0026-895X/94/040612-06$03.00/0

MOLECULAR PHARMACOLOGY, 46:612-617, 1994.

Induction of Aggregation and Augmentation of Protein Kinase-Mediated Phosphorylation of Purified Vimentin Intermediate Filaments by Withangulatin A

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Received April 18, 1994; Accepted July 21, 1994

SUMMARY

Purified assembly-competent vimentin, an intermediate filament protein, was obtained from bovine lens in this study. The effects of withangulatin A on vimentin assembly with or without phosphorylation were examined by negative-stain electron microscopy. Soluble tetrameric vimentin was assembled into irregular fibrils with lateral associations or short filaments after pretreatment with 50 or 100 μM withangulatin A, respectively. Incubation of assembled vimentin filaments with withangulatin A at 50 or 100 μM resulted in the formation of aggregates, and the degree of aggregation was concentration dependent. The appearance of vimentin filaments was slightly altered after treatment with cAMP-dependent protein kinase or protein kinase C; however, phosphorylation of filamentous vimentin by the protein kinases in the presence of withangulatin A resulted in higher degrees of aggregation of the filaments, compared with those treated only with the drug. Moreover, the level of phosphorylation of filamentous vimentin by the protein kinases was augmented in the presence of withangulatin A. Experimental results indicated that withangulatin A directly and specifically affects the conformation of the vimentin molecules, thereby resulting in alterations in assembly behavior and reactivity toward cAMP-dependent protein kinase and protein kinase C. The data observed further imply that withangulatin A, which directly causes aggregation of vimentin filaments, is a vimentin intermediate filament-targeting drug.

IFs are composed of proteins that are members of a large multigene family whose expression is developmentally regulated, as well as being tissue and cell specific (1, 2). In cells of mesenchymal origin and most cells in culture, IFs are primarily composed of a single-subunit protein referred to as vimentin, which is a class III IF protein (2). Like many other IF proteins, phosphorylation of vimentin is important in the structural reorganization of vimentin IFs. Most notably, the reorganization of vimentin IFs into a juxtanuclear cap during mitosis is due to a phosphorylation process mediated by cdc2 kinase (3–5). Additionally, simultaneous reorganization and enhanced phosphorylation of vimentin have been reported in cells responding to hormones (6–9), protein phosphatase inhibitors (10–13), WA (14), and heat (15). The vimentin-containing IF network makes contact with the nuclear envelope, interacts with the nuclear lamina through the nuclear pore, and associates with the membrane skeleton at the cell periphery (16). Therefore, the system may be responsible for conveying mechanical as well as molecular signals from the cell surface to the nucleus and vice versa (17, 18). More recently, it has been suggested that vimentin acts as a phosphate sink in cells experiencing a disturbance of protein phosphorylation/dephosphorylation (19). However, the physiological functions of IFs have not been thoroughly elucidated, due to the lack of an IF-targeting drug.

We previously reported that WA (Fig. 1), a biologically active compound isolated from the Chinese antitumor herb *Physalis angulata* (20), can induce a typical stress response in 9L rat brain tumor cells (21). The compound is cytotoxic and is capable of suppressing general protein synthesis and inducing the synthesis of a small set of proteins, including those generated by heat-shock treatment (21). Additional studies indicated that in WA-treated cells the vimentin IF network collapses and clusters around the nucleus, concomitantly with a series of biochemical alterations of the vimentin molecules, including augmentation of phosphorylation level, retardation of electrophoretic mobility, and decrease in detergent extractability (14). In this study, the direct molecular actions of WA were further characterized by examing the effects of WA on vimentin assembly and phosphorylation *in vitro*.

This work was supported by Republic of China National Science Council Grant NSC 83-0203-B007-003 to Y.-K.L.

ABBREVIATIONS: IF, intermediate filament; MBP, myelin basic protein; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; SDS, sodium dodecyl sulfate; WA, withangulatin A.

