Biochimica et Biophysica Acta, 602 (1980) 285—298

BBA 78984

CHEMOTACTIC FACTOR-INDUCED MEMBRANE POTENTIAL CHANGES IN RABBIT NEUTROPHILS MONITORED BY THE FLUORESCENT DYE 3,3'-DIPROPYLTHIADICARBOCYANINE IODIDE

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(Received May 1st, 1980)

Key words: Membrane potential; Na^+ permeability; Membrane hyperpolarization; Chemotactic peptide; Complement; (Fluorescent probe)

Summary

Rabbit neutrophil leucocytes take up the cationic, fluorescent dye 3,3'-dipropylthiadicarbocyanine iodide (DiS-C_3-(5)). Treatment with valinomycin and K^+ then produces characteristic changes in suspension fluorescence that indicate that the dye enters the cells in a potential-dependent fashion and that the resting membrane potential lies between —66 and —86 mV. The peptide, *N*-fMet-Leu-Phe, a potent chemoattractant for neutrophils, added to stained cell suspensions, induces fluorescence intensity changes. These occur over an 8—10 min period. The time course of this response is profoundly affected by the omission of Ca^{2+} from the medium. When this ion is present (1.26 mM) a small, transient increase in intensity is observed, superimposed on a sustained decrease. On the other hand, in the absence of added Ca^{2+} a large, transient increase is observed. The ED_{50} for this is $1.1 \cdot 10^{-10}$ M. These changes are not elicited by *N*-fMet-Phe (10^{-9} M) and are inhibited by the antagonist Boc-Leu-Phe-Leu-Phe. However, a component of zymosan-activated rabbit plasma, which is complement-derived, induces identical fluorescence changes that are not inhibited by the antagonist, confirming that neutrophil activation by complement operates through an independent receptor.

The fluorescence responses to the chemotactic peptide and the activated-plasma component may be interpreted in terms of changes in neutrophil membrane potential brought about by alterations in cell ionic permeability at an

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Abbreviations: PAPA, plasma activation permeability agent; DiO-C_5-(3), 3,3'-dipentyloxacarbocyanine iodide; DiS-C_3-(5), 3,3'-dipropylthiadicarbocyanine iodide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; ED_{50}, effective dose giving 50% of the maximum response.

early stage during activation. The transient increase corresponds to a depolarisation that may be associated with a change in Na^+ permeability, while the sustained decrease corresponds to a membrane hyperpolarisation.

Introduction

The processes by which neutrophil leucocytes recognise and ultimately destroy bacteria commence with a chemotactic response directed towards either bacterial protein factors or certain components of complement that have been released at sites of inflammation. Subsequent events include phagocytosis of opsonised bacteria and exocytosis of lysosomal enzymes [1]. Neutrophil chemotaxis may be studied in vitro by observing the migration of cells up a concentration gradient of a chemoattractant. In both human and rabbit neutrophils, exposure to chemotactic agents produces a series of rapid responses. For instance, *N*-fMet-Leu-Phe, the most potent of a series of synthetic chemoattractants [2,3], has been shown to bind rapidly to specific, high-affinity sites on human neutrophils [4] and to produce a transient rise in intracellular cyclic AMP that reaches a maximum after only 30 s [5]. The binding, which may be directly visualised using a fluorescent chemoattractant, is followed by internalisation within 5 min [6]. Other events that occur within 60 s following treatment of rabbit neutrophils with chemotactic agents include protein carboxymethylation [7] and mobilisation of intracellular Ca^{2+} [8]. Naccache et al. [9] have used radioactive-tracer techniques to measure ion fluxes in rabbit neutrophils. Their results show that *N*-fMet-Leu-Phe at a chemotactically effective dose brings about a rapid increase in Na^+ influx, stimulation of the (Na,K)-pump and changes in intracellular Ca^{2+} levels. They suggest that the increased Na^+ influx may cause the plasma membrane to depolarise. On the other hand, stimulation of the (Na,K)-pump could have an opposite effect. Korchak and Weissman [10,11] have used the lipid-soluble cation, triphenylmethylphosphonium, to monitor the membrane potential of human neutrophils treated with a number of agents including *N*-fMet-Leu-Phe. The time course of the potential change induced by *N*-fMet-Leu-Phe over a 10 min period following treatment is triphasic, commencing with a rapid hyperpolarisation [12]. Seligmann and Gallin [13,14], on the other hand, have used the potential-sensitive fluorescent dye, 3,3′-dipentyloxacarbocyanine iodide (DiO-C_5-(3)) to investigate the effect of *N*-fMet-Leu-Phe on human neutrophils. Their results indicate an initial depolarisation.

Carbocyanine dyes have been used extensively to monitor potential changes in a wide variety of cells [15]. We have investigated the early events in the response of rabbit neutrophils to *N*-fMet-Leu-Phe and a component of zymosan-activated plasma (plasma activation permeability agent (PAPA)) by observing changes in the fluorescence of the potential-sensitive probe, 3,3′-dipropylthiadicarbocyanine iodide (DiS-C_3-(5)) [16]. Our findings indicate that the resting membrane potential of rabbit neutrophils is close to their K^+ equilibrium potential. Similar fluorescence changes occur when the cells are treated with *N*-fMet-Leu-Phe or PAPA but the nature of these changes is strongly dependent on the presence of extracellular Ca^{2+}.

