Biophysical Properties and Microfilament Assembly in Neutrophils: Modulation by Cyclic AMP

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Abstract. The microfilament lattice, composed primarily of filamentous (F)-actin, determines in large part the mechanical (deformability) properties of neutrophils, and thus may regulate the ability of neutrophils to transit a microvascular bed. Circulating factors may stimulate the neutrophil to become rigid and therefore be retained in the capillaries. We hypothesized that cell stiffening might be attenuated by an increase in intracellular cAMP. A combination of cell filtration and cell poking (mechanical indentation) was used to measure cell deformability. Neutrophils pretreated with dibutyryl cAMP (db-cAMP) or the combination of prostaglandin E_2 (PGE$_2$, a stimulator of adenylate cyclase) and isobutylmethylxanthine (IBMX, an inhibitor of phosphodiesterase) demonstrated significant inhibition of the n-formyl-methionyl-leucyl-phenylalanine (fMLP)-inducing stiffening. The inhibition of cell stiffening was associated with an increase in intracellular cAMP as measured by enzyme-linked immunoassay (EIA) and an increase in the activity of the cAMP-dependent kinase (A-kinase). Treatment with PGE$_2$ and IBMX also resulted in a decrease in the F-actin content of stimulated neutrophils as assayed by NBD-phallacidin staining and flow cytometry or by changes in right angle light scattering. Direct addition of cAMP to electropermeabilized neutrophils resulted in attenuation of fMLP-induced actin assembly. Neutrophils stimulated with fMLP demonstrated a rapid redistribution of F-actin from a diffuse cortical location to a peripheral ring as assessed by conventional and scanning confocal fluorescence microscopy. Pretreatment of neutrophils with the combination of IBMX and PGE$_2$ resulted in incomplete development and fragmentation of the cortical ring. We conclude that assembly and redistribution of F-actin may be responsible for cell stiffening after exposure to stimulants and that this response was attenuated by agents that increase intracellular cAMP, by altering the amount and spatial organization of the microfilament component of the cytoskeleton.

Actin, a ubiquitous protein present within the cytoplasm of nearly all cells, is the major component of the microfilament lattice and exists in two forms, globular (G)-actin and filamentous (F)-actin, that rapidly interconvert (see review by Korn, 1982). In response to a variety of stimuli such as the chemotactic peptide fMLP (n-formyl-methionyl-leucyl-phenylalanine), the neutrophil rapidly assembles (polymerizes) actin filaments with the conversion of G to F-actin (see review by Omann et al., 1987). Microfilaments are involved in many neutrophil functions, including chemotaxis, chemokinesis, granule secretion, and receptor mobility (reviewed by Sha'afi and Molski, 1988). In addition, the microfilament lattice determines in large part the mechanical properties of leukocytes, which may be important in the process of secretion (Lui et al., 1987) and have recently been related to the ability of leukocytes to deform in order to transit capillaries (Worthen et al., 1989)

The factors that determine the ability of circulating leukocytes to transit the capillaries are incompletely understood. The fact that the neutrophil cell diameter is larger than the mean capillary diameter mandates that these cells must deform to transit the capillaries (reviewed by Hogg, 1987). To the extent that the cell is unable to deform, it will be retained in the capillary for varying periods of time. When stimulated by a variety of agents, neutrophil deformability is decreased and the cells are sequestered in various microvascular beds for prolonged periods of time (Doerschuk et al., 1989). This microvascular sequestration may be related to changes in cellular mechanical properties which in turn may be related to changes in the amount and organization of the actin cytoskeleton (Worthen et al., 1989).

In addition to their role in host defense, neutrophils may injure normal tissues by the release of toxic oxygen radicals and proteolytic enzymes (see review by Sandborg and Smo-

len, 1988). Neutrophil-mediated tissue injury has been implicated in the pathogenesis of myocardial infarction (Engler, 1983), acute lung injury (including the adult respiratory distress syndrome; see Tate and Repine, 1983), and ischemia-reperfusion injury of various organs, including the kidney (Flores et al., 1972) and brain (Ames et al., 1968). Thus, the factors determining neutrophil localization in a microvascular bed are important in the understanding of host defense as well as neutrophil-mediated tissue injury.

The destructive potential of neutrophils can be regulated in different ways. Agents that increase intracellular cAMP have been shown to inhibit a variety of neutrophil functions such as chemotaxis as well as to antagonize many potentially injurious functions of neutrophils such as oxygen radical production and enzyme secretion (Rivken et al., 1975). By extension, we reasoned that similar effects might be exerted on the microfilament reorganization and cell stiffening observed after exposure to inflammatory stimuli. Indeed, increases in intracellular cAMP have been shown to affect the cytoskeleton of mesangial cells (Kreisberg et al., 1985), MDCK cells (Mills and Lubin, 1986), platelets (Fox et al., 1987), and astrocytes (Goldman and Abramson, 1990), perhaps by phosphorylation of key cytoskeletal proteins.

In this report, cell filtration and direct measurement of deformability by "cell poking" (measurement of cellular resistance to indentation using a microprobe) were employed to study the effect of increased intracellular cAMP and activation of the A-kinase on cellular mechanical properties of human neutrophils. In addition, light scattering, flow cytometry, fluorescence and confocal microscopy were utilized to quantify changes in the amount and distribution of F-actin and these changes were related to alterations in cellular mechanical properties. We demonstrate that increases in neutrophil cAMP modulate the reorganization of the microfilament lattice and the consequent changes in cellular mechanical properties after exposure to the chemoattractant fMLP. This cAMP-dependent modulation may function under normal circumstances as a negative feedback signal to limit cytoskeletal changes after cell stimulation or, after exposure to endothelial-derived prostanoids (Frangos et al., 1985), may contribute to the modulation of neutrophil stiffening that would allow the neutrophil to undergo transendothelial migration.

Materials and Methods

Reagents

Percoll was obtained from Pharmacia Inc. (Montreal, P.Q.). Reagents for Krebs Ringers Phosphate Dextrose Buffer (KRPD) including NaCl, KCl, Na$_2$HPO$_4$, K$_2$HPO$_4$, MgCl$_2$, and CaCl$_2$ were obtained from Mallinckrodt Inc. (Paris, KY). Glucose, Hepes, β-glycerophosphate, ATP, and Digitonin, EGTA, fMLP, Hepes, 1-isobutyl-methylxanthine (IBMX)[1], cAMP, db-cAMP, and ATP (K + salt) were obtained from Sigma Chemical Co. (St. Louis, MO). NBD-phalloidin, rhodamine phalloidin, Bodipy-phalloidin, and propidium iodide were obtained from Molecular Probes Inc. (Eugene, OR). Lyso-phosphatidylcholine (lyso PC) was obtained from Avanti Polar Lipids (Pelham, AL). Protein kinase substrate peptide (Kemptide) was obtained from Gibco BRL (Gaithersburg, MD). γ-^{32}P ATP was obtained from ICN Radiochemical (Costa Mesa, CA), and PGE$_2$ from The Upjohn Co. (Kalamazoo, MI).

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1 *Abbreviations used in this paper*: EIA, enzyme-linked immunoassay; IBMX, isobutylmethylxanthine; PGE$_2$, prostaglandin E$_2$.

Cell Isolation

Human neutrophils (>98% pure) were isolated from citrated whole blood obtained by venipuncture using dextran sedimentation and discontinuous plasma-Percoll gradients as previously described in detail (Haslett et al., 1985). Neutrophils obtained from these gradients were labeled with ^{111}In (New England Nuclear, Boston, MA) by incubating 20 μCi of ^{111}InCl/10^6 neutrophils with 5 × 10^{-4} M tropolonate (Fluka, Ronkonkoma, NY) for 5 min in KRPD followed by a wash with KRPD. The separation and labeling procedure required 2 h and the cells were used immediately after isolation for the experiments described. The functional integrity and nonactivated state of neutrophils isolated and labeled in this manner has been extensively validated in previous publications (for example, Downey et al., 1988).

Filtration Apparatus

As described previously (Usami et al., 1975; Downey and Worthen, 1988), polycarbonate filters (Poretics Corporation, Livermore, CA) with a mean pore size of 6.5 μm (range 6.0–7.0 μm; coefficient of variation <10%) in diameter, polypropylene chambers and siliconized plastic intravenous tubing were protein coated by incubation in 20% heat-inactivated human plasma at 37°C for 2 h to minimize cell adhesion to the tubing and chambers. A multichannel infusion pump (Harvard Apparatus Co., Millis, MA) was used to provide a constant flow rate of the buffer across the filters. Immediately upstream of each filter chamber, pressure was continuously measured by a pressure transducer connected to a strip chart recorder. This apparatus allowed three filtration systems to be run and monitored simultaneously.

