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Low Concentration of Reserpine Accelerates Actin Polymerization via Interaction with G-Actin

SEIJI NAKAMURA, KAZUHIRO OHMI, and YOSHIAKI NONOMURA

Department of Pharmacology, Faculty of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo, Japan (S.N., K.O., Y.N.) and Department of Toxicology and Microbial Chemistry, Faculty of Pharmaceutical Sciences, Science University of Tokyo, Ichigaya, Shinjuku-ku, Tokyo, Japan (K.O.)

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SUMMARY

The effect of reserpine on actin polymerization was examined by measurement of the changes in high shear viscosity and by electron microscopic observation of the actin solution. In the presence of low concentrations of reserpine, the time course of actin polymerization was accelerated dose dependently (up to approximately 0.5 nM), without affecting the final level of viscosity. The effect of reserpine rather decreased with dosages over this concentration. The binding of reserpine to actin was tested by developing the mixture of G- or F-actin and [^{3}H]reserpine through a Sephadex G-50 column. A portion of the reserpine coeluted with G-actin, but little reserpine did with F-actin. This means that reserpine bound to G-actin but scarcely to F-actin. The binding of reserpine to G-actin was also confirmed using the method of photoaffinity labeling. After the irradiation of the mixed G-actin and [^{3}H]reserpine by ultraviolet light, they were subjected to SDS-PAGE followed by fluorography. It was demonstrated that reserpine was bound to G-actin covalently by the ultraviolet light irradiation. This indicated the close interaction of reserpine with G-actin. Thus, the effect of reserpine on actin polymerization seemed to be exerted via interaction with G-actin.

Reserpine is one of the typical antihypertensive drugs, which has been clinically used for a long time. Reserpine blocks the entry of dopamine, the precursor of norepinephrine, from cytosol to storage vesicles of catecholamine in the terminal portion of sympathetic nerves, leading to the depletion of norepinephrine from the storage vesicles (1). This is the main basis of the pharmacological mechanism of reserpine. However, the detailed mechanism at the molecular level is not completely elucidated.

Reserpine has some well known side effects, such as the production of psychic depression (2), gastrointestinal ulceration, and a variety of other actions; its intraarterial administration produced peripheral vasodilation in both normal and sympathectomized human extremities. Another example was the depression of several parameters of myocardial function (3). Morphological changes in myocardium were also reported in the case of its chronic administration in small doses. Thus, reserpine seems to have a wide spectrum of target organs and a variety of actions.

These observations lead to the hypothesis that part of the action of reserpine is executed via interaction with molecules widely distributed among many kinds of organs or tissues. We have investigated a protein, actin, as a candidate for this kind of molecule. This protein was originally identified as one of the major contractile proteins in skeletal muscle (4) and now it is well known to be a ubiquitous protein widely distributed among many kinds of muscle and nonmuscle cells (5).

In nonmuscle cells, actin is regarded as one of the major constituents of cytoskelton, playing an important role in cell motility. It is quite possible that actin has other kinds of functions, because this protein is conserved in almost all kinds of cells and can interact with various kinds of molecules (6). The length distribution of actin filaments is in a dynamic equilibrium in a given condition and is influenced by molecules such as gelsolin (7) and gelsolin-like factors (8, 9). The interaction of this kind of molecules with actin can be Ca^{2+} dependent, especially in nonmuscle cells. Some chemicals might also modify the actin length distribution through direct interaction with actin or through interaction with some actin binding proteins. We have examined the effect of reserpine on the polymerization of actin *in vitro* and also its interaction with actin.

Materials and Methods

Chemicals and equipment. Reserpine was purchased from Wako Chemicals Co. (Osaka, Japan). It was dissolved in ethanol or 2.5% acetic acid just before use. ^{3}H-labeled reserpine ([*benzoyl*-^{3}H]reserpine, 20.0 Ci/mmol, 50 μM, dissolved in ethanol) and Aquasol-2 were obtained

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ABBREVIATIONS: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CBB, Coomassie brilliant blue.

from New England Nuclear Co. (Boston, MA). Fluorographic reagent Amplify was purchased from Amersham Co. (Arlington Heights, IL). Ostwald type microviscometer (minimal sample volume, 0.15 ml) for measurement of high shear viscosity was produced by Kimura Glass Co. Ltd. (Tokyo, Japan), according to the design of Dr. I. Mabuchi.

Preparation of actin and tropomyosin. Actin was prepared from chicken breast muscle according to Spudich and Watt (10) and further purified by gel filtration through a Sephadex G-100 column with depolymerization buffer containing 0.2 mM $CaCl_2$, 0.2 mM ATP, and 5 mM Tris·HCl (pH 7.5 at 4°) to eliminate minor amounts of contaminating proteins. The actin thus purified exists as a monomeric form (G-actin). F-actin (polymerized fibrous actin) was prepared by incubating G-actin in the polymerization buffer (50 mM KCl, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.5 mM ATP, and 10 mM Tris·HCl, pH 7.5 at 25°) at 25° for 1 hr. Tropomyosin was prepared from chicken gizzard as mentioned previously (11).

