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AGONIST-STIMULATED Cl^- EFFLUX FROM HUMAN NEUTROPHILS

A COMMON PHENOMENON DURING NEUTROPHIL ACTIVATION

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Abstract—When human peripheral blood neutrophils were stimulated with various agonists which activate and/or prime neutrophils, we found that Cl^- efflux was enhanced with a dramatic (50%) loss of intracellular Cl^-. Interestingly, the Cl^- efflux was enhanced by both agonists which induce a rapid transient increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [class I, e.g. *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), interleukin-8 (IL8), platelet-activating factor, leukotriene B_4 and C5a] and those which do not induce such an $[Ca^{2+}]_i$ elevation [class II, e.g. tumor necrosis factor α (TNF) and granulocyte-macrophage colony-stimulating factor (GM-CSF)]. The time course of agonist-stimulated Cl^- efflux differed depending on the agonist. Class I agonists such as IL8 and fMLP exhibited a 1 min lag phase before the onset of Cl^- efflux; class II agonists such as GM-CSF and TNF displayed a 2 and 5 min lag phase, respectively. Both IL8 (class I)- and TNF (class II)-stimulated Cl^- efflux exhibited similar sensitivity to inhibition by different types of ion transport inhibitors [ethacrynic acid (EA), amiloride, 4-acetamido-4′-isothiocyanato-stilbene-2,2′-disulfonic acid, anthracene-9-carboxylic acid, and 4-4′-diisothiocyanatostilbene-2,2′-disulfonic acid]. On the other hand, natural Cl^- efflux, which is thought to be mainly mediated by Cl^-/Cl^- self exchange, was not inhibited by EA (0.5 mM) or amiloride (0.3 mM). These results imply that both class I and class II agonist-stimulated Cl^- efflux occurs via a common Cl^- transporter which is different from that reported previously in resting human neutrophils. Although all agonists which induced a Cl^- efflux also induced shape change of neutrophils, there did not appear to be a causal relationship between shape change and agonist-stimulated Cl^- efflux. However, a temporal correlation was found to exist between agonist-stimulated Cl^- efflux and intracellular alkalinization following agonist stimulation. Agonist-stimulated Cl^- efflux therefore seems to be a common phenomenon activated by several agonists which act through different signal transduction pathways.

Neutrophils, the most numerous of the white blood cells in man, respond to a vast range of different agonists [e.g. *N*-formyl-methionyl-leucyl-phenylalanine (fMLP†), interleukin-8 (IL8), platelet-activating factor (PAF), leukotriene B_4 (LTB_4), tumor necrosis factor α (TNF) and granulocyte-macrophage colony-stimulating factor (GM-CSF)] with a diverse array of receptors on the cell surface. All the agonists however activate a similar series of responses that result in cell functions such as shape change, chemotaxis, adhesion, priming, phagocytosis and release of inflammatory mediators and oxidants [1–4]. Although the precise mechanisms of neutrophil activation are unknown, evidence suggests the involvement of many signal transduction-related proteins and second messengers, including an important role for inorganic ion movements in the generation of neutrophil responses (see Ref. 5 for review). There have been many studies investigating Na^+, H^+, K^+ and Ca^{2+} ion movement [5–7], intracellular pH (pH_i) change [8–12], and membrane potential changes [13] during neutrophil activation. Recently, it has been shown that chloride movements, which occur via Cl^- channels or a Cl^- transporter, have many physiological roles in various cells, such as pH control [14, 15], cell volume control [14, 16–18], stabilizing membrane potential [19], transepithelial transport (see Ref. 20 for review) and contraction of vascular smooth muscle cells [21, 22].

The neutrophil is unusual in that chloride is not passively distributed according to membrane potential, but is actively maintained at a value 4-fold higher than equilibrium [23]. However, although chloride content and Cl^- movement in the resting neutrophil have been measured [15, 23], little is known about Cl^- movement following neutrophil activation, and there is no information about agonist-activated Cl^- transporters in human neutrophils. In this paper, we have investigated the effect of several

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† Abbreviations: fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; rh, recombinant human; IL8, interleukin-8; TNF, tumor necrosis factor α; PAF, platelet-activating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor; LTB_4, leukotriene B_4; PMA, phorbol myristate acetate; LPS, lipopolysaccharide; EA, ethacrynic acid; 9-AC, anthracene-9-carboxylic acid; SITS, 4-acetamido-4′-isothiocyanato-stilbene-2,2′-disulfonic acid; CHC, α-cyano-4-hydroxycinnamic acid; Cyt B, cytochalasin B; DIDS, 4-4′-diisothiocyanatostilbene-2,2′-disulfonic acid; DMSO, dimethyl sulfoxide; BSS, balanced salt solution; BSA, bovine serum albumin; CMS, calcium- and magnesium-free BSS.

kinds of agonist, which activate and/or prime neutrophils, on Cl^- movement from the cells. Almost all of the agonists induced a marked Cl^- efflux accompanied by a dramatic loss (50%) of intracellular Cl^- (Cl_i^-). We have also investigated the nature of the Cl^- transporter which mediates the agonist-stimulated Cl^- efflux, and the possible relationship between the Cl^- efflux, shape change and pH_i changes.

