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12 May 1978; revised 26 September 1978

Chemotactic Factor–Induced Release of Membrane Calcium in Rabbit Neutrophils

Abstract. *The interaction of chemotactic factors (fMet-Leu-Phe and C5a) with rabbit neutrophils leads to rapid and specific release of membrane calcium, as evidenced by changes in the fluorescence of cell-associated chlorotetracycline. These two structurally different stimuli appear to interact with the same pool of membrane calcium.*

Chemotactic and secretory stimuli alter the ionic permeability of the plasma membrane and the intracellular concentrations of exchangeable calcium in neutrophils (*1*, *2*). The molecular mechanisms that cause these effects are of interest for an understanding of neutrophil physiology in particular and the contractile activities of nonmuscle cells in general. We reported recently that the interaction of chemotactic factors with neutrophil membranes leads to release of calcium from previously bound stores, and we postulated that the plasma membrane or other membranous cell components may act as such calcium stores (*2*). This tentative conclusion was based on indirect evidence derived from studies dealing with the effect of chemotactic factors on the movement of ^{45}Ca across rabbit neutrophil membranes. We report here the results of experiments that implicate membrane-associated calcium, and its release by chemotactic factors, in the initial events involved in neutrophil activation.

The fluorescence characteristics of the chelate probe chlorotetracycline are extremely sensitive to the concentrations of divalent cations within the hydrophobic environment into which it is preferentially partitioned. Chlorotetracycline fluoresces more intensely when complexed to divalent cations, and its emission and excitation spectra can be used to differentiate between its Ca^{2+} and Mg^{2+} chelates (*3*).

We took advantage of these fluorescence characteristics of chlorotetracycline, particularly the dependence on Ca^{2+}, to investigate the effects of chemotactic factors on the fluorescence of chlorotetracycline-loaded neutrophils. Rabbit peritoneal polymorphonuclear leukocytes (neutrophils) were incubated for 45 to 60 minutes at 37°C in the presence of 100 μM chlorotetracycline (Sigma Chemical Co., St. Louis, Mo.) in Hanks balanced salt solution containing 10 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (pH, 7.3), 1.7 mM Ca^{2+}, and no Mg^{2+}, to which glucose and bovine serum albumin were added at 1 mg/ml each. The desired number of cells were then washed once, resuspended in Hanks balanced salt solution without Mg^{2+} and bovine serum albumin and with or without 0.5 mM Ca^{2+}, and transferred to a Perkin-Elmer MPF 2A fluorescence spectrophotometer equipped with a temperature control cuvette holder and stirrer. All the experiments were performed at 37°C with a cell density of 2.5×10^6 cells per milliliter. The viability of the cells, as measured by lactate dehydrogenase release, and their functional responsiveness, as indicated by their ability to release lysosomal enzymes in the presence of cytochalasin B and fMet-Leu-Phe (*4*), were found not to be affected by these experimental manipulations. Excitation and emission wavelengths were 390 and 520 nm, respectively. The excitation and emission slits were adjusted to maximize the signal-to-noise ratio. These experimental conditions are essentially the same as those described by other workers who used chlorotetracycline as a calcium probe (*3*). The synthetic chemotactic factor fMet-Leu-Phe was obtained as previously described (*5*). Its competitive antagonist Boc-Phe-Leu-Phe-Leu-Phe (*4*) was provided by R. J. Freer, Medical College of Virginia, Richmond. Partially purified C5a, the low-molecular-weight chemotactic fragment of the fifth component of complement, was generated by trypsin treatment of C5 as described by Cochrane and Müller-Eberhard (*6*). These preparations of C5a exhibited maximal biological activity (lysosomal enzyme release) at a dilution of 1 to 1000.