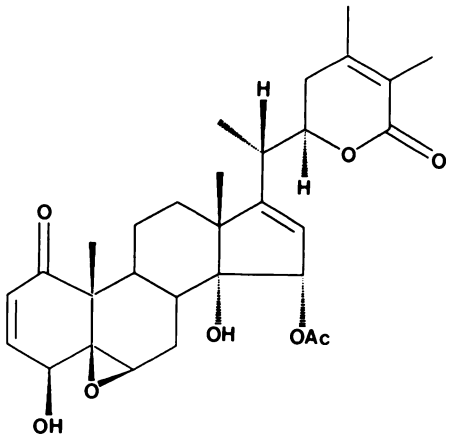

Fig. 1. Structural formula of WA, a stress response inducer isolated from *P. angulata* (20). *Ac*, acetyl.

Experimental Procedures

Materials. WA was isolated from *P. angulata* as described (20). It was dissolved in dimethylsulfoxide at a concentration of 20 mM, stored in the dark at 4°, and diluted to appropriate concentrations before use. Catalytic subunits of PKA and PKC were purchased from Promega. Histone II-A, histone III-S, and MBP were obtained from Sigma. [γ-^{32}P]ATP (specific activity, >5000 Ci/mmol) was purchased from Amersham. Chemicals for electrophoresis were purchased from Bio-Rad. General chemicals were supplied by Sigma or Merck.

Purification of vimentin and *in vitro* assembly of vimentin IFs. Vimentin was purified from bovine lens by urea extraction, anion exchange chromatography, and chromatofocusing (22, 23). Fractions containing vimentin were identified by SDS-polyacrylamide gel electrophoresis (24), pooled, and concentrated by ultrafiltration (Amicon) when necessary. The protein concentration of the purified samples was adjusted to 1.5–3.0 mg/ml, and the samples were stored in 5 mM Tris·HCl, pH 8.4, containing 1 mM dithiothreitol (storage buffer), at −70° until use. Protein concentration was determined by the method of Bradford (25), using purified vimentin as the standard. *In vitro* assembly of soluble tetrameric vimentin into a filamentous form was accomplished by altering the buffer strength to physiological ionic strength at neutral pH (26). Soluble tetrameric vimentin in storage buffer (0.8–1.2 mg/ml, 50 μl) was dialyzed against 3 × 150 ml of 25 mM Tris·HCl, pH 7.5, containing 160 mM NaCl and 1 mM dithiothreitol (assembly buffer), in an oscillatory microdialysis system (Biotech) for a total of 4 hr at room temperature. The samples were directly applied to 300-mesh Formvar-coated copper grids (Electron Microscopy Sciences) and allowed to set for 1 min. The grids were then negatively stained with 1% (w/v) uranyl acetate at room temperature and examined with an Hitachi H-600 electron microscope at 75 kV.

***In vitro* phosphorylation of purified vimentin.** Phosphorylation of purified vimentin was performed as described by Inagaki *et al.* (27). For each reaction, 12 μg of purified vimentin (in tetrameric or filamentous form) were incubated with 6.25 μg/ml PKA (90 units), 0.1 mM ATP, 0.3 mM $MgCl_2$, 30 mM NaCl, and 25 mM Tris·HCl, pH 7.0, or with 1.25 μg/ml PKC (90 units), 0.1 mM ATP, 0.3 mM $MgCl_2$, 0.8 mM $CaCl_2$, 30 mM NaCl, 50 mg/ml phosphatidylserine, and 25 mM Tris·HCl, pH 7.0, in a total volume of 40 μl. After 30 min of incubation, the phosphorylated tetrameric vimentin preparations were dialyzed against assembly buffer and processed for electron microscopy, whereas the phosphorylated filamentous vimentin preparations were directly processed for electron microscopy.

The effectiveness of the phosphorylation procedure was monitored by using radioactive ATP under identical conditions, except that the reactions were stopped by the addition of equal volumes of 2× SDS sample buffer (24), and samples were analyzed by SDS-polyacrylamide gel electrophoresis using 10% gels (24). WA (20–100 μM) was included in the phosphorylation reaction mixture to examine the effect of WA on the phosphorylation of tetrameric or filamentous vimentin. Subsequent steps were carried out exactly as described above. Furthermore, other proteins, i.e., histone II-A, histone III-S, and MBP, were used in parallel experiments to assess the specificity of the effect of WA on protein phosphorylation by PKA and PKC. The reaction products were analyzed on 12.5% SDS gels and the levels of phosphorylation of different proteins by PKA and PKC in the presence of WA were monitored by autoradiography. The optical densities of the autoradiographs were quantitated with a Molecular Dynamics computer densitometer, using ImageQuant software.

