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Actin-Binding Specificity of Marine Macrolide Toxins, Mycalolide B and Kabiramide D[1]

Shun-ichi Wada,* Shigeki Matsunaga,* Shin-ya Saito,† Nobuhiro Fusetani,* and Shugo Watabe*,[2]

Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657; and †Department of Veterinary Pharmacology, Iwate University, Morioka, Iwate 020-8550

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An actin-depolymerizing marine natural product, mycalolide B, and a related compound, kabiramide D, were labeled with biocytin, a biotin derivative, and used to specify target molecules in cultured rat 3Y1 fibroblasts. Mycalolide B exhibited the ability to bind to various intracellular proteins, probably through the Michael addition of a sulfhydryl group to C5 of mycalolide B. However, no intracellular proteins other than actin apparently reacted with biocytinylated kabiramide D, demonstrating that the binding of kabiramide D to actin was highly specific. Cells treated with biocytinylated kabiramide D followed by staining with fluorescein isothiocyanate-conjugated avidin showed that biocytinylated kabiramide D bound to stress fibers composed of F-actin, although the staining intensity was weaker than the fluorescent phalloidin staining. The assay for the binding of kabiramide D to actin, which had previously been treated with other actin-depolymerizing agents, showed that the actin-binding site for kabiramide D was the same as that for bistheonellide A, but not those for latrunculin A and cytochalasin D.

Key words: actin, biocytin, kabiramide D, marine natural product, mycalolide B.

Actin is known as a contractile and structural protein, and regulation of its polymerization from globular (G-) actin monomers to fibrous (F-) actin is primarily relevant to its functions. Cytochalasins, fungal secondary metabolites, have been used as sole actin-depolymerizing agents since the discovery of their ability to disorder actin-filaments early in the 1970s (*1*). For example, cytochalasins have been used as tools to investigate the kinetic behavior of actin polymerization (*1*). Recently, the demand for actin-depolymerizing agents for cell biology further increased, since the dynamic reorganization of actin filaments has been demonstrated to be closely related to the cell cycle and other actin-associated cellular events. In addition, signal transduction pathways stimulated by cell adhesion and the addition of growth factors have been found to proceed when microfilaments of F-actin are formed (*2-4*).

Cytochalasins, however, seem to have two disadvantages when used as molecular probes for investigating intracellular actin functions (*1*). First, the specificity of cytochalasins as to actin is not well established. Cytochalasin B inhibits actin polymerization, but is also known as an inhibitor of monosaccharide transportation without any actin-related reaction (*1*). Although other types of cytochalasins, including cytochalasin D, do not have such an actin-unrelated function, it seems difficult to exclude their possible interaction with proteins other than actin. Second, cytochalasins are incapable of depolymerizing F-actin into monomers, the actins remaining in an oligomer form. Cytochalasins rather promote the nucleation of actin polymerization (*5*). These features make it difficult to precisely determine the functions of microfilaments in cellular dynamics when cytochalasins are used as research tools.

In the mid-1980s, latrunculins isolated from a marine sponge were demonstrated to be potent inhibitors of actin polymerization (*6, 7*). Since then, several bioactive compounds of marine origin have been identified as actin-depolymerizing agents (*8-12*). These compounds are conventionally classified into three groups according to their structures. The first group is represented by latrunculins, which consist of a 2-thiazolidinone macrolide with no long side chains (*13*). The second group of compounds has a macrolide structure with a single side chain. Tolytoxin (*14*), mycalolide B (*15*), and aplyronin A (*16*) belong to this group. Although the structures of their macrolide rings are quite different from each other, their side chains are similar. The third group is composed of macrodiolides including swinholide A (*17*) and bistheonellide A (*18*). They have symmetric structures with two side chains in contrast to the one side chain found in the second group.

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[2] To whom correspondence should be addressed. Tel: + 81-3-3812-2111 (Ext. 7520), Fax: +81-3-5684-0622, E-mail: awatabe@hongo.ecc.u-tokyo.ac.jp

Abbreviations: BSA, bovine serum albumin; ECL, enhanced chemiluminescence; FITC, fluorescein isothiocyanate; PVDF, polyvinylidene difluoride; TRITC, tetramethylrhodamine isothiocyanate.

