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Effect of inhibitors of Na^+/H^+-exchange and gastric H^+/K^+ ATPase on cell volume, intracellular pH and migration of human polymorphonuclear leucocytes

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1 Stimulation of chemotaxis of human polymorphonuclear leucocytes (PMNs) with the chemoattractive peptide fMLP (N-formyl-Met-Leu-Phe) is paralleled by profound morphological and metabolic alterations like changes of intracellular pH (pH_i) and cell shape. The present study was performed to investigate the interrelation of cell volume (CV) regulatory ion transport, pH_i and migration of fMLP stimulated PMNs.

2 Addition of fMLP to PMNs stimulated directed migration in Boyden chamber assays and was accompanied by rapid initial intracellular acidification and cell swelling.

3 Inhibition of the Na^+/H^+ exchanger suppressed fMLP stimulated cell migration, accelerated the intracellular acidification and inhibited the fMLP-induced cell swelling.

4 Step omission of extracellular Na^+ caused intracellular acidification, which was accelerated by subsequent addition of gastric H^+/K^+ ATPase inhibitor SCH 28080, or by omission of extracellular K^+ ions. In addition Na^+ removal caused cell swelling, which was further enhanced by fMLP.

5 H^+/K^+ATPase inhibitors omeprazole and SCH 28080 inhibited stimulated migration and blunted the fMLP-induced increase in CV.

6 Increasing extracellular osmolarity by addition of mannitol to the extracellular solution caused cell shrinkage followed by regulatory volume increase, partially due to activation of the Na^+/H^+ exchanger. In fMLP-stimulated cells the CV increase was counteracted by simultaneous addition of mannitol. Under these conditions the fMLP stimulated migration was inhibited.

7 The antibacterial activity of PMNs was not modified by Hoe 694 or omeprazole.

8 Western analysis with a monoclonal anti gastric H^+/K^+ATPase β-subunit antibody detected a glycosylated 35 kD core protein in lysates of mouse and human gastric mucosa as well as in human PMNs.

9 The results indicate that fMLP leads to cell swelling of PMNs due to activation of the Na^+/H^+ exchanger and a K^+-dependent H^+-extruding mechanism, presumably an H^+/K^+ ATPase. Inhibition of these ion transporters suppresses the increase in CV and precludes PMNs from stimulated migration.

Keywords: Leucocyte; cell migration; chemotaxis; cell volume; intracellular pH; Na^+/H^+ exchanger; Hoe 694; Hoe 642; K^+/H^+ATPase; omeprazole; SCH 28080

Introduction

Polymorphonuclear neutrophil leucocytes (PMNs) migrate to sites of infection upon activation by various chemoattractants including the bacterial peptide fMLP (N-formyl-Met-Leu-Phe). The activation of PMNs with fMLP is paralleled by profound metabolic changes like generation of intracellular H^+ ions (Borregaard *et al*., 1984; Grinstein & Furuya, 1986) and reactive oxygen intermediates (Nathan, 1987), as well as morphological alterations such as cell shape change and cell swelling (Grinstein & Furuya, 1984; Rosengren *et al*., 1994; Worthen *et al*., 1994).

The directed locomotion of cells requires the generation and coordinated action of contractile and protrusive forces. Actively migrating cells polarize by forming a leading lamella at the site of receptor activation, guiding the cells towards increasing concentrations of the chemoattractant. The molecular mechanisms during generation of the leading lamella include dynamic cycles of actin assembly-disassembly, substrate adhesion, endo-exocytosis, membrane flow and ruffling, shuttling of chemoattractant receptors as well as myosin-actin contraction (Zigmond & Sullivan, 1979; Singer & Kupfer, 1986; Stossel, 1993). Even though these mechanisms have been investigated in detail, the nature of the protrusive forces pushing and directing the leading lamella are still unclear, and different hypotheses have been proposed to explain this phenomenon (Oster & Perelson, 1987; Bray & White, 1989; Egelhoff & Spudich, 1991; Lee *et al*., 1993; Stossel, 1993). The 'solation-expansion' model explains membrane protrusion by localized osmotic swelling due to local depolymerization of the cortical actin filament network at the tip of the cell. The focal weakening of the actin filaments causes cortical gel-sol transition and enables the isotropic forces created by actin-myosin contraction and/or osmotic swelling to protrude the cell membrane at this point, whereas the neighbouring structures retain intact gel properties, thus

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resisting deformation by the increasing intracellular pressure. Subsequent reconstitution of the actin gel stabilizes the cell at the advanced position.

According to this model, cell migration is expected to be accompanied by water fluxes across the cell membrane and hence changes of cell volume. Indeed, cell swelling has been observed in PMNs migrating into inflamed tissue *in vivo* (Worthen *et al*., 1994) as well as *in vitro* following stimulation with fMLP or phorbol esters. This cell volume increase has been attributed to activation of the Na^+/H^+ exchanger (Grinstein & Furuya, 1984; Rosengren *et al*., 1994).

The Na^+/H^+ exchangers comprise a family of cell membrane bound proteins, termed NHE-1, 2, 3, 4 and β-NHE, which mediate the exchange of extracellular Na^+ for intracellular H^+ ions. NHEs are involved in the regulation of intracellular pH, cell volume, transcellular transport, cell proliferation, cell differentiation, cell adhesion, as well as cell migration (Grinstein *et al*., 1989; Noël & Pouysségur, 1995; Wakabayashi *et al*., 1997; Lang *et al*., 1998) and are thought to play an important role in pathological processes like myocardial reperfusion injury (Scholz *et al*., 1993; 1995; Xue *et al*., 1996) and stenosis formation after injury of arterial vessels (Kranzhofer *et al*., 1993) or malignant cell transformation (Ritter & Wöll, 1996). The Na^+/H^+ exchanger expressed in human neutrophils has been identified as the NHE-1 isoform (Fukushima *et al*., 1996). In these cells activation of the Na^+/H^+ exchanger occurs following stimulation with phorbol esters and chemoattractants (Grinstein *et al*., 1986), extracellular matrix proteins and during cell spreading (Demaurex *et al*., 1996), as well as after crosslinking of Fcγ- and complement receptors during phagocytosis (Fukushima *et al*., 1996). During these processes, intracellular H^+ ions are generated by the NADPH oxidase and hexose monophosphate shunt pathway (Borregaard *et al*., 1984; Grinstein & Furuya, 1986; Nanda & Grinstein, 1991; Nanda *et al*., 1992; 1993). The action of the Na^+/H^+ exchanger compensates for the resulting intracellular acidosis (Simchowitz, 1985a, b; Simchowitz & Cragoe, 1986). Another consequence of Na^+/H^+ exchanger activation is cell swelling due to net uptake of Na^+ ions and osmotically obliged water (Grinstein & Furuya, 1984). In shrunken cells the Na^+/H^+ exchanger is activated to restore the initial cell volume, a process called regulatory volume increase (RVI) (Grinstein & Foskett, 1990; Lang *et al*., 1998). The functional significance of cell swelling after stimulation of PMNs is less understood. Earlier studies have shown that inhibition of the Na^+/H^+ exchanger inhibits migration of PMNs. In addition, osmotic cell swelling has been shown to facilitate neutrophil migration (Simchowitz & Cragoe, 1986; Rosengren *et al*., 1994). Oscillatory cycles of K^+ channel activation associated with oscillations of the cell volume have been suggested to promote migration of transformed MDCK-F cells (Schwab *et al*., 1994; Schwab & Oberleithner, 1996). The bulk of evidence points to a close engagement of cell volume and cell migration. Therefore, the present study was performed to investigate further the involvement of cell volume regulatory ion transport mechanisms in the regulation of fMLP-stimulated PMN migration.

Methods

Cell preparation

Polymorphonuclear leucocyte (PMN) suspensions were isolated from heparin-treated blood of healthy volunteers or from buffy coats (Innsbruck University Hospital Blood Bank) by Ficoll-Paque (ICN Flow, Costa Mesa, CA) discontinuous density gradient centrifugation and washed in Hank's balanced salt solution with subsequent hypotonic lysis of contaminating erythrocytes (>95% PMNs by morphology in Giemsa stains, >98% viability by trypan dye exclusion). The PMN suspensions were resuspended in RPMI 1640 medium containing 0.5% bovine serum albumin (BSA; Sigma, St. Louis, MO) for chemotaxis experiments or in experimental solution (see below) for all other experiments.