Results

Effects of WA on the assembly competence of tetrameric vimentin or the assembly state of filamentous vimentin. The assembly competence of the vimentin preparations was examined by negative-stain electron microscopy, which revealed formation of extensive smooth filaments with a diameter of 10–12 nm (Fig. 2A). When the sample was pretreated with 50 μM WA for 30 min and then processed for assembly, the tetrameric vimentin polymerized into irregular fibrils with lateral associations (Fig. 2B). Moreover, when the concentration of WA was increased to 100 μM, the assembly of tetrameric vimentin into filaments was incomplete and only short filaments were formed (Fig. 2C). To analyze the effect of WA on the structural organization of filamentous vimentin, assembled vimentin filaments were incubated with WA and then examined. After 30 min of incubation with 50 μM WA, the filamentous structure was still preserved but loose aggregates were formed (Fig. 2D). When the sample was treated with 100 μM WA, however, dense aggregates resulted (Fig. 2E).

Effects of WA on the assembly states of PKA- or PKC-phosphorylated filamentous vimentin. The effect of phosphorylation on the assembly state of the filamentous vimentin was examined. Those results indicated that the filamentous structure was only slightly altered by the phosphorylation step. That is, neither PKA nor PKC exerted significant effects on the structural organization of the filaments (Fig. 3, A and D). Subsequently, phosphorylation of the assembled vimentin filaments by PKA and PKC was carried out in the presence of WA. Also, the effect of WA on the structural organization of the phosphorylated filamentous vimentin was characterized. Experimental results indicated that filamentous vimentin phosphorylated by PKA in the presence of 50 μM WA formed dense aggregates (Fig. 3B), which were similar to the vimentin filaments treated with 100 μM WA (Fig. 2E). When PKA-mediated phosphorylation of filamentous vimentin was performed in the presence of 100 μM WA, massive aggregates were formed and the structure appeared to consist of a higher order assembly of filaments (Fig. 3C). In contrast, when vimentin filaments were phosphorylated by PKC in the presence of 50 μM WA (Fig. 3E) or 100 μM WA (Fig. 3F), the structural organization observed was similar to that of filaments treated with WA alone. No further aggregation of the filaments occurred, as indicated from a comparison of Fig. 3, E and F, with Fig. 2, D and E.

Effects of WA on phosphorylation of vimentin *in vitro*. Although the tetrameric vimentin was successfully phosphorylated by PKA or PKC, the level of phosphorylation remained relatively constant when WA was included in the phosphorylation reaction at 0–100 μM (Fig. 4, A, B, E, and F). When filamentous vimentin was used as a substrate in the same reactions, however, the phosphorylation of vimentin filaments

