

reporting the effects of outer hair cell forces in the absence of forward mechano-electrical transduction. Cochlear amplification switches in only when these movements are fed back to augment the sensory input to the hair cells^{4,6}. Nevertheless, the cochlear responses reported here show comparable tuning to those induced by sound in a similar preparation²² where a heterodyne interferometer, constructed around an optical sectioning microscope and sensitive to velocity, detected light backscattered from the cochlear partition. Our results support the hypotheses^{4,6,20} that outer hair cells are responsible for amplification within the cochlea as well as for electrically evoked otoacoustic emissions from the intact cochlea²³, and show that it is possible to bridge the gap between *in situ*^{24,25} experiments and *in vivo*²⁶⁻²⁸ observations. □

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1. Brownell, W. E., Bader, C. R., Bertrand, D. & de Ripabuerie, Y. *Science* **227**, 194-196 (1985).
2. Dallos, P., Evans, B. N. & Hallworth, R. *Nature* **350**, 155-157 (1991).
3. Ashmore, J. F. *J. Physiol.* **388**, 323-347 (1987).
4. Neely, S. T. & Kim, D. O. *J. acoust. Soc. Am.* **79**, 1472-1480 (1986).
5. Dallos, P., in *Auditory Function: Neurobiological Bases of Hearing* (eds Edelman, G. M., Gall, W. E. & Cowan, W. M.) 153-188 (Wiley, New York, 1988).

6. Hubbard, A. *Science* **259**, 68-71 (1993).
7. Davis, H. *Hearing Res.* **9**, 79-90 (1983).
8. Mammann, F. & Ashmore, J. F. *J. Physiol.* **452**, 169P (1992).
9. Dallos, P. *Hearing Res.* **14**, 281-291 (1984).
10. Ashmore, J. F. & Meech, R. W. M. *Nature*, **322**, 368-371 (1986).
11. Evans, B. N., Hallworth, R. & Dallos, P. *Soc. Neurosci. Abstr.* **14**, 800 (1988).
12. Evans, E. F., Wilson, J. P. & Borerwe, T. A. *Tinnitus: Ciba Foundation Symposium* **85**, 108-138 (1981).
13. Stypulkowski, P. H. *Hearing Res.* **46**, 113-143 (1990).
14. McFadden, D. & Plattsmier, H. S. *J. acoust. Soc. Am.* **76**, 443-448 (1984).
15. Shehata, W. E., Brownell, W. E. & Dieler, R. *Acta otolar.* **111**, 707-718 (1991).
16. Santos-Sacchi, J. *J. Neurosci.* **9**, 2954-2962 (1989).
17. Santos-Sacchi, J. *J. Neurosci.* **11**, 3096-3110 (1991).
18. Davis, H. *Laryngoscope* **68**, 359-382 (1958).
19. Kolston, P. J., de Boer, E., Viergever, M. & Smoorenberg, G. *J. acoust. Soc. Am.* **86**, 133-140 (1989).
20. Mammann, F. & Nobili, R. *J. acoust. Soc. Am.* **93**, 3320-3332 (1993).
21. Greenwood, D. D. *J. acoust. Soc. Am.* **87**, 2592-2605 (1990).
22. Ulfendahl, M., Khanna, S. M. & Flock, A. *Hearing Res.* **40**, 55-64 (1989).
23. Hubbard, A. E. & Mountain, D. C. *Science* **222**, 510-512 (1983).
24. Reuter, G. & Zenner, H. P. *Hearing Res.* **43**, 219-230 (1990).
25. Reuter, G., Gitter, A., Thurm, U. & Zenner, H. P. *Hearing Res.* **60**, 236-246 (1992).
26. Sellick, P. M., Patuzzi, R. B. & Johnstone, B. M. *J. acoust. Soc. Am.* **72**, 131-141 (1982).
27. Ruggiero, M. & Rich, N. J. *Neurosci.* **11**, 1057-1067 (1991).
28. Nuttall, A. L., Dolan, D. F. & Avinash, G. *Hearing Res.* **51**, 203-214 (1991).