MATERIALS AND METHODS

Reagents. Reagents were obtained from the following sources: recombinant human (rh) IL8, rhTNF, rh platelet-derived growth factor (PDGF), β-chain homodimer (Bachem Inc., Essex, U.K.); rhGM-CSF, rh transforming growth factor (TGF) β1 (British Bio-technology Products Ltd, U.K.); fMLP, C5a, PAF, LTB_4, A23187, phorbol myristate acetate (PMA), lipopolysaccharide (LPS) purified from *Escherichia coli* K235, amiloride, ethacrynic acid (EA), anthracene-9-carboxylic acid (9-AC), 4-acetamido-4′-isothiocyanato-stilbene-2,2′-disulfonic acid (SITS), α-cyano-4-hydroxycinnamic acid (CHC), 4-4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), cytochalasin B (Cyt B), heparin, bovine serum albumin (BSA; essentially fatty acid free), fura-2-AM (Sigma Chemical Co., Poole, U.K.); BCECF-AM (Molecular Probes, Eugene, OR, U.S.A.); $Na^{36}Cl$ (sp. act. of $^{36}Cl^-$ was 12–14 mCi/g Cl^-) (Amersham International plc, Amersham, U.K.); ionomycin (Calbiochem, Nottingham, U.K.). The standard medium used throughout this study had the following composition (per litre): BSS (balanced salt solution: 137 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose 1 g, 10 mM HEPES, pH 7.4), BSA–BSS (5 mg/mL BSA containing BSS), CMF (calcium- and magnesium-free BSS).

Isolation of human peripheral blood neutrophils. Neutrophils were obtained from heparinized venous blood from healthy adult donors and purified by standard techniques as described previously [24]. Briefly, fresh human blood (about 80 mL) was obtained by venepuncture and collected into heparin (10 U/mL blood). Neutrophils were isolated from red cells by sedimentation with Dextraven 110 (Fisons plc, Loughborough, U.K.) (one part Dextraven 110:three parts blood) for 45 min at 37°. The leucocyte-rich supernatant was washed in CMF (300 g for 10 min) and the pellet was resuspended in 16 mL CMF. The cell suspension was divided into equal volumes, layered on four 6-mL Ficoll-Paque cushions (Pharmacia, Piscataway, NJ, U.S.A.) and spun at 300 g for 40 min at room temperature. After removing the red cell layer (bottom layer of the pellets) carefully with a micro tip, the remaining pellet, containing neutrophils, was collected and washed once in BSS before finally resuspending the cells in BSA–BSS. Neutrophil number and purity were assessed by counting the cells diluted 1:10 in Turk's white cell counting fluid on a modified Neubauer haemocytometer. Neutrophil purity was between 95 and 98% as assessed by cytospin and Giemsa-May Grunwald differentiating stain and the average yield was 1–2 × 10^8 cells/80 mL blood. Cell viability as assessed by Trypan blue exclusion was >99%.

Measurement of $^{36}Cl^-$ *efflux.* To measure $^{36}Cl^-$ efflux, the technique described by Simchowitz and De Weer [23] was used. For the efflux studies, neutrophils were first suspended at 2 × 10^7 cells/mL and incubated with $^{36}Cl^-$ (2.5 μCi/mL) for 2 hr at 37°. Before the addition of agonists the cells were washed twice in BSA–BSS prewarmed at 37° and then resuspended in unlabeled BSA–BSS. At stated intervals, duplicate 100 μL aliquots were layered on 0.7 mL of silicone oil (Versilube F-50, GE Silicones, Waterford, NY, U.S.A.), contained in 1.5 mL plastic tubes, and spun at 8000 g for 30 sec in a microcentrifuge (5415C, Eppendorf, Hamburg, Germany). The supernatants were dispersed separately in 6 mL of Ultima Gold (Packard, Downers Grove, IL, U.S.A.) and counted for 5 min in a liquid scintillation counter (2500TR, Packard). The percentage efflux was calculated as follows: [(cpm of supernatant of sample) − (cpm of supernatant at zero time)]/[(total cpm of cell suspension) − (cpm of supernatant at zero time)] × 100.

To test the effects of ion transport inhibitors, chemicals were added to the cells (2–4 × 10^6 cells/assay) 5 min before addition of agonists. Then, we compared the $^{36}Cl^-$ efflux of inhibitor-treated cells with non-treated cells at 7.5 (for IL8, fMLP and GM-CSF) and 15 (for TNF) min after agonist stimulation. To assess the effects of the above chemicals on $^{36}Cl^-$ efflux from unstimulated neutrophils (natural efflux), we compared the $^{36}Cl^-$ efflux from the cells at 30 min with and without inhibitors. Chemicals were dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 0.25% (v/v). At this concentration, DMSO alone did not interfere with agonist-stimulated Cl^- efflux. The percentage efflux inhibition at the stated time point was calculated as follows: % inhibition = 100 × [(cpm of agonist-stimulated efflux) − (cpm of agonist stimulated efflux with inhibitor)]/[(cpm of agonist-stimulated efflux) − (cpm of natural efflux)]. This allowed a correction to be made for any differential effects of the inhibitors on natural versus agonist induced efflux.