Experimental Protocol

For the experiments, 1 × 10^5 neutrophils were used per filter which represents a ratio of approximately 1 leukocyte/4 pores (the number of pores in the filter was taken from the manufacturer's specifications). ^{111}In-labeled neutrophils in suspension were injected as a bolus over 2 s into the port of the filtration system, the effluent collected into 50-ml polypropylene containers and subsequently transferred into plastic scintillation vials for counting. The cells in suspension were allowed to flow through the filtration system until a total of 5 ml was collected after which time flow was interrupted, the tubing disconnected from the filter chambers and the filters removed. The filters, proximal and distal chambers were also placed in plastic scintillation vials and counted, along with the effluent, in a gamma well counter (Gama 7000; Beckman Instruments). All values were expressed as a percent of total radioactivity recovered. All filtration experiments were carried out at room temperature.

Direct Measurement of Cell Deformability

The stiffness of the isolated leukocytes was measured using the Cell Poker as described (Petersen et al., 1982). This instrument measures the deformability of the free surface of cells adherent to the bottom surface of a coverslip. The surface was indented by a glass microprobe (tip diameter $\cong$ 2 μm) attached to a flexible glass beam of known bending constant. The cell poker was programmed to execute a singular triangular waveform with a velocity of 5.2 μm/s, a total amplitude of 2.6 μm, and a total duration of 1.5 s. The degree of bending of the beam was used to calculate the force with which a cell resists indentation as a function of the depth of indentation. The initial slope (in mdyn/μm) of the ingoing limb of the force-displacement curve after the point of contact of the poker tip with the cell was computed by least squares and taken to be to the stiffness of the cell. Deformability measurements were done on cells adherent to poly-2-hydroxyethyl methacrylate [poly(HEMA)] to prevent cell spreading as previously described (Worthen et al., 1989).

Light Scattering Determinations

Right angle light scattering was determined using a Hitachi F2000 fluorescence spectrometer and was monitored at 550 nm, as described by Sklar et al. (1984). The data are expressed as percent of the initial scattering of untreated cells.

Permeabilization Procedure

Neutrophils were permeabilized by electroporation essentially as described (Grinstein and Furuya, 1988; Downey et al, 1990). Briefly, 8 × 10^6 cells were sedimented and resuspended in 1 ml of ice-cold permeabilization medium (140 mM KCl, 1 mM MgCl$_2$, 10 mM glucose, 1 mM ATP, 10
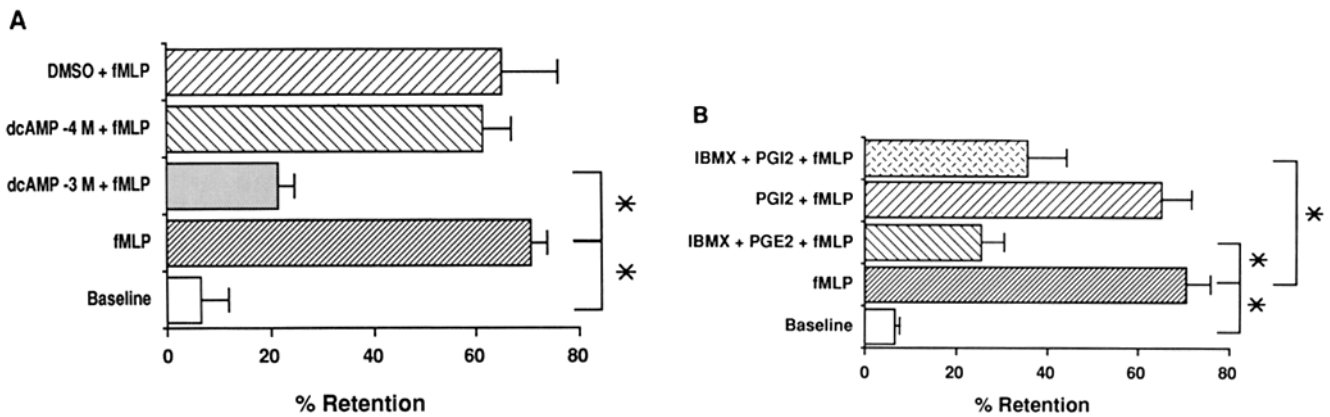
A

DMSO + fMLP

dcAMP -4 M + fMLP

dcAMP -3 M + fMLP

fMLP

Baseline

% Retention

B

IBMX + PGI2 + fMLP

PGI2 + fMLP

IBMX + PGE2 + fMLP

fMLP

Baseline

% Retention

Figure 1. (A) Effect of pretreatment with dibutyryl cAMP (dcAMP, 10^{-3} or 10^{-4} M as specified) or the vehicle control (0.1% DMSO) for 5 min at 37°C on fMLP-induced retention of neutrophils in filters of 6.5 μm pore diameter. Neutrophils in suspension were pretreated with dibutyryl cAMP or DMSO and then exposed to 10^{-8} M fMLP for 1 min before filtration. For the experiments shown the flow rate was 1 ml/min. Each value represents the mean ± SD of $n = 4$ observations. * indicates $p < 0.05$ for the comparisons indicated as determined by analysis of variance for repeated measures with correction for multiple comparisons (Sheffe). *(B)* Effect of pretreatment of neutrophils with isobutyl methyl xanthine (IBMX: 10^{-4} M for 10 min at 37°C) and then either PGE$_2$ or PGI$_2$ (10^{-6} M for 2 min at 37°C) on fMLP-induced retention of neutrophils in filters of 6.5 μm pore diameter. Also illustrated is the effect of pretreatment with 10^{-6} M PGI$_2$ alone on subsequent exposure to fMLP. Neutrophils in suspension were pretreated as indicated and then exposed to 10^{-8} M fMLP for 1 min before filtration. For the experiments shown the flow rate was 1 ml/min. Each value represents the mean ± SD of $n = 4$ observations. * indicates $p < 0.05$ for the comparisons indicated as determined by analysis of variance for repeated measures with corrections for multiple comparisons (Sheffe).

mM Hepes, pH 7.0, 1 mM EGTA, 0.193 mM CaCl$_2$ to give the final free Ca^{2+} concentration of 100 nM as calculated by the method of Fabiato and Fabiato [1979]). Aliquots of this suspension (0.8 ml) were transferred to a Gene Pulser cuvette and subjected to two discharges of 2 kV from a 25 μF capacitor using the Gene Pulser (Bio-Rad Laboratories, Richmond, CA). The cells were sedimented and resuspended in fresh ice-cold medium between pulses. Finally, the cells were equilibrated for 30 s in the indicated medium at 37°C before stimulation and measurement of actin polymerization.

Flow Cytometry

Neutrophil content of polymerized actin (F-actin) was determined by NBD-phallacidin staining of fixed and permeabilized cells according to the method of Howard and Meyer (1984), modified as previously described (Downey et al., 1989). This fluorescence method has been shown to correlate well with biochemical measurements of F-actin (Howard and Meyer, 1984; Wallace et al., 1984). The stained cells were analyzed on an Epics 5 or an Epics Profile fluorescence-activated cell sorter (Coulter, Hialeah, FL). Cells were excited with an argon laser at 488 nm and emission recorded at 520 nm with band pass and short pass filters. Gating was done on the forward angle and right angle light scatter only to exclude debris and cell clumps. A minimum of 10,000 cells was measured per condition and all values are expressed as relative fluorescence index (RFI). For experiments carried out on the Epics 5, the RFI was calculated according to the formula RFI = $2^{((b-a)/26)}$ where a = mean channel number of the control cell population, b = mean channel number of the cell population in question, and 26 = number of channels representing a doubling of fluorescence intensity. For experiments carried out on the Profile, the RFI was calculated using the ratio of the linearized mean fluorescence of the cell populations in questions, as provided by the data processing software.

Fluorescence and Confocal Microscopy

Rhodamine phalloidin and Bodipy-phallacidin were used to stain filamentous actin distribution in neutrophils as they were more resistant to bleaching than NBD-phallacidin. Neutrophils (200 μl of a suspension containing 2 × 10^6 cells) were exposed to fMLP or the appropriate amount of DMSO (0.001%), and at the end of the time period specified the cells were fixed with an equal volume of 4% phosphate-buffered paraformaldehyde (final concentration 2%) for 10 min. After fixation, the cells were allowed to settle on coverslips that were previously coated with 0.03% poly-L-lysine. After 20 min., the coverslips were gently washed with KRPD buffer and then per-

meabilized by incubation with 0.02% Tween 20 for 5 min followed by a wash with KRPD. The cells were then stained by incubation with 1.65 × 10^{-7} M rhodamine-phalloidin for 10 min at 37°C. Where indicated, 10^{-6} M propidium iodide was added with the rhodamine phalloidin to stain the nuclei. The coverslips were then mounted on slides in a 1:1 solution of PBS/glycerol with p-phenylenediamine (0.1%) as a quenching agent, and the edges sealed with nail polish. The slides were viewed using an Olympus Vanox or a BioRad 500 laser scanning confocal microscope mounted on a Nikon inverted microscope.

cAMP Assay

Intracellular levels of cAMP were measured by an enzyme-linked immuno-assay as described (Pradelles et al., 1989).