Cell migration measurements

Chemotaxis of PMNs was measured as a modified Boyden chamber assay (Wilkinson, 1988; Wiedermann *et al*., 1993) in 48-well microchemotaxis chambers (Neuroprobe, Bethesda, MD) using 5 μm pore nitrocellulosenitrate filters. PMNs (50000/well) were placed in the upper compartment and fMLP (10 nmol l^{-1}) was added to the lower compartment. Where indicated, Hoe 694, Hoe 642, ethylisopropyl-amiloride (EIPA), omeprazole, SCH 28080 or mannitol were added to the lower and/or upper compartment at the concentrations given. After a 45 min incubation period (humidified air, 37°C, 5% CO_2) migration was assessed as penetration depth (in μm) of cells into the filter by microscopically measuring the distance from the surface of the filter to the leading front of the cells.

Determination of intracellular pH (pH_i)

Measurements were carried out with BCECF (2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein). Suspended PMNs were incubated for 15 min with 3 μmol l^{-1} BCECF-acetoxymethylester (Molecular Probes, Eugene, OR, U.S.A.) and allowed to adhere to uncoated glass coverslips. Thereafter, the glass coverslip was mounted into a heated perfusion chamber (37°C) allowing for fluid exchange and the cells were continuously superfused with control or experimental solution. Adherent cells were allowed to adapt for at least 5 min to yield a stable pH_i. Changes in pH_i and cell shape due to adhesion and spreading have been shown to be complete within this period of time (Demaurex *et al*., 1996). Measurements were made under an inverted microscope (IM-35, Zeiss, Oberkochen, FRG) equipped for epifluorescence and photometry as described earlier (Wöll *et al*., 1993). To reduce the region from which fluorescence was collected, a pinhole was placed in the image plane of the phototube (limitation to a circular area of 60 μm diameter). Fluorescence values were corrected for cellular autofluorescence. pH_i was calibrated with the high potassium/nigericin technique (Ganz *et al*., 1986).

Cell volume (CV) measurements

Electronic cell sizing Measurements were performed at 34–37°C using a Schärfe cell analyser (CASY1 TT, Schärfe, Reutlingen, FRG) as described earlier (Ritter *et al*., 1992; 1993). Before the CV measurements, the cells were suspended either in control solution and allowed to adapt for ~5 min or in Na^+ free isotonic solution without time of adaptation. Where indicated, fMLP, Hoe 694, Hoe 642, omeprazole or SCH 28080 were added at the concentrations given, or extracellular osmolarity was increased by addition of mannitol.

Optical sectioning and three-dimensional reconstruction by confocal microscopy Fluorescence optical measurements of CV were performed using calcein as fluorescent probe. This

dye is insensitive to changes in pH_i and intracellular calcium, it is well retained in viable cells, does not interfere with leucocyte chemotaxis or superoxide production (De Clerck *et al*., 1994; Denholm & Stankus, 1995) and changes in cell shape have been shown to be without effect on cell fluorescence (Mandeville *et al*., 1995). Calcein was utilized to monitor CV changes (Crowe *et al*., 1995). Suspended PMNs were incubated for 15 min with 3 μmol l^{-1} calcein-acetoxymethylester (Molecular Probes, Eugene, OR, U.S.A.) and allowed to adhere to uncoated glass coverslips. Thereafter, the glass coverslip was mounted into a heated perfusion chamber superfused as described above. Measurements were performed on a Zeiss LSM 410 inverted laser scanning microscope (Carl Zeiss, Oberkochen, FRG). Light from an argon ion laser (488 nm, 15 mW) was directed through the objective (Plan Neofluar $40\times/1.3$ oil). Emitted fluorescence was directed through a long pass filter (LP515, Zeiss) and detected by a photomultiplier. The first series of z-sections was made to determine the CV under control conditions. Subsequently, the control solution was switched to experimental solution and the measurement was repeated after 20 min. No intermediate measurements were performed to avoid bleaching of the dye. The images were stored on the hard disc and analysed utilizing the integrated system software. The individual cross-sectional areas were calculated by counting the pixels within the focal plane area of the cell. The image was subjected to threshold filtering to delete pixels of faint brightness and the outer circumference of the resulting image defined the single slice area. The single slice volume was calculated by multiplication of the slice area with the height of a single z-step (1 μm) and CV was assessed by addition of all slice volumes. The CVs obtained by this method are expected to be overestimated if the cells have a flattened shape, and neighbouring single slice areas differ considerably in size. This was observed in almost all of the cells analysed.

E. coli *preparation and* E. coli *killing assay*

For preparation of *E. coli* Δ1060 a 4 h culture in LB medium (Gibco, Paisley, U.K.) with 3% DMSO was stored at $-70°C$. After determination of the colony forming unit (CFU) count on nutrient agar plates (Trypcase Soja Agar, Bio Merieux, Lyon, France), bacteria were centrifugated at $600\times g$ for 10 min. Sedimented *E. coli* were resuspended in Krebs-Ringer bicarbonate buffer (KRDP, Sigma, Munich, FRG) and opsonized by incubation with fresh human serum at 37°C for 30 min, diluted with 1 ml phosphate-buffered saline (PBS) and centrifugated at $600\times g$ for 15 min. The supernatant was discarded and the bacteria were resuspended in PBS containing 40% human serum to a concentration suitable for mixing with 10^7 PMN ml^{-1} at a PMN ml^{-1}: $CFU_{E.coli}$ ml^{-1} ratio of 1 : 1, 1 : 10 or 1 : 50. Where indicated, 10 nmol l^{-1} fMLP, Hoe 694, omeprazole (10 μmol l^{-1} each) or the respective solvents were added. Optimal evaluation of colony growth was usually observed at a ratio of 1 : 1. Thereafter, the suspensions were incubated at 37°C for 2 h while shaking, lysed with 0.1% Trixon X-100 in PBS and various dilutions between 10^{-2} and 10^{-5} of the lysates were placed onto nutrient agar plates. The CFU of viable bacteria were counted after overnight incubation at 37°C. Bacterial suspensions treated identically but without addition of PMNs served to determine CFU formation in the absence of PMNs. The bacterial killing activity of PMNs under the various experimental conditions was calculated as the difference between the corresponding $CFU_{E.coli}$ ml^{-1} counts in the absence and presence of PMNs.

Immunoblotting

Tissue and cell preparation Human gastric fundus or corpus mucosa was obtained by biopsy from patients who underwent gastroscopy for diagnostic reasons. Only normal mucosa was accepted for experiments. Mouse gastric mucosa was obtained from C57/BL6 mice (supplied by Charles Riever). Mouse and human gastric mucosa were homogenized by an ultra-turrax in lysis buffer (1 mmol l^{-1} EGTA, 1% Nonidet P 40, 0.1% SDS, 1 mmol l^{-1} PMSF, 20 μg ml^{-1} leupeptin, 20 μg ml^{-1} pepstatin A, 20 μg ml^{-1} antipain and 20 μg ml^{-1} aprotinin in PBS, pH 7.5) (Stuart-Tilley *et al*., 1994), PMNs and HepG2 cells were homogenized in lysis buffer with a 26-gauge needle. The homogenate was clarified by centrifugation at $12000\times g$ for 2 min.

Treatment of proteins with peptide N-glycosidase Protein lysate containing 60 μg protein was boiled for 10 min in denaturing buffer (0.5% SDS, 1% β-mercaptoethanol). One tenth volumes of 0.5 mol l^{-1} sodium-phosphate buffer, pH 7.5, and peptide N-glycosidase F (final concentration 83 u μl^{-1}, New England BioLabs) were added. The mixture was incubated for 1 h at 37°C. The preparation was then subjected to the immunoblotting protocol (Callaghan *et al*., 1995).

SDS-PAGE and Western analysis Cell and tissue lysates were solubilized in Laemmli sample buffer, heated to 100°C for 3 min and resolved on a SDS-PAGE (13% polyacrylamide) by the method of Laemmli (Laemmli, 1970). Proteins were electrotransferred (Sambrook *et al*., 1989) onto nitrocellulose membrane (Amersham), stained with Ponceau S (Sigma) after blotting and photographed. Membranes were blocked in Tris-buffered saline (TBS) containing 1% Tween and 5% nonfat dry milk for 1 h at room temperature, followed by incubation with primary antibody (monoclonal anti mouse proton pump β-subunit antibody 2B6 (MBL 2B6 No. D032-3, Nagoya, Japan) diluted in TBST (2 μg antibody ml^{-1}) for 4 h at room temperature. After washing extensively with TBST, the nitrocellulose membranes were incubated with secondary antibody (horseradish peroxidase-conjugated sheep anti-mouse IgG or horseradish peroxidase-conjugated donkey anti rabbit IgG antibody, Amersham Life Science, Braunschweig, FRG) for 1 h at 1 : 5000 dilution in TBST. Excess secondary antibody was washed and the bound secondary antibody was detected by enhanced chemiluminescence (ECL-System, Amersham).

