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ENDOTHELIN-INDUCED ACTIVATION OF NEUTROPHIL MIGRATION

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Abstract—Migration of rabbit peritoneal neutrophils was stimulated by endothelin-1 (ET-1) up to 2.10^{-8} M. Higher concentrations inhibited random migration. The stimulating effect of ET-1 was inhibited by BQ-123, a specific antagonist of the ET_A receptor. A checkerboard assay showed that the stimulating effect of ET-1 on neutrophil migration was chemokinetic rather than chemotactic. Extracellular Ca^{2+} was required for the activating effect of ET-1. Non-selective calcium channel blockers such as econazole and La^{3+} strongly inhibited ET-1-activated migration but had little effect on fMLP-activated migration, underlining the importance of Ca^{2+} influx for ET-1-activated migration. Studies with electroporated neutrophils showed that the increase in migration was most pronounced at calcium concentrations between 100 nM and 1 μM. ET-1-activated migration of electroporated cells was completely blocked by low concentrations of calcium-channel blockers such as verapamil and nitrendipine. Migration by intact cells was inhibited by the same concentration of verapamil, but to a lesser degree; nitrendipine had little effect on migration of intact cells. This suggests that calcium derived from intracellular stores is required for migration activated by ET-1. Protein kinase C, protein tyrosine kinase, and phosphatase activity were involved in the activating effect of ET-1 on neutrophil migration. ET-1 did not induce exocytotic enzyme release, in neither the presence nor the absence of cytochalasin B.

Key words: endothelin-1; neutrophil; calcium; migration; chemotaxis; chemokinesis

ET-1† is a vasoactive peptide produced by a variety of tissues, but especially by vascular endothelial cells. Besides its vasoconstrictor action it promotes the proliferation of several cell types, increases intracellular Ca^{2+} level and activates protein kinase C and phospholipase C [1–3]. ET-1 plasma levels are enhanced after clinical insults such as myocardial infarction, septic shock, diabetes, and kidney failure [4].

A few properties of neutrophils are altered by ET-1. Although it was not able to stimulate superoxide generation by itself, it enhanced chemotactic peptide-activated superoxide production [5]. ET-1 induced an enhancement of cytosolic free calcium concentration [6, 7]. This enhancement was inhibited by L-arginine, which was interpreted as an involvement of NO-related systems [7].

Neutrophil migration is a relevant property of these cells. It is of importance both for the body's defence reaction against infection and for the pathogenesis of inflammatory disorders. Because previous studies [5–7] have shown that neutrophils apparently possess binding sites for ET-1, we decided to study the effect of ET-1 on neutrophil migration.

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† Abbreviations: ET-1, endothelin 1; fMLP, formyl-methionyl-leucyl-phenylalanine; AMG-C_{16}, 1-*O*-hexadecyl-2-methyl-*sn*-glycerol; BQ-123, cyclo(-D-Trp-D-Asp-Pro-D-Val-Leu).

MATERIALS AND METHODS

Isolation of neutrophils. Neutrophils were isolated from the peritoneal cavity of the rabbit [8]. Four hours after intraperitoneal injection with 200 mL isotonic saline containing 1.5 mg/mL glycogen, the exudate was collected by flushing the peritoneal cavity with isotonic saline containing citrate (0.4%, pH 7.4). The cells were centrifuged and washed with medium. The medium used consisted of 140 mM NaCl, 5 mM KCl, 10 mM glucose, 0.5% bovine serum albumin and 20 mM HEPES, pH 7.3. Unless otherwise stated the medium was supplemented with 1 mM Ca^{2+} and 1 mM Mg^{2+}. The final cell suspension during the experiments contained 3×10^6 neutrophils per mL.

Migration measurements. Cell migration was measured with the Boyden chamber technique, as described by Boyden [9], and modified by Zigmond and Hirsch [10]. The two compartments of the chamber were separated by a cellulose acetate Millipore filter with a pore size of 3 μm. Neutrophils were placed in the upper compartment of the chamber and incubated for 35 min at 37°. After migration the filters were fixed and stained and the distance travelled in μm into the filter was determined according to the leading front technique [10]. The assays were carried out in triplicate and the migration distance of the neutrophils was determined at five different filter sites. The effects of ET-1 on migration

were compared with those of fMLP, which has well-known chemotactic effects on neutrophil migration.

