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The involvement of protein kinase G in stimulation of neutrophil migration by endothelins

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Abstract

Activation of human neutrophil migration by endothelin-1 and endothelin-3 is inhibited by guanylate cyclase inhibitors, by antagonists of protein kinase G (G-kinase), and by KT-5823, an inhibitor of G-kinase. Although no direct effect of endothelins on cGMP level could be established, these results suggest that the effect of these endothelins on migration is mediated by cGMP, and that the effect of cGMP proceeds via a G-kinase. There was little or no effect of guanylate cyclase inhibitors and G-kinase antagonists on endothelin-2-activated migration, indicating that the role of cGMP and G-kinase in endothelin-2-induced activation was either absent or at least different from that of the other endothelins. As compared with other activators, the role of G-kinase in formyl-methionyl-leucyl-phenylalanyl-(fMLP-)activated migration resembled that of endothelin-activated migration, while the role of G-kinase in interleukin-8- or leukotriene B_4-activated migration was less pronounced.

Keywords: Neutrophil; Endothelins; Chemotaxis; Migration; cGMP; G-kinase

1. Introduction

Endothelins are a group of vasoactive peptides, which possess a large number of actions. They are produced by vascular endothelial cells and a number of other cell types. In addition to a vasoconstrictive effect, they have a large number of other effects in several cell types: they increase intracellular Ca^{2+} level, and they activate protein kinase C and phospholipase C (Rubanyi and Polokoff, 1994; Simonson, 1993; Änggård et al., 1990). The levels of endothelins, especially of endothelin-1, in plasma are enhanced after clinical insults as myocardial infarction, septic shock, diabetes, and kidney failure (Battistini et al., 1993).

A number of neutrophil functions are affected by endothelins. Endothelin-1 primed neutrophils for enhanced superoxide production by chemotactic peptide (Ishida et al., 1990). Endothelin-1 and endothelin-2 induced an enhancement of the concentration of cytosolic free Ca^{2+} (Elferink and De Koster, 1994, 1996; Lopez Farre et al., 1991). The enhancement induced by endothelin-1 was inhibited by L-arginine, which was interpreted as an involvement of NO-related systems (Lopez Farre et al., 1991). Recently we and others found that endothelins modified neutrophil migration (Elferink and De Koster, 1994, 1995, 1996; Wright et al., 1994). The effect was dependent on the concentration: low concentrations of endothelin stimulated migration, while high concentrations inhibited migration stimulated by chemotactic activators.

There are several indications that cyclic GMP is important in neutrophil migration. Agents which cause an increase of cGMP level of neutrophils often stimulate migration (see Elferink and VanUffelen, 1996 for review); there are however, some exceptions. Addition of low concentrations of cGMP to permeabilized cells caused an increase of migration, however, higher cGMP concentrations inhibited chemotaxis (Elferink and De Koster, 1993). There is circumstantial evidence from literature, that endothelins might exert a number of cell functions via a change in the level of cyclic nucleotides. Endothelin-1 gave an increase of cGMP production in a neuronal cell line via production of nitric oxide (Reiser, 1990). Endothelin-3 enhanced cGMP level in mesangial cells (Owada et al., 1994), and rat cerebellar slices (Shraga-Levine et al., 1994), possibly via nitric oxide formation. Endothelin-stimulated tyrosine phosphorylation in platelets was inhibited by cGMP and

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especially by cAMP (Catalán et al., 1995). It was also found that (only) in the presence of L-arginine, endothelin-1 induced an enhancement of cGMP level in neutrophils (Riesco et al., 1993).

In this study we considered the role of cGMP in stimulation of neutrophil migration by endothelins, and especially in the target system of the cyclic nucleotides: cGMP-dependent protein kinase (G-kinase). We could not detect any changes in cGMP level in neutrophils, but this might be associated with the difficulty in measuring small changes in cGMP level, rather than an absence of a change. Furthermore, we have found that cGMP level changes in the neutrophil, induced by other agents, was rapidly regulated to resting level so that a level found is more indicative for the experimental procedure followed to measure cGMP than a reflection of its importance. In addition, there are some indications that the effect of cyclic nucleotides is compartmentalized. This implicates that the absence of a measured increase of cGMP level does not necessarily mean that cGMP is not involved in a given process. For that reason we made a different approach to study the role of cGMP in endothelin-stimulated migration by using specific inhibitors of guanylate cyclase, and specific antagonists of G-kinase.

