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Inhibition of Interleukin-8-activated Human Neutrophil Chemotaxis by Thapsigargin in a Calcium- and Cyclic AMP-dependent Way

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ABSTRACT. Chemotactic migration of human neutrophils, induced by interleukin-8 (IL-8) or other activators, was inhibited by thapsigargin in the high nanomolar range. The degree of inhibition depended on the type of activator. Other inhibitors of Ca^{2+}-ATPases associated with intracellular calcium stores, such as cyclopiazonic acid and 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone, equally inhibited IL-8-activated migration. Inhibition of migration by thapsigargin and the other ATPase inhibitors occurred only in the presence of extracellular Ca^{2+}; migration was not inhibited in the presence of EGTA. La^{3+} reversed thapsigargin-induced inhibition to a large degree; other calcium channel blockers gave a partial reversal (econazole, verapamil, and SK&F 96365) or had no effect (gadolinium chloride and Ni^{2+}). Using electroporated cells and Ca buffers, it was shown that inhibition started at about 0.2 μM and was complete at a cytosolic Ca concentration of about 2 μM. It appears that under certain conditions the thapsigargin-induced influx of extracellular calcium, causing relatively high local calcium concentrations, initiates or permits a process which may be detrimental to chemotactic migration. Cyclic AMP (cAMP; adenosine 3',5'-cyclic monophosphate) is probably involved in this process, because thapsigargin increased the cAMP level and cAMP inhibited IL-8-activated migration in a calcium-dependent way. The hypothesis that cAMP is involved in the effect of thapsigargin on migration is supported by the finding that very low concentrations of thapsigargin stimulate neutrophil migration in the absence of other chemoattractants. The results suggest that thapsigargin causes a (compartmentalized) increase in cAMP, which results in a calcium-dependent modulation of migration. BIOCHEM PHARMACOL **59**;4:369–375, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. neutrophil; thapsigargin; chemotaxis; calcium; cAMP

Calcium plays a role in the cellular signal transduction pathways of several neutrophil functions, but its precise role is still obscure [1–4]. The neutrophil may derive its cytosolic calcium from two sources: via influx of extracellular Ca^{2+} or via release of Ca^{2+} from intracellular stores. Some time ago a calciosome, a sarcoplasmic reticulum-like organelle, was identified in neutrophils [5,6]. The difference in Ca^{2+} concentrations between store and cytoplasm is maintained by Ca^{2+} pumps in the form of Ca^{2+}-ATPases. Some agents which specifically inhibit the Ca^{2+}-ATPases of intracellular calcium stores have been identified. Several studies have dealt with the effects of these agents on changes in cytosolic free calcium. Tg,† CPA and DBHQ specifically inhibit the Ca^{2+}-ATPase of endoplasmic or sarcoplasmic reticulum, but do not interfere with the Ca^{2+}-ATPase of the plasma membrane [7–10]. They cause an increase in the concentration of cytosolic free Ca^{2+} [Ca^{2+}]: in several cell types, including neutrophils [11–15]. The increase is not only due to the release of Ca^{2+} from intracellular membrane-bound stores, but also to the influx of extracellular Ca^{2+} into the cell as a consequence of calcium-induced Ca^{2+} influx.

The role of calcium ions in neutrophil migration has been the subject of a number of studies. Although changes in the concentration of free cytosolic Ca^{2+} are not always a prerequisite for migration to occur, removal of all calcium from the neutrophil stores causes an inhibition of neutrophil movement, possibly via an effect on adhesion [16–18]. On the other hand, a number of agents which have in common the ability to cause an increase in cytosolic free calcium inhibit neutrophil chemotaxis. These agents, which include ATP, ryanodine, cytochalasin B, hexachlorocyclohexane, ionophores, polyamines, and leukocyte inhibitory factor [19–25], inhibit migration while mostly stimulating or priming the respiratory burst or exocytosis. They all cause an influx of extracellular calcium into the neutrophil. We considered the possibility that under certain circumstances an increase in intracellular calcium may

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† *Abbreviations:* IL-8: interleukin-8; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; Tg, thapsigargin; DBHQ, 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone; CPA, cyclopiazonic acid; and cAMP, adenosine 3',5'-cyclic monophosphate.

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affect neutrophil migration and thus studied the effect of thapsigargin on said migration.

MATERIALS AND METHODS

Isolation of Human Neutrophils

Neutrophils were isolated from the buffy coat of blood of healthy donors. The buffy coat was diluted with a 4-fold volume of heparinized medium and layered on top of Ficoll-amidotrizoate (d = 1.077). After centrifugation, the pellet was resuspended and starch was added to sediment erythrocytes. After sedimentation, the neutrophil-containing supernatant was collected and centrifuged. The remaining erythrocytes were removed by hypotonic hemolysis and the neutrophils suspended in medium. The cells comprised 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% BSA, 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.