Exocytotic enzyme release. Exocytosis was measured as the release of the granule-associated enzyme lysozyme in the absence of a significant release of the cytoplasmic enzyme lactate dehydrogenase (LDH). Neutrophils were incubated for 35 min at 37°. Subsequently, the mixture was centrifuged and the supernatant analysed. Lysozyme was measured by determining the rate of lysis of *Micrococcus lysodeikticus*. LDH release, measured as the conversion of NADH into NAD^+ during the conversion of pyruvate into lactate, was determined as a measure for cell damage. Under the conditions of our experiments LDH release was negligibly low (less than 5%). Enzyme release was expressed as a percentage of a maximum value, obtained by treating the cells with 0.05% Triton X-100 for 35 min at 37°.

Electroporation of neutrophils. Neutrophils were electroporated according to the method of Grinstein [11], with minor modifications. The electropermeabilization procedure was carried out at room temperature. When permeabilization was carried out at 0° the cells did not migrate. Neutrophils (3×10^6 per mL) in permeabilization medium (135 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM HEPES pH 7.0, 10 mM glucose and 0.5% BSA), and reagents as indicated, were placed in the cuvette of a BioRad Gene Pulser. The cells were exposed to two discharges of 4.75 kV/cm from a 25 μF capacitor. Between discharges the cell suspension was stirred with a plastic pipette. After permeabilization and mixing 0.2 mL of the suspension was placed in the upper compartment of the Boyden chamber.

Statistical analysis. Experiments were carried out with cells from one animal. Three separate experiments were performed. For each experiment migration was determined at five different places on the filter. Values given are arithmetical means ± SEM. The results presented are representive for experiments with cells from three different animals. Because different cell batches gave differences in absolute values of migration, but not in qualitative changes when different agents or actions were compared, the results presented are based on experiments with cells from one animal. Significances were calculated with the Student's *t*-test; a value of $P < 0.01$ was considered as statistically significant.

Materials. ET-1, fMLP, AMG-C_{10} and calphostin C were obtained from Sigma (St. Louis, NY, U.S.A.). Staurosporine and okadaic acid were from Boehringer Mannheim (Germany). Calyculin A, tyrphostin A25 and lavendustin were obtained from Calbiochem-Novabiochem (Bad Soden, Germany). Genistein was from ICN Chemicals (Cleveland, OH, U.S.A.). BQ-123 [cyclo(-D-Trp-D-Asp-Pro-D-Val-Leu)] was obtained from Bachem (Bubendorf, Switzerland).

RESULTS

ET-1 vs random migration and fMLP-activated chemotaxis

ET-1 caused an increase in neutrophil migration in the concentration range 2.10^{-10}–2.10^{-8} M in the absence of other migration activators. Higher concentrations inhibited random migration. The enhancement of migration was most pronounced when ET-1 was present in both the upper and lower compartment of the Boyden chamber (Fig. 1). BQ-123, a specific antagonist of the ET-1 receptor, inhibited the effect of ET-1 on migration completely (Fig. 2). In contrast, both random migration and fMLP-activated migration were slightly stimulated by BQ-123.

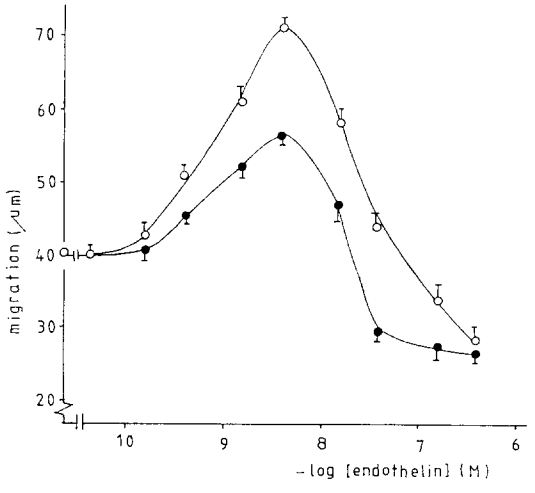

Fig. 1. The effect of increasing concentrations of endothelin on neutrophil migration. ET-1 was present either in both compartments of the Boyden chamber (○) or in the lower compartment only (●). Under these conditions the chemotactic agent fMLP caused a migration of 101.8 ± 1.3 μm when present in the lower compartment only, and a migration of 51.9 ± 1.1 μm when present in both compartments.

