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The stimulation of human neutrophil migration by angiotensin II: its dependence on Ca^{2+} and the involvement of cyclic GMP

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1 Angiotensin II had a bimodal effect on human neutrophil migration. Low concentrations of angiotensin II stimulated random migration. At a concentration of 10^{-10} M it caused a maximal increase of migration; migration increased from 47.2 ± 2.1 μm in the absence of angiotensin II, to 73.1 ± 2.2 μm with 10^{-10} M angiotensin II present in the lower compartment of the Boyden chamber ($n=5$, $P<0.001$). Stimulation of migration by angiotensin II was partly chemotactic and partly chemokinetic. Angiotensin II concentrations of 10^{-8} M and higher inhibited chemotactic peptide-stimulated chemotaxis.

2 The stimulant effect of angiotensin II on migration was completely dependent on extracellular Ca^{2+}. In the presence of 1 mM Ca^{2+}, angiotensin II stimulated migration to 76.1 ± 1.7 μm, while migration in the absence of Ca^{2+} was 42.2 ± 1.9 μm ($n=4$, $P<0.001$). Different types of calcium channel blockers either moderately or strongly inhibited angiotensin II-activated migration. Stimulation of migration by angiotensin II in intact cells required higher concentrations of Ca^{2+} than in electroporated cells. This supports the view that there is an influx of Ca^{2+} through the plasma membrane, and a requirement of calcium for an intracellular target.

3 Angiotensin II-stimulated migration was inhibited by pertussis toxin; from 71.6 ± 2.0 μm in the absence, to 43.6 ± 1.5 μm in the presence of pertussis toxin ($n=4$, $P<0.001$). Migration of electroporated neutrophils stimulated by angiotensin II was synergistically enhanced by GTPγS. This suggests that one or more G-proteins are involved in the activating effect of angiotensin II.

4 Inhibitors of soluble guanylate cyclase and antagonists of cyclic GMP-dependent kinase strongly inhibited the activating effect of angiotensin II. The results suggest that the activating effect of angiotensin II is mediated by cyclic GMP and by cyclic GMP-dependent kinase.

Keywords: Angiotensin II; neutrophil migration; cyclic GMP; G-kinase; calcium; chemotaxis; chemokinesis; G-protein

Introduction

Migration by neutrophils is an important function of these cells, allowing them to reach tissues where chemotactic agents are released. Because the neutrophil plays a central role in the defence reaction of the body as well as in inflammation, modulation of migration might also affect these processes.

A number of physiologically active peptides with a primary effect on the vascular system, are of importance for the immune system because they have effects on neutrophils and other phagocytes. Vasoactive peptides such as endothelins and atrial natriuretic peptide have priming actions on the respiratory burst of neutrophils (Ishida *et al*., 1990; Widermann *et al*., 1992). We and others have found that these peptides modulate migration (Wright *et al*., 1994; Elferink & de Koster, 1994; 1995a,b; 1996). Recently, it was found that angiotensin III, which acts on the same receptor as angiotensin II, was chemotactic for neutrophils and that the other angiotensins have the same effect (Yamamoto *et al*., 1993).

There is some evidence that guanosine 3′:5′-cyclic monophosphate (cyclic GMP) plays a role in the effect of endothelins, natriuretic peptide and angiotensin II. For natriuretic peptide this seems evident, because it is a well-known activator of particular guanylate cyclase. For the endothelins and the angiotensins the evidence for the involvement of cyclic GMP is rather circumstantial. Angiotensin II increases cyclic GMP content in endothelial cells (Buonassisi & Venter, 1976), in neuroblastoma cells (Chaki & Inagami, 1992) and in the rat carotid artery (Caputo *et al*., 1995). Cyclic GMP has been found to be involved in neutrophil migration (Elferink & de Koster, 1993). Agents which cause an increase of cyclic GMP, mostly cause a potentiation of chemotactic migration. In electroporated cells cyclic GMP strongly increased random migration at low concentrations, and inhibited chemotactic peptide-activated migration at high concentrations. The aim of our study was two fold. First, we wanted to characterize the effect of angiotensin II on human neutrophil migration with regard to the nature of the effect and the role of calcium. Secondly, we wanted to determine the possible involvement of G-proteins and of cyclic GMP in the effect of angiotensin II on neutrophil migration.

