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Jasplakinolide, a Cytotoxic Natural Product, Induces Actin Polymerization and Competitively Inhibits the Binding of Phalloidin to F-actin*

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Jasplakinolide, a naturally occurring cyclic peptide from the marine sponge, *Jaspis johnstoni*, has both fungicidal and antiproliferative activity. We now report that this peptide is a potent inducer of actin polymerization *in vitro*. The peptide has a much greater effect on Mg^{2+}-actin than on Ca^{2+}-actin. Competitive binding studies using rhodamine-phalloidin suggest that jasplakinolide binds to F-actin competitively with phalloidin with a dissociation constant of approximately 15 nM. This compares favorably to the previously reported IC_{50} of 35 nM for the antiproliferative effect of jasplakinolide on PC3 prostate carcinoma cells. The binding curve suggests that nearest neighbor positive cooperativity influences the binding of jasplakinolide (and perhaps also phalloidin) to F-actin. These results imply that jasplakinolide may exert its cytotoxic effect *in vivo* by inducing actin polymerization and/or stabilizing pre-existing actin filaments.

Jasplakinolide is a cyclic peptide with a 15-carbon macrocyclic ring containing three amino acid residues: L-alanine, *N*-methyl-2-bromotryptophan, and β-tyrosine (1, 2). The function of the native molecule in the organism from which it is isolated, the marine sponge, *Jaspis johnstoni*, is unknown, but recent studies using purified jasplakinolide have demonstrated both fungicidal and antiproliferative activity (3, 4). In the current work, we show that jasplakinolide dramatically decreases the critical concentration of rabbit skeletal muscle actin and competes with rhodamine-phalloidin for F-actin. We suggest that jasplakinolide and phalloidin bind to F-actin by similar mechanisms. The pharmacologic and biochemical implications of these findings are discussed.

EXPERIMENTAL PROCEDURES

Materials—Rabbit skeletal muscle actin was prepared from frozen muscle (Pel-Freez, Rogers, AR) as previously described and stored in a buffer containing 2.5 mM imidazole, 0.2 mM ATP, 0.2 mM dithiothreitol, 0.1 mM $CaCl_2$, and 0.01% sodium azide, pH 7.8 (buffer G) (5). Pyrenyl-labeled actin[1] was prepared and found to contain 0.97 mol of label/mol of protein using the method of Kouyama and Mihashi (6). Both unlabeled and pyrenyl-labeled actins were further purified by gel filtration on Sephacryl HR-300 (Pharmacia Biotech). Jasplakinolide (NSC 613009) was provided to the Drug Synthesis and Chemistry Branch, NCI, by Dr. Phillip Crews (University of California, Santa Cruz).

Effect of Jasplakinolide on the Time Course of Actin Polymerization and Steady State Actin Concentration—Pyrenyl-labeled Ca^{2+}-actin (2.2 μM) was converted to Mg^{2+}-actin by the addition of 50 μM $MgCl_2$ and 125 μM EGTA. After 12 min, various concentrations of jasplakinolide were added and the time course of fluorescence change was monitored at an excitation wavelength of 366 nm and emission wavelength of 386 nm. Time-averaged fluorescence emission intensity was measured at 90° from incident light with a Spex Fluorolog 212 spectrofluorimeter using 0.95-ml samples. Photobleaching was minimized by use of a timed shutter. In one sample, jasplakinolide was added directly to pyrenyl-labeled Ca^{2+}-actin. At 24 h, the steady state fluorescence intensity was compared with the fraction of actin that pelleted in 30 min at 30 p.s.i. in an Airfuge (Beckman Instruments). The concentration of F-actin was calculated as total actin minus the actin in the supernatant as determined by densitometry after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7).

Competitive Binding between Rhodamine-Phalloidin and Jasplakinolide—Unlabeled Mg^{2+}-actin (final concentration, 1.1 μM), prepared as for pyrenyl-labeled actin except that only 5 min was allowed for exchange of cation, was polymerized with $MgCl_2$ (final concentration, 2 mM). In one series of experiments, 0.79 μM rhodamine-phalloidin was added to F-actin and allowed to equilibrate for 20 min before the addition of various concentrations of jasplakinolide. In a second series, jasplakinolide was added first, followed 20 min later by the addition of rhodamine-phalloidin. In both series of experiments, the fluorescence intensity was monitored with excitation at 550 nm and emission at 580 nm, and steady state values were obtained after 2 h. The increment in fluorescence caused by binding of rhodamine-phalloidin to F-actin was determined in separate control experiments in which the fluorescence of 0.79 μM rhodamine-phalloidin was determined in the presence and absence of excess F-actin. With this information, the steady state fluorescence data for the experimental samples could be converted to the amount of rhodamine-phalloidin that was bound to F-actin (8).