Solutions and chemicals

The control solution was composed of (in mmol l^{-1}): NaCl 131, KCl 5.4, $MgCl_2$ 0.8, $CaCl_2$ 1.2, Na_2HPO_4 0.8, NaH_2PO_4 0.2, glucose 5.5 and 5.0 Tris; adjusted to pH 7.4 with HCl. In Na^+ free solutions Na_2HPO_4/NaH_2PO_4 was removed and NaCl was replaced isoosmotically by choline chloride. In solutions containing 5 mmol l^{-1} $BaCl_2$, NaCl or choline chloride was reduced to 124 mmol l^{-1} and in K^+ free solutions KCl was omitted at the expense of choline chloride. Hoe 694 (3-methylsulphonyl-4-piperidinobenzoyl-guanidine methanesulphonate), Hoe 642 (4-isopropyl-3-methylsulphonylbenzoyl-guanidine methanesulphonate), both NHE1 selective inhibitors of the Na^+/H^+ exchanger and ethylisopropyl-amiloride, a nonselective inhibitor of the Na^+/H^+ exchanger, were supplied by Hoechst AG (Frankfurt, FRG). The K^+ competitive inhibitor of gastric H^+/K^+ ATPase SCH 28080 (3-(cyanomethyl) - 2 - methyl-8-(phenylmethoxy)imidazo(1,2α)-pyridine) was kindly provided by Schering-Plough Research

Inst. (Kenilworth, N.J., U.S.A.) and omeprazole ([5-methoxy-2-(((4-methoxy-3,5-dimethyl-2-pyridyl)methyl)sulphinyl)-1H-benzimidazole]) (Losec) was obtained from Astra Ges.m.b.H. (Linz, Austria). For experiments with omeprazole PMNs were preincubated for 30 min with the drug. All the other reagents were obtained from Sigma (Munich, F.R.G.).

Statistical analysis

All experiments were carried out at least three times with PMNs from different donors. The data are given as arithmetic means ± s.e.mean. Statistical analysis was made by paired and unpaired *t* test, where applicable. Where appropriate, differences between groups were analysed by one way analysis of variance (ANOVA) and correction for multiple comparisons was made by Bonferroni's multiple comparisons test. Statistically significant differences were assumed at $P<0.05$.

Results

Cell migration

Effect of fMLP and Na^+/H^+ exchange inhibitors Hoe 694 (Figure 1a), Hoe 642 (Figure 1b) and ethylisopropyl-amiloride (EIPA, Figure 1c) Under control conditions PMNs exhibited a mean random migration (i.e. migration in the absence of drugs) of 58 ± 9 μm ($n=15$), 72 ± 6 μm ($n=5$) and 56 ± 4 μm ($n=10$), respectively. Addition of fMLP (10 nmol l^{-1}) led to a stimulation of cell migration to 86 ± 4 μm ($n=15$), 109 ± 10 μm ($n=5$) and 73 ± 7 μm ($n=10$), respectively. In the presence of Hoe 694, Hoe 642 or EIPA (10 μmol l^{-1} each) the stimulated migration was suppressed (64 ± 4 μm, $n=15$; 91 ± 9 μm, $n=5$ and 56 ± 9 μm, $n=10$; respectively). The random migration was slightly blunted by Hoe 694 (48 ± 3 μm, $n=15$), but unaffected by Hoe 642 or EIPA (68 ± 5 μm, $n=5$ and 49 ± 5 μm, $n=10$, respectively). Significant inhibition of

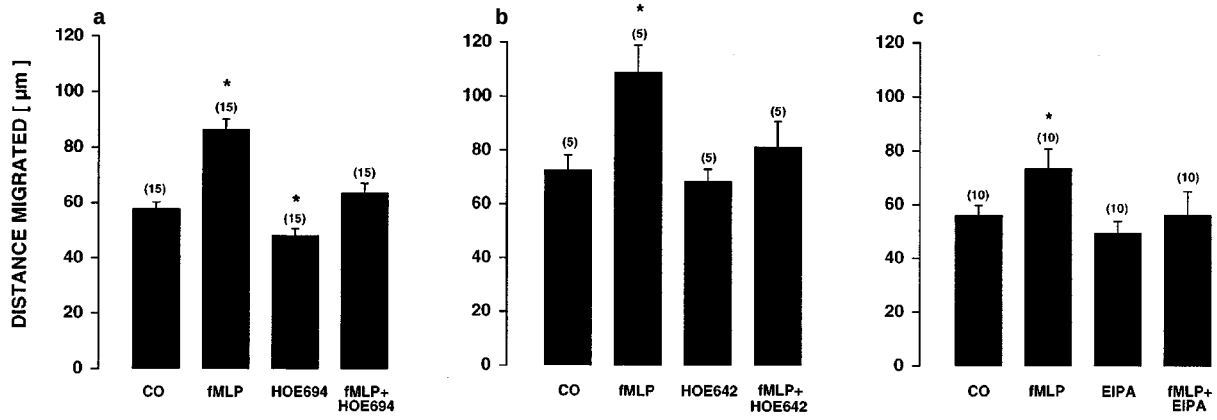

Figure 1 Migration of polymorphonuclear leucocytes (PMNs) under control conditions (CO), after stimulation with 10 nmol l^{-1} fMLP (fMLP), in the presence of 10 μmol l^{-1} of the Na^+/H^+ exchange inhibitors Hoe 694 (a), Hoe 642 (b) or ethylisopropylamiloride (EIPA, c), as well as in the presence of both fMLP and Hoe 694 (a), Hoe 642 (b) or EIPA (c). Migration under control conditions, i.e. in the absence of drugs is referred to as random migration throughout the text. Data are expressed as means ± s.e.mean. Numbers in parentheses indicate the number of individual observations. Asterisks indicate statistically significant difference from the respective control condition ($P<0.05$).

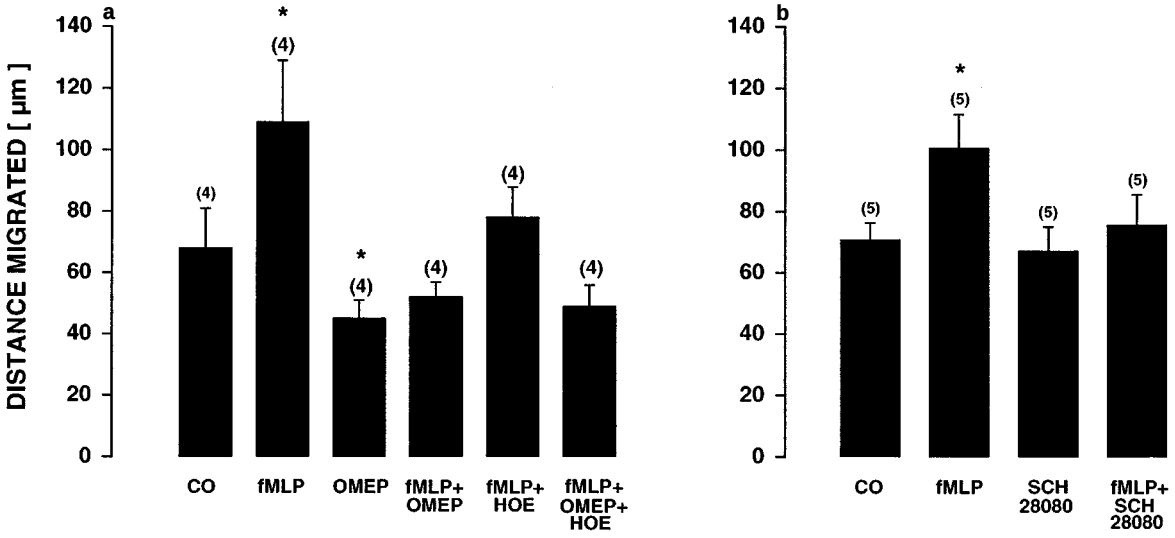

Figure 2 Migration of PMNs under control conditions (CO), after stimulation with 10 nmol l^{-1} fMLP, in the presence of gastric H^+/K^+ATPase inhibitors omeprazole (OMEP, a) or SCH 28080 (b), in the presence of both fMLP and omeprazole (a) or SCH 28080 (b), in the presence of fMLP and 1 μmol l^{-1} Hoe 694 (fMLP + HOE, a) as well as in the presence of fMLP, omeprazole and Hoe 694 (fMLP + OMEP + HOE, a). Data are expressed as means ± s.e.mean. Numbers in parentheses indicate the number of individual observations. Asterisks indicate statistically significant difference from the respective control condition ($P<0.05$).

chemotaxis by Hoe 694 could be observed at a concentration of 1 μmol l^{-1} (Figure 2a). The solvent (dimethylsulphoxide 1 : 10000) did not alter migration of unstimulated (58 ± 3 μm, $n = 6$) or fMLP stimulated cells (83 ± 7 μm, $n = 6$).

