Inhibition of Actin Regulatory Activity of the 74-kDa Protein from Bovine Adrenal Medulla (Adseverin) by Some Phospholipids*

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A 74-kDa protein (adseverin) derived from adrenal medulla severs actin filaments and nucleates actin polymerization in a Ca"+-dependent manner but does not form an EGTA-resistant complex with actin monomers, which is different from the gelsolin-actin interaction. The dissociation of gelsolin-actin complexes by phosphatidylinositol 4,5-bisphosphate (PIP2) and the inhibitory effect on actin filament severing by gelsolin was recently reported. This study shows that the activity of adseverin is inhibited not only by PIP2 but also by some common phospholipids including phosphatidylinositol (PI) and phosphatidylserine (PS). Other phospholipids such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) showed no effect. The addition of PC or PE to PI diminished the inhibitory effect of PI. Triton X-100 and neomycin were also found effective in suppressing the effect of PI, suggesting that the arrangement of polar head groups is important in exerting the inhibitory effect. Ca"+-dependent binding of adseverin to PS liposomes but not to PC or PE liposomes was observed by a centrifugation assay.

Adrenal medullary chromaffin cells are thought to be an excellent system to study the mechanism of secretion (1). Chromaffin granules have been extensively studied because they are easy to obtain as a homogeneous population (2). Chromaffin cells can be maintained in primary culture and represent a convenient model to study the functional aspect of secretion because influences on catecholamine secretion can be rigidly controlled (3-5). A dense network of actin filaments at the cell periphery has been observed in many types of cells, and the idea that these filaments have to be reorganized or disassembled to allow secretory granules access to exocytotic sites was first considered for pancreatic ß-cells (6) and recently the idea has been extended to a variety of secretory cells including chromaffin cells (7-10). The organization and its change were thought to be caused by the action of various actin regulatory proteins, and Ca"+-dependent regulatory proteins are thought to be physiologically important because of the absolute requirement of Ca"+ ions in secretion (11). Recently, Bader et al. (12) showed the presence of Ca"+-dependent actin-severing activity in a sample prepared from adrenal medulla using a DNase I affinity column which has been established to be useful for the isolation of many actin regulatory proteins. They also presented immunological evidence that a gelsolin-like protein is contained in the adrenal chromaffin cells. Following this work, purification of gelsolin from adrenal medulla was reported (13). Recent studies on the regulatory mechanisms of secretion in adrenal medulla demonstrated that there is a Ca"+-dependent binding of gelsolin to PS liposomes and that the inhibitory effect of gelsolin on actin filament severing was recently reported. This study shows that the activity of gelsolin is inhibited not only by polyphosphoinositides such as phosphatidylinositol 4,5-bisphosphate (PIP2) and phosphatidylserine (PS) but also by some kind of acidic phospholipids such as phosphatidylinositol 4-monophosphate (PIP) and tightly bound actin molecule was shown to be released from gelsolin with PIP2.

In the previous study, we reported the purification and characterization of another Ca"+-dependent actin-severing protein from adrenal medulla (18). This protein, with a molecular weight of 74,000, showed a Ca"+-dependent actin filament severing and barbed end capping activity. Physicochemical and immunological characterization showed that this protein differs from gelsolin. Furthermore, the 74-kDa protein did not form a tight complex with actin monomer as revealed by a gel filtration experiment. This suggested that the mode of action of the 74-kDa protein-actin interaction was different from that of the gelsolin-actin interaction. It is, therefore, interesting to study the effect of PIP2 and related molecules on the activity of the 74-kDa protein.

In this study we show that the actin regulatory action of the 74-kDa protein is inhibited not only by polyphosphoinositides but also by some kind of acidic phospholipids such as phosphatidylinositol (PI) and phosphatidylserine (PS). This result, combined with the other characteristics described above, shows that the 74-kDa protein is different from gelsolin. We, therefore, call this protein "adseverin" from its original source (adrenal medulla) and actin-severing activity.