Measurement of intracellular $^{36}Cl^-$ *content.* To determine the net movement of $^{36}Cl^-$ (balance of $^{36}Cl^-$ efflux and influx), we measured the change in intracellular $^{36}Cl^-$ content ($^{36}Cl_i^-$). Neutrophils were suspended at 4 × 10^7 cells/mL and incubated with $^{36}Cl^-$ (2.5 μCi/mL) containing BSA–BSS for 2 hr at 37° to equilibrate the $^{36}Cl^-$ concentration between intracellular and extracellular solution. We used this cell suspension for the following experiments without washing. After addition of agonists, at different times, duplicate aliquots (50 μL) were collected, diluted into 0.5 mL of BSA—BSS layered on 0.7 mL of silicone oil and centrifuged at 8000 g for 30 sec. After removing the supernatants, the pellets were isolated and counted as above. To account for any background $^{36}Cl^-$ which was trapped between intercellular spaces in the cell pellet, we centrifuged the cells immediately after addition of $Na^{36}Cl$ and counted the radioactivity associated with the pellet. The percentage of remaining $^{36}Cl_i^-$ was calculated as follows: % remaining $^{36}Cl_i^-$ = 100 × [(cpm of

sample pellet) − (cpm of control pellet)]/[(cpm of control pellet) − (cpm of background level)].

Measurement of intracellular Ca^{2+} elevation. Neutrophils (2×10^7 cells) were loaded with fura-2 by incubation with 5 μg/mL fura-2-AM, as described previously [25]. The cells were washed twice in BSA–BSS, and kept in the same buffer at room temperature until use. The agonist induced fluorescence changes of a 2-mL stirred neutrophil suspension (2×10^6 cells/mL) kept at 37° were monitored with a LS-50B luminescence spectrometer (Perkin Elmer, Buckinghamshire, U.K.), using 340 and 380 nm excitation wavelengths and 510 nm emission wavelength.

Measurement of intracellular pH. Neutrophils (2×10^7 cells) were loaded with BCECF by incubation with BCECF-AM (3 μg/mL) for 30 min at 37°. After washing twice in BSA–BSS the cells were resuspended (2×10^6 cells/mL) and agonist-induced fluorescence changes at Ex 485 nm and Em 540 nm measured. Calibration was performed as described previously [26].

Shape change assay. Purified neutrophils were incubated at 37° with various agonists for varying time points (30 sec to 20 min). After the incubation period the neutrophils were fixed by the addition of an equal volume of BSS containing 0.5% glutaraldehyde for 10 min at room temperature. The cells were then examined under the microscope (Labophot-2A, Nikon, Japan) and scored for shape changed cells as described [27].

RESULTS

Time course and dose dependence of agonist-stimulated Cl^- efflux

We first investigated the effect of neutrophil-activating and/or priming agonists on Cl^- movement in neutrophils. When we measured $^{36}Cl^-$ efflux from neutrophils after agonist stimulation, we found that most of the agonists we examined, such as fMLP, IL8, C5a, PAF, LTB_4, TNF and GM-CSF, enhanced the $^{36}Cl^-$ efflux from neutrophils compared with natural efflux, in a time- and dose-dependent manner (Figs 1 and 2). These agonists could be divided into two types based on their ability to induce a rapid transient $[Ca^{2+}]_i$ elevation (Table 1). Class I agonists, such as IL8, fMLP, PAF and LTB_4, induced $[Ca^{2+}]_i$ elevation dose dependently, whereas class II agonists TNF and GM-CSF did not (Fig. 3). This indicates that the agonist-stimulated Cl^- efflux does not necessarily require such a $[Ca^{2+}]_i$ elevation before stimulation. In addition, the concentrations which stimulated a half-maximal response (ED_{50}) were different for class I and class II. The ED_{50} for class II agonists (2×10^{-11}–5×10^{-11} M) was one to two orders of magnitude lower than that of class I agonists (5×10^{-10}–4×10^{-9} M; Fig. 2, Table 1). This suggests that TNF and GM-CSF are more potent stimulators of Cl^- efflux than well-known chemoattractants such as C5a and IL8. It is important to note that these agonists stimulate Cl^- efflux even at a priming dose, which does not stimulate strong superoxide and/or arachidonic acid release.