The compounds in these three groups are similar to each other in terms of the inhibition of actin-polymerization, although they exhibit some differences in F-actin severing activity. They bind to actin monomers in a molar ratio of 1:1 or 1:2. Since those of the third group have a symmetric structure with two side chains extending from the central macrodiolide, it is plausible that they bind to two actin monomers rather than one dimer of actin. They exhibit high ability to sequester actin molecules from polymer actins, thus depolymerizing actin-filaments drastically. Since the intracellular levels of actin filaments are regulated by various actin-binding proteins, it might be impossible to completely depolymerize them even with these notable actin-depolymerizing agents. However, these agents effectively disrupt stress fibers in cultured cells with 1/10~1/1,000 concentrations of cytochalasins (*6, 8, 10, 11*). Marine actin-depolymerizing agents are thus expected to be useful for a wide range of actin-related cellular research. For example, latrunculin A has already shown the involvement of actin-filaments in fertilization and early development in the sea urchin and mouse (*19*), and in determination of the cell polarity in yeast (*20, 21*). However, these compounds also possibly interact with proteins other than actin, and such a possibility has not been satisfactorily examined yet.

In this study, we labeled mycalolide B and a related compound, kabiramide D, with biocytin, an analogue of biotin, and then examined their interactions with intracellular proteins including actin, in comparison with other actin-depolymerizing agents.

MATERIALS AND METHODS

Materials—Mycalolide B, bistheonellide A, and kabiramide D were isolated from a marine sponge, *Mycale* sp. (*15*), a sponge, *Theonella* sp. (*18*), and egg masses of the nudibranch mollusk, *Hexabranchus* sp. (*22*), respectively. Latrunculin A and cytochalasin D were obtained from Wako Pure Chem. (Osaka). They were dissolved in methanol and diluted when used for the following experiments. Other agents, unless indicated otherwise, were of analytical grade and obtained from either Sigma or Wako.

Biocytinylation of Mycalolide B and Kabiramide D—Mycalolide B was hydrolyzed at 60°C for 1 h in a solution comprising 0.5 N HCl and 50% acetonitrile to yield the terminal aldehyde. The aldehyde derivative was partitioned between water and ethyl acetate, and the organic layer was concentrated *in vacuo*. It was then dissolved in methanol at a concentration of 1 mg/100 μl, and then 1 mg of biocytin dissolved in 100 μl of 50% methanol and 2 mg of $NaCNBH_3$ were added. The mixture was left to stand at room temperature for 1 h. The reaction mixture was separated to give biocytinylated mycalolide B by HPLC on an ODS column (Cosmosil 5C18-MS, 1×25 cm) equilibrated with 75% methanol. The molecular weight of the biocytinylated mycalolide B was determined to be 1,364 by FAB mass spectrometry, which was consistent with the expected molecular formula of $C_{66}H_{99}N_7O_{20}SNa$ (M+Na) (*15*). Biocytinylation of kabiramide D by the same procedure resulted in a labeled compound with a molecular weight of 1,236 on FAB mass spectrometry, this being in good agreement with the expected formula of $C_{61}H_{95}N_7O_{16}SNa$ (M+Na) (*22*).

Cell Culture—The rat embryonic fibroblast cell line, 3Y1 B Clone 1-6 (*23*), was obtained from the Japanese Cancer Research Resources Bank. Cells were grown in Dulbecco's modified Eagle medium (Gibco BRL) supplemented with 10% fetal calf serum (BioWhittaker) and an antibacterial-antimycotic cocktail consisting of penicillin G sodium, streptomycin, amphotericin B and gentamicin (Gibco BRL). They were incubated under a humidified atmosphere of 5% CO_2: 95% air at 37°C.

Preparation of Proteins—All procedures were performed at 4°C. Forty microliters of exponentially growing cells (density 1.5×10^7) was collected and homogenized in 250 μl of a Triton lysis buffer comprising 10 mM Tris-HCl (pH 7.2), 1% Triton X-100, 0.15 M NaCl, 2 μg/ml chymostatin, 2 μg/ml E-64, and 0.4 mM phenylmethylsulfonyl fluoride. The soluble fraction was obtained by centrifugation at 15,000×*g* for 20 min after extraction under shaking for 30 min. For the precipitation assay described below, the soluble fraction was gel-filtered on a Sepharose 6B column (1×5 cm) equilibrated with a high salt-Triton lysis buffer containing 1 M NaCl instead of 0.15 M NaCl.