Methods

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 heparin-treated medium and layered on top of Ficoll-amidotrizoate (d = 1.077). After centrifugation (20 min, 580 *g*) the pellet was resuspended in 5 ml heparin-treated 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 *g*). The remaining erythrocytes were removed by hypotonic haemolysis and the neutrophils suspended in medium. The cells consisted of more than 95% 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 ml^{-1}.

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Migration measurements

Cell migration was measured with the Boyden chamber technique (Elferink & 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 μm into the filter was determined according to the leading front technique (Zigmond & Hirsch, 1973). Chemotactic assays were carried out in duplicate and the migration distance of the neutrophils was determined at five different filter sites.

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 ml^{-1}) 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) 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 the cell suspension had been permeabilized and mixed, 0.2 ml 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.

Determination of intracellular calcium

Neutrophils (1×10^7 cells ml^{-1}) were incubated in medium with 1 μM Fura-2/AM for 30 min at 37°C, in the presence of 1 mM Ca^{2+}. After being washed the cells were resuspended in medium and used at a concentration of 3×10^6 cells ml^{-1}. Fluorescence measurements were performed in the medium as described before, with 1 mM Mg^{2+} present and with or without the additional presence of 1 mM Ca^{2+}. Fura-2 fluorescence was measured in a Perkin Elmer LS50B fluorescence spectrophotometer, equipped with a thermostated cuvette compartment and a mixing device. Fluorescence (emission wavelength 510 nm) was recorded at two excitation wavelengths (340 nm and 390 nm), and the data were used to calculate the concentrations of cytoplasmic free calcium (Grynkiewicz *et al*., 1985). As a positive control we used 10 nM formyl methionyl leucyl phenylalanine (fMLP), which gives a strong signal, both in the absence but especially in the presence of extracellular calcium.

Cyclic GMP assay

Neutrophils (final concentration 2×10^7 cells ml^{-1}) 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 r.p.m. To 100 μl of the supernatant 50 μl of radioactive cyclic GMP and 50 μl antibody from the radioimmunoassay kit (Amersham, U.K.) were added. After the solution had been mixed it was kept on ice for 90 min, after which time 1 ml ice-cold 60% $(NH_4)_2SO_4$ was added. The solution was mixed, kept on ice for a further 10 min and centrifuged. The supernatant was carefully removed and the residue taken up to 1.1 ml water; 1 ml of this solution was mixed with 4 ml scintillation fluid (299, Packard) and counted in the scintillation counter. Known amounts of cyclic GMP were treated in the same way as the cells and were used for the calibration curve.

Cyclic AMP assay

Neutrophils (final concentration 2×10^7 cells ml^{-1}) 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 r.p.m. to 50 μl of the supernatant 50 μl of radioactive cyclic AMP and 100 μl antibody from the radioimmunoassay kit (Amersham, U.K.) were added. After the solution had been mixed it was kept on ice for 120 min, after which time 100 μl ice-cold charcoal suspension was added. The solution was mixed, kept on ice for a further 2 min and centrifuged. To 200 μl of the supernatant 10 ml scintillation fluid (299, Packard) was added and the mixture was counted in the scintillation counter. Known amounts of cyclic AMP were treated in the same way as the cells and were used for the calibration curve.

Statistical analysis

All values for the chemotactic assays are arithmetical means $\pm$ s.e.mean of four different experiments. In those cases where random migration or activated migration was considerably different for different cell batches, values are 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.

Materials

Angiotensin II, formyl-methionyl-leucyl-phenylalanine (fMLP), and methylene blue were purchased from Sigma Chemical Co. Interleukin-8 (IL-8) was obtained from R & D Systems Europe (Abingdon, U.K.). The compound LY-83583 (6-anilino-5,8-quinolinedione) was obtained from Calbio chem (Bierges, Belgium). The G-kinase antagonists R_p-pCPT-cGMPS (R_p-8-(4-chlorophenylthio-guanosine-3′, 5′-cyclic monophosphorothioate) R_p-Br-cGMPS (R_p-8-(4-bromoguanosine-3′,5′-cyclic monophosphorothioate) were from Biolog (Bremen, Germany). The other chemicals were obtained from Sigma Chemical Co (Saint Louis, Missouri, U.S.A.) and were of the highest purity available.