Effect of H^+/K^+ ATPase inhibitors

Omeprazole (Figure 2a) The mean random migration under control conditions was 68 ± 13 μm ($n = 4$). Addition of fMLP (10 nmol l^{-1}) led to a stimulation of cell migration to 109 ± 20 μm ($n = 4$). In the presence of 10 μmol l^{-1} omeprazole the random migration was reduced to 45 ± 6 μm ($n = 4$) and the fMLP-stimulated migration was completely suppressed (52 ± 5 μm, $n = 4$). Addition of Hoe 694 (1 μmol l^{-1}) blunted fMLP stimulated migration (78 ± 10 μm, $n = 4$). In the presence of Hoe 694 (1 μmol l^{-1}), the inhibitory effect of omeprazole (10 μmol l^{-1}) was not augmented (49 ± 7 μm, $n = 4$).

SCH 28080 (Figure 2b) The mean random migration under control conditions was 71 ± 6 μm ($n = 5$). Addition of fMLP (10 nmol l^{-1}) led to a stimulation of cell migration to 101 ± 11 μm ($n = 5$). In the presence of 10 μmol l^{-1} SCH 28080, the random migration was 67.8 μm ($n = 5$) and the fMLP stimulated migration was inhibited (76 ± 10 μm, $n = 5$).

Effect of increasing extracellular osmolarity (Figure 3) In these experiments the random migration was 68 ± 13 μm ($n = 5$) and fMLP-stimulated migration by 40 ± 6 μm ($n = 5$). Establishing hypertronic conditions by addition of mannitol (100 mmol l^{-1}) to the upper (cell containing) compartment of the Boyden chamber did not alter the random migration (by 6 ± 1 μm, $n = 5$), whereas under hypertronic conditions the fMLP stimulated migration was blunted (18 ± 2 μm, $n = 5$).

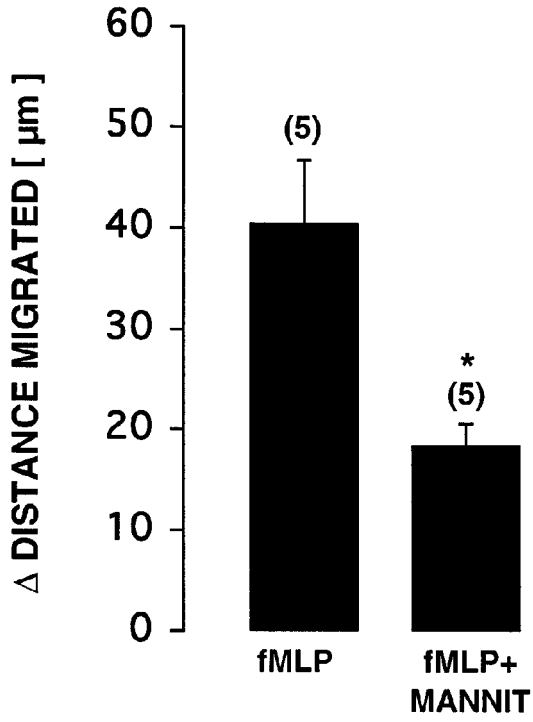

Figure 3 The fMLP (10 nmol l^{-1}) stimulated migration of PMNs under isotonic conditions (fMLP) was blunted after establishing hypertonic conditions by addition of 100 mmol l^{-1} mannitol to the upper compartment of the Boyden chamber (fMLP + MANNIT). Data are expressed as means ± s.e.mean. Numbers in parentheses indicate the number of individual observations. Asterisk indicates statistically significant difference ($P < 0.05$).

Intracellular pH

Effect of fMLP and Hoe 694 (Figure 4) Under control conditions pH_i of PMNs was 7.51 ± 0.04 ($n = 4$). Addition of fMLP (10 nmol l^{-1}) led to a rapid decrease of pH_i to 7.15 ± 0.05 by 0.36 ± 0.04 pH units ($n = 4$). In the presence of Hoe 694 (10 μmol l^{-1}), pH_i was 7.52 ± 0.16 ($n = 5$) which was not significantly different from pH_i under control conditions. Stimulation of these cells with fMLP led to a decrease of pH_i to 6.80 ± 0.12 bt 0.72 ± 0.10 pH units ($n = 5$), which was significantly lower than the respective value in the absence of the inhibitor.

Effect of Na^+ ommission and SCH 28080 (Figure 5a) In one series extracellular Na^+ was removed to abolish Na^+/H^+ exchange-dependent proton extrusion. In these cells pH_i decreased from 7.39 ± 0.07 to 7.19 ± 0.06 pH units ($\Delta pH_i = 0.20 \pm 0.08$; $n = 6$). Subsequent addition of H^+/K^+ATPase inhibitor SCH 28080 (10 μmmol l^{-1}) led to a further acidification to 6.87 ± 0.05 ($\Delta pH_i = 0.53 \pm 0.07$, $n = 6$).

Effect of Na^+ and K^+ omission (Figure 5b) In these experiments barium was added to block K^+ channels. In the presence of 5 mmol l^{-1} $BaCl_2$ pH_i was 7.17 ± 0.03 ($n = 6$). Removal of extracellular Na^+ led to a decrease of pH_i to

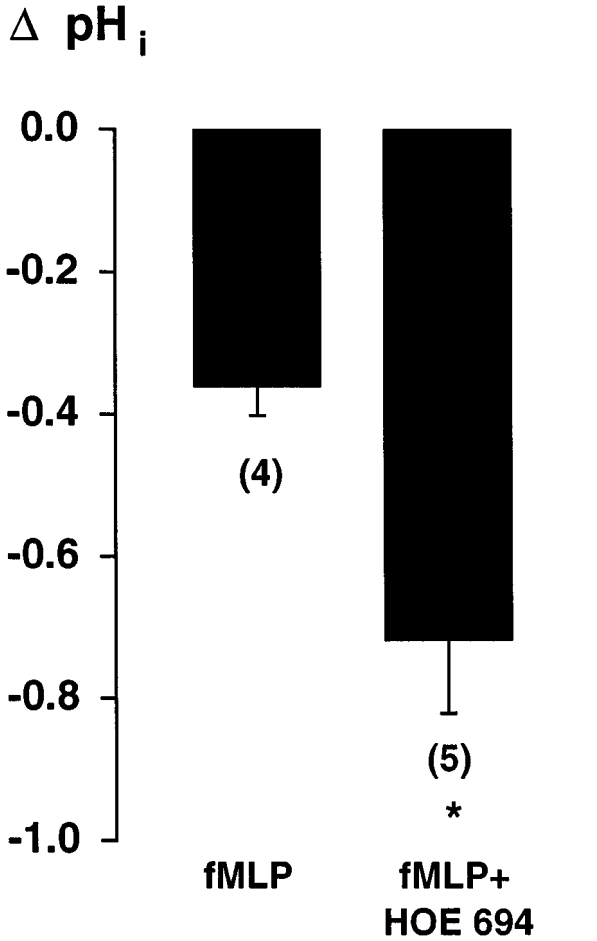

Figure 4 Decrease in intracellular pH (ΔpH_i) of PMNs upon stimulation with 10 nmol l^{-1} fMLP in the absence (fMLP) and in the presence of 10 μmol l^{-1} of Hoe 694 (fMLP + HOE). Data are expressed as means ± s.e.mean. Numbers in parentheses indicate the number of individual observations. Asterisk indicates statistically significant difference ($P < 0.05$).

7.08 ± 0.03 ($\Delta pH_i = 0.10 \pm 0.01$, $n = 6$). Subsequent omission of extracellular K^+ caused a further decrease in pH_i to 6.98 ± 0.04 ($\Delta pH_i = 0.19 \pm 0.04$, $n = 6$).

Cell volume (CV)-electronic cell sizing

Effect of fMLP and Na^+/H^+ exchange inhibitors (Figures 6a and b) The mean CV of PMNs was 338 ± 4 fl ($n = 25$). The fMLP induced intracellular acidification was paralleled by cell swelling: addition of fMLP (10 nmol l^{-1}) led within 5 min to a rapid increase in CV to $111 \pm 1\%$ ($n = 12$). In the presence of Hoe 694 or Hoe 642 (10 μmol l^{-1} each) CV of PMNs was 336 ± 5 fl ($n = 10$) and 301 ± 9 fl ($n = 5$), respectively. Under these conditions the fMLP-induced increase in CV was significantly blunted ($103 \pm 1\%$, $n = 10$ and $106 \pm 1\%$, $n = 5$; respectively). Approximatley half-maximal inhibition was achieved at a concentration of 1 μmol l^{-1} Hoe 694.