Actin was purified from rabbit fast skeletal muscle according to the method described by Spudich and Watt (*24*), using buffer G comprising 2 mM Tris-HCl (pH 8.0), 0.2 mM $CaCl_2$, 0.2 mM ATP, and 0.5 mM β-mercaptoethanol.

Detection of Proteins Bound to Mycalolide B and Kabiramide D—To the soluble fraction of 1.5×10^7 3Y1 cells was added 0.5 μM biocytinylated mycalolide B or biocytinylated kabiramide D in 250 μl of the Triton lysis buffer at 0°C for 1 h. To remove excess unbound agents, the reaction mixture was dialyzed twice against 250 mM Tris-HCl (pH 7.2) containing 0.775 M NaCl at 0°C for 3 h. Bound proteins were treated with an equal volume of SDS-PAGE sample buffer comprising 50 mM Tris-HCl (pH 6.8), 2% SDS, and 5% glycerol, and then boiled for 5 min prior to SDS-PAGE (*25*). Proteins in SDS-PAGE gels were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore), and blocked with 5% skim milk for 2 h at room temperature followed by the addition of 1 μg/ml horseradish peroxidase-conjugated streptavidin (Jackson Immuno Research Laboratories). The proteins covalently bound to biocytinylated mycalolide B or biocytinylated kabiramide D were detected with an enhanced chemiluminescence system (ECL: Amersham).

Proteins non-covalently bound to biocytinylated agents were detected by the following precipitation method. To 2 ml of the Triton lysis buffer containing 0.5 μM biocytinylated kabiramide D was added the gel-filtered soluble fraction of 1.5×10^7 3Y1 cells described above. The reaction mixture was treated with 100 μl of agarose-conjugated streptavidin (Calbiochem) and then incubated at 0°C for 1.5 h. This reaction mixture was centrifuged at 100×*g* for 1 min, and the resulting precipitate was washed with the high salt-Triton lysis buffer. After repeating the same washing procedure two more times, the bound proteins in the precipitate were solubilized with an equal volume of the SDS-PAGE sample buffer, subjected to SDS-PAGE, and then detected by silver staining. In the case of purified actin, the same methods were performed using 0.5 nmol actin. For the competition assay, actin was pretreated with a 10-fold molar concentration of kabiramide D, bistheonellide A, latrunculin A, or cytochalasin D at 0°C for 1.5 h. The following reactions with biocytinylated kabiramide D and

subsequently with agarose streptavidin were carried out as described above in actin-containing mixtures.

Interaction between Actin-Depolymerizing Agents and Cysteine—Mycalolide B, bistheonellide A, latrunculin A, and cytochalasin D were dissolved at 25 nmol/100 μl in 10 mM Tris-HCl (pH 7.2) containing 0.15 M NaCl, to which was added 0.5 μmol cysteine-HCl. Each solution was immediately neutralized with 0.5 μmol NaOH and then stirred for 1.5 h at room temperature. Reaction products were fractionated by HPLC on the same ODS column equilibrated with 78% methanol as used for the preparation of biocytinylated mycalolide B.