The time courses of these agonist-stimulated $^{36}Cl^-$ effluxes also differed depending on class of agonist; that is, class I agonists stimulated $^{36}Cl^-$ efflux within 1 min of agonist stimulation whereas class II agonists reproducibly exhibited a longer lag time. For example, fMLP and IL8 stimulated Cl^- efflux within about 1 min of agonist stimulation, whereas GM-

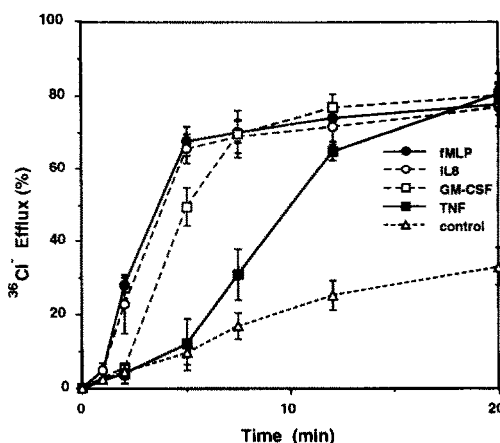

Fig. 1. Effect of several agonists on $^{36}Cl^-$ efflux from cells. After incubation of the cells for 2 hr in $^{36}Cl^-$-containing BSA–BSS, the cells were washed twice and then resuspended with unlabeled BSA–BSS. At the zero time point, we stimulated the cells with IL8 (10^{-8} M), fMLP (10^{-8} M), GM-CSF (7×10^{-9} M) or TNF (6×10^{-9} M). At stated times, the supernatants were isolated and counted for radioactivity as described in Materials and Methods. Results are the means ± SEM for N = 4 cell preparations.

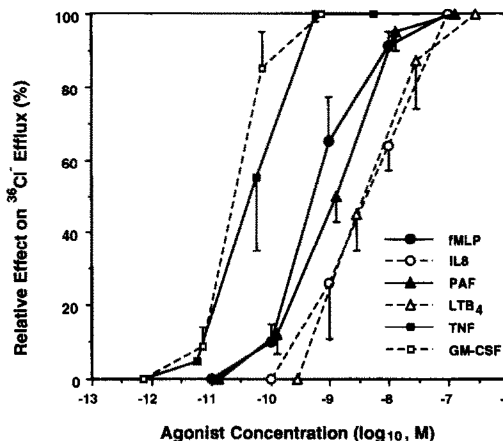

Fig. 2. Dose dependence of agonist-stimulated Cl^- efflux from neutrophils. The $^{36}Cl^-$-labeled neutrophils were suspended in unlabeled BSA–BSS and stimulated with varying concentrations of agonists. $^{36}Cl^-$ efflux was measured 7.5 min after stimulation (IL8, fMLP, PAF, LTB_4 and GM-CSF) or 15 min after stimulation (TNF). Relative $^{36}Cl^-$ effluxes at stated doses are represented as percentages of the maximum response after subtraction of natural $^{36}Cl^-$ efflux. Results are the means ± SEM for N = 3 cell preparations.

Table 1. Effect of various agonists on $^{36}Cl^-$ efflux, $[Ca^{2+}]_i$ elevation and shape change

Agonist	(ED_{50})	Cl^- efflux	$[Ca^{2+}]_i$	Shape change
Class I agonist				
fMLP	(5×10^{-10} M)	+	+	+
C5a	(3×10^{-9} M)	+	+	+
IL8	(4×10^{-9} M)	+	+	+
PAF	(10^{-9} M)	+	+	+
LTB_4	(4×10^{-9} M)	+	+	+
Class II agonist				
TNF	(5×10^{-11} M)	+	−	+
GM-CSF	(2×10^{-11} M)	+	−	+
Other				
LPS	[2 μg/mL]	−	−	−
PDGFβ	[10^{-8} M]	−	−	−
TGFβ1	[10^{-8} M]	−	−	−

We re-confirmed the agonist-stimulated $[Ca^{2+}]_i$ elevation using fura-2-loaded neutrophils as described in Materials and Methods. Change in $[Ca^{2+}]_i$ was monitored for 10 min after agonist stimulation. Measurements of Cl^- efflux and shape change were made 20 min after agonist stimulation.

In round brackets are ED_{50} for Cl^- efflux. In square brackets are maximum concentrations which did not stimulate Cl^- efflux.

Results are representative of at least three experiments. +, enhanced Cl^- efflux, $[Ca^{2+}]_i$ elevation and shape change. −, no effect.

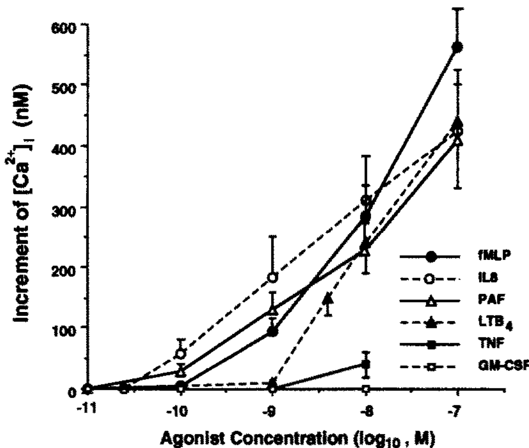

Fig. 3. Dose dependence of agonist-induced $[Ca^{2+}]_i$ elevation in neutrophils. Neutrophils loaded with fura-2 were resuspended in BSA–BSS as described in Materials and Methods and stimulated with various concentrations of several agonists. Increment of $[Ca^{2+}]_i$ was calculated by subtracting the basal concentration (188 ± 12 nM) from the maximum increased levels obtained within 10 min of stimulation. Results are the means ± SEM for N = 3 cell preparations.

