Phallloidin-induced actin polymerization in the cytoplasm of cultured cells interferes with cell locomotion and growth

(microfilaments/microtubules/tonofilaments/movement/immunofluorescence microscopy)

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ABSTRACT Phallloidin, the toxic drug from the mushroom Amanita phalloides, was injected into the cytoplasm of tissue culture cells and the changes in intracellular actin distribution were followed by immunofluorescence microscopy with actin antibody. At low concentrations, phallloidin recruits the non- or less highly polymerized forms of cytoplasmic actin into stable “islands” of aggregated actin polymers and does not interfere with the preexisting thick bundles of microfilaments (stress fibers). Differential focusing shows that these islands of phallloidin-induced actin polymers occur at a level in the cytoplasm that is above the submembranous bundles of microfilaments present on the adhesive side of the cells. The pattern of cytoplasmic microtubules remains unaffected by the injection of phallloidin; however, filamin, a protein usually associated with actin in the cytoplasm, is also recruited into the islands. At higher phallloidin concentrations, contraction of the cell is observed. These results are discussed in the light of previous biochemical studies by Wieland and Faulstich and their coworkers (for a review see Wieland, T. (1977) Naturwissenschaften 64, 363-369) on the in vitro interaction of phallloidin with muscle actin, which have documented that phallloidin reacts stoichiometrically with actin, promotes actin polymerization, and stabilizes actin polymers.

In addition, we show that microinjection of phallloidin interferes in a concentration-dependent manner with cell locomotion and cell growth. These results indicate that a well-balanced controlled reversible equilibrium between different polymerization states of actin may be a necessary requirement for the cell locomotion and may also influence other cellular functions such as growth.

Actin is the major structural protein of the cytoplasm of eukaryotic cells. The structural organization and polymerization of cytoplasmic actin is still poorly understood (for a review see ref. 1). Although in muscle cells actin is exclusively organized in nearly crystalline ordered arrays of thin filaments, the actin organization in nonmuscle cells can be rather diverse. Actin is the major protein of the thick bundles of microfilaments (stress fibers) typical for a variety of fibroblastic, epithelial, and other cells (2-4), but less ordered actin polymerization occurs in the ruffling edge of these cells, and many other cell types show only thin microfilamentous structures (2, 3, 5, 6). In addition, the Amanita phalloides, was introduced as an actin-specific drug in vitro. Wieland, Faulstich, and their coworkers have documented in several studies that phallloidin reacts stoichiometrically with muscle actin (for a review see ref. 7). Of particular interest are their findings that phallloidin strongly promotes actin polymerization and that phallloidin stabilizes polymerized actin.

Here we report on the action of phallloidin on the distribution and organization of actin in cells in tissue culture. Phallloidin was introduced into cells by microinjection, and the actin distribution was followed by immunofluorescence microscopy using antibodies against actin (3). When phallloidin is injected into cells it recruits the less highly polymerized forms of actin and/or G actin, to form “islands” of aggregated actin in a focal plane above the stress fibers. Furthermore, we show that phallloidin interferes with cell locomotion and cell growth.

MATERIALS AND METHODS

Cells of the established cell lines PtK2 (rat kangaroo, Potorous tridactyla) and 3T3 (mouse) were grown on square glass cover slips subdivided into 0.25-mm² squares as described (8, 9). Cells were microinjected by the procedure of Graessmann and Graessmann (10), using a microscope at a magnification of 300 with a micromanipulator and microcapillaries having a diameter of approximately 0.5 μm at the tip.

