Attachment to Fibronectin or Vitronectin Makes Human Neutrophil Migration Sensitive to Alterations in Cytosolic Free Calcium Concentration

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Abstract. Transient increases in cytosolic free calcium concentration, $[Ca^{2+}]_i$, appear to be required for the migration of human neutrophils on poly-D-lysine–coated glass in the presence of dilute serum (Marks, P. W., and F. R. Maxfield. 1990. *J. Cell Biol.* 110:43–52). In contrast, no requirement for $[Ca^{2+}]_i$ transients exists when neutrophils migrate on albumin-coated glass in the absence of serum. To determine the mechanism that necessitates $[Ca^{2+}]_i$ transients on poly-D-lysine in the presence of serum, migration was examined on substrates consisting of purified adhesive glycoproteins. In the absence of external Ca^{2+}, a treatment which causes the cessation of $[Ca^{2+}]_i$ transients, migration on fibronectin (fn) and vitronectin (vn) was significantly inhibited. Migration was also inhibited in Ca^{2+}-buffered cells on these substrates, indicating that this effect was the result of an alteration of $[Ca^{2+}]_i$. In the absence of external Ca^{2+}, the inhibition of migration on fn or vn was more pronounced when soluble fn or vn was added to cells migrating on these substrates. This effect of soluble adhesive glycoprotein was specific: in the absence of external Ca^{2+}, soluble fn did not affect the migration of cells on vn, and soluble vn did not affect the migration on fn. No additional inhibition of migration was observed in Ca^{2+}-buffered cells with the addition of soluble adhesive glycoprotein. These data indicate that $[Ca^{2+}]_i$ transients are involved in continued migration of human neutrophils on fn or vn, proteins which are part of the extracellular matrix that neutrophils encounter in vivo.

NEUTROPHILS play a major role in host defense against infection by most bacteria (Smolen and Boxer, 1990). To carry out their effector functions, neutrophils must migrate from the blood to sites of infection or inflammation. Depending on the situation, this may involve adherence to and penetration of the vascular endothelium, migration through the surrounding basement membrane, and migration through the connective tissue stroma.

The adhesive glycoproteins fibronectin (fn)[1] and vitronectin (vn) are major soluble proteins in blood, each present at a concentration of a few hundred micrograms per milliliter (Grinnell and Phan, 1983; Shaffer et al., 1984). The vascular basement membrane and the connective tissue stroma also each contain their own complement of adhesive glycoproteins: the basement membrane is rich in laminin (Martin and Timpl, 1987), and the connective tissue stroma is rich in fn and vn (Hayman et al., 1983; Ruoslahti, 1988). Over the past few years it has been found that neutrophils possess specific adhesion receptors which may be involved in each of the steps of emigration from the bloodstream into the tissues outlined above. Many of these receptors have been found to belong to the integrin family of adhesion receptors (Hynes, 1987).

The integrins comprise a family of proteins involved in cell–matrix and cell–cell adhesive interactions (Hynes, 1987). They are heterodimers consisting of α and β chains. Classification of these receptors into subfamilies has been based on the identification of a number of common β chains, each of which may be associated with a variety of α chains. The ligand specificity of many of these molecules remains under investigation, and cells apparently possess mechanisms by which they can regulate the specificity of a given integrin (Lotz et al., 1990). Integrins require divalent cations for ligand binding, and at least in some cases this requirement is preferentially fulfilled by Mg^{2+} (Gailit and Ruoslahti, 1988; Hogg, 1989).

One group of integrins that has been the subject of extensive investigation is the CD11/CD18 subfamily, which is apparently restricted to leukocytes (Hogg, 1989; Nathan et al., 1989). The CD11/CD18 subfamily is comprised of three different members, all of which are present in neutrophils. Between them, members of the CD11/CD18 subfamily have been implicated in the mediation of a variety of adhesive interactions in leukocytes, including adhesion to endothelium, chemotaxis, and phagocytosis (Arnaout et al., 1983; Keizer

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1. *Abbreviations used in this paper:* $[Ca^{2+}]_i$, cytosolic free calcium concentration; fn, fibronectin; vn, vitronectin.

et al., 1987). In addition to the CD11/CD18 subfamily, neutrophils possess other integrins: an fn receptor, a vn receptor, and an apparently novel receptor with broad ligand specificity which has been described recently (Gresham et al., 1989).

In vivo, neutrophils must migrate through substrates containing adhesive glycoproteins, and it has been suggested that fn may play a role in the modulation of adherence during migration (Proctor, 1987). However, over the past two decades movement has most frequently been studied by examining cells migrating in response to chemoattractant through cellulose-based filters or on plain glass surfaces in the presence of albumin (Elferink and Deierkauf, 1985; Meshulam et al., 1986; Zigmond et al., 1988). Although such studies have contributed much to our understanding of the mechanisms of cell motility, they have excluded from consideration potentially important specific neutrophil–substrate interactions.

Previously we have observed that transient increases in cytosolic free calcium concentration ($[Ca^{2+}]_i$) appear to be required for the migration of human neutrophils on poly-D-lysine in the presence of dilute serum (Marks and Maxfield, 1990). This requirement stems from an inability of the cells to detach from where they are anchored to the substrate rather than an inability of the cells to put forth pseudopodia. In the absence of external Ca^{2+} and the presence of EGTA, the basal $[Ca^{2+}]_i$ dropped from ∼150 to ∼50 nM, and both the $[Ca^{2+}]_i$ transients and migration ceased (Marks and Maxfield, 1990). This effect was reversible. Cells unable to migrate in the absence of external Ca^{2+} were able to do so after the addition of external Ca^{2+} to the medium. $[Ca^{2+}]_i$-buffering cells with high intracellular concentrations of quin-2 or BAPTA damped or eliminated $[Ca^{2+}]_i$ transients, and basal $[Ca^{2+}]_i$ was 100 ± 30 nM (Marks and Maxfield, 1990). When $[Ca^{2+}]_i$ buffered, the number and velocity of cells migrating in the presence of Ca^{2+} was significantly decreased. This suggested that the suppression of the $[Ca^{2+}]_i$ transients caused the inhibition of migration when external Ca^{2+} was removed.

We had observed that neutrophils were capable of migration on poly-D-lysine in response to the chemoattractant N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) in the absence of such transients when albumin rather than serum was present. We therefore hypothesized that the adhesive glycoproteins present in serum, adhering to the poly-D-lysine–coated surface of the chambers, were responsible for the apparent requirement of $[Ca^{2+}]_i$ transients for migration. In this paper, we tested this hypothesis by examining the random migration (chemokinesis) of neutrophils in response to the chemoattractant present in serum on glass coated with different substrates. We found that migration on fn- and vn-coated glass was completely inhibited under conditions that suppressed $[Ca^{2+}]_i$ transients. We then investigated whether the same inhibition could be reproduced in the absence of serum. To do this, the Ca^{2+} dependence of chemokinesis in response to fMLP on purified fn and vn substrates in the absence of soluble protein was examined. Under these conditions, a significant but incomplete reduction in the percentage of cells that migrate was observed when $[Ca^{2+}]_i$ transients were damped or eliminated. We then tested whether adding purified soluble components from serum would lead to a more complete inhibition of migration

in the absence of $[Ca^{2+}]_i$ transients. We found that soluble fn or vn could further decrease the percentage of migrating cells in the absence of $[Ca^{2+}]_i$ transients on fn- or vn-coated glass, respectively, essentially reproducing the inhibition of migration observed in the absence of external Ca^{2+} on poly-D-lysine–coated glass in the presence of serum.