CSF and TNF exhibited a 2 and 5 min lag time, respectively, before the onset of the Cl^- efflux. Since different concentrations of agonist, (e.g. 10^{-7}, 10^{-8} and 10^{-9} M IL8, or 6×10^{-8}, 6×10^{-9} and 6×10^{-10} M TNF) did not change the time of onset (data not shown), the difference between the times onset may be a feature of differences in the signal transduction pathways utilized by each agonist, which ultimately lead to activation of the Cl^- transporter.

Non-physiological neutrophil activators such as calcium ionophores, A23187 and ionomycin, and PMA which activate Ca^{2+}/phospholipid-dependent enzyme protein kinase C also enhanced the Cl^- efflux (data not shown). In contrast, LPS (at doses from 2 to 0.01 μg/mL) and PDGF (at doses from 10^{-7} to 10^{-10} M), which have been reported to be neutrophil-activating agents [1, 28], and TGF β1 (at doses from 10^{-8} to 10^{-13} M), a neutrophil chemoattractant [29], did not stimulate Cl^- efflux within 20 min (Table 1).

Cl^- *efflux is accompanied by intracellular* Cl^- *loss.*

To ascertain whether the agonist-stimulated Cl^- efflux is associated with a Cl_i^- loss, or is only the result of activating Cl^-/Cl^- self-exchange, we measured the change in Cl_i^- after agonist stimulation (Fig. 4). Before addition of agonists, neutrophils were incubated with $^{36}Cl^-$-containing BSA–BSS for 2 hr to equilibrate, and used without washing. In contrast to unstimulated cells, fMLP-, IL8- and TNF-stimulated cells showed a dramatic (about 50%) loss of Cl_i^- for up to 15 to 20 min. The time course of $^{36}Cl^-_i$ decrease correlates well with that of $^{36}Cl^-$ efflux. For example, in the case of TNF stimulation, the lag time before $^{36}Cl^-_i$ decreases was equivalent to the lag prior to detectable $^{36}Cl^-$ efflux (Figs 1 and 4). In addition, the magnitude of $^{36}Cl^-$ efflux was equivalent to the decrease in $^{36}Cl^-_i$ at the same point. This indicates that agonist-stimulated Cl^- efflux was through either unidirectional Cl^- efflux or unequivalent Cl^- exchange (i.e. efflux ≫ influx) and not through activation of the Cl^-/Cl^- self-exchanger. As shown in Fig. 4, the losses of Cl_i^- were restored to original Cl_i^- levels by 35–45 min after the onset of Cl_i^- loss suggesting the presence of a re-uptake mechanism for Cl^-. Subsequent re-stimulation of the cells again induced the Cl_i^- loss (data not shown).

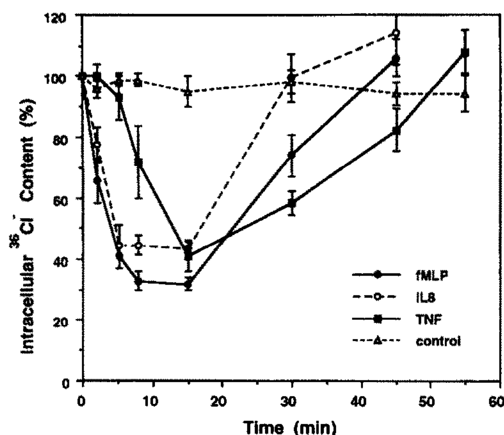

Fig. 4. Time course of intracellular $^{36}Cl^-$ loss from neutrophils after agonist stimulation. The cells were preincubated for 2 hr in $^{36}Cl^-$-containing BSA–BSS to equilibrate, and then used without washing. At the zero time point, we stimulated the cells with IL8 (10^{-8} M), fMLP (10^{-8} M) or TNF (6×10^{-9} M). At stated times, samples were taken and the cell pellets were counted for radioactivity as described in Materials and Methods. Results are the means ± SEM for N = 3 cell preparations.

Agonist-stimulated Cl^- efflux is not mediated by the Cl^-/Cl^- exchanger.

It is known that neutrophils have an anion exchanger which mediates natural Cl^-/Cl^- exchange and Cl^-/HCO_3^- exchange under alkaline-loaded conditions [15, 23]. Although the unequivalent movement of Cl^- indicated that agonist-stimulated Cl^- efflux was not mediated by Cl^-/Cl^- exchanger, the Cl^-/HCO_3^- exchanger could mediate Cl^- efflux. We therefore used known inhibitors of Cl^- movement [15, 23] to investigate whether agonist-stimulated Cl^- efflux as mediated by this exchanger or not. As shown in Table 2, both IL8- and TNF-stimulated Cl^- efflux were strongly inhibited by 0.5 mM EA (loop diuretic) and 0.3 mM amiloride (an inhibitor of Na^+/H^+ exchange). However, natural efflux was not inhibited by EA or amiloride (Table 2) as shown previously by Simchowitz and co-workers [15, 23]. These results indicate that the agonist-simulated Cl^- efflux is mediated by a Cl^- transporter other than the anion exchanger.