cAMP-dependent Kinase Assay

Neutrophils (2.5 × 10^6) isolated as described herein, were first treated with 10^{-4} M IBMX for 10 min at 37°C followed by exposure to PGE$_2$ (10^{-6} M) for 2 min at 37°C. FMLP (10^{-8} M) stimulation was for 5 min at 37°C. Buffer (KRPD) alone was added at appropriate times for experimental controls resulting in a final volume of 1 ml in polypropylene tubes. After stimulation, 40 μl of neutrophils (1 × 10^5) were transferred to 1.5 ml polypropylene Eppendorf micro centrifuge tubes (Brinkmann Instrument Co., Westbury, NY) containing 40 μl of 2× permeabilization buffer (137 mM NaCl, 5.4 mM KCl, 0.3 mM Na$_2$HPO$_4$, 0.4 mM K$_2$HPO$_4$, 1 mg/ml Glucose, 20 mM Hepes, 25 mM β-glycerophosphate, pH 7.2, 10 mM MgCl$_2$, 100 μM ATP, 5 mM EGTA, pH 7.0, 2.5 mM CaCl$_2$, 50 μg/ml Digitonin, 100 μM Kemptide, and 50 μCi γ-^{32}P ATP (all concentrations final in 80 μl total volume). The neutrophils were permeabilized for 10 min at 37°C and the reaction was terminated with 20 μl of 25% (wt/vol) TCA (Fisher Scientific Co.; Fair Lawn, NJ). Experimental treatments were run in duplicate and 40 μl of terminated reaction mix was spotted on 2 × 2 cm phosphocellulose strips (P-81; Whatman; Hillsboro, OR) and washed 3× in 75 mM phosphoric acid solution as described (Roskoski, 1983), followed by one acetone wash. The filter strips were allowed to air dry and radioactivity counted in a Beckman LS 5000TD beta counter measuring Cerenkov radiation in the absence of scintillant. Peptide phosphorylation results are expressed as pmol/min/mg protein.

Statistics

All data are reported as mean ± SD unless otherwise specified. Results

Table I. Effects of Prostaglandins and Phosphodiesterase Inhibitors on Neutrophil Retention in Filters

Condition	Retention (% infused)	SD
Baseline	6.4	0.9
PGE$_2$	6.1	0.9
PGE$_2$ + fMLP	64	4.5
PGI$_2$	6.2	1.0
Theophylline	6.4	1.0
Theophylline + fMLP	70	4.4
IBMX	7.1	1.7
IBMX + fMLP	66.8	4.4

Effect of prostaglandins PGE$_2$ (10^{-6} M for 2 min at 37°C) and PGI$_2$ (10^{-6} M for 2 min at 37°C), theophylline (10^{-4} M for 10 min at 37°C), and IBMX (10^{-4} M for 10 min at 37°C) on retention of neutrophils in filters with 6.5 μm pore diameter at a flow rate of 1 ml/min in the presence or absence of fMLP 10^{-8} M. Data are the mean ± SD of at least $n = 3$ experiments.

were analyzed by analysis of variance (ANOVA) with corrections for multiple comparisons (Sheffes) where appropriate. For the analysis of the data from the cell poker experiments, a nonparametric test (Wilcoxon Rank Sum) was used because the data were not normally distributed.

Results

Does Increased Intracellular cAMP Attenuate Changes in Mechanical Properties Induced by Cell Stimulation?

Cell Filtration. We first quantified the effects of the chemoattractant fMLP, on neutrophil mechanical properties as assayed using cell filtration (Chien et al., 1984). Fig. 1 *A* illustrates that exposure to fMLP at a concentration of 10^{-8} M, resulted in significant cell stiffening as assayed by retention of neutrophils in the pores of filters. Pretreatment of the neutrophils with a membrane permeant analogue of cAMP, db-cAMP, did not affect baseline (unstimulated) retention (data not shown) but significantly attenuated the fMLP-induced retention at a concentration of 10^{-3} M (Fig. 1 *A*).

To exclude nonspecific membrane effects of high (millimolar) concentrations of db-cAMP, we studied the effects of chemically unrelated agents known to increase intracellular cAMP. Treatment with either prostaglandin E$_2$ (PGE$_2$) or prostaglandin I$_2$ (PGI$_2$), agents that are known to stimulate adenylate cyclase, in doses as high at 10^{-5} M had no significant effect on cell filterability either unstimulated or after stimulation with fMLP (Table I). Next, we examined the effects of inhibitors of phosphodiesterase, theophylline, and IBMX. As shown in Table I, treatment with either of these compounds altered neither baseline nor fMLP-induced retention.

As it was possible that either the peak level or the duration of the rise in cAMP induced by either of the prostaglandins (PGs) or by the phosphodiesterase inhibitors alone was insufficient to influence cell mechanical properties, we next studied the effects of the combination of a stimulator of adenylate cyclase with an inhibitor of the phosphodiesterase. In the cells pretreated with IBMX (to inactivate phosphodiesterase) followed by treatment with either PGE$_2$ or PGI$_2$ (direct stimulators of the cyclase), fMLP-induced retention in the filters was almost totally inhibited (Fig. 1 *B*). This effect was apparent at concentrations of either PGE$_2$ or PGI$_2$ as low as 10^{-8} M (not shown).

Direct Measurement of Neutrophil Stiffness: The Cell Poker. The filtration assay may be affected by factors other than cell deformability such as cell surface adhesive properties (Worthen et al., 1989). Therefore, we felt it important to measure cell stiffness directly with the cell poker. Fig. 2 illustrates that neutrophils exposed to fMLP became significantly more stiff and that this stiffening was inhibited by pretreatment with either dibutyryl cAMP or by the combination of PGE$_2$ and IBMX. Neither PGE$_2$ nor IBMX alone prevented fMLP-induced stiffening (not shown).

Does Increased Intracellular cAMP Attenuate Changes in the Microfilaments Induced by Cell Stimulation?

Light Scattering. Polymerization of actin is associated with a change in the light scattering properties of a suspension of neutrophils which can be used as a real-time assay (see Yuli and Snyderman, 1984). Addition of fMLP to intact cells was followed almost immediately by a decrease in light scattering corresponding to an increase in actin polymerization (Fig. 3 *A*). At 37°C, the change in scattering peaked at 15–30 s and was followed by a more sustained, smaller decrease lasting several minutes. In intact cells pretreated with IBM and PGE$_2$, the magnitude of the light scattering response was diminished by ~40%. Pretreatment with either IBMX or PGE$_2$ alone had no effect on the light scattering response induced by fMLP (data not shown).

Quantification of Changes in F-actin by NBD-Phallacidin Staining and Flow Cytometry. As changes in light scattering may reflect changes in properties other than the state of microfilament assembly (Kraus and Niederman, 1990), the changes in F-actin content were measured directly using the specific F-actin binding compound NBD-phallacidin. In agreement with earlier observations, Fig. 3 *B* demonstrates that exposure to fMLP resulted in an increase in F-actin that peaked between 30 and 60 s and returned to a steady state by 5 min that was higher than baseline. This increase in F-actin was attenuated by pretreatment of the cells with IBMX and PGE$_2$ (Fig. 3 *B*).

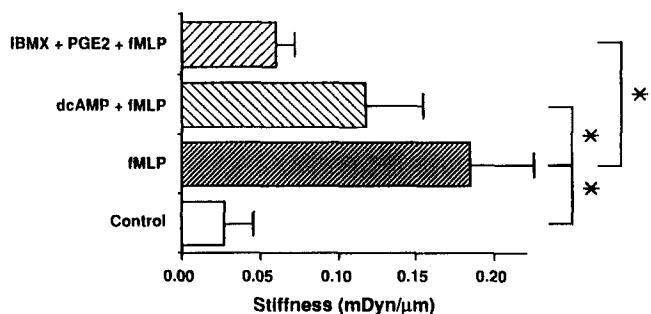To introduce cAMP (which is relatively membrane imper-