Materials and Methods

Cell and Serum Preparation

Human whole blood was obtained from healthy volunteers. Blood was collected using a syringe and was immediately transferred to a sodium-heparin-containing polypropylene tube. When necessary, a portion of blood was placed into a siliconized glass tube containing no additive for use in preparing serum. This tube was centrifuged at room temperature within 15 min of obtaining blood to obtain fresh serum, which was then kept on ice until use.

Polymorphonuclear leukocytes were isolated by single-step separation over a ficoll-hypaque solution of density 1.114 (Mono-Poly Resolving Medium; Flow Laboratories, Inc., McLean, VA) according to the instructions of the manufacturer. This was followed by 30–40 s hypotonic lysis of contaminating erythrocytes. Cells were then washed and resuspended in PBS, followed by a wash with incubation medium (150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM Hepes, 10 mM glucose, pH 7.4). After cells were isolated, they were kept in incubation medium on ice until use. The final cell preparation consisted of >97% polymorphonuclear leukocytes. Only neutrophils in the polymorphonuclear leukocyte cell fraction were examined, and these cells were distinguished under the microscope from the small number of eosinophils and basophils present by their smaller, less conspicuous granules.

Ca^{2+} Buffering and Depletion of Neutrophils

For Ca^{2+}-buffering experiments, neutrophils were loaded with a 50 μM quin-2/acetoxymethyl ester (Molecular Probes Inc., Junction City, OR) solution. The solution was prepared as follows: 3 μl of a 50 mM quin-2 stock solution in anhydrous DMSO was mixed with 4 μl of a 25% wt/vol solution of Pluronic-F127 (Molecular Probes Inc.) in DMSO. This was mixed with 60 μl of heat-inactivated FCS, and then 2.9 ml of incubation medium was added with mixing. 1–5 × 10^6 cells were then tumbled for 30 min in 1 ml of this solution.

After loading, cells were rinsed twice in incubation medium and resuspended in the same. For experiments in which Ca^{2+} depletion of neutrophils with quin-2 was performed, the same loading procedure was used, except that Ca^{2+}-free incubation medium containing 1 mM EGTA was added to the loading mixture instead of the usual incubation medium. The cells were then washed and resuspended in Ca^{2+}-free incubation medium containing 1 mM EGTA. Whenever quin-2–buffering experiments were performed, control cells were mock loaded with the same loading mixture described above without the quin-2.

The concentration of quin-2 loaded into the cytosol was determined using a homogenization procedure which has been described previously (Ratan et al., 1986). The cytosolic quin-2 concentration determined by this method was ∼0.8 mM. That acetoxymethylester loading the cells to high concentrations was not in and of itself toxic was checked by loading cells to 3.9 mM with quene-1/acetoxymethyl ester, a pH-sensitive relative of quin-2 (Rogers et al., 1983). No detectable differences in the migration of mock- and quene-1-loaded cells were observed.

Experimental Chambers

For studying the migration of neutrophils on glass, poly-D-lysine, or gelatin, the experimental chamber consisted of a 35-mm tissue culture dish which had a No. 1 glass coverslip attached beneath a 12-mm hole which had been punched in the bottom. For studies on albumin, fn, and vn, the same type of chamber was used except that a 7-mm hole was punched in the bottom. In both cases, the glass coverslips which were attached to the dishes were first cleaned using Nochromix (Godax Laboratories Inc., New York, NY) in concentrated sulfuric acid according to the directions of the manufacturer.

The glass surface of the chambers was coated with different substances. For some experiments the glass surface of the chambers was coated with

100 μg/ml poly-D-lysine (150,000–300,000 mol wt; Sigma Chemical Co., St. Louis, MO) in water or 0.1% gelatin (Porcine, 300 bloom; Sigma Chemical Co.) in PBS for 1 h at room temperature. After coating the glass, the surface was washed thoroughly with water (for poly-D-lysine) or PBS (for gelatin) and allowed to dry at room temperature.

For other experiments the glass surface was coated with 1 mg/ml bovine albumin (essentially globulin and fatty acid free; Sigma Chemical Co.) for 1 h. After coating of the glass, the surface was rinsed four times with PBS and covered with PBS until use.

Initially, a variable inhibition of migration in the absence of external calcium on albumin-coated glass was found. Since it seemed likely that globulin- and fatty acid–free albumin might contain contaminating adhesive glycoproteins, it was further purified using a Sephacryl S-300 column (Pharmacia Chemical Co., Uppsala, Sweden). The fractions of highest purity, determined by SDS-PAGE and Coomassie blue staining, were pooled and concentrated to 20 mg/ml in a microconcentrator (Amicon Corp., Danvers, MA). This was followed by glass bead affinity chromatography to remove known adhesive glycoproteins (Barnes et al., 1984). This purified albumin was used in all experiments examining migration.

In other experiments the glass surface was coated with various concentrations of fn (New York Blood Center, New York, NY) or vn (Telios Pharmaceuticals, San Diego, CA) in PBS. Unless otherwise indicated, fn was used at a concentration of 100 μg/ml, and vn was used at a concentration of 10 μg/ml. Some experiments were performed by first coating the glass with 1 mg/ml fn or 10 μg/ml vn for 1 h, rinsing the dishes with PBS, and then coating the surface with 1 mg/ml albumin. fn was used at a higher concentration because albumin has been demonstrated to displace fn attached to a surface (Grinnell, 1986). Using lower concentrations of fn before albumin coating produced greater variability in results. In experiments where soluble fn or vn was used, fn or vn was allowed to coat the glass surface until use, at which time the surface was rinsed as described above.

The homogeneity of the fn and vn coatings of the glass surfaces was confirmed with a monoclonal anti-fn antibody or a polyclonal anti-vn antibody (Telios Pharmaceuticals). Fluorescently labeled second antibodies were then used to visualize the distribution of either the fn/antibody complexes or the vn/antibody complexes. Fluorescence microscopy indicated that both the fn and vn coatings were uniform.

Microspectrofluorometry was used to compare the effects of different coating protocols upon the relative amounts of fn or vn on the surface. We were able to detect fn on glass surfaces coated with between 10 μg/ml and 1 mg/ml fn. The intensity of the fluorescence varied with the concentration of the fn coating. vn was detectable with varying intensities on glass surfaces coated with between 1 and 10 μg/ml vn. Serum-coated glass did not show any detectable fn or vn by this assay. If the glass was previously coated with 100 μg/ml poly-D-lysine, the addition of serum resulted in little fn fluorescence but vn was detected; the fluorescence intensity was equivalent to having coated the glass with between 1 and 10 μg/ml vn. Glass coated with 0.1% gelatin and then exposed to serum showed a small amount of vn and a large amount of fn. The intensity of the fn fluorescence was equivalent to the fluorescence signal obtained by coating the glass with 100 μg/ml fn.