Electroporation of Neutrophils

Neutrophils were electroporated as described previously [26]. The electropermeabilization procedure was carried out at room temperature. When permeabilization was performed at 0°, 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% BSA) were placed in the cuvette of a Biorad Gene Pulser. The cells were exposed to two discharges of 14.75 kV/cm 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 was placed in the upper compartment of a Boyden chamber. When electroporated neutrophils were compared with control neutrophils, the latter cells were also suspended in permeabilization buffer. Unless otherwise indicated, 1 mM Ca^{2+} was included in the medium during the experiments, because this concentration of Ca^{2+} did not require the use of calcium buffers, and migration is optimal at this concentration.

Neutrophil Migration

Cell migration was measured with the Boyden chamber technique, as described by Boyden [27] and modified by Zigmond and Hirsch [28]. The two compartments of the chamber were separated by a cellulose acetate Millipore filter with a pore size of 3 μm. Medium supplemented with 1 mM Ca^{2+}, 1 mM Mg^{2+}, and 0.5% BSA was present in both the upper and lower compartments, unless otherwise indicated. Neutrophils were placed in the upper compartment of the chamber, followed by incubation for 40 min at 37°. 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 [28]. Chemotactic assays were carried out in duplicate, and the migration distance of the neutrophils was determined at five different filter sites. The following chemoattractants were used: fMLP; IL-8; leukotriene B_4 (LTB_4); phorbol 12-myristate 13-acetate (PMA); dioctanoylglycerol (diC8), and the diacylglycerol kinase inhibitor R59022. The chemoattractants were prepared as a stock solution in DMSO; before the experiment a dilution in medium was made. The final concentration of DMSO was always lower than 0.4%.

Cyclic AMP Assay

Neutrophils (final concentration 2×10^7 cells per mL) were exposed to reagents at 37° for the indicated times. 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°) $NaHCO_3$. After 10 min, the mixture was centrifuged for 3 min at 1100 *g*. To 50 μL of the supernatant 50 μL of radioactive cAMP and 100 μL antibody from the radioimmunoassay kit (Amersham) were added. After mixing, the solution was kept on ice for 120 min, after which 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 (Emulsifier Scintillator Plus, Packard) was added, and the mixture was counted in the scintillation counter. Known amounts of cAMP were treated in the same way as the cells and were used for the calibration curve.

Statistical Analysis

All mean values for the chemotactic assays are arithmetical means ± SEM of four experiments. In those cases where random or activated migration was considerably different for different cell batches, values were expressed as percentages of control. Significances were calculated with the Student's *t*-test; a value of $P < 0.05$ was considered statistically significant.

RESULTS

Effect on Migration

Thapsigargin inhibited IL-8-activated chemotaxis in the nanomolar range, with the level of random migration being reached at a concentration of 1 μM Tg. Chemotaxis activated by fMLP was also inhibited by Tg, but here higher concentrations of Tg were required to obtain the same degree of inhibition as with IL-8 (Fig. 1). Various groups have shown that in addition to the classical receptor-mediated chemoattractants such as IL-8, fMLP, and leukotriene B_4 (LTB_4), activators of protein kinase C may induce chemotaxis [29–31]. Migration induced both by receptor-mediated chemoattractants and by protein kinase C-medi-

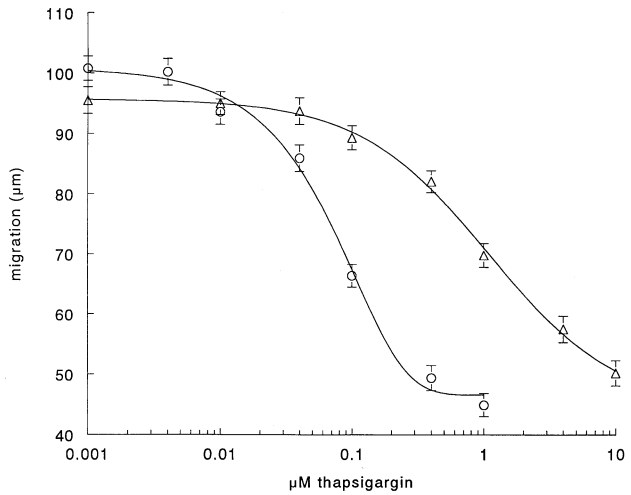

FIG. 1. The effect of increasing concentrations of thapsigargin on IL-8 (–○–)-or fMLP (–△–)-activated migration. Random migration was 50.1 ± 2.1 µm. Values for thapsigargin concentrations higher than 0.04 µM are significant different ($P < 0.05$) from control for IL-8; values for thapsigargin concentrations higher than 0.4 µM are significantly different ($P < 0.05$) from control for fMLP. Values represent ne means ± SEM, N = 4.