2. Materials and methods

2.1. Isolation of human neutrophils

Neutrophils were isolated from the buffy coat of blood of healthy donors. The buffy coat (5 ml) was diluted with a four-fold volume of heparinized medium, and layered on top of Ficoll-amidotrizoate ($d = 1.077$). After centrifugation (20 min, $580 \times g$) the pellet was resuspended in 5 ml heparinized medium, and starch (6% poly(*O*-2-hydroxyethyl)starch in 0.9% NaCl, 4 ml) was added to sediment erythrocytes. After sedimentation the neutrophil-containing supernatant was collected and centrifuged (3 min, $480 \times g$). The remaining erythrocytes were removed by hypotonic hemolysis, and the neutrophils were suspended in medium. The cells consisted of more than 95% of neutrophils, and were more than 99% viable, as determined with Trypan blue exclusion. 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+} during the experiments. The final cell suspension during the experiments contained 3×10^6 neutrophils per ml.

2.2. Migration measurements

Cell migration was measured with the Boyden chamber technique (Elferink and De Koster, 1993), as described by Boyden (1962), and modified by Zigmond and Hirsch (1973). 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, followed by incubation for 35 min at 37°C. After migration the filters were fixed and stained and the distance travelled in micrometers into the filter was determined according to the leading front technique (Zigmond and Hirsch, 1973). Chemotactic assays were carried out in duplicate and the migration distance of the neutrophils was determined at five different filter sites.

2.3. Electroporation of neutrophils

Neutrophils were electroporated according to the method of Grinstein and Furuya (1988), with minor modifications. The electro-permeabilization procedure was carried out at room temperature. When permeabilization was carried out at 0°C the cells were not able to 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% bovine serum albumin), were placed in the cuvette of a BioRad Gene Pulser. The cells were exposed to two discharges of 14.75 kV cm^{-1} from a 25 μF capacitor. Between the two discharges the cell suspension was stirred with a plastic pipette. After permeabilization and mixing 0.2 ml of the suspension were placed in the upper compartment of the Boyden chamber. When electroporated neutrophils were compared with control neutrophils the latter cells were also suspended in permeabilization buffer.

2.4. Cyclic GMP assay

Neutrophils (final concentration 2×10^7 cells per ml) were exposed to reagents at 37°C for the indicated time. Subsequently 1 ml 3.5% perchloric acid was added, and the resulting mixture was stored overnight in the freezer. The solution was neutralized by adding 0.5 ml saturated (22°C) $NaHCO_3$. After 10 min the mixture was centrifuged for 3 min at 2000 rpm. To 100 μl of the supernatant 50 μl of radioactive cGMP and 50 μl antibody from the radioimmunoassay kit (Amersham, England) were added. After mixing, the solution was kept on ice for 90 min, after which 1 ml icecold 60% $(NH_4)_2SO_4$ was added. The solution was mixed, and kept on ice further for 10 min, and centrifuged. The supernatant was carefully removed, and the residue was taken up in 1.1 ml water. One milliliter of the solution was mixed with 4 ml scintillation fluid (299, Packard), and counted in the scintillation counter. Known amounts of cGMP were treated in the same way as the cells, and were used for the calibration curve.

2.5. Statistical analysis

All mean values for the chemotactic assays are arithmetical means ± S.E.M. of four different experiments. In those cases where random migration or activated migration was considerable different for different cell batches, values

were expressed as percentage of control. Significances were calculated with Student's *t*-test for paired data; a value of $P < 0.05$ was considered as statistically significant.

2.6. Materials

The endothelins were from Sigma (St. Louis, MO, USA). The compound LY-83583 (6-anilino-5,8-quinolinedione) was obtained from Calbiochem (Bierges, Belgium). The G-kinase inhibitor KT-5823 was obtained from Calbiochem. The G-kinase antagonists R_p-pCPT-cGMPS (R_p-8-(4-chlorophenylthio-guanosine-3′,5′-cyclic monophosphorothioate) and R_p-Br-PET-cGMPS (R_p-8-bromo-β-phenyl-1,N^2-ethenoguanosine-3′,5′-cyclic-monophosphorothioate) were from Biolog (Bremen, Germany). The other chemicals were obtained from Sigma and were of the highest purity available.

