lation in normal embryos to that in embryos that were UV-irradiated as zygotes to block subcortical rotation. The IP_3 mass at the 256-cell stage was not significantly different in UV-irradiated (100.2 ± 10.8 fmol per embryo) and control embryos (103.6 ± 9.0 fmol per embryo) (*16*). Subcortical rotation is therefore not a prerequisite for IP_3 accumulation in the cleavage stage embryo, and our data do not support the suggestion that zygotic UV irradiation should globally enhance PI cycle activity during mesoderm induction (*7*).

Combined with the demonstration of rescue from Li^+-induced teratogenesis by provision of exogenous *myo*-inositol (*6*), our data documenting PI cycle inhibition and depression of IP_3 masses by teratogenic Li^+ treatments strongly support the hypothesis that the effects of lithium on *Xenopus* development depend in large measure on its ability to inhibit the PI cycle signal transduction system. Lithium is also a documented teratogen in human embryos (*17*), in which its mechanism of action remains unknown.

The PI cycle probably functions in normal development, as suggested by both the abrupt rise in IP_3 mass, which is coincident with the onset of mesoderm induction, and the effect of PI cycle inhibition on axis determination. As we shall report (*18*), when mammalian serotonin type 1C receptors (which activate the embryonic PI cycle when stimulated) are expressed dorsally and are activated during mesoderm induction, dorsoanterior specification is blocked in intact embryos, and both convergent extension and transcription of the cardiac actin gene (indicators of mesoderm induction) are inhibited in explants from the animal pole treated with exogenous activin A. Thus, the PI cycle seems to be part of a complex suite of signal transducers involved in induction. One role of the PI cycle, which the data reported here and elsewhere (*6*) suggest, could be as a mediator of the negative feedback signal recently postulated to modulate the induction process (*19*).