Materials and Methods

Neutrophils were obtained from albino rabbits by intraperitoneal injection of 150—250 ml of 0.1% glycogen in sterile, physiological saline. Drainage of the peritoneal exudate was carried out after 4 h into heparinised beakers. The cells were then spun down and washed three times in presoaked polycarbonate tubes using a modified Hank's balanced salt solution. This medium, which lacked Ca^{2+} salts, had the following composition: 137 mM NaCl, 5.36 mM KCl, 0.44 mM KH_2PO_4, 0.49 mM $MgCl_2$, 0.4 mM $MgSO_4$, 4.1 mM $NaHCO_3$, 0.42 mM Na_2HPO_4, 1 mg/ml glucose, 1 mg/ml bovine serum albumin and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonate (Hepes buffer) titrated with NaOH to give a pH of 7.3 at 37°C. Cell viability was assessed by observing ethidium bromide exclusion and was routinely greater than 90%. The red blood cell content of the working suspensions was less than 10% of the neutrophil count. On occasions, however, red cells were present at higher levels. In these circumstances, the washed cells were subjected to osmotic shock to lyse the erythrocytes. This was carried out by diluting 5 ml of the suspension with 13.5 ml of distilled water and, after 45 s, adding 1.5 ml of 10-times strength phosphate-buffered saline. The cells were then washed twice more in the Hank's medium. Cell viability following this procedure was not adversely affected. Before experimentation, the washed neutrophils were incubated in plastic tubes at 37°C for at least 30 min. In those experiments where Ca^{2+} was present in the medium, $CaCl_2$ was added (1.26 mM) before incubation.

Rabbit red blood cells, obtained by venipuncture, were collected in heparinised tubes and washed three times in the Hank's medium. They were also incubated with or without Ca^{2+} for 30 min (1.26 mM).

The fluorescent dye, DiS-C_3-(5), was provided by Dr. Alan Waggoner, Amherst College, U.S.A. It was dissolved in ethanol at a concentration of $1.32 \cdot 10^{-4}$ M. This stock solution was stored at 4°C. Fluorescence measurements were made with an Aminco Bowman Spectrofluorometer (Model SPF, American Instrument Co., Silver Spring, MD, U.S.A.) fitted with a red-sensitive photomultiplier tube (9670B, EMI Ltd., Ruislip, U.K.). 2-mm slits were used at 620 nm (excitation) and 670 nm (emission). A plastic film polariser (Polacoat 105 UVWRMR, 3M U.K. Ltd., London, U.K.) was employed to polarise the incident beam in the horizontal direction to reduce light scattering. 10-mm pathlength glass cuvettes were used and all measurements were carried out at 37°C. In each experiment, neutrophils were suspended at $2.5 \cdot 10^6$/ml in 1.5 ml of the Hanks' medium with or without added Ca^{2+}. Additions of dye and other compounds were made with an adjustable micropipette (usually 5 μl). After each addition, the suspension was stirred by pipetting two or three times. No effect was observed due to cell sedimentation during the course of the fluorescence measurements. The phototube shutter was closed during each addition. The final concentration of dye was 440 nM. At this concentration, it had no effect on the viability of the cells as estimated by ethidium bromide exclusion.

The vascular permeability inducing agent, PAPA, is a component extracted from zymosan-activated rabbit plasma. This material and a control extract that did not induce permeability were prepared and provided by Drs. T.J. Williams

and P.J. Jose, Royal College of Surgeons, London. The control material was obtained from plasma that had not been treated with zymosan but had undergone otherwise identical procedures [17,18]. Characterisation of PAPA indicates that it is a component of complement [18], probably C5a (Williams, T.J. and Jose, P.J., personal communication). It also has the property of inducing neutrophil recruitment in vivo (Williams, T.J., personal communication). Moreover, in the presence of 5 μg/ml of cytochalasin B, PAPA (260 ng/ml) induced the secretion of β-glucuronidase from rabbit neutrophils, measured by the method of Showell et al. [3]. The control substance was inactive. These effects confirm that the active material is complement-derived. Both PAPA and its control were dissolved in 0.9% saline/pH 3.8 sodium acetate buffer (0.2 M) at a protein concentration of 26 and 11.4 μg/ml respectively. Thus, addition of 5 μl to 1.5 ml yielded final protein concentrations of 87 and 38 ng/ml, respectively.

N-fMet-Leu-Phe, *N*-fMet-Phe, valinomycin, ouabain and EGTA were obtained from Sigma London Ltd., Poole, U.K. Boc-Leu-Phe-Leu-Phe was provided by Dr. Richard Freer, Medical College and Virginia, Richmond, U.S.A. Valinomycin was dissolved in ethanol, ouabain in pH 4 saline and *N*-fMet-Leu-Phe, *N*-fMet-Phe and Boc-Leu-Phe-Leu-Phe in Me_2SO. EGTA was dissolved in water and titrated to pH 7.2 with NaOH. Final concentrations of either ethanol or Me_2SO were always less than 1% (v/v). Addition of ethanol, Me_2SO, pH 4 or pH 3.8 saline had no effect on the fluorescence of the stained cell suspensions.

Results

When the cationic dye, DiS-C_3-(5), is added to a cell suspension, it enters the cells in a potential-dependent fashion [15]. The emission properties of the intracellular dye are strongly modified by binding to cell contents and by the formation of non-fluorescent dimers [16,19,20]. The spectral shifts and quenching that result cause the emission at 670 nm, excited at 620 nm, to decrease. When DiS-C_3-(5) is added to rabbit neutrophil suspensions, the fluorescence signal that is obtained rapidly decays to less than 50% of its initial value, as shown in Fig. 1. A stable level of fluorescence is obtained within 6—7 min. This high uptake of dye by rabbit neutrophils suggests that they possess a significant, negative membrane potential. When the chemotactic peptide, *N*-fMet-Leu-Phe, is added to a cell suspension previously equilibrated with dye, fluorescence changes typified by those shown in Fig. 2 are observed. The final *N*-fMet-Leu-Phe concentration is 10^{-9} M. Although the shape of these changes is reproducible, the magnitude of the responses varies with time as the cells age, increasing at first but decreasing later. The reasons for this are not clear but may be related to the tendency for cells that are less responsive to adhere preferentially to the walls of the plastic tubes. Thus, aliquots removed for each experiment may become richer in the more responsive cells as time proceeds. Cell adherence also affects the total number of cells delivered to each cuvette, causing fluctuations in the dye-to-cell ratio, a factor towards which the performance of carbocyanine dyes is very sensitive [16].