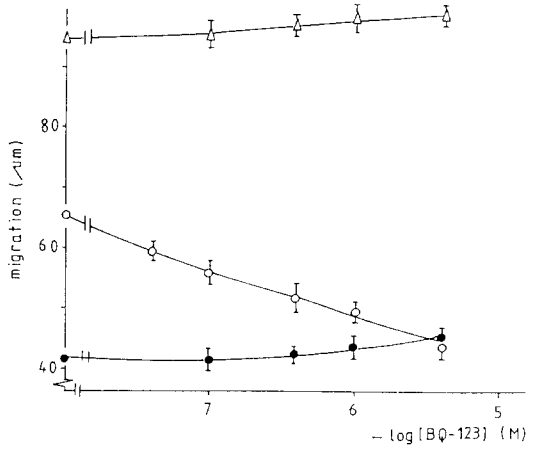

Fig. 2. Inhibition of ET-1-activated migration by the endothelin antagonist BQ-123. As a comparison the effect of BQ-123 on random migration and on fMLP-activated migration is included. —○—: Activation by 4×10^{-9} M ET-1, present in both compartments; —●—: random migration; —△—: activation by fMLP (10^{-9} M), present in the lower compartment of the Boyden chamber.

Table 1. Migration of neutrophils in different absolute concentrations and concentration gradients of ET-1

ET-1 concentrations (M) in upper compartment	ET-1 concentrations (M) in lower compartment			
	0	4×10^{-10}	1.2×10^{-9}	4×10^{-9}
0	49.8 ± 0.8	54.7 ± 1.4(52)	59.7 ± 1.5 (55)	65.6 ± 1.4(57)
4×10^{-10}	57.7 ± 1.5(60)	62.9 ± 1.5	65.4 ± 1.2(65)	69.7 ± 1.5(68)
1.2×10^{-9}	65.9 ± 1.8(68)	70.0 ± 1.3(71)	72.9 ± 1.1	75.6 ± 1.3(76)
4×10^{-9}	74.0 ± 1.3(76)	78.1 ± 1.3(79)	80.9 ± 1.0(81)	83.7 ± 1.4

Values given represent the migration in μm into the filter after an incubation time of 40 min in the presence of the indicated concentrations of ET-1 below and above the filter. The values between brackets are the migration values to be expected on the basis of effects on chemokinesis alone (calculated according to Zigmond and Hirsch [10]).

To determine whether the enhancing effect was chemokinetic or chemotactic, a checkerboard assay was carried out (Table 1), with different ET-1 concentrations in the upper and lower compartment [12]. The enhancing effect appeared larger than might be expected on the basis of chemokinesis alone, although the difference was small. The presence of a predominant chemokinetic effect is most evident from the extreme situation where ET-1 is present together with the cells (ET-1 in upper compartment only), as opposed to ET-1 only present in the lower compartment. A high value in the former case suggests chemokinesis, and in the latter chemotaxis. In contrast, the well-known chemotactic peptide fMLP is predominantly chemotactic. Tested in a separate experiment fMLP gave an enhancement of 44.6 ± 1.2 (random migration) to 51.9 ± 1.1 when 10^{-9} M fMLP was present in both compartments, and to 101.8 ± 1.0 when the same concentration was present in the lower compartment only.

The role of Ca^{2+} *in ET-1-activated migration*

The presence of extracellular Ca^{2+} was necessary for the activating effect of ET-1. The presence of magnesium alone had no effect, but magnesium slightly enhanced the effect of ET-1 in the presence of Ca^{2+}. The Ca^{2+} requirement of ET-1-activated migration was completely different from that of random migration and that of fMLP-activated migration where divalent cations had only a slight effect (Fig. 3).

To study the importance of intracellular (cytoplasmic free) calcium in ET-1-activated migration we considered the effect of ET-1 on electroporated neutrophils, where the calcium concentration was imposed with calcium-EGTA buffers. As a comparison the Ca^{2+}-dependence of fMLP-activated chemotaxis was measured. As reported previously [13], the effect of fMLP was hardly dependent on Ca^{2+}. ET-1-activated migration was strongly dependent on intracellular free calcium, with