REFERENCES AND NOTES

1. E. A. Jones and H. R. Woodland, *Development* **101**, 557 (1987); R. L. Gimlich and J. C. Gerhart, *Dev. Biol.* **104**, 117 (1984).
2. H. Grunz and L. Tacke, *Wilhelm Roux Arch.* **195**, 467 (1986).
3. J. M. W. Slack, B. G. Darlington, J. K. Heath, S. F. Godsave, *Nature* **326**, 197 (1987).
4. L. Dale and J. M. W. Slack, *Development* **100**, 279 (1987).
5. D. Kimelman *et al.*, *Science* **242**, 1053 (1988); T. J. Musci, E. Amaya, M. W. Kirschner, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 8365 (1990); D. Kimelman and M. W. Kirschner, *Cell* **51**, 869 (1987); J. M. W. Slack and H. V. Isaacs, *Development* **105**, 147 (1989); G. Thomsen *et al.*, *Cell* **63**, 485 (1990); M. Asashima *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6511 (1991); R. A. Shiurba *et al.*, *Development* **113**, 487 (1991); L. L. Gillespie *et al.*, *ibid.* **106**, 203 (1989); E. Amaya, T. J. Musci, M. W. Kirschner, *Cell* **66**, 257 (1991); A. J. M. van den Eijnden–Van Raaij *et al.*, *Nature* **345**, 732 (1990); J. C. Smith, B. M. J. Price, K. Van Nimmen, D. Huylebroeck, *ibid.*, p. 729; M. Asashima *et al.*, *Roux's Arch. Dev. Biol.* **198**, 330 (1990); J. M. W. Slack, *Development* **113**, 661 (1991).
6. W. B. Busa and R. L. Gimlich, *Dev. Biol.* **132**, 315 (1989).
7. M. J. Berridge *et al.*, *Cell* **59**, 411 (1989).
8. M. J. Berridge, *Biochem. J.* **220**, 345 (1984); Y. Nishizuka, *Nature* **308**, 693 (1984).
9. L. Hallcher and W. Sherman, *J. Biol. Chem.* **255**, 10896 (1980); M. J. Berridge, C. P. Downes, M. R. Hanley, *Biochem. J.* **206**, 587 (1982); S. R. Nahorski, C. I. Ragan, R. A. J. Challiss, *Trends Pharmacol. Sci.* **12**, 297 (1991).
10. K. R. Kao, Y. Masiu, R. P. Elinson, *Nature* **322**, 371 (1986); K. R. Kao and R. P. Elinson, *Dev. Biol.* **127**, 64 (1988).
11. Two chromatographic fractions (*12*) were assayed: one contained *myo*-inositol and the other contained inositol mono- and higher phosphates. Because the assay is insensitive to inositol tetrakis and higher phosphates under the conditions used here (*12*), the "total inositol phosphates" reported represent only inositol mono- through trisphosphates, of which the mono- and bisphosphates constitute the vast majority.
12. J. A. Maslanski and W. B. Busa, in *Methods in Inositide Research*, R. F. Irvine, Ed. (Raven, New York, 1990), pp. 113–126. Neutralized perchloric acid embryo extracts were separated into distinct inositol phosphate–containing fractions on Sep-Pak Accell Plus QMA cartridges (Waters) by elution with step gradients of triethylammonium hydrogen carbonate (TEAB). Fractions were then vacuum-dried to remove TEAB and were dephosphorylated with alkaline phosphatase. The *myo*-inositol (the product of the dephosphorylation reactions) was quantified by sequential reactions with inositol dehydrogenase and diaphorase; the latter reaction used as substrate the nonfluorescent electron acceptor resazurin (purified by thin-layer chromatography), which was stoichiometrically converted to fluorescent resorufin and quantified at 565- and 585-nm excitation and emission wavelengths on a spectrofluorometer. Calibration curves were determined from internal standards added to some samples just before homogenization of tissues.
13. D-*myo*-inositol 1,4,5-trisphosphate [^{3}H] assay system (Amersham). Assay reliability was assessed by three means. Potentially interfering compounds in extracts were evaluated by assaying serial dilutions of cell extract supplemented with unlabeled IP_3; results were linear over a tenfold range of dilutions (correlation coefficient = 0.99). Undiluted extracts were supplemented with 0, 0.5, 2, or 5 pmol of unlabeled IP_3, and a plot of picomoles determined versus picomoles added was again linear (correlation coefficient = 0.97). Triplicate determinations of a single extract supplemented with 20 pmol of IP_3 yielded a value of 20.7 ± 1.0 pmol after correction for endogenous IP_3.
14. J. A. Maslanski, unpublished data.
15. J. Gerhart *et al.*, *Development (suppl.)* **107**, 37 (1989).
16. Mean ± SEM (n = 15) for embryos of five females; $P > 0.05$, Student's t test. Zygotes were irradiated in a silica dish above a UV transilluminator (254 nm) for 2 min, beginning 20 min after fertilization. Groups of 30 irradiated or nonirradiated embryos (256-cell stage) were extracted and assayed as described in Fig. 2. Average DAI scores for siblings were 0.8 ± 0.1 (n = 66) for irradiated embryos and 5.2 ± 0.1 (n = 73) for nonirradiated controls.
17. T. H. Shepard, *Catalog of Teratogenic Agents* (Johns Hopkins Univ. Press, Baltimore, MD, ed. 5, 1986), pp. xxi–xxii and 344–346.
18. K. T. Ault, A. Galione, P. L. Harger, W. B. Busa, in preparation.
19. J. Cooke, *Curr. Top. Dev. Biol.* **25**, 45 (1991).

20. Supported by NIH grants HD22879 and HD27546. We thank C. Frasier (Waters Division of Millipore, Incorporated) for generous provision of supplies and equipment.

16 December 1991; accepted 27 February 1992

Diacylglycerol-Stimulated Formation of Actin Nucleation Sites at Plasma Membranes

Aneesa Shariff and Elizabeth J. Luna

Diacylglycerols, which are generated during phospholipase-catalyzed hydrolysis of phospholipids, stimulated actin polymerization in the presence of highly purified plasma membranes from the cellular slime mold *Dictyostelium discoideum*. The increased rate of actin polymerization apparently resulted from de novo formation of actin nucleation sites rather than uncapping of existing filament ends, because the membranes lacked detectable endogenous actin. The increased actin nucleation was mediated by a peripheral membrane component other than protein kinase C, the classical target of diacylglycerol action. These results indicate that diacylglycerols increase actin nucleation at plasma membranes and suggest a mechanism whereby signal transduction pathways may control cytoskeletal assembly.

Changes in cell shape and rapid increases in actin polymerization are early cellular responses to stimulation by chemoattractants or growth factors (*1*, *2*). Although the signaling pathway is unclear, it may include phospholipid metabolism, especially phosphatidylinositide (PI) turnover. One theory is that phosphatidylinositol bisphosphate (PIP_2) in the plasma membrane (PM) directly controls actin assembly through interactions with PI-sensitive actin regulatory proteins, such as profilin and gelsolin (*3*). PIP_2 induces the dissociation of these proteins from actin in vitro potentiating actin assembly. One inconsistency of this mechanism is that in vivo PIP_2 concentrations are at a minimum shortly after chemotactic stimulation when actin polymerization from newly generated free barbed ends is increas-

Cell Biology Group, Worcester Foundation for Experimental Biology, Shrewsbury, MA 01545.

ing (4). Also, changes in the amounts of actin-gelsolin complexes do not always accompany PIP_2 turnover and actin assembly (5).