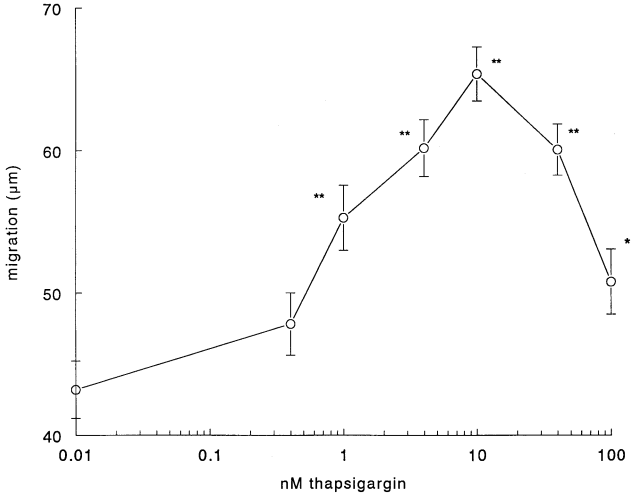

FIG. 3. Stimulation of migration by low concentrations of thapsigargin. Thapsigargin was present in the lower compartment of the Boyden chamber only. When 10 nM thapsigargin was present in both compartments of the Boyden chamber, migration was 51.8 ± 2.1 µm. *, $P < 0.05$; **, $P < 0.01$, as compared with control (no thapsigargin). Values represent the means ± SEM, N = 4.

ated activators was inhibited by Tg. Of all activators, fMLP-induced chemotaxis proved the most resistant against Tg (Fig. 2). Remarkably, very low concentrations of Tg gave a slight but significant enhancement of neutrophil migration. Stimulation of migration by Tg (in the absence of other chemoattractants) was maximal at a concentration of 10 nM Tg decreasing again at higher Tg concentrations (Fig. 3). The effect of Tg was time-dependent. Without preincubation of cells with Tg, there was little inhibition of IL-8-activated migration by a concentration of Tg which gave a moderate inhibition after 15 min. Preincubation of the cells for 15 min gave an inhibition which only slightly increased upon prolonged preincubation. Inhibition was considerably lower when Tg was removed after preincubation, i.e. before the chemotaxis experiment. The degree of inhibition was not dependent on the presence of calcium during preincubation (results not shown). Other inhibitors of Ca^{2+}-ATPase, namely CPA and DBHQ, also inhibited IL-8-activated chemotaxis, but only at relatively high concentrations (Fig. 4). Even a concentration of 1 µM CPA or 2.5 µM DBHQ gave less inhibition than 0.1 µM Tg.

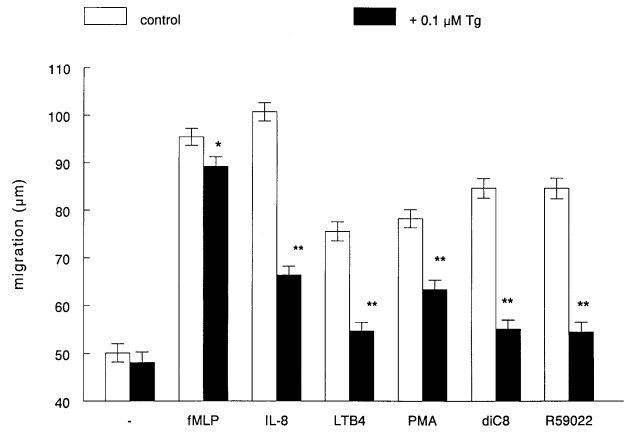

FIG. 2. Inhibition of migration, activated by different chemoattractants, by 0.1 µM thapsigargin. Concentrations (activator present in the lower compartment of the Boyden chamber): fMLP, 10^{-9} M; IL-8, 4.10^{-9} M; leukotriene B_4 (LTB_4), 2.10^{-9} M; phorbol 12-myristate 13-acetate (PMA), 10^{-10} g/mL; dioctanoylglycerol (diC8), 50 µM; R59022, 50 µM. *, $P < 0.05$; **, $P < 0.01$, as compared with control (no thapsigargin). Values represent the means ± SEM, N = 4.