Polymerization of G-actin and measurement of high shear viscosity. To start the polymerization of actin, 5 or 10 μl of G-actin (3 mg/ml) in depolymerization buffer was added to 150 μl of polymerization buffer containing various amounts of reserpine. The final concentration of actin was either 0.1 or 0.2 mg/ml. In most of the experiments using reserpine, it was first dissolved in ethanol, and 0.2% ethanol at maximum was carried into the polymerization buffer with reserpine. Exceptionally, in the experiments illustrated in Figs. 1 and 2, reserpine was first dissolved in 2.5% acetic acid and then added to the polymerization buffer; these experiments were closely interrelated, and in the experiment in Fig. 2 we observed the distribution of actin filaments by electron microscope after staining the samples with uranyl acetate. The staining was hardly possible in the presence of 0.2% ethanol. The final concentration of acetic acid was 3.6×10^{-4}%. Therefore we added a suitable amount of ethanol or acetic acid to every polymerization buffer including that of control experiments to adjust the final concentration of ethanol or acetic acid in the buffer to 0.2% or 3.6×10^{-4}%, respectively. Immediately after starting the polymerizing reaction, the mixture was transferred to the microviscometer set up in the water bath at 25°. The viscosity was measured every minute until it reached the plateau level.

Binding experiment of reserpine to actin—gel filtration method. [^{3}H]Reserpine was mixed with 50 μg of either G-actin or F-actin in 100 μl of depolymerization buffer or polymerization buffer, respectively. The final concentration of actin was 12.5 μM (0.5 mg/ml) and that of reserpine was 12.5 nM. The mixture was loaded on a Sephadex G-50 column (bed volume, 5 ml) and eluted with the same solution as that of the sample at the speed of 12 ml/hr. Every 0.3-ml aliquot was collected, of which 0.2 ml was mixed with the same volume of 2 N NaOH and 10 ml of Aquasol-2. Radioactivity was measured by liquid scintillation counter (Beckman LS 1800). The protein concentration of each fraction was also measured.

Binding experiment of reserpine to actin—photoaffinity labeling method. A total of 0.5 mg/ml of G-actin in depolymerization buffer or 1.0 mg/ml of gizzard tropomyosin in 0.1 M KCl and 10 mM Tris·HCl (pH 7.5 at 4°) was mixed with 1 μM of [^{3}H] reserpine in the wells of a microtest plate (Falcon no. 3072). The final volume was 50 μl, and 2% ethanol was contained in the mixture. It was placed on ice and irradiated at a distance of approximately 10 cm for 60 min with ultraviolet light (peak wave length, 253.7 nm, 2.82 W). The irradiated sample was subjected to SDS-PAGE using a slab gel according to Laemmli (12). The acrylamide concentration of the stacking gel was 5% and that of the separation gel was 13.5%. After electrophoresis, the gel was stained with CBB and destained by several changes of 7% acetic acid. Then it was transferred to a plastic box containing the fluorographic reagent Amplify and gently shaken for 30 min at room temperature. After drying the gel, it was subjected to fluorography at −80° for 1 week.

Measurement of protein concentration. Protein concentration was determined either by the dye binding method (13) or by measurement of A_{290} (in the case of G-actin, $E^{1\%}_{290} = 6.5$).

Observation of actin filaments by electron microscopy. The actin solution was applied to carbon-coated collodion grids, stained with 1% uranyl acetate, and examined by a JEM-1200 EX electron microscope.

Results

To examine the effect of reserpine on actin polymerization, we have measured the time courses of actin polymerization in the presence of various concentrations of reserpine by monitoring the changes in high shear viscosity as mentioned in Materials and Methods. Fig. 1 shows the typical result of the experiments. In this experiment, the final concentration of actin was 0.1 mg/ml (2.4 μM) and that of reserpine was 0.24 nM. At 0 min, G-actin was added to either polymerization buffer or that containing 0.24 nM reserpine, and the mixed solution was immediately transferred to a microviscometer. The buffer flow time was 33.5 sec in both cases. The changes in specific viscosity are illustrated in Fig. 1. Without reserpine, the viscosity started to increase at around 20 min, reaching the plateau level at about 40 min. The final value of specific viscosity was 0.13. On the other hand, with 0.24 nM reserpine, the viscosity started to increase at around 12 min and reached the plateau level at about 30 min. The final value of specific viscosity was 0.15. Thus reserpine at this concentration clearly accelerated actin polymerization. The final value of specific viscosity was a little higher in the presence of reserpine than in the absence of it, but this was not always the case. The final values had no meaningful difference in other experiments.