Chemokinesis Assays

Two microscopes were used: a microscope equipped with phase-contrast optics (Diavert; E. Leitz Inc., Wetzlar, Germany), or a microscope equipped with Nomarski differential interference contrast optics (Axiovert; Zeiss, Oberkochen, Germany). A 25× objective was used on both microscopes. Video microscopy was used to follow migration of cells in the chambers on the microscope stage at 37°C. Images of cells were recorded using a video camera (Newvicon; Dage-MTI Inc., Wabash, MI) onto an optical memory disk recorder (Panasonic; Matsushita Electronics Corp., Osaka, Japan) for later analysis.

To examine chemokinesis, polymorphonuclear leukocytes (10^3–10^4 cells) were plated onto the coverslip area of the experimental chamber and allowed to attach at 37°C for 5 min. For experiments examining chemokinesis in response to the chemoattractant present in human serum (Jungi, 1977), a 10% solution of freshly obtained human serum in incubation medium or Ca^{2+}-free incubation medium containing 5 mM EGTA was then added. After 5 min, single frames were recorded every 10 s for a 3–5-min period. Since similar percentages of cells were observed migrating for at least 20 min after the addition of chemoattractant, three different fields were routinely examined in each chamber. The free Ca^{2+} concentration in human plasma is ~1.3 mM (Aurbach et al., 1985). The free Mg^{2+} concentration in the Ca^{2+}-free incubation medium containing 5 mM EGTA was ~0.7 mM. Free Mg^{2+} concentrations were calculated using the published

stability constants for the metal ion binding of EGTA of Martell and Smith (1974).

Experiments examining chemokinesis in response to 10 nM N-fMLP on purified protein substrates were performed similarly, except that after plating the cells for 5 min, the attached cells were allowed to incubate for 5 min in incubation medium or Ca^{2+}-free incubation medium containing 1 mM EGTA. The extra incubation time was used to insure that $[Ca^{2+}]_i$ transients in cells in Ca^{2+}-free medium had ceased before recordings were made (Marks and Maxfield, 1990). After this incubation period, fMLP was added to the cells at 10-nM final concentration. After 5 min further incubation, the chamber was placed on the microscope stage, and recordings of migration were made as described above. The free Mg^{2+} concentration in the Ca^{2+}-free incubation medium containing 1 mM EGTA was ~0.9 mM.

In experiments where soluble fn or vn was used, these proteins were added to the incubation medium at the same time as the 10 nM fMLP. Soluble fn was used at 100 μg/ml and soluble vn was used at 10 μg/ml.

Analysis of Motility

For counting the number of migrating cells in a given field, outlines were made of the cells on the video screen using a marker, and the optical memory disk recorder was stepped through 3 min of successive frames. Migrating cells were defined as those in which both the leading edge and tail of the cell were observed to move at least 7 μm from their initial position during this time. For determining the velocity of migrating cells, images were digitized onto an image processor (model IP8400; Vicom-Gould, Fremont, CA). The cursor was positioned at the centroid of a cell and its position was recorded in successive frames covering a 3-min period. The values obtained were then used to calculate the distance and velocity of migration.

Many experiments were repeated over several days. To control for day-to-day variations in cell migration, the percent migration for each condition is compared with a matched control on the same day. The control was a $+Ca^{2+}$ condition matched in substrate, chemoattractant, and soluble factors. This allowed the statistical analysis of the effects of the various $-Ca^{2+}$ treatments while minimizing interference from the day-to-day variation in cell motility.

Measurement of [Ca²⁺]

$[Ca^{2+}]_i$ was measured in neutrophils using fura-2 and microspectrofluorometry as previously described (Marks et al., 1988; Marks and Maxfield, 1990).

Results

To determine which proteins in serum were responsible for the $[Ca^{2+}]_i$ effects on migration, the Ca^{2+} dependence of migration was investigated under a number of conditions on a variety of substrates. Removal of external Ca^{2+} was used for screening the Ca^{2+} dependence of migration on different substrates because of its ability to eliminate the occurrence of $[Ca^{2+}]_i$ transients (Marks and Maxfield, 1990). To further investigate whether effects observed were the result of altering $[Ca^{2+}]_i$, Ca^{2+}-buffered cells were also examined.

External Ca²⁺ Is Required for Migration in the Presence of Dilute Serum on Gelatin, fn, and vn

We first investigated whether coatings of the glass surface other than poly-D-lysine could result in a similar Ca^{2+} dependence of migration. A chemokinesis assay was used to examine the migration of neutrophils in the presence of 10% human serum on a number of substrates. The chemoattractant activity of human serum has been described (Jungi, 1977).

Simply coating the glass surface with 20% serum and adding 10% serum in incubation medium to stimulate chemokinesis resulted in migration that was essentially independent of the presence or absence of external Ca^{2+} (Table I). How-

Table I. Ca^{2+} Dependence of Chemokinesis on Various Substrates in the Presence of Serum

Substrate	Cells migrating	
	$+Ca^{2+}$	$-Ca^{2+}$
	%	
Poly-D-lysine (100 μg/ml)	25	0
Serum (20%)	94	80
Gelatin (0.1%)	80	0
fn (100 μg/ml)	50	0
vn (10 μg/ml)	34	0

Chemokinesis in the presence of 10% human serum was observed on the different substrates at 37°C. Cells were allowed 5 min to attach to the substrate, and then 10% serum in either incubation medium or Ca^{2+}-free incubation medium containing 5 mM EGTA was added. After 5 min, images were recorded onto videodisc every 10 s for 3 min. Cells were scored as migrating when both leading edge and tail were observed to move at least 7 μm from their initial position during this time. Data are from experiments on each substrate performed on different days. At least 20 cells were examined for each condition.

ever, as shown in Table I, when the glass surface was first coated with 0.1% gelatin, migration in response to dilute serum was absolutely dependent on the presence of external Ca^{2+}. In addition, a greater percentage of cells migrated on gelatin than poly-D-lysine.

That poly-D-lysine or gelatin coating of the glass was necessary in order to observe migration that was Ca^{2+} dependent suggested that these surfaces might be acting to aid in the attachment of proteins present in serum to the substratum. Poly-L-lysine has been shown to aid in the attachment of fn to a glass surface (Klebe et al., 1981), and the ability of gelatin to bind fn has been well characterized (Vartio, 1983; Ruoslahti, 1988). Such help is probably necessary for the fn present in serum to adhere to the substratum because the amount of albumin present in serum is capable of significantly diminishing its attachment to plain glass (Grinnell, 1986). This might also be the case for vn. In fact, using immunofluorescence we found no detectable fn or vn on plain glass when it was treated with dilute serum. However, when gelatin or poly-D-lysine-coated glass surfaces were treated with dilute human serum, we were able to detect fn adherent to the gelatin-coated surface, and vn adherent to both the gelatin and poly-D-lysine-coated surfaces (data not shown).