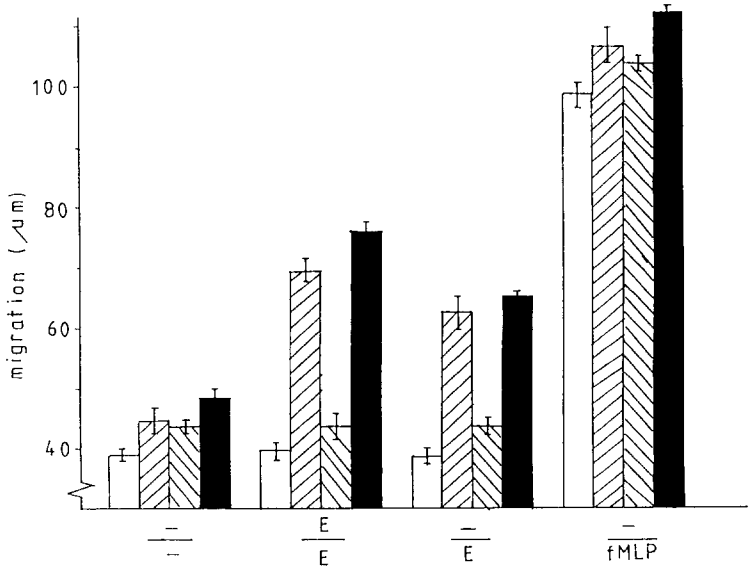

Fig. 3. The effect of divalent cations on random migration (-/-), ET-1-activated migration, and fMLP-activated migration. ET-1 (4×10^{-9} M) was present either in both compartments (E/E) or in the lower compartment only (-/E). The chemotactic peptide fMLP (10^{-9} M) was present in the lower compartment only. □: 1 mM EDTA; ▨: 1 mM Ca^{2+}; ▧: 1 mM Mg^{2+} (+50 μM EGTA); ■: 1 mM Ca^{2+} + 1 mM Mg^{2+}.

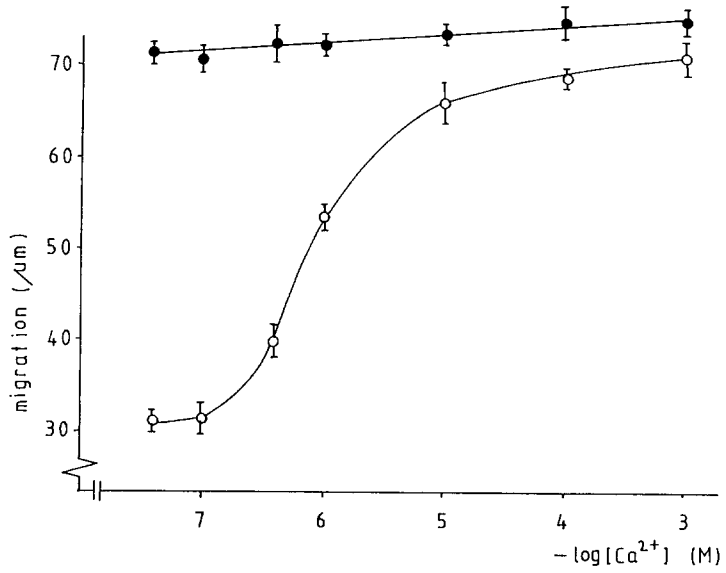

Fig. 4. The effect of increasing Ca^{2+} concentrations on migration of electroporated neutrophils in the presence of 4×10^{-9} M ET-1 in both compartments (—○—), and towards 10^{-9} M fMLP in the lower compartment (—●—). Ca^{2+} concentrations between 10 nM and 10 μM were prepared by mixing EGTA and EGTA-Ca^{2+} solutions, thus obtaining buffered calcium concentrations [13, 14]. Higher concentrations of Ca^{2+} were not buffered.

the strongest increase in migration at calcium concentrations between 100 nM and 1 μM (Fig. 4). Three different inhibitors of calmodulin (trifluoperazine, W-7 and calmidazolium) inhibited ET-1-activated migration of electroporated cells (Table 2) at lower concentrations than those required for intact cells. Migration by fMLP, however, was also inhibited under the same conditions.

Migration of ET-1-activated neutrophils was also inhibited by verapamil, but higher concentrations were required than with electroporated cells (Table 3). Nitrendipine gave little inhibition with intact cells. Migration of fMLP-activated neutrophils was slightly inhibited by verapamil, both in the presence and in the absence of extracellular Ca^{2+} (Table 3).