To confirm that the specific viscosity reflected the length distribution of F-actin filaments, we examined the time course of actin polymerization using electron microscopy. In this case we have performed the same kind of experiments as mentioned above except that the mixture was incubated in test tubes at 25°. At 20 and 25 min, aliquots from each mixture were taken, stained with uranyl acetate, and observed by electron microscope. To observe a general view of a large area, a relatively positive-stained area was selected. As is shown in Fig. 2, F-actin filaments formed in the presence of reserpine were longer and more abundant, compared with those formed in the absence of reserpine both at 20 and 25 min. At 0 min, aliquots from both kinds of specimens contained no noticeable F-actin fila-

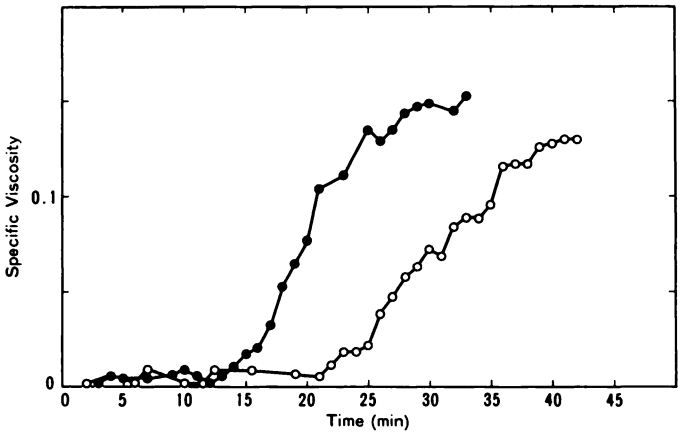


Fig. 1. Time course of actin polymerization in the presence and absence of reserpine. At 0 min, 0.1 mg/ml of G-actin was added to the polymerization buffer with (●) or without (○) reserpine. Final concentration of reserpine was 0.24 nM. The mixture was immediately transferred to the Ostwald type microviscometer and changes in viscosity were followed.