Results

Activation of migration

Angiotensin II caused a strong increase in neutrophil migration when it was present in the lower compartment of the Boyden chamber (Figure 1). The activation occurred over a rather small concentration range. The concentration-response curve was bell-shaped: up to a concentration of 10^{-10} M angiotensin II increased migration, at higher concentrations migration decreased sharply. Stimulation of migration occurred when angiotensin II was present in either the lower compartment the upper compartment or both compartments. However, for all concentrations of angiotensin II the strongest stimulation occurred when angiotensin II was present in the lower compartment only (Table 1).

Inhibition of fMLP- or IL-8-induced chemotactic migration

Because of the bimodal character of the stimulation by angiotensin II we considered the possibility that high concentrations of angiotensin II might be inhibitory. Angiotensin II at concentrations of 10^{-8} M and higher inhibited fMLP- and IL-8-stimulated chemotaxis moderately (Figure 2). At a concentration of 10^{-6} M the inhibition of IL-8-activated migration was slightly stronger than that of fMLP-activated migration.

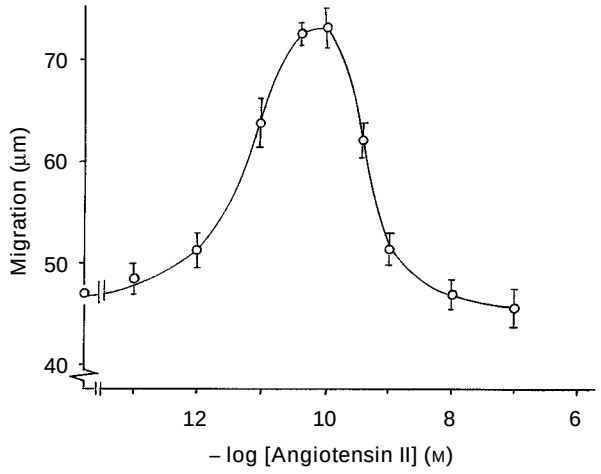

Figure 1 The effect of increasing concentrations of angiotensin II on neutrophil migration. The indicated concentrations of angiotensin II was present in the lower compartment of the Boyden chamber only. Vertical lines show s.e.mean.

Table 1 Dependence of stimulation of migration by angiotensin II on its localization in the Boyden chamber

	Localization and migration (μm)		
Angiotensin concentration M	*Lower*	*Upper and lower*	*Upper compartment*
0		51.7±1.7	
10^{-11}	68.2±1.5	63.3±1.7	58.0±1.8
10^{-10}	76.1±1.9	70.6±2.0	65.9±1.8
5×10^{-10}	61.5±2.0	57.4±1.6	55.7±1.9

The role of Ca^{2+}

The stimulating effect of angiotensin II was absolutely dependent on the presence of extracellular Ca^{2+}; in the absence of this ion stimulation was absent (Figure 3). This was in contrast to the effect of the chemotactic peptide fMLP, which stimulated migration by a mechanism largely independent of extracellular Ca^{2+}. Mg^{2+} could not replace calcium in the stimulation of migration by angiotensin II, but slightly increased both random and stimulated migration when it was present together with Ca^{2+}. Different types of calcium channel blockers inhibited angiotensin-activated migration, though inhibition was not complete (Table 2).

We did not find that angiotensin II induced an increase of cytoplasmic free calcium, as measured by the Fura-technique, under conditions where fMLP and IL-8 gave a strong fluorescence peak. Different concentrations of angiotensin II (10^{-10}, 10^{-9}, 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M) were tested, but none induced an increase (results not shown).

