjection

In addition to the effects on cell morphology, the present studies have detailed complex changes in the actin MF networks after elevation of A-kinase activity. In the case of smooth muscle cells there is substantial evidence indicating a role for A-kinase, MLCK, and myosin light chain in regulating the contractility of actomyosin (2, 4, 22, 29, 39, 57, 66). The enzyme MLCK, which requires Ca $^{++}$ and calmodulin for its kinase activity, catalyzes the phosphorylation of the regulatory or MLC (2, 21, 29, 36; reviewed in reference 1, 39). This phosphorylation of the MLC has been correlated with increases in both the actin-dependent Mg-ATPase activity of myosin (reviewed in 22, 35) and the avidity of the whole myosin complex for actin (68), resulting in a concomitant stimulation of actomyosin contraction and/or certain types of cell motility (3, 6, 24, 67). Because phosphorylated MLC has a short half-life in cells (2, 7, 22, 39, 57, 66), phosphorylation/dephosphorylation may provide for rapid changes in the contractility of the actomyosin system. Interestingly, the enzyme MLCK has been colocalized with myosin along the length of the stress fibers, and, as such, would appear to be adequately situated to effect such a regulatory role (28). Since the phosphorylation of MLCK by A-kinase can inhibit MLCK activity *in vivo* (26, 60), presumably via inhibiting the binding of Ca $^{++}$ /calmodulin to MLCK (1, 3, 21, 37, 47), it has been suggested that cAMP plays a role in the regulation of smooth muscle contractility as opposed to a regulatory mechanism involving solely Ca $^{++}$ /calmodulin (22, 39, 66).

We have shown that injection of A-kinase into living non-muscle cells results in an increased phosphorylation of MLCK, a decreased incorporation of phosphate into MLC and a loss of actin MF bundles. Such data provides strong *in vivo* evidence for a role of cAMP and A-kinase in the regulation of actin MF assembly in nonmuscle cells through the increased phosphorylation of MLCK and a concomitant decreased phosphorylation of the myosin light chain. However, it remains possible that as yet other unidentified phosphorylation/dephosphorylation events may occur after injection of the purified A-kinase that may aid in the disassembly of the actin filaments. Indeed we did observe numerous other changes in the phosphorylation status of various proteins after injection of A-kinase. Although the injected A-kinase seems to cause a complete disassembly of the MFs, it is possible that short, single MFs remain but are undetectable under the fixation conditions used here. We are currently addressing this issue using fluorescent probes in living cells.

Alterations in MF Integrity and Protein Phosphorylation after Microinjection of Antibodies Directed against MLCK

To address more directly the possible role of MLCK in the regulation of the MFs, we have examined the effect of specifically inactivating MLCK *in vivo* through the injection of inhibitory antibodies directed against it and have found that this inactivation is in of itself sufficient to cause both the dephosphorylation of MLC and the concomitant dissolution of the actin MF bundles. We introduced a number of antibodies into living cells in addition to the antibodies directed against MLCK. Only affinity-purified anti-MLCK antibodies, which also inhibit MLCK activity *in vitro*, led to the reorganization of MF and MLC dephosphorylation, whereas a number of other purified antibodies directed against other MF-associated proteins had no such effect. These data strongly suggest that the disassembly of MF after anti-MLCK injection results from the specific inactivation of MLCK and the consequent dephosphorylation of MLC as opposed to an effect involving solely the binding of the antibodies to the MLCK present in the MF bundles. These data, coupled to the results obtained after injection of A-kinase, appear to link the dephosphorylation of the MLC with the disassembly of the actin MFs in nonmuscle cells. While the molecular details governing the relationship between MLC phosphorylation and the integrity of the actin MF bundles are not completely clear, we can offer some speculation. If dephosphorylation of MLC has effects similar to those described in smooth muscle (i.e., reduces the myosin ATPase activity and avidity of the myosin head for actin), then we might suggest that the filament would become destabilized by the loss of phosphorylated MLC, thereby leading to the disassembly of the actomyosin-containing MFs. However, while these data imply the involvement of MLC phosphorylation in MF assembly/disassembly, they do not exclude the potential role of any of the other MF-associated proteins in this process. Indeed, the dephosphorylation of MLC (after injection of either A-kinase or the inhibitory antibodies) or the injection of the inhibitory antibodies, may well perturb the function or binding of any of the other MF-associated proteins (56) and thereby contribute to the destabilization of the actin microfilaments.

Relationship of the Effects of Drugs that Elevate Endogenous A-Kinase with that of Microinjection of Exogenous A-Kinase

While most of the cellular changes elicited by the drugs that elevate endogenous cAMP levels appeared similar to those observed after A-kinase injection, we did observe one discrepancy. As discussed above, in those cells microinjected with the enzyme, a reduced level of MLC phosphorylation was observed. Interestingly, however, in drug-treated cells the level of MLC phosphorylation was only slightly reduced or, in some experiments, unchanged (data not shown). This latter finding is consistent with that of Bayley and Rees (7), who showed that db-cAMP treatment of fibroblasts leads to only a small and highly transient decrease in the level of MLC phosphorylation. While there are many possible explanations for this transient effect of the drugs on the level of phos-

phorylation of the MLC, we suspect that this may be due to the phosphorylation of the MLC by kinases other than the MLCK, that are somehow affected or stimulated by the pharmacological agents. For example, at least four other protein kinases have been reported to phosphorylate MLC at different sites *in vitro* (61). We are currently performing peptide mapping analysis of the phosphorylated MLC to determine the sites of phosphorylation under the different conditions that lead to a dissolution of the actin MFs (i.e., drug-treated vs. A-kinase-injected cells).

In addition to the changes in MLC and MLCK phosphorylation detailed above, very obvious changes in the phosphorylation status of vimentin also were observed in cells injected with A-kinase (Fig. 7, B-D). It is of interest to note that immunofluorescence analysis revealed the vimentin-containing intermediate filaments to rapidly collapse in and around the nucleus, and in some types dissolve altogether, shortly after A-kinase injection (data not shown). Since others have implicated changes in the organization of intermediate filaments as a function of vimentin phosphorylation (12, 14, 17, 30, 31, 33, 41, 63), we are pursuing the possibility that the phosphorylation of vimentin, catalyzed via A-kinase, may serve to regulate intermediate filament integrity in these cells. Finally, with respect to the change in cell morphology, injection of A-kinase induced cell rounding, while injection of the anti-MLCK had no obvious effect on the morphology of the cells. Nevertheless, both treatments effectively induced the disassembly of the actin MFs. Taken together with the effect of A-kinase on intermediate filaments, these results indicate, not too surprisingly, that cell shape is not determined solely by the integrity of the MFs but perhaps involves the contribution of both MF and intermediate filament networks.

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