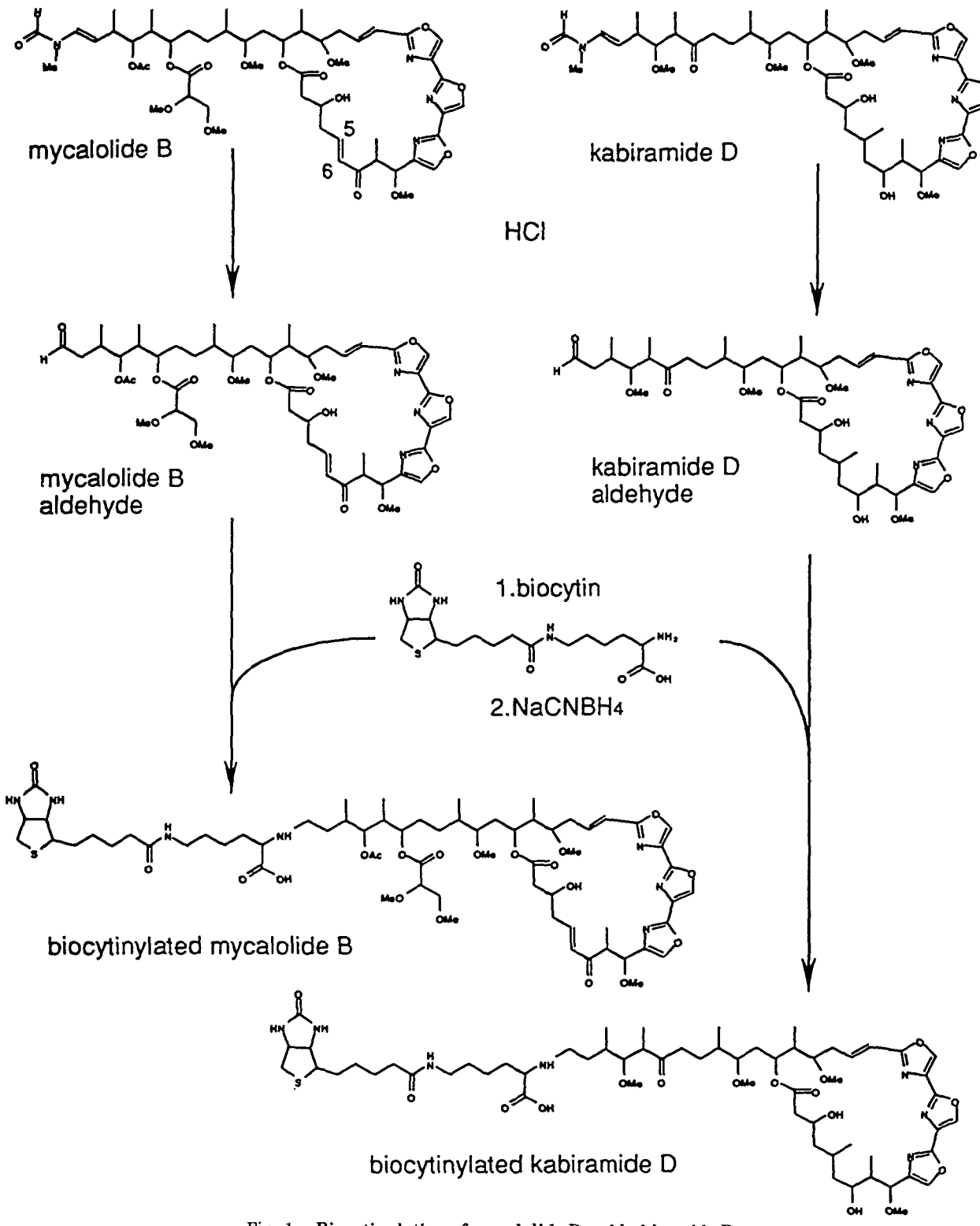

Fig. 1. **Biocytinylation of mycalolide B and kabiramide D.**

Fluorescence Staining of Intracellular Proteins—Cells were seeded on a glass coverslip in a 8-cm² Petri dish at 6.0×10^4 cells/2 ml. After incubation for 24 h at room temperature, they were fixed with 3.7% formaldehyde for 20 min and then permeabilized with phosphate-buffered saline (PBS) containing 0.25% Triton X-100 for 5 min. After washing twice with PBS, the cells were blocked with PBS containing 2% bovine serum albumin (BSA) for 1 h and subsequently 0.1% BSA for 10 min. Biocytinylated mycalolide B or biocytinylated kabiramide D at 5 μM was then applied to the fixed cells, followed by incubation for 2 h. To the cells was added fluorescein isothiocyanate (FITC)-conjugated avidin at 7.5 μg/ml, and then the mixture was left to stand for 2 h. Excess reagents were removed by washing three times for 5 min with Tris-buffered saline (TBS) containing 250 mM Tris-HCl (pH 8.0), 4.5% NaCl, and 0.05% Tween 20. Fixed cells were observed under a Nikon Labophoto-2 light microscope equipped with a Nikon HB-10101 AF fluorescence apparatus. Cells stained with tetramethylrhodamine B isothiocyanate (TRITC)-conjugated phalloidin were observed, as a reference, to compare actin-staining patterns (*11*).

