An Actin-nucleating Activity in Polymorphonuclear Leukocytes Is Modulated by Chemotactic Peptides

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Abstract. We examined the actin-nucleating activity in polymorphonuclear leukocyte lysates prepared at various times after chemotactic peptide addition. The actin nucleation increases two- to threefold within 15 s after peptide addition, decays to basal levels within 90 s, and is largely independent of cytoplasmic calcium fluxes. The peptide-induced nucleation sites behave as free barbed ends and therefore may increase the level of polymerized actin in vivo. The new nucleation sites may also determine the cellular sites of actin polymerization. This localization of actin polymerization could be important for the directional extension of lamellipodia during chemotaxis.

ADDING chemotactic peptides to polymorphonuclear leukocytes (PMNs)[1] induces actin polymerization (F-actin) (9, 11, 12, 23, 27, 35). Concomitantly, PMNs spread and extend ruffles over their surface. Both responses peak within 30 s. At later times some of the ruffles withdraw and the amount of F-actin decreases to an intermediate level. Cytochalasin D (CD), which blocks the peptide-induced actin polymerization, inhibits the cell shape change (35, 38). Consequently, actin polymerization appears to be required for the protrusion of surface ruffles in PMNs. The extension of filopodia on platelets, of the acrosomal process on thyone sperm, and of microvilli on fertilized sea urchin eggs have been previously shown to correlate with actin polymerization in similar studies (8, 10, 32).

The cell must have mechanisms for preventing complete actin polymerization. Actin, in the ionic conditions and at the concentrations present within a cell, would always be 99% polymerized in the absence of regulation (13, 30). However, by a number of criteria (actin sedimentation, inhibition of DNAase activity, nitrobenzoxadiazole phallacidin staining), 50–70% of the actin is unpolymerized in an unstimulated PMN (9, 23). This state could be maintained by regulatory proteins that bind free actin monomers with high affinity, thus preventing their polymerization, and by proteins that cap filament ends, thus blocking polymerization.

Proteins with these properties, able to regulate actin polymerization, have been isolated from PMNs (13, 30). Profilin binds monomeric actin, preventing monomer addition to the nonpreferred (pointed) end of actin filaments but not to the preferred (barbed) end (5, 33, 34). Profilin could maintain a large pool of unpolymerized actin in PMNs in conjunction with a capping protein such as gelsolin, which blocks polymerization on the barbed end of an actin filament, (15, 39). Chemotactic peptides, therefore, could increase the fraction of actin polymerized by releasing actin monomers, by uncapping pre-existing filaments, or by creating new barbed end polymerization sites.

Studies of isolated actin regulatory proteins suggest that none of the receptor-generated signals would yield the appropriate regulation of actin polymerization. The binding of chemotactic peptide to its receptor causes a transient decrease in the intracellular concentration of phosphatidylinositol 4,5-bisphosphate as well as a transient increase in the intracellular concentration of calcium (2, 8, 29, 37). Phosphatidylinositol 4,5-bisphosphate is reported to destabilize the profilin–actin complex (16), while calcium activates gelsolin to sever pre-existing filaments and to cap the barbed ends of filaments (15, 39). In addition, activated gelsolin can nucleate filaments that grow at the pointed end. Therefore, the net effect of a transient decrease in phosphatidylinositol 4,5-bisphosphate and transient increase in calcium should be actin depolymerization and not the observed increase in actin polymerization.

To examine the regulation of actin polymerization by chemotactic peptides, we compared lysates prepared from nontreated and peptide-treated cells. We examined the effect of peptide on the number of actin polymerization sites (actin nuclei) by monitoring the rate of pyrene actin polymerization in PMN lysates. The rate of polymerization is proportional to the number of actin nuclei and the actin monomer concentration (13). Consequently, if the monomer actin concentration is constant, the elongation rate depends only on the number and the type of filament ends.

We now report that lysates prepared from cells treated with peptide have a two- to threefold higher nucleation activity than lysates prepared from unstimulated cells. The peptide-

1. *Abbreviations used in this paper*: CD, cytochalasin D; fNorleLeuPhe, *N*-formylnorleucylleucylphenylalanine; PMN, polymorphonuclear leukocyte.

stimulated increase in polymerization appears to be due to an increase in the number of free barbed ends. Calcium does not appear to be necessary for this response.