EXPERIMENTAL PROCEDURES

Materials—Phosphatidyicholine (PC), phosphatidylethanolamine (PE), PS, phosphatidic acid, PI (from bovine liver and from soybean), PIP, PIP2, inositol triphosphate, 1,2-dioleoyl-2-acetyl-sn-glycerol, L-a-glycerophosphoinositol, neomycin, Triton X-100, heparin sulfate, and poly-l-glutamate were obtained from Sigma. PS (beef brain) and PI (purified from bovine adrenal medulla) were purchased from Serdary Research Laboratories (London, Ontario, Canada). Phospholipids that were supplied as a solution in chloroform/methanol were dried under a stream of nitrogen gas. Water was added onto the dried samples to a concentration of 1 mg/ml, mixed by a Vortex mixer, and further sonicated for 30 s at 70 watts in a sonicator (Ohtake, Japan). These liposome suspensions were divided into aliquots and frozen at -80°C until use.

Protein Preparations—Purification of adseverin from bovine adrenal medulla and of actin from rabbit skeletal muscle was performed as described previously (18). Modification of muscle actin with N-ethylmaleimide was carried out by a modified method (19) of Koyama and Mihasi (20).

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1 The abbreviations used are: PIP2, phosphatidylinositol 4,5-bisphosphate; Mes, 2-(N-morpholino)ethanesulfonic acid; BSA, [glycine and glutamic acid]; PMSF, 2,3-[-N-morpholino]ethanesulfonic acid; EGTA, [ethylenediaminetetraacetic acid]; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIP2, phosphatidylinositol 4-monophosphate; PS, phosphatidylserine.
Adseverin, a Novel Actin Severing Protein

Fluorescence Measurement—Fluorescence was measured in a temperature-controlled cuvette chamber using a Hitachi 650-10S fluorospectrophotometer. The excitation and emission wavelengths were 365 and 407 nm, respectively. To measure the nucleation activity of adseverin on actin polymerization, monomeric actin solution containing 5% pyrene-labeled actin (2.4 μM) was added, and the increase of fluorescence was monitored. The activity of adseverin to sever actin filaments was measured as described previously by Janmey and Stossel (14) using an actin sample containing 50% pyrene-labeled actin. The order of addition of various components was always fixed unless otherwise specified. Generally, the buffer solution into which monomeric actin of F-actin was to be diluted (solution A: 10 mM Mes-KOH, 100 mM NaCl, 2 mM MgCl2, 0.1 mM dithiothreitol, 0.2 mM EGTA, 0.4 mM CaCl2, pH 6.8) was added to an Eppendorf tube followed by lipids and other additives, if necessary, and vortexed. After 1 min at 37 °C, adseverin was added and vortexed. One min later, the mixture was added to a cuvette containing polymerized actin to dilute the concentration of actin solution from 5 to 0.2 μM, and the change of fluorescence was monitored at 37 °C.

RESULTS

Effects of various phospholipids on the activity of adseverin was studied, and PIP2 was shown to be effective in inhibiting both the nucleation and the severing activities of adseverin. Interestingly, PI was also effective on both activities (Fig. 1, A and B). In the following studies we analyzed mainly the inhibitory effect of phospholipids on the nucleation activity of adseverin, because this activity is easy to quantify and is fairly dose-dependent (see below). The presence or absence of Mg2+ ions had little effect on the inhibitory activity; hence, all assays described below were done in the presence of Mg2+ ions. Dose-response curves of the inhibitory activities of PI and PIP2 showed that the inhibitory effect was not derived from contaminating PIP2 in the PI sample or contaminating PI in the PIP2 fraction (Fig. 1C); PIP2 showed some inhibitory effect, but the extent was lower than these phospholipids (Fig. 1A and B). No difference was observed in PI samples derived from calf brain, pig brain, or soybean, suggesting that whether the hydrocarbon chain of fatty acids is saturated or not is of no importance in the inhibitory effect. Other phospholipids such as PC, PE, and phosphatidic acid in addition to inositoltrisphosphate, 1-oleoyl-2-acetyl-sn-glycerol, and L-a-glycerophosphatidylinositol showed no effect except for PS which showed the same level of inhibitory effect as PI (Fig. 1D).