Figure 2. Direct measurement of cell stiffness using the cell poker. Effect of pretreatment of neutrophils with dcAMP (10^{-3} M for 5 min at 37°C) or the combination of IBMX (10^{-4} M for 10 min at 37°C) and PGE$_2$ (10^{-6} M for 2 min at 37°C) on cell stiffening due to subsequent exposure to 10^{-8} M fMLP. Each value represents the median ± SD of $n = 4$ observations from separate donors. * indicates $p < 0.05$ for the comparisons indicated as determined by analysis of variance for repeated measures with correction for multiple comparisons (Sheffe).
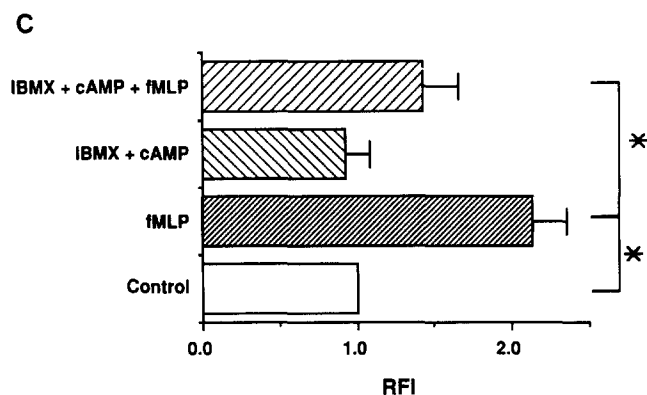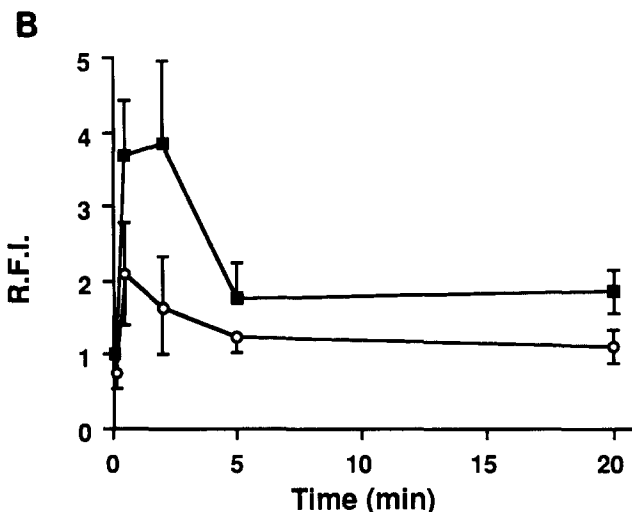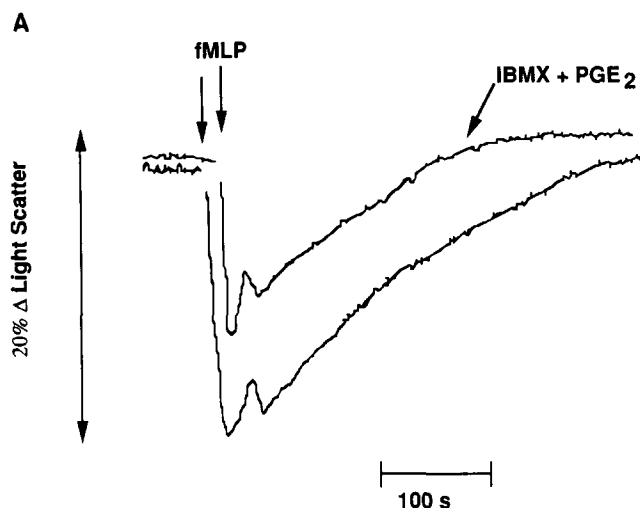
A

fMLP

IBMX + PGE$_2$

20% Δ Light Scatter

100 s

B

R.F.I.

5
4
3
2
1
0

0   5   10   15   20

Time (min)

C

IBMX + cAMP + fMLP

IBMX + cAMP

fMLP

Control

0.0   1.0   2.0

RFI

Figure 3. (*A*) Relative time courses of changes in right angle light scatter of a suspension of neutrophils in response to exposure to 10^{-8} M fMLP. The top trace represents cells that were pretreated with IBMX (10^{-4} M for 10 min at 37°C) and PGE$_2$ (10^{-6} M for 2 min at 37°C) as indicated. The arrows indicate the time at which the fMLP was added. The discontinuity of the traces represents the artifact created by opening the lid of the sample compartment of the fluorimeter which closes the shutter of the photomultiplier tube. Each trace is representative of at least four determinations using cells from different donors. (*B*) Effect of pretreatment with IBMX (10^{-4} M for 10 min at 37°C) and PGE$_2$ (10^{-6} M for 2 min at 37°C) on fMLP-induced actin assembly as assayed by staining with NBD-phallacidin of fixed and permeabilized cells at the specified time points after addition of the stimulus. Data are expressed as the RFI, relative to control neutrophils incubated in a comparable concentration of DMSO (≤0.1%). The top line (*solid squares*) represents cells exposed only to 10^{-8} M fMLP. The lower line (*open circles*) represents cells that were pretreated with IBMX (10^{-4} M for 10 min at 37°C) and PGE$_2$ (10^{-6} M for 2 min at 37°C) before exposure to 10^{-8} M fMLP. Each value represents the mean ± SD of 4 determinations. The two groups are significantly different ($p < 0.05$) as determined by analysis of variance for repeated measures with correction for multiple comparisons (Sheffe). (*C*) Measurement of actin assembly in neutrophils permeabilized by electroporation as assayed by staining with NBD-phallacidin. Where indicated, neutrophils were pretreated with IBMX (10^{-4} M for 10 min at 37°C) to inactivate phosphodiesterase followed by electroporation and the direct addition of cAMP (10^{-4} M). Where indicated, cells were subsequently exposed to 10^{-8} M fMLP for 1 min followed by fixation and staining. Also illustrated is the effect of the addition of fMLP (10^{-8} M) alone. Data are expressed as the RFI, relative to control neutrophils incubated in a comparable concentration of DMSO (≤0.01%). Each value represents the median ± SD of n = 4 observations from separate donors. * indicates $p < 0.05$ for the comparisons indicated as determined by analysis of variance for repeated measures with correction for multiple comparisons (Sheffe).

meant) directly into the cytosol we employed the technique of electroporation. This technique has been demonstrated to allow the rapid equilibration of solutes of M_r ≤ 700, while the cells retain the ability to respond to fMLP (Grinstein and Furuya, 1988). Fig. 3 *C* demonstrates that in electropermeabilized neutrophils pretreated with IBMX, the addition of cAMP significantly attenuated fMLP-induced actin assembly, thus supporting the conclusion that the effects of treatment with either dibutyryl cAMP or with IBMX and PGE$_2$ were due to an increase in intracellular cAMP.

Does Treatment with IBMX and PGE$_2$ Increase Intracellular cAMP and Activate A-Kinase?

To confirm that treatment of neutrophils with the combination of IBMX and PGE$_2$ under the conditions of the experiments outlined above in fact led to an increase in intracellular cAMP, we measured intracellular cAMP directly with an enzyme-linked immunoassay (EIA) (Pradelles et al., 1989).

As has been reported by others (Smolen et al., 1980; Rivken et al., 1975), treatment with either PGE$_2$ or IBMX resulted in a small but significant increase in intracellular cAMP (Fig. 4 *A*) that was sustained for at least 5 min (data not shown). By comparison, treatment with the combination of IBMX and PGE$_2$ resulted in a threefold increase in intracellular cAMP (Fig. 4 *A*). Further, Fig. 4 *B* illustrates that exposure of the neutrophils to fMLP resulted in a small and transient increase in intracellular cAMP and that treatment with the combination of IBMX and PGE$_2$ potentiated the fMLP-induced increase in cAMP as has been reported by others (Hatch et al., 1977; Keller et al., 1979; Jackowski and Sha'afi, 1979). Lastly, it is important to note that the elevated cAMP levels were sustained for at least 5 min (Fig. 4 *B*), corresponding to the time frame for the measurement of cell deformability either by filtration or with the cell poker.