Alternatively, PIP_2 could control actin assembly through one or more second messengers. Second messengers generated from PIP_2 during both chemotaxis and mitogenesis include inositol trisphosphate (IP_3) and diacylglycerol (DG). Although the increased concentrations of cytoplasmic Ca^{2+} from IP_3 production are not required for actin polymerization (6), DG could provide the relevant signal. Membrane-permeant DGs induce actin polymerization and cytoskeletal changes in lymphocytes, neutrophils, and basophilic leukemia cells (7, 8). The increased amounts of F-actin are associated with surface projections at the cell periphery (7), suggesting an involvement of the PM. Neutrophils stimulated with chemoattractant produce two peaks of endogenous DG, one at ~10 s after stimulation and another, prolonged peak at 1 to 5 min (9). These peaks correspond temporally with peaks of actin assembly after chemotactic stimulation (2) and may arise from different signaling pathways (9, 10). DG production from phospholipids other than PIP_2 may account for observations that PI turnover can be uncoupled from actin polymerization (11).

We found that membrane-permeant DGs also trigger actin polymerization in living *Dictyostelium* amoebae. Both 1,2-dioctanoyl-*sn*-glycerol (diC8) and 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) transiently increased the cellular F-actin content with a sharp peak at 60 s (Fig. 1), about the same time as the second peak of chemoattractant-induced actin assembly (2). The optimal DG concentration was ~1.0 μM, which increased the amount of cellular F-actin to 162 ± 12% (diC8) or 156 ± 13% (OAG) of that in untreated cells.

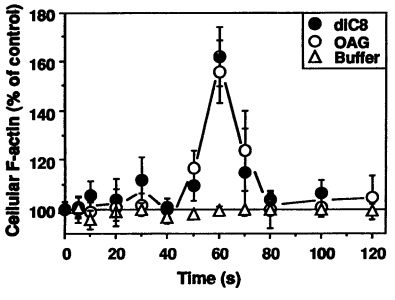

Fig. 1. Time course of actin polymerization after stimulation of log-phase *D. discoideum* amoebae with 1.0 μM DG. The F-actin content of fixed, permeabilized cells (2 × 10^6 per assay) was determined with NBD-phallacidin (23) at each time after addition of DG in 20 mM 1,4-piperazinediethanesulfonic acid (pH 7.0). Cellular F-actin content is the ratio of the fluorescence of DG-stimulated cells to that of unstimulated cells (t = 0). Error bars represent the SD for stimulated cells (24) and buffer controls (n = 5).

Actin-free *Dictyostelium* PMs (12) were used to study DG-mediated actin assembly in vitro. Micromolar concentrations of DGs increased the rate of PM-mediated actin polymerization by an average of 68% (diC8) or 59% (OAG) over the increase of 1.8- to 1.9-fold observed with PM alone (Fig. 2A). By contrast, the unphysiological 1,3-isomer of diC8 (1,3-diC8) had no apparent effect. Unlike cells, purified PMs appear not to degrade, or adapt to, exogenous DG because the rate of actin polymerization was unchanged after preincubation of PM with DG for up to 10 min before assay.