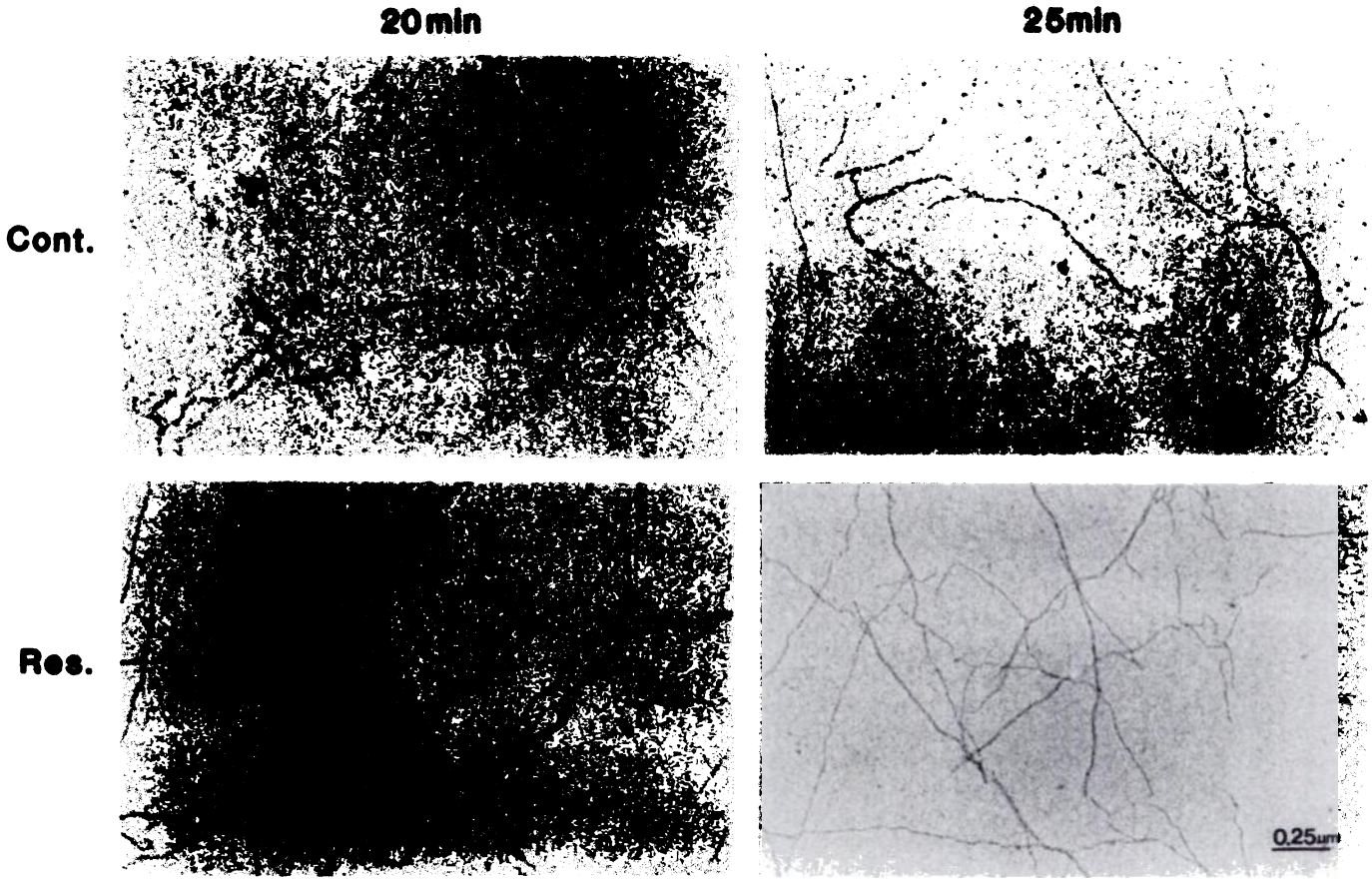


Fig. 2. Electron microscopic observation of actin filaments. The experimental conditions were the same as those in Fig. 1, except that the mixtures were incubated in the test tubes at 25°, instead of being transferred to the microviscometer. At 20 and 25 min, aliquots were removed, stained with uranyl acetate, and observed by electron microscope. *Cont.*, actin solution without reserpine, *Res.*, actin solution with 0.24 nM reserpine.

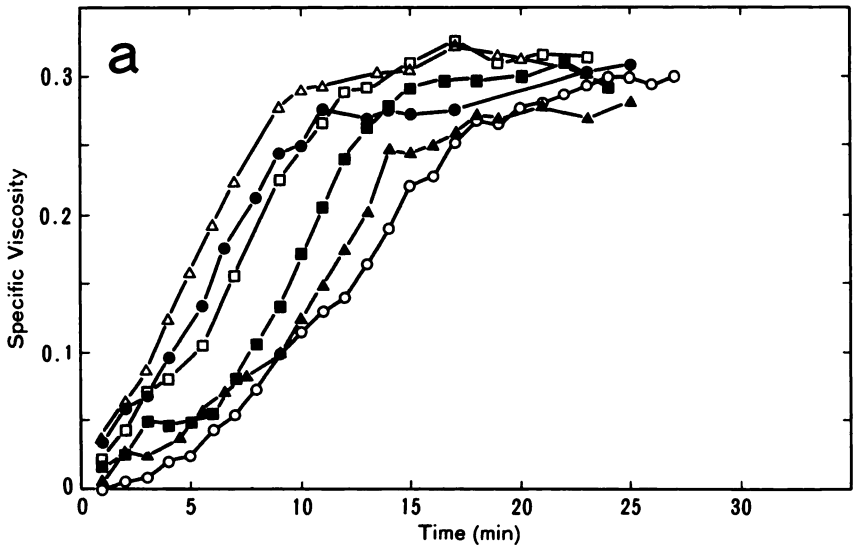
ments. At 35 min, both aliquots contained many long F-actin filaments; the degree of F-actin formation in the two specimens seemed almost the same and was more pronounced than that in the specimen with reserpine at 25 min (data not shown). This observation confirmed that reserpine accelerated polymerization of actin.

Then we examined the dose dependence of the effect of reserpine on actin polymerization. Various amounts of reserpine ranging from 0.048 nM to 0.48 μM (in terms of molar ratio to actin, they corresponded to $1/10^5$ and 1/10, respectively) were mixed with polymerization buffer and the changes in viscosity after addition of G-actin were followed. In this series of experiments, the final concentration of actin was 0.2 mg/ml (4.8 μM), twice the concentration used in the previous experiments. The experimental condition with 0.48 μM reserpine was different from those of the other experiments in that the final concentration of ethanol was 2% instead of 0.2%.

The results of a series of experiments are shown in Fig. 3a. To illustrate the results in a more understandable way, the specific viscosity of each sample at 8 min, when that of the control sample reached about one third of the plateau level, was plotted against the concentrations of reserpine in the solution (Fig. 3b). The accelerating effect of reserpine on actin polymerization increased dose dependently up to the concentration of about 0.5 nM (the molar ratio to actin is about $1/10^4$). Above this concentration range of reserpine, however, the effect of reserpine decreased along with the increased dosage. Reserpine at 0.48 μM had little, if any, effect on actin polymerization. The electron microscopic observation of the samples with different concentrations of reserpine (0, 0.48, and 48 nM) at 8 min revealed that the nature of the actin filaments was the same in all cases; an abnormal structure such as branching was never observed. We have examined the dose dependence of the action of reserpine with 0.1 mg/ml of actin, and the result was essentially the same as that with 0.2 mg/ml of actin (data not shown).