Figure 1. $[Ca^{2+}]_i$ changes measured during migration on an fn substrate. The glass surface was treated with 100 μg/ml fn before the plating of neutrophils. Cells were plated in incubation medium and allowed 5 min to adhere to the substrate. The cells were then washed with incubation medium or with Ca^{2+}-free incubation medium containing 1 mM EGTA. After 5-min incubation in medium alone, 10 nM fMLP in incubation medium or in Ca^{2+}-free incubation medium containing 1 mM EGTA was added to stimulate chemokinesis. After 4-5 min in the presence of fMLP, measurement of $[Ca^{2+}]_i$ was performed. (*a*) Cell migrating in the presence of external Ca^{2+}. (*b*) Cell migrating in the absence of external Ca^{2+}. Note that $[Ca^{2+}]_i$ transients, defined here as increases of 50 nM or greater (Marks and Maxfield, 1990), occur in the presence of external Ca^{2+}, whereas no such increases occur in its absence. The transients observed in the presence of external Ca^{2+} on fn are similar to those observed on poly-D-lysine in the presence of serum (Marks and Maxfield, 1990).

Table II. Chemokinesis of Neutrophils on Various Substrates in Response to the Chemoattractant fMLP in the Absence of Soluble Protein

Substrate	Migrating cells
	%
Glass	14
Albumin (globulin and fatty acid free, *1 mg/ml*)	47
Albumin (S-300 and glass bead purified, *1 mg/ml*)	44
Gelatin (*0.1%*)	23
fn (*100 μg/ml*)	51
vn (*10 μg/ml*)	50

Chemokinesis was observed on the various substrates in response to 10^{-8} M fMLP in incubation medium on the microscope stage at 37°C. Single frames were recorded onto videodisc every 10 s. Cells were scored as migrating when both leading edge and tail were observed to move at least 7 μm from their initial position during a 3-min period. Data are from multiple experiments performed on different days. A minimum of 40 cells were examined for each condition.
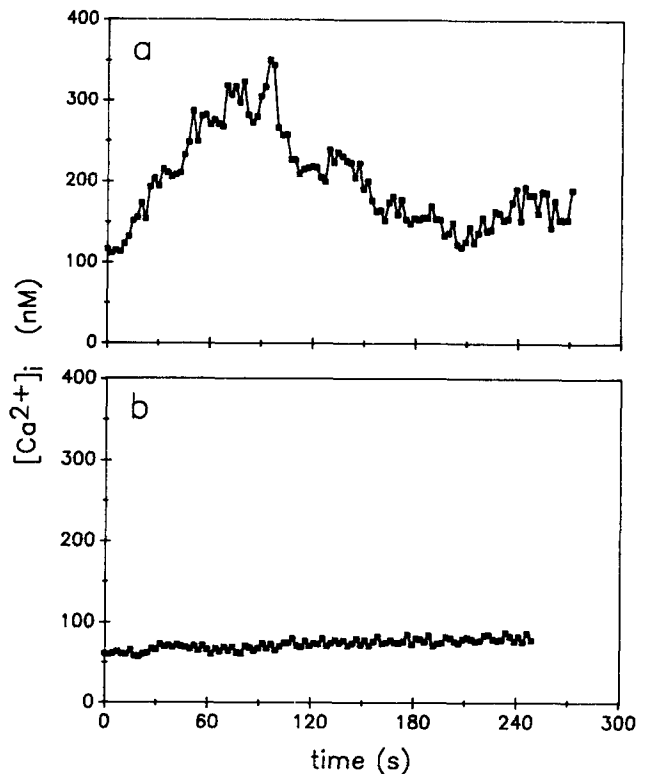
The inhibition of migration in the absence of external Ca^{2+} could be reproduced when glass was first coated with either purified fn or vn (Table I). Similar results were obtained on fn and vn when 10 nM fMLP was added to the serum as an additional chemokinetic stimulus. The results presented in Table I indicate that adherent fn and vn are involved in the Ca^{2+} dependence of migration on poly-D-lysine in the presence of serum.

Migration on Purified Substrates

Having observed that migration on fn- or vn-coated glass in the presence of serum was Ca^{2+} dependent, we wanted to determine whether this effect could be reproduced in the absence of serum on these substrates. We characterized the Ca^{2+} dependence of migration in the absence of serum on a number of substrates, including fn and vn. The capability of a number of substrates to support migration was first tested.
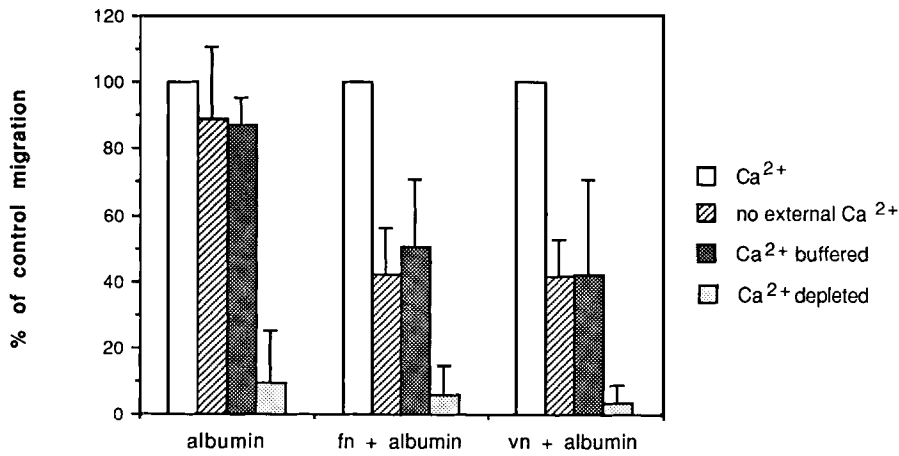
120
100
80
60
40
20
0

% of control migration

albumin   fn + albumin   vn + albumin

□ Ca^{2+}
▨ no external Ca $^{2+}$
■ Ca^{2+} buffered
▨ Ca^{2+} depleted

Figure 2. Migration on purified substrates. Chemokinesis in response to 10 nM fMLP was observed on glass coated with 1 mg/ml albumin, 1 mg/ml albumin + 1 mg/ml fn, or 1 mg/ml albumin + 10 µg/ml vn. Coating the glass with the substrates and the chemokinesis assays were performed as described in Materials and Methods. The cells were scored as migrating when both leading edge and tail were observed to move at least 7 µm from their initial position. In the absence of fMLP, <1% of the cells moved on these substrates. After the plating and stimulation of the cells with 10 nM fMLP, three fields of about 15 cells were examined on each dish for a period of 3 min. Each condition was examined in one to three trials (separate dishes) and repeated in three to eight experiments done on different days. A minimum of 97 cells were analyzed for each condition. The data is presented as the mean percent of control movement and the error bars indicate the SD between experiments done on different days. Control migration is defined as the fMLP-stimulated migration on the specified substrate in the presence of calcium. Migration in the absence of external Ca^{2+} or in Ca^{2+}-buffered cells or in the Ca^{2+}-depleted cells was measured and compared with a matched Ca^{2+} control done on the same day. The observed inhibitions of migration in the absence of external Ca^{2+} and in Ca^{2+}-buffered cells on fn + albumin and on vn + albumin substrates were at least significant at the level $p < 0.05$ in t test when compared to matched controls. Only the Ca^{2+}-depleted cells were significantly different from control on the albumin substrate. After fMLP stimulation, 44% of the cells were observed to migrate on albumin, while 58% of the cell migrated on fn + albumin, and 55% of the cells migrated on vn + albumin.