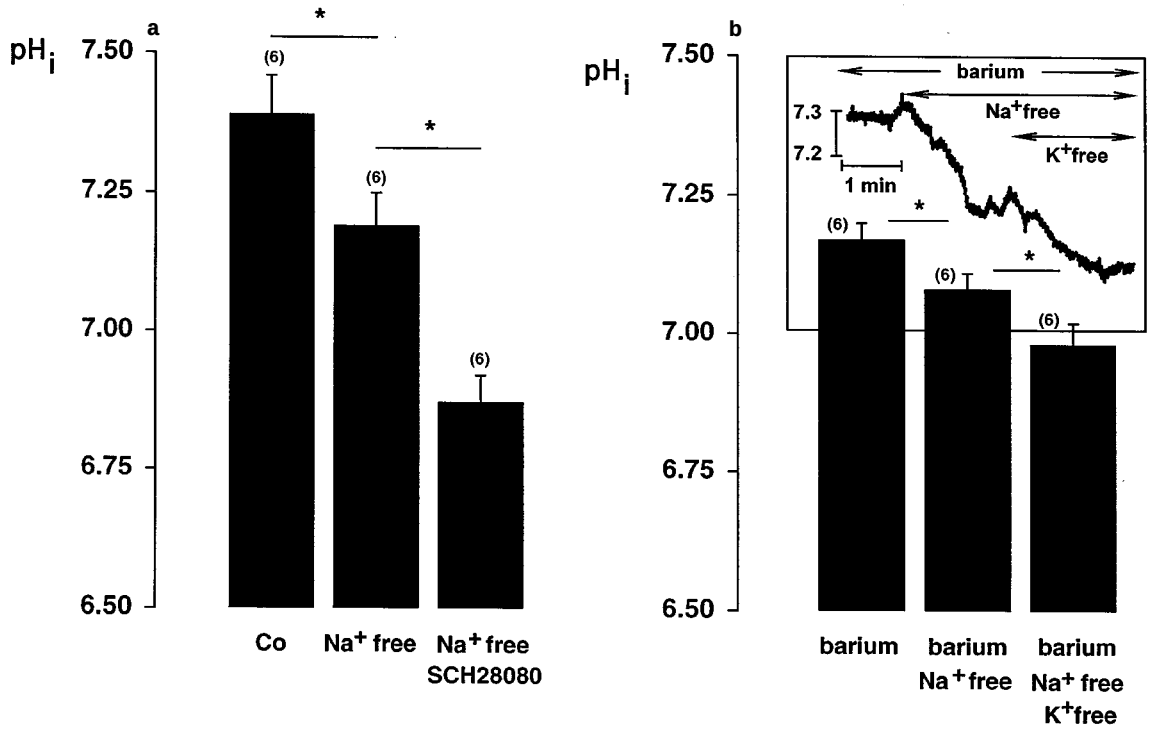

Figure 5 (a) Intracellular pH (pH_i) of PMNs under control conditions (CO), after omission of extracellular Na^+ (Na^+free) and after subsequent addition 10 μmol l^{-1} SCH 28080 (Na^+free/SCH28080). (b) pH_i of PMNs in the presence of 5 mmol l^{-1} $BaCl_2$ (barium), after omission of extracellular Na^+ (barium/Na^+free) and after subsequent omission of extracellular K^+ (barium/Na^+free/K^+free). Insert: redrawn original tracing from BCECF-fluorescence measurements representative for 6 similar experiments. Data are expressed as means $\pm$ s.e.mean. Numbers in parentheses indicate the number of individual observations. Asterisks indicate statistically significant difference between the conditions compared as indicated by the bars ($P < 0.05$).

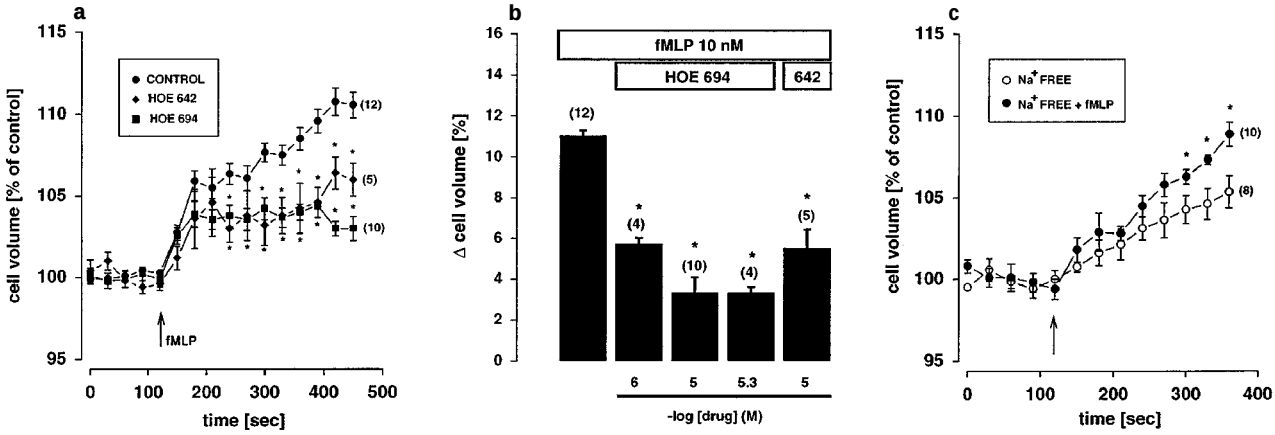

Figure 6 (a) Cell volume of suspended PMNs (as % of the mean initial volume of unstimulated cells) upon stimulation with 10 nmol l^{-1} fMLP (arrow) in the absence of inhibitors (control) and in the presence of 10 μmol l^{-1} Hoe 694 or Hoe 642. (b) Inhibitory effect of Hoe 694 (1, 10 and 50 μmol l^{-1}) and Hoe 642 (10 μmol l^{-1}) on fMLP-induced cell swelling. (c) Cell volume of PMNs suspended in Na^+free solution at time 0. These cells gradually increased their volume without further treatment. Addition of 10 nmol l^{-1} fMLP (arrow) further enhanced this cell swelling. (a–c) Data are expressed as means $\pm$ s.e.mean. Numbers in parentheses indicate the number of individual observations. Asterisks indicate statistically significant difference from the corresponding value in the absence of inhibitors (a and b) or fMLP (c), respectively ($P < 0.05$).

Effect of Na^+ omission (Figure 6c) In the nominal absence of extracellular Na^+ CV of PMNs was 337 ± 3 fl ($n=18$), but gradually increased without further treatment to $105 \pm 1\%$ ($n=8$) within 6 min. This cell swelling was still enhanced by fMLP within 4 min after addition to $109 \pm 1\%$ ($n=10$).

Effect of H^+/K^+ ATPase inhibitors (Figure 7a–c) In the presence of the H^+/K^+ATPase inhibitors omeprazole (10, 100 and 1000 μmol l^{-1}) or SCH 28080 (10 μmol l^{-1}), the CVs were 339 ± 9 fl ($n=5$), 342 ± 2 fl ($n=10$), 356 ± 3 fl ($n=4$) and 329 ± 6 fl ($n=7$), respectively. In the absence of inhibitors fMLP led to cell swelling to $111 \pm 1\%$ ($n=10$) and $112 \pm 2\%$ ($n=10$), respectively. This cell swelling was blunted by 10 μmol l^{-1} and 100 μmol l^{-1} omeprazole ($107 \pm 1\%$, $n=5$ and $102 \pm 0.4\%$, $n=10$; respectively) as well as by 10 μmol l^{-1} SCH 28080 ($105 \pm 1\%$, $n=5$). In the presence of both 10 μmol l^{-1} omeprazole and 1 μmol l^{-1} Hoe 694 the fMLP-induced increase in CV was completely inhibited ($101 \pm 1\%$, $n=4$).

Effect of increasing extracellular osmolarity (Figure 8) Addition of 100 mmol l^{-1} mannitol to the extracellular solution led to rapid cell shrinkage to $91 \pm 3\%$ ($n=5$) followed by regulatory volume increase (RVI) to $99 \pm 1\%$ ($n=5$) within 5 min. In the presence of Hoe 694 RVI was slower and significantly blunted ($96 \pm 1\%$, $n=7$), thus disclosing the activation of the Na^+/H^+ exchanger. Simultaneous addition of fMLP and mannitol counteracted the hypertonicity-induced cell shrinkage ($102 \pm 1\%$, $n=6$).