The experiments mentioned so far were carried out in the presence of 0.5 mM ATP. ADP was not included in the original polymerization buffer. We further examined the effect of reserpine on actin polymerization in the presence of ADP. Actin was added to the polymerization buffer containing 0.5 nM reserpine and either 0.1 or 0.5 mM ADP. The presence of ADP in the polymerization buffer per se had no remarkable effect on actin polymerization. When reserpine was also present in the buffer in addition to ADP, the polymerization process was markedly suppressed (Fig. 4). The suppression was more pronounced in the presence of 0.5 mM ADP than in the presence of 0.1 mM ADP (data not shown). Thus the effect of reserpine on actin polymerization in the presence of additional ADP was just opposite to the effect of reserpine in the absence of ADP.

In any event, reserpine has been proved to have a remarkable influence on actin polymerization at a low concentration range,



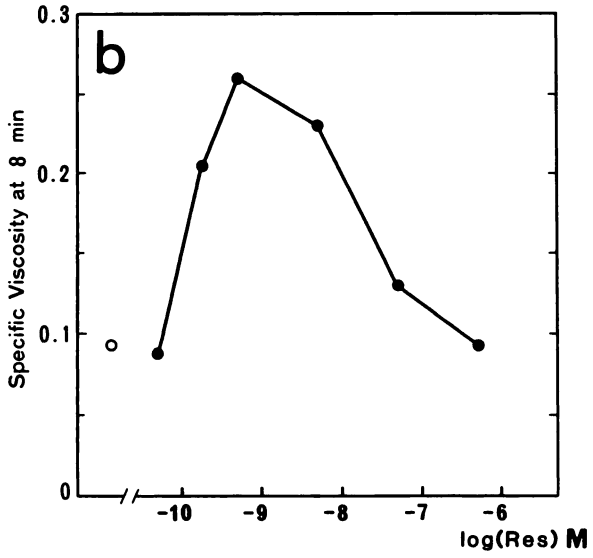


Fig. 3. Dose dependence of the effect of reserpine on actin polymerization. The same kind of experiments as those shown in Fig. 1 were performed with various concentrations of reserpine in the polymerization buffer. The final concentration of actin was 0.2 mg/ml (4.8 μM). a, The time courses of the changes in viscosity. The concentrations of reserpine in the polymerization buffer were ○, 0 nM; □, 0.16 nM; △, 0.48 nM; ●, 4.8 nM; ■, 48 nM; and ▲, 0.48 μM. b, The viscosity of an individual actin solution at 8 min plotted against the concentration of reserpine in the polymerization buffer. ○, the viscosity of actin solution without reserpine at 8 min.

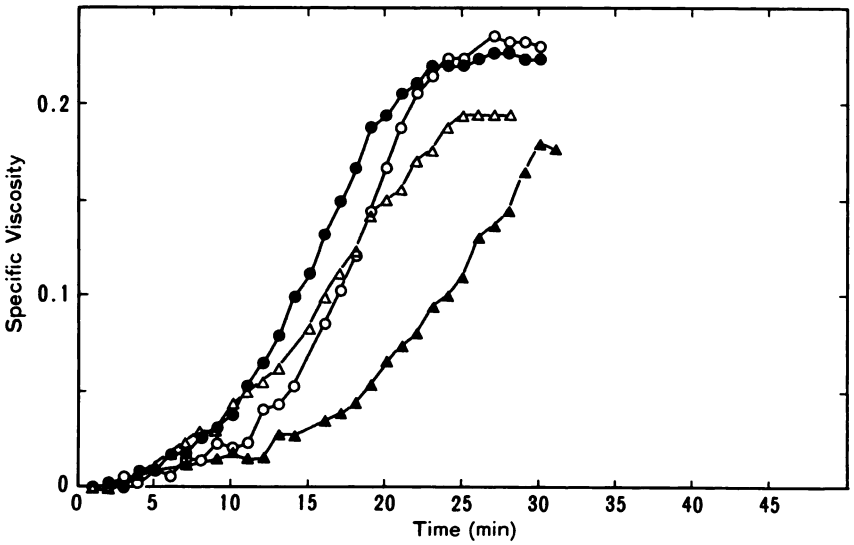


Fig. 4. The effect of reserpine on the actin polymerization in the presence of ADP. The same kind of experiments as in Fig. 1 were performed in the presence of 0.5 mM ADP. Final concentrations of actin and reserpine were 0.2 mg/ml (4.8 μM) and 4.8 nM, respectively. ○, Experiment without ADP and reserpine; ●, experiment without ADP but with reserpine; △, experiment with ADP but without reserpine; and ▲, experiment with ADP and reserpine.

although the effect in the presence of ADP was completely opposite to that in the absence of it. We therefore proceeded to examine the binding ability of reserpine to actin.