Role of Extracellular Calcium

IL-8-activated chemotaxis was possible in the absence of extracellular Ca^{2+}. Migration was only slightly less —about 5 µm— than in the presence of extracellular Ca^{2+}, and this is probably a reflection of the effect of divalent ions on random migration; random migration in the absence of Ca^{2+} was also slightly less than in the presence of this ion, while the stimulation by IL-8 was constant. It appeared that inhibition by Tg only occurred in the presence of extracellular calcium, while stimulation by IL-8 in the absence of calcium was only slightly affected. The same phenomenon occurred with inhibition by CPA and DBHQ (Fig. 4). Pretreatment of neutrophils with Tg in the absence of Ca^{2+}, followed by chemotaxis in the presence of calcium, gave about the same inhibition as when the preincubation was carried out in the presence of Ca^{2+}: migration went down from 96.5 ± 2.1 µm (no Tg) to 54.4 ± 1.8 µm (with 0.1 µM Tg). The role of extracellular calcium in the inhibition of migration by Tg was specific for calcium;

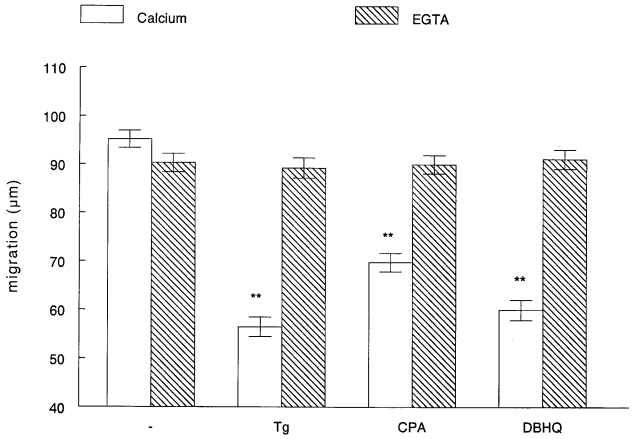

FIG. 4. The role of extracellular Ca^{2+} during inhibition of IL-8-activated migration by inhibitors of Ca^{2+}-ATP-ase. Cells were preincubated with the indicated concentration of inhibitor for 15 min, after which they were placed in the upper compartment of the Boyden chamber. IL-8 (4×10^{-9} M) was present in the lower chamber. Tg, thapsigargin; CPA, 1 μM cyclopiazonic acid; DBHQ, 2.5 μM 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone. Random migration was 53.7 ± 2.0 μm in the presence of Ca^{2+} and 49.6 ± 1.9 μm in the presence of EGTA. In all cases, 1 mM Mg^{2+} was present during the experiment. **, $P < 0.01$, as compared with control (no thapsigargin). Values represent the means ± SEM, N = 4.

inhibition of migration did not occur when only magnesium was present (Fig. 4) or when calcium was replaced by strontium or barium, two ions which can replace calcium in some neutrophil functions [26] (Table 1). A number of calcium channel blockers were tested to see whether the inhibitory effect of Tg could be prevented. All agents tested had only a slight effect on IL-8-activated migration. La^{3+} nearly completely prevented the inhibitory effect of Tg: econazole, verapamil, and SK&F 96365 gave a moderate prevention, while gadolinium chloride and Ni^{2+} were only slightly effective (Table 2). Neutrophils retained the capacity to migrate (at least to a large extent) when they were electroporated at room temperature. This enabled us to study the importance of intracellular (cytosolic free) calcium in Tg-induced inhibition of IL-8-activated migration.

TABLE 1. Comparison of the effect of calcium and other divalent cations on inhibition of IL-8-activated migration by thapsigargin

	Migration (μm)	
	–	+0.1 μM Tg
EGTA	90.3 ± 1.9	89.2 ± 2.0
Ca^{2+}	95.2 ± 2.1	56.5 ± 1.8
Sr^{2+}	92.8 ± 2.0	92.5 ± 1.9
Ba^{2+}	95.7 ± 2.1	95.5 ± 2.1

Cells were preincubated with or without thapsigargin (Tg) for 15 min at 37° and then placed in the upper compartment of the Boyden chamber. The concentration of EGTA was 50 μM, while that of the divalent cations was 1 mM; in all cases 1 mM Mg^{2+} was present. IL-8 (4×10^{-9} M) was present in the lower compartment of the Boyden chamber. Random migration under these conditions was 51.1 ± 1.9 μm.

TABLE 2. The effect of some calcium channel blockers on inhibition of IL-8-activated migration by thapsigargin

	Migration (μm)	
	–	+0.1 μM Tg
–	100 ± 2	49 ± 2
La^{3+} (20 μM)	96 ± 3	92 ± 2
Econazole (30 μM)	96 ± 2	62 ± 3
Gadolinium chloride (1 μM)	94 ± 1	54 ± 2
SK&F 96365 (30 μM)	98 ± 2	67 ± 1
Ni^{2+} (100 μM)	97 ± 2	50 ± 2
Verapamil (10 μM)	99 ± 1	65 ± 3

Cells were preincubated in the presence of the calcium channel blockers with or without thapsigargin (Tg) for 15 min at 37°, and then placed in the upper compartment of the Boyden chamber. IL-8 (4×10^{-9} M) was present in the lower compartment of the Boyden chamber. Values are expressed as a percentage of migration towards IL-8 without additions.