Table II shows that neutrophils are capable of migrating on a variety of substrates in response to 10 nM fMLP in the absence of soluble protein. fn- and vn-coated glass proved to be good substrates for migration. As observed on poly-D-lysine in the presence of serum (Marks and Maxfield, 1990), using fura-2 and microspectrofluorometry, we observed that Ca^{2+} transients occurred in cells migrating in response to fMLP on fn- and vn-coated glass when Ca^{2+} was present (Fig. 1 *a*), and that detectable transients did not occur after 10 min in the absence of external Ca^{2+} and the presence of 1 mM EGTA (Fig. 1 *b*).

In preliminary experiments, we found that migration on albumin-coated glass was inhibited to roughly 50% of control migration in the absence of external Ca^{2+}, although the extent of the inhibition was variable. Since essentially globulin-free albumin still contains a variety of contaminants including adhesive glycoproteins, albumin which was essentially globulin and fatty acid free was further purified as described in the Materials and Methods.

The migration of neutrophils on glass coated with this purified albumin is essentially independent of the presence or absence of external Ca^{2+} (Fig. 2). Additionally, quin-2–buffering cells in the presence of external Ca^{2+} had no discernible effect on the fraction of cells migrating. No statistically significant differences were observed in the velocities of cells migrating on albumin under these three conditions (Table III). However, in cells examined in the absence of external Ca^{2+} which had been Ca^{2+} depleted to a $[Ca^{2+}]_i$ of <10 nM by loading with quin-2 in the absence of external Ca^{2+} (Marks and Maxfield, 1990), chemokinesis was found to be significantly inhibited. Only 3% of the Ca^{2+}-depleted cells were observed to migrate in comparison to 44% of control cells (Fig. 2). When compared with control cells, Ca^{2+}-depleted cells appeared to be highly adherent to the substrate. Cells that did not move appeared anchored in place to the substrate, yet were capable of repeatedly extending small pseudopodia.

In the next series of experiments we wanted to determine

Table III. Velocities of Neutrophils Migrating under Different Conditions of Ca^{2+}

Substrate	$+Ca^{2+}$	Velocity Ca^{2+} buffered	$-Ca^{2+}$
		µm/min	
Albumin	5.4 ± 0.37 (15)	4.6 ± 0.40 (15)	5.8 ± 0.54 (15)
fn	5.4 ± 0.31 (19)	4.9 ± 0.39 (21)	5.6 ± 0.26 (26)
fn, with soluble fn	7.6 ± 0.35 (67)	6.2 ± 0.33 (39)	4.5 ± 0.27 (21)

Analysis of migration was performed as described in Materials and Methods on recordings made of cells undergoing chemokinesis in response to 10 nM fMLP. Cells were Ca^{2+} buffered with quin-2 as described in Materials and Methods. Purified albumin (1 mg/ml) or fn (100 µg/ml) was used to coat the glass surface of the chamber. Soluble fn, when present, was used at a concentration of 100 µg/ml. Values represent the mean ±SEM. The number of cells observed is in parentheses. The neutrophils in the $+Ca^{2+}$ condition migrated more rapidly on fn when soluble fn was present than when it was absent ($p < 0.01$). When soluble fn was present, the velocity of the $+Ca^{2+}$ cells was greater than the $-Ca^{2+}$ cells on an fn substrate ($p < 0.01$). Other differences were not significant at $p < 0.01$. The velocities were analyzed using a 3×3 (Substrate × Calcium) analysis of variance with differences between individual means determined by Tukey honest significant difference test.
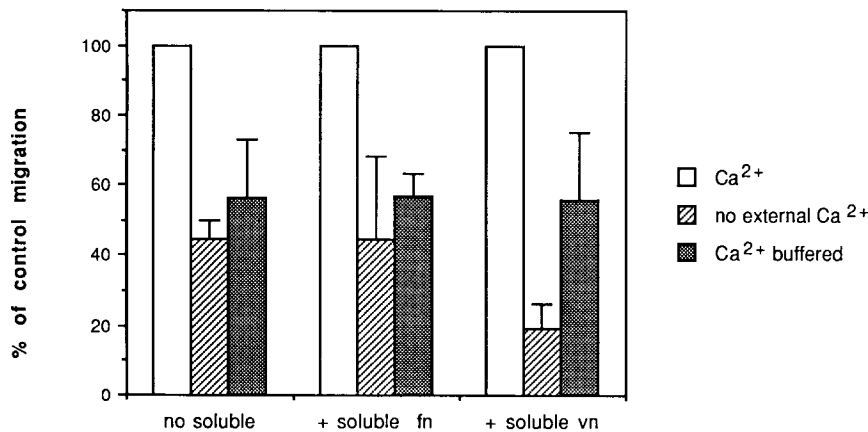
A. Fibronectin substrate

☐ Ca²⁺
▨ no external Ca²⁺
▦ Ca²⁺ buffered

B. Vitronectin substrate

☐ Ca²⁺
▨ no external Ca²⁺
▦ Ca²⁺ buffered
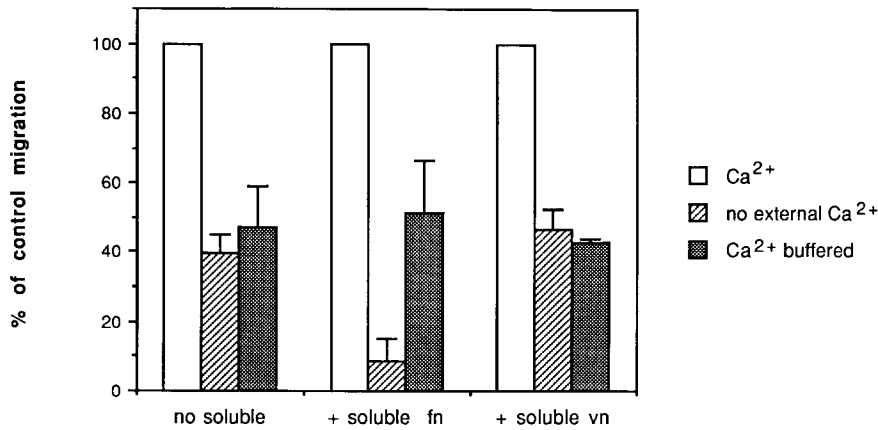
Figure 3. Effect of soluble fn and vn on fMLP-induced chemokinesis. (*A*) Effect of 100 μg/ml soluble fn or 10 μg/ml soluble vn on migration on an fn substrate. (*B*) Effect of 100 μg/ml soluble fn and 10 μg/ml soluble vn on migration on a vn substrate. Coating of the glass with either 100 μg/ml fn or 10 μg/ml vn and the chemokinesis assays were performed as described in the Materials and Methods. In the absence of fMLP, migration was <1% of the cells on these substrates. The addition of soluble fn or vn had no effect on migration in the absence of fMLP. After the plating and stimulation of the cells with 10 nM fMLP, three fields of about 15 cells were examined on each dish for a period of 3 min. The fMLP-induced migration in the absence of external Ca²⁺ or in Ca²⁺-buffered cells was measured on each substrate and with each soluble factor using separate dishes. Each condition was examined in one to three trials (dishes) compared with the appropriate Ca²⁺ control and repeated on two to six different days. A minimum of 113 cells were analyzed for each condition. The data is presented as the mean percent of control movement and the error bars indicate the SD. The control migration is the migration in the presence of Ca²⁺ in response to 10 nM fMLP. Migration on fn alone in response to 10 nM fMLP was 51%, when soluble vn was present it was 49%, and when soluble fn was present it was 63%. Migration on vn alone in response to 10 nM fMLP on vn was 50%, when soluble vn was present it was 50%, and when soluble fn was present it was 44%.