Data were fit using an infinite linear lattice model with competition between binding sites to determine the dissociation constant of jasplakinolide and F-actin (K_D^J) (9). A K_D^P of 40 nM was assumed for the interaction between rhodamine-phalloidin and F-actin with stoichiometry of 1:1 (8) (see Ref. 10 for alternative binding scheme). The critical concentration of actin was assumed to be zero in 0.79 μM phalloidin (11). Using the conditional probability method of McGhee and von Hippel (12), additional variation in the theoretical binding curve could be explicitly determined for varying K_D^J and ω, an interaction term describing the effect on the binding constant if a nearest neighbor along the one-start actin filament already has its jasplakinolide binding site occupied by jasplakinolide (*i.e.* $\omega = 1$ if there is no effect on the nearest neighbors and $\omega > 1$ if nearest neighbor binding is influenced by positive cooperativity).

RESULTS

Time Course of Actin Polymerization and Steady State Concentration of F-actin—Addition of 0.5–7.4 μM jasplakinolide increased the fluorescence of 2.2 μM pyrenyl-labeled Mg^{2+}-actin, indicative of polymerization, without any additional monovalent or divalent cations, *i.e.* under nominally non-polymerizing conditions (Fig. 1). The rate of fluorescence increase was greater with increasing concentrations of drug. All samples reached steady state fluorescence intensity by 24 h. The

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[1] The abbreviation used is: pyrenyl-labeled actin, *N*-pyrenylcarboxyamidoethyl actin.

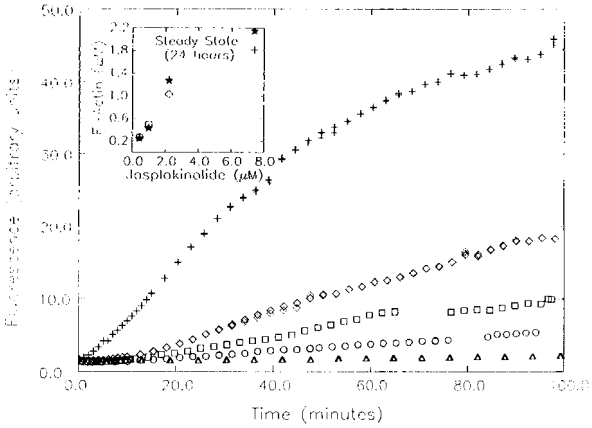

FIG. 1. **Time course of actin polymerization as monitored by fluorescence intensity.** Data are shown for 0.5 (○), 1.0 (□), 2.2 (◇), and 7.4 (+) μM jasplakinolide with 2.2 μM pyrenyl-labeled Mg^{2+}-actin, except for one sample with 2.2 μM jasplakinolide and 2.2 μM Ca^{2+}-actin (△). The *inset* shows the steady state fluorescence intensity for the Mg^{2+}-actin samples after correlation of fluorescence intensity with F-actin concentration and the corresponding amounts of pelletable F-actin (★). All samples were in a "non-polymerizing" buffer containing 0.25 imidazole, 0.2 mM ATP, 0.2 mm dithiothreitol, 0.1 mM $CaCl_2$, and 0.01% sodium azide, pH 7.8.

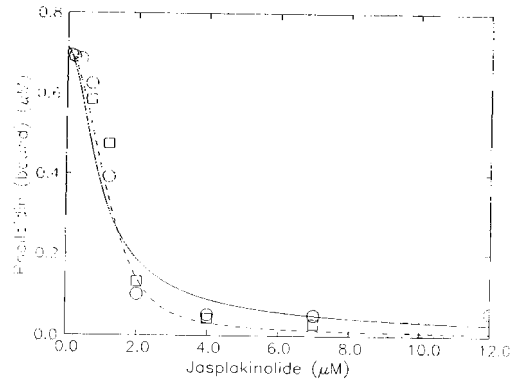

FIG. 2. **Competitive binding of jasplakinolide and rhodamine-phalloidin for F-actin.** All samples contained 1.1 μM Mg^{2+}-F-actin, 0.79 μM rhodamine-phalloidin, and jasplakinolide at the indicated concentrations in the same buffer as in Fig. 1 plus 2 mM $MgCl_2$. Samples to which rhodamine-phalloidin was added first are shown by *circles*; samples to which jasplakinolide was added first are shown by *squares*. The *solid curve* represents the best fit for competitive binding with K_D^J = 15 nM. The *dashed curve* shows that a better fit can be obtained when nearest neighbor cooperativity, ω, is 10 and K_D^J is 300 nM.