As can be seen in Fig. 2, the shape of the fluorescence changes in response to *N*-fMet-Leu-Phe depends on the presence of external Ca^{2+}. At 1.26 mM, here-

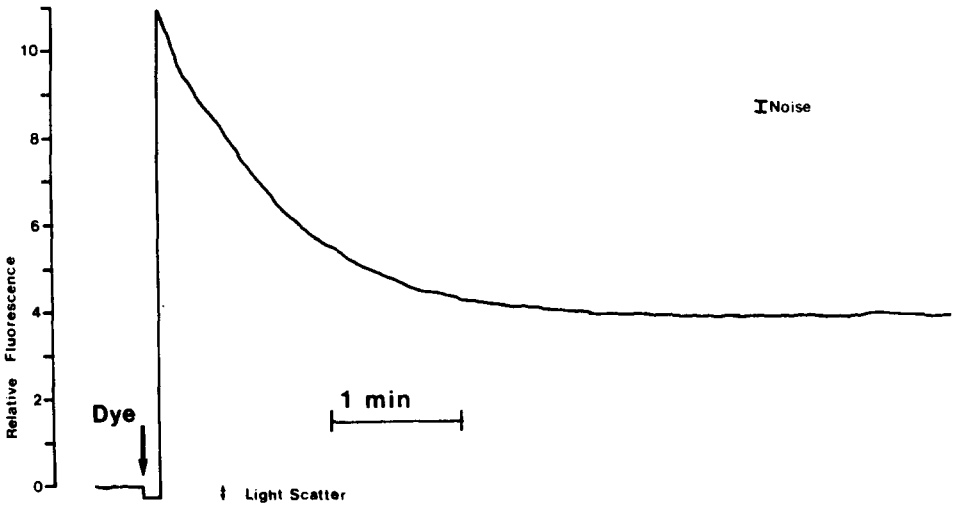

Fig. 1. Effect of adding 5 μl of $1.32 \cdot 10^{-4}$ M DiS-C_3-(5) solution to a neutrophil suspension ($2.5 \cdot 10^6$ cells/ml). The final dye concentration was equivalent to 440 nM. The fluorimeter shutter was closed during the addition and stirring. The average noise level is indicated and the small signal seen before the addition of dye is due to light scattering.

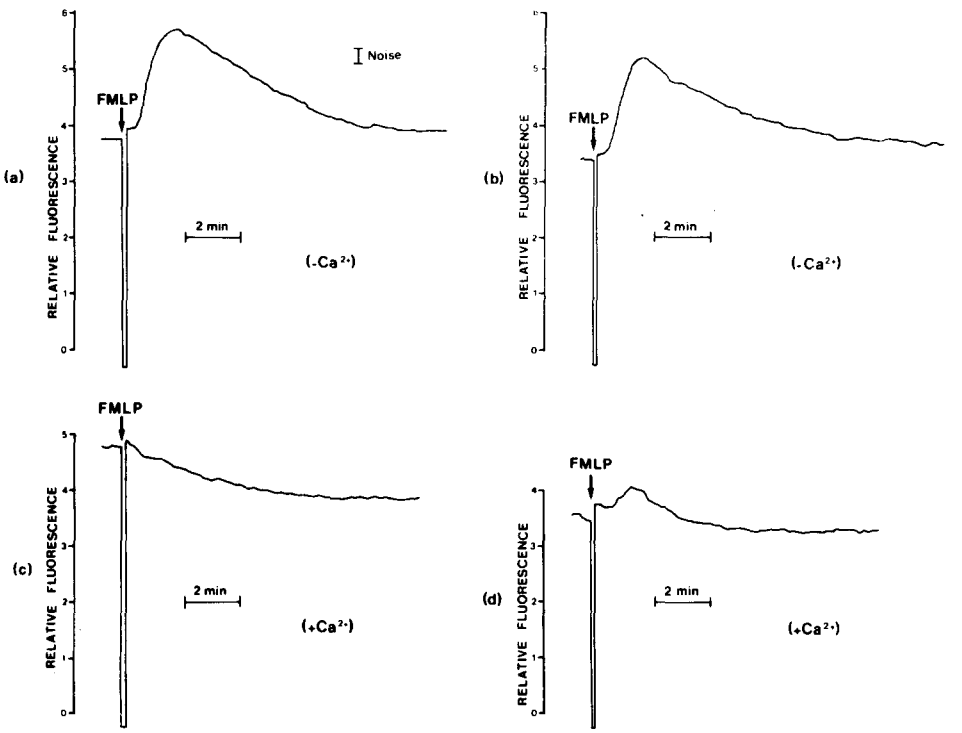

Fig. 2. Fluorescence changes observed on adding *N*-fMet-Leu-Phe (FMLP) (10^{-9} M) to neutrophils suspended in Hanks' medium with and without added Ca^{2+}. Suspensions were previously equilibrated with dye. The average noise level throughout is indicated in a. Each trace was obtained from a different cell preparation, a and b in the absence of added Ca^{2+}, c and d in the presence of 1.26 mM Ca^{2+}.

after referred to as (+Ca^{2+}), there are two components to the response. The most prominent is a slow decrease in fluorescence that reaches a steady value in 6—8 min. Superimposed upon this signal is a transient increase that peaks between 1 and 2 min after *N*-fMet-Leu-Phe addition. The magnitude of the overall fluorescence decrease is about 20% ($\Delta F/F = -0.2 \pm 0.1$ (S.D.), $n = 9$ preparations). The transient component varies from a distinct intensity increase (approx. 5%) to a mere inflection in the fluorescence decay (Fig. 2). In the absence of added Ca^{2+}, hereafter referred to as (—Ca^{2+}), a quite different response is observed. After a lag period of about 20 s there is a sharp increase in emission, reaching a maximum at about $1\frac{1}{2}$ min, corresponding in time to the transient peak described above. After reaching this maximum the fluorescence dies away slowly, approaching its initial level about 10 min after the addition of *N*-fMet-Leu-Phe. The maximum increase in fluorescence is approx. 50% ($\Delta F/F = 0.5 \pm 0.2$ (S.D.), $n = 8$ preparations). This response shows a marked dose dependence as shown in Fig. 3. In order to obtain these data, special measures were taken to reduce variations in the dye-to-cell ratio between each run. Sufficient cells for each dose-response curve were made up to $2.5 \cdot 10^6$/ml and stained in bulk at 37°C for 6—7 min. 1.5-ml aliquots of this suspension were then transferred to cuvettes and treated with different doses of *N*-fMet-Leu-Phe in the fluorimeter by adding 5 µl of the appropriate stock solution. This procedure also avoided the long delays that would have occurred if each sample had been stained and equilibrated separately. Each of the curves in Fig. 3 was obtained from a separate cell preparation. Since the proportion of responding cells in each preparation was different, the responses are plotted as fractions of the maximal fluorescence change. Thus, the response to *N*-fMet-Leu-Phe shows

