Reorganization of actin in platelets stimulated by thrombin as measured by the DNase I inhibition assay

( actin assay/platelet actin polymerization during stimulation/Ca2+ effects on actin in extracts)

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ABSTRACT The effect of thrombin stimulation on actin organization in human platelets has been analyzed by using the DNase I inhibition assay, which is selective for unpolymerized and filamentous actin. The results provide biochemical evidence for the suggestion that stimulation leads to rapid polymerization of actin. The measurements also reveal changes in the polymerization state of actin occurring after cell lysis. These changes are influenced by the concentration of free calcium in the extracts.

Platelets respond to a number of stimuli by extensive shape changes. The stimulating agents can be proteolytic enzymes, macromolecular aggregates, or low molecular weight effectors. The primary response to all these agents is a transformation of the platelets within seconds from smooth discs to spheres with long thin protrusions. This shape change is followed by aggregation of the cells and secretion of granules (1-4). In the presence of plasma the stimulated platelets become enmeshed in a fibrin clot and are thought to provide force for clot retraction (5-7).

The early morphological changes are accompanied by a reorganization of the circular ring of microtubules (1, 8, 9). Actin filament bundles appear in the cell body and as cores of the protrusions formed after stimulation (7, 10-12). Several investigators have proposed that the actin filaments are formed from unpolymerized precursors (7, 10, 12). Platelets are one of several systems in which actin filaments have been suggested to form in situ (13-18), but it has not been possible to study this process by biochemical techniques.

Nonmuscle cell extracts made in isotonic buffer contain actin in both unpolymerized and filamentous states (for reviews see refs. 19 and 20). Purification of unpolymerized actin from several cell types, using DNase inhibition as an actin assay, results in isolation of actin complexed to profilin—an inhibitor of actin polymerization (ref. 21; unpublished observations). Thus, at least part of the unpolymerized actin in these cell extracts is accounted for by this complex, but the exact proportion remains to be established. It also remains to be elucidated whether and how this unpolymerized actin functions as a precursor to filaments in situ.

Extracts of platelets contain a large fraction of the actin in an unpolymerized form (22-27) and the isolation of profilin from platelets has been reported (25, 26). In the present investigation we have characterized, by using the DNase inhibition assay (27), the polymerization state of actin in extracts of unstimulated platelets in comparison with platelets stimulated with thrombin. This assay discriminates between unpolymerized and filamentous actin in model systems and distinguishes two pools of actin in cell extracts (27). We use the terms "unpolymerized" and "filamentous" for these pools, even though this may represent an oversimplification. Our results provide biochemical evidence for the hypothesis that stimulation leads to a rapid polymerization of actin. A preliminary account of the reorganization of actin in platelets has been presented elsewhere (28).

MATERIALS AND METHODS

Human platelets were obtained from the Blood Center, University Hospital, Uppsala. Platelet concentrates in citrated plasma were prepared either as for clinical use (at pH 7.0-7.1) or at a lower pH (6.5). Platelet preparations were freed of contaminating red blood cells and leukocytes by centrifugation for 10-15 min at 150 X g at room temperature. The suspension of platelets (10-20 ml) was then immediately chromatographed on a Sepharose 2B column (2.5 X 30 cm) equilibrated with 126 mM NaCl/5.4 mM KCl/0.05 mM CaCl2/1 mM MgCl2/14.5 mM Tris-HCl/1 mg of glucose per ml/16 mg of human serum albumin (KABI, Stockholm) per ml. The pH of this solution was adjusted to 6.9 with NaOH after the albumin had been dissolved. This pH value is somewhat lower than that in the description by Tangen et al. (39); this modification was found to decrease morphological changes and aggregation occurring during the chromatography. The purified cell suspension was left on a rocking shaker at pH 6.9 and samples were adjusted to pH 7.4 just before each experiment.

The morphology of the cells was checked by light microscopy. Batches in which the majority of cells eluted from the column had transformed from discs to spiky spheres were not used. With platelet concentrates prepared at the lower pH (6.5), fewer batches had to be discarded.