Do IL8 and TNF activate the same Cl^- transporter?

To assess whether class I and class II agonists stimulate the same Cl^- transporter, we investigated the sensitivity of IL8- and TNF-stimulated Cl^- efflux to several ion transport inhibitors. Both IL8- and TNF-stimulated Cl^- efflux were strongly inhibited by the Cl^- transport inhibitors EA, 9-AC, and DIDS, and by amiloride. However, other anion transport inhibitors, namely SITS and CHC, caused only moderate inhibition or no inhibition of the Cl^- efflux, respectively. As shown in Table 2, the extent to which the IL8- or TNF-stimulated Cl^- efflux was inhibited remained equivalent if the same inhibitor was used for both agonists. This suggests that both class I and class II agonists activate the same transporter via different type of receptor activation (see discussion).

The causal relationship between shape change and agonist-stimulated Cl^- efflux

All agonists tested caused both enhanced Cl^- efflux and shape change of neutrophils (Table 1), suggesting the possibility that there is a relationship between the two responses. Figure 5 shows the time course of shape change of neutrophils after agonist stimulation with the time point of onset of Cl^- efflux. In the case of GM-CSF and TNF, the onset of shape change was clearly slower than that induced by IL8 and fMLP as was the case for Cl^- efflux. As shown in Fig. 5, with all agonists tested, shape change preceded Cl^- efflux. In addition, amiloride and 9-AC had no effect on IL8- and TNF-stimulated shape changes (data not shown). These results imply that Cl^- efflux is not the cause of shape change. On the

Table 2. Inhibitory effect of various ion transport inhibitors on agonist-stimulated Cl^- efflux and natural Cl^- efflux

		IL8-stimulated (%)	TNF-stimulated (%)	Natural efflux (%)
DMSO	(0.25%)	5 ± 5	4 ± 6	2 ± 5
CHC	(1.7 mM)	12 ± 12	7 ± 10	14 + 5
EA	(0.5 mM)	108 ± 3	91 ± 6	8 ± 17
DIDS	(1.0 mM)	131 ± 17	119 ± 11	37 ± 5
SITS	(1.0 mM)	38 ± 15	57 ± 4	55 ± 5
9-AC	(1.0 mM)	110 ± 16	131 + 20	51 ± 3
Amilo	(0.3 mM)	119 ± 12	100 ± 15	4 ± 5
Cyt B	(1 μg/mL)	2 ± 14	0 ± 12	2 ± 7

The $^{36}Cl^-$-labeled cells were preincubated with the inhibitors for 5 min at 37°, and then stimulated with the agonist (IL8 10^{-8} M, TNF 6×10^{-9} M).

Results are expressed as percentage inhibition of agonist-stimulated Cl^- efflux, which was calculated as described in Materials and Methods.

Mean values ± SEM were obtained with three different neutrophil preparations, each tested in duplicate using the inhibitors at the concentrations indicated.

Amilo, amiloride.

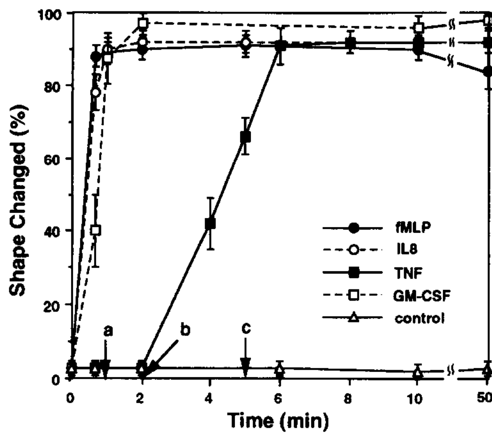

Fig. 5. Time course of cell shape change after agonist stimulation. The cells were preincubated for 2 hr in BSA–BSS and then stimulated with fMLP (10^{-8} M), IL8 (10^{-8} M), GM-CSF (7×10^{-9} M) or TNF (6×10^{-9} M). At stated times samples were taken and fixed with glutaraldehyde. The cells were then scored for shape change as described in Materials and Methods. Results are expressed as percentages of shape changed cells and represent means ± SEM of four separate experiments. Arrows indicate the onset time of agonist-stimulated Cl efflux derived from Fig. 1. (a) fMLP and IL8, (b) GM-CSF, (c) TNF.

other hand, membrane deformation has been shown to allow ion permeability via mechanosensitive channels [30] suggesting that shape change might be the trigger for Cl^- efflux. However, as shown in Table 2, inhibition of shape change with cytochalasin B (Cyt B; which acts by disturbing microfilament formation) had no effect on either natural Cl^- efflux or agonist-stimulated Cl^- efflux. This suggests that agonist-induced Cl^- efflux is not directly linked to shape change. This is further supported by the fact that whereas the Cl^- efflux was transient, and Cl_i^- was restored to original level by 35–50 min after agonist stimulation (Fig. 4), shape change persisted for prolonged periods (Fig. 5).