Phallloidin (Boehringer, Mannheim, FRG) was dissolved in dimethylsulfoxide (Me2SO) at 50 mM and kept at −20 °C. Just prior to injection, the phallloidin was diluted into 0.14 M KCl to a final concentration between 0.05 and 1 mM. Corresponding dilutions of phallloidin-free Me2SO into 0.14 M KCl were used as controls to assess the specificity of the action of phallloidin. Essentially identical results were obtained when phallloidin was diluted into a phosphate buffer (0.048 M K2HPO4/0.014 M NaH2PO4-2H2O/0.0045 M KH2PO4, pH 7.2). All cells were injected into the cytoplasm close to the nucleus. The cover slips were returned to the incubator for various lengths of time and then processed for indirect immunofluorescence microscopy. The rabbit anti-actin antibody (3, 9), the monospecific rabbit anti-tubulin antibody (11, 12), and the fluorescein-labeled goat anti-rabbit antibody (9), as well as the details of the immunofluorescence microscopy (9, 15), have been described previously. Filamin from chicken gizzard (14, 15) was purified to homogeneity and antibodies were raised in rabbits.

Cell growth of PtK2 cells was measured by counting the number of cells present on a defined area of a glass cover slip using a phase microscope for up to 5 days. Neighborin areas were freed of other cells by use of a micromanipulator. The number of cells originally present in this area was approximately 100.

Locomotion of 3T3 cells was followed on marked cover slips coated with 0.1% poly (l-lysine). Approximately 20 cells were microinjected either with phallloidin solution or with control

Abbreviation: Me2SO, dimethylsulfoxide.

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FIG. 1. 3T3 cells (A–C) and PtK2 cells (D–F) 3 hr after microinjection of a phalloidin solution—0.2 mM in 0.14 M KCl, 0.4% in Me2SO (B, C, E, and F)—or the control solution without phalloidin (A and D) processed by indirect immunofluorescence microscopy using antibody against actin (A, B, and D–F) or tubulin (C). E and F show the same cell in different focal planes to document that the phalloidin-induced islands of actin aggregates are present predominantly above the stress fibers, which are present submembranously on the adhesive side of the cell. (A, B, E, and F are ×800; C and D are × 950.)
solution. After microinjection the position of the cells was recorded by a phase contrast photograph. Eight hours later the identical area was again photographed to record the cell displacement as a measure of cell locomotion.

RESULTS

Phalloidin Induces Islands of Aggregated Actin. Mouse 3T3 and rat kangaroo PtK2 cells were microinjected with phalloidin solutions (0.2 or 1 mM) in 0.14 M KCl (MeSO4 concentration 0.4 or 2%). Control experiments were performed using the same solvents without phalloidin. After microinjection the cells were incubated in growth medium for times between 1 hr and 3 days.

The distribution of cytoplasmic actin obtained by immunofluorescence microscopy 3 hr after injection into the cytoplasm of phalloidin solution (0.2 mM) together with control experiments to demonstrate the specificity of the phalloidin interaction with actin are shown in Fig. 1. Fig. 1A shows a typical pattern of stress fibers decorated by the actin antibody in a 3T3 cell 3 hr after injection with the control solvent. The actin fiber system resembles that of normal 3T3 cells (3, 4, 6, 9, 16) and injection of the control solvent does not induce actin polymerization. Fig 1B shows the corresponding result when phalloidin was injected at 0.2 mM. The stress fibers are still present, but in addition numerous islands of aggregated actin can be detected. Differential focusing shows that these islands are located in a different focal plane of the cytoplasm than the stress fibers and occur at a higher level in the cell than the stress fibers, which are located submembranously at the adhesive side of the cell (3, 4). That phalloidin does not induce unspecific protein precipitation is shown in Fig. 1C, because the pattern of cytoplasmic microtubules in 3T3 cells (see refs. 12 and 13) remains unchanged after the introduction of phalloidin. When cells that do not contain strong bundles of microfilaments (actin cables) (6) such as HeLa cells, Simian virus 40-transformed 3T3 cells, or Chinese Hamster Ovary (CHO) cells are injected with phalloidin, the same islands of actin aggregates are visualized (data not shown).