Materials and Methods

Actin Purification and Labeling

Actin was prepared from rabbit skeletal muscle according to the method of Spudich and Watt (31) modified as described by Murray et al. (20). Briefly, actin was extracted from the acetone powder into 50 μM ATP, 200 μM $CaCl_2$, 100 μM CaEDTA, 10 mM Tris, pH 8.0, for 30 min. G-actin was polymerized by the addition of 2 mM $MgCl_2$ and 0.1 M KCl. Polymerization proceeded at room temperature for 30 min followed by 30 min at 15°C. Before centrifugation, the KCl concentration was raised to 0.8 M primarily to remove troponin-tropomyosin complexes that remained associated with actin filaments. Pyrene actin was prepared according to the method of Kouyama and Mihashi (14) with the following modifications. G-actin (40 μM) was polymerized by the addition of 2 mM $MgCl_2$ and 0.1 M KCl. Pyrenyliodoacetamide, dissolved in DMSO at 1 mg/ml, was added at a final concentration of 2.5 μM immediately after the KCl addition. The pyrene actin mixture was polymerized for 90 min at 15°C before centrifugation. The pyrene actin prepared by this method increased its fluorescence 25–30-fold upon polymerization. Pyrene actin was chromatographed through Bio-Gel P150 (16). Early fractions that contain traces of actin-binding proteins and high levels of nucleating activity were discarded. Pyrene actin was stored either at 4°C in the elution buffer and used within a period of 2–4 wk, or frozen in liquid nitrogen and stored at −80°C. The actin concentrations were calculated as outlined by Seldon and Estes (25): mM actin = $(OD_{290} - 0.33\ OD_{344})/24.9\ mM^{-1}cm^{-1}$. Actin was labeled to ≥90% and used in all experiments undiluted by unlabeled actin.

The critical concentration is the actin concentration that must be exceeded for polymerization to occur (13). This value was determined for each pyrene actin preparation by measuring the extent of polymerization of increasing actin concentrations (0.1–2.5 μM). Critical concentration values varied between 0.1 and 0.2 μM in different actin preparations.

Fluorescence and Kinetic Measurements

Fluorescence was measured in a double-beam fluorimeter (model MPF-2L; Perkin-Elmer Corp., Pomona, CA). Excitation/emission wavelengths were 370/410 nm for pyrene actin. Light scattering effects were minimized with a 390-nm cut-off filter in the emission path. The actin samples were exposed only intermittently to exciting light during recording in order to minimize photobleaching.

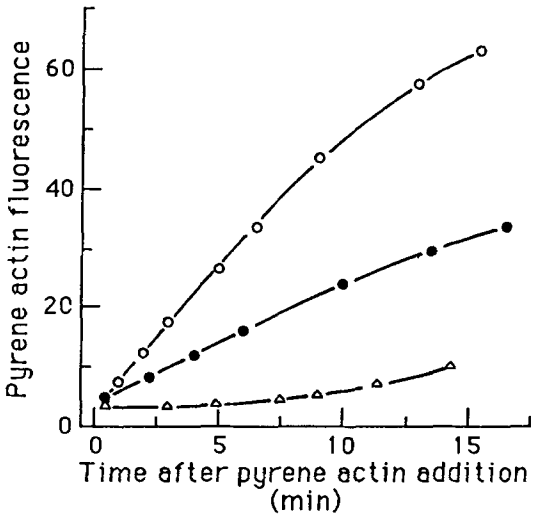

Figure 1. Time course of pyrene actin polymerization in the absence of cell lysates (*open triangles*) and in the presence of lysates prepared from unstimulated cells (*solid circles*), or lysates prepared from cells stimulated for 10 s with 10^{-7} M fNorleLeuPhe before lysis (*open circles*). Lysates were prepared from a final concentration of 4 × 10^5 cells/ml. Fluorescence is expressed in arbitrary units.

Cell Preparation

Rabbit peritoneal PMNs (≥95% pure) obtained as previously described (40) were washed in saline and treated with 5 mM diisofluorophosphate as described by Amrein and Stossel (1). Cells were suspended at 2 × 10^6 cells/ml in 138 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 10 mM imidazole, pH 7.4, and stored on ice until use. EGTA (1 mM) was included in all solutions unless otherwise specified. Diisofluorophosphate-treated cells in this medium exhibited stimulated locomotion and morphological responses upon the addition of chemotactic peptides during filmed visual assays (Carson, M., unpublished results; reference 1). Storing cells on ice allowed assays to extend over 4–5 h without alterations in cell response.

Nucleation Assay

Cells which had been stored on ice were warmed for 5 min at 37°C before *N*-formylnorleucylleucylphenylalanine (fNorleLeuPhe) addition. At various times, after fNorleLeuPhe treatment, 0.2 ml of cell suspension was diluted into 1 ml of lysis medium at 27°C containing 0.2% Triton X-100 in the suspension buffer or into a high KCl buffer (0.5 M KCl, 2 mM $MgCl_2$, 1 mM ATP, 10 mM imidazole, pH 7.4, 0.2% Triton X-100). The final cell concentration was 4 × 10^5 cells/ml. After lysis (2.5 s), a final concentration of 2 μM pyrene actin was added to the sample from a stock solution of 20–30 μM. The initial rate of fluorescence increase at 27°C was measured and used as an indicator of the number of actin nuclei present.