As the primary effect of a rise in intracellular cAMP is activation of the A-kinase (Li et al., 1977), we next assayed the
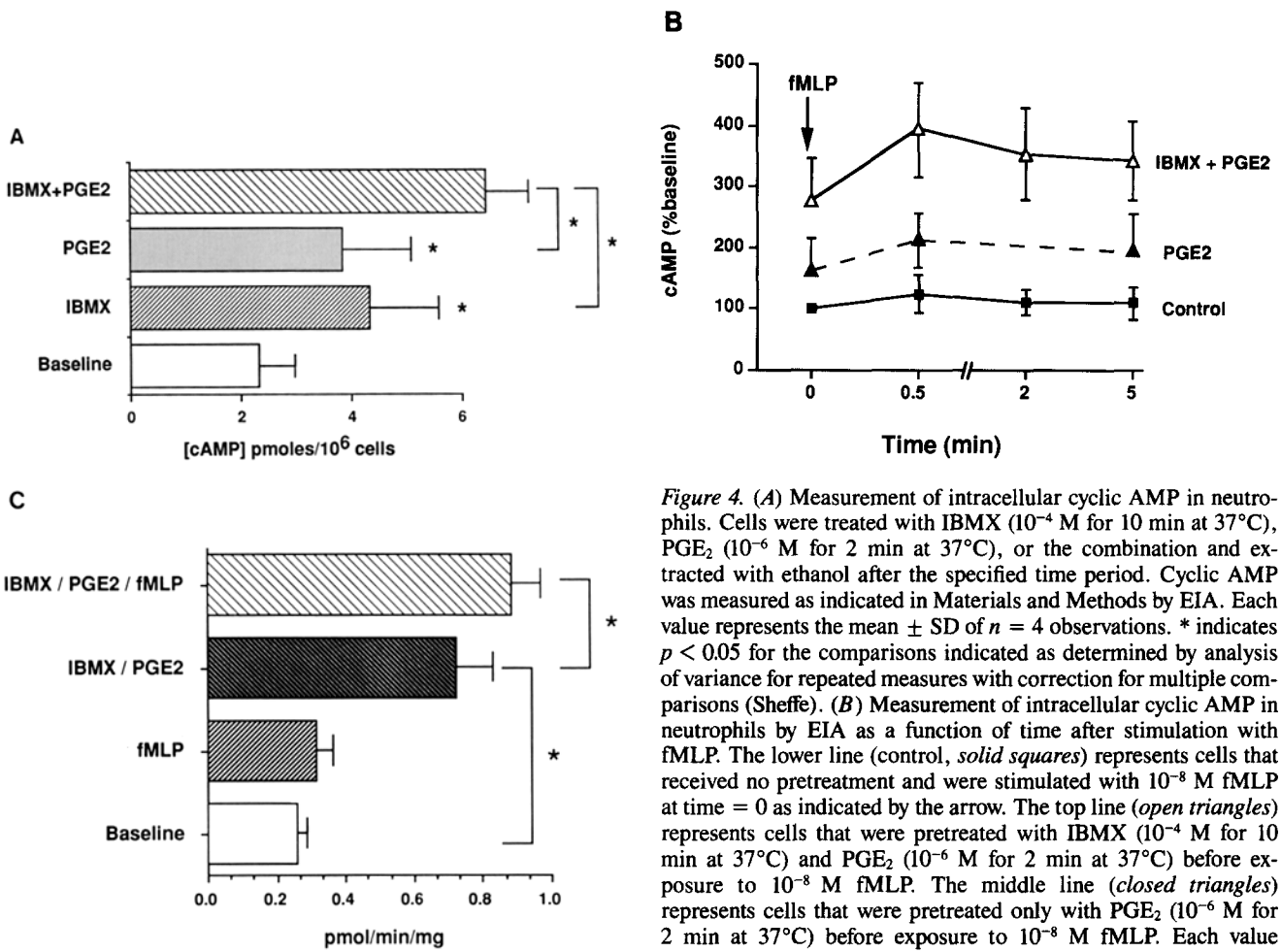
A

IBMX+PGE2

PGE2 *

IBMX *

Baseline

0   2   4   6

[cAMP] pmoles/10^6 cells

B

fMLP

500

400

cAMP (%baseline)

300 ⟶ IBMX + PGE2

200 ⟶ PGE2

100 ⟶ Control

0

0   0.5   2   5

Time (min)

C

IBMX / PGE2 / fMLP

*

IBMX / PGE2

fMLP *

Baseline

0.0   0.2   0.4   0.6   0.8   1.0

pmol/min/mg

Figure 4. (*A*) Measurement of intracellular cyclic AMP in neutrophils. Cells were treated with IBMX (10^{-4} M for 10 min at 37°C), PGE$_2$ (10^{-6} M for 2 min at 37°C), or the combination and extracted with ethanol after the specified time period. Cyclic AMP was measured as indicated in Materials and Methods by EIA. Each value represents the mean ± SD of $n = 4$ observations. * indicates $p < 0.05$ for the comparisons indicated as determined by analysis of variance for repeated measures with correction for multiple comparisons (Sheffe). (*B*) Measurement of intracellular cyclic AMP in neutrophils by EIA as a function of time after stimulation with fMLP. The lower line (control, *solid squares*) represents cells that received no pretreatment and were stimulated with 10^{-8} M fMLP at time = 0 as indicated by the arrow. The top line (*open triangles*) represents cells that were pretreated with IBMX (10^{-4} M for 10 min at 37°C) and PGE$_2$ (10^{-6} M for 2 min at 37°C) before exposure to 10^{-8} M fMLP. The middle line (*closed triangles*) represents cells that were pretreated only with PGE$_2$ (10^{-6} M for 2 min at 37°C) before exposure to 10^{-8} M fMLP. Each value represents the mean ± SD of four determinations. The three groups are significantly different ($p < 0.05$) as determined by analysis of variance for repeated measures with correction for multiple comparisons (Sheffe). (*C*) Measurement of the activity of the cyclic AMP-dependent kinase in neutrophils at 37°C. Cells were treated for 5 min with fMLP 10^{-8} M, or pretreated with IBMX (10^{-4} M for 10 min) and PGE$_2$ (10^{-6} M for 2 min), and then exposed to fMLP or buffer for an additional 5 min and the activity of the cyclic AMP-dependent kinase measured as outlined in Materials and Methods. Each value represents the mean ± SD of four determinations.

activity of this kinase under the conditions of our experiment. Fig. 4 *C* illustrates that treatment with IBMX and PGE$_2$ increased the activity of the A-kinase over threefold and that pretreatment with IBMX and PGE$_2$ potentiated the fMLP-induced activation. Treatment with IBMX or PGE$_2$ alone failed to significantly activate the kinase (data not shown). These observations mirror the results with cAMP and may explain the observation that treatment with IBMX or PGE$_2$ alone did not attenuate the fMLP-induced stiffening.

What are the Morphologic Changes in the Microfilaments in Response to Cell Stimulation and Are These Altered by an Increase in Intracellular cAMP?

To correlate alterations in cellular mechanical properties with alterations in the structure of the actin cytoskeleton, we studied alterations in the subcellular distribution of the microfilaments in response to stimulation and their modulation under conditions where intracellular cAMP was elevated. To visualize the microfilaments, fluorescence staining with rhodamine phalloidin or Bodipy-phalloidin and both

conventional (Figs. 5 and 6) and confocal fluorescence microscopy (Fig. 7) were employed. In the quiescent state, F-actin was observed to be distributed diffusely and homogeneously throughout the cytoplasm with a slight perinuclear concentration noted in some cells (Figs. 5, *a* and *b*). As has been reported by others (Wallace et al., 1984), in response to stimulation with fMLP, the F-actin rapidly redistributed to form a peripheral submembranous ring within 15-30 s (Fig. 5, *c* and *d*). This was followed by a marked change in shape of the cells with the development of pseudopodia and the colocalization of F-actin to their bases (Fig. 5, *e* and *f*). In cells pretreated with IBMX and PGE$_2$, the diffuse homogeneous distribution of F-actin characteristic of unstimulated cells was unaltered (not shown). However, in response to fMLP, the development of the peripheral ring of F-actin was markedly attenuated and aggregates of F-actin formed (Fig. 6, *a* and *b*). At later time points, in cells pretreated with IBMX and PGE$_2$ and then stimulated with fMLP, there was coalescence of the aggregates of F-actin that yielded multipolar structures (Fig. 6, *c* and *d*) as compared to the cells treated with fMLP alone where the aggregates of F-actin coalesced to only one or, less commonly, two poles.
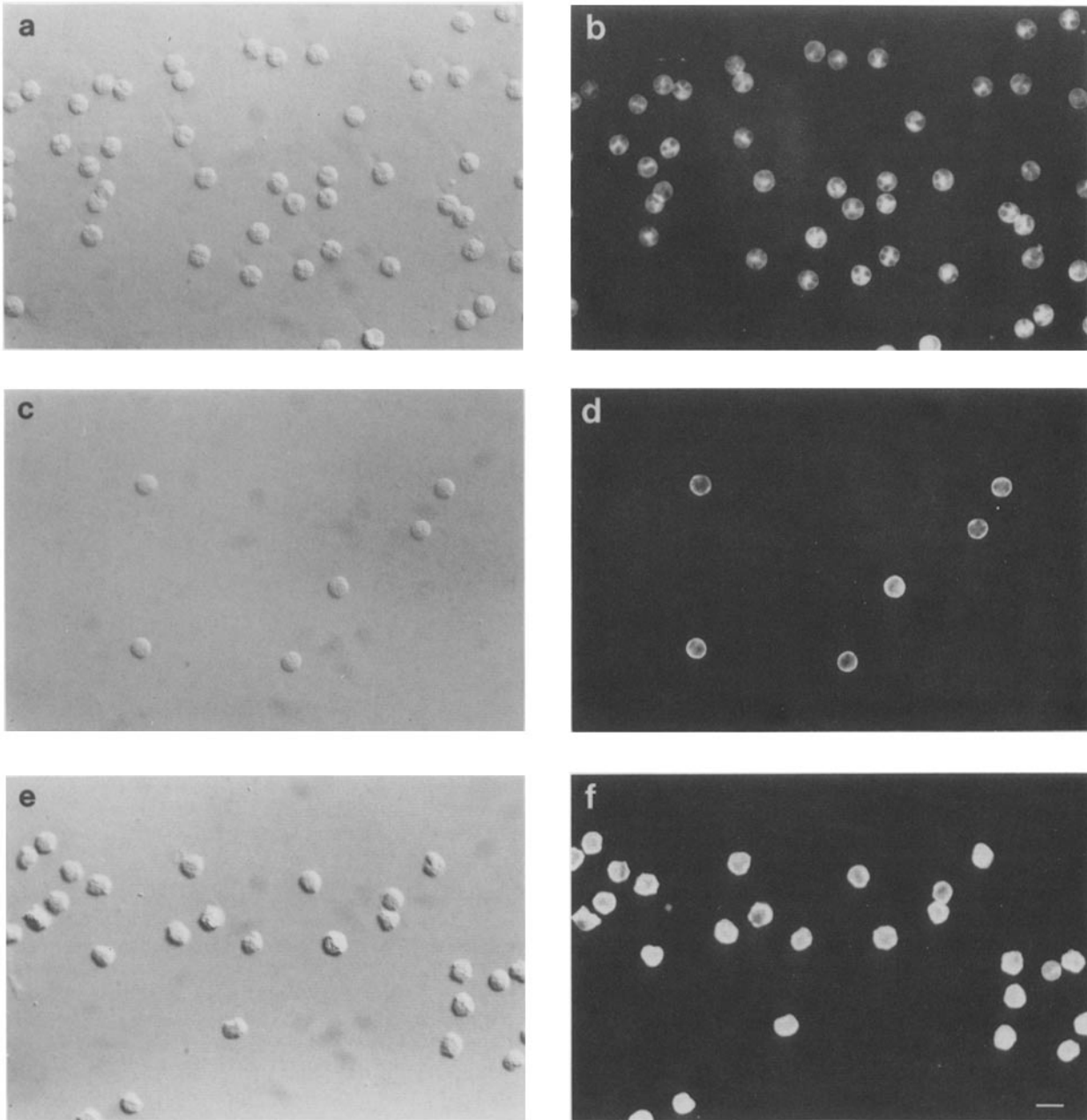
Figure 5. (*a-f*) Nomarski and fluorescence micrographs of quiescent and fMLP-stimulated neutrophils fixed and stained with rhodamine-phalloidin to identify the spatial distribution of F-actin. *a, c,* and *e* represent Nomarski images and *b, d,* and *f* represent fluorescence images. The exposure time for each fluorescence image was the same (30 s) and thus the intensity of the fluorescence is approximately proportional to the amount of F-actin. (*a* and *b*) Quiescent (control) cells. (*c-f*) Cells stimulated with 10^{-8} M fMLP for 30 s (*c* and *d*) or 60 s (*e* and *f*) at 37°C. Bar, 10 μm.