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Inhibition of contractile vacuole function *in vivo* by antibodies against myosin-I

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MYOSIN-I is thought to supply the force for movement of cell membranes relative to actin filaments (reviewed in refs 1, 2), but confirmation of this hypothesis has been difficult because of the presence of multiple isoforms of myosin-I and other unconventional myosins in most cells³. We report here the first evidence that a myosin-I isoform is essential for a specific class of intracellular membrane movements *in vivo*. In *Acanthamoeba*, the contractile vacuole is an autonomous structure which fuses with the plasma membrane to control the water content of the cell. Because myosin-IC is the only myosin-I isoform concentrated in the contractile vacuole complex^{4,5}, and a protein antigenically related to myosin-IC is located on or near the *Dictyostelium* (slime mould) contractile vacuole⁶, we thought this organelle might provide the best opportunity to demonstrate a relationship between myosin-I and membrane motility. Antibodies that inhibit the activity of *Acanthamoeba* myosin-IC *in vitro* interfere with expulsion of excess water by the contractile vacuole *in vivo*, leading to overfilling of this organelle and cell lysis. Myosin-IC may generate the force required to contract the vacuole and may also be involved in transfer of water to the contractile vacuole during refilling.

Antibodies raised against a synthetic peptide with the sequence of the myosin-IC phosphorylation site⁴ inhibited myosin-IC activity *in vitro* in three assays (Table 1): (1) phosphorylation of myosin-IC by the myosin-I heavy chain kinase (phosphorylation is required for activation of *Acanthamoeba* myosin-I ATPase activity^{7,8} and motility *in vitro*^{9,10}); (2) actin-activation of the Mg²⁺-ATPase activity of prephosphorylated myosin-IC; and (3) crosslinking of actin filaments by myosin-IC, indicating that the ATP-sensitive actin binding site (which

TABLE 1 Antibody inhibition of myosin I activities *in vitro*

Experiment	Antibody concentration (μM)	Activity (% of control)		
		ATPase	Crosslinking	Phosphorylation
Myosin-IC	0.01	100	95	100
	0.06	68	—	100
anti-myosin-IC	0.11	—	0	—
	0.63	0	—	87
	2.50	—	—	41
	3.13	—	—	3
	3.65	—	—	0
	Myosin-IB	0.01	100	84
anti-myosin-IB	0.06	32	—	—
	0.11	—	0	—
	0.63	0	0	—

Anti-myosin-IC IgG and anti-myosin-IB IgG were tested for inhibition of three activities: actin-activated Mg²⁺-ATPase activity; crosslinking of F-actin; phosphorylation of myosin-I heavy chain. Myosin-I and myosin-I heavy chain kinase were purified by the method of Lynch *et al.*²⁷. Actin was purified by the method of Spudich and Watt²³. The Mg²⁺-ATPase activity of 0.2 μM myosin-I was assayed as described^{24,25} in the presence and absence of 4 μM F-actin, with the basal activity in the absence of F-actin being subtracted to give the actin-activated Mg²⁺-ATPase activity (control activity: 14.8 s⁻¹). Crosslinking of F-actin (4 μM) by myosin-I (0.2 μM) was measured by a pelleting assay using a low centrifugal force (18,500g) which was just sufficient for pelleting only crosslinked F-actin²⁶. The mixture before pelleting and the supernatant after pelleting were analysed by SDS-PAGE and scanning densitometry to calculate the proportion of myosin-I present as F-actin crosslinks (control value: 100%). Myosin-IC (2 μM) was phosphorylated by autophosphorylated myosin-I heavy chain kinase (60 nM); autophosphorylation is required for kinase activity²⁶. Kinase was autophosphorylated by a 30 min incubation at 30 °C in 2.5 mM ATP, 3.5 mM MgCl₂, 2 mM EGTA, and 50 mM imidazole, pH 7.0. Myosin-I was phosphorylated in the same buffer supplemented with [γ-³²P]ATP (control activity: 1 mol P/mol myosin-I). Relative phosphorylation levels were determined by densitometer scans of autoradiograms after SDS-PAGE.

is near the antigenic epitope in the globular head) was blocked.

We used syringe loading¹¹ to introduce these antibodies into living *Acanthamoeba* cells. We added rhodamine-dextran to the loading solution, and isolated the loaded cells by fluorescence-activated cell sorting (Fig. 1). The combination of these two techniques is considerably easier than microinjection, particularly with *Acanthamoeba*¹², and rapidly yields a large population of loaded cells amenable to statistical analysis. Syringe-loaded anti-myosin-IC IgG was associated with the contractile vacuole

and plasma membrane after fixation of unlysed loaded cells (Fig. 2h).