Phosphorylase a Assay

Assays were conducted as described by Slonczewski et al. (28). Briefly, cells suspended in Hanks' media were warmed for 30 min at 37°C and then treated with 3 × 10^{-8} M fNorleLeuPhe for various times. Samples were stopped by immediately chilling on ice in a medium containing 250 mM NaF, 25 mM EGTA, 25 mM EDTA, 250 mM Pipes, pH 7.0. The cells were broken by sonication. Phosphorylase a activity was assayed in the sonicated samples by monitoring ^{14}C glucose-1-phosphate incorporation into glycogen for 60 min at 30°C.

Results

fNorleLeuPhe Increases the Actin-nucleating Activity in PMNs

Fig. 1 illustrates a time course of the polymerization of pyrene actin in the presence and the absence of cell lysates. In the absence of cell lysates, the polymerization of gel-filtered pyrene actin occurs after a lag resulting from the slow rate of spontaneous actin nucleation. Adding lysates prepared from control PMNs to pyrene actin eliminated the lag suggesting the presence of actin nuclei in the lysates. The maximal polymerization rate increased an additional 2–3-fold in lysates prepared from PMNs stimulated with 10^{-7} M fNorleLeuPhe for 10 s before lysis. The magnitude of the lysate- and fNorleLeuPhe-induced effects varied between cell preparations.

The fNorleLeuPhe-induced increase in the nucleation activity was transient. The activity was maximal 10–12 s after fNorleLeuPhe treatment, and declined to basal levels within 60 s (Fig. 2 *a*). 10^{-9} M fNorleLeuPhe is sufficient to stimulate maximal actin nucleation (Fig. 2 *b*). This dose–response curve approximates the fNorleLeuPhe dose–response for cell locomotion and actin polymerization. To compare several samples from different cell preparations, data are expressed as the peptide-induced increase in the maximal polymerization rate (rate in peptide-treated cell sample/rate in untreated cell sample).

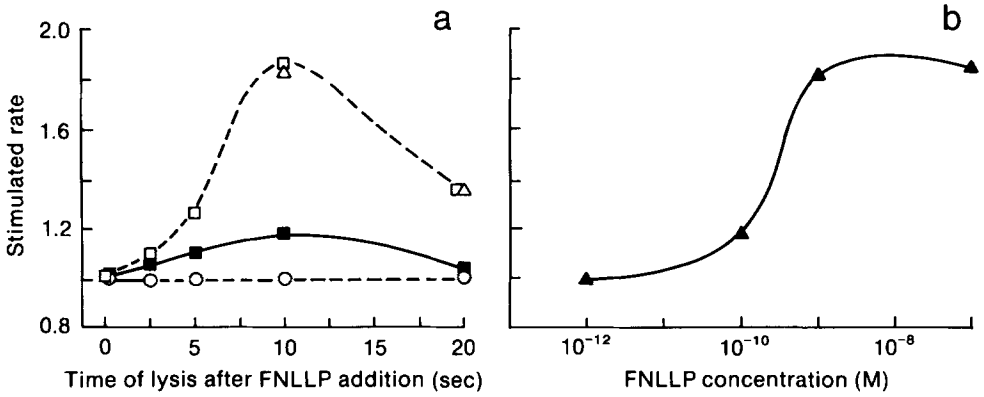

Figure 2. Time course and dose dependence of the fNorleLeuPhe (FNLLP)-induced nucleation. (*a*) Cells are lysed at various times after fNorleLeuPhe addition (10^{-12} M [*open circles*]; 10^{-10} M [*solid squares*]; 10^{-9} M [*open triangles*]; 10^{-7} M [*open squares*]). The data is expressed as the increase in the rate of polymerization in lysates prepared from stimulated cells relative to the rate in lysates prepared from unstimulated cells (rate sample x/rate in unstimulated cell lysate). (*b*) Dose dependence of fNorleLeuPhe for cells lysed 10 s after fNorleLeuPhe addition.

Several controls were run to insure that these fluorescence increases reflected actin polymerization. The lysate-induced fluorescence was pelletable under conditions that would pellet F-actin (Table I). The fluorescence remaining in the supernate is likely to be due to small actin filaments (5–10 monomers) that do not pellet under these conditions (6). Assuming this to be true, the remaining fluorescence would account for ~0.05 μM F-actin. Increasing the actin concentration with unlabeled G-actin caused this fluorescence to pellet after copolymerization with the unlabeled actin. Similar values were obtained with pyrene actin polymerized in the absence of cell lysates. Consequently, it is unlikely that the nonpelleting fluorescence reflected nonspecific lysate-induced changes in pyrene actin fluorescence independent of polymerization.