Electroporated cells retained their ability to migrate when electroporation was carried out at room temperature and angiotensin II stimulated migration of electroporated cells. Stimulation of migration by angiotensin II in electroporated cells strongly increased when calcium concentrations in the low micromolar range were applied. Stimulation of migration by angiotensin II in intact cells required higher concentrations of Ca^{2+} (Figure 4).

For the inhibition of fMLP- or IL-8-activated chemotaxis by angiotensin II, Ca^{2+} also played a role but this was less clear. Inhibition of fMLP-activated chemotaxis was inhibited less in the absence of Ca^{2+} than in its presence, while IL-8-activated chemotaxis was not inhibited at all in the absence of extracellular Ca^{2+} (Table 3).

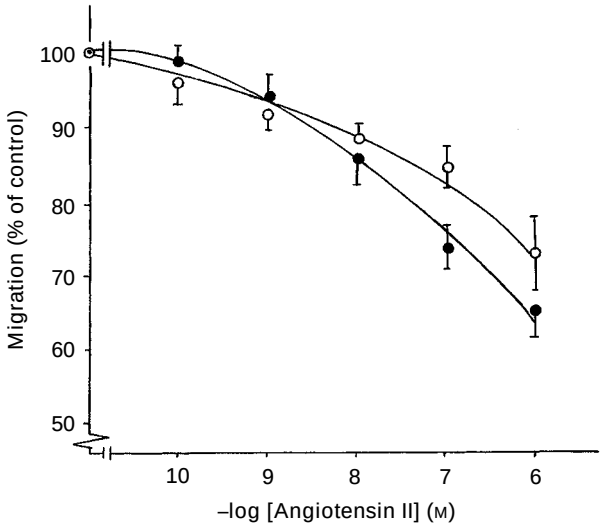

Figure 2 Inhibition of fMLP- (○)- and IL-8 (●)-induced chemotaxis by relatively high concentrations of angiotensin II. Values are expressed as a percentage of the migration in the absence of angiotensin II (with either 10^{-9} M fMLP or 4×10^{-9} M IL-8 present in the lower compartment of the Boyden chamber). Vertical lines show s.e.mean.

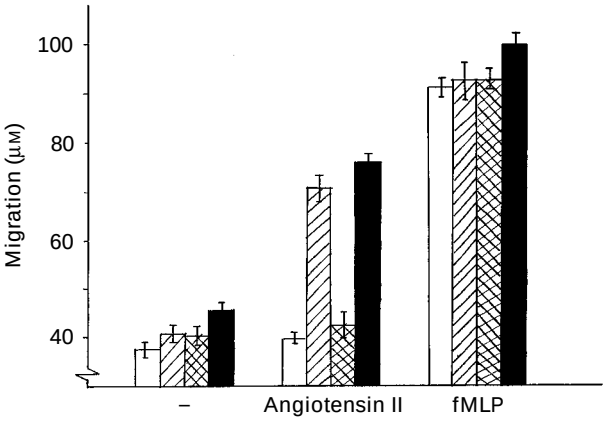

Figure 3 The effect of divalent cations on random migration (–), angiotensin-activated migration, and fMLP-activated migration. Angiotensin II (10^{-10} M) and the chemotactic peptide fMLP (10^{-9} M) were present in the lower compartment. Open columns, 1 mM EDTA; hatched columns, 1 mM Ca^{2+} ; cross-hatched columns, 1 mM Mg^{2+} (+50 μM EGTA); solid columns 1 mM Ca^{2+} + 1 mM Mg^{2+}.

Table 2 Inhibition of migration activated by angiotensin II by some calcium channel blockers

	Migration (μm)
Control	72.4±1.9
Verapamil (5 μM)	66.3±2.4
Econazole (20 μM)	66.6±2.2
La^{3+} (20 μM)	59.8±1.9
Gadolinium chloride (0.5 μM)	54.0±2.0
Ni^{2+} (200 μM)	52.9±2.3

Cells were preincubated with the agents indicated for 10 min at 37°C, and then placed in the upper compartment of the Boyden chamber. Angiotensin II (10^{-10} M) was present in the lower compartment of the Boyden chamber. Under the same experimental conditions random migration was 46.8±1.8 μm. All values of the experiments with calcium channel blockers were significantly different ($P<0.001$) from the control value (without calcium blocker).