Acanthamoeba cells loaded with anti-myosin-IC IgG lysed in response to osmotic stress, as judged by microscopic observation (Fig. 3a). Lysis was directly related to osmotic stress, because cells loaded with anti-myosin-IC antibodies and incubated in isotonic growth medium lysed at a much lower frequency than those incubated in water (Fig. 3a). The fraction of cells that lysed directly depended on the fraction of cells above a specific fluorescence level; therefore, lysis occurred when a threshold dose of antibody was introduced into the cell. We determined empirically that a fluorescence level of 700 (arbitrary units) was the minimum required for lysis (Fig. 3a). Assuming the same quantum yield for fluorescence from rhodamine-dextran loaded amoebae and quantitative flow cytometry standards, proportional uptake of IgG and rhodamine-dextran, and the myosin-IC content previously estimated⁵, the threshold fluorescence level was equivalent to an intracellular stoichiometry of about 0.5 IgG binding sites per myosin-IC heavy chain. Control populations loaded with anti-myosin-IB IgG, purified preimmune IgG, bovine serum albumin, or inactivated anti-myosin-IC IgG lysed at low levels and, in each of these cases, the fraction of lysed cells was unrelated to the extent of loading (Fig. 3a). The small percentage of cells that lysed in these control samples may have done so because of damage during the loading procedure irrespective of the amount of protein loaded.

Release of loaded IgG into the supernatant is another way to measure the number of lysed cells (Fig. 3b). The time course of IgG release suggested that all loaded populations contained some damaged cells which lysed within 10 minutes and which accounted for almost all of the lysed cells in the negative control samples. Cells loaded with anti-myosin-IC IgG had a second, larger population that lysed more slowly over 3–4 hours in response to osmotic stress.

DAPI (4',6-diamidino-2-phenylindole) staining combined with flow cytometry is a third method for measuring lysis of

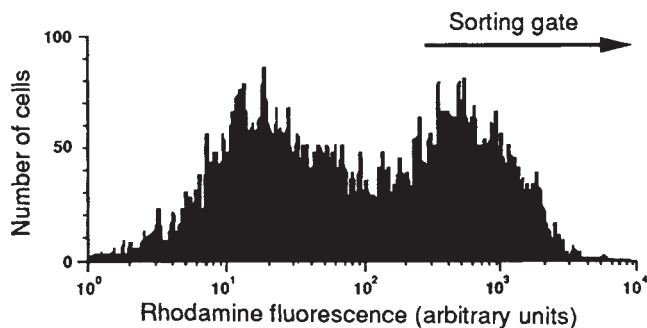


FIG. 1 Fluorescence distribution of cells after loading with immunoglobulins in buffer containing rhodamine-labelled dextran. Note the bimodal distribution of fluorescence. After sorting, no cells with fluorescence below 50 fluorescence units remained in the sample.

METHODS. *Acanthamoeba* cells were grown to stationary phase and concentrated to 10^7 cells ml^{-1} in loading buffer¹² containing 2 mg ml^{-1} purified immunoglobulins and 20 mg ml^{-1} rhodamine-labelled dextran (M_r 73,100; Sigma) before loading. Cells were syringe-loaded^{d1} with five slow passes through a 30-gauge needle. The syringe plunger was drawn back to the 1 ml mark for filling, and the loading suspension was forced out of the syringe at roughly 0.2 ml s^{-1} . The suspension was centrifuged for 1 min at $5,000g$, the loading buffer was removed, and the cells were washed briefly in growth medium. The cells were resuspended in 1–4 ml growth medium and sorted on a Becton-Dickinson FACStar fluorescence-activated cell sorter (FACS). The population was screened for viable cells by forward and side light scattering and the live cells were sorted on the basis of rhodamine fluorescence in the same FACS run. Cells with >10 times the median fluorescence of control cells were accepted as loaded.

cells loaded with anti-myosin-IC IgG after osmotic shock (Fig. 3c). DAPI is a DNA-binding fluorescent dye that does not cross the plasma membrane of intact cells during short incubations and therefore stains only DNA from lysed cells in this assay. Lysed cells, nuclei, and possibly mitochondria have high DAPI fluorescence and low rhodamine fluorescence, intact cells have low DAPI and high rhodamine fluorescence, and membrane fragments and other organelles have low fluorescence from both DAPI and rhodamine. The largest population of control cells loaded with anti-myosin-IB IgG and osmotically stressed consisted of intact cells (49%, Fig. 3d) that had light-scattering characteristics similar to unloaded intact cells (data not shown). The remaining major groups were composed of lysed cells and nuclei (17%), and small organelles and membrane fragments (29%). The ratio of these three groups was dramatically different for anti-myosin-IC-loaded osmotically shocked cells. Less than 3% of the cells remained intact and the two major populations (Fig. 3c) consisted of: (1) lysed cells, nuclei and possibly mitochondria (49%); and (2) membrane fragments and other organelles (44%).