RESULTS

We chose biotin for labeling mycalolide B, since biotin-labeled compounds, which are easily detected with avidin/streptavidin conjugates, have been proved to be useful for various experiments. However, intact mycalolide B was not applicable to labeling with biotin. Therefore, an aldehyde derivative of mycalolide B was first prepared by removing the *N*-methyl formamide group in the terminal side chain. From among a few amine-containing derivatives of biotin, we employed biocytin for coupling with the mycalolide B aldehyde (Fig. 1).

Then biocytinylated mycalolide B was examined for its specificity as to binding to actin by using a whole cell lysate of 3Y1 fibroblasts. Since mycalolide B was observed to bind to actin covalently in our preliminary experiments, we analyzed, by SDS-PAGE, proteins which were bound to biocytinylated mycalolide B even after treatment with 2% SDS and 5% β-mercaptoethanol (Fig. 2). While a few bands were detected after treatment with peroxidase-conjugated streptavidin only (lane 4), various proteins as well as actin reacted with biocytinylated mycalolide B (lane 5). However, no differences in the staining pattern were observed between a control (lane 4) and biocytinylated kabiramide D (lane 6). It was considered that such a complex staining pattern with biocytinylated mycalolide B might result from a non-specific covalent interaction between mycalolide B and proteins, probably *via* thiol groups in the latter which could form a Michael adduct in the α,β-unsaturated moiety of mycalolide B (see Fig. 1). To examine this, mycalolide B and cysteine were mixed in 10 mM Tris-HCl (pH 7.2) containing 0.15 M NaCl, and the reaction products were analyzed by HPLC (Fig. 3). Intact mycalolide B appeared as peak #1, whereas other peaks eluted were identified as being due to the buffer components (Fig. 3A). When mycalolide B was mixed with cysteine, peak #1 corresponding to mycalolide B almost disappeared, and instead two peaks, #2 and #3, were newly observed in addition to a large peak containing cysteine (Fig. 3B). However, latrunculin A and bistheonellide A, which each have an α,β-unsaturated ester moiety, did not react with cysteine in the above system (data not shown). Although mycalolide B is a powerful tool for depolymerizing F-actin *in vitro*, its specificity toward actin in the presence of other proteins *in vivo* is questionable.

Kabiramide D (*22*), another macrolide, is an analogue of mycalolide B, but does not contain any reactive ketone group (Fig. 1). No proteins in the whole cell lysate of 3Y1 fibroblasts were found to bind to biocytinylated kabiramide D in the presence of 2% SDS, as described above (see Fig. 2). Furthermore, kabiramide D was found not to be reactive with cysteine, as judged on HPLC (data not shown). Biocytinylated kabiramide D specifically bound to actin in

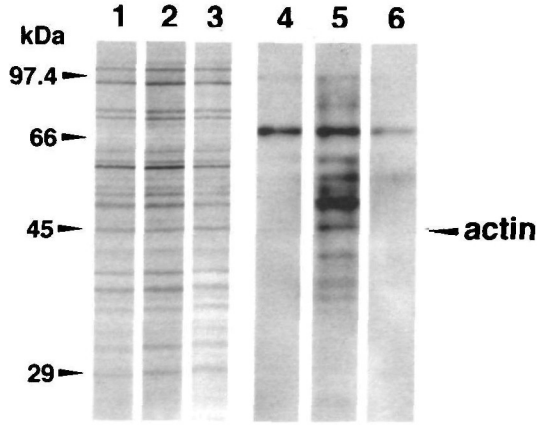

Fig. 2. **Interactions of mycalolide B and kabiramide D with proteins in 3Y1 cells.** A whole cell lysate of 3Y1 fibroblasts, 1.5×10^7 cells/250 μl, was reacted with 1% methanol (lanes 1 and 4), 0.5 μM biocytinylated mycalolide B (lanes 2 and 5), and 0.5 μM biocytinylated kabiramide D (lanes 3 and 6), and then subjected to SDS-PAGE followed by silver staining (lanes 1-3). Alternatively, proteins in the gels were transferred to PVDF membranes and then reacted with peroxidase-conjugated streptavidin. Proteins bound to the biotinylated compounds were visualized with an ECL system (lanes 4-6).