To determine the requirement for intracellular Ca^{2+}, cells were depleted of intracellular calcium by treatment with relatively high concentrations quin2-AM. Loading of cells with quin2-AM in the absence of Ca^{2+}, followed by chemotaxis in the presence of Ca^{2+}, resulted in nearly complete inhibition of the effect of angiotensin II (Table 4). Loading of cells with quin2-AM in the presence of Ca^{2+}, followed by chemotaxis in the presence of Ca^{2+}, resulted in a strong inhibition of the effect of angiotensin II. Under the same conditions fMLP-activated migration was also inhibited, but to a lesser extent than angiotensin-activated migration (Table 4).

The role of cyclic GMP and cyclic AMP

Pretreatment of neutrophils with two inhibitors of cyclic GMP accumulation, methylene blue or 83583 (Gruetter *et al*., 1981; Schmidt *et al*., 1985; Mülsch *et al*., 1988), resulted in a strong reduction of angiotensin-induced enhancement of migration (Figure 5). Pretreatment of neutrophils with two antagonists of G-kinase (Butt *et al*., 1990; 1994), R_p-pCPT-cGMPS and R_p-Br-cGMPS, had an even stronger inhibitory effect on angiotensin-stimulated migration (Figure 5). Under the conditions of our experiments, we could not demonstrate a significant increased cyclic GMP level of the cells at incubation times of 0.5 min or more with angiotensin II. Neither a significant increased nor a decreased cyclic AMP level was observed after application of 10^{-10} M angiotensin at time intervals of 0.5, 1 and 2 min (results not shown).

Involvement of G-proteins

Preincubation of neutrophils with pertussis toxin abolished the stimulating effect of angiotensin II. The effect of pertussis toxin on angiotensin II activation of migration resembled its effects on fMLP- or IL-8-activated migration, where pertussis toxin inhibited activation equally (Table 5), though the effect of fMLP was less affected than that of IL-8. GTPγS increased migration of electroporated neutrophils. When angiotensin II (either a suboptimal or optimal concentration) was combined with GTPγS, a synergistic increase of migration was observed (Table 6). In this regard, angiotensin II resembled the effect of suboptimal concentrations of fMLP, which also acted synergistically with GTPγS (but differed from protein kinase C activators, which caused an additional effect with GTPγS

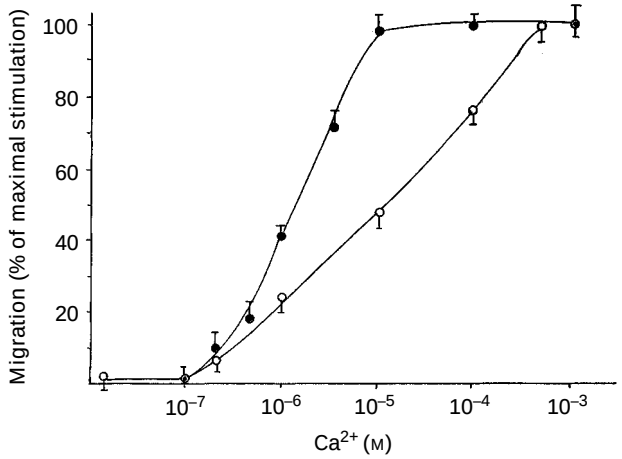

Figure 4 The effect of increasing calcium concentrations on stimulation of migration by angiotensin II in (–●–) electroporated cells and (–○–) intact cells. Migration is expressed as a percentage of maximal stimulation, thus migration in the presence of angiotensin II and 1 mM Ca^{2+} minus the value for random migration. The random migration for electroporated cells was 27.1 ± 1.8 μm and migration in the presence of angiotensin II and 1 mM Ca^{2+} was 66.0 μm. The random migration for intact cells was 45.1 ± 1.8 μm and migration in the presence of angiotensin II and 1 mM Ca^{2+} was 75.5 ± 2.1 μm. Calcium concentrations lower than 100 μM were established with calcium-EGTA buffers, as described by Tatham and Gomperts (1990).