The reactivity of purified platelets was routinely checked (at pH 7.4) by exposure of the cells to thrombin. The experiments reported here were performed with Topostatin (0.6-1 NIH unit/ml; Hoffman-La Roche). Similar results have been seen with cells exposed to purified human thrombin (KABI, Sweden). With acceptable batches, small aggregate of cells were visible after 20-30 sec, and within 90 sec these had turned into a large flocculent precipitate.

Cells in chromatography buffer were lyed by addition of 0.1 vol of 10% Triton X-100/20 mM MgCl2/20 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)/2 mM ATP/5 mM dithioerythritol (pH 7.4) with or without 19 mM CaCl2. When stated in the text, 0.2 mM phenylmethylsulfonyl fluoride (PMSF) was added to this solution just before the start of an experiment. The final buffer conditions are referred to as Ca buffer and EGTA buffer, respectively. Free Ca2+ concentrations were calculated to be about 1.0 nM in the EGTA buffer and 1.2 μM in the Ca buffer, using a value of 3.25 X 105 M-1 for the binding constant of Ca2+ to EGTA at pH 7.4 (30). It is difficult to assess the influence of the

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride.

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cell contents on these Ca\(^{2+}\) concentrations. The cell suspensions used contained about 5 \times 10^7 platelets per ml, and release of all the Ca\(^{2+}\) from cells at this concentration would increase the total Ca\(^{2+}\) concentration by about 0.2 mM (31). If this is added to the buffers described above, the free Ca\(^{2+}\) concentrations become 4.4 nM and 0.15 mM in the EGTA and Ca buffers, respectively. These figures should represent maximal values in the extracts.

Measurement of unpolymerized and total actin was performed with the DNase inhibition assay as described (27). Filamentous actin inhibits DNase I, but at a slower rate than unpolymerized actin does. In the experiments described here the DNase inhibitor activity measured in the absence of guanidine-HCl represents the amount of unpolymerized actin in a sample and the activity after treatment of the sample with guanidine-HCl measures total actin. DNase I (DN-100, Sigma) was dissolved in 10 mM Tris-HCl, pH 7.5/150 mM NaCl/0.5 mM CaCl\(_2\)/0.1 mM PMSF and further purified by chromatography on Sephadex G-100 in the same buffer without PMSF (32). The peak fractions were pooled and diluted with buffer to 0.05 mg of DNase per ml, and 10 \(\mu\)l of this solution was used in the DNase inhibition assay. With this batch of DNase, an inhibitor activity of 10,000 units/ml corresponded to about 0.1 mg of actin per ml. Total actin concentrations were determined after treatment of samples at 0°C with an equal volume of 1.5 M guanidine-HCl in 1.0 M sodium acetate/1 mM CaCl\(_2\)/1 mM ATP/20 mM Tris-HCl, pH 7.5.

RESULTS

Characterization of the lysis conditions

In preliminary experiments we used platelets suspended in whole plasma to avoid unnecessary handling of the cells. Extracts of unstimulated cells contained 90-100% of the total actin in an unpolymerized form. After stimulation 25% of the actin, all of which was unpolymerized, was found in the soluble part of the lysate. The remainder, trapped in or bound to the fibrin clot, was recovered in an unpolymerized form after repeated and vigorous homogenization of the clot. It was found that purified F actin depolymerized rapidly and extensively in lysis buffer containing plasma, suggesting the presence of depolymerizing factor(s) in plasma. Because of the difficulties in dealing with the fibrin clot and the instability of F actin in plasma, subsequent experiments were performed with platelets separated from plasma by gel filtration. Although actin depolymerization occurs also in extracts of purified platelets, it can be reduced to some extent by lowering the concentration of free Ca\(^{2+}\) (28). Detailed investigation of the stability of platelet actin pools in buffers with two different concentrations of free Ca\(^{2+}\) is illustrated in Figs. 1 and 2. The buffers designated as Ca and EGTA buffer contained >1 \(\mu\)M and <5 nM free Ca\(^{2+}\), respectively. The minimum free Ca\(^{2+}\) concentration that promotes rapid depolymerization of the actin in extracts is 0.01-0.1 \(\mu\)M (not illustrated).