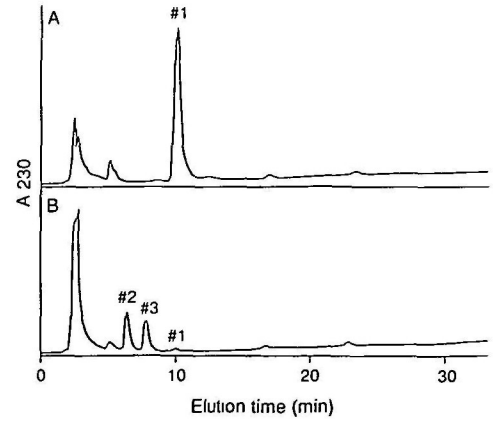

Fig. 3. **HPLC patterns showing the interaction between mycalolide B and cysteine.** Mycalolide B (25 nmol) was dissolved in 10 mM Tris-HCl (pH 7.15) containing 0.15 M NaCl (A), and then 0.5 μmol cysteine was added (B). Intact mycalolide B was eluted as peak #1, whereas reaction products appeared as peaks #2 and #3. The injection volume was 45 μl. Other HPLC conditions are given in the text.

the whole cell lysate was confirmed when the protein fraction bound to biocytinylated kabiramide D was precipitated with agarose-conjugated streptavidin and subsequently analyzed by SDS-PAGE (Fig. 4, lane 2). Other bands in the range of 55–70 kDa probably represented proteins bound to agarose-conjugated streptavidin nonspecifically, since these bands were also observed for the control treated with 1% methanol in the absence of biocytinylated kabiramide D (Fig. 4, lane 1).

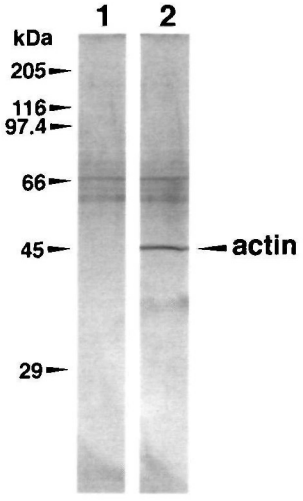

Fig. 4. **Interaction of biocytinylated kabiramide D with actin.** Whole cell lysates of 3Y1 fibroblasts, 1.5×10^7 cells/ 250 μl, were reacted with 1% methanol (lane 1) and 0.5 μM biocytinylated kabiramide D (lane 2). Proteins bound to kabiramide D were separated as a precipitate with agarose-conjugated streptavidin, and then subjected to SDS-PAGE followed by silver staining.

The profiles of intracellular proteins bound to mycalolide B and kabiramide D were also examined with fixed cells using biocytinylated derivatives (Fig. 5). 3Y1 cells fixed with formaldehyde were reacted with mycalolide B followed by biocytin and FITC-conjugated avidin, and are shown as a control in panel A. Only faint fluorescence was observed around nuclei. In panel B, proteins bound to mycalolide B were visualized by staining with the biocytinylated derivative and FITC-avidin. Filamentous structures similar to stress fibers could be seen in peripheral regions of 3Y1 cells, whereas intense fluorescence was observed in the central regions including nuclei. The

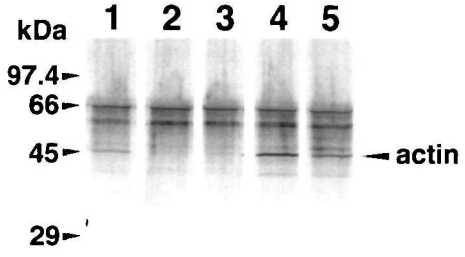

Fig. 6. **Inhibition of actin-binding to biocytinylated kabiramide D by other actin-depolymerizing agents.** Actin (0.5 nmol) purified from rabbit skeletal muscle was reacted with 1% methanol (lane 1), 5 nmol kabiramide D (lane 2), 5 nmol bistheonellide A (lane 3), 5 nmol latrunculin A (lane 4), and 5 nmol cytochalasin D (lane 5). After biocytinylated kabiramide D (1 nmol) had been added to the reaction mixtures, actin bound to it was separated as a precipitate with agarose-conjugated streptavidin and then subjected to SDS-PAGE followed by silver staining.