Table 3 The effect of extracellular Ca^{2+} on inhibition of fMLP- or IL-8-induced chemotaxis by angiotensin II

	Migration (μm)	
	Control	*+ Angiotensin II 1 μM*
Ca^{2+}	48.0 ± 1.9	50.4 ± 2.1
Ca^{2+}, fMLP	97.5 ± 2.0	73.7 ± 1.8
Ca^{2+}, IL-8	104.2 ± 1.8	74.4 ± 2.0
EGTA	43.7 ± 1.9	42.9 ± 2.1
EGTA, fMLP	90.8 ± 2.0	79.8 ± 1.9
EGTA, IL-8	99.3 ± 1.9	99.4 ± 2.1

Cells were preincubated without or with 1 μM angiotensin II ml^{-1} for 10 min, in the presence of 1 mM Ca^{2+} or 1 mM EGTA and subsequently placed in the upper compartment of the Boyden chamber. In the lower compartment no activator (Control), fMLP (10^{-9} M) or IL-8 (4×10^{-9} M) were present.

Table 4 Inhibition of angiotensin II- or fMLP-activated migration in Ca^{2+} -depleted cells

	Migration (μm)		
	Control	*Angiotensin II*	*fMLP*
–	46.4 ± 1.9	80.9 ± 1.8	97.8 ± 2.1
Quin2-AM (loaded in the absence of Ca^{2+})	44.9 ± 2.0	49.4 ± 1.9	70.4 ± 1.9
Quin2-AM (loaded in the presence of Ca^{2+})	45.8 ± 2.1	59.4 ± 2.0	81.8 ± 1.4

Cells were loaded in the absence or presence of 1 mM Ca^{2+}, with 10 μM quin2-AM for 30 min at 37°C (in the absence of Ca^{2+}, the incubation medium was supplemented with 50 μM EGTA). Subsequently the cells were centrifuged and added to the new medium. In all cases 1 mM Ca^{2+} was present during the chemotaxis experiments.

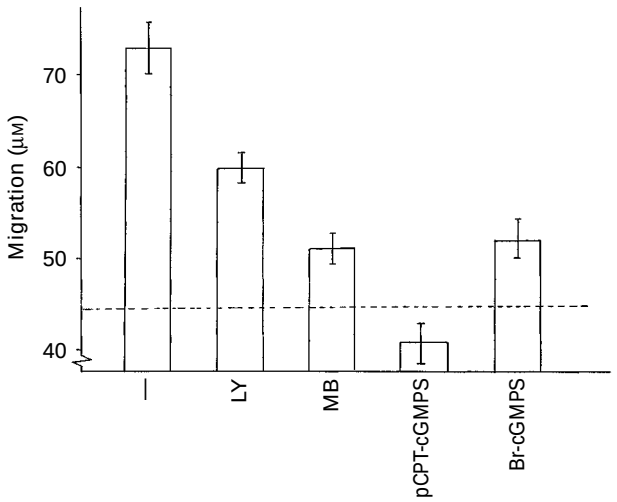

Figure 5 Inhibition of activated neutrophil migration by pretreatment with inhibitors of cyclic GMP accumulation or with G-kinase antagonists. Cells were preincubated without reagents (–), with 5 μM LY 83583 (LY), with 20 μM methylene blue (MB), with 4 nM R_p-pCPT-cGMPs, or with 100 nM R_p-Br-cGMPs for 30 min at 37°C. Subsequently the cells were placed in the upper compartment of the Boyden chamber. In the lower compartment 10^{-10} M angiotensin II was present. The dotted line represents the level of random migration. The values of the experiments with LY 83583, methylene blue, pCPT-cGMPs and Br-cGMPs were all significantly different ($P < 0.001$) from the control value.