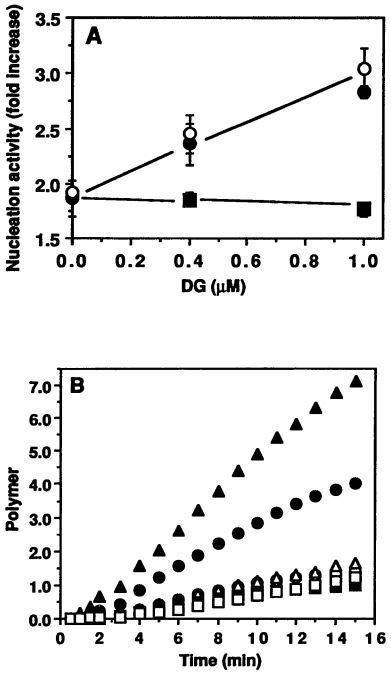

Fig. 2. DG-mediated increases in the actin nucleation activity of purified *D. discoideum* PM. (**A**) Fold increases in nucleation activity as a function of DG concentration were obtained from curves such as those shown in (B). Actin + diC8 + PM (●); actin + OAG + PM (○); actin + 1,3-diC8 + PM (■). We obtained the fold increase in actin nucleation activity by dividing the maximal rate of actin polymerization of each time course by the maximal rate of polymerization observed for actin alone (15). (**B**) Time course of actin polymerization after addition of actin (3 μM, 10% labeled with pyrene) to actin polymerization buffer with or without purified *D. discoideum* PM (10 μg/ml) or diC8 (1.0 μM). Actin + PM + diC8 (▲); actin + PM (●); actin + diC8 (△); actin only (○); actin + vesicles reconstituted from *Dictyostelium* lipid + diC8 (□); actin + PM denatured by disulfide reduction and alkylation + diC8 (■). Lipid vesicles from *Dictyostelium* PM, PM, and reduced and alkylated PM were prepared as described (15). DG was added to assay mixtures immediately before the addition of actin and measurement of actin polymerization. Fluorescence of pyrene actin was normalized on a scale from 0 to 10, with 10 being the fluorescence value of each sample after 24 hours (13).

Active DGs reduced the delay before onset of actin polymerization and increased the maximal rate of polymerization (Fig. 2B), indicating that the number of actin nucleation sites had increased (13). Therefore, DG may potentiate one of the two rate-limiting processes associated with actin assembly—activation of actin monomers or monomer oligomerization into elongation-competent nuclei (14). Alternatively, DG might unmask ends from preexisting filaments by releasing F-actin capping proteins (2). Because DG did not promote actin polymerization in the absence of PM (Fig. 2B), DG appears not to have a direct effect on monomer activation or filament stability. Thus, DG apparently affects actin nucleation by enhancing either monomer oligomerization or the dissociation of capping proteins.

One or more functional membrane proteins were required for the DG-mediated increase of actin nucleation activity (Fig. 2B). No stimulation by DG was observed in the presence of vesicles containing only lipids from *Dictyostelium* PM. In addition, reduction of disulfides with dithiothreitol and alkylation with *N*-ethylmaleimide eliminated both the DG-stimulated and basal actin nucleation activities.

One of the proteins responsible for the effect of DG appeared to be a tightly bound peripheral membrane protein (Fig. 3). Although extraction of loosely bound proteins with 1 M KCl had little effect on either the DG-mediated or basal activity, extraction

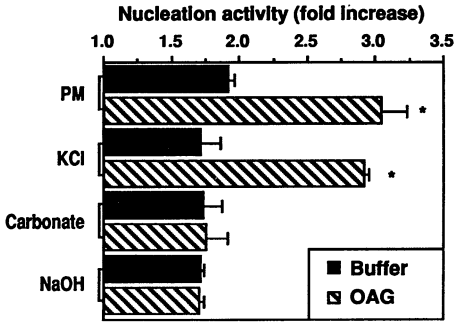

Fig. 3. Relation between the DG-mediated increase in actin nucleation activity and prior treatment of PM. The rate of actin polymerization in the presence of PM (10 μg/ml) with or without OAG (1.0 μM) was determined as shown in Fig. 2. The plasma membranes were extracted with 1 M KCl, pH 8.0 (12), 1 M sodium carbonate (pH 10.5) (25), or 0.1 N NaOH, 1 mM dithiothreitol, pH 12.5 (12, 15). Asterisks indicate statistically significant differences between fold increases observed with and without OAG; $P \leq 0.01$ (24). Similar results were obtained in experiments performed with and without diC8.

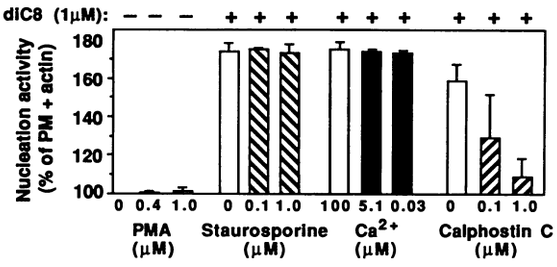

Fig. 4. Summary of experiments indicating that a classical protein kinase C is not required for DG potentiation of actin nucleation activity. All samples contained PM (10 μg/ml) and actin (3 μM). Calcium concentrations were controlled with EGTA/Ca^{2+} buffers. Actin polymerization was measured as described in Fig. 2. Only the inhibition by 1.0 μM calphostin C was significant ($P < 0.02$).

of more tightly bound proteins with basic solutions, pH 10.5 or 12.5, essentially abolished the response to DG. Consistent with previous reports (*15*), membranes extracted with basic solutions retained 90 ± 4% of the basal actin nucleation activity.