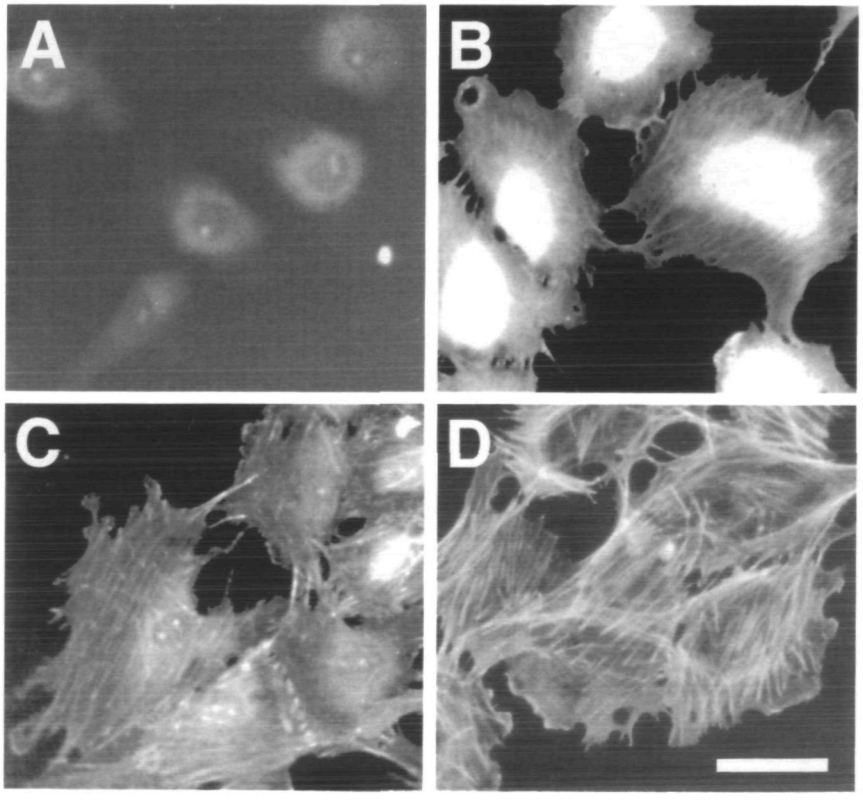

Fig. 5. **Localization of proteins bound to biocytinylated mycalolide B and biocytinylated kabiramide D.** Exponentially growing 3Y1 cells were fixed with 3.7% formaldehyde, and then reacted with 5 μM mycalolide B and 5 μM biocytin (A), 5 μM biocytinylated mycalolide B (B), 5 μM biocytinylated kabiramide D (C), and 0.16 μM rhodamine-phalloidin (D). FITC-conjugated avidin was employed for visualization of the bound proteins in A–C. The bar represents 50 μm.

staining pattern with biocytinylated kabiramide D was different from that with biocytinylated mycalolide B, there being a fluorescent reaction along the stress fibers but not around nuclei (Fig. 5C). Staining with TRITC-phalloidin as a control showed typical F-actin localization (Fig. 5D).

Since it seems that biocytinylated kabiramide D is a better probe than biocytinylated mycalolide B, we examined whether or not the binding site of kabiramide D for actin is the same as those of other actin-depolymerizing agents. Actin purified from rabbit fast skeletal muscle was pretreated with 1% methanol as a reference, or a 10-fold molar concentration of each of kabiramide D, bistheonellide A, latrunculin A, and cytochalasin D (Fig. 6). To the reaction mixtures was added biocytinylated kabiramide D, and then actin that bound to it was separated as a precipitate with agarose-streptavidin. Actin bound to kabiramide D and bistheonellide A could hardly react with biocytinylated kabiramide D. However, actin pretreated with methanol, latrunculin A, or cytochalasin D bound to biocytinylated kabiramide D. These results suggest that kabiramide D has the same binding site as bistheonellide A, but that this site is different from those of latrunculin A and cytochalasin D. It was noted that actin pretreated with latrunculin A apparently exhibited the highest affinity to actin in the present analytical system.

DISCUSSION

Marine toxins endowed with actin-depolymerizing ability have been kinetically studied with pyrene-labeled actin, whereas they have been effectively used as probes to investigate actin-associated events in cultured cells (*6–12*). Kinetic studies on the interaction of marine toxins with actin revealed that they bind to actin in a molar ratio of 1:1 or 1:2, and that some of them exhibit F-actin severing activity. However, the specificity of these agents as to actin has remained unclear.