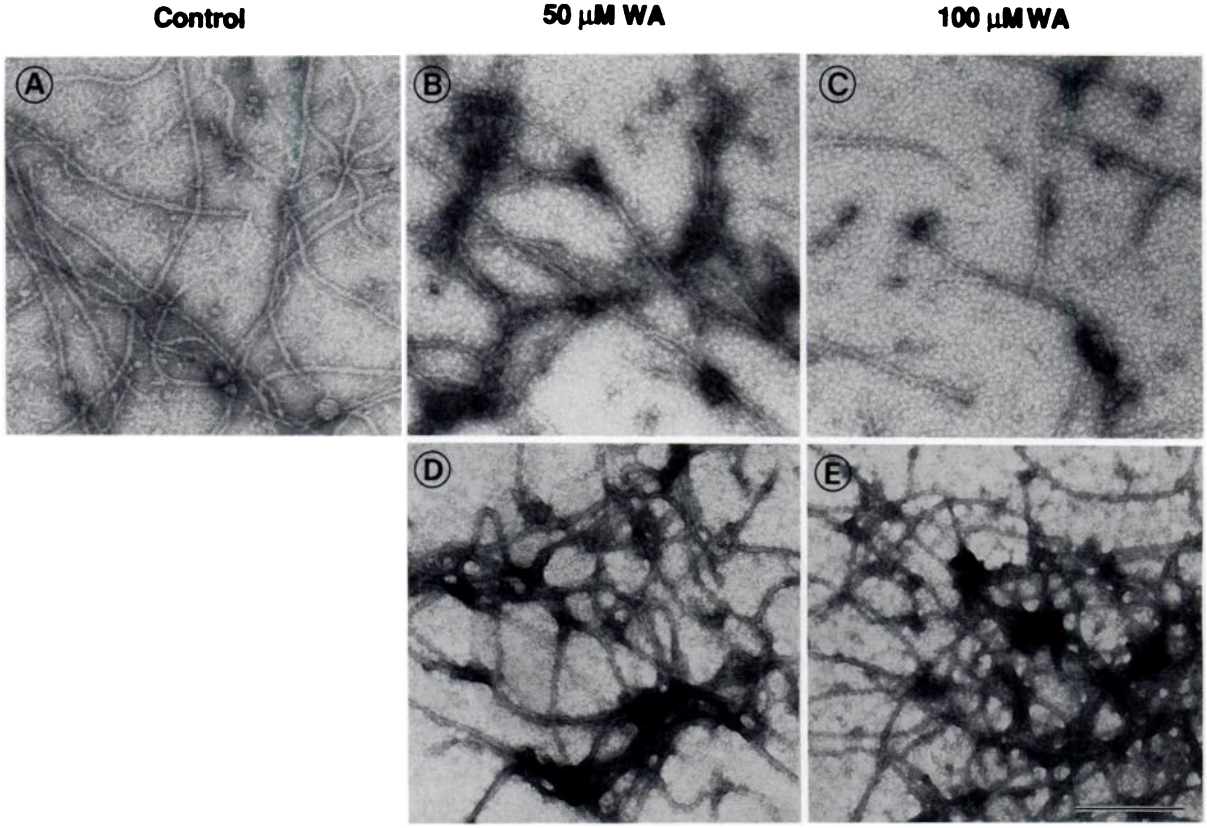

Fig. 2. Effect of WA on the assembly competence of tetrameric vimentin or the assembly state of filamentous vimentin. Soluble tetrameric vimentin was assembled into filamentous form and examined by negative-stain electron microscopy (A). Treatments with WA were performed before or after the assembly step. Shown are the structural organization of filamentous vimentin assembled from tetrameric vimentin pretreated with 50 μM WA (B) or 100 μM WA (C) and the structural reorganization of preformed vimentin filaments treated with 50 μM WA (D) or 100 μM WA (E). *Bar*, 200 nm.

by PKA or PKC in the presence of WA was enhanced in a concentration-dependent manner (Fig. 4, C-F). In PKA-treated samples, enhanced phosphorylation of vimentin reached a maximum level with 60 μM WA, in the presence of which a 3-fold increase in the level of phosphorylation was observed (Fig. 4C). In contrast, the maximum level of phosphorylation was increased by approximately 2-fold when PKC was used (Fig. 4F). Other substrates for PKA and PKC, i.e., histone II-A, histone III-S, and MBP, were used in addition to vimentin in parallel experiments. Those results indicated that WA did not affect the phosphorylation levels of histone II-A and MBP when they were used as substrates for PKA (Fig. 5, A and C). Similar results were obtained when histone III-S and MBP were used as substrates for PKC (Fig. 5, B and D). The data described above suggested that WA did not affect the activity of the protein kinases but specifically increased the reactivity of vimentin toward the enzymes.

Discussion

We have demonstrated that treatment with WA can alter the assembly behavior of tetrameric vimentin and induce aggregation of preformed vimentin filaments *in vitro*. It is known that the assembly of monomeric vimentin into filamentous form involves a sequential series of interactions of vimentin molecules, from coiled-coiled dimers to antiparallel tetramers and to long, 2–3-nm-wide protofilaments, eight of which then assemble around a central axis to form the typical IF structure (2). Hydrophobicity of the rod domain of the vimentin molecules plays a major role in these interactions, and it has been reported that cholesterol is capable of binding to the rod domain of vimentin molecules (28). Therefore, WA may conceivably exert its effects on vimentin assembly by direct binding, because this compound is a steroidal lactone containing a sterol backbone that closely resembles the structure of cholesterol (Fig. 1). Such binding may disturb the conformation of the vimentin molecules, thereby resulting in abnormal assembly of tetrameric vimentin and aggregate formation of filamentous vimentin. However, it is noteworthy that binding of cholesterol to the vimentin filaments did not alter the assembly state of the filaments (28).