One shortfall of conventional fluorescence microscopy is that three dimensional objects are viewed in two dimensions and thus multiple structures are superimposed to yield a final composite image. To obtain optical sections through the middle of the cell and thus improve the resolution of the fluorescent imaging, scanning confocal microscopy was employed. Fig. 7 *a.* (*top frame*) shows that using confocal microscopy and Bodipy-phallacidin to stain F-actin, and propidium iodide to stain the nucleus, F-actin was distributed diffusely and homogeneously throughout the cytoplasm. In response to fMLP, F-actin rapidly redistributed to form a peripheral submembranous ring that was external to the nucleus (Fig.

7 *b, middle frame*). Finally, in the cells pretreated with IBMX and PGE$_2$ and then stimulated with fMLP, confocal microscopy confirmed that the multipolar aggregipolar aggregates of F-actin colocalized to the pseudopodia and were external to the nucleus (Fig. 7 *c, bottom frame*). Nomarski optics demonstrated that these aggregates were immediately subjacent to the plasma membrane (Fig. 6 *c*).

Discussion

It has been shown that intracellular cAMP regulates a variety of neutrophil functions including oxygen radical production
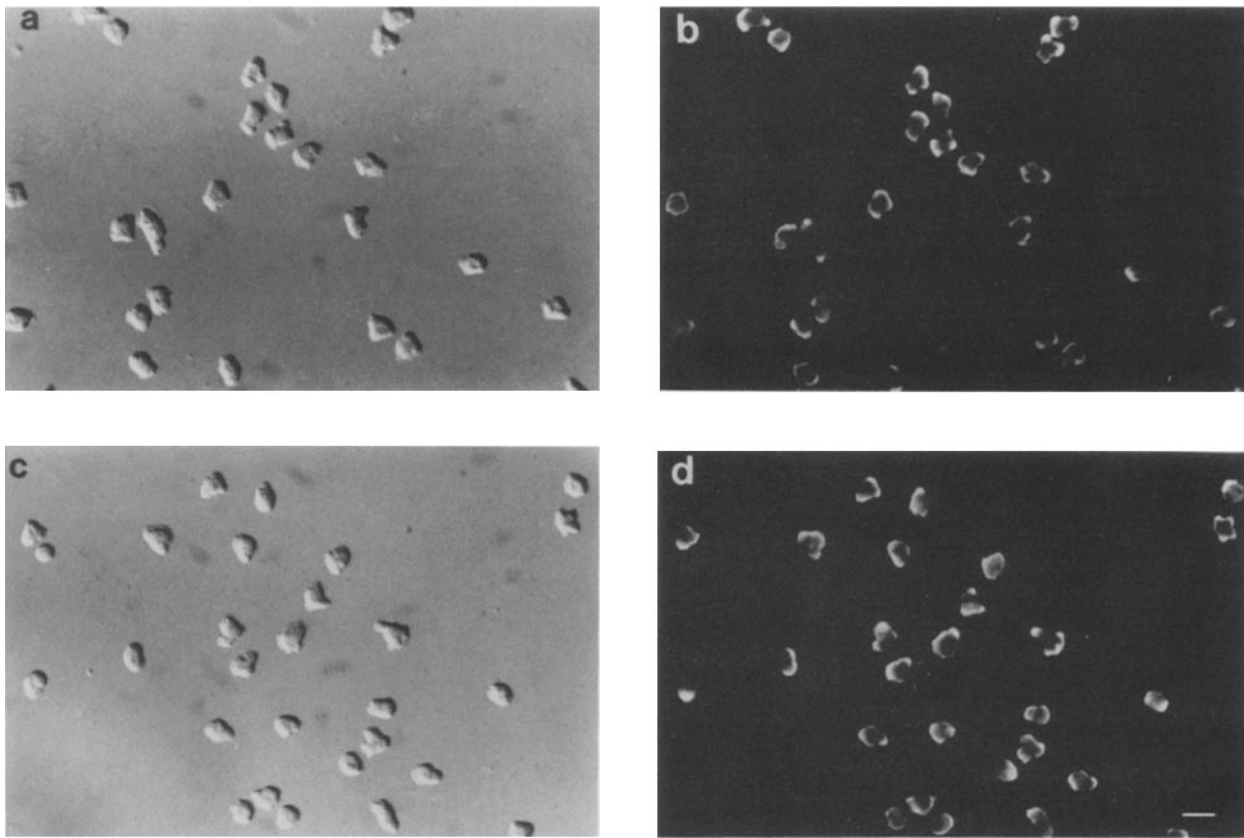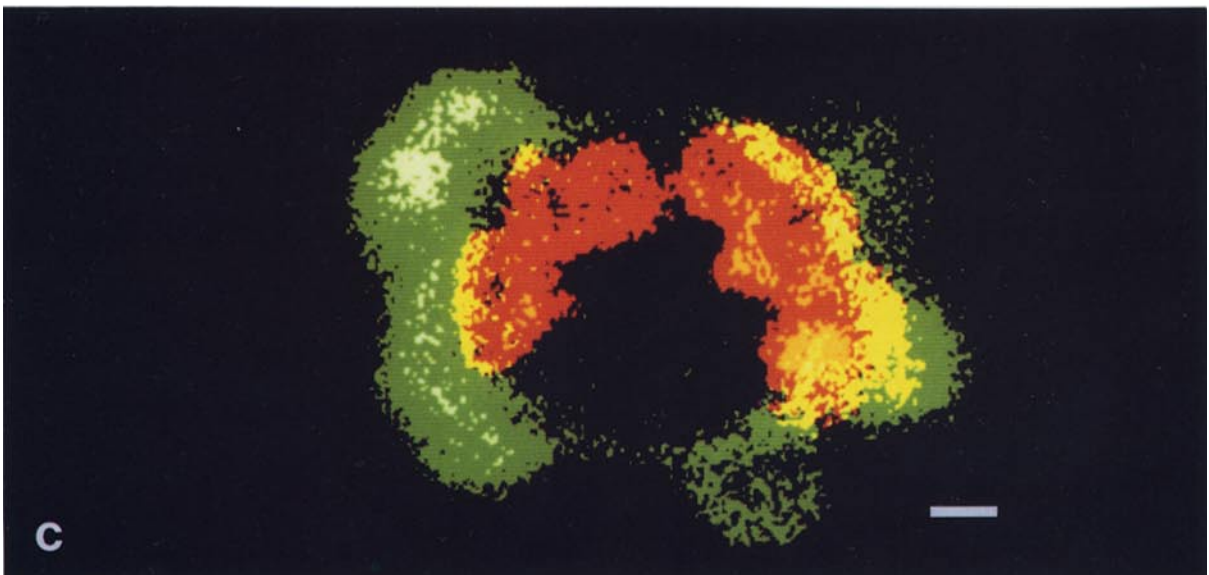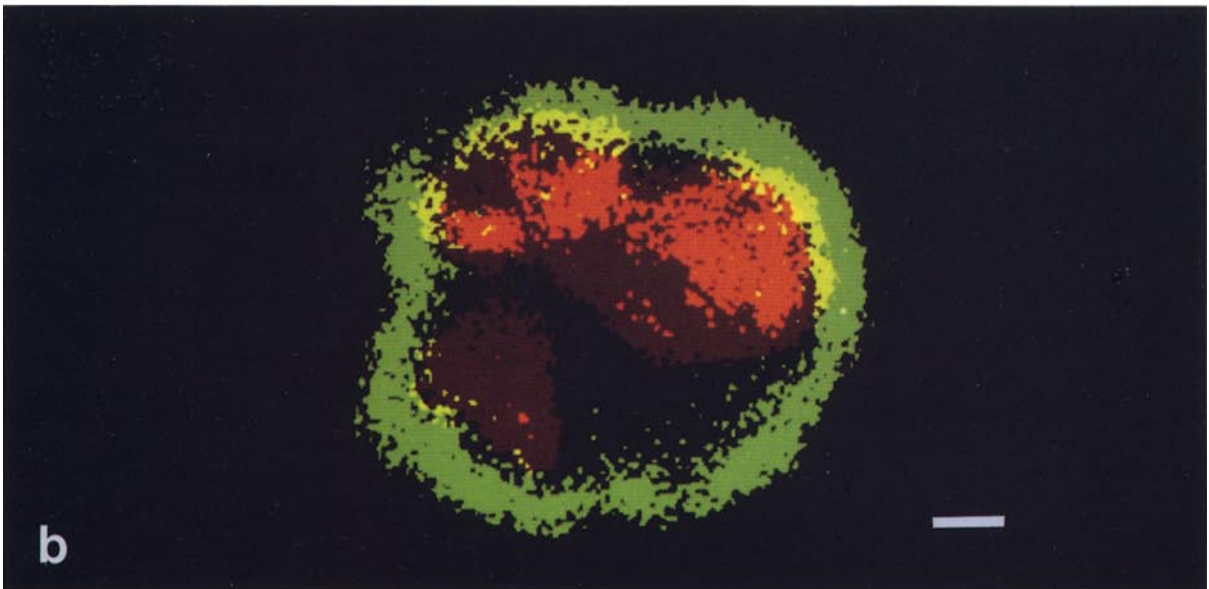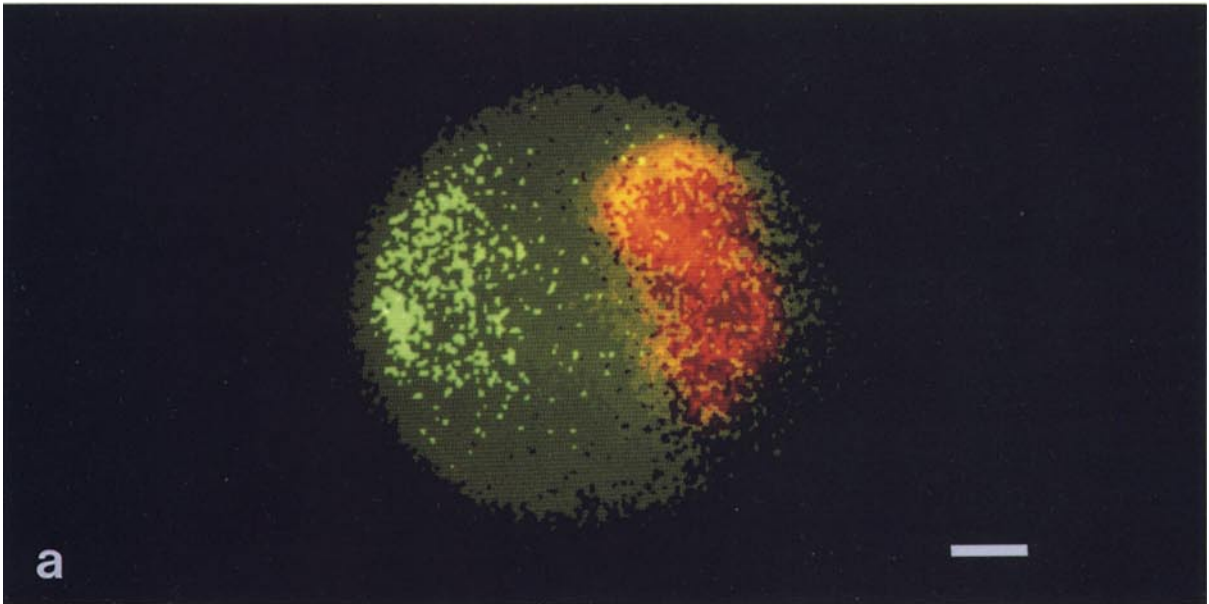
Figure 6. (a–d) Nomarski and fluorescence micrographs of neutrophils pretreated with IBMX and PGE$_2$ before exposure to fMLP. Neutrophils were fixed and stained with rhodamine-phalloidin to identify the spatial distribution of F-actin. *a* and *c* represent Nomarski images and *b* and *d* represent fluorescence images. The exposure time for each fluorescence image was the same (30 s) and thus the intensity of the fluorescence is approximately proportional to the amount of F-actin. Cells were pretreated with IBMX (10^{-4} M for 10 min at 37°C) and PGE$_2$ (10^{-6} M for 2 min at 37°C) and then stimulated with fMLP for 30 s (*a* and *b*) or 60 s (*c* and *d*). Treatment with IBMX and PGE$_2$ did not alter the actin cytoskeleton and these cells were indistinguishable from control (not shown). Bar, 10 μm.