Dependence of the Nucleation Activity on Cell Concentration

Fig. 3 shows the effect of cell concentration on the rate of pyrene actin polymerization. In lysates prepared from cell concentrations equal to or greater than 4×10^6 cells/ml, the rate of actin polymerization began to decrease after a smaller fraction of the actin had polymerized than in lysates prepared from lower cell concentrations. This limitation on the rate of actin polymerization could be due to increasing concentration of a factor that either sequesters monomers or capped the barbed end of filaments. This decrease in the rate of polymerization at high cell concentrations was not seen if the cells were lysed into high KCl concentrations (0.5 M). High KCl did not alter the fNorleLeuPhe-induced increase in nucleation. In paired samples, the fNorleLeuPhe-induced increase in nucleation was the same in 0.5 and 0.138 M KCl (a 2.4-fold increase). The polymerization rate in lysates prepared from the lower cell concentrations (4×10^5 cells/ml) was also unaffected by KCl concentration. We suggest that high KCl may decrease the affinity of the hypothesized factor for actin.

The final level of pyrene actin polymerization (measured after 12 and 24 h at room temp) was affected only slightly by the presence of cell lysates. The critical concentration of pyrene actin in the absence of cell lysates ranged from 0.1 to 0.2 μM, while the critical concentration ranged from 0.1 to 0.4 μM in the presence of cell lysates. The critical concentration in the presence of the lysate was independent of peptide treatment. This critical concentration (0.1 μM) is characteristic of filaments with free barbed ends. Complete capping of all the barbed ends should raise the critical concentration to 0.8–1.0 μM in the ionic conditions used here. Therefore, the observed critical concentration suggests that in the presence of lysate at least some of the free filament ends must be barbed ends. The critical concentration is relatively insensitive to changes in the percentage of free barbed ends between at least 50 and 100% (21). Thus we cannot determine by this means whether most of the free ends are barbed or pointed.

Table I. Actin Sedimentation

Pyrene Actin	Total fluorescence (at 24 h)	Fluorescence in supernate	Fluorescence in supernate after addition of 10 μM unlabeled actin	Fluorescence in supernate after second centrigation
1 μM	127.1 ± 2.5	9.0	25	0.10
2 μM	374.0 ± 8.0	9.1	25	0.08
3 μM	552.0 ± 4.6	9.0	25	0.08

All actin samples were polymerized in the presence of 4×10^6 cells/ml. After 24 h, samples were centrifuged in a Beckman airfuge for 30 min at 35 psi. 10 μM unlabeled actin (from a 170-μM stock) was added to the supernate, and allowed to polymerize for 24 h. The sample was again centrifuged. Similar values were obtained with actin samples which had been polymerized in the absence of cell lysates.

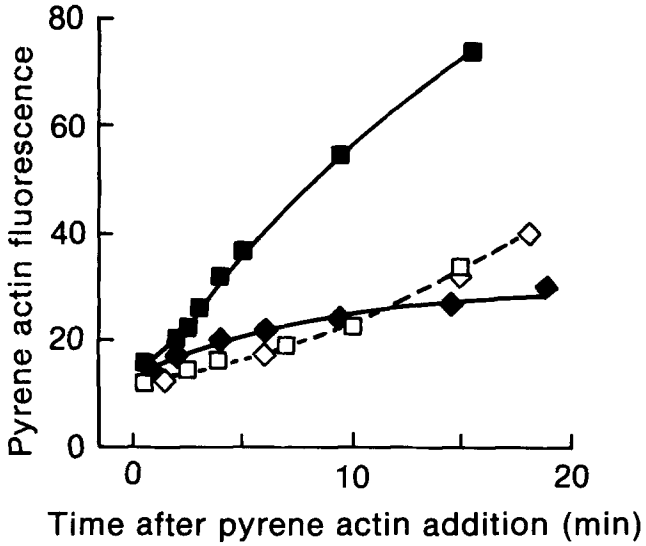

Figure 3. The effect of cell concentration on pyrene actin polymerization. Lysates prepared from a final cell concentration of 4×10^6 cells/ml (*solid symbols*) or 4×10^5 cells/ml (*open symbols*) were lysed in the presence of a final KCl concentration of 0.138 M (*diamonds*) or 0.5 M (*squares*).

fNorleLeuPhe Treatment Increases Free Barbed Ends in PMN Lysates

Although cytochalasin D (CD) has multiple effects, in vitro it has been shown to bind primarily to the barbed end of actin filaments (4, 17, 18, 28). In vivo, CD blocks the peptide-induced increase in actin polymerization (35). Treating PMNs with CD either before or during cell lysis (10 μg/ml final concentration in lysate) completely inhibited the fNorleLeuPhe-induced increase in nucleation (Fig. 4). This result suggests that fNorleLeuPhe stimulation increases free barbed end nuclei in PMN lysates. CD also decreased the polymerization rate in lysates prepared from unstimulated cells (Fig. 4 *b*). Thus, free barbed ends also probably exist in the control lysate preparation.