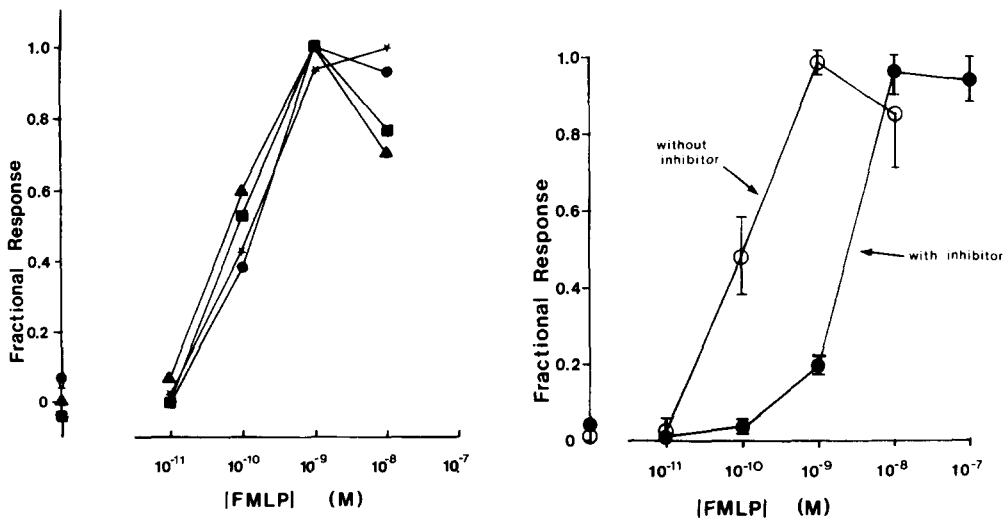

Fig. 3. The effect of *N*-fMet-Leu-Phe (FMLP) dose on the transient fluorescence increase observed in the absence of added Ca^{2+}. Data from four different cell preparations expressed, in each case, as a fraction of the maximum response, $(\Delta F/F)/(\Delta F/F)_{max}$.

Fig. 4. The effect of the inhibitor Boc-Leu-Phe-Leu-Phe on the response to *N*-fMet-Leu-Phe (FMLP) in the absence of added Ca^{2+}. (○) Mean data from Fig. 3 ($n = 4$). (●) Inhibitor present at $5 \cdot 10^{-6}$ M ($n = 3$). Error bars represent S.D. Ordinate as in Fig. 3.

a sharp onset with increasing dose. The mean ED_{50} is $1.1 \cdot 10^{-10}$ M (S.D. = $0.4 \cdot 10^{-10}$ M). It was not possible to obtain similar data for cells in the presence of added Ca^{2+} because the longer duration of each trace caused cumulative delays and a consequent loss of reproducibility. However, significant responses to *N*-fMet-Leu-Phe were observed at doses as low as 10^{-10} M.

A number of compounds are able to inhibit both the binding of *N*-fMet-Leu-Phe to neutrophils and its biological activity [21,22]. These antagonists may themselves be weakly chemotactic such as *N*-fMet-Phe [3], or may be more potent, competitive inhibitors of *N*-fMet-Leu-Phe action such as the pentapeptide, Boc-Phe-Leu-Phe-Leu-Phe [7,8,23], and the tripeptide, Boc-Phe-Leu-Phe [24]. The analogous compound, Boc-Leu-Phe-Leu-Phe, is also a competitive antagonist (Freer, R.J., personal communication) and its addition to stained cell suspensions, prior to *N*-fMet-Leu-Phe treatment, reduces the magnitude of the subsequent fluorescence changes. With the same cell preparation that was used to obtain Fig. 2c, 10^{-9} M *N*-fMet-Leu-Phe produced a fluorescence decrease ($+Ca^{2+}$) of 21%, but when the inhibitor was present at $5 \cdot 10^{-6}$ M, the same concentration of *N*-fMet-Leu-Phe produced a decrease of only 9%. This experiment was repeated after 40 min and the same result was observed. When Ca^{2+} is omitted from the medium, the effects of the inhibitor peptide are even more apparent. Fig. 4 shows the effect of $5 \cdot 10^{-6}$ M Boc-Leu-Phe-Leu-Phe on the dose response to *N*-fMet-Leu-Phe ($-Ca^{2+}$). At this concentration the ED_{50} is increased to $2.5 \cdot 10^{-9}$ M.