The calcium concentration was imposed with calcium–EGTA buffers [32]. As a comparison the Ca^{2+} dependence of IL-8-activated chemotaxis was measured. It appeared that the effect of IL-8-activated migration was hardly dependent on Ca^{2+}. Tg-induced inhibition of IL-8-activated migration was strongly dependent on intracellular free calcium, with the strongest increase in inhibition calcium concentrations between 100 nM and 1 μM (Fig. 5).

Involvement of cAMP

Because cAMP is known to be a potential inhibitor of neutrophil migration, we considered the possibility that it is

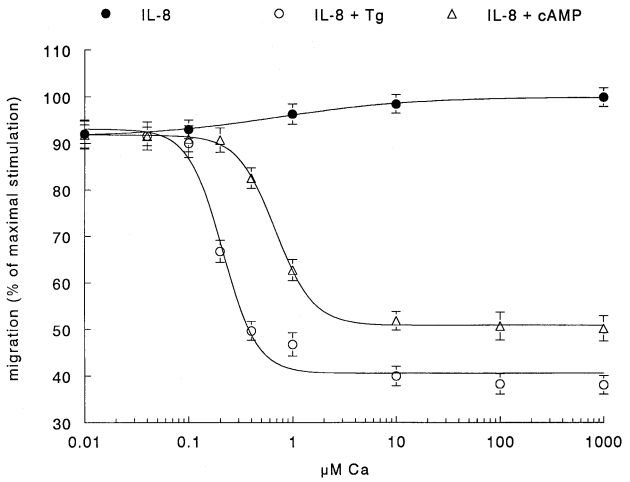

FIG. 5. The effect of increasing Ca^{2+} concentrations on IL-8-activated migration of electroporated neutrophils in the absence of inhibitors (–●–), in the presence of 0.1 μM thapsigargin (Tg) (–○–), or in the presence of 20 μM cAMP (–△–). Ca^{2+} concentrations between 10 nM and 10 μM were prepared by mixing EGTA and EGTA–Ca^{2+} solutions, thus obtaining buffered calcium concentrations. Higher concentrations of Ca^{2+} were not buffered. Migration in the presence of 1 mM Ca^{2+} (maximal migration) was 75.8 ± 2.1 μm; random migration was 25.6 μm in the absence of Ca^{2+} and 31.3 μm in the presence of 1 mM Ca^{2+}. Values represent the means ± SEM, N = 4.

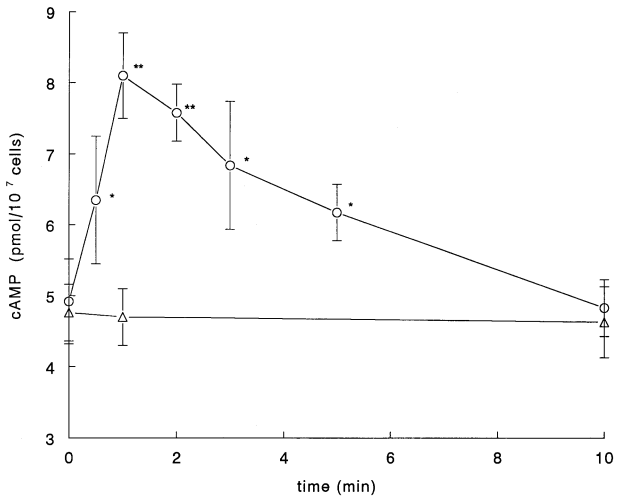

FIG. 6. Time–course of the effect of thapsigargin on the cAMP level in the neutrophil. (–○–) 0.1 μM thapsigargin; (–△–): control. *: $P < 0.05$; **: $P < 0.01$, as compared with control (no thapsigargin). Values represent the means ± SEM, N = 4.

involved in the inhibitory effect of Tg. A transient increase in the cAMP level resulted upon treatment of neutrophils with Tg (Fig. 6). The increase was maximal after 1 min, and decreased slowly with time. Previously, we found that cAMP inhibited migration of electroporated neutrophils (although very low concentrations were stimulating) [33]; electroporated cells were thus used herein to enable cAMP to enter the cell. We considered the calcium-dependency of cAMP-induced inhibition, and found that this dependency resembled to some degree that of Tg (Fig. 5). The difference in dependency observed might depend on the concentration or compartmentalization [34] of cAMP. We considered the possibility that other divalent cations such as Sr^{2+} and Ba^{2+} could substitute for Ca^{2+}. IL-8 gave stimulation of migration in the presence of Sr^{2+} and Ba^{2+}, with this stimulation not being significant inhibited by cAMP (Table 3).