The results obtained with extracts from unstimulated cells varied somewhat from one cell batch to another. Extracts from one cell type contained a relatively high (70-90%) and stable proportion of unpolymerized actin in Ca buffer at room temperature (Fig. 1a); slight depolymerization was observed if these extracts were transferred to 0°C immediately after lysis. In EGTA buffer, the pool of unpolymerized actin constituted about 70% of the total and decreased to 45-50% within 30-40 min after lysis (Fig. 1c), indicating polymerization of the actin. This process was slower if extracts were incubated at 0°C. Addition of CaCl\(_2\) to the EGTA extracts initiated a rapid release of inhibitor activity, up to nearly the same level as that seen when cells were lysed directly in Ca buffer. On the other hand, EGTA added to extracts made in Ca buffer did not induce polymerization of actin (not illustrated).

Extracts from the second type of "unstimulated" cells were characterized by a lower relative concentration of unpolymerized actin when assayed immediately after lysis (between 45 and 75% of the total actin). Here the actin depolymerized in Ca buffer and remained stable in EGTA buffer (Fig. 2a).

A third pattern of changes in actin pools was seen in extracts of platelets stimulated with thrombin. In Ca buffer only a small difference between stimulated and unstimulated cells (about 10%) was detected in the relative amounts of unpolymerized actin. A clear difference was observed when PMSF was included in the Ca buffer. As shown in Fig. 1b, the extract of stimulated cells prepared in the presence of PMSF had only 60%
of its actin in the unpolymerized form soon after lysis. This fraction increased to 80% during 15 min of incubation, suggesting depolymerization of actin filaments. When extracts were made in EGTA buffer there was a pronounced difference between unstimulated and stimulated cells, especially in the measurements made early after lysis (Fig. 1d). The concentration of unpolymerized actin increased with time of incubation also in these extracts, again suggesting depolymerization of actin. This contrasts with the polymerization seen in EGTA extracts of unstimulated cells (Fig. 1c). Addition of CaCl₂ to the extracts of stimulated cells accelerated the depolymerization. Addition of PMSF had no effect on the actin pools in this case.

The total amount of actin measured as DNase inhibitor activity in the presence of guanidine-HCl was nearly the same in all extracts, except for stimulated cells lysed in EGTA buffer (Fig. 1d). In the latter case it was more difficult to recover all activity. Vigorous agitation of the cells on a Vortex mixer during stimulation and incubation of the EGTA extracts at 0°C before treatment with guanidine-HCl improved the recovery of inhibitor activity. In samples in which Ca-induced depolymerization had occurred, all activity was recovered with guanidine-HCl.

Time course of actin reorganization

In examination of the time course of the stimulation-dependent reorganization of actin, we analyzed the actin pools in extracts made in both Ca and EGTA buffers with PMSF present. Extracts were kept at 0°C to get maximal activity with guanidine-HCl. Fig. 2 illustrates the changes in actin pools in cell extracts prepared at various times after stimulation with thrombin. The analyses in both Ca and EGTA buffer show that there is a thrombin-dependent decrease in the amount of unpolymerized actin. As in Fig. 1 the effect of stimulation on the actin pools was most pronounced in EGTA extracts and in the measurements made soon after lysis. The maximal amount of actin recorded by the assay after guanidine-HCl treatment was the same throughout the experiment. This demonstrates that there was an increase in filamentous actin concomitant with the decrease in the pool of unpolymerized actin. Electron microscopy of negatively stained extracts of unstimulated and stimulated cells (not illustrated) supported this interpretation that many more filaments could be seen in the stimulated sample.