We also observed cells loaded with anti-myosin-IC antibodies by time-lapse video microscopy after osmotic shock. Lysis in these cells appeared to occur by two distinct mechanisms. Many cells lysed because of overextension of the plasma membrane by the distended contractile vacuole (Fig. 2a–g), with the vacuole occasionally remaining intact even after total rupture of the plasma membrane (Fig. 2d). In another group of cells, the vacuole suddenly stopped increasing in size after contraction, followed by swelling of the cytoplasm and lysis.

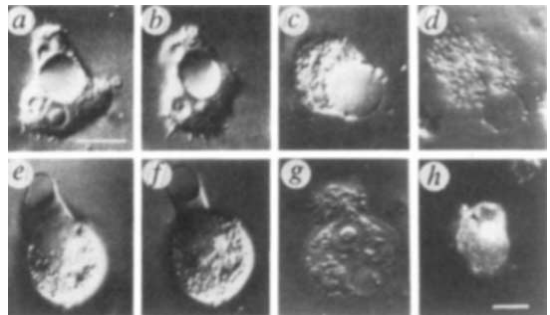
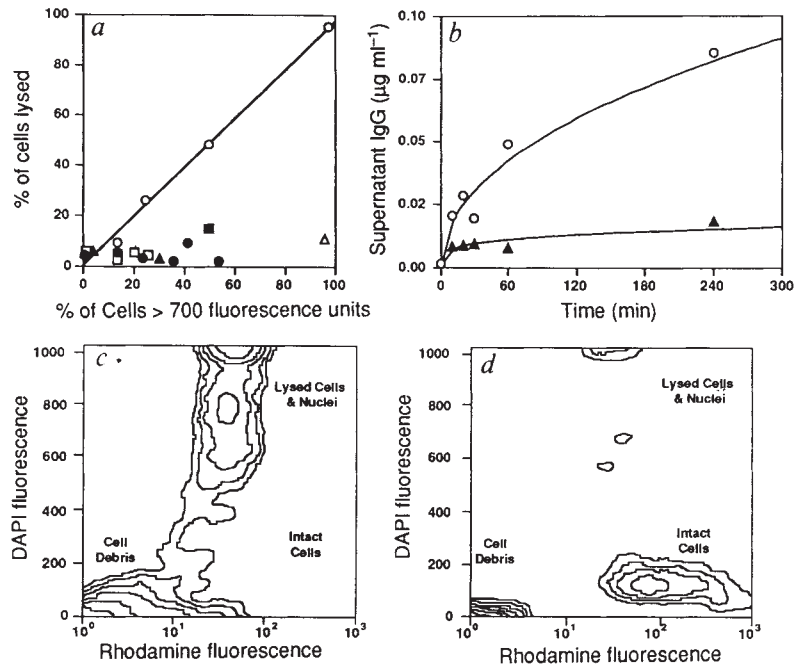


FIG. 2 Video microscopy of cell lysis. a–d, Time course of lysis of a single cell over 35 min (a, 3 min 30 s; b, 17 min 20 s; c, 30 min 5 s; d, 35 min). The plasma membrane ruptured (c), whereas the contractile vacuole remained substantially intact. e–g, Time course of lysis of a single cell over 12 min (e, 1 min 15 s; f, 7 min 15 s; g, 12 min 5 s). The enlarged contractile vacuole distended the plasma membrane and ruptured, followed by lysis of the cell. Scale bar in a, $15 \mu\text{m}$. h, Immunofluorescence detection of anti-myosin-IC IgG in loaded cells fixed after incubation in growth medium and stained with fluorescein conjugated goat anti-rabbit IgG. The anti-myosin-IC antibody labels the contractile vacuole complex (arrowhead) and plasma membrane. Scale bar, $10 \mu\text{m}$. **METHODS.** Cells were syringe-loaded with anti-myosin-IC IgG and sorted by FACS (see Fig. 1). After sorting, the cells were resuspended in $250 \mu\text{l}$ growth media and pipetted onto a glass coverslip. After 15 min for the cells to attach, the excess media was wicked off and the cover slip was inverted onto a glass microscope slide with a drop of distilled water within a ring of petroleum jelly. The cells were observed on a Zeiss Axiocvert 135TV microscope equipped with a Plan-Neofluar $\times 100$ objective using differential interference contrast optics (courtesy of D. B. Murphy). The image was collected with a Hamamatsu C2400 television camera and controller and was processed with a Hamamatsu Argus-10 image processor. The processed images were photographed using a Polaroid freeze frame video recorder and 35 mm camera adapter with Kodak TMax 100 film. For immunofluorescence, loaded cells without FACS sorting were allowed to settle in growth medium onto poly-L-lysine coated microscope slides for 20 min before fixation, permeabilization in 100% acetone and reduction with sodium borohydride as described^{4,5}. Anti-myosin-IC IgG was detected by goat anti-rabbit IgG conjugated to fluorescein ($1.0 \mu\text{g ml}^{-1}$; Molecular Probes).