TABLE 3. Comparison between the effect of calcium and other divalent cations on inhibition of IL-8-activated migration of electroporated cells by cAMP

	Migration (μm)		
	A	B	C
	—	IL-8	IL-8 + 20 μM cAMP
EGTA	29.3 ± 2.1	68.0 ± 1.9	67.6 ± 2.1
Ca^{2+}	31.7 ± 2.0	71.4 ± 2.3	35.7 ± 2.0
Sr^{2+}	21.8 ± 2.0	63.6 ± 1.8	59.9 ± 2.3
Ba^{2+}	24.8 ± 2.5	56.0 ± 2.2	55.5 ± 1.9

The following parameters were measured: A, random migration; B, IL-8-activated migration; and C, IL-8-activated migration in the presence of an inhibitory concentration (20 μM) of cAMP. The concentration of EGTA was 50 μM, while that of the divalent cations was 1 mM; in all cases 1 mM Mg^{2+} was present. IL-8 (4×10^{-9} M) was present in the lower compartment of the Boyden chamber.

DISCUSSION

The results clearly show that Tg can inhibit neutrophil migration and that this inhibitory effect is associated with the ability of Tg to inhibit the Ca^{2+}-ATP-ase of intracellular calcium stores. This can be deduced from the observation that besides Tg, compounds with a completely different chemical structure such as CPA and DBHQ also inhibit neutrophil migration. The specificity of Tg, CPA, and DBHQ as inhibitors of the Ca^{2+}-ATPase of calcium stores and the similarity in the pattern of inhibition of migration by these agents indicate that the first step of inhibition of migration is the blockade of this Ca^{2+}-ATPase. The inhibition of Ca^{2+}-ATPase by Tg causes calcium to be released into the cytosol and the stores to be depleted. The depletion of the stores results in an influx of extracellular Ca^{2+}, or store-regulated calcium influx [9–15]. The inhibitory effect of Tg on migration was absolutely dependent on extracellular Ca^{2+} (either derived from the influx in intact cells or application of a calcium buffer in electroporated cells), indicating that this ion played a decisive role in the process. Extracellular calcium is required inside the cell, because inhibition of calcium fluxes with calcium blockers prevented Tg-induced inhibition of migration. Furthermore, the experiment with electroporated neutrophils showed that inhibition by Tg could only be achieved by relatively high calcium concentrations, which cannot be attained by the release of Ca^{2+} from internal stores alone. The effect is quite specific to Ca^{2+}, because similar ions such as Sr^{2+} or Ba^{2+} have no effect, although these ions are able to substitute for Ca^{2+} in some neutrophil functions such as exocytosis [26]. Although the experiment with calcium channel blockers is indicative of a role for calcium influx, the sequence of the magnitude of reversing Tg-induced inhibition is somewhat surprising. Both gadolinium chloride and imidazoles block Tg-induced Ca^{2+} influx quite effectively [35], but they were less effective than La^{3+} in our experiments in reversing Tg-induced inhibition of migration; in contrast, verapamil had some effect on Tg-induced inhibition of migration but little effect on Ca^{2+} influx.

Under certain conditions, an enhancement of calcium in the cell is associated with inhibition of migration. This view is further supported by a number of data from the literature, which show that a variety of substances with completely different structures all have the ability to enhance cytosolic free Ca^{2+} and inhibit migration in neutrophils, while at the same time priming or stimulation exocytosis and respiratory burst [19–25]. However, calcium alone is not inhibitory; the results with electroporated cells show that neutrophils migrate maximally at high calcium concentrations. Furthermore, many chemotactic agents cause an increase in cytosolic free calcium, although the maximal effect mostly occurs at concentrations which are higher than those required for optimal chemotaxis. In the experiment with electroporated cells, there was only an inhibition of chemotaxis at a given calcium concentration