Because protein kinase C (PKC) is a major intracellular target for DG (*16*), we investigated the possibility that this peripheral membrane protein mediated the DG-promoted actin assembly. However, phorbol 12-myristate 13-acetate (PMA), an activator of PKCs in *Dictyostelium* and mammalian cells (*17*), did not stimulate membrane-mediated actin nucleation activity (Fig. 4). Furthermore, staurosporine, a potent inhibitor of the catalytic activity of both mammalian and *Dictyostelium* PKCs (*17, 18*), did not block the effect of DG. The DG-induced increase in actin nucleation activity also was independent of free Ca^{2+} concentrations from 0.03 to 100 μM. Finally, we observed no DG-mediated phosphorylation of actin or PM proteins with γ-^{32}P-labeled adenosine triphosphate (ATP), either under our standard actin nucleation assay conditions or in assay buffer containing the phosphatase inhibitors okadaic acid (1 μM) or phenylarsine oxide (10 μM). Thus, PKC appeared not to be involved in the DG-induced actin assembly response.

The peripheral protein mediating the effect of DG may contain a DG-binding site similar to that in PKC. Calphostin C, a molecule that interacts with the lipid binding site in the regulatory domain of PKC (*19*), inhibited the DG-induced increase in actin nucleation activity (Fig. 4). Further, the median inhibitory concentration (IC_{50}) for this inhibition (~100 nM) was comparable to that reported for calphostin C inhibition of mammalian PKC (IC_{50} = 50 nM).

We also investigated whether the peripheral protein mediating the effect of DG was residual membrane-bound actin or a capping protein bound to actin. Both possibilities were precluded because not enough actin is present on the PM to account for the observed increase in actin nucleation activity. Consistent with previous observations (*20*), cross-linked actin trimers (~25 ng/ml), the smallest effective actin nuclei, increased the rate of actin polymerization to about the same extent as did PM (10 μg/ml) in the absence of DG. Thus, at least 0.25% of the membrane protein would have had to be actin trimers to account for the basal activity, and ~0.15% would have had to be trimers to support the DG-invoked increase. Protein blots probed with an antibody to actin (anti-actin) showed that the PM contained ≤0.01% actin (Fig. 5). Therefore, the membranes used in these experiments contained less than 7% of the actin required for the observed DG-mediated increase in actin nucleation activity, eliminating the possibility that this activity is caused by the uncapping of actin filaments. Increased nucleation due to the severing of actin filaments by proteins such as *Dictyostelium* severin or gelsolin (*21*) was ruled out because the DG effect did not require micromolar concentrations of Ca^{2+} (Fig. 4). Thus, regulation of membrane-mediated actin nucleation by PIP_2-binding proteins such as gelsolin and profilin is likely to be indirect, through the control of DG formation.

Our observations indicate that DG, acting through an unknown signal transduction pathway, promotes the generation of new actin nuclei at (or near) the *Dictyostelium* PM. DG might modulate the basal actin nucleation activity of ponticulin (*15*), an integral membrane protein that binds directly to F-actin (*22*). Alternatively, DG might augment the activity of some other actin-nucleating protein. In either case, DG may be the common denominator by which diverse signal transduction pathways affect the organization of the actin-based cytoskeleton.

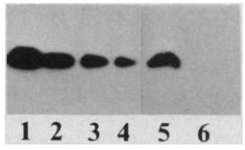

Fig. 5. Protein blot probed with anti-actin. Membrane proteins (40 μg per lane) were separated on 10 to 20% gradient polyacrylamide SDS gels, blotted to nitrocellulose, and probed with a polyclonal antibody to actin from sea urchin eggs and with ^{125}I–protein A (0.1 μCi/ml). Autoradiograph shows the signal generated against (lanes 1 to 4) a dilution series of purified *Dictyostelium* actin (1000, 100, 72, and 26 ng per lane); (lane 5) PM with ~0.12% residual endogenous actin (*15*); and (lane 6) PM. Plasma membrane extracted with salt or basic solutions also lacked detectable actin.