Addition of the low chemotactic activity peptide, *N*-fMet-Phe, to stained cell suspensions at 10^{-9} M produces only small fluorescence changes. In a single experiment, sustained decreases of 6% ($-Ca^{2+}$) and 5% ($+Ca^{2+}$) were observed. No transient increase in signal was detected. The same cell preparation treated with *N*-fMet-Leu-Phe (10^{-9} M) yielded a fluorescence increase of 32% ($-Ca^{2+}$) and a decrease of 13% ($+Ca^{2+}$). On the other hand, the vascular permeability-inducing agent, PAPA, isolated from zymosan-activated rabbit plasma, produces fluorescence changes very similar to those induced by *N*-fMet-Leu-Phe as shown in Fig. 5. The maximal fluorescence increase ($-Ca^{2+}$) is about 40% ($\Delta F/F = 0.40 \pm 0.13$ (S.D.), $n = 3$ preparations) and the overall decrease ($+Ca^{2+}$) is about 17% ($\Delta F/F = -0.17 \pm 0.06$ (S.D.), $n = 3$ preparations). Addition of the control plasma extract (see Materials and Methods), either produces a small intensity decrease ($-Ca^{2+}$) or has no effect ($+Ca^{2+}$). Pretreatment of the stained cell suspensions with $3.3 \cdot 10^{-5}$ M Boc-Leu-Phe-Leu-Phe has no effect on the fluorescence responses ($\pm Ca^{2+}$) to PAPA. This dose of inhibitor, however, completely suppresses the responses ($\pm Ca^{2+}$) to *N*-fMet-Leu-Phe (10^{-9} M).

The large initial uptake of dye during the staining process suggests that the cell membrane potential (E_m) may approach the K^+ equilibrium potential (E_K). This was tested by adding the K^+-specific ionophore, valinomycin. Fig. 6a shows the effect of valinomycin (10^{-5} M) on a stained neutrophil suspension ($-Ca^{2+}$). After an abrupt rise in emission, which we discuss below, a small amount of dye is taken up by the cells indicating a small hyperpolarisation. Serial additions of molar KCl then produce successive increases in fluorescence as the cells depolarise. Since in some preparations there is a small percentage of red blood cells which also take up dye when treated with valinomycin (data not shown), it is necessary to ensure that their presence does not account for

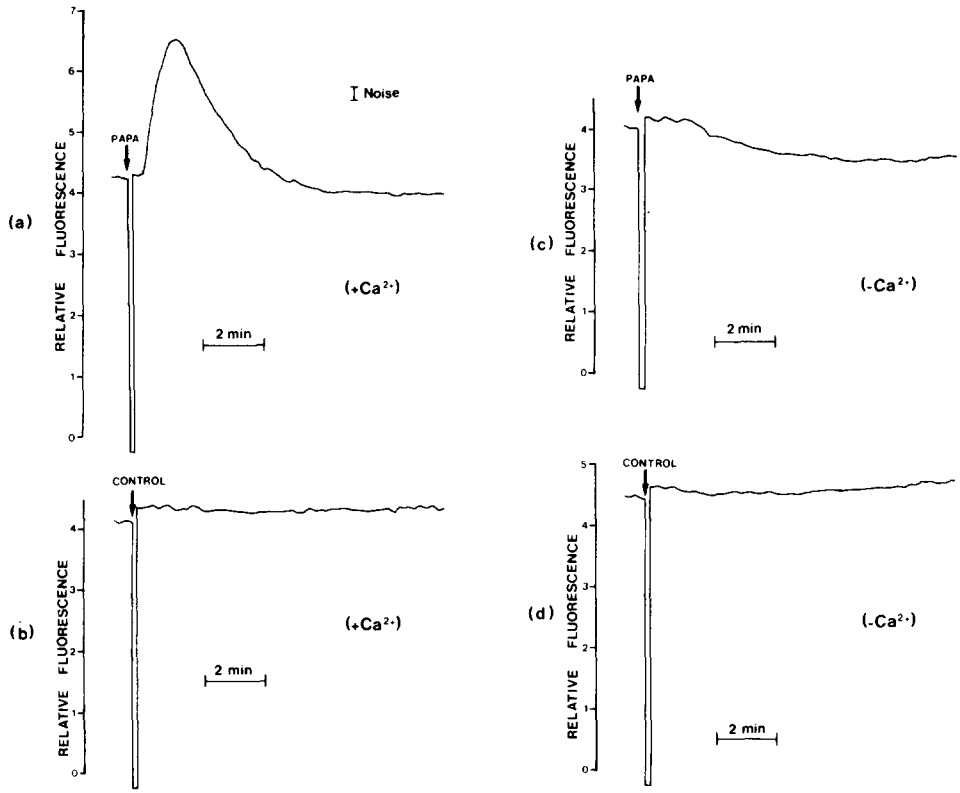

Fig. 5. The effect of the zymosan-activated plasma component PAPA on suspension fluorescence compared with non-activated control in the presence and absence of added Ca^{2+}. In a and b, $[Ca^{2+}]$ = 1.26 mM and in c and d Ca^{2+} was omitted from the medium.

the observed hyperpolarisation. Their contribution was assessed by adding known quantities to an erythrocyte-free neutrophil suspension. Fig. 6b shows that there was no effect on the size of the valinomycin response when the erythrocyte contamination was 10% of the neutrophil count. When equal numbers of red cells and neutrophils were present, however, the response was increased by 70% as shown in Fig. 6c. Since in our preparations the erythrocyte contamination was less than 10%, we may use the data of Fig. 6 and similar data obtained in the presence of Ca^{2+} (not shown) to estimate the resting potential of neutrophils by the null-point method. That is, if we assume that at this concentration of ionophore the cells are highly permeable to K^+, then their potential may be calculated from the Nernst equation. If, however, sufficient K^+ is added to the suspension to restore the fluorescence to its orignal level, and if the osmotic contribution of the added ions is not significant, then the Nernst equation will provide an estimate of E_m in the absence of ionophore. The null-point method has been used to estimate E_m for erythrocytes [25] and lymphocytes [26]. With or without added Ca^{2+}, the volume of 1 M KCl that was sufficient to restore the fluorescence to its original level was between 5 and 10 μl. The intracellular K^+ concentration of rabbit neutrophils has been found to be 110 mmol/l cells [9] and if we assume a cell water content of 75% then