when Tg was present. The results of the experiments with electroporated cells also make it unlikely that negative interaction between Tg and chemotactic factors [36], by interfering with calcium pumping or with store-operated Ca^{2+} entry, is the cause of Tg-induced inhibition of migration. Neutrophils are apparently quite able to migrate when there is a high cytosolic free calcium concentration. Thus, it is clear that calcium is not the direct cause of inhibition, but only permits another agent to act as inhibitor. Therefore, there must be another effect of Tg, in addition to its effect on the cellular calcium level, which, in combination with calcium, is responsible for inhibition. Tg causes an increase in the cellular cAMP level. Cyclic AMP is known to cause inhibition of migration, although the precise mechanism of action is still unknown, in particular the extent to which compartmentalization of cAMP is involved [33,34,37]. Inhibition of migration by cAMP is calcium-dependent, and resembles the calcium dependency of Tg-induced inhibition. It is tempting to speculate that cAMP is involved in Tg-induced inhibition of migration. According to this hypothesis, Tg would cause an increase in the cAMP level and a release of calcium from intracellular stores. This would, in turn, casue an influx of extracellular calcium which would be large enough to make an inhibition of IL-8-activated migration by cAMP possible. This hypothesis is supported by the finding that very low concentrations of Tg stimulate neutrophil migration significantly, albeit not to a large extent. A comparable effect is observed for cAMP: low concentrations give a slight stimulation of migration (in the absence of other chemoattractants), while high concentrations inhibit migration activated by chemoattractants [33]. This view is further supported by the observation that neither thapsigargin nor cAMP inhibits IL-8-activated migration in the presence of Sr^{2+} or Ba^{2+}.

References

1. Alteraifi AM and Zhelev DV, Transient increase of free cytosolic calcium during neutrophils motility responses. *J Cell Sci* **110:** 1967–1977, 1997.
2. Malawista SE and de Boisfluery Chevance A, Random locomotion and chemotaxis of human blood polymorphonuclear leukocytes (PMN) in the presence of EDTA: PMN in close quarters require neither leukocyte integrins nor external divalent cations. *Proc Natl Acad Sci USA* **94:** 11577–11582, 1997.
3. Mandeville JT, and Maxfield FR, Calcium and signal transduction in granulocytes. *Currt Opin Hematol* **3:** 63–70, 1996.
4. Maxfield FR, Regulation of leukocyte locomotion by Ca^{2+}. *Trends Cell Biol* **3:** 386–391, 1993.
5. Krause KH, Pittet D, Volpe P, Pozzan T, Meldolesi J and Lew DP, Calciosome, a sarcoplasmic reticulum-like organelle involved in intracellular Ca^{2+}-handling by non-muscle cells: Studies in human neutrophils and HL-60 cells. *Cell Calcium* **10:** 351–361, 1989.
6. Lew DP, Receptor signalling and intracellular calcium in neutrophil activation. *Eur J Clin Invest* **19:** 338–346, 1989.
7. Thastrup O, Thapsigargin, a tumor promoter, dicharges intracellular Ca^{2+} stores by specific inhibition of the endoplasic reticulum Ca^{2+}-ATPase. *Proc Natl Acad Sci USA* **87:** 2466–2470, 1990.
8. Thastrup O, Role of Ca^{2+}-ATPases in regulation of cellular Ca^{2+} signalling, as studied with the selective microsomal Ca^{2+}-ATPase inhibitor, thapsigargin. *Agent Actions* **29:** 8–15, 1990.
9. Törnquist K, Activation of calcium entry by cyclopiazonic acid in thyroid FRTL-5 cells. *Cell Calcium* **14:** 411–417, 1993.
10. Muallem S, Loessberg P, Sachs G and Wheeler LA, Agonist-sensitive and -insensitive intracellular Ca^{2+} pools. Separate Ca^{2+}-releasing mechanisms revealed by manoalide and benzohydroquinone. *Biochem J* **279:** 367–375, 1991.
11. Demaurex N, Lew DP and Krause KH, Cyclopiazonic acid depletes intracellular Ca^{2+} stores and activates an influx pathway for divalent cations in HL-60 cells. *J Biol Chem* **267:** 2318–2324, 1992.
12. Robinson IM, Cheek TR and Burgoyne RD, Ca^{2+} influx induced by the Ca^{2+}-ATPase inhibitors 2,5-di-(*t*-butyl)-1,4-benzohydroquinone and thapsigargin in bovine adrenal chromaffin cells. *Biochem J* **288:** 457–463, 1992.
13. Llopis J, Chow SB, Kass GE, Gahm A and Orrenius S, Comparison between the effects of the microsomal Ca^{2+} translocase inhibitors thapsigargin and 2,5-di-(*t*-butyl)-1,4-benzohydroquinone on cellular calcium fluxes. *Biochem J* **277:** 553–556, 1991.
14. Rotness JS and Iversen JG, Thapsigargin reveals evidence for fMLP-insensitive calcium pools in human leukocytes. *Cell Calcium* **13:** 487–500, 1992.
15. Foder B, Scharff O and Thastrup O, Ca^{2+} transients and Mn^{2+} entry in human neutrophils induced by thapsigargin. *Cell Calcium* **10:** 477–490, 1989.
16. Meshulam T, Proto P, Diamond RD and Melnick DA, Calcium modulation and chemotactic response: Divergent stimulation of neutrophil chemotaxis and cytosolic calcium response by the chemotactic peptide receptor. *J Immunol* **137:** 1954–1960, 1986.
17. Elferink JG and Deierkauf M, The effect of quin2 on chemotaxis by polymorphonuclear leukocytes. *Biochim Biophys Acta* **846:** 364–369, 1985.
18. Marks PW and Maxfield FR, Transient increases in cytosolic free calcium appear to be required for the migration of adherent human neutrophils. *J Cell Biol* **110:** 43–51, 1990.
19. Elferink JG, de Koster BM, Boonen GJ and de Priester W, Inhibition of neutrophil chemotaxis by purinoceptor agonists. *Arch Int Pharmacodyn Ther* **317:** 93–106, 1992.
20. Zigmond SH and Hirsch JG, Effects of cytochalasin B on polymorphonuclear leucocyte locomotion, phagocytosis and glycolysis. *Exp Cell Res* **73:** 383–393, 1972.
21. Kaplan SS, Zdziarski UE, Kuhns DB and Basford RE, Inhibition of cell movement and associated changes by hexachlorocyclohexanes due to unregulated intracellular calcium increases. *Blood* **71:** 677–683, 1988.
22. Wilkinson PC, Leucocyte locomotion and chemotaxis. The influence of divalent cations and cation ionophores. *Exp Cell Res* **93:** 420–426, 1975.
23. Elferink JG and de Koster BM, Ryanodine as inhibitor of chemotactic peptide-induced chemotaxis in human neutrophils. *Biochem Pharmacol* **50:** 975–979, 1995.
24. Walters JD, Miller TJ, Cario AC, Beck FM and Marucha PT, Polyamines found in the gingival fluid inhibit chemotaxis by human polymorphonuclear leukocytes *in vitro*. *J Periodontol* **66:** 274–278, 1995.
25. Borish L, O'Reilly D, Klempner MS and Rocklin RE, Leukocyte inhibitory factor (LIF) potentiates neutrophil responses to formyl-methionyl-leucyl-phenylalanine. *J Immunol* **137:** 1897–1903, 1986.