Either G- or F-actin was mixed with [*benzoyl*-^{3}H]reserpine in 100 μl of the depolymerization or polymerization buffer, respectively, as mentioned in Materials and Methods. The mixture was loaded on the Sephadex G-50 column and developed. As a control experiment [^{3}H]reserpine alone was developed with either depolymerization or polymerization buffer. The results were essentially the same in both cases so only the experiment with depolymerization buffer is illustrated in Fig. 5a. The peak fractions of reserpine were from nos. 20 to 25. When G-actin was mixed with [^{3}H]reserpine and developed, part of the labeled reserpine coeluted with G-actin as is shown in Fig. 5b. When the concentration of [^{3}H]reserpine in the sample was raised, the amount of reserpine that coeluted with G-actin also increased (data not shown). On the other hand, when [^{3}H]reserpine was mixed with F-actin and developed, the labeled reserpine scarcely coeluted with F-actin (Fig. 5c). These results strongly suggested that reserpine has a binding affinity for G-actin, but has little, if any, affinity for F-actin.

The interaction of reserpine with actin was also examined with a different method, which utilized the formation of covalent bonds between these molecules by ultraviolet light irradiation (photoaffinity labeling method). The results are shown in Fig. 6. The *upper panel* is the CBB-stained SDS-PAGE pattern of the samples and the *lower panel* is the fluorography of the gel. Only when the mixture of G-actin and reserpine was irradiated by ultraviolet light, a clear band just corresponding to the position of actin was observed. The mixture of G-actin and reserpine without ultraviolet light irradiation yielded no band on fluorography. The concentration of [^{3}H]reserpine used in this experiment was high (1 μM). The experiment with 12.5 nM [^{3}H]reserpine was also performed (data not shown). In this case, the mixture of [^{3}H]reserpine and G-actin, after irradiation with ultraviolet light, showed a clear (but faint) band located at the position of actin on fluorography, after very long exposure (−80°, 2 months). We have performed the same experiment with 1 μM [^{3}H]reserpine only to clearly show the band. This observation supported the view that reserpine interacts with G-actin.

As a control, the same experiments were performed with gizzard tropomyosin. In this case no bands were detected on fluorography.

The ionic strength of the depolymerization buffer was very low and there remained the possibility that the interaction between reserpine and G-actin was nonspecific in character. To exclude this possibility, we performed the following experiment: 0.5 mg/ml of F-actin was mixed with 1 μM of [^{3}H] reserpine in 50 μl of polymerization buffer. After ultraviolet light irradiation, the mixture was centrifuged at 90,000 × *g* for 1 hour to separate long F-actin from the small amount of coexisting G-actin (contained in the F-actin solution at critical concentration) and short F-actin. The supernatant (containing G-actin and short F-actin) and the pellet (containing long F-actin) were separately subjected to SDS-PAGE followed by fluorography. Only the actin contained in the supernatant showed a clear band on fluorography (data not shown).

It was concluded from this experiment that, under the conditions used, reserpine hardly bound to long F-actin, although it is quite possible that reserpine bound to only one of the two terminals of F-actin. On the other hand, reserpine did bind to the mixed solution of G-actin and short F-actin under the condition of rather high ionic strength. Thus the interaction of reserpine with G-actin seemed to be specific.

Discussion

Using the highly purified actin from chicken breast muscle, we have shown that reserpine exerted the accelerating effect on actin polymerization dose dependently, in the low concentration range (up to approximately 0.5 nM), based on the measurement of the changes in high shear viscosity and on the observation by electron microscopy of F-actin formation. The accelerating effect of reserpine gradually diminished in concentrations higher than about 0.5 nM, and very little effect was observed in the highest concentration examined (0.48 μM). The "dose dependence experiments" were performed with reserpine dissolved in ethanol. In the case of the experiment with 0.48 μM reserpine, the final concentration of ethanol was 2%. We did not examine the effect of reserpine above this concentration to avoid carrying higher concentrations of ethanol into the mixture. Experiments with higher concentrations of reserpine (dissolved in acetic acid) up to 5 μM showed that neither an accelerating nor a suppressive effect was exerted by reserpine on actin polymerization (data not shown). Thus, reserpine has proved to be one of the reagents that affect the process of actin polymerization. The effective concentration was quite low. The gel filtration experiments with ^{3}H-labeled reserpine showed that reserpine bound to G-actin but hardly to F-actin.

The ultraviolet light irradiation induces covalent bond formation between some kinds of molecules located very close to each other. For example, the formation of covalent bonds between protein and nucleotide by ultraviolet light irradiation has been used to analyze the interaction between protein and nucleotide or DNA (14–16). We have used this method to examine the molecular interaction between reserpine and G-actin. After the mixture of reserpine and G-actin in depolymerization buffer was irradiated by ultraviolet light, they comigrated on SDS-PAGE, which was revealed by comparison of the CBB-stained SDS-PAGE pattern with that of fluorography. When reserpine by itself was treated in the same way, no band was detected on the CBB-stained SDS-PAGE gel as well as on fluorography (data not shown). These results strongly suggested the formation by ultraviolet light irradiation of covalent bond(s) between G-actin and reserpine and supported the view that reserpine interacts with G-actin. The chemical reaction leading to the covalent bond formation between reserpine and actin is unknown at present, but it is possible that free radical(s) formed in the reserpine molecule by the ultraviolet light irradiation reacted with the actin molecule (17).