FIG. 3 *Acanthamoeba* cells loaded with antibodies against myosin-IC lyse when incubated in distilled water. **a**, Percentage of cells that lyse within 2 h of incubation as a function of fluorescence level. ○, Cells loaded with anti-myosin-IC IgG and incubated in water; ■, cells loaded with anti-myosin-IC IgG and incubated in growth medium; ▲, cells loaded with preimmune IgG and incubated in water; △, cells loaded with anti-myosin-IB IgG and incubated in water; □, cells loaded with inactivated anti-myosin-IC IgG and incubated in water; ●, cells loaded with BSA and incubated in water. **b**, Time course of the release of IgG from lysing cells. ○, Cells loaded with anti-myosin-IC IgG and incubated in water; ▲, cells loaded with preimmune IgG and incubated in water. **c, d**, Flow cytometry analysis of cell lysis. As cells lyse, they lose the rhodamine fluorescence signal and increase in DAPI fluorescence. As analysed by light scattering, the population with high rhodamine fluorescence is primarily made up of intact cells, whereas the high DAPI/low rhodamine population is a mixture of lysed cells and free nuclei (and perhaps mitochondria) of lysed cells. The population with both low DAPI and low rhodamine signals is made up of small cell fragments with no DNA content. **c**, Cells loaded with anti-myosin-IC IgG and incubated in water. **d**, Cells loaded with anti-myosin-IB IgG and incubated in water.

METHODS. Cells were syringe-loaded and sorted by FACS (see Fig. 1). The distribution of fluorescence level in the cell population was determined by flow cytometry during sorting, and the percentage of cells above 700 arbitrary fluorescence units was calculated. Immediately after sorting, the cells were allowed to recover in 1 ml growth medium for at least 30 min. The data shown in **a** were obtained by incubating the cells for 15 min on tissue culture slides (Miles Scientific, Naperville, IL) in growth medium to allow the cells to attach to the surface. The growth medium was gently removed and either distilled water or additional growth medium was added. After 2 h at least 500 cells in each sample were examined by each of two observers, and phase transparent cells were scored as lysed. The flow cytometer was calibrated with fluorescent standards of known rhodamine content. For data shown in **b**, loaded and sorted cells were allowed to attach to wells in a 16-well tissue culture plate for 15 min. The growth medium was gently removed and the cells covered with



Contraction of the vacuole was severely inhibited in cells loaded with anti-myosin-IC IgG, but not those loaded with anti-myosin-IB IgG. We observed at least 100 vacuole filling and contraction cycles in both loaded and unloaded cells. The average time between contractions after osmotic shock was increased from 92 s for unloaded cells and 105 s for cells loaded with anti-myosin-IB, to 237 s for cells loaded with anti-myosin-IC. Abortive partial contractions accounted for 18% of the observed contractions in anti-myosin-IC-loaded cells; no partial contractions were observed in either unloaded cells or anti-myosin-IB-loaded cells. Contraction of isolated contractile vacuoles *in vitro* requires ATP (data not shown).

These observations strongly suggest that myosin-IC is essential for active contraction of the vacuole. The exact mechanism remains unclear, but it is apparent that it is an active process involving an actomyosin-I interaction, rather than a passive mechanism of membrane rupture and concomitant contractile vacuole collapse¹³. Myosin-IC may also be a motor for movement of spongione vesicles during filling of the vacuole. It remains a possibility that anti-myosin-IC IgG disrupts water regulation at the plasma membrane, leading to overloading of the contractile vacuole system, but in that case we would expect that the time between vacuole contractions would decrease before cell lysis rather than dramatically increase as we observed. Also, this possibility does not explain the presence of 'frozen' non-contracting contractile vacuoles observed only in anti-myosin-IC-loaded cells.