Depolymerization studies support these conclusions. Pyrene actin samples which had reached complete polymerization (at 24 h) were depolymerized in the presence and the absence of the barbed end capping protein villin (37 nM) (21, 36). Depolymerization was initiated by diluting the total actin concentration from 2 μM to 0.04 μM, approximately twofold below the critical concentration of actin. Villin inhibited the depolymerization rate of pyrene actin polymerized in lysates prepared from stimulated or unstimulated PMNs (Fig. 5). The depolymerization rates of pyrene actin polymerized in lysates prepared from stimulated (for 10 s) or unstimulated cells were indistinguishable. Pyrene actin polymerized in the presence of the lysate depolymerized much more slowly than pyrene actin polymerized in the absence of the cell lysate. However, all samples reached identical endpoints upon completion of depolymerization, as assayed after 18 h.

All villin depolymerization studies were done in the presence of an estimated 10 μM $CaCl_2$, the calcium concentration required to activate the capping but not the filament-severing activity of villin (21, 36). As PMN lysates have a calcium-activated barbed end capping activity, lysates were prepared in EGTA. Pyrene actin was allowed to polymerize in the EGTA/lysate solution and then the lysate/pyrene actin sample was diluted into the calcium solution. At the cell concentrations used, the depolymerization rate in the presence of low calcium was indistinguishable from the rate in EGTA (data not shown).

The Actin-nucleating Activity Pellets

Brief centrifugation of the cell lysate (25 s in Eppendorf microfuge) before the addition of pyrene actin removed most of the nucleating activity from the supernate (Fig. 6). The residual nucleating activity in the supernate was equal in lysates from control and fNorleLeuPhe-treated cells. Resuspension of the pellet recovered 75% of the fNorleLeuPhe-modulated activity and did not effect the relative peptide-in-

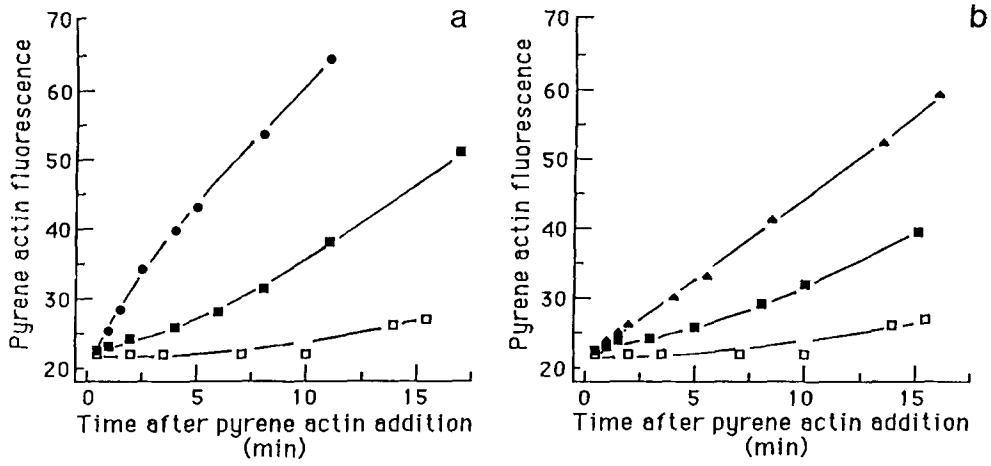

Figure 4. The effect of CD on pyrene actin polymerization. (*a*) Pyrene actin was polymerized in the presence (*squares*) or the absence (*solid circles*) of CD (10 μg/ml). Pyrene actin was polymerized in the absence (*open symbols*) or the presence of lysates prepared from cells lysed 10 s after the addition of 10^{-7} M fNorleLeuPhe (*solid symbols*). (*b*) Pyrene actin was polymerized in the presence (*squares*) or the absence (*solid triangles*) of CD (10 μg/ml). Pyrene actin was polymerized in the absence (*open symbols*) or the presence of lysates prepared from unstimulated cells (*solid symbols*).

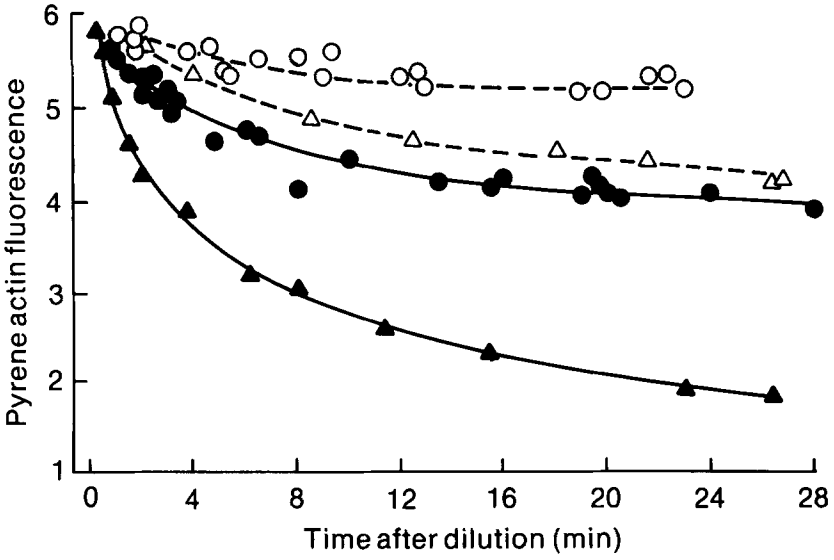

Figure 5. Time course of pyrene actin depolymerization. Pyrene actin (2 μM) was polymerized to completion in the absence of cell lysates (*triangles*) or in the presence of cell lysates (*circles*). Depolymerization was initiated by diluting the pyrene actin samples to 0.04 μM in 138 mM KCl, 1 mM ATP, 2 mM $MgCl_2$, 1 mM EGTA, 1.1 mM $CaCl_2$, pH 7.4. Solid symbols represent samples depolymerized in the presence of 37 nM villin.