Fig. 3 displays the amount of unpolymerized actin determined from the first measurements made after lysis of cells stimulated for various lengths of time. The result clearly demonstrates that stimulation leads to a progressive decrease in the fraction of actin in the unpolymerized form. During an early phase of the stimulation all the actin was readily recovered as DNase inhibitor by treatment with guanidine-HCl, both in Ca and EGTA extracts. After this period, the recovery of total actin was reduced in EGTA extracts unless the cells were Vor-
Stimulation of platelets with thrombin leads to a decrease in the concentration of unpolymerized actin and a simultaneous increase in filamentous actin present in cell extracts soon after lysis. This strongly suggests a precursor-product relationship between these two forms of actin. The rearrangement of actin apparently requires the action of thrombin on intact cells, because addition of thrombin to extracts of unstimulated cells does not produce a corresponding rearrangement of the actin. The same seems true for the change seen in the filamentous actin, late in the stimulation process, from a form readily depolymerized by guanidine-HCl to a form more resistant to this treatment. We have not determined the actin pools earlier than 2–3 min after cell lysis, so that the possibility of rapid changes occurring in extracts during the first minutes cannot be excluded. The decrease in the fraction of unpolymerized actin resulting from stimulation follows a time course similar to that of the initial morphological changes in which pseudopods containing actin filament bundles are formed (1).

The variation in the stability of actin pools in extracts of unstimulated platelets may reflect inadvertent stimulation occurring both during purification and on storage of the cells. It is clear from morphological observations that some cell batches are affected more than others by the purification procedure. In some instances cell suspensions gave rise to extracts with a high ratio of unpolymerized-to-filamentous actin immediately after chromatography and, 1–2 hr later, to extracts with a significantly decreased level of unpolymerized actin.

The concentration of monomeric actin in the extracts is well above the critical concentration for filament formation with purified actin (ref. 24; unpublished results). This suggests the presence of factor(s) that keep the actin in the unpolymerized form. It is likely that profilin is such a factor affecting the polymerizability of actin, but other modifications of the actin cannot be excluded. The F actin detected by the assay must be in some kind of equilibrium with the pool of monomeric actin, but it is impossible at present to evaluate this relationship. Neither the polymerization of actin seen in EGTA extracts of unstimulated cells nor the depolymerization in Ca extracts appears to be explained by the properties of purified actin and profilin (the profilin–actin complex). Control experiments showed that both rabbit skeletal muscle F actin and calf spleen profilin (at 0.2 mg/ml) are stable over at least 30 min at room temperature and at 0°C, in both Ca and EGTA buffer, and neither is affected by the addition of thrombin. Thus the changes in actin organization may reflect interaction of actin with other components in the extracts. Free Ca²⁺ at micromolar concentrations is known to affect various cellular processes (33–37), several of which may interfere with the distribution of actin between different organizational states.

The conversion of unpolymerized to filamentous actin in EGTA extracts of unstimulated cells might be an in vitro corollary to the process initiated by stimulation of intact cells. It is interesting to note that EGTA added to extracts made in the presence of Ca²⁺ did not induce polymerization of actin. Another possibility is that the polymerization seen reflects the effect of detergent-released proteolytic enzymes on the unpolymerized actin. Brief treatment of purified profilin with trypsin or carboxypeptidase A induces polymerization of the actin in the presence of salt (unpublished observations). On the other hand, protease digestion could cause depolymerization of filamentous actin (38). A small inhibitory effect of PMSF was seen on the depolymerization occurring in extracts made in Ca buffer.

During stimulation of platelets an increased conversion of the metabolic pool of ATP to IMP and hypoxanthine takes place (39). Furthermore, it is known that ATP can be broken down within minutes in cell extracts (40). Removal of the bound nucleotide from purified actin results in a rapid and irreversible loss of polymerizability (41, 42). Thus, extensive degradation of ATP in platelet extracts could affect actin organization. As a first approach to test this, we increased the final concentration of ATP in the lysis buffer from 0.2 mM to 2 mM. This decreased the rate of polymerization in extracts of unstimulated cells lysed in EGTA buffer, but no effect was observed on other types of extract.

The present report gives evidence for the conversion of unpolymerized to filamentous actin concomitant with the induction of cell movement. It remains to be established whether the profilin–actin complex participates in this process and so constitutes the precursor to actin filaments. The availability of a rapid biochemical assay for actin should facilitate continued investigation of the filament precursor and of the factors involved in filament formation and organization.

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