Table 5 Inhibition of angiotensin II -activated migration by pertussis toxin

	Migration (μm)	
	Control	*Pertussis toxin-treated*
–	47.9±1.9	39.8±2.1
Angiotensin II	71.6±2.0	43.6±1.5
fMLP	99.3±2.1	59.1±1.9
IL-8	95.2±1.8	41.0±2.1

Cells were preincubated with pertussis toxin 50 ng^{-1} for 30 min and subsequently placed in the upper compartment of the Boyden chamber. In the lower compartment either no activator (–), angiotensin II (10^{-10} M), fMLP (10^{-9} M) or IL-8 (4×10^{-9} M) was present.

Table 6 The effect of GTPyS on angiotensin II and fMLP-activated migration in electroporated and intact cells

	Migration (μm)	
	Control	*+GTPyS*
Electroporated neutrophils:		
–	32.0±1.9	48.6±2.1
Angiotensin II (10^{-12} M)	42.1±2.2	64.9±1.8
Angiotensin II (10^{-10} M)	60.3±2.0	79.5±1.9
fMLP (10^{-11} M)	42.6±2.1	88.8±1.8
fMLP (10^{-9} M)	69.1±1.8	44.3±2.2
Intact neutrophils		
–	46.8±1.9	48.9±2.1
Angiotensin II (10^{-10} M)	72.4±2.1	54.1±1.6
fMLP (10^{-9} M)	89.1±2.1	61.2±2.2

Cells were electroporated in the presence of GTPyS and subsequently placed in the upper compartment of the Boyden chamber. The indicated concentration (either suboptimal or optimal) of angiotensin II or fMLP was present in the lower compartment. The concentration of GTPyS was 50 μM.

(Boonen *et al*., 1993). In contrast, whereas the effects of optimal concentrations of fMLP were inhibited by GTPγS, the migration induced by both suboptimal and optimal concentrations of angiotensin II was enhanced by GTPγS. Angiotensin- or fMLP-activated migration of intact cells was inhibited by GTPγS.

Discussion

The results show that, dependent on the concentration, angiotensin II may have a stimulating or inhibitory effect on neutrophil migration. The stimulating effect strongly resembled that of angiotensin III, as described previously (Yamamoto *et al*., 1993). The effect is bimodal, with an maximal stimulating effect at 10^{-10} M, while the experiments with varying concentrations in the upper and lower compartment of the Boyden chamber showed that the activating effect is composed of a chemokinetic and a chemotactic component. This may be concluded from the observation that the strongest stimulation occurred when angiotensin II was present in the lower compartment only, when the concentration of angiotensin II in the filter was lower than that applied in the lower compartment.

The signal transduction pathways which regulate neutrophil migration can proceed without a change in cytosolic free calcium or an influx of extracellular calcium. This is apparent from the fMLP-activated migration of intact cells, which can proceed in the absence of extracellular calcium, and from the fMLP-activated migration of electroporated cells, which can migrate in a calcium-free medium (Elferink *et al*., 1992a). The activating effect of angiotensin II on migration was found to be strongly calcium-dependent. Somewhat surprisingly, angiotensin II did not induce a measurable increase of cytoplasmic free calcium. In other cell types angiotensin II is known to increase the cytoplasmic free calcium concentration. The observation that at its maximal stimulating concentration angiotensin II did not increase cytosolic free calcium does not mean that there is no influx of Ca^{2+} at all. The fura-fura/Ca^{2+} system functions as a calcium buffer, making it impossible to see small changes in cytosolic free calcium. In a comparable situation, we found that, in neutrophils, endothelin-2 causes no increase in cytoplasmic free calcium at a concentration where migration is enhanced. However, for endothelin-2 we demonstrated that intracellular calcium, derived from the influx of extracellular calcium, was required for its effect on migration (Elferink & de Koster, 1996). For angiotensin II there are a few indications that the calcium required for its effect on migration needs to be inside the cell and is not a surface phenomena. The inhibition by calcium channel blockers suggests that calcium influx plays a role. Additional support for this hypothesis is provided by the difference in calcium requirement for angiotensin II activation between electroporated and intact cells. The real calcium concentration in the electroporated cells during the chemotaxis experiment with angiotensin II is not the concentration represented in Figure 4, which is the concentration applied to the cells. The cells close again after a few minutes, after which time the Ca^{2+} concentration will be lowered due to pumping of calcium pumps. However, it is clear that the activation of electroporated cells occurs at lower Ca^{2+} concentrations than in intact cells, indicating an intracellular target. The experiment where intracellular calcium stores were depleted by treatment with quin2-AM shows that in addition to the influx of extracellular Ca^{2+}, release of Ca^{2+} from intracellular stores is important; this also points to an intracellular action of Ca^{2+}. However, the evidence presented is largely indirect, thus the possibility that Ca^{2+} has an extracellular effect, such as a requirement for angiotensin II binding to its putative receptor, cannot be excluded. The nature of the Ca^{2+}-dependent target remains to be discovered. Apparently there is a Ca^{2+}-dependent process which is required for the effect of angiotensin II, but which is not required for fMLP-activated migration. This implies that for the effect of angiotensin II more steps are required than for the classical chemoattractants. However, remarkably we found a comparable situation for the effect of endothelins (Elferink & de Koster, 1996).