The effect of reserpine on actin polymerization is characterized by the low effective dosage, acceleration of the time course of polymerization, and the unaltered final viscosity level. These characteristics suggest that the effect of reserpine was on both nucleation and elongation steps among the four steps of actin polymerization: activation, nucleation, elongation, and annealing (6).

Cytochalasin B is the representative reagent known to accelerate the nucleation reaction (18). Actin polymerization in the presence of cytochalasin B, monitored by the changes in specific viscosity, is characterized by the initial acceleration and lower steady state level of viscosity.

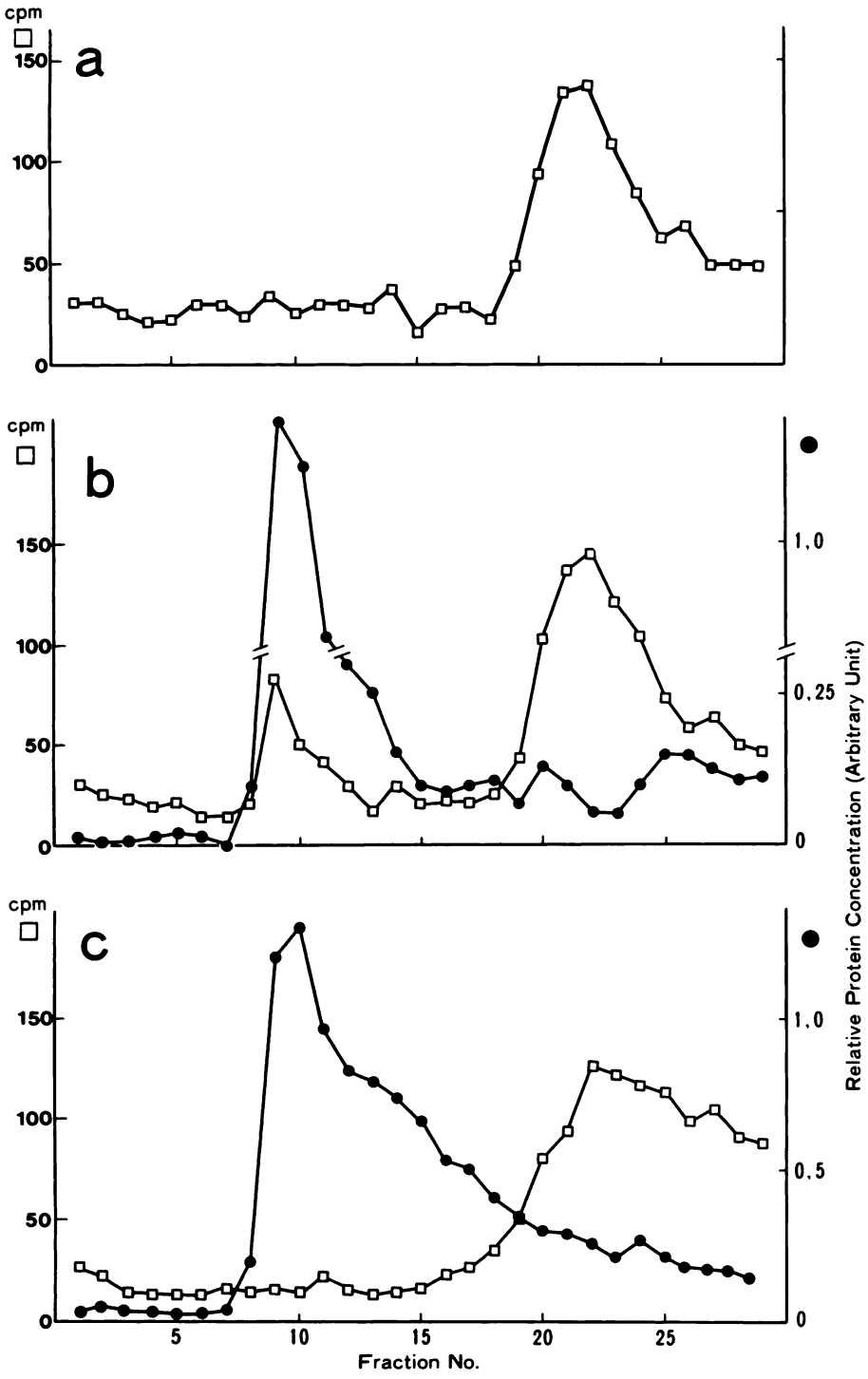


Fig. 5. Binding study of reserpine to actin by gel filtration method. G- or F-actin was mixed with [*benzoyl*-^{3}H]-reserpine in 100 μl of depolymerization or polymerization buffer, respectively, as was mentioned in Materials and Methods. The mixtures were separately loaded on the Sephadex G-50 column and developed with the respective solution. Fractions (0.3 ml) were collected and protein concentration and radioactivity of each fraction were measured. a, Elution profile of [^{3}H]reserpine with depolymerization buffer; b, elution profile of the mixture of G-actin and [^{3}H]reserpine with depolymerization buffer; c, elution profile of the mixture of F-actin and [^{3}H]reserpine with polymerization buffer. □, Radioactivity in cpm; ●, protein concentration.