Immediately after the addition of either fMet-Leu-Phe or C5a, a rapid decrease in the fluorescence of chlorotetracycline-loaded neutrophils was observed (Fig. 1). Comparison of Fig. 1, A to C, shows that essentially similar results were obtained whether or not extracellular calcium was present at the time of stimulation. This indicates that the fluorescence signal reflects one of the initial molecular events that follows the binding of the chemotactic factor to its receptor rather than the net influx of calcium that occurs when chemotactic factors are added in the presence, but not in the absence, of extracellular Ca^{2+} (*1*, *2*). The dependence of the fluorescence changes on the concentration of the chemotactic factors is illustrated in Fig. 1B. Fluores-

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cence changes can be detected at fMet-Leu-Phe concentrations as low as $10^{-10}M$ and reach a maximum around $10^{-8}M$. These are similar to the fMet-Leu-Phe concentrations that exhibit biological activity under the same experimental conditions—that is, in the absence of divalent cations (*7*). The ability of the neutrophils to respond to a second challenge with $10^{-8}M$ fMet-Leu-Phe is dependent on the concentration of the initial stimulus—that is, the response appears to become saturated around $10^{-8}M$ fMet-Leu-Phe or the cells become desensitized to this stimulus (Fig. 1B). Furthermore, the addition of $10^{-9}M$ fMet-Leu-Phe causes a 5-nm shift toward shorter wavelengths in the excitation and emission spectra of cell-associated chlorotetracycline (data not shown). Spectral shifts such as these have been shown, by comparing the fluorescence of chlorotetracycline in cell membranes and in methanol in the presence of Ca^{2+} or Mg^{2+}, to be strongly indicative of selective release of membrane calcium (*3*).

There is evidence that chemotactic factors such as fMet-Leu-Phe and C5a act through specific but distinct receptors on the plasma membrane of the neutrophils (*8*, *9*). Competitive antagonists of the binding and biological activity of fMet-Leu-Phe and other similarly active synthetic peptides have been described (*10*). These inhibitors are without effect on C5a-induced biological responses (*8*, *10*). One such antagonist is Boc-Phe-Leu-Phe-Leu Phe. As shown in Fig. 2, the fluorescence response to suboptimal concentrations of fMet-Leu-Phe, but not to equivalent concentrations of C5a, can be effectively blocked by Boc-Phe-Leu-Phe-Leu-Phe. The antagonist, by itself, has no effect on the fluorescence of the cells. These results show that the decrease in fluorescence induced by fMet-Leu-Phe is mediated through the binding of the chemotactic factor to its receptors and thus show the specificity of the observed chemotactic factor–induced fluorescence changes. No antagonist to the binding of C5a is available. The dissociation constant for Boc-Phe-Leu-Phe-Leu-Phe, which can be calculated from the shift caused by this antagonist in the concentration-response curve of the fluorescence changes (data not shown), is $4 \times 10^{-7}M$. This is in good agreement with values derived from binding and biological assays (*11*), which range from 2.5×10^{-7} to $8 \times 10^{-7}M$.

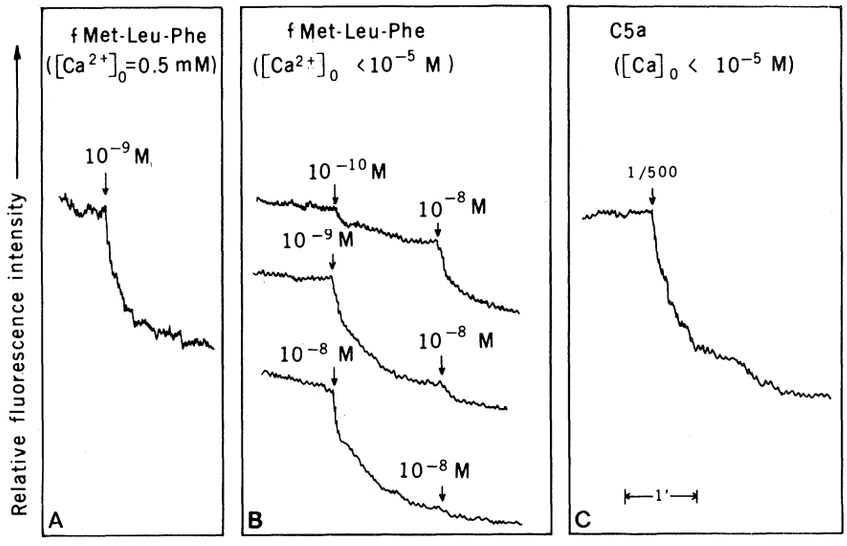

Fig. 1. Effects of chemotactic factors on the fluorescence of chlorotetracycline-loaded neutrophils. The cells were prepared as described in the text. The arrows indicate the time of addition of the chemotactic factors. (A) Addition of $10^{-9}M$ fMet-Leu-Phe to cells resuspended in the presence of 0.5 m*M* Ca^{2+}. (B) Addition of fMet-Leu-Phe at the indicated concentrations to cells resuspended in the absence of added Ca^{2+}. (C) Addition of C5a to cells resuspended in the absence of added Ca^{2+}. The tracings are taken from representative experiments performed on different days, hence the magnitudes of the fluorescence decreases cannot be directly compared.