In Table 4 the effects of some non-specific calcium channel blockers on ET-1-activated migration and of fMLP-activated migration are shown. It appears that some blockers, notably econazole and La^{3+}, strongly inhibited ET-1-activated migration and had little effect on fMLP-activated migration. We also considered the effect of these compounds on changes in intracellular free calcium, measured as Indo-1 fluorescence. Here the situation was rather complicated. Econazole gave an enhancement of both ET-1-induced and fMLP-induced Indo fluorescence. The metal ions (at the highest concentration indicated in Table 4) reduced fMLP-enhanced fluorescence by approximately 50%, and gave a nearly complete inhibition of ET-1-activated enhancement of fluorescence.

In addition to Ca^{2+} dependence, the use of electroporated neutrophils demonstrated another difference between ET-1-activated and fMLP-activated migration. Permeabilization of neutrophils resulted in a reduction of both random migration

Table 2. Inhibition of migration of electroporated neutrophils by calmodulin inhibitors and calcium channel blockers

	Migration (μm)		
	—	ET-1	fMLP
—	36.1 ± 1.1	53.7 ± 1.8	72.8 ± 1.8
Calmidazolium (1 μM)	33.7 ± 1.4	34.6 ± 1.8**	58.1 ± 1.3**
W-7 (5 μM)	35.2 ± 1.8	44.2 ± 1.7**	40.6 ± 1.5**
Trifluoperazine (10 μM)	34.5 ± 1.7	45.8 ± 1.0**	62.6 ± 2.1**
Verapamil (1 μM)	28.3 ± 1.7**	31.9 ± 1.9**	39.5 ± 2.0**
Nitrendipine (1 μM)	32.1 ± 1.5**	32.6 ± 1.8**	32.5 ± 2.4**

Cells were preincubated with the indicated concentration of inhibitor and subsequently electropermeabilized. ET-1 (4×10^{-9} M) was present in both compartments and fMLP (10^{-9} M) was present in the lower compartment of the Boyden chamber. **$P < 0.01$ as compared with the value for (activated) migration in the absence of inhibitor.

Table 3. The effect of calcium channel blockers on migration of (intact) neutrophils

	Migration (μm)		
	—	ET-1	fMLP
—	45.9 ± 1.7	64.1 ± 1.9	99.5 ± 1.8
Verapamil (1 μM)	45.4 ± 1.4	55.7 ± 1.7	96.3 ± 1.4
Verapamil (10 μM)	42.5 ± 2.1	51.5 ± 1.4	92.7 ± 1.6
Nitrendipine (1 μM)	44.5 ± 1.8	62.1 ± 1.3	99.8 ± 1.8
Nitrendipine (10 μM)	44.4 ± 1.8	61.9 ± 1.6	96.9 ± 1.2

Cells were preincubated with verapamil or nitrendipine for 10 min at 37° and then placed in the upper compartment of the Boyden chamber. ET-1 (4×10^{-9} M) was present in both compartments while fMLP (10^{-9} M) was present in the lower compartment of the Boyden chamber. In the absence of extracellular Ca^{2+} (with 50 μM EGTA present to complex adherent calcium) migration towards fMLP was 94.8 ± 1.3 μm. In the presence of 10 μM verapamil the distance was 90.0 ± 1.7 μm, and in the presence of 10 μM nitrendipine the distance was 90.7 ± 2.2 μm. Inhibition of migration towards fMLP by verapamil was statistically significant.

and fMLP-activated migration by approx. 30%. At optimal Ca^{2+} concentration activation by ET-1 was hardly diminished; while activated migration by ET-1 in intact cells remained far behind that of fMLP, there was very little difference between stimulation by these two agents in permeabilized cells (at high Ca^{2+} concentrations) (Fig. 4).

Involvement of kinases and phosphatases

ET-1-activated migration was strongly inhibited by the protein kinase C inhibitors staurosporine AMG-C_{16} and, especially, calphostin C. Migration activated by fMLP was also inhibited by staurosporine and calphostin, albeit to a somewhat lesser degree; AMG-C_{16} was not effective in inhibiting fMLP-activated migration (Table 5).

To study the possible involvement of protein tyrosine kinase in ET-1 activated migration the effect of three inhibitors of this enzyme (genistein, tyrphostin, and lavendustin A) were considered. Both ET-1-and fMLP-activated migration were inhibited, especially by genistein. Two inhibitors of protein phosphatases, okadaic acid and caliculin A, were more effective in inhibiting fMLP-activated migration than ET-1-activated migration (Fig. 5).