In several cell systems G-proteins are involved in the action of angiotensin II, especially for the AT_1 receptor subtype. The inhibition of angiotensin-activated migration by pertussis toxin can be explained as a coupling of the angiotensin II receptor to the signal transduction system via a trimeric G-protein. However, it is possible that there is more than one G-protein involved in the activation of migration and that inhibition by pertussis toxin refers to another G-protein. The involvement of more than one G-protein in the regulation of neutrophil migration has been suggested previously (Boonen *et al*., 1993). One (or more) of them is associated with actin polymerization; the existence of a different G-protein, associated with the fMLP-receptor, has been previously suggested on the basis of the difference between the synergistic stimulation by GTPγS and suboptimal concentrations of fMLP, and the additive effect between GTPγS and protein kinase C activators of migration (Boonen *et al*., 1993). The synergistic enhancement of migration was also found for GTPγS and angiotensin II, which could imply that in both cases, fMLP and angiotensin II, the receptor is coupled to a G-protein, in addition to the G-protein associated with actin polymerization. Because nucleotides like GTPγS have an inhibitory effect on (activated but not random) migration via an interaction with purinoceptors on the cell surface (Table 6, and Elferink *et al*., 1992b), the synergistic stimulation, as shown in Table 6, is probably underestimated and is larger than the data shown. However, a difference was also found between the effects of fMLP and the angiotensin II. For fMLP the effect of a sub-

optimal concentration was enhanced by GTPγS, while the effect of an optimal fMLP concentration was inhibited. For angiotensin II both optimal and suboptimal concentrations were enhanced by GTPγS.

There are some indications which point to an involvement of cyclic GMP in the effect of angiotensin II on neutrophil migration, in spite of the absence of an effect on the cyclic GMP level by angiotensin II. The enhancement of cyclic GMP induced by other agents in the neutrophil is transient and rather small, indicating that the level is rapidly regulated and that small changes are not observed. Unfortunately, this cannot be modulated by phosphodiesterase inhibitors. Furthermore, there are indications that the effects of cyclic nucleotides are compartmentalized (Pryzwanski *et al*., 1990; Harvath *et al*., 1991). Support for the involvement of cyclic GMP is the observation that inhibitors of guanylate cyclase also inhibited angiotensin-activated migration. The strongest evidence for the hypothesis that cyclic GMP mediates the effect of angiotensin on migration, is the inhibition of angiotensin-activated migration by low concentrations of specific antagonists of G-kinase. This not only implies that the effect of angiotensin II on migration is mediated by cyclic GMP, but also that the effect is controlled by G-kinase.

At present the physiological relevance of the effect of angiotensin II (and of other vasoactive peptides which have an effect on migration which resembles that of angiotensin II) remains to be determined. The concentration of angiotensins in the blood is extremely low (about 0.04 nM (Wilkes *et al*., 1991)), but this cannot be related to higher concentrations formed where they are secreted and where they are effective on their target system. Because the K_D of the angiotensin receptors is round 1 nM, it seems that both the stimulating and the inhibitory effects of angiotensin II occur at concentrations which might have some significance for the situation *in vivo*.

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