Comparison of agonist-induced Cl^- efflux and intracellular pH change

Of the agonists tested which induce Cl^- efflux, it is known that fMLP, LTB_4 and GM-CSF also induce a biphasic change in pH_i [8–11]. However, it is not known if IL8 or TNF also cause a change in pH_i. Since all of these agents induced both shape change and Cl^- efflux it was of interest to ascertain if change in pH_i was also a common feature of their signalling pathways. The time courses of agonist induced pH_i changes are shown in Fig. 6. A biphasic response to all agonists was evident with an initial acidification followed by an alkalinization. However, with GM-CSF, as has been described previously by McColl *et al.* [10], acidification was not always apparent prior to alkalinization. Furthermore, we found that TNF behaves in a similar manner with very slight acidification preceding alkalinization. Regardless of the degree of acidification, alkalinization was consistently produced by all agonists (Fig. 6). The time of onset of alkalinization correlated well with the onset of agonist-induced Cl^- efflux for both class I and class II agonists.

DISCUSSION

Agonist-stimulated Cl^- efflux

The major finding of the present study is that agonists which activate and/or prime neutrophils induce loss of Cl_i^-. These agonists can be divided into two classes depending on their ability to induce $[Ca^{2+}]_i$ elevation and the time at which they induce Cl^- efflux. The receptors for class I agonists, which elevate $[Ca^{2+}]_i$ and induce a relatively rapid Cl^- efflux, belong to the family of G-protein-coupled seven-transmembrane-segment receptors [31]. On the other hand, class II agonists, which do not elevate $[Ca^{2+}]_i$ and have a more protracted lag phase before Cl^- efflux, can be divided into two further sub-classes since GM-CSF and TNF (the class II agonists) have receptors which belong to separate families [31]. The time courses of Cl^- efflux induced by these agonists also differed (Fig. 1). However, not all agonists which have been demonstrated to

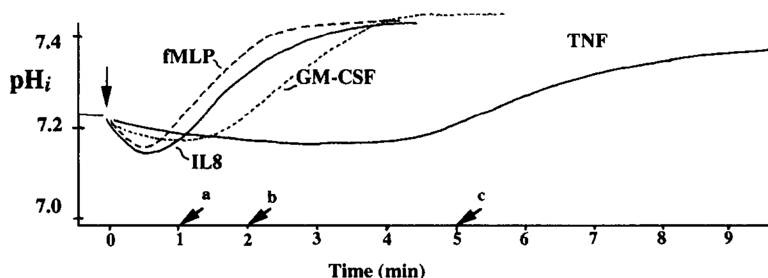

Fig. 6. Time course of intracellular pH change after agonist stimulation. BCECF-loaded cells were stimulated with several agonists. The final concentrations of agonists were as follows; fMLP (10^{-8} M), IL8 (10^{-8} M), GM-CSF (10^{-9} M) and TNF (10^{-9} M). The traces are representative of at least five experiments that yielded similar results using blood from different donors. Arrows indicate the onset time of agonist-stimulated Cl^- efflux derived from Fig. 1. (a) fMLP and IL8, (b) GM-CSF, (c) TNF.

influence neutrophil function caused Cl^- efflux since LPS [28], PDGF [1] and TGF-β1 [29] had no effect on Cl^- movement. This suggests that these agonists are using alternative signal transduction pathways of neutrophil activation which are not linked to Cl^- efflux.

In contrast to the measurement of agonist-stimulated arachidonic acid release or superoxide release, which require the use of Cyt B-treated neutrophils [24], Cl^- efflux can be measured without such an unphysiological treatment. Therefore, agonist-stimulated Cl^- efflux provides a useful marker for characterizing the state of neutrophil activation as a complement to the established procedures.

Characterization of the Cl^- transporter which mediates agonist-stimulated Cl^- efflux

In resting neutrophils, the bulk of chloride influx and efflux is due to Cl^-/Cl^- self-exchange using an anion exchanger. This anion exchanger also mediates Cl^-/HCO_3^- exchange under alkaline-loaded conditions. Under these conditions, it removes internal HCO_3^- in exchange for external Cl^- [15, 23]. It has also been observed that extracellular alkalinization raises the rate of Cl^-/Cl^- or Cl^-/HCO_3^- exchange (Cl^- in, HCO_3^- out) [32], indicating that this anion exchanger may be important in regulating the pH of unstimulated neutrophils. However, the present results indicate that agonist-stimulated Cl^- efflux is via a different Cl^- transporter from that used in resting cells. The evidence for this is that, firstly, the marked decrease in $^{36}Cl_i^-$ after agonist stimulation cannot be explained by Cl^-/Cl^- self-exchange and, secondly, the sensitivity of agonist-stimulated Cl^- efflux to several ion transport inhibitors is distinctly different from that of the natural efflux (Table 2).