Table 4. The effect of some non-selective calcium channel blockers on migration activated by ET-1 or fMLP

	Migration (μm)	
	ET-1	fMLP
—	69.5 ± 1.5	97.4 ± 1.8
Econazole (20 μM)	47.7 ± 2.2	97.5 ± 1.7
La^{3+} (20 μM)	49.1 ± 1.1	80.6 ± 1.9
Cd^{2+} (100 μM)	65.6 ± 1.7	96.9 ± 2.1
Cd^{2+} (500 μM)	43.4 ± 1.2	41.7 ± 1.5
Ni^{2+} (50 μM)	68.6 ± 1.4	97.1 ± 1.6
Ni^{2+} (200 μM)	68.6 ± 1.7	62.5 ± 1.9

Cells were preincubated with the agents indicated for 10 min at 37° and then placed in the upper compartment of the Boyden chamber. ET-1 (4×10^{-9} M) was present in both compartments, while fMLP (10^{-9} M) was present in the lower compartment of the Boyden chamber.

Enzyme release

Because a large number of migration-stimulating agents stimulate exocytotic enzyme release from neutrophils we considered the effect of ET-1 on LDH release and on lysozyme release. A concentration of ET-1 that caused maximal stimulation of migration had no enzyme-releasing effect. Neither the enhancement of ET-1 concentration by a factor of 10 nor the addition of cytochalasin B had an effect on enzyme release (results not shown). As a comparison the effect on enzyme release of comparable concentrations of fMLP was tested; here a strong lysozyme release was induced in the presence of cytochalasin B.

DISCUSSION

The results show that ET-1 may have two effects on migration: a stimulating effect at low concentrations, and an inhibitory effect at higher concentrations. Both effects occur at concentrations which are much higher than those measured in serum *in vivo*, but coincide with those used to produce a pharmacologic effect in experimental systems. The effect of ET-1 is mediated by the ET_A receptor, because BQ-123, an antagonist selective for the ET_A receptor [15], inhibits ET-1-activated migration while having no effect on fMLP-activated migration. The checkerboard assay [12] shows that the stimulating effect is predominantly chemokinetic rather than chemotactic. Both the comparison of the experimental values and the calculated values and the mere observation that ET-1 in the upper compartment is more effective than in the lower compartment support this view. In this regard it differs from most migration-promoting substances (such as fMLP, which was included in a number of experiments for comparison) where the chemotactic component is predominant.

A number of biological actions of ET-1 were found to depend on calcium fluxes across the plasma membrane. Random migration and chemotaxis activated by fMLP (which is most thoroughly studied in this regard) is largely independent of calcium fluxes across the plasma membrane, because

Table 5. Inhibition of ET-1-activated migration by staurosporine, calphostin C, and AMG-C_{16}

	Migration (μm)		
	—	ET-1	fMLP
—	54.3 ± 1.8	68.5 ± 1.6	100.2 ± 2.1
Staurosporine (200 nM)	40.5 ± 1.1	59.6 ± 1.3	81.1 ± 1.8
Calphostin C (500 nM)	34.2 ± 1.3	39.9 ± 1.1	52.5 ± 1.2
AMG-C_{16} (50 μM)	44.8 ± 1.6	56.3 ± 1.3	100.2 ± 1.4

Cells were preincubated with staurosporine, calphostin C or AMG-C_{16} for 15 min at 37° and then placed in the upper compartment of the Boyden chamber. ET-1 (4×10^{-9} M) was present in both compartments, while fMLP (10^{-9} M) was present in the lower compartment of the Boyden chamber.

migration is possible in the absence of extracellular Ca^{2+}. ET-1-induced enhancement of migration is absolutely calcium dependent. The experiments with the non-specific calcium channel blockers econazole and La^{3+} on migration support the view that influx of extracellular Ca^{2+} is more important for ET-1-activated migration than for fMLP-activated migration. Speculating that calmodulin might be the target of the extracellular calcium required for activation of migration by ET-1, we tested three different calmodulin inhibitors in electroporated neutrophils. It appears that these agents, at lower concentrations than usually required for intact cells, inhibit ET-1-induced activation of migration, with calmidazolium being especially effective. Remarkably, however, fMLP-activated migration was also affected by these inhibitors. Because fMLP-activated migration is possible in the absence of cytoplasmic calcium these results are enigmatic. The results with verapamil and nitrendipine in electroporated cells are clearer. ET-1-activated migration is dependent on Ca^{2+} derived from intracellular stores, because activated migration can be completely blocked by treatment with low concentrations of calcium channel blockers. A comparable result was found for fMLP-activated migration [13]. Where this calcium is required in the cell remains a subject for further investigation, especially as concerns fMLP. Migration of intact cells activated by ET-1 is also inhibited by verapamil, but inhibition was less effective than with electroporated cells. Inhibition of ET-1-activated migration by monocytes was also found to be inhibited by relatively high (5 μM) calcium channel blockers. This was interpreted as interference with calcium influx across the plasma membrane [16]. This explanation could also apply to neutrophils. However, because fMLP-activated migration is also inhibited by verapamil, even in the absence of extracellular Ca^{2+}, it is possible that the effect of calcium channel blockers on intact cells is at least