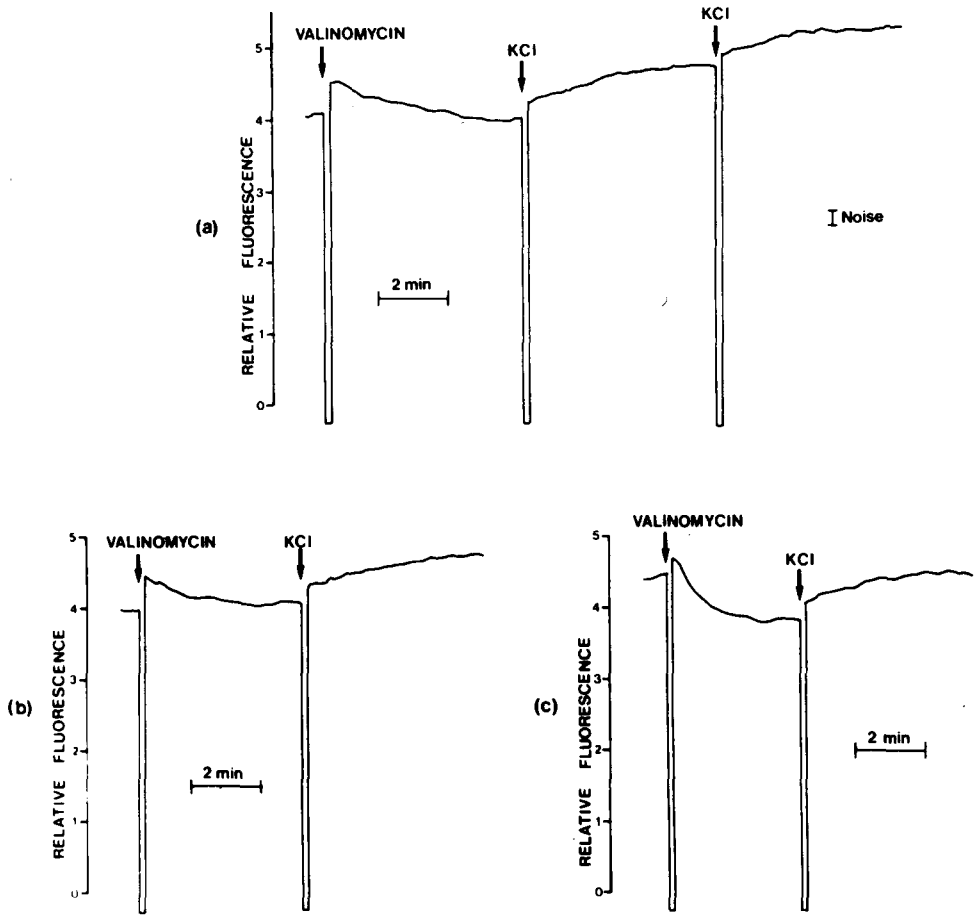

Fig. 6. The effect of valinomycin and KCl on suspension fluorescence. The final ionophore concentration was 10^{-5} M and KCl was added in 5-μl quantities from a 1 M stock solution. (a) A red cell-free neutrophil suspension. (b) A cell suspension containing $2.5 \cdot 10^6$ neutrophils/ml and $2.5 \cdot 10^5$ erythrocytes/ml. (c) A cell suspension containing $2.5 \cdot 10^6$ neutrophils/ml and $2.5 \cdot 10^6$ erythrocytes/ml. Average noise level throughout is shown in a.

$[K^+]_{cell}$ = 147 mmol/kg cell water. The extracellular K^+ concentration is normally 5.8 mM and at the null-point is between 9.1 and 12.5 mM, whence E_K = —86 mV and E_m = —66 to —74 mV. Out of a total of 12 preparations in which the cells reacted characteristally to *N*-fMet-Leu-Phe, only eight demonstrated a valinomycin-induced hyperpolarisation. The remainder showed no fluorescence decrease and in two cases small but significant increases in fluorescence were produced by adding 1 M KCl in the absence of ionophore. A lack of response to valinomycin would be observed in cells that are already K^+ permeable. We therefore limit our conclusion to an estimate of the resting potential of between —66 and —86 mV.

We have also investigated the effect of valinomycin on the response of the cells to *N*-fMet-Leu-Phe. In the presence of 10^{-5} M ionophore, *N*-fMet-Leu-Phe (10^{-9} M) produced a fluorescence decrease of 9% ($+Ca^{2+}$) and an increase of 16% ($-Ca^{2+}$), showing that although the responses were somewhat reduced, the