3. Results

3.1. The role of cGMP formation

All endothelins caused activation of migration, as found previously by us and others (Elferink and De Koster, 1994, 1995, 1996; Wright et al., 1994); stimulation was predominantly chemokinetic for endothelin-1, and chemotactic for endothelin-2 and endothelin-3. Optimal stimulation was 4×10^{-9} M for endothelin-1 (present in both compartments of the Boyden chamber), 4×10^{-10} M for endothelin-2 and 1.6×10^{-8} M for endothelin-3 (endothelin-2 and endothelin-3 present in the lower compartment only); these concentrations were used in all experiments, unless otherwise indicated. The results obtained with human neutrophils strongly resembled those of rabbit neutrophils; only the effect of endothelin-2 was somewhat less (in a quantitative sense) for human neutrophils than it was for rabbit neutrophils (Elferink and De Koster, 1994, 1995, 1996). A number of experiments were carried out with electroporated cells, because some of the agents tested did not readily cross the intact cell membrane. Neutrophils retained their ability to migrate when they were electroporated, allowing the introduction of chemicals into the cell interior in a given concentration. Electroporation had to be carried out at room temperature, and both random migration and activated migration were somewhat lower than in intact cells.

LY83583, an inhibitor of guanylate cyclase, strongly inhibited endothelin-1 and endothelin-3-activated migration, but had little effect on endothelin-2-activated migration. 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), another inhibitor of guanylate cyclase, also strongly inhibited endothelin-1 and endothelin-3-activated migration, but only in electroporated cells (Fig. 1). In intact cells the inhibition by ODQ was less and variable (results not shown), which could mean that the compound had some difficulty in entering the neutrophil; for that reason both ODQ and LY83583 were tested on electroporated neutrophils.

3.2. G-kinase antagonists and inhibitors

Two specific antagonists of G-kinase, R_p-Br-PET-cGMPS and R_p-pCPT-cGMPS, strongly inhibited endothe-

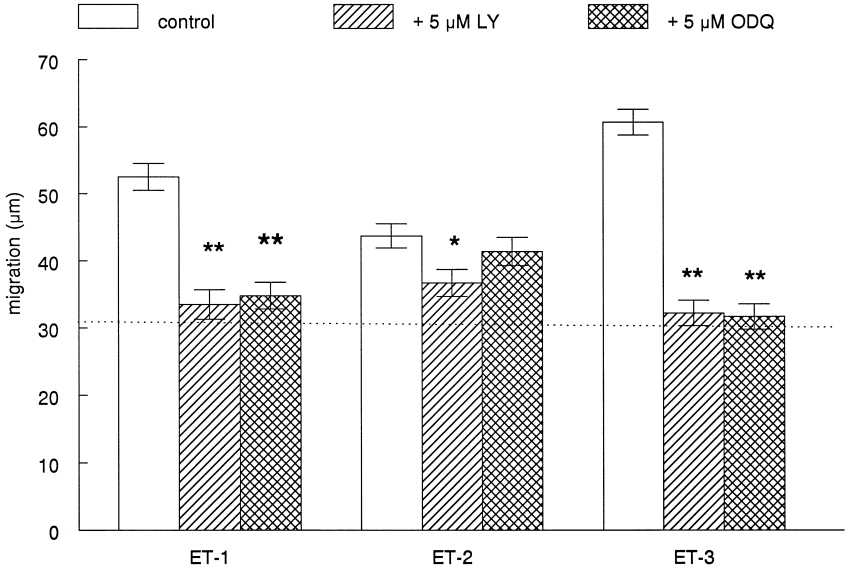

Fig. 1. The effect of the guanylate cyclase inhibiters LY83583 and ODQ on migration of electroporated neutrophils activated by endothelins. The level of random migration is indicated with a dotted line. $*$: $P < 0.05$; $**$: $P < 0.01$, as compared with activator without inhibitor. Concentrations: endothelin-1: 4×10^{-9} M (present in both compartments of the Boyden chamber), endothelin-2: 4×10^{-10} M; endothelin-3: 1.6×10^{-8} M (endothelin-2 and endothelin-3 present in the lower compartment only).