whether coating the surface of the glass with fn or vn in addition to albumin could render migration Ca²⁺ sensitive. Fig. 2 shows that by coating the surface with either fn or vn, before coating with albumin, migration became dependent on the presence of external Ca²⁺. The migration of Ca²⁺-buffered cells was reduced to a similar extent as that observed when external Ca²⁺ was removed. The inhibition of the migration of Ca²⁺-buffered cells on fn or vn was 50 and 42% of control, respectively. These results with Ca²⁺-buffered cells are consistent with the interpretation that on fn- and vn-coated glass the removal of external Ca²⁺ is effective in inhibiting migration because it causes the cessation of [Ca²⁺]ᵢ transients.

Migration in the Presence of Soluble fn or vn

Although the inhibitions observed by altering Ca²⁺ described above are significant, they do not reproduce the complete inhibition observed in the presence of serum. Preliminary attempts to achieve such a complete inhibition involved simultaneously coating the glass substrate with both fn and vn. These attempts were unsuccessful, and the inhibition of migration observed in the absence of external Ca²⁺ or in

Ca²⁺-buffered cells was never more than that observed on fn alone.

In the presence of serum a large number of proteins might be responsible for the complete inhibition of migration observed in the absence of [Ca²⁺]ᵢ transients. Since adhesive glycoproteins are present in serum, we tested the possibility that these soluble glycoproteins might be required for the complete Ca²⁺ dependence of migration. Migration of neutrophils was observed on purified adhesive glycoprotein substrates in the presence of soluble adhesive glycoprotein. A greater absolute percentage of cells was observed to migrate in the presence of 100 μg/ml soluble fn than in its absence (63 vs. 51%). These cells also had a somewhat higher velocity than cells migrating in the absence of soluble fn or on albumin: 7.6 ± 0.35 vs. 5.41 ± 0.31 (mean ± SD; Table III). When external Ca²⁺ was removed, the inhibition approached the absolute inhibition observed on fn in the presence of serum (Fig. 3). Although the velocity of the migrating cells was reduced when Ca²⁺ was removed in the presence of soluble fn (Table III), the major effect was a dramatic reduction in the percentage of migrating cells (Fig. 3).

If soluble fn was present during incubation with Ca²⁺-free incubation medium containing 10 nM fMLP and was
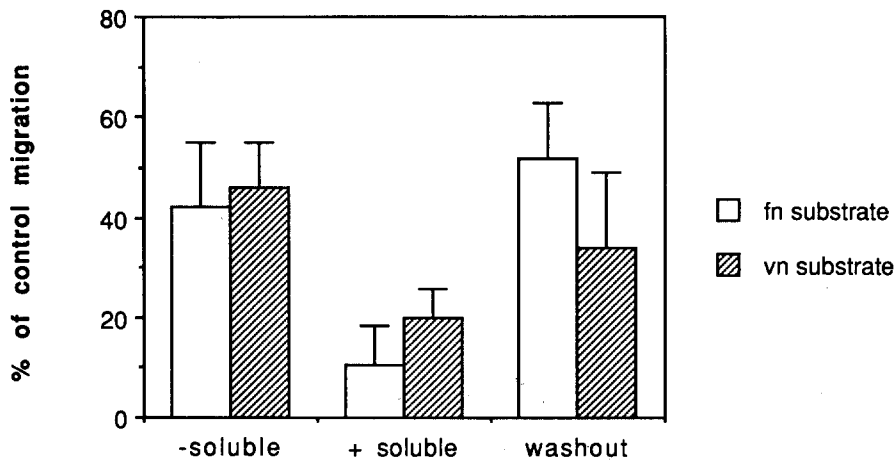
Figure 4. Reversibility of the additional inhibition of migration in the absence of external Ca^{2+} caused by the addition of soluble fn or vn. Glass was coated with fn or vn. Migration was examined in response to 10 mM fMLP in the absence of external Ca^{2+}. The addition of 100 μg/ml soluble fn to cells migrating on fn substrates increased the inhibition in the absence of Ca^{2+} ($p < 0.01$). When soluble fn was removed (washout), the additional inhibition was fully reversible. The difference between soluble fn and the washout condition was significant at $p < 0.01$. A smaller decrease in motility was observed with the addition of soluble vn to cells on vn substrates ($p < 0.01$). However, with vn the washout condition was not significantly different from either the − soluble condition or the + soluble condition. Each condition was examined in 6 to 15 trials (dishes) spread over 4 to 10 separate experiments (days). In each experiment the percent of control movement was determined by comparing each condition with the appropriate Ca^{2+} control. The movement of the control cells is the same as reported in Fig. 3. The data are presented as the mean percent of control with the error bars indicating the SD. Statistics were analyzed for each substrate by using analysis of variance with the significant differences between treatment means determined by the Tukey honest significant difference test.

then washed away just before examining migration in Ca^{2+}-free incubation medium containing 10 nM fMLP, the inhibition of migration observed returned to the levels observed in cells that had not been treated with soluble fn (Fig. 4). This indicates that this effect is due to soluble fn and is rapidly reversible.

The additional inhibition of migration observed in the presence of soluble fn is not reproduced by Ca^{2+} buffering the cells (Fig. 3), indicating that this phenomenon may be due to the removal of external Ca^{2+}. Alternatively, the inability of this treatment to further inhibit migration could be the result of an insufficient inhibition of the Ca^{2+} transients by quin-2 buffering (Marks and Maxfield, 1990). The morphology of the cells in the Ca^{2+}-buffered condition, however, was qualitatively different from nonbuffered cells. Ca^{2+}-buffered cells often had a more flattened morphology and frequently had prominent tails trailing behind them (Fig. 5).

Similar results were obtained with vn (Fig. 3), although the inhibition of migration observed on this substrate in the absence of external Ca^{2+} when soluble vn was present was consistently less pronounced than that observed with fn. In addition, the reversibility of the soluble vn inhibition was not as clear as the soluble fn inhibition (Fig. 4).