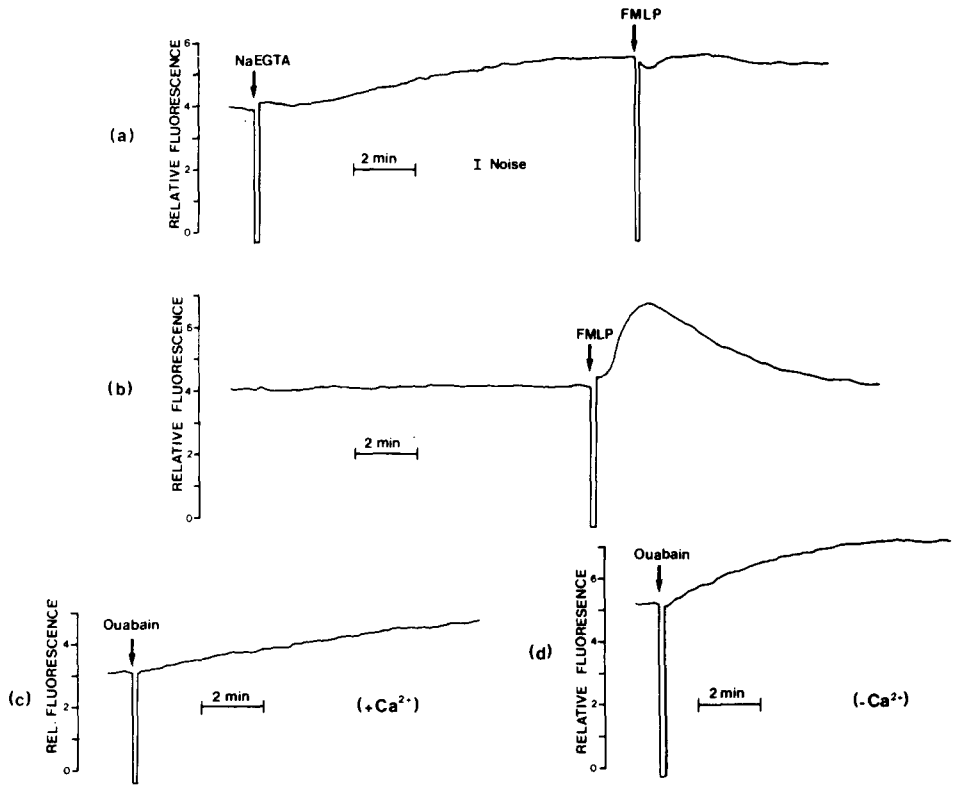

Fig. 7. The effects of EGTA and ouabain on suspension fluorescence. (a) 0.05 mM NaEGTA followed by 10^{-9} M *N*-fMet-Leu-Phe (FMLP) (Ca^{2+} omitted). (b) As a but without NaEGTA. (c) $3.3 \cdot 10^{-5}$ M ouabain in the presence of 1.26 mM Ca^{2+}. (d) $3.3 \cdot 10^{-5}$ M ouabain, Ca^{2+} omitted. Average noise level throughout is indicated in a.

characteristic fluorescence changes were not suppressed.

Since omitting Ca^{2+} from the medium does not ensure its complete absence from the extracellular environment, we have also investigated the effect of adding the chelating agent, EGTA, to stained cell suspensions. Fig. 7 shows the effect of adding EGTA (50 μM) and subsequently *N*-fMet-Leu-Phe (10^{-9} M). Over an 11 min period, the chelating agent produced a 35% fluorescence increase. Similar effects were seen when EGTA was added at 100 μM and 3 mM (not shown). In all these cases, after this slow fluorescence change was complete, *N*-fMet-Leu-Phe produced rather small and indistinct effects. It appears therefore that even at the lowest concentration, EGTA depolarises the cells. Similar slow changes were observed when cells were treated with $3.3 \cdot 10^{-5}$ M ouabain, also shown in Fig. 7. This suggests that the slow depolarisation may be a result of inhibition of the (Na,K)-pump. Treatment with *N*-fMet-Leu-Phe after ouabain still produced fluorescence changes, although the size and shape of these responses were rather variable. Neither $3.3 \cdot 10^{-5}$ M ouabain nor up to 10^{-6} M *N*-fMet-Leu-Phe had any significant effect on the fluorescent emission of rabbit red cell suspensions treated with dye under conditions in which valinomycin caused a large hyperpolarisation (not shown).

The small, instantaneous changes in fluorescence that occurred from time to

time following the addition of various agents and stirring of the suspensions were also observed when stirring alone was carried out. Since these effects occurred more frequently when the cells had been stored for a few hours, we assume that they are related to cell damage caused by the stirring procedure, giving rise to a rapid release of a small amount of dye. We are unable to account for the somewhat larger increases that were generally observed when valinomycin was added, since these also occurred with freshly prepared cells.

Discussion

The cationic, lipophilic dye, DiS-C_3-(5), is taken up by rabbit neutrophils in a potential-dependent fashion. The equilibration time of 7 min is comparable to that observed in guinea-pig neutrophils [27] and to that of the dye, DiO-C_5-(3), in human neutrophils [14]. After equilibration, the fluorescent emission intensity of the suspension responds to changes in cell membrane potential, as shown by the effects of valinomycin and K^+ (Fig. 6a). The fluorescence changes produced by adding either *N*-fMet-Leu-Phe or PAPA to cell suspensions equilibrated with dye are almost equal (Figs. 2 and 5). However, the shape of these fluorescence responses is profoundly affected by the omission of Ca^{2+} from the medium. There appear to be two components in the fluorescence changes that we observe: (a) a transient increase in intensity that peaks at 90 s and (b) an intensity decrease that reaches a steady value in 6—8 min. In the absence of added Ca^{2+} only (a) is observed, while in the presence of this ion at 1.26 mM contributions from both components are present, although the sustained component b is predominant, having a small and rather variable amount of a superimposed upon it. The following evidence supports the conclusion that these changes are associated with chemotaxis.

(1) They are not elicited by *N*-fMet-Phe (10^{-9} M) which has a chemotactic activity 6000-times less than *N*-fMet-Leu-Phe [3].

(2) The doses of *N*-fMet-Leu-Phe that evoke a dye response are very similar to those that induce cell migration. We observe, in the absence of added Ca^{2+}, an ED_{50} of $1.1 \cdot 10^{-10}$ M. This may be compared with an ED_{50} for chemotaxis of rabbit neutrophils of $7.0 \cdot 10^{-11}$ M [3].

(3) Treatment with the competitive inhibitor, Boc-Leu-Phe-Leu-Phe ($5 \cdot 10^{-6}$ M), shifts this dose response to *N*-fMet-Leu-Phe to higher doses (Fig. 4) increasing the ED_{50} to $2.5 \cdot 10^{-9}$ M. Although we do not have dose-response data for cells in the presence of Ca^{2+}, the same concentration of Boc-Leu-Phe-Leu-Phe does inhibit the fluorescent response in the presence of this ion. The dissociation constant for the inhibitor calculated from the dose ratio of *N*-fMet-Leu-Phe (ED_{50} ratio at $5 \cdot 10^{-6}$ M Boc-Leu-Phe-Leu-Phe) is $2.3 \cdot 10^{-7}$ M. This is very close to the value of $4 \cdot 10^{-7}$ M for Boc-Phe-Leu-Phe-Leu-Phe estimated by Naccache et al. [8].

(4) The response to PAPA is not affected by the presence of this inhibitor at $3.3 \cdot 10^{-5}$ M, a dose that completely suppresses the effects of *N*-fMet-Leu-Phe (10^{-9} M). This is compatible with the finding that *N*-fMet-Leu-Phe and C5a interact with neutrophils through different receptors [22].