duced increase in fNorleLeuPhe nucleation. fNorleLeuPhe induced a 2.33-fold increase in nucleation in both control samples and samples which had been pelleted and resuspended. Therefore, the fNorleLeuPhe-modulated nucleation activity does not appear to be a soluble factor such as free gelsolin.

The fNorleLeuPhe-stimulated Nucleation Is Unstable in Solution

The stability of the fNorleLeuPhe-modulated nucleating activity was examined by adding pyrene actin to lysates at various times after lysis. The fNorleLeuPhe-stimulated nucleation decayed 50% within 50 s and to unstimulated levels within 90 s. In no case did the nucleation levels decay below the levels present in unstimulated lysates. This level was stable for at least 18 h. If the fNorleLeuPhe-stimulated lysate was centrifuged, the nucleation activity in the pellet was stabilized. The pellet could be stored for at least 60 min without loss of activity. Upon resuspension, the activity decayed to basal levels within 90 s.

The fNorleLeuPhe-modulated Nucleation Has Calcium-insensitive Components

The intracellular calcium pools mobilized by fNorleLeuPhe can be depleted by incubating cells with the calcium ionophore A23187 (10^{-6}) and EGTA (1 mM) (22, 28). Depletion of calcium pools in each experiment was monitored by following the ability of fNorleLeuPhe to activate a calcium-sensitive intracellular enzyme: glycogen phosphorylase. In cells incubated with 1 mM EGTA alone in calcium-free medium for 15 min, fNorleLeuPhe induced a twofold increase in phosphorylase activity (Fig. 7). In cells incubated with A23187 and EGTA for 15 min, fNorleLeuPhe caused no change in phosphorylase activity.

Incubating cells with A23187 (10^{-6} M) and EGTA (1 mM) for 15–60 min did not block the fNorleLeuPhe-induced increase in nucleation. The calcium-depleted cells retained between 75 and 100% of the nucleation activity present in control cells (Fig. 8). The ionophore/EGTA treatment had no effect on the nucleating activity in lysates prepared from unstimulated cells. Cytoplasmic calcium levels also could be

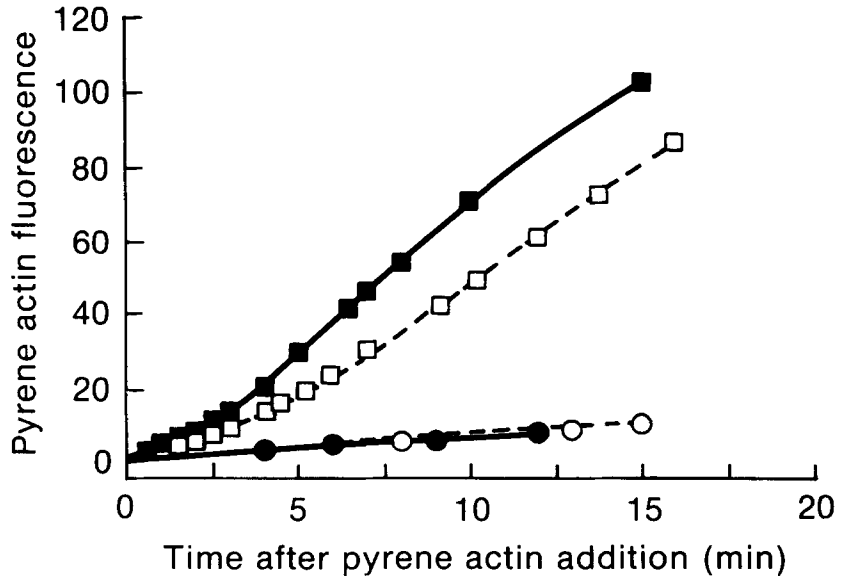

Figure 6. The effect of centrifugation and resuspension of the cell lysates. Lysates were centrifuged in an Eppendorf microfuge for 25 s. Pellets were resuspended in the original volume with fresh buffer. Polymerization was monitored in the resuspended pellet (*open squares*), in the supernates (*circles*), and in the untreated lysate (*solid squares*). Filled circles represent the supernate of centrifuged lysates prepared from cells stimulated for 10 s, unfilled circles represent the supernate of centrifuged lysates prepared from unstimulated cells.