Reserpine, on the other hand, accelerates the polymerization but does not change the steady state level. The mode of action of reserpine on the actin polymerization seems to be also different from any other proteins so far reported that influence actin polymerization. The detailed mechanism of action at the molecular level remains to be elucidated.

The reason that the effect of reserpine was diminished when the concentration was greater than about 0.5 nM is not clear. It is possible that reserpine has more than two modes of action with opposite effects on actin polymerization and that the final effect of a certain concentration of reserpine on actin polymerization is determined by the balance of these actions. However, with the maximum concentration of reserpine examined, no suppressive effect was observed.

The effect of reserpine in the presence of ADP was completely opposite to that in the absence of ADP. The reason for the apparently opposite effects of reserpine under these conditions is unknown at present. Further study is necessary to elucidate this phenomenon on the basis of more systematic experiments using ATP and ADP.

Reserpine bound to G-actin but hardly to F-actin with long length. It is reasonable to assume that reserpine binds to a

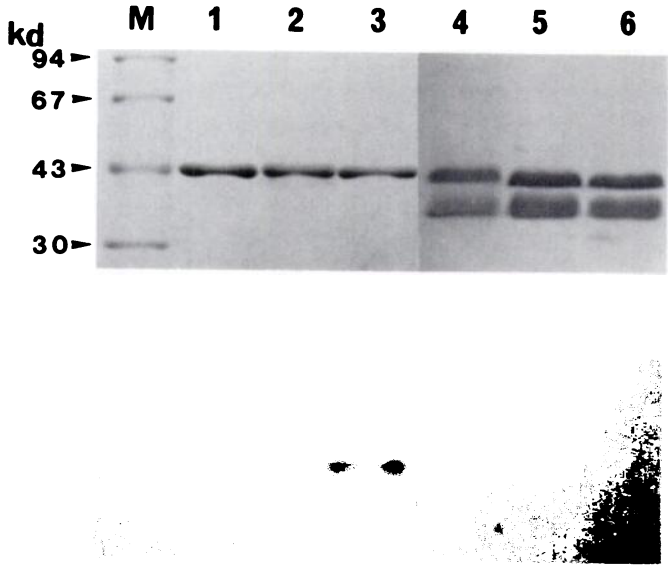


Fig. 6. Binding study of reserpine to actin molecule by photoaffinity labeling method. G-actin or gizzard tropomyosin was mixed with [^{3}H] reserpine and irradiated with ultraviolet light as mentioned in Materials and Methods. The SDS-PAGE patterns (*upper panel*) and corresponding fluorographic patterns (*lower panel*) are shown. *M*, Molecular weight marker; 1, G-actin without [^{3}H]reserpine, nonirradiated; 2, G-actin mixed with [^{3}H]reserpine, nonirradiated; 3, G-actin mixed with [^{3}H]reserpine, irradiated; 4, tropomyosin without [^{3}H]reserpine, nonirradiated; 5, tropomyosin mixed with [^{3}H]reserpine, nonirradiated; 6, tropomyosin mixed with [^{3}H]reserpine, irradiated.

particular region of the actin molecule and that it cannot gain access to the region after polymerization of actin molecules. In that case, the possible binding site of reserpine to F-actin might be only either of the two terminals of F-actin. Thus, the relative amount of reserpine that binds to actin may well depend on its length, and consequently could be influenced by proteins such as gelsolin (7), actin length regulator (8), and so on. Ca^{2+} could also influence the amount of reserpine that binds to actin because the interaction of such kind of proteins with actin is often Ca^{2+} dependent.

From the results of our *in vitro* experiments we cannot know whether the effect of reserpine on actin polymerization has something to do with the pharmacological effects and/or side effects of this reagent. However, the low concentration range of reserpine in which it works on actin *in vitro* and the easy accessibility of reserpine inside the cells suggest that actin is really one of the target molecules on which reserpine works, *in vivo*.

Actin in nonmuscle cells is considered mainly to play a role as cytoskeleton, but there remains the possibility that it also is engaged in other functions, suggested by its interactions with a variety of molecules (6). For example it was recently demonstrated that actin is very likely to participate in the transcription reaction (19). The elucidation of unidentified functions of actin would also shed light on the unclarified pharmacological mechanism of reserpine.

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Send reprint requests to: Seiji Nakamura, Department of Pharmacology, Faculty of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo, Japan.