Cell volume-optical sectioning by confocal microscopy

The mean CV of PMNs adherent to uncoated glass cover slips assessed by optical sectioning with confocal microscopy was 451 ± 26 fl ($n=74$). Addition of 10 nmol l^{-1} fMLP led to an increase in CV to $126 \pm 3\%$ ($n=27$) within 20 min. This cell swelling was inhibited in the presence of 10 μmol l^{-1} Hoe 694 ($104 \pm 5\%$, $n=11$), 10 μmol l^{-1} SCH 28080 ($103 \pm 4\%$, $n=17$) or both Hoe 694 and SCH 28080 ($94 \pm 2\%$, $n=10$). Untreated cells did not significantly change their CV within 20 min ($102 \pm 1\%$, $n=9$) (Figure 9).

E. coli *killing*

Plating of an *E. coli* suspension prepared in the absence of PMNs on agar plates yielded $8.78 \pm 2.59 \times 10^7$ ($n=8$) $CFU_{E.coli}$ ml^{-1} after overnight cultivation. A 2 h incubation of *E. coli* with PMNs resulted in a marked reduction of bacterial colony formation to $4.10 \pm 2.21 \times 10^7$ by $4.66 \pm 1.12 \times 10^7$ $CFU_{E.coli}$ ml^{-1} ($n=8$). Stimulation of the cells with 10 nmol l^{-1} fMLP did not modify their anti-

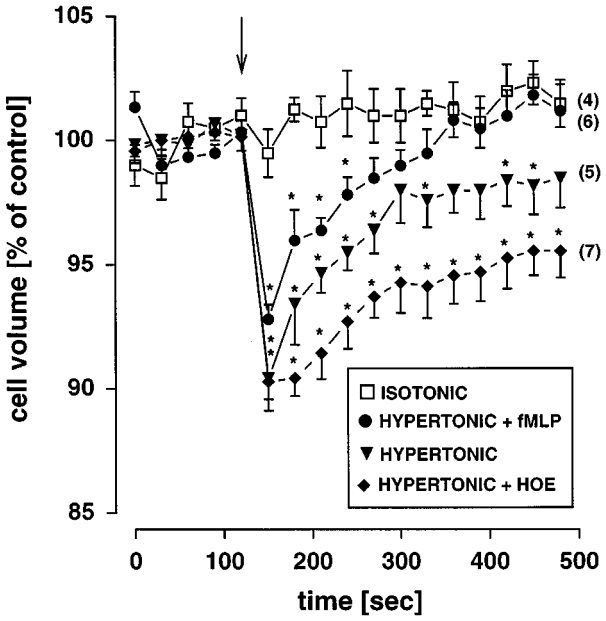

Figure 8 Cell volume of PMNs (as % of the mean initial volume of untreated cells) under isotonic conditions and after induction of cell shrinkage by addition of 100 mmol l^{-1} mannitol (arrow) in the absence of drugs, in the presence of 10 μmol l^{-1} Hoe 694 or in the presence of 10 nmol l^{-1} fMLP. Data are expressed as means-±s.e.mean. Numbers in parentheses indicate the number of individual observations. Asterisks indicate statistically significant difference from the corresponding value under isotonic conditions ($P<0.05$).

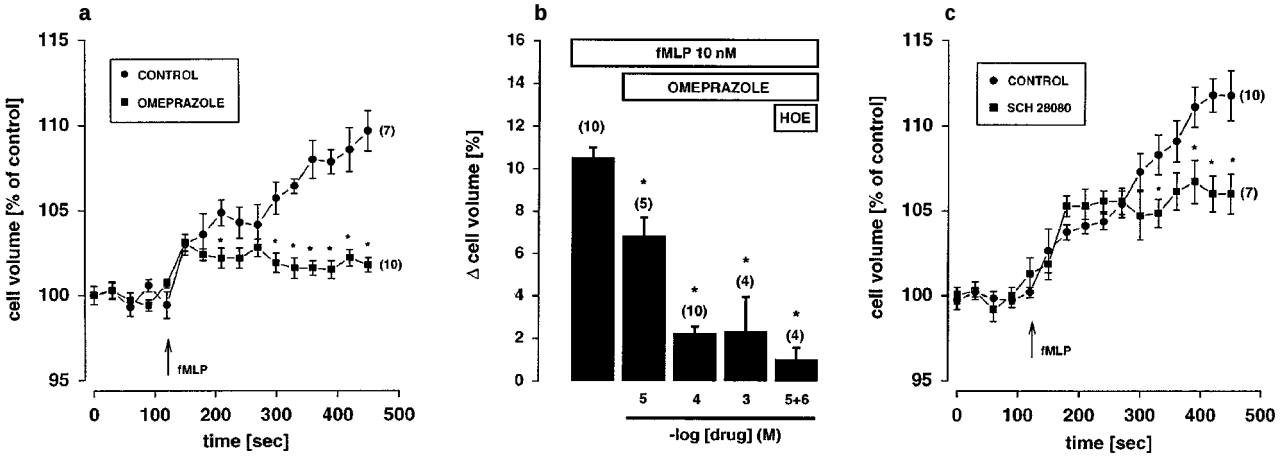

Figure 7 (a) Cell volume change of PMNs (as % of the mean initial volume of unstimulated cells) upon stimulation with 10 nmol l^{-1} fMLP (arrow) under control conditions (solvent polyethylenglycole 400, 1 : 1000) and in the presence of 100 μmol l^{-1} omeprazole. (b) Inhibitory effect of omeprazole (10, 100 and 1000 μmol l^{-1}) and both omeprazole (10 μmol l^{-1}) and Hoe 694 (1 μmol l^{-1}) on fMLP-induced cell swelling. (c) Cell volume change of PMNs upon stimulation with fMLP (arrow) under control conditions and in the presence of 10 μmol l^{-1} SCH 28080. (a–c) Data are expressed as means±s.e.mean. Numbers in parentheses indicate the number of individual observations. Asterisks indicate statistically significant difference from the corresponding value in the absence of inhibitors ($P<0.05$).

bacterial activity. The $CFU_{E.coli}\,ml^{-1}$ were reduced to $4.08 \pm 2.24 \times 10^7$ by 4.71 ± 1.11 ($n=8$) $CFU_{E.coli}\,ml^{-1}$ under these conditions. No significant inhibition could be observed in fMLP treated cells upon addition of 10 μmol l^{-1} Hoe 694 or 10 μmol l^{-1} omeprazole. The $CFU_{E.coli}\,ml^{-1}$ were reduced to $4.4 \pm 2.14 \times 10^7$ by 4.37 ± 1.21 ($n=8$) and to $4.27 \pm 2.17 \times 10^7$ by 4.51 ± 1.13 ($n=8$), respectively. The respective solvents were without effect on the bactericidal function of PMNs.

Identification of the H^+/K^+ ATPase β-subunit (Figure 10)

Western analysis was performed to test for a possible expression of H^+/K^+ATPase in PMNs using the anti gastric H^+/K^+ATPase β-subunit antibody 2B6 supplied by MBL (Nishio *et al*., 1994; Callaghan *et al*., 1995). Lysates from mouse and human gastric mucosa served as positive controls and lysates from HepG2 cells, a human liver derived cell line, as negative control. To protect proteins during preparation of the cell lysates from degradation by proteolytic enzymes present in granulocytes, various protease inhibitors were added to the lysis buffer. The lysates were resolved on SDS-PAGE, electrotransferred onto nitrocellulose membranes and the proteins were stained with Poncau S. As shown in the upper panels of Figure 10, multiple protein bands were present which distributed over the whole range of the membranes. Incubation of the membranes with he antibody 2B6 yielded a broad smear of immunoreactive proteins in the range between 60 and 80 kD in human and mouse gastric mucosa. A faint smear was also detected within this range in lysates of human PMNs on 10% SDS-PAGE. On 13% SDS-PAGE this signal appeared as a band. No signal could be detected in lysates of HepG2 cells (Figure 10a, lower panel).

Treatment of the lystates with peptide N-glycosidase F led to the appearance of a band with an apparent molecular mass of ~35 kD in mouse and human gastric mucosa, as well as in human PMNs. This band is likely to represent the deglycosylated form of an H^+/K^+ATPase β-subunit. The signal at 60 to 80 kD disappeared nearly completely in PMNs and human gastric mucosa. In mouse gastric mucosa a band at about 70 kD persisted and probably represents a partially deglycosylated H^+/K^+ATPase β-subunit.