Mycalolide B, one of the most characterized actin-depolymerizing agents, has an α,β-unsaturated ketone moiety which is highly reactive with the thiol groups in cysteine residues. The present study clearly demonstrated that mycalolide B reacted with cysteine (see Fig. 3) and biocytinylated mycalolide B bound to proteins other than actin in 3Y1 cells, possibly *via* their cysteine residues (see Fig. 2). The two peaks observed on HPLC (see Fig. 3) suggest the presence of two optical isomers of the bound complex. Such unexpected reactivity of mycalolide B with proteins was first recognized at a slightly high pH (7.8), where the reactivity of the thiol groups was enhanced (data not shown). When corresponding experiments were performed under physiological pH conditions (7.2), the same results were obtained. Recently, we isolated a C5 glutathione adduct of mycalolide B, through a sulfur atom, from the marine sponge, *Mycale* sp. (data to be published elsewhere), indicating the possibility that Michael addition takes place in living cells. In cultured cells fixed with formaldehyde, intense localization of biocytinylated mycalolide B was observed around nuclei (see Fig. 5). Since stress fibers are not particularly concentrated in this area, it seems reasonable to assume that proteins other than actin were also stained. Mycalolide B induced the same morphological changes of cultured cells as those with other actin-depolymerization agents, giving rise to cellular shrinkage and many arborescent processes with comparable doses (data not shown). Therefore, it is suggested that actin has specific site(s) for binding to mycalolide B other than cysteine residues, and other proteins bound do not interfere with such actin-binding to mycalolide B. On the other hand, kabiramide D, an analogue of mycalolide B lacking the ability to react with thiol groups, could bind to actin with high specificity (see Fig. 4). Such high specificity was further examined by light microscopy, biocytinylated kabiramide D being observed to react only with stress fibers composed of actin (see Fig. 5). However, the staining intensity was weaker than that with TRITC-phalloidin, suggesting that kabiramide D has a lower affinity to F-actin than phalloidin, although it is well known that the fluorescence of FITC is weaker than that of TRITC.

It was found that actin-depolymerizing agents with long side chains, including mycalolide B (data not shown), kabiramide D and bistheonellide A, have a common site for actin binding, differing from those of latrunculin A and cytochalasin D. The actin-depolymerizing activity of kabiramide D is now under investigation using pyrene-labeled actin. So far, it is suggested that kabiramide D shows not only actin-sequestering but also actin-severing activity (data not shown). Biocytinylated kabiramide D reacted with actin bound to latrunculin A and cytochalasin D, but not with that bound to kabiramide D and bistheonellide A. Actin treated with latrunculin A showed increased affinity to biocytinylated kabiramide D (see Fig. 6), probably due to the complete depolymerization of the actin sample with latrunculin A.

It has been reported that Arg183, Asp184, Arg210, Asp211, Lys213, Glu214, and Lys215 of the yeast actin molecule are important for binding with latrunculin A (*20*). However, Val139 and Ala295 of actin in KB cells are reactive with cytochalasin B (*26*). Actin is well-known as a conserved protein. Therefore, such reported data, together with the present results, indicate that latrunculins, cytochalasins, and other actin-depolymerizing agents with functional side chain(s), such as kabiramide D and bistheonellide A, recognize different amino acids of the actin molecule from each other. Since it seems that actin is the sole common target protein for the above agents, combined adoption of these agents is highly useful for elucidating unknown actin-associated cellular events.

We could label mycalolide B and kabiramide D with biocytin by removing the *N*-methyl formamide group in the terminal side chain. While mycalolide B covalently bound not only to actin but also to various intracellular proteins, the binding of kabiramide D to actin was noncovalent and highly specific. In addition, it was suggested that in the actin molecule, kabiramide D bound to a site different from those for latrunculin A and cytochalasin D. Various actin-related experiments involving biocytinylated kabiramide D are now in progress in our laboratory. Since latrunculin A can be chemically synthesized (*27*), its application as an actin-depolymerizing agent in cell biology will increase further. Aquatic organisms potentially contain various actin-depolymerizing agents, whereas macrolides belonging to the group with long side chain(s) have many analogues (*e.g.* Refs. *15, 17, 18,* and *28*). It would be interesting to examine their different profiles as to interaction with actin, which would thus disclose the physiological roles of actin in cellular events more clearly.

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