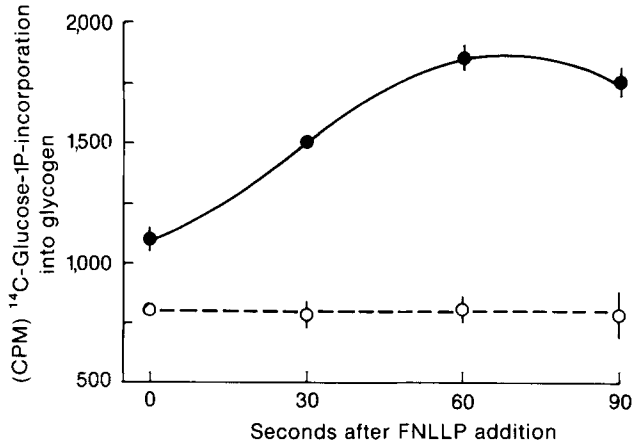

Figure 7. The time course of glycogen phosphorylase a activation by 10^{-7} M fNorleLeuPhe (FNLLP). Cells were incubated for 15 min in calcium-free Hanks' balanced salt solution containing 1 mM EGTA in the presence (*open circle*) or the absence (*solid circle*) of 10^{-6} M A23187 before the addition of fNorleLeuPhe. The y axis represents the counts of ^{14}C-glucose incorporated into glycogen by the cell sonicate over a 60-min period.

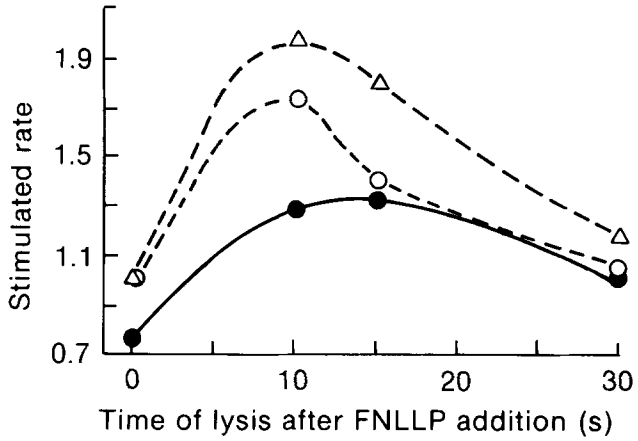

Figure 8. The effect of cellular calcium depletion on the fNorleLeu-Phe (FNLLP)-induced nucleation. Lysates were prepared from untreated cells (*open triangle*), from cells treated with 10^{-6} M A23187 and 1 mM EGTA (*open circle*) and from cells treated with 10^{-6} M A23187 and 1 mM $CaCl_2$ for 15 min (*solid circles*). All cells were either lysed immediately or treated with 10^{-7} M fNorleLeuPhe for various times and then lysed.

raised by treating the cells with A23187 (10^{-6} M) in the presence of calcium (1 mM) for 15 min. Nucleation activity from these cells was assayed in an EGTA buffer. This treatment with ionophore and calcium lowered the unstimulated nucleation activity (time 0, Fig. 8). Yet fNorleLeuPhe still increased nucleation almost twofold over the lowered basal nucleation.

Discussion

We have examined whether chemotactic peptides alter either the number or the nature of polymerization sites in PMNs. We find that lysates prepared from cells treated with fNorleLeuPhe for 10 s have a two- to threefold higher nucleation activity for pyrene actin than lysates prepared from unstimulated cells. This peptide-induced increase in nucleation results in an increase in free barbed ends in the lysate. Depletion of intracellular calcium could inhibit only 25% of the peptide-induced nucleation response.

The fNorleLeuPhe-stimulated increase in the rate of pyrene actin polymerization is unlikely to be due to changes in the free actin monomer concentration in PMN lysates. The total amount of actin contributed by the cell lysate (prepared from 4×10^5 cells/ml) is ~0.1 μM (13, 30). Even if all the cellular actin was in the monomer form, the cellular actin would constitute <5% of the total actin present in the assays. Any changes in the levels of cellular actin in free monomer form in the lysate would be too small to account for the two- to threefold changes in nucleation. Consequently, these results demonstrate an increase in actin nuclei in PMN lysates or possibly a lysate-induced increase in the pyrene nucleation rate. The experiments do not eliminate the possibility that the increase in nucleation results from a primary change in the free actin monomer levels occurring in vivo before lysis.

The peptide-induced nucleating activity is unstable within the lysate while the nucleation activity in lysates of unstimulated cells is stable within the lysate for up to 18 h. This differential stability indicates that chemotactic peptides induce a qualitative as well as a quantitative change in nucleation. Both peptide-induced and basal nucleation activities pellet after brief microcentrifugation. The peptide-induced activity is stable while in the pellet for at least 60 min. These results are consistent with the peptide-induced nucleating activity being in the form of growing filaments at the time of our measurements.