and lysosomal enzyme release (Lad et al., 1985; Zurier et al., 1974). In this study we have demonstrated that increasing intracellular cAMP and consequent activation of the A-kinase can attenuate chemoattractant-induced cell stiffening (i.e., decreased cell deformability) as measured by both cell filtration and cellular resistance to indentation (cell poking). Increased intracellular cAMP and activation of A-kinase also attenuated the increased actin microfilament assembly and modified the alterations in the microfilament organization induced by the chemoattractant. Together, these data support the notion that an increase in the number of actin microfilaments and a change in the organization of the microfilament system contribute to the stiffening of neutrophils triggered by chemoattractant.

The physiological significance of these observations arises from the fact that neutrophils must deform repeatedly as they recirculate through the capillaries of microcirculatory beds

during their 4–6 h sojourn in the blood stream (reviewed by Hogg, 1987). In the presence of various inflammatory stimuli derived either from a local site or present within the circulation, neutrophils are sequestered in these microvessels. This microvascular trapping of neutrophils can result from a decrease in cell deformability (Worthen et al., 1989). After exposure to a variety of stimuli intracellular cAMP increases in neutrophils (Hatch et al., 1977; Keller et al., 1979; Jackowski and Sha'afi, 1979). This increase could serve to limit cytoskeletal changes that lead to stiffening and thus diminish microvascular sequestration which would be detrimental to the function of the organ. Moreover, while in the capillaries, the neutrophils are in close contact with endothelial cells which are known to produce and release prostaglandins such as PGI$_2$ (Crutchley et al., 1983). This exposure to exogenous prostaglandins (plus an additional stimulus which would substitute for IBMX) might also serve as a mechanism

Figure 7. (a–c) Photographs of scanning confocal images of neutrophils fixed and stained with Bodipy-phallacidin (illustrated in green pseudocolor) to identify the distribution of F-actin and propidium iodide (illustrated in red pseudocolor) to stain the nucleus. (Fig. 6 *a*.) Quiescent (control) cell. (Fig. 6 *b*) Cell stimulated with 10^{-8} M fMLP for 60 s. (Fig. 6 *c*) Cell pretreated with IBMX (10^{-4} M for 10 min at 37°C) and PGE$_2$ (10^{-6} M for 2 min at 37°C) and then stimulated with fMLP for 60 s. The intensity of the confocal images was adjusted using the image processing software to illustrate the distribution of the F-actin, and in these images the apparent intensity of staining is not proportional to the intensity of the actual fluorescence images. Bar, 1 μm.