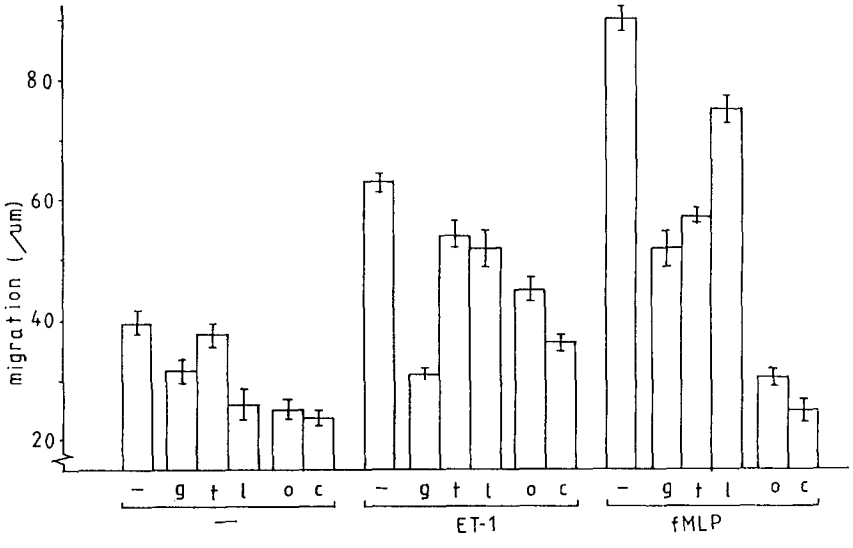

Fig. 5. Inhibition of migration by tyrosine kinase inhibitors and by serine/threonine phosphatase inhibitors. —: No inhibitor; g: 200 μM genistein; t: 20 μM tyrphostin; l: 20 μM lavendustin A; o: 1 μM okadaic acid; c: 20 nM calyculin A. Cells were preincubated with or without inhibitor for 10 min, and subsequently placed in the upper compartment of the Boyden chamber. ET-1 (4×10^{-9} M) was present in both compartments of the Boyden chamber, while fMLP (10^{-9} M) was present in the lower compartment.

partly due to interference with calcium channels in membranes of intracellular calcium stores, which cannot be readily reached in intact cells.

Protein kinase C activation has been implicated in the effects of ET-1 [17]. The experiments with staurosporine, AMG-C_{16} and calphostin show that protein kinase C plays a predominant role in ET-1-activated migration. Inhibition by calphostin is of particular importance, because this substance has been reported to be a highly specific inhibitor of protein kinase C [18] in contrast to staurosporine, which inhibits several protein kinases. Endothelin has been found to stimulate tyrosine phosphorylation [19]. The effects of genistein and other inhibitors show that tyrosine phosphorylation plays a role in migration but one which is not specific for ET-1 because inhibition also occurs with fMLP to the same degree.

Okadaic acid and caliculin A, two inhibitors of types 1 and 2A protein phosphatases [20, 21], strongly inhibit fMLP-activated migration, indicating that dephosphorylation plays a crucial role in chemotaxis. This also applies to ET-1-activated migration, but because the percentage inhibition is less than for fMLP-activated migration it seems that dephosphorylation is not as important in ET-1-activated migration as in fMLP-induced chemotaxis.

Migration-promoting agents such as fMLP, interleukin-8 and leukotriene B_4 are capable of inducing exocytosis in neutrophils. In most cases, however, the presence of cytochalasin B is required to express the secretory property. In contrast to these agents endothelin has no secretory properties: neither in the absence nor in the presence of cytochalasin B does ET-1 cause any enzyme release. This is another indication that the effect of ET-1 on neutrophil functions differs in essential ways from other migration-promoting substances.

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