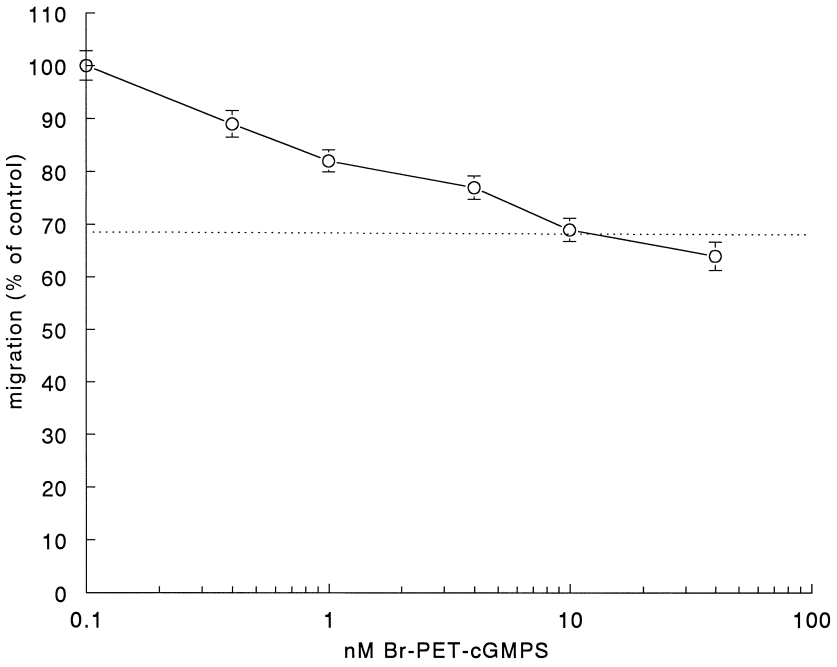

Fig. 2. Inhibition of ET-1-activated migration of neutrophils by increasing concentrations of the G-kinase antagonist R_p-Br-PET-cGMPS. The dotted line represents the level of random migration. The concentration of endothelin-1 was 4×10^{-9} M (present in both compartments of the Boyden chamber).

lin-1 and endothelin-3-activated migration, but had little (Br-PET-cGMPS) or no (pCPT-cGMPS) effect on endothelin-2-activated migration (Figs. 2 and 3). The effect of G-kinase antagonists (the term antagonist is reserved for compounds which have structural resemblance with cGMP) on endothelin-activated migration was compared with that on migration stimulated by other chemotactic activators. Migration activated by fMLP was strongly inhibited by Br-PET-cGMPS. In contrast, interleukin-8- or leukotriene B_4-activated migration was only slightly affected under the same conditions (Fig. 4); even a tenfold higher concentration of Br-PET-cGMPS gave little additional inhibition (results not shown). KT-5823, an inhibitor of G-kinase (Jin et al., 1993; Kase et al., 1987), did inhibit endothelin-1 and endothelin-3-activated migration. fMLP-activated migration was also strongly inhibited, while interleukin-8-

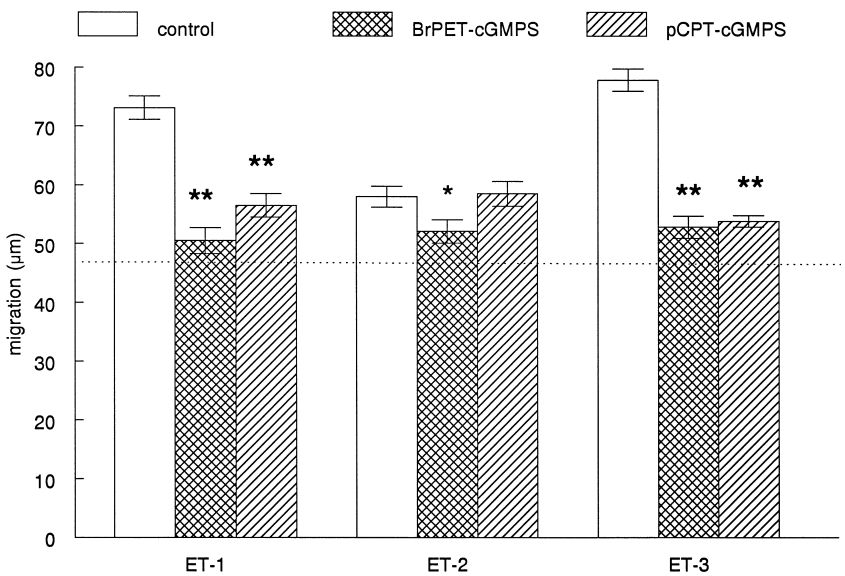

Fig. 3. The effect of 10 nM Br-PET-cGMPS and 10 nM pCPT-cGMPS on migration of neutrophils activated by endothelins. The level of random migration is indicated with a dotted line. $*$: $P < 0.05$; $**$: $P < 0.01$, as compared with activator without antagonist.