The present results also imply that both class I and class II agonists utilize the same Cl^- transporter. This was suggested by the observation that several ion transport inhibitors caused different levels of inhibition of Cl^- efflux from the cells. However, the level of inhibition caused by each separate inhibition was the same whether IL8 or TNF was used to stimulate Cl^- efflux (Table 2). Moreover, both agonists induced a dramatic amount of Cl^- efflux within approximately the same period (Fig. 4) suggesting that they use Cl^- transporters of similar conductivity. Such a dramatic (30–50%) loss of intracellular Cl^- is known, in the case of rat parotid or submandibular acini, to occur via the apical Cl^- channel, which can be activated by muscarinic agonists [33, 34]. Thus, although we are presently undertaking an electrophysiological study to confirm the identity of the Cl^- transporter used by class I and class II agonists, it is plausible to expect that it will be common to both.

The mechanism(s) of Cl^- transporter activation

The mechanism of agonist-induced Cl^- efflux is at present unknown. Although in the case of class I agonists the dose–response curves of Cl^- efflux and $[Ca^{2+}]_i$ elevation are comparable (see Figs 2 and 3), class II agonists did not induce rapid transient $[Ca^{2+}]_i$ elevation within 10 min of agonist stimulation. These results suggest that such a $[Ca^{2+}]_i$ elevation is not a prerequisite for Cl^- efflux. It is noteworthy however that amiloride inhibits agonist-stimulated Cl^- efflux. Amiloride is known to block several other kinds of ion transport pathways, including Na^+/H^+ exchange and Na^+/Ca^{2+} exchange [7, 30, 35] although it has not been reported previously that amiloride can block Cl^- transport. It is known that the Na^+/H^+ transporter is the principal mechanism by which neutrophil agonists such as fMLP, LTB_4 and GM-CSF induce intracellular alkalinization [8–11]. Taken together, the finding of a temporal correlation between Cl^- efflux and intracellular alkalinization (Fig. 6), and the observation that amiloride inhibits agonist-stimulated Cl^- efflux suggest that the two phenomena may be linked. It is possible that Cl^- efflux is triggered by Na^+/H^+ exchange activation via Na^+ influx and/or resulting intracellular alkalinization. Further work is necessary to clarify the link between Na^+/H^+ exchange and agonist-stimulated Cl^- efflux.

Since, with all agonists tested, shape change precedes Cl^- efflux, we also investigated the possibility that shape change with accompanied membrane deformation is a trigger of agonist-stimulated Cl^- efflux. Although we cannot rule out this possibility completely, our data did not support it.

Possible physiological significance of the agonist-stimulated Cl^- efflux

Since almost all neutrophil agonists which activate or prime neutrophils stimulate Cl^- efflux, it is possible that Cl^- efflux has an important physiological role during neutrophil activation. Some agonists such as fMLP, C5a, PAF and LTB_4 can induce respiratory burst in neutrophils [36], but GM-CSF [37] and IL8 [24, 38] do not significantly activate it by themselves, although they do act as priming agents. However, Cl^- effluxes were generated to the same extent by all of these agonists, so it seems unlikely that there is a relationship between the Cl^- efflux and the respiratory burst. Moreover, there does not appear to be a relationship between Cl^- efflux and the induction of priming in neutrophils (data not shown).

It is known that fMLP, IL8 and TNF stimulate a small amount of degranulation by themselves, namely 1–3% of azurophilic granule marker release and 6–10% of specific granule marker release [39]. In the case of fMLP and IL8, these levels of degranulation were increased several-fold (up to 60–70%) in the presence of Cyt B and/or with pretreatment with TNF [39]. However, when measuring agonist-stimulated Cl^- efflux, about 50% loss of intracellular Cl^- content was found with each agonist in the absence of Cyt B (Figs 1 and 4), and Cyt B alone had no effect on the Cl^- efflux (Table 2). These results suggest that Cl^- efflux is not the result of release of intracellular contents accompanied by degranulation. This is supported by the fact that amiloride does not inhibit agonist-stimulated granule enzyme release [40].

In other cell types Cl^- efflux is known to be associated with cell volume decrease with a

concomitant loss of K† and cell water (see Ref. 14 for review), and it has been proposed that this cell shrinkage triggers a catabolic pattern of cellular metabolism [16]. Therefore, it is plausible that agonist-stimulated Cl^- efflux may cause cell volume decrease, which would induce a catabolic state in the neutrophils required to supply the energy for chemotaxis and/or phagocytosis. Recent data suggest that $[Cl^-]_i$ regulates glycogen synthase phosphatase in swollen hepatocytes [41], so the decrease in $[Cl^-]_i$ may have a similar effect in neutrophils. We are currently investigating this possibility in an attempt to elucidate further the physiological role of agonist-stimulated Cl^- efflux in neutrophils.

In summary, our results provide the first demonstration of an agonist-stimulated Cl^- efflux from neutrophils. The agonist-stimulated Cl^- efflux seems to be a common phenomenon during neutrophil activation irrespective of the type of agonist and may play an important role in regulating neutrophil responses following stimulation.

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