To test whether the effect of soluble fn or vn was specific we examined cells migrating on fn in the presence of soluble vn and on vn in the presence of soluble fn. Fig. 3 shows that on fn, soluble vn does not further inhibit the migration of neutrophils in the absence of Ca^{2+}, whereas soluble fn does. These findings indicate that the effects of these soluble proteins are specific.
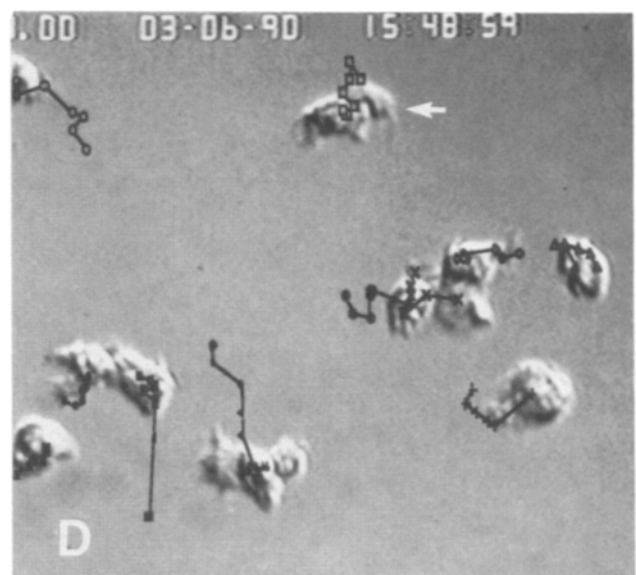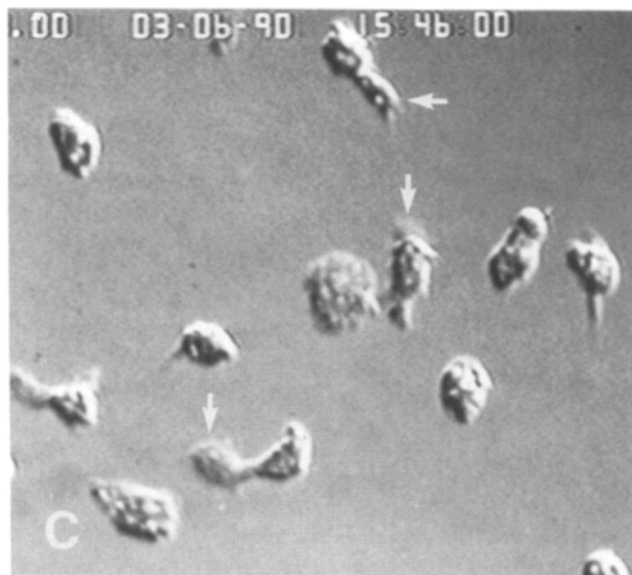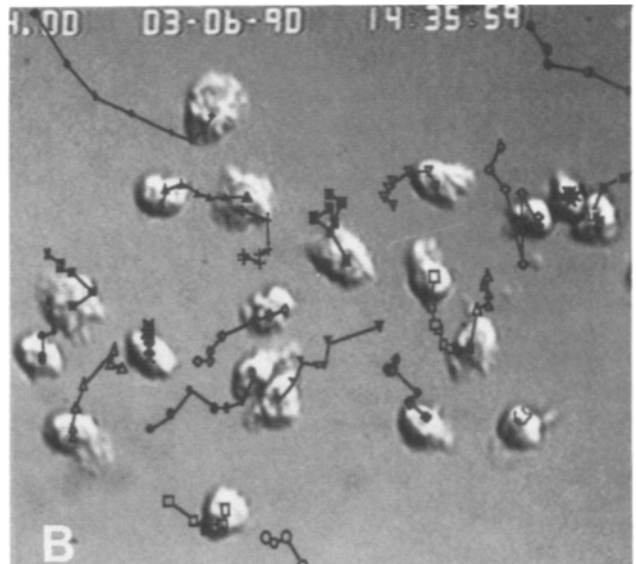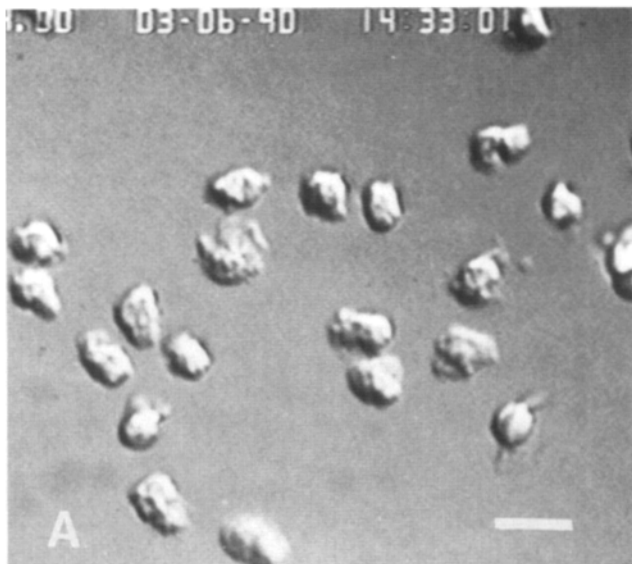
Discussion

In this work we sought to identify the components(s) of serum that made the migration of human neutrophils dependent on $[Ca^{2+}]_i$ transients. Starting from observations of migration on different substrates in the presence of serum,

we proceeded to examine migration on purified substrates in its absence, and to identify proteins apparently responsible for the inhibition observed.

In this paper (Fig. 1), and in a previous paper (Marks and Maxfield, 1990), we have shown that removal of external Ca^{2+} causes a complete suppression of $[Ca^{2+}]_i$ transients within 10 min, and resting $[Ca^{2+}]_i$ drops to a stable value at ∼50 nM. During this period the neutrophils gradually stop migrating. Since external Ca^{2+} drops immediately, but $[Ca^{2+}]_i$ transients decay over a period of a few minutes, the inhibition of migration is not simply due to the absence of external Ca^{2+}. Ca^{2+} buffering the cells also causes a significant, yet incomplete, damping of the $[Ca^{2+}]_i$ transients. We have found that the migration of human neutrophils on substrates that contain the adhesive glycoproteins fn or vn is significantly inhibited under conditions that suppress $[Ca^{2+}]_i$ transients. Mg^{2+} was present at a concentration of ∼0.9 mM in the Ca^{2+}-free incubation media so the reported divalent cation requirement of the integrins for ligand binding should have been satisfied (Gailit and Ruoslahti, 1988; Hogg, 1989). Since an inhibition of migration was also observed on these substrates in Ca^{2+}-buffered cells in the presence of 1 mM external Ca^{2+}, we conclude that the inhibition is the result of alterations in $[Ca^{2+}]_i$ regulation. No inhibition of migration was observed in neutrophils migrating in the absence of external Ca^{2+} on albumin, nor was there an inhibition of migration of Ca^{2+}-buffered cells on this substrate.

Our previous results (Marks and Maxfield, 1990) and those reported here indicate that $[Ca^{2+}]_i$ changes play an important role in neutrophil migration on the adhesive glycoproteins fn and vn. Transient increases in $[Ca^{2+}]_i$ appear to be involved in migration when these purified glycoproteins are present. Since both EGTA treatment and the $[Ca^{2+}]_i$ buffers reduce both transient increases in $[Ca^{2+}]_i$ and reduce basal $[Ca^{2+}]_i$ from 150 to between 50 and 100 nM, we cannot unequivocally demonstrate that it is the suppression of $[Ca^{2+}]_i$ transients and not the reduction in basal Ca^{2+} that is responsible for the inhibition of migration. However, the in-

hibition of transients is much more pronounced than the reduction in basal $[Ca^{2+}]_i$. In either case, migration on adherent substrates appears dependent on $[Ca^{2+}]_i$. Such a role for $[Ca^{2+}]_i$ changes is physiologically relevant because neutrophils must migrate in vivo through environments containing these molecules. Adhesive glycoproteins may play a major role in facilitating the emigration of neutrophils from the bloodstream to sites of infection.