Our observation that osmotic shock is required to reveal the phenotype of myosin-IC deficient cells has more general implications. The actomyosin system in motile cells is quite robust under

water. The supernatant was sampled at various time points, and IgG was quantified by ELISA. For data in **c, d**, loaded and sorted cells were allowed to recover in growth medium, and then collected by centrifugation for 5 min at 1,000g and resuspended in water. After a 40 min incubation, DAPI (4',6'-diamidino-2-phenylindole, Molecular Probes) was added to the suspension, which was incubated for 5 min, and then analysed by dual wavelength flow cytometry. DAPI undergoes a 20-fold increase in fluorescence on binding to DNA, and does not cross intact cell membranes during short incubations. Anti-myosin-IC and -IB polyclonal antibodies were previously described^{4,5}. Inactivated antibodies were biotinylated with four to five moles of biotin per mole of antibody. The biotinylated IgG did not recognize myosin-I on protein immunoblots, nor did it stain cells by indirect immunofluorescence.

laboratory culture conditions, with cells surviving even after inhibition or genetic elimination of major cytoskeletal proteins^{12,14-22}. Subjecting these cells to severe stress analogous to osmotic shock might reveal interesting new phenotypes. □

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- Pollard, T. D., Doberstein, S. K. & Zot, H. G. *A. Rev. Physiol.* **53**, 653-681 (1991).
- Hammer, J. A. III *Trends Cell Biol.* **1**, 50-56 (1991).
- Cheney, R. E. & Mooseker, M. S. *Curr. Opin. Cell Biol.* **4**, 27-35 (1992).
- Baines, I. C. & Korn, E. D. *J. Cell Biol.* **111**, 1895-1904 (1990).
- Baines, I. C., Brzeska, H. & Korn, E. D. *J. Cell Biol.* **119**, 1193-1203 (1992).
- Zhu, Q. & Clarke, M. *J. Cell Biol.* **118**, 347-358 (1992).
- Maruta, H. & Korn, E. D. *J. Biol. Chem.* **252**, 8329-8332 (1977).
- Lynch, T. J., Brzeska, H., Miyata, H. & Korn, E. D. *J. Biol. Chem.* **264**, 19333-19339 (1989).
- Albanesi, J. P. et al. *J. Biol. Chem.* **260**, 8649-8652 (1985).
- Zot, H. G., Doberstein, S. K. & Pollard, T. D. *J. Cell Biol.* **116**, 367-376 (1991).
- Clarke, M. S. F. & McNeil, P. L. *J. Cell Sci.* **102**, 533-541 (1992).
- Sinard, J. H. & Pollard, T. D. *Cell Motil. Cytoskel.* **12**, 42-53 (1989).
- Wigg, D., Bovee, E. C. & Jahn, T. L. *J. Protozool.* **14**, 104-108 (1967).
- Titus, M. A., Wessels, D., Spudich, J. A. & Soll, D. *Molec. Biol. Cell* **4**, 233-246 (1993).
- Wessels, D., Murray, J., Jung, G., Hammer, J. A. & Soll, D. R. *Cell Motil. Cytoskel.* **20**, 301-315 (1991).
- Jung, G. & Hammer, J. H. III *J. Cell Biol.* **110**, 1955-1964 (1990).
- Witke, W., Schleicher, M. & Noegel, A. A. *Cell* **68**, 53-62 (1992).
- Cox, D. *J. Cell Biol.* **116**, 943-955 (1992).
- DeLozanne, A. & Spudich, J. A. *Science* **236**, 1086-1091 (1987).
- Knecht, D. A. & Loomis, W. F. *Science* **236**, 1081-1086 (1987).
- Wessels, D. et al. *Dev Biol.* **128**, 164-177 (1988).
- Furukawa, R., Butz, S., Fleischmann, E. & Fehcheimer, M. *Protoplasma* **169**, 18-27 (1992).
- Spudich, J. A. & Watt, S. *J. Biol. Chem.* **246**, 4866-4871 (1971).
- Pollard, T. D. & Korn, E. D. *J. Biol. Chem.* **248**, 4682-4690 (1973).
- Albanesi, J. P., Lynch, T. J., Fujisaki, H. & Korn, E. D. *J. Biol. Chem.* **261**, 10445-10449 (1986).
- Brzeska, H., Lynch, T. J. & Korn, E. D. *J. Biol. Chem.* **265**, 3591-3594 (1990).
- Lynch, T. J., Brzeska, H., Baines, I. C. & Korn, E. D. *Meth. Enzym.* **196**, 12-23 (1990).

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