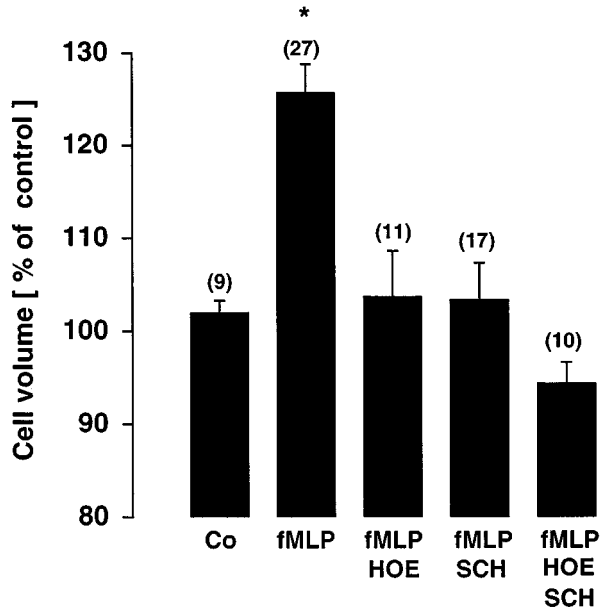

Figure 9 Cell volume measurements of PMNs adherent to glass surface by optical sectioning with confocal microscopy. The first measurement was performed to determine the cell volume before treatment and a second measurement was made 20 min after superfusing the cells with control solution alone (Co) or with the same solution containing 10 nmol l^{-1} fMLP, fMLP and 10 μmol l^{-1} Hoe 694 (HOE), fMLP and 10 μmol l^{-1} SCH 28080 (fMLP, SCH) or fMLP, SCH 28080 and Hoe 694 (fMLP, HOE, SCH). Changes in cell volume are given as % of the initial volume of untreated cells. Data are expressed as means ± s.e.mean. Numbers in parentheses indicate the number of individual observations. Asterisks indicate statistically significant difference from the corresponding value under control conditions ($P<0.05$).

Discussion

The present data confirm previous findings demonstrating dependence of stimulated migration of polymorphonuclear leucocytes (PMNs) on Na^+/H^+ exchange (Simchowitz & Cragoe, 1986; Rosengren *et al*., 1994). As shown in Figure 1, the fMLP-stimulated migration of PMNs was completely inhibited in the presence of EIPA, Hoe 694 or Hoe 642, potent and selective inhibitors of the NHE1 isoform of the Na^+/H^+ exchanger (Wöll *et al*., 1993; Counillon *et al*., 1993; Scholz *et al*., 1993; 1996; Noël & Pouysségur, 1995). In contrast, there was only a minute or even absence of an effect on the random migration. A similar observation was made by Demaurex *et al*. (1996) who observed that the random migration was even more pronounced in Na^+-free media. Obviously lack of activity of the Na^+/H^+ exchanger does not preclude cells from random migration, whereas stimulated migration requires full activity of the exchanger. Stimulation of PMNs with fMLP or TPA leads to formation of H^+ ions due to activation of the NADPH oxidase and hexose monophosphate shunt pathway (Borregaard *et al*., 1984; Grinstein & Furuya, 1986; Nanda & Grinstein, 1991; Nanda *et al*., 1992; 1993). The resulting intracellular acid load is compensated by activation of the Na^+/H^+ exchanger, leading to restoration of the initial pH_i and a secondary intracellular alkalinization. In the presence of Hoe 694 (Figure 4), amiloride or in the nominal absence of Na^+ the acidification is stronger and the secondary alkalinization is abolished (Grinstein & Furuya, 1984), pointing to the crucial role of the exchanger in extruding cellular excess acid. Similar molecular mechanisms lead to activation of the Na^+/H^+ exchanger in PMNs during adhesion and spreading of the cells and during phagocytosis. However, in contrast to stimulated migration Na^+/H^+ exchange activity is not required for cell adhesion, spreading or phagocytosis, since these processes proceeded normally in the presence of Hoe 694 (Demaurex *et al*., 1996; Fukushima *et al*., 1996). Similarly, we found that the bactericidal activity of PMNs remained unaffected in the presence of Hoe 694. From earlier studies, showing inhibition of superoxide production in media devoid of Na^+ (Korchak & Weissmann, 1978) or in the presence of amiloride (Berkow *et al*., 1987), it has been inferred that the activation of the Na^+/H^+ exchanger is essential for a normal bactericidal response in PMNs. However, the inability of Hoe 694 to suppress *E. coli* killing suggests that alternative mechanisms can compensate for the NHE activity during bacterial killing.

Another consequence of Na^+/H^+ exchange activation is cell swelling (Grinstein *et al*., 1986; Grinstein & Foskett, 1990; Lang *et al*., 1998). PMNs rapidly swell upon stimulation with fMLP (Grinstein & Furuya, 1984; Rosengren *et al*., 1994; Worthen *et al*., 1994), an effect that can be in part inhibited by Hoe 694 or Hoe 642 or by replacement of extracellular Na^+ by $choline^+$ (Figure 6). Assessment of the subcellular distribution

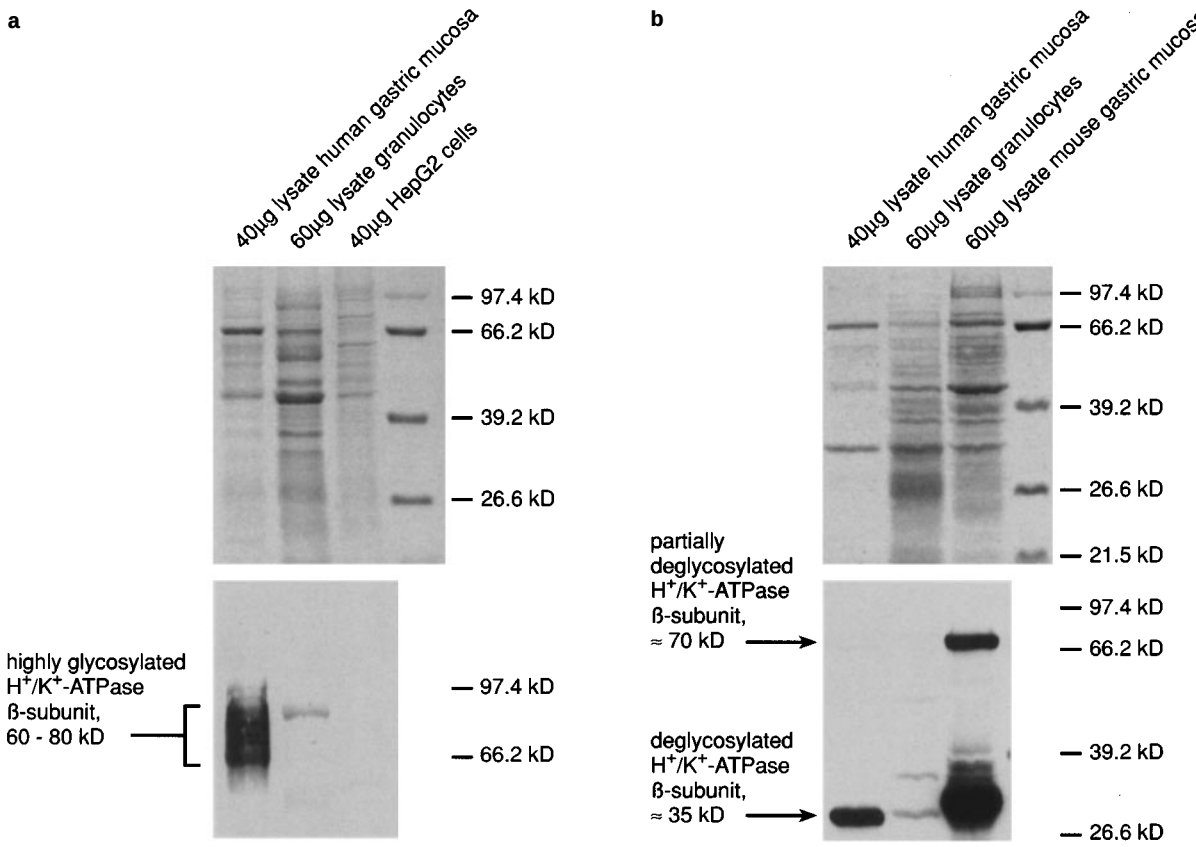

Figure 10 Identification of a human PMN protein that binds to a monoclonal anti-mouse proton pump β-subunit antibody (2B6) supplied by MBL. (a) Lysates of human gastric mucosa, PMNs and HepG2 cells were separated in a 13% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was incubated with monoclonal antibody 2B6. A broad smear is seen between 60 and 80 kD in human gastric mucosas and a smaller band in PMNs. No signal was detected in HepG2 cells (negative control). (b) Lysates from human gastric mucosa, PMNs and mouse gastric mucosa (positive control) were treated with protein N-glycosidase F before electrophoresis. A band at about 35 kD was detected in all lanes, which is likely to represent the deglycosylated form of the H^+/K^+ATPase β-subunit. The signal at 60 to 80 kD disappeared nearly completely in PMNs and human gastric mucosa after deglycosylation. In mouse gastric mucosa, a band at about 70 kD probably represents a partially deglycosylated H^+/K^+ATPase β-subunit. The upper panels show the Ponceau S stained membranes, the lower panels the corresponding Western blots.