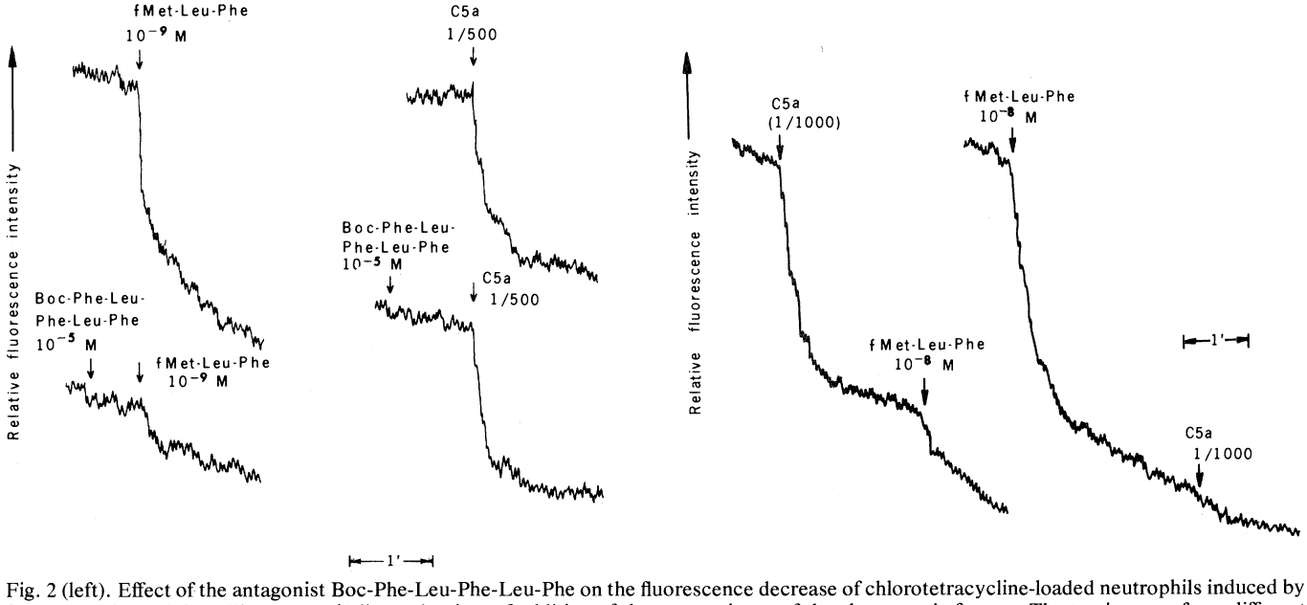

Fig. 2 (left). Effect of the antagonist Boc-Phe-Leu-Phe-Leu-Phe on the fluorescence decrease of chlorotetracycline-loaded neutrophils induced by fMet-Leu-Phe and C5a. The arrows indicate the time of addition of the antagonist or of the chemotactic factors. The tracings are from different experiments, hence the magnitudes of the fluorescence decreases induced by C5a and fMet-Leu-Phe cannot be quantitatively compared. Fig. 3 (right). Effect of consecutive addition of fMet-Leu-Phe and C5a on the fluorescence of chlorotetracycline-loaded neutrophils. The arrows indicate the time of addition of the chemotactic factors.

Additional insight into the mechanism of action of fMet-Leu-Phe and C5a was obtained by consecutive addition of the two to the same sample of chlorotetracycline-loaded neutrophils (Fig. 3). The addition of even a large concentration of either of the chemotactic factors following a saturating concentration of the other has no further effect on the fluorescence of the cells, irrespective of which chemotactic factor is added first. These results strongly suggest that, although initiated by their binding to different receptors (*10*), the actions of fMet-Leu-Phe and C5a are mediated, at least in part, through the release of a common pool of membrane calcium.