The results obtained with PtK2 cells are shown in Fig. 1D-F. Fig. 1D shows that injection of the control solvent does not induce actin polymerization. Fig. 1E and F shows the actin distribution in two different focal planes in the same PtK2 cell after injection of phalloidin at 0.2 mM. Fig. 1E and F documents that the islands of actin aggregates are in a focal plane above that of the stress fibers. Control experiments on PtK2 cells show that the system of cytoplasmic microtubules and the system of tonofilaments typical for this cell line (8) are not changed by the injection of phalloidin (data not shown). However, when PtK2 cells injected with a 0.2 mM solution of phalloidin are stained with antibody against filamin, again islands of aggregates as well as stress fibers are clearly visualized by immunofluorescence microscopy (Fig. 2). These filament aggregates are not found in cells, injected with control solutions. Such cells show a distribution of filament indistinguishable from the normal actin distribution as reported by Singer and co-workers for other cell lines (14, 15).

Phalloidin-induced actin aggregates can occasionally be
observed after injection of PtK2 cells with a 0.05 mM solution of phalloidin. The typical islands become clearly visible in the majority of injected cells (90%) at 0.1 mM solution. At 0.2 mM the islands are clearly seen 1 hr after injection (earlier times have not been tested). The induced structures are stable and are well preserved even after 24 or 36 hr, indicating a strong binding of phalloidin.

When phalloidin solutions of higher concentration (1 mM) are injected into PtK2 cells, one can notice a contraction of the cells, which does not occur directly, but approximately 15 min after the injection, and leads to a change in cell morphology. Extensive formation of islands of aggregated actin is again seen. However, in addition the cell morphology is changed and in some cells the stress fibers are no longer visible. Under these conditions, the actin aggregates seem to correspond to structures that can also be detected by phase contrast microscopy (Fig. 3 A and C). Under these conditions, the actin aggregates seem to correspond to structures that can also be detected by phase contrast microscopy (Fig. 3 A and C).

**Phalloidin Interferes with Locomotion and Cell Growth.** Locomotion of mouse 3T3 cells measured as cell displacement over an 8-hr interval averaged 90 μm (70–110 μm). This value was not noticeably changed for cells injected as controls with 0.14 M KCl, 1% in MeSO. When phalloidin was present in the same solvent at 0.2 mM, average cell displacement dropped to 15 μm (5–20 μm). Increase of the phalloidin concentration in the same solvent to 0.5 mM gave no noticeable cell displacement during the same time period. Thus, phalloidin seems to interfere with cell locomotion in a concentration-dependent manner.

Cell growth was followed by phase microscopy of approximately 100 PtK2 cells on a defined area of a glass cover slip. The results are summarized in Fig. 4. Under our experimental conditions cells grow with a doubling time of approximately 35 hr (Fig. 4, curve A), and this behaviour was not changed when phalloidin was present at 0.1 mM in the growth medium (0.2% MeSO, curve B). Other experiments showed that phalloidin present in the medium at 0.1 mM does not influence cell growth or actin distribution for a variety of established cell lines including mouse 3T3 cells, Simian virus 40-transformed 3T3 cells, and HeLa cells. PtK2 cells injected as controls showed, after an initial lag of 1 day, nearly normal growth (curves C and D). Clearly, cells injected with phalloidin at 0.2 mM in the same solvent show a much longer lag period after injection (curve E). Such cells contain islands of actin polymers that can be detected both at early times (Fig. 1 E and F) and at times up to 2–3 days after the application of the drug. Cells injected with higher phalloidin concentrations (1 mM in 2% MeSO) and which in the immunofluorescence studies showed indication of morphological change and contraction (see Fig. 3) did not resume growth during the observation period of 120 hr, although an occasional cell division was seen (curve F).

**DISCUSSION**

Microinjection of phalloidin has been used in conjunction with immunofluorescence microscopy to assess the direct and indirect functions of cytoplasmic actin in its different stages of polymer formation. The use of phalloidin was previously limited, because most tissue culture cell lines are resistant to the drug applied in the medium (see Results). The possibility of a permeation barrier for a majority of cell types was expected, because phalloidin poisoning of animals showed that the liver was the specific target of the drug and pathological changes in other tissues were not observed (17, 18).