of the Na^+/H^+ exchanger in fibroblasts revealed focal concentration of the protein along the border of lamillipodia and near the edge of cell processes (Grinstein *et al*., 1993; Goss *et al*., 1994). Accordingly, cell swelling due to water influx at the leading edge would support directed cell membrane protrusion. To distinguish whether PMN migration requires cell swelling or other effects due to activation of the Na^+/H^+ exchanger, the antiporter was activated by increasing extracellular osmolarity. This leads to cell shrinkage which is followed by regulatory volume increase (RVI) and intracellular alkalinization (Grinstein *et al*., 1992). Both the increase in pH_i and RVI were inhibited by Hoe 694 and are therefore mediated by Na^+/H^+ exchange (Figure 8). Osmotic activation of the Na^+/H^+ exchanger by addition of mannitol did not stimulate cell migration *per se* or alter the random migration of the cells but effectively suppressed fMLP-stimulated migration (Figure 3). As shown in Figure 8, the fMLP-induced CV increase was counteracted under these conditions. These results suggest that cell swelling is a prerequisite for stimulated cell migration and are in agreement with those obtained by Rosengren *et al.* (1994), who demonstrated that inhibition of neutrophil chemotaxis by amiloride or dimethyl-amiloride could be overcome in hypoosmolar media.

However, the activation of the Na^+/H^+ exchanger does not seem to be the only mechanism mediating cell volume increase after stimulation with fMLP since it cannot be completely suppressed by inhibition of the antiporter, and significant cell swelling occurs even in the nominal absence of Na^+ as measured by electronic cell sizing (Figure 6). Volume measurements performed by three-dimensional reconstruction of PMNs migrating in collagen matrices (Rosengren *et al*., 1994) or of PMNs adherent to glass surface (Figure 9) on the other hand yielded a more complete inhibition of fMLP-mediated CV increase by impairment of the Na^+/H^+ exchanger. This might be due to different behaviour of adherent and suspended cells. Nathan described different patterns of H_2O_2 release in suspended neutrophils and those adherent to glass or several constituents of biological surfaces (Nathan, 1987). For pH_i comparable results have been obtained in suspended and adherent PMNs in response to fMLP or TPA (Grinstein & Furuya, 1984; Nanda & Grinstein, 1991; Nanda *et al*., 1992; Rosengren *et al*., 1994; Demaurex *et al*., 1996). To test whether PMNs adherent to glass surface behaved like cells in suspension with respect to CV changes, we performed optical sectioning measurements by confocal microscopy. The results obtained by this method are in good agreement with the findings from electronic cell sizing and confirm that fMLP-induced cell swelling is sensitive to blockers of the Na^+/H^+ exchanger and gastric H^+/K^+ATPase (Figure 9). However, the absolute CVs obtained with three-dimensional reconstruction were $\sim$30% higher than those measured by electronic cell sizing. While this might be attributed in part

to overestimation of the cell volume (see Methods), an increase in CV is expected to occur following activation of the Na^+/H^+ exchanger during adhesion and spreading (Demaurex *et al*., 1996; Fukushima *et al*., 1996).

Nanda and Grinstein have calculated that the pH_i reached during respiratory burst in neutrophils with inhibited Na^+/H^+ exchange activity is about 5 pH units above the value predicted from the rate of H^+ generation. They have shown the existence of a protein kinase C activated H^+ (equivalent) conductance and bafilomycin-sensitive acid extrusion following insertion of V-type H^+ATPase bearing vesicles into the plasma membrane after agonist stimulation (Nanda & Grinstein, 1991; Nanda *et al*., 1992; 1996). As shown in Figure 5, PMNs exhibit agonist-independent intracellular acidification upon step omission of extracellular Na^+, most likely due to reversal of the Na^+/H^+ exchanger (Demaurex *et al*., 1996). This manoeuvre was chosen to exclude Na^+-dependent and to minimize H^+ATPase-dependent H^+-extrusion. The acidification was significantly enhanced upon addition of SCH 28080, a selective K^+-competitive antagonist of gastric H^+/K^+ ATPase (Sachs *et al*., 1995). To determine whether this acidification is a K^+-dependent process, K^+ ions were removed from the extracellular fluid and Ba^{2+} was added to block K^+ channels, thus preventing eventual recirculation of cellular K^+ ions. As shown in Figure 5b, the acidification upon withdrawal of Na^+ could be further enhanced by step omission of extracellular K^+. Moreover, the CV increase elicited by fMLP was significantly inhibited by omeprazole or SCH 28080 and completely inhibited by addition of both Hoe 694 and omeprazole at concentrations which exert approximately half-maximal inhibitory effects when administered alone (Figure 7b). In addition, cell migration was inhibited by omeprazole (Figure 2a) (Wandall, 1992) and SCH 28080 (Figure 2b), inhibitors which act on distinct sites on the ion transporter (Sachs *et al*., 1995). These results indicate the existence of a K^+-dependent SCH 28080/omeprazole-sensitive H^+ transporting mechanism mediating cell swelling. Given the specificity of the inhibitors, the results point to a possible involvement of H^+/K^+ATPase. This ion transporter catalyzes the uptake of K^+ ions in exchange for H^+ ions. This is expected to result in increased CV if cellular accumulation of K^+ ions is supported by a hampered exit of the ions due to a decreased K^+ conductance of the cell membrane, which is probably caused by the intracellular acidosis (Oberleithner *et al*., 1988). However, even if the concentrations used are expected to specifically inhibit H^+/K^+ATPase, nonspecific inhibition of V-type H^+ ATPase cannot completely be ruled out, since both omeprazole and SCH 28080 have been shown to block this ion transporter at albeit high (>100 μmol l^{-1}) concentrations (Sabolic *et al*., 1994). It is tempting to speculate that such a mechanism would support migration of the cells under conditions that are unfavourable for Na^+/H^+ exchange activity, such as in the highly acidic milieu of abscesses (Swallow *et al*., 1990), by providing an independent mechanism for CV increase. In contrast—as observed for inhibition of the Na^+/H^+ exchanger—the bactericidal activity of PMNs was not affected by 10 μmol l^{-1} omeprazole or SCH 28080.

Immunoblotting experiments with lysates of human PMNs and of mouse and human gastric mucosa using the monoclonal anti gastric H^+/K^+ATPase β-subunit antibody 2B6 (Callaghan *et al*., 1995) detected a protein with an apparent molecular mass between 60 and 80 kD in lysates of human and mouse gastric mucosa. A smaller signal was also detected within this range in lysates of human PMNs. This corresponds to the native molecular mass reported for gastric H^+/K^+ATPase β-subunit. Since the distribution of the stained proteins within this range of molecular mass has been shown to result from the high degree of glycosylation of the H^+/K^+ATPase β-subunit, the lysates were treated with N-glucosidase F to remove N-linked carbohydrates. This resulted in a reduction of the molecular mass of the immunoreactive protein in PMNs as well as in human and mouse gastric mucosa to an apparent molecular mass of $\sim$35 kDa, which is identical with the core protein of H^+/K^+ATPase β-subunit expressed in stomach and kidney (Rabon & Reuben, 1990; Wingo & Smolka, 1995). This was paralleled by the nearly complete disappearance of the signal at 60 to 80 kD. In mouse gastric mucosa a band at about 70 kD persisted and most likely represents partially deglysosylated H^+/K^+ATPase β-subunit. Similar results have been shown for distal tubular H^+/K^+ATPase using the same antibody (Callaghan *et al*., 1995). Given the specificity of the 2B6 antibody, the results indicate the existence of a protein in human PMNs which is related to gastric and renal H^+/K^+ATPase (Nishio *et al*., 1994; Sachs *et al*., 1995; Callaghan *et al*., 1995). It remains to be investigated whether this protein is part of functional active H^+/K^+ATPase, which could account for the K^+-dependent H^+-extrusion and cell swelling of stimulated PMNs observed in this study.

In conclusion, PMNs respond to the chemoattractant fMLP with rapid cell swelling due to activation of the Na^+/H^+ exchanger and a K^+-dependent omeprazole/SCH28080 sensitive H^+ ion extruding mechanism, presumedly an H^+/K^+ATPase. Prevention of fMLP-induced cell swelling by inhibition of either ion transporter or osmotically counteracting the increase in cell volume preclude PMNs from stimulated migration.

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