amount of pelletable actin correlated well with the fluorescence intensity and varied linearly with the jasplakinolide concentration, at least until the molar stoichiometry of jasplakinolide to actin reached 1:1 (Fig. 1, *inset*). A sample of 2.2 μM pyrenyl-labeled Ca^{2+}-actin and equimolar jasplakinolide increased in fluorescence much more slowly than the equivalent sample of Mg^{2+}-actin and jasplakinolide (Fig. 1, compare *triangles* with *diamonds*). At steady state, 0.6 μM of the Ca^{2+}-actin had polymerized (data not shown) compared to 1 μM of Mg^{2+}-actin (Fig. 1, *inset*). Samples with only 5% pyrenyl-labeled actin gave the same relative increases in fluorescence intensity as the samples with 100% pyrenyl-labeled actin (data not shown).

To confirm that the increase in fluorescence intensity reflected actin polymerization, samples were negatively stained with uranyl acetate and examined by electron microscopy. Numerous 9-nm-wide filaments were present in the samples with jasplakinolide, and their appearance was indistinguishable from that of filaments polymerized in 2 mM $MgCl_2$ in the absence of jasplakinolide. Filaments were not present in actin samples to which neither jasplakinolide nor $MgCl_2$ had been added (data not shown).

Actin filaments made by the addition of equimolar jasplakinolide were resistant to depolymerization. After dilution of these filaments into buffer G to a concentration of 100 nM (from a 10 μM stock solution of pyrenyl-labeled Mg^{2+}-F-actin), the fluorescence intensity decreased <2% in 60 s and <8% after 5 min. A control sample of pyrenyl-labeled Mg^{2+}-F-actin (in 2.0 mM $MgCl_2$) diluted in the same manner had a 65% decrease in fluorescence intensity in the first 60 s and was completely depolymerized after 5 min.

Competitive Binding Study—Control samples demonstrated that binding of rhodamine-phalloidin to rabbit muscle actin resulted in a 15.4-fold increase in fluorescence intensity. Increasing concentrations of jasplakinolide decreased the fluorescence intensity observed at a fixed concentration of F-actin (1.1 μM) and rhodamine-phalloidin (0.79 μM) (Fig. 2). The order of addition of rhodamine-phalloidin and jasplakinolide had no effect on the results, suggesting that the decrease in fluorescence was due to an equilibrium reaction. Assuming a K_D^P of 40 nM for the interaction of rhodamine-phalloidin with F-actin, the best fit to a simple competitive binding model was obtained with K_D^J (the dissociation constant of jasplakinolide from F-actin) equal to 15 nM (Fig. 2, *solid line*). This value compares favorably to the IC_{50} of 35 nM for PC3 prostate carcinoma cells (4). A better fit to the data could be obtained by assuming that jasplakinolide interacts with the nearest neighbor binding sites to increase their affinity for jasplakinolide (Fig. 2, *dashed line*). The earliest portions of the binding curve (jasplakinolide concentration < 1 μM) could be fit only by a still more complicated model in which rhodamine-phalloidin also exerted nearest neighbor positive cooperativity for the binding of additional rhodamine-phalloidin (theoretical curve not shown).

To determine whether the loss of rhodamine fluorescence in the presence of jasplakinolide was, in fact, due to competitive binding rather than, for example, quenching of fluorescence, rhodamine-phalloidin-labeled F-actin was pelleted from solutions containing 0.2 and 2.0 μM jasplakinolide. Only the pellet from the solution containing 0.2 μM jasplakinolide was darkly colored (from the bound rhodamine-phalloidin). When unlabeled F-actin was added to the supernatant of the solution originally containing 2.0 μM jasplakinolide, the fluorescence intensity increased to approximately 50% of the original fluorescence in the absence of jasplakinolide. Addition of F-actin to the supernatant of the solution originally containing 0.2 μM jasplakinolide resulted in no significant fluorescence change (data not shown). These results indicate that the higher concentration of jasplakinolide competitively removed rhodamine-phalloidin from the F-actin so that rhodamine-phalloidin was available to bind to a fresh aliquot of F-actin.