↑ cAMP

G-actin

↓ polymerizability

↑ activity of A-kinase

Phosphorylation → Cytoskeletal-membrane linking protein

• MAP-2
• Vimentin

Inhibition of MLCK

Actin binding protein

Collapse of intermediate filaments or microtubules

Severing of links of actin cytoskeleton to plasma membrane

↓ MLC phosphorylation

↓ Ability to interact with microfilaments

Dissolution of crosslinked actin gel

Aggregates of F-actin

Dissociation of MLC → Destabilization and disassembly of Microfilaments

↓ Contractile ring

↓ Stiffening

MAP-2: microtubule associated protein-2
MLCK: myosin light chain kinase
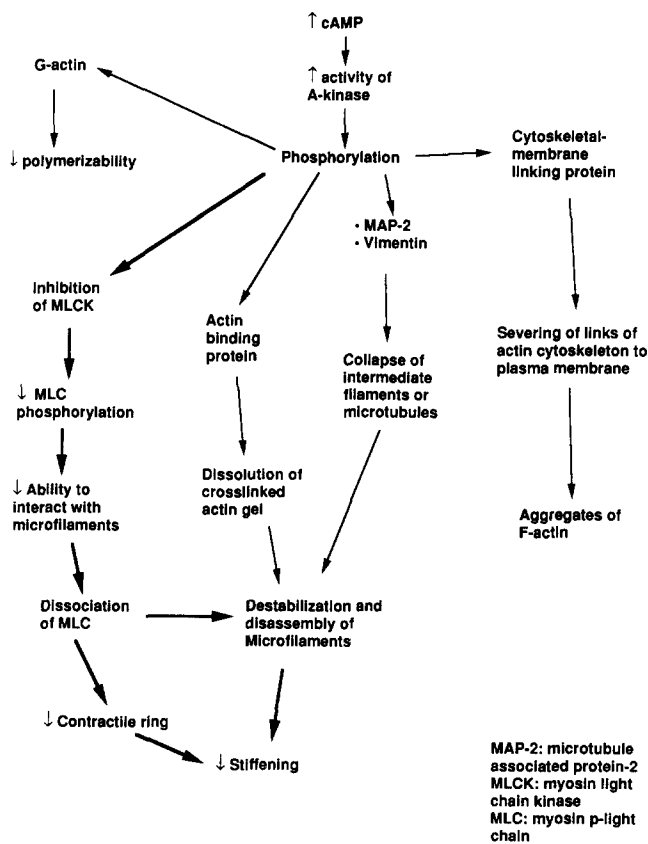MLC: myosin p-light chain

Figure 8. A model to explain the effects of activation of the A-kinase on the neutrophil cytoskeleton and summarizes possible targets of the A-kinase.

to limit microvascular sequestration of neutrophils and subsequent neutrophil-mediated tissue damage. Lastly, to emigrate from the vascular space into the interstitial spaces of tissues, neutrophils must significantly deform as they traverse intercellular junctions. Accordingly, prostaglandins produced by the endothelial cells might contribute to a limitation of cell stiffening, thus allowing the neutrophil to deform and facilitate emigration.

The magnitude of the rise in the intracellular concentration of cAMP was important in the attenuation of cell stiffening. While treatment with either PGE₂ or PGI₂ alone resulted in a twofold rise in cAMP that was sustained during the time of measurement of cell stiffness (5 min), this did not affect the actin cytoskeleton or cell stiffness. In contrast, the combination of IBMX and PGE₂ or PGI₂, which increased intracellular cAMP and the activity of the A-kinase by threefold, was sufficient to alter the actin cytoskeleton and attenuate the chemoattractant-induced cell stiffening. These data suggest that activation of the A-kinase is essential to produce the observed mechanical and morphological effects, that it is the maximum level of intracellular cAMP rather than the duration of the rise that is of paramount importance, and that the concentration of intracellular cAMP must exceed a threshold for activation of the kinase to occur. This threshold effect may be explained by the presence of multiple cAMP binding sites on the regulatory domain of the A-kinase (reviewed by Glass and Krebs, 1980).

The combination of cell poker and filtration measurements

indicates that treatment by fMLP changes the material properties of the neutrophils (i.e., their viscoelasticity) and that these changes are attenuated by increased intracellular cAMP. It has previously been shown that the viscoelasticity of several types of cells including neutrophils is dominated by the actin cytoskeleton (Lui et al., 1987; reviewed by Elson, 1988). Furthermore, actin polymers form networks of high rigidity in vitro (shear modulus 283 Pa) when compared to intermediate filaments of microtubules (shear moduli ≈34 Pa: Janmey et al., 1991). Therefore, although the phosphorylation of components of the microtubule and intermediate filaments by A-kinase has been observed (e.g., Sloboda et al., 1975; Escribano and Rozengurt, 1988), we shall focus our attention on the actin microfilament system.

Information now available is sufficient to determine the mechanisms and molecules responsible for the fMLP-induced stiffening of neutrophils or its attenuation by activation of the A-kinase. Nevertheless, there is precedent for supposing that conventional nonmuscle myosin might be involved. It has been shown that isometric tension developed by conventional myosin is responsible for stiffening of the amoeba *Dictyostelium discoideum* during the capping of crosslinked cell surface proteins (Pasternak and Elson, 1989). Similarly, it has also been shown that stiffening of *Dictyostelium* during the "cringe" response upon exposure to a chemoattractant and in the rear of cells chemotaxing up a chemoattractant gradient also depend on the presence of conventional myosin (Pasternak, C., and E. L. Elson. 1990. *J. Cell Biol.* 111:7A). In neutrophils, the effect of increasing intracellular cAMP would most plausibly be exerted by A-kinase-mediated phosphorylation of the myosin light chain kinase (MLCK). Phosphorylation of MLCK by A-kinase can reduce its interaction with calmodulin and thereby interfere with the phosphorylation of the myosin P-light chain which is essential for the activation of smooth muscle and nonmuscle myosin (reviewed by Sellers and Adelstein, 1987). It has been shown that inhibition of the activity of MLCK by A-kinase-mediated phosphorylation with a consequent loss of myosin activity can destabilize the microfilament lattice and cause it to disassemble in fibroblasts (Lamb et al., 1988). Hence, both the increase in stiffness triggered by fMLP and its inhibition by increased intracellular cAMP can be explained in terms of effects on myosin.

There are, however, many other ways in which phosphorylation of proteins by A-kinase could modulate cellular stiffness. In principle, an increase in the number or length of actin microfilaments, in their degree of crosslinking, or in the isometric tension exerted on the microfilaments all could increase the cell stiffness. We have shown that there is an increase in actin microfilaments in response to fMLP and that this increase is attenuated by increased cAMP. This latter effect could result from the phosphorylation of actin by the A-kinase. Actin is a substrate of the A-kinase in vitro, and phosphorylated actin polymerizes less readily than the unphosphorylated form (Ohta et al., 1987). Nevertheless, simply increasing the amount of polymerized actin seems insufficient to produce the measured changes in cell stiffness. (Increasing the concentration of polymer molecules is likely to have a larger effect on the viscosity than on the elasticity of a system, at least for slow deformations.) Increasing the elastic resistance of a cell to deformation could result from crosslinking actin microfilaments with a protein such as actin

binding protein (ABP; Stossel et al., 1985), which increases the tensile strength and decreases the deformability of microfilament matrices in vitro (Janmey et al., 1990). Both ABP and the structurally similar protein filamin are substrates of the A-kinase (Stossel et al., 1985; Wallach et al., 1978). Moreover, the phosphorylated form of ABP is less efficient at crosslinking microfilaments (Ausiello and Hartwig, 1985), thus contributing to destabilization of the actin cytoskeleton. More indirect effects on the microfilament system could result from disassembly of either intermediate filaments or microtubules due to phosphorylation by A-kinase of vimentin (Escribano and Rozengurt, 1988) or of microtubule-associated protein-2 (MAP-2; Sloboda et al., 1975). Since both intermediate filaments and microtubules indirectly stabilize actin microfilaments (Bershadsky and Vasiliev, 1988), activation of the A-kinase could promote disassembly of the actin cytoskeleton indirectly by these effects. Activation of the A-kinase could also result in an inhibition of membrane phospholipid metabolism (Takenawa et al., 1986) which has in turn been linked to regulation of actin assembly (reviewed by Stossel, 1989). Finally, it is possible that phosphorylation of proteins which link the actin cytoskeleton to the cell membrane (analogous to glycoprotein $1b_\beta$ in platelets: Fox et al., 1987) could disrupt this linkage, thereby promoting the formation of aggregates of F-actin seen in the cells treated with PGE_2 and IBMX (Figs. 6 and 7). A model which summarizes potential effects of the activation of the A-kinase on the neutrophil cytoskeleton is shown in Fig. 8.

In summary, we have demonstrated that agents that increase intracellular cAMP and activate the A-kinase can attenuate chemoattractant-induced neutrophil stiffening. It is likely that this response reflects a physiologically important mechanism for regulating cell stiffness and therefore, the ability of neutrophils to traverse the capillary microcirculation and to exit from capillaries into the extravascular spaces. We have shown that this effect is correlated with the inhibition of actin assembly and to an alternation in the chemoattractant-induced changes in the spatial organization of the actin cytoskeleton. The molecular mechanisms responsible for these effects on cellular mechanical properties remain to be determined. Activation of neutrophil myosin by fMLP and inhibition via phosphorylation of the myosin light chain kinase by cAMP-dependent kinase suggest one plausible mechanism. Increases in the extent of actin polymerization and of microfilament cross linking could also be contributory factors.

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