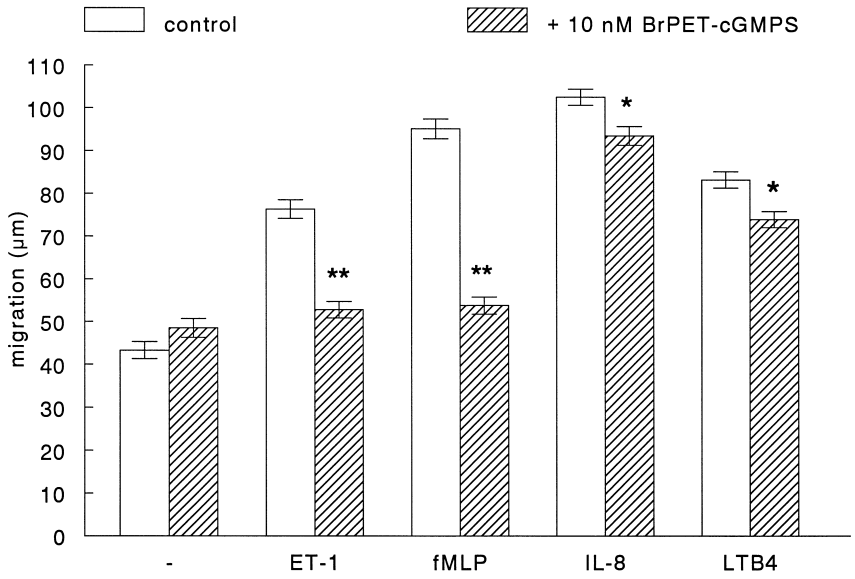

Fig. 4. The effect of Br-PET-cGMPS on migration of neutrophils stimulated by different activators. $*$: $P < 0.05$; $**$: $P < 0.01$, as compared with activator without antagonist. Concentrations (optimal concentrations, activator present in the lower compartment of the Boyden chamber): fMLP: 10^{-9} M; interleukin-8 (IL-8): 4.10^{-9} M; leukotriene B_4 (LTB_4): 2.10^{-9} M.

activated migration was less inhibited, and leukotriene B_4-activated migration was hardly affected (Fig. 5).

3.3. Cyclic GMP measurements

The average control value for cGMP level of resting cells was variable, and depended on the batch of cells. Values varied from 2.29 ± 0.46 to 4.48 ± 0.32 pmol$/10^7$ cells. Because of this variation, values were expressed as a percentage of control. Cells exposed to 4×10^{-9} M endothelin-1 for 0, 1, 2, 3 or 5 min were not significantly different from control. This observation was also made by Riesco et al. (1993). As a positive control *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (40 μM) was used; this gave an increase of cGMP level with $170 \pm 12\%$ after exposure for 2 min.