DISCUSSION

The bicyclic phalloidin peptides were first isolated in 1937 (13). Other than the chemically similar virotoxins (both phalloidin and the virotoxins are heptapeptides isolated from poisonous *Amanita* mushrooms), no other chemicals have been previously shown to stabilize actin filaments. The identification of jasplakinolide as a drug that binds F-actin competitively with phalloidin may provide insight regarding the mechanism by which cyclic peptides bind to actin, and subsequently, perhaps explain why these peptides stabilize actin filaments. Chemically, the 15-carbon macrocyclic ring of jasplakinolide bears little resemblance to that of the virotoxins or phalloidin except for the presence of an L-alanine residue followed by *N*-methyl-2-(bromo)-L-tryptophan in jasplakinolide compared with alanine followed by 2-(methylsulfonyl)-L-tryptophan (virotoxin) or L-tryptophan linked to cysteine by a sulfide bridge

(phalloidin) (1, 2, 14). The hydroxyl groups in the peptide residues preceding alanine that are critical for virotoxin and phalloidin activity (15) are absent from jasplakinolide.

Although phalloidin and jasplakinolide have similar affinities for F-actin, jasplakinolide seems to stabilize filaments more effectively. For example, the results reported in Estes *et al.* (16) imply that Ca^{2+}-actin in 100 μM phalloidin (1:1, actin to phalloidin) has an apparent critical concentration of more than 50 μM, whereas we estimate a critical concentration of 1.6 μM for 2.2 μM Ca^{2+}-actin in the presence of equimolar jasplakinolide. Although the much greater effect of jasplakinolide on Mg^{2+}-actin than on Ca^{2+}-actin has not been reported for phalloidin, it might be predicted from the suggestion of Estes *et al.* (16) that phalloidin stabilizes F-actin by decreasing the rate of dissociation of actin oligomers. As the nucleation rate of Mg^{2+}-actin is faster than that of Ca^{2+}-actin and the elongation rates are similar (17), phalloidin would be expected to enhance the rates of polymerization of Mg^{2+}-actin and Ca^{2+}-actin in proportion to their relative nucleation rates. An alternative explanation derives from the fact that phalloidin inhibits the dissociation of P_i from F-actin, but not the hydrolysis of ATP to ADP-P_i on F-actin (18). Because the ratio of the dissociation rate of ADP-actin subunits to that of ADP-P_i-actin subunits is lower for Mg^{2+}-actin than for Ca^{2+}-actin (17), the prevention of loss of P_i would be expected to have a greater effect on Mg^{2+}-actin than Ca^{2+}-actin. These various possibilities can be tested by quantitative evaluation of nucleation and elongation rate constants in the presence of phalloidin and jasplakinolide.

The pharmacologic usefulness of phalloidin and virotoxin has been extremely limited because, in general, cells (both in culture and *in vivo*) are impermeable, and therefore insensitive to them (19, 20). Hepatocytes are an unfortunate exception in which uptake is mediated by a membrane-bound bile acid-transporter resulting in severe hepatotoxicity (21). Jasplakinolide, in contrast to phalloidin and virotoxins, has demonstrated cytotoxicity to a number of tumor-derived cell lines, including breast and prostatic cancers (3, 4).

Previous studies (4) demonstrated that exposure of living cells to jasplakinolide markedly altered the distribution of rhodamine-phalloidin (a specific label for filamentous actin) applied after fixation and permeabilization. Since the current results show that phalloidin and jasplakinolide interact competitively, further studies are necessary to clarify the ultrastructural basis for the change in cellular distribution of actin label. Nevertheless, the present experiments identify F-actin as a molecular target for jasplakinolide. (The increased permeability of cells to jasplakinolide relative to phalloidin suggests that fluorescent derivatives of jasplakinolide may prove to be a valuable tool for the study of the distribution of actin in cells that have not been subjected to harsh permeabilization procedures.) In summary, although a number of chemotherapeutic agents have activities directed at the microtubule-based cytoskeleton, the results reported in this paper suggest that jasplakinolide may be a useful pharmacologic agent by virtue of its ability to induce actin polymerization and inhibit the depolymerization of actin filaments.

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