The inhibition observed on fn or vn substrates was more pronounced when soluble fn or vn was present. This inhibitory effect of soluble fn or vn in the absence of Ca^{2+} was apparently specific, since only soluble fn was able to further decrease migration on fn, and only soluble vn was able to decrease migration on vn. This additional inhibition of migration observed in the absence of external Ca^{2+} was not reproduced in Ca^{2+}-buffered cells. This further inhibition is therefore either due to an effect of external Ca^{2+}, or is the result of insufficiently buffering $[Ca^{2+}]_i$ with quin-2. This second possibility is plausible because small $[Ca^{2+}]_i$ transients have been shown to occur even in Ca^{2+}-buffered cells (Marks and Maxfield, 1990). Further work will be necessary to distinguish between these two possibilities.

In a previous study, we found that the velocity of Ca^{2+}-buffered cells was significantly reduced on poly-D-lysine in the presence of serum (Marks and Maxfield, 1990). In studying the motility of the fMLP-stimulated neutrophils on fn, we found that the percentage of migrating cells was strongly dependent on regulation of $[Ca^{2+}]_i$. However, we found no significant effect on the velocity of the cells that did move on fn when they were Ca^{2+} buffered (Table III). There was a small, statistically significant effect on the velocity of the cells migrating on fn in the presence of soluble fn when Ca^{2+} was removed. These data indicate that on purified fn the percentage of migrating cells is decreased when $[Ca^{2+}]_i$ transients are suppressed, but the velocity of the migrating cells is only slightly altered. The basis for the difference in the Ca^{2+} dependence of velocity for serum-stimulated migration on poly-D-lysine vs. fMLP-stimulated migration on fn is not known.

A number of conflicting reports have been published on the role of Ca^{2+} in neutrophil migration. These have ranged from those indicating an absolute requirement of Ca^{2+} influx for neutrophil migration (Boucek and Snyderman, 1976) to those reporting that the migration of Ca^{2+}-depleted neutrophils in the absence of external Ca^{2+} is essentially unimpaired (Zigmond et al., 1988). In between these two extremes, a number of studies have demonstrated a decrease in the migratory capability of Ca^{2+}-buffered cells (Elferink and Deierkauf, 1985; Meshulam et al., 1986). Although there are other possible reasons for the discrepancy between

studies, one explanation for the differences is that in each case the substrate was coated with a different type or quantity of adhesive glycoprotein. Since Boucek and Snyderman (1976) examined migration toward activated serum, it is possible that as the gradient in the modified Boyden chamber developed, the adhesive glycoproteins present in serum adhered to the filters that were used, rendering migration sensitive to alterations in $[Ca^{2+}]_i$. In the studies of Elferink and Deierkauf (1985) and Meshulam et al. (1986), in which a partial inhibition of migration was observed when soluble albumin was present, it is possible that contaminating adhesive glycoproteins present in the albumin made migration sensitive to $[Ca^{2+}]_i$.

The Integrins, $[Ca^{2+}]_i$ Transients, and Migration

$[Ca^{2+}]_i$ changes are not required for a number of motile processes in neutrophils. Actin polymerization, shape changes, and migration on albumin (Sha'afi et al., 1986; Meshulam et al., 1986; Zigmond et al., 1988) can occur without increases in $[Ca^{2+}]_i$. Even Ca^{2+}-depleted neutrophils are capable of spreading on a surface and repeatedly extending pseudopodia (Marks and Maxfield, 1990).

As previously outlined (Marks and Maxfield, 1990), a number of mechanisms can be proposed to explain the apparent requirement of $[Ca^{2+}]_i$ transients for neutrophil migration on adhesive glycoprotein substrates. One possible role would be that $[Ca^{2+}]_i$ transients are required for a contractile event so that neutrophils can retract their tails which are firmly attached to the substratum. A second possibility is that $[Ca^{2+}]_i$ transients are required for the disruption of specialized contacts with the substratum. This could be mediated through the activation of gelsolin (Stossel et al., 1985) which disrupts actin filaments that are present in focal contacts in other cell types (Burridge et al., 1988). Neither of these two mechanisms is exclusive of the other.

A third possibility is that $[Ca^{2+}]_i$ transients are involved in the modulation of the affinity of the integrins for adhesive glycoproteins, perhaps causing a transition from a higher to a lower affinity state. Such a function for the transients would explain many of our observations. For example, preventing $[Ca^{2+}]_i$ transients in cells could keep the integrins in a higher affinity state preventing release from fn or vn. The cells would therefore become stuck to the substrate, as we have observed. This would prevent migration even if other force-generating mechanisms continued to operate. It should be stressed that the role for $[Ca^{2+}]_i$ via this third mechanism would not exclude its role in either of the other two possible functions outlined above.

Regardless of the precise mechanism, our results show

Figure 5. Micrographs of neutrophils migrating on fn in the presence of soluble fn. Recordings were made on optical memory disc of cells migrating in response to 10 nM fMLP on 100 μg/ml fn in the presence of 100 μg/ml soluble fn as described in the Materials and Methods. (*A* and *B*) Migration of neutrophils in the presence of 1 mM external Ca^{2+}; (*C* and *D*) migration of quin-2–buffered cells in the presence of 1 mM external Ca^{2+}; (*E* and *F*) migration of cells in the absence of external Ca^{2+} and the presence of 1 mM EGTA. Cell movement was monitored for a 3-min period starting in *A*, *B*, or *C* and ending in *B*, *D*, or *F*, respectively. The black lines in *B*, *D*, and *F* track the movement of the cells over the 3-min period; the symbols indicate the position of the cell centroid at 30-s intervals. The nonbuffered neutrophils migrate rapidly in the presence of external Ca^{2+}. In contrast, only a few quin-2–buffered cells migrate from their starting position (*C* and *D*) and several have a flattened or elongated morphology with tails that remain attached to the substrate (*arrows*). In the absence of external Ca^{2+}, a nearly complete inhibition of migration is observed (*E* and *F*; see also Fig. 3). Bar, 10 μm.

that $[Ca^{2+}]_i$ transients play a role in the modulation of the migration of neutrophils on adhesive glycoprotein substrates. Further studies should help elucidate the molecular basis for this modulation. While our studies have focused on neutrophil migration, many cell types must repeatedly bind and release extracellular components through integrins. It is possible that $[Ca^{2+}]_i$ transients, which have now been observed in many cell types, may affect integrin binding by similar mechanisms. For example, in lymphocytes, modulation of cell-cell adhesion is required, and lymphocytes have been shown to produce series of transient $[Ca^{2+}]_i$ increases (Wilson et al., 1987). Insights gained in studies of neutrophils may be applicable to a variety of cell types.

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