The instability of the fNorleLeuPhe-induced increase in nucleation might be due to depolymerization of actin filaments in the lysate. Depolymerization would occur if the cellular F-actin was diluted below the critical concentration (0.2 μM) as in the lysate solution but not in the lysate pellet (13). The stability of the nucleation activity from control cells might be due to stabilization of the actin filaments. F-actin binding proteins such as tropomyosin could greatly decrease the monomer off-rate. Alternatively, this activity may reflect nonspecific nucleation or nucleation independent of previous F-actin formation.

CD blocked the peptide-stimulated activity and decreased the unstimulated nucleation activity whether it was added either before or during cell lysis. These results suggest that peptide stimulation is due to an increase in free barbed end nuclei. CD could be acting by blocking free barbed ends generated in vivo.

The presence of free barbed end nuclei in lysates was further indicated by the ability of villin to inhibit the depolymerization of actin filaments polymerized in the lysates of both stimulated and unstimulated cells. The extent of the inhibition by villin suggested that many of the filaments had free barbed ends. Our data were not good enough to determine the proportion of barbed ends or to resolve a difference in the rates of depolymerization of filaments polymerized in the lysates of stimulated or unstimulated cells.

It is difficult to determine the proportion of filaments that are capped at one or both ends in the lysate of unstimulated PMNs. As the critical concentration of pyrene actin in the lysate varied only slightly from the critical concentration of pyrene actin alone, and as villin (a barbed end capping protein) inhibited the depolymerization of lysate polymerized

actin filaments, some free barbed ends must exist within the lysate. However, at least 50% of the barbed ends would need to be capped to increase the critical concentration of an actin sample, and 90–97% may need to be capped to maximally increase the critical concentration (13, 21).

Pyrene actin polymerized in the lysate depolymerized much more slowly than control pyrene actin polymerized in the absence of the lysate. As the depolymerization rate depends on the number and type of free filament ends, lysate polymerized pyrene actin may have either fewer filaments, a greater proportion of free pointed ends or a high proportion of capped filaments. It is unlikely that the number of filaments was significantly less since the maximal polymerization rate in the lysate was nearly that observed in the control pyrene actin. It is also possible that other proteins in the lysate such as tropomyosin, which bind along the sides of actin filaments, have stabilized the actin filaments and decreased the monomer off rate (13).

The polymerization rate began to decrease when a lower fraction of the actin had polymerized in lysates prepared from 4×10^6 cells/ml than in lysates prepared from 4×10^5 cells/ml. Concentrated PMN lysates (2×10^7 cells/ml) also induced a transient depolymerization of polymerized actin (data not shown). Neither of these treatments significantly altered the critical concentration of pyrene actin. High KCl treatment blocked these high cell concentration effects.

This data suggests the presence of a capping protein that is only partially effective at high concentrations, but is ineffective upon dilution or in the presence of high salt. The proposed capping activity might not alter the critical concentration if the capper deteriorates with time. Yet, the polymerization rate in lysates prepared from unstimulated PMNs was unaffected by an 18-h storage. Alternately, the polymerization rate is sensitive to the capping of 0–100% of filament ends, but the critical concentration may not be sensitive to the capping of at least 50% of filament ends. These data also suggest that dilution of cellular components after cell lysis inactivates some modes of regulation used within the cell. Thus, the physiological modes of actin regulation detected in lysate assays are limited (19).

Calcium regulates many actin regulatory proteins and thus could modulate the actin nucleation response in vivo. Two results suggest that calcium is not the sole or primary mediator of the peptide-induced nucleation. Depleting calcium pools did not inhibit the peptide-induced response by more than 25%. Increasing the cytoplasmic calcium levels also did not prevent a peptide response. Similar conclusions were reached by two independent groups. Sklar et al. (27) showed that buffering intracellular calcium levels with Quin 2 inhibited the peptide-induced increase in intracellular calcium without inhibiting the initial actin polymerization induced by chemotactic peptides. Sha'afi et al. (26) were able to demonstrate that treating PMNs with quinacrine prevented the chemotactic peptide-stimulated rise in intracellular calcium without preventing the increase in cytoskeletal-associated actin. Cytoskeletal-associated actin was used as a measure of actin polymerization.

The in vivo consequences of the increase in actin nucleation could be of major importance for cell locomotion. The new nucleation sites may determine the cellular sites of actin polymerization. PMNs extend lamellipodium toward the higher concentrations of chemotactic factors (24, 40). The localization of actin polymerization could be important for determining this directional response. The increase in nucleation may contribute to the increase in the fraction of actin polymerized. The state of actin in an unstimulated cell may be balanced by profilin complexing monomeric actin, and partial capping of the barbed ends of filaments. A slight increase in the number of barbed ends may be sufficient to increase the level of actin polymerization. If the increase in nucleation is due to the severing of pre-existing filaments, this event also could be a mechanism for increasing cytoplasmic fluidity.

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