REFERENCES AND NOTES

1. K. L. Carraway and C. A. C. Carraway, *Biochim. Biophys. Acta* **988**, 147 (1989).
2. P. N. Devreotes and S. H. Zigmond, *Annu. Rev. Cell Biol.* **4**, 649 (1988); J. Condeelis *et al.*, *Dev. Genet.* **11**, 333 (1990).
3. I. Lassing and U. Lindberg, *Nature* **314**, 472 (1985); T. P. Stossel, *J. Biol. Chem.* **264**, 18261 (1989); P. J. Goldschmidt-Clermont *et al.*, *Science* **251**, 1231 (1991).
4. M. Carson, A. Weber, S. H. Zigmond, *J. Cell Biol.* **103**, 2707 (1986); A. L. Hall, V. Warren, S. Dharmawardhane, J. Condeelis, *ibid.* **109**, 2207 (1989).
5. C. Y. Dadabay, E. Patton, J. A. Cooper, L. J. Pike, *ibid.* **112**, 1151 (1991).
6. G. M. Omann *et al.*, *J. Biol. Chem.* **264**, 16355 (1989); G. P. Downey *et al.*, *J. Cell Biol.* **110**, 1975 (1990).
7. A. Zimmermann, P. Gehr, H. U. Keller, *J. Cell Sci.* **90**, 657 (1988); H. U. Keller, V. Niggli, A. Zimmermann, *ibid.* **93**, 457 (1989).
8. P. D. Phatak, C. H. Packman, M. A. Lichtman, *J. Immunol.* **141**, 2929 (1988); J. R. Apgar, *J. Cell Biol.* **112**, 1157 (1991).
9. J. Reibman *et al.*, *J. Biol. Chem.* **263**, 6322 (1988).
10. J. H. Exton, *ibid.* **265**, 1 (1990).
11. T. Bengtsson *et al.*, *ibid.* **263**, 17385 (1988); M. Eberle, A. E. Traynor-Kaplan, L. A. Sklar, J. Norgauer, *ibid.* **265**, 16725 (1990).
12. E. J. Luna, C. M. Goodloe-Holland, H. M. Ingalls, *J. Cell Biol.* **99**, 58 (1984).
13. J. A. Cooper, S. B. Walker, T. D. Pollard, *J. Muscle Res. Cell Motil.* **4**, 253 (1983).
14. F. Oosawa and M. Kasai, *J. Mol. Biol.* **4**, 10 (1962); C. Frieden, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6513 (1983).
15. A. Shariff and E. J. Luna, *J. Cell Biol.* **110**, 681 (1990).
16. Y. Nishizuka, *Science* **233**, 305 (1986); R. M. Bell and D. J. Burns, *J. Biol. Chem.* **266**, 4661 (1991).
17. M. E. E. Ludérus, R. G. Van der Most, A. P. Otte, R. Van Driel, *FEBS Lett.* **253**, 71 (1989).
18. T. Tamaoki *et al.*, *Biochem. Biophys. Res. Commun.* **135**, 397 (1986).
19. E. Kobayashi *et al.*, *ibid.* **159**, 548 (1989).
20. A. A. Lal, E. D. Korn, S. L. Brenner, *J. Biol. Chem.* **259**, 8794 (1984).
21. K. Yamamoto *et al.*, *J. Cell Biol.* **95**, 711 (1982); H. L. Yin, *BioEssays* **7**, 176 (1987).
22. L. J. Wuestehube and E. J. Luna, *J. Cell Biol.* **105**, 1741 (1987); C. P. Chia, A. L. Hitt, E. J. Luna, *Cell Motil. Cytoskeleton* **18**, 164 (1991).
23. A. L. Hall, A. Schlein, J. S. Condeelis, *J. Cell Biochem.* **37**, 285 (1988); T. H. Howard, and W. H. Meyer, *J. Cell Biol.* **98**, 1265 (1984).
24. Unless stated otherwise, all experiments were performed in triplicate. Graphs show means ± SD. Paired Student's *t* tests were used in statistical analyses.
25. M. P. Tranter, S. P. Sugrue, M. A. Schwartz, *J. Cell Biol.* **109**, 2833 (1989).

26. We thank E. Bonder, Rutgers University, for the antibody to sea urchin egg actin, T. Tamaoki, Kyowa Hakko Kogyo Co., for calphostin C, and J. Fallon, D. Fishkind, A. Hitt, R. O'Connell, R. Vallee, and Y.-L. Wang for comments. Supported by NIH grant GM-33048 and American Cancer Society grant CD-387A.

21 October 1991; accepted 28 January 1992