Does phalloidin act specifically on cytoplasmic actin when microinjected into cells? Currently we have several lines of evidence that argue that it does. First, the induction of islands of actin aggregates is not observed when injection is performed with MeSO buffer without phalloidin present. Second, the pattern of cytoplasmic microtubules in 3T3 and in PtK2 cells stays normal in cells injected with phalloidin and cannot be distinguished by immunofluorescence microscopy from the usual pattern (8, 9, 12, 13). Third, the tonofilament system typical for PtK2 cells (8) is not changed upon microinjection of phalloidin. These results, as well as previous biochemical studies on the stoichiometric interaction of muscle actin with phalloidin in vitro (for a review see ref. 7) and the finding that phalloidin poisoning induces the extensive formation of membrane-bound actin-like filaments in the liver (17, 18), argue strongly that phalloidin and actin interact in vitro with a high degree of specificity.

Polymerized actin, at least in the form of stress fibers, is accompanied by several accessory proteins of the microfilament system, as shown by immunofluorescence microscopy. Currently myosin (19), tropomyosin (16), α-actinin (16), and filamin (14) have been identified as accessory proteins. In the case of filamin, recent experiments have shown that its intracellular distribution parallels that of actin and that it is also found in ruffling membrane (15). Our finding of filamin in islands of phalloidin-injected cells indicates that other accessory proteins of the microfilament system may also be incorporated in the phalloidin-induced actin aggregates. Electron microscopical analysis of liver membranes from phalloidin-poisoned animals has shown that phalloidin-induced actin filaments can still interact with heavy meromyosin (20).

Our results show that phalloidin interferes with cell locomotion and cell growth. Microinjection does not necessarily lead to irreversible cell damage, as had been shown previously (see for instance refs. 10 and 21). We have found a similar recovery of cells from the microinjection event (Fig. 4). Thus, the concentration-dependent reductions in cell locomotion and in cell growth observed with phalloidin-injected cells are most likely due to the formation of phalloidin-induced stable actin aggregates observed by immunofluorescence microscopy (Figs. 1–3). Assuming that phalloidin changes the mechanism of actin polymerization in the cytoplasm and that phalloidin influences actin in vivo and in vitro in a similar manner, two properties of the phalloidin–actin interaction documented previously by biochemical studies are probably very important (for a review see ref. 7). First, phalloidin drastically promotes actin polymerization. Second, the resulting phalloidin-induced F-actin,
as well as the F actin previously present, acquire, by their binding of phalloidin, a higher degree of stability than the F actin normally present. Thus, it is tempting to suggest that in normal cells a well-balanced, controlled reversible equilibrium between different states of actin polymerization states exists in the cytoplasm and may be required for cell locomotion. Phalloidin could interfere with this equilibrium in a concentration-dependent way by shifting it dramatically towards the induced actin polymers and/or the stabilized actin polymers. These, because they are more stable than normal F actin, could then be removed from the normal cellular control over actin polymerization and depolymerization and the normal actin pool would be depleted. The reduction in cellular growth observed with phalloidin-injected cells could be due either to a general change in the cells physiology because of the formation of exceptionally stable induced F actin polymers, or to the absence of a specific, more subtle, cellular process(es) that requires the normal actin distribution of the cell. Further experiments are necessary to decide between these possibilities and to determine if the time-dependent recovery of cell growth observed at the lower phalloidin concentrations is due to newly synthesized actin.

In summary, our results show that phalloidin seems to influence in a concentration-dependent manner the intracellular actin distribution by inducing the formation of stable cytoplasmic islands of actin aggregates. Because the drug also interferes in a concentration-dependent manner with cell locomotion and growth, it is possible that a well-balanced, controlled reversible equilibrium between different normal polymerization states of actin is a requirement for locomotion and may also be a requirement for other cellular functions, such as cell growth.

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