26. Boonen GJ, VanSteveninck J and Elferink JGR, Strontium and barium induce exocytosis in electropermeabilized neutrophils. *Biochim Biophys Acta* **1175:** 155–160, 1993.
27. Boyden SV, The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leukocytes. *J Exp Med* **115:** 453–466, 1962.
28. Zigmond SH and Hirsch JG, Leukocyte locomotion and chemotaxis. New methods for evaluation and demonstration of a cell-derived chemotactic factor. *J Exp Med* **137:** 387–410, 1973.
29. Wright TM, Hoffman RD, Nishijima J, Jakoi L, Snyderman R and Shin HS, Leukocyte chemoattraction by 1,2-diacylglycerol. *Proc Natl Acad Sci USA* **85:** 1869–1873, 1988.
30. Boonen GJ, de Koster BM, VanSteveninck J and Elferink JG, Neutrophil chemotaxis induced by the diacylglycerol kinase inhibitor R59022. *Biochim Biophys Acta* **1178:** 97–102, 1993.
31. Gabler WL, Bullock WW and Creamer HR, Phorbol myristate acetate induction of chemotactic migration of human polymorphonucelar neutrophils. *Inflammation* **17:** 521–530, 1993.
32. Elferink JG, Boonen GJ and de Koster BM, The role of calcium in neutrophil migration: The effect of calcium and calcium antagonists in electroporated neutrophils. *Biochem Biophys Res Commun* **182:** 864–869, 1992.
33. Elferink JG and de Koster BM, The effect of cyclic GMP and cyclic AMP on migration by electroporated human neutrophils. *Eur J Pharmacol* **246:** 157–161, 1993.
34. Harvath L, Robbins JD, Russell AA and Seamon KB, cAMP and human neutrophil chemotaxis. Elevation of cAMP differentially affects chemotactic responsiveness. *J Immunol* **146:** 224–232, 1991.
35. Wenzel-Seifert K, Krautwurst D, Musgrave I and Seifert, R, Thapsigargin activates univalent- and bivalent-cation entry in human neutrophils by a SK&F 96365- and Gd^{3+}-sensitive pathway and is a partial secretagogue: Involvement of pertussis-toxin-sensitive G-proteins and protein phosphatases 1/2A and 2B in the signal-transduction pathway. *Biochem J* **314:** 679–686, 1996.
36. Montero M, Garcia-Sancho J and Alvarez J, Transient inhibition by chemotactic peptide of a store-operated Ca^{2+} entry pathway in human neutrophils. *J Biol Chem* **268:** 13055–13061, 1993.
37. Elferink JG and VanUffelen BE, The role of cyclic nucleotides in neutrophil migration. *Gen Pharmacol* **27:** 387–393, 1996.