The inhibitory effect of PI was shown to be weakened by the addition of other phospholipids such as PC or PE (Fig. 1E). This result suggests that some kind of polyanionic state of the lipid vesicle surface is important in the inhibitory effect of PI. Other polyanions such as heparin sulfate or poly-L-glutamate, however, showed no effect on the activity of adseverin. The importance of the arrangement of the polar head groups was further supported by the inhibitory effects of Triton X-100 and neomycin, which is known to bind to the polar head group of inositol phospholipids (Fig. 2). The binding constant of adseverin to PI vesicles was estimated from the inhibition of the nucleation activity in the presence of PI vesicles, supposing that the binding of the protein to the vesicle directly results in the inhibition of the activity. Fig. 3A shows that the nucleation activity is linearly related to the amount of adseverin up to some extent. This result enabled us to get a binding curve of adseverin to PI vesicles (Fig. 3B). A Scatchard analysis showed that the association constant between adseverin and PI molecules was about $1 \times 10^6$ M$^{-1}$ with about 1000 moles of PI needed to bind 1 mole of adseverin (this means the association constant between adseverin and PI vesicles is more than $1 \times 10^6$ M$^{-1}$) (Fig. 3C). The binding of adseverin to liposomes in the presence of Ca2+ ions was confirmed by a sedimentation experiment. Fig. 3D reveals that adseverin cosediments with PS liposome in a Ca2+-dependent fashion. The same result was obtained with liposomes of PI, PIP2, and PIP3. The binding of adseverin to these liposomes became evident above pCa 7, the same Ca2+ concentration at which the actin severing activity of adseverin was activated as described previously (18). When PS liposomes were replaced with PC or PE liposomes, only a slight increase in cosedimentation was observed in the presence of Ca2+ ions (Fig. 3D).

![Fig. 1. Inhibition of nucleation and severing activities of adseverin with some phospholipids. A, nucleation activity of adseverin (37 nm) for actin polymerization was studied in solution A in the presence of various phospholipids (0.04 mg/ml) (a–d, f, +adseverin; e, −adseverin; a, −phospholipid; b, +PA or PC or PE; c, +PI; d, +PIP2; f, +PIP3). Polymerization of actin was monitored with the change of fluorescence (FL) of pyrene-labeled actin at 37 °C. B, various phospholipids (0.04 mg/ml) were added to adseverin (46 nm) in solution A, and polymerized actin was diluted with the above solution, and the change of fluorescence was monitored at 37 °C (a–f, the same as in A). C, the nucleation activity of adseverin (24 nm) for actin polymerization was studied under various amounts of PI (●) or PIP2 (○) and expressed as the percentage of the value obtained without phospholipids. D, dose-dependent inhibition of the nucleation activity of adseverin (18 nm) by PI (●) and PS (○). E, the inhibitory activity of PI (0.04 nm) on the nucleation activity of adseverin (21 nm) was observed after mixing with various amounts of PC (●) or PE (○).](http://www.jbc.org/content/109/3/10941/F1.large.jpg)

![Fig. 2. Recovery of the nucleation activity of adseverin by the addition of various amounts of Triton X-100 (●) or neomycin (○) to PI (0.04 nm). Inhibition of the PI activity on adseverin by Triton X-100 or neomycin was expressed as percent.](http://www.jbc.org/content/109/3/10941/F2.large.jpg)
FIG. 3. A, a dose-dependent effect of adseverin on actin nucleation. The increase in fluorescence after 1 min of incubation in the presence of various amounts of adseverin was plotted against the amount of adseverin added. B, a binding curve of adseverin to PI vesicles. In the presence of a constant amount of PI (0.01 mM), various amounts of adseverin were added, and the nucleating activity of actin polymerization was measured. The amount of free protein was calculated from the curve shown in A. C, a Scatchard analysis of the binding of adseverin to PI vesicles shown in B. B, F, and PI represent molar concentrations of bound (B) and free (F) adseverin molecule to phosphatidylinositol molecule (PI), respectively. D, binding of adseverin to phospholipids. Adseverin (180 nM) was mixed with each phospholipid 0.25 mg/ml in solution A containing 2 mM EGTA (EGTA) or solution A (Ca). After 10 min of incubation at 35 °C, the mixture was spun at 150,000 x g for 20 min at 35 °C, and the supernatant (s) and pellets (p) were electrophoresed in the presence of sodium dodecyl sulfate using a 10% acrylamide gel (18).