Phosphorylation of vimentin by intracellular protein kinases is thought to play a major role in the reorganization of vimentin IFs in cells during mitosis or during responses to external stimulation. Under these conditions, vimentin IFs are reorganized into different forms of aggregates (3–15). Increased phosphorylation of the vimentin molecules by specific protein kinases has been claimed to be responsible for the organizational changes (3, 6, 8, 9, 29). In this investigation, however, the results from *in vitro* phosphorylation by individual protein kinases indicate otherwise. Filamentous vimentin phosphorylated by PKA or PKC was found either to be depolymerized (27, 30) or to remain unaffected (Refs. 22 and 27 and the present study). An organizational change of preformed vimentin filaments into aggregates by protein kinases has not been observed *in vitro*. These results suggest that phosphorylation of vimentin by individual kinases may not account for the

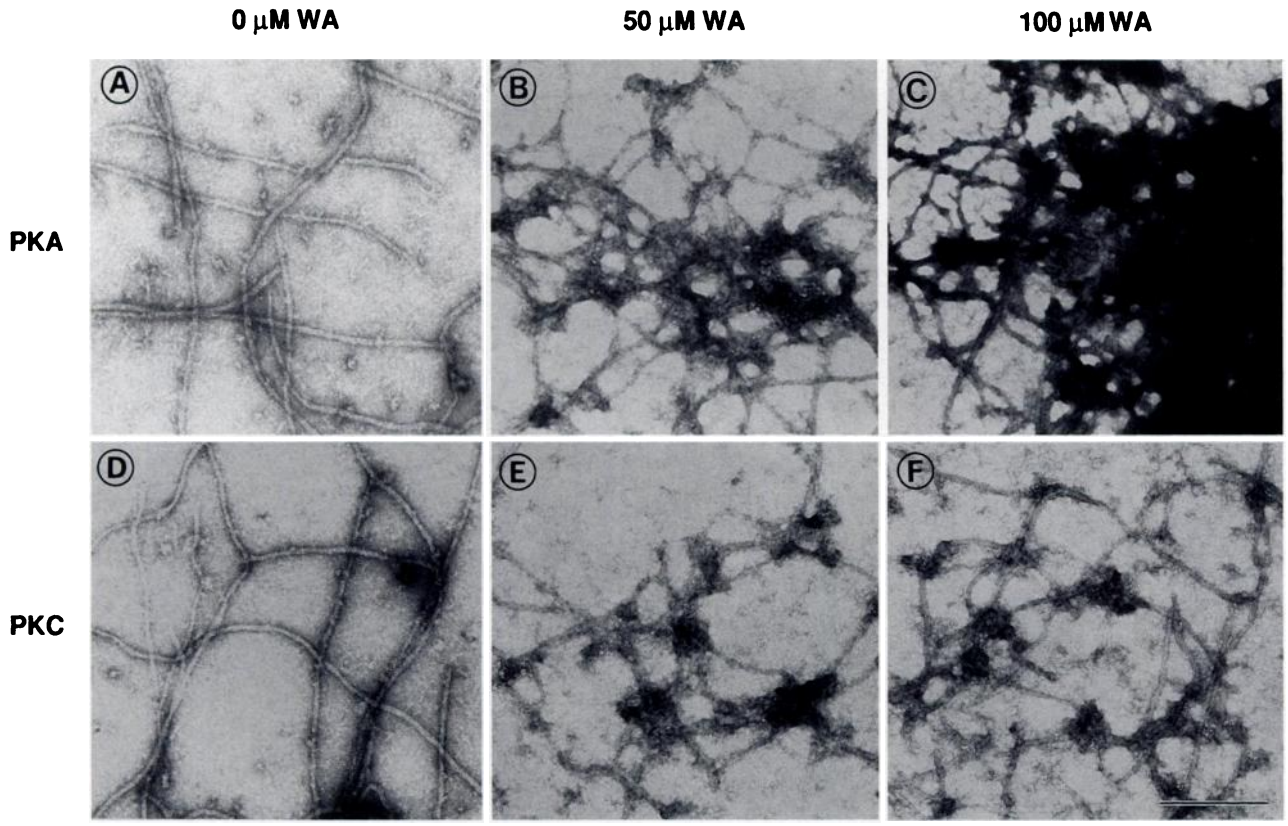

Fig. 3. Effect of WA on the assembly state of phosphorylated filamentous vimentin. Preformed vimentin filaments were phosphorylated by PKA or PKC in the presence of WA and the samples were prepared for negative-stain electron microscopy. Shown is the structural organization of filamentous vimentin phosphorylated by PKA and PKC, respectively, in the presence of 0 (A and D), 50 (B and E), or 100 μM WA (C and F). *Bar*, 200 nm.

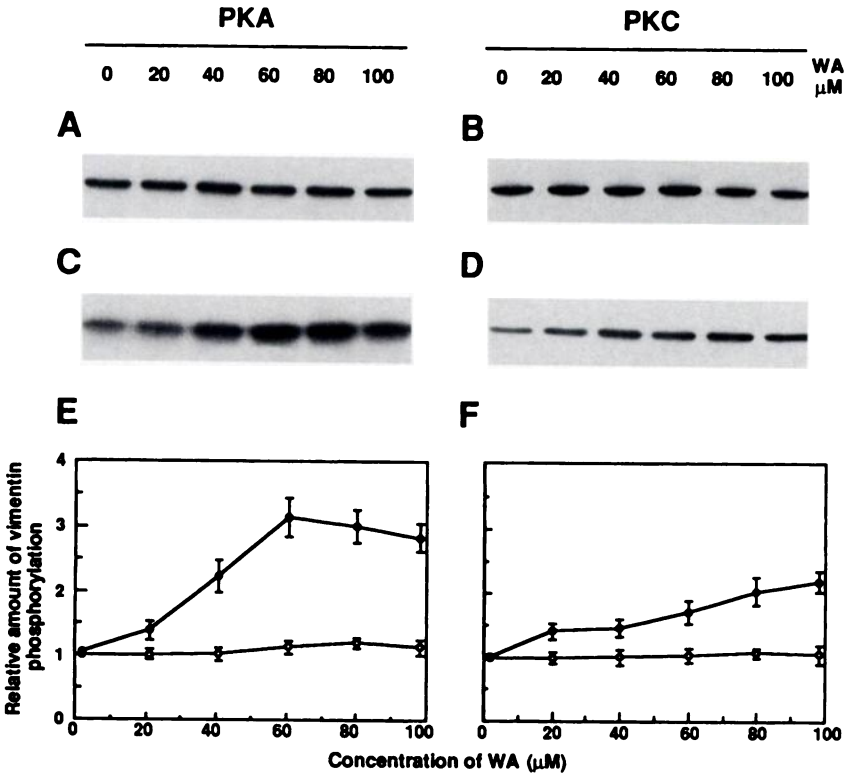

Fig. 4. Effect of WA on the *in vitro* phosphorylation of vimentin. Tetrameric (A and B) or filamentous (C and D) vimentin was phosphorylated by PKA or PKC in the presence of 0–100 μM WA. The phosphorylation procedure was performed as described in Experimental Procedures, and the relative levels of phosphorylation of tetrameric (○) or filamentous (●) vimentin were quantitated (E and F). The data are means ± standard deviations from three independent experiments.