The chemotactic factor-induced decrease of the fluorescence of chlorotetracycline-loaded neutrophils thus satisfies the following conditions for functional significance: (i) the two very different chemotactic factors tested induced a decrease in cell-associated chlorotetracycline fluorescence; (ii) the time course of the change in fluorescence is consistent with that of the biological effects of the chemotactic factors; (iii) the concentrations of chemotactic factors required to elicit biological responses and changes in chlorotetracycline fluorescence are similar; and (iv) the binding of fMet-Leu-Phe, the biological functions it induces, and the decrease in chlorotetracycline fluorescence can all be competitively inhibited by the same concentrations of Boc-Phe-Leu-Phe-Leu-Phe. To our knowledge these results represent the first direct experimental evidence for the postulated (*2*) involvement of membrane calcium in transmembrane signal transduction in the neutrophils. The factors modulating the chemotactic factor-induced decrease in the fluorescence of chlorotetracycline—or more specifically the underlying release of membrane calcium by chemotactic factors—remain to be elucidated. One attractive possibility is that this release of membrane calcium is related to the recently demonstrated chemotactic factor-induced protein carboxymethylation in neutrophils (*12*). Elucidation of these mechanisms should help in mapping the sequence of events that occur between the binding of the stimulus (chemotactic factor) to its specific receptors and the activation of the contractile apparatus of the neutrophils.

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6 November 1978

Local Foveal Inhibitory Effects of Global Peripheral Excitation

Abstract. *Global excitation produced by oscillating a peripheral square-wave grating back and forth through one-half cycle inhibits the visibility of an incremental test flash only when the flash is presented in the foveal region of the visual field. This finding is discussed in the context of the neurophysiological periphery effect and shift-effect and their possible role in saccadic suppression.*

When a contour shifts or a grating consisting of black and white bars oscillates back and forth in the periphery of the visual field it can decrease the visibility of a central incremental light flash (*1-3*). This psychophysical effect is probably caused by an inhibition of the signal generated by the test flash (*3*) and therefore suggests that activity in one region of the visual field can be inhibited by excitation in another, remote region. We have found that the test flash inhibition produced by global peripheral excitation is obtained only locally at the fovea. Besides offering neurophysiological and functional interpretations of these results, we also suggest some implications for future neurophysiological and neuroanatomical studies.

The stimulus display (Fig. 1) was front projected from three projectors onto a white matte posterboard screen located about 230 cm from the subject. The display subtended an area of 24° by 19.5°. One projector supplied the outer surround consisting of either a vertical grating, as shown, or a uniform white field with a luminance equal to the space-averaged luminance of the black and white bars. From the grating, a disk-shaped central region 7.0° in diameter was deleted by an appropriate mask. In its place was projected from a second projector a uniformly illuminated background disk, onto which in turn was projected an incremental, 0.38° diameter test spot from a third projector.

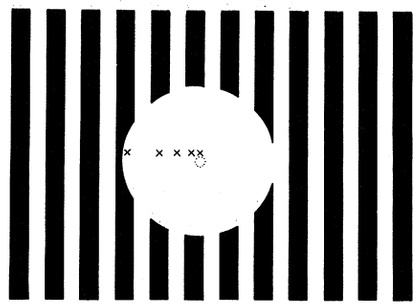

Fig. 1. The stimulus display. The central, incremental test flash is indicated by a stippled circle. The positions of the fixation point are indicated by x's.

The widths of the white and black bars of the grating were each 0.94°, corresponding to a spatial frequency of 0.53 cycle per degree; their respective luminances, L_{max} and L_{min}, were 11.10 cd/m^2 and 0.41 cd/m^2. The contrast of the grating, according to the formula $(L_{max} - L_{min})/(L_{max} + L_{min})$ was 0.93. The luminance of the uniform outer surround was $(L_{max} + L_{min})/2 = 5.75$ cd/m^2. The grating was oscillated at a previously determined optimal frequency of 4 Hz (*2*) by means of a mirror placed in the beam of the first projector and mounted on an electromagnet which, in turn, was driven by a function generator (Wavetek model 133). The grating was oscillated in a square-wave manner through one bar width producing a luminance modulation equivalent to pattern reversal. On the basis of an earlier study (*3*), the luminance of the background disk was set at optimal levels for each of

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