Treatment of the cells with the K^+ ionophore, valinomycin, and KCl indicates that their resting potential is close to the K^+ equilibrium potential, sug-

gesting that their relative permeability to this ion is high. Carbocyanine dyes, such as DiS-C_3-(5), at high concentrations have been shown to block Ca^{2+}-activated K^+ channels in red blood cell ghosts [28,29] and also to depolarise lymphocytes [26]. Consequently, it is unlikely that the high K^+ permeability that we observe is an artifact caused by the dye in our experiments. Naccache et al. [9] have reported high K^+ fluxes in rabbit neutrophils and a high, relative permeability to this ion would also be consistent with the finding that elevated external K^+ enhances neutrophil chemotactic sensitivity through a mechanism that involves ion translocation [30].

We do not have sufficient information to explain in detail the mechanisms that give rise to the fluorescence changes produced by *N*-fMet-Leu-Phe and PAPA. However, we do have evidence that suggests that they too result from membrane potential fluctuations that can be accounted for by changes in cell ionic permeability. The biphasic response to these agonists in the absence of added Ca^{2+} corresponds to a membrane depolarisation followed by a hyperpolarisation. Such a depolarisation could be caused by an increase in Na^+ permeability or a decrease in K^+ permeability. However, similar albeit somewhat reduced, responses are observed in the presence of a concentration of valinomycin that would render the cells permanently permeable to K^+, indicating that an Na^+ movement is responsible for the depolarisation. Indeed, Naccache et al. [9] have shown that *N*-fMet-Leu-Phe produces an enhanced Na^+ influx in rabbit neutrophils. The subsequent hyperpolarisation may then be due to the cells recovering their normal Na^+ permeability by natural processes or, alternatively, due to dye interacting with, and blocking, the open Na^+ channels. Such an interaction would be analogous to that observed with open K^+ channels in red cell membranes (see above).

The fluorescence responses in the presence of Ca^{2+} contain an additional component that corresponds to a sustained membrane hyperpolarisation. Once again, valinomycin does not prevent the appearance of this feature, suggesting that it is not caused by an increase in K^+ permeability per se. However, it may be due to stimulation of (Na,K)-pump. Naccache et al. [9] have shown that *N*-fMet-Leu-Phe increases (Na,K)-pump activity in rabbit neutrophils. In order for such a process to be electrogenic, it must transport Na^+ and K^+ at a ratio not equal to unity. If this pump stoichiometry favours the extrusion of Na^+, then stimulation would generate a membrane hyperpolarisation. Such an effect in red blood cells has been reported. The pump was stimulated by external K^+ and the resultant, ouabain-sensitive hyperpolarisation was detected using DiS-C_3-(5) [31]. The absence of a sustained fluorescence decrease in experiments in which Ca^{2+} was omitted from the medium might then be ascribed to a failure of the pump to develop under these conditions. Some support for this hypothesis is offered by the similar effects of EGTA and ouabain, both of which produce a slow fluorescence increase, presumably inhibiting basal pump activity and allowing the inward leak of Na^+ to depolarise the cells (Fig. 7).

The other principal difference between the responses with and without Ca^{2+} in the medium is the extent of the contribution of the transient component a. This feature gives rise to large fluorescence changes without Ca^{2+} but quite small effects in its presence. We have no explanation for this but we note that Marasco et al. [32] report that directed locomotion of rabbit neutrophils on

glass is 25—50% more rapid when Ca^{2+} is omitted from the medium.

Korchak and Weissmann [10,11] have used the lipophilic cation, triphenylmethylphosphonium, to investigate the effects of various stimuli on human neutrophils. Although this radioactive probe has a very long equilibration time in these cells (approx. 75 min [10]), an initial hyperpolarisation in response to *N*-fMet-Leu-Phe has been reported [11,12] which is in contrast to the findings of Seligmann and Gallin [14]. The effect of *N*-fMet-Leu-Phe on the uptake of the cationic dye, DiO-C_5-(3), by human neutrophils, reported by these workers, is similar to the response that we observe without added Ca^{2+}. This may be due to the fact that the human cells were kept in a Ca^{2+}-free medium until immediately before the addition of dye and *N*-fMet-Leu-Phe. Under these conditions, they may have had insufficient time to equilibrate their internal calcium levels with respect to the external concentration.

Korchak and Weissmann [10,12] have also estimated the resting potential of human neutrophils from the distribution of triphenylmethylphosphonium between cells and medium. They report a value of —26 mV, which is considerably different from the value that we have obtained for rabbit cells, although in earlier work a potential of —68 mV was deduced from ratios of ouabain-insensitive K^+ fluxes [33]. Utsumi et al. [27] have used DiS-C_3-(5) to investigate guinea-pig neutrophils. Although their experiments were mostly concerned with the effects of concanavalin A, their data show that the null-point in the presence of valinomycin occurs at between 10 and 17 mM K^+, which is similar to the concentration we have estimated for rabbit cells. If the potential deduced from K^+ fluxes in human cells is incorrect, then there is evidence that the membrane potential in man differs from that in the rabbit and guinea-pig. Alternatively, the radioactive, cationic probe may underestimate neutrophil membrane potential.

Our results provide additional evidence that changes in plasma membrane potential occur at an early stage in the response of neutrophils to chemotactic agents. The advantage of using carbocyanine dye fluorescence to monitor the potential is that it is possible to follow the time course of the changes. The nature and sequence of the events that give rise to the fluctuations are still not clear, but the use of dyes such as DiS-C_3-(5) to provide a continuous monitor of membrane potential, will help in the identification of the factors that give rise to the changes at each stage.

Acknowledgements

We wish to thank Dr. Alan Waggoner for providing the dye used in these studies, Dr. Richard Freer for the inhibitor and Drs. Timothy Williams and Peter Jose for the permeability agonist. We are indebted to Drs. Bastien Gomperts and Trevor Powell for their advice and to Dr. Jonathan Bennett who also carried out the secretion measurements. We thank Professor Tait for his interest.

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