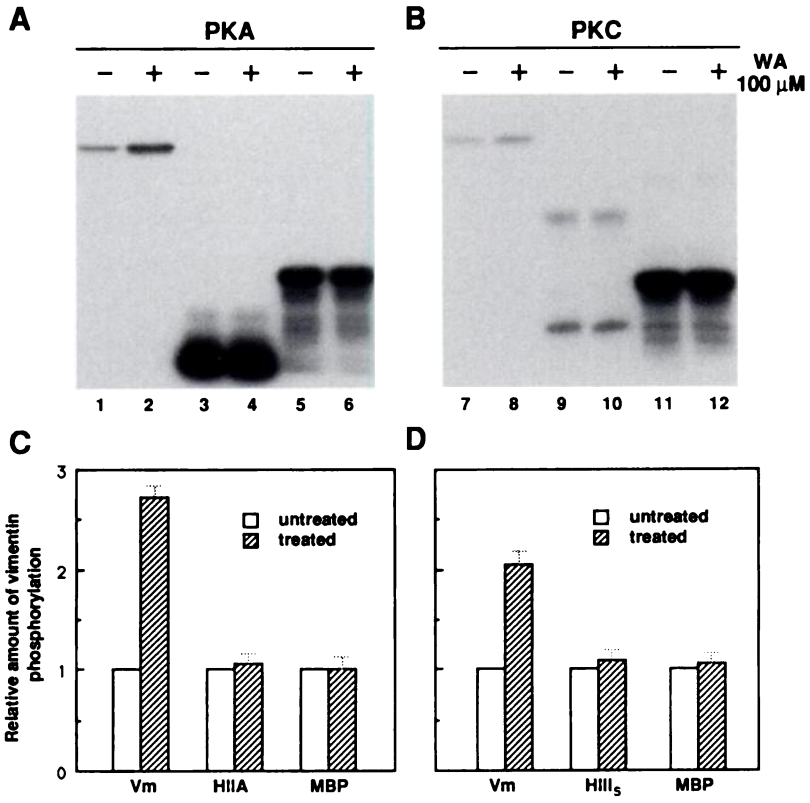

Fig. 5. Specificity of WA effects on the *in vitro* phosphorylation of various proteins. In addition to filamentous vimentin (*Vm*), histone II-A (*HIIA*), histone III-S (*HIII$_S$*), and MBP were phosphorylated by PKA (A) or PKC (B) in the presence (+) or absence (−) of 100 μM WA. *Lanes 1*, *2*, *7*, and *8*, filamentous vimentin; *lanes 3* and *4*, histone II-A; *lanes 5*, *6*, *11*, and *12*, MBP; *lanes 9* and *10*, histone III-S. The relative levels of phosphorylation of the substrates were quantitated (C and D). The data are means ± standard deviations from three independent experiments.

reorganization of vimentin IFs observed *in vivo*. On the other hand, although PKA or PKC individually does not seem to affect the structural organization of the filamentous vimentin, PKA but not PKC can greatly intensify the effect of WA on vimentin aggregation. This difference and the different effects of PKA and PKC on the assembly competence of tetrameric vimentin may be due to different enzymatic specificities of the protein kinases, which act on different serine residues of the vimentin molecule (31). Moreover, phosphorylation of filamentous, but not tetrameric, vimentin by PKA and PKC is significantly augmented in the presence of WA. Because the phosphorylation of other substrates by the protein kinases is not affected by WA, the effect of WA on vimentin phosphorylation is not due to stimulation of the enzymatic activity but instead is due to a change of substrate reactivity of the vimentin molecules. One plausible explanation for the synergistic effect of WA and PKA on the induction of massive aggregation of vimentin filaments is provided as follows. WA directly induces conformational changes that lead to aggregate formation of filamentous vimentin. The WA-mediated conformational changes may also result in the exposure of more accessible sites, thereby increasing reactivity towards the protein kinase. Finally, the phosphorylation process further intensifies the WA-mediated aggregation of filamentous vimentin into a higher order structure.

In summary, we have demonstrated that WA, which induces vimentin modifications *in vivo* (14), can also affect the assembly state and phosphorylation potential of filamentous vimentin *in vitro*. Experimental results indicate that WA, by itself, can directly induce conformational changes in vimentin molecules and the process is reflected in the changes in their assembly behavior and reactivities toward PKA and PKC. Additional studies with radioactive or fluorescently labeled WA would be necessary to determine the binding site and the precise mode of action of this compound. Nevertheless, our experiments suggest that WA can be considered a vimentin IF-targeting drug, which causes aggregation of vimentin filaments, and may be used as a tool to study the physiological functions of vimentin IFs in living cells.

Acknowledgments

The authors would like to thank Drs. A.-S. Chiang and W.-C. Lee for their constructive criticism. Special thanks go to Mr. K. Mueller and Mr. T. Knoy for their assistance in preparing the manuscript.

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