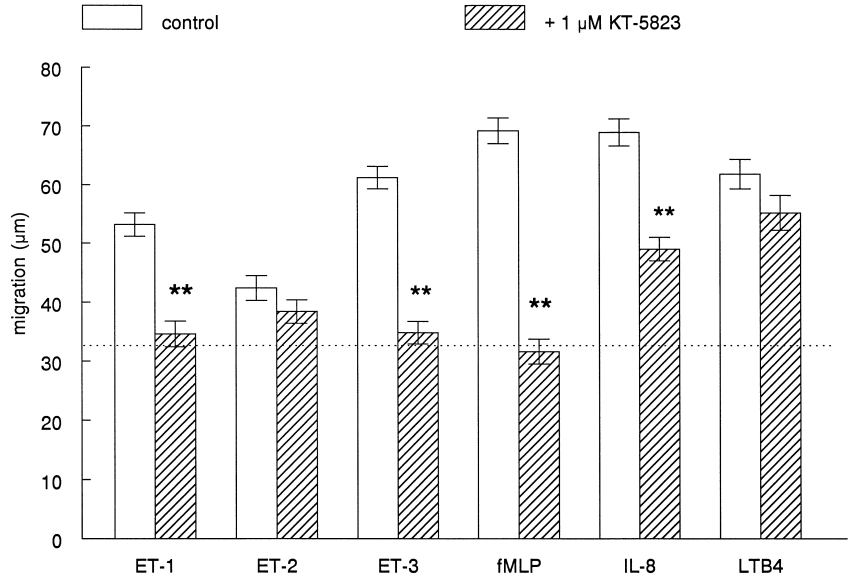

Fig. 5. The effect of the G-kinase inhibitor KT-5823 on migration of electroporated neutrophils activated by endothelins, fMLP, interleukin-8 (IL-8), and leukotriene B_4 (LTB4). The level of random migration is indicated with a dotted line. $*$: $P < 0.05$; $**$: $P < 0.01$, as compared with activator without inhibitor. Concentrations: endothelin-1: 4×10^{-9} M (present in both compartments of the Boyden chamber), endothelin-2: 4×10^{-10} M; endothelin-3: 1.6×10^{-8} M; fMLP: 10^{-9} M; interleukin-8 (IL-8): 4.10^{-9} M; leukotriene B_4 (LTB_4): 2.10^{-9} M (endothelin-2, endothelin-3, fMLP, interleukin-8 and leukotriene B_4 present in the lower compartment only).

4. Discussion

There are several indications in literature that the effects of endothelins, especially those which proceed via the endothelin ET_B receptor, are mediated by cGMP (Riesco et al., 1993; Owada et al., 1994; Dockrell et al., 1996; Gagnet et al., 1996). We found previously that both the endothelin ET_A as well as the endothelin ET_B receptor are involved in activation of neutrophil migration by endothelins (Elferink and De Koster, 1995, 1996). Only under specific conditions (in the presence of L-arginine) an endothelin-induced cGMP enhancement can be found in neutrophils; under normal conditions, an increase of cellular cGMP can not be observed when endothelin-1 is applied to neutrophils (Riesco et al., 1993). The failure to measure cGMP enhancement after application of endothelins to neutrophils was confirmed by us. However, the failure to measure an increase in cGMP level does not mean that under those experimental conditions cGMP is not involved: the enhancement of cGMP could be below the detection level, could be compartmentalized or could be rapidly regulated to basal level. The results show that cGMP plays an important role in migration activated by endothelin-1 and endothelin-3. This may be concluded from the observation that inhibitors of guanylate cyclase also inhibit migration activated by these endothelins, and from the inhibitory effect of the G-kinase antagonists and the G-kinase inhibitor KT-5823 on endothelin-activated migration. In contrast with endothelin-1 and endothelin-3, the role of cGMP in endothelin-2-activated migration is different from, and less pronounced than that of endothelin-1 and of endothelin-3. Because endothelin-1 is chemokinetic, and both endothelin-2 and endothelin-3 are chemotactic, it means that in this case no clear difference in the signalling between chemotaxis and chemokinesis exists.

The cGMP-mediated effect of endothelin-1 and of endothelin-3 proceeds via a G-kinase, as appears from the inhibition of migration by G-kinase antagonists and by KT-5823. Especially the G-kinase antagonists R_p-Br-PET-cGMPS and R_p-pCPT-cGMPS are selective inhibitors of G-kinase without major effects on cAMP-dependent protein kinase (Butt et al., 1994, 1995). The presence of G-kinase in neutrophils has been established by Pryzwansky et al. (1990) who found that this enzyme was transiently co-localized to intermediate filaments, and phosphorylated vimentin upon exposure of neutrophils to fMLP. For fMLP the role of cyclic nucleotides has been studied previously. Besides cGMP and G-kinase, also cAMP and A-kinase are positively involved in regulating migration (Ydrenius et al., 1997). With regard to the involvement of G-kinase there are some remarkable resemblances and differences with other chemotactic agents. A resemblance is apparent between the role G-kinase in endothelin-1 or endothelin-3 as compared with fMLP-activated migration. Activation of migration by two other important physiological chemotactic agents, interleukin-8 and leukotriene B_4, are only slightly affected by G-kinase antagonists. Here the role of G-kinase is less if present, indicating that the G-kinase is of differential importance for chemoattractant-stimulated migration. Because fMLP. interleukin-8 and leukotriene B_4 all act via the same type of receptor and are mediated via a pertussis toxin-sensitive G-protein, it seems likely that the G-kinase involvement is downstream of the receptor events.

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