**DISCUSSION**

In this report, we show that the activity of adseverin is suppressed by some kinds of acidic phospholipids. Regulation of the actin modulating activity is important because actin filaments are thought to be distributed underneath the cell membrane not only defining the cell shape but also supporting the spatial distribution of various membrane proteins such as ion channels and transporters (21, 22). Inhibition of the activity of adseverin by membrane phospholipids means to limit the working place of this protein. Transient increase in the Ca²⁺ concentration is thought to be brought about by an inward flow of Ca²⁺ from outside the cell through Ca²⁺ channels or by the release of Ca²⁺ from intracellular vesicles containing Ca²⁺. This increase in the Ca²⁺ concentration will cause the activation of adseverin and result in the reorganization of actin filaments. Unused activated adseverin will be quickly inactivated by binding to membrane phospholipids; the working place and time will be thus highly limited. Decrease of the intracellular Ca²⁺ concentration results in the dissociation of Ca²⁺ from the protein and the release of the protein from the membrane to the soluble fraction. Immuno-staining of this protein in adrenal chromaffin cells using a monospecific antibody described in the previous paper (18) showed no clear localization of this protein, suggesting its free distribution in the cytoplasm, although much more work is needed to determine the intracellular role of this protein.

One mole of adseverin was calculated to bind about 1000 mol of PI in vesicles. This molar ratio of binding was much higher than the values estimated in the case of gelsolin binding (100 mol of lipids/1 mol of protein) or profilactin binding (10 mol/1 mol of protein) to PIP₂ (14, 23). In the case of synthetic and egg yolk phosphatidyicholine, the smallest size of the liposome is known to be 20 nm in diameter, and our electron microscopic observation of negatively stained PI vesicles also showed the presence of small vesicles of this size. The surface area of a phospholipid is known to be in the range of 0.5 and 0.7 nm² (24, 25). From these values, one liposome is calculated to contain more than 2500 phospholipid molecules. This means one liposome can bind at least two adseverin molecules. Considering the high binding constant and fairly high content of PS and PI in the membrane (about 20% of all phospholipids) and no inhibitory effect of Mg²⁺ ions on the activity, it is suggested that the modulation of the activity of adseverin does occur in vivo. A two-dimensional polyanionic surface seemed to be important for the inhibitory effect since the activity was inhibited by the presence of Triton X-100 or neomycin and since other polyanions such as heparin sulfate or poly-L-glutamate did not suppress the activity.

The calcium-dependent lipid binding behavior of adseverin resembles that of the annexin/lipocortin/calsequestrin family (26, 27). The latter proteins are known to bind to phenyl-Sepharose in the presence of a Ca²⁺ ion and elute with an EGTA wash. Using adrenal medulla extract we confirmed a Ca²⁺-dependent binding of the annexins. Western blotting of the EGTA eluate using a specific antibody against adseverin, however, showed no binding of this protein to this column. This experiment also showed no immunological cross-reactivity of adseverin with annexins. These results, combined with the fact that the Ca²⁺ sensitivity of adseverin described in the previous report and in this paper is much higher than for the annexins, shows that adseverin is not an annexin.

Recent studies on the molecular structure of gelsolin revealed the functional and regulatory domain of the molecule (28, 29). Such studies on adseverin will be useful in understanding its molecular structure and may show how the regulatory mechanisms of these proteins are different.

**REFERENCES**

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