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DIRECT DEMONSTRATION OF INCREASED INTRACELLULAR CONCENTRATION OF FREE CALCIUM IN RABBIT AND HUMAN NEUTROPHILS FOLLOWING STIMULATION BY CHEMOTACTIC FACTOR

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SUMMARY: An increase in the level of intracellular free calcium concentration in rabbit and human neutrophils stimulated by chemotactic factors has been demonstrated directly using the calcium-sensitive fluorescent probe quin-2. Addition of f-Met-Leu-Phe (10^{-9} M), $C5_a$ ($3x10^{-9}$ M) or leukotriene B_4 (6×10^{-8} M) to the neutrophils induces a rapid increase in the intracellular concentration of free calcium that reaches a maximum value 15 seconds following stimulation. At concentrations of f-Met-Leu-Phe less than 10^{-8} M the enhancement is dose dependent with an ED_{50} of 8×10^{-11} M and is significantly reduced in the presence of EGTA in the suspending medium.

It is commonly hypothesized that neutrophil activation by chemotactic factors is mediated by a change in the concentration of intracellular free calcium (1-7). A great deal of effort has thus been devoted to demonstrating experimentally that chemotactic factors do indeed cause an increase in the intracellular level of free calcium. However, because of the limitations inherent in the previously available techniques, only an indirect deduction of the presence of such an increase has proven possible so far. The recent synthesis of the calcium sensitive fluorescent probe quin-2 and its permeable non-polar ester derivate quin-2/AM (8-10) provides

ABBREVIATIONS

5(S), 12(R) dihydroxy-6,8,11,14(cis,trans,trans,cis) eicosatetraenoic acid: leukotriene B_4
N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid: HEPES
Polymorphonuclear neutrophilic leukocytes: neutrophils
Formyl-Methionyl-Leucyl-Phenylalanine: f-Met-Leu-Phe
Ethyleneglycol-bis-(β-amino-ethyl-ether)N,N',-tetra-acetic acid: EGTA
Nanometer: nm
Nanomolar: nM

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an opportunity to measure directly the change in the intracellular level of free calcium in neutrophils following stimulation.

In this paper we wish to report experiments showing directly that the chemotactic factors formyl-methionyl-leucyl-phenylalanine (f-Met-Leu-Phe), $C5_a$ and leukotriene B_4, rapidly, and at physiological concentrations, increase the concentration of intracellular free calcium in rabbit and human neutrophils.

METHODS AND MATERIALS

Rabbit peritoneal neutrophils (4-12h exudates) were obtained, washed and handled as previously described (11). Human neutrophils were isolated on Ficoll-Hypaque gradients as described by English and Anderson (12). The cells were resuspended in protein and magnesium free modified Hanks' Balanced Salt Solution, the composition of which was (mM): NaCl, 124; KCl, 4; $NaHPO_4$, 0.64; K_2HPO_4, 0.66; $NaHCO_3$, 15.2; HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid), 10.0; and glucose 5.56; pH 7.4.

Loading with quin-2/AM

Quin-2/AM at a final concentration of 100 µM was added to washed cells suspended (10^8 cells/ml) in the modified Hanks' solution containing Ca^{2+} (1.6 mM). The cells were incubated at this concentration for 10 min in a rotary shaker (37°C) and were then diluted ten fold with thermally equilibrated (37°C) buffer either with or without Ca^{2+} (8) and then allowed to incubate for different periods of time. At the end of the incubation period, the desired number of cells were then washed once, resuspended in Hanks' balanced solution containing either calcium (1.6 mM) or 2 mM EGTA, and transferred to a cuvette for fluorescence measurements. The suspensions were allowed to stand for 10 minutes before fluorescence measurement was initiated. This was necessary to achieve a stable baseline.

Fluorescence measurements

Fluorescence measurements were performed using an SLM (Model 8000) fluorescence spectrophotometer with temperature controlled cuvette and magnetically driven stirrer. The excitation and emission wave lengths were 339 and 492 nm respectively, and the excitation and emission slits were adjusted to maximize the signal to noise ratio. Fluorescence recordings were processed with a Hewlett Packard HP85 desk top computer.

Cell fluorescence was sampled every one second. The stimulus was added directly to the cuvette through a special hole without interrupting monitoring. The final concentration of DMSO did not exceed 0.2%. The intracellular concentration of free calcium in rabbit neutrophils was calculated according to the following equation (8):

$$[Ca^{2+}]_i = K_d (F - F_{min})/(F_{max} - F) \quad (1)$$

Where $[Ca^{2+}]_i$ represents the concentration of intracellular free calcium; K_d is the apparent dissociation constant of quin-2 for calcium; F is the fluorescence signal in arbitrary units, and F_{min} and F_{max} are the fluorescence at very low and very high calcium, respectively. They represent the fluorescence signals levels attained when the intracellular quin-2 is released by Triton X-100 (1%) and exposed to less than 5 nM and 1 mM calcium, respectively (8).

Materials

EGTA (Ethylene glycol-bis-(β-amino-ethyl-ether) N',N'-tetra-acetic acid), Hepes and f-Met-Leu-Phe were all purchased from Sigma Chem. Co. (St. Louis,

Mo.). Quin-2/AM was purchased from Lancaster Synthesis Ltd., England. Leukotriene B_4 [5(S),12(R)dihydroxy-6,8,11,14(cis,trans,trans,cis)-eicosa tetraenoic acid] was prepared from swine blood, as previously described (13).

RESULTS AND DISCUSSION

Upon penetration into the neutrophils, quin-2/AM is rapidly hydrolyzed thereby generating, and trapping in the cytoplasm, the impermeant quin-2. The two forms of the probe can be differentiated experimentally on the basis of their emission peaks (432 nm and 492 nm for quin-2/AM and quin-2, respectively). The time course of the uptake of quin-2/AM by the neutrophils and its conversion to quin-2 can therefore be examined by monitoring the fluorescence at 492 nm. The results illustrated in figure 1 demonstrate that the uptake of the dye can indeed be observed as a time-dependent increase in the fluorescence signal of the rabbit neutrophil suspension. The intracellular concentration of free calcium in unstimulated rabbit neutrophils calculated from the fluorescence of cell-associated quin-2 as described in Materials and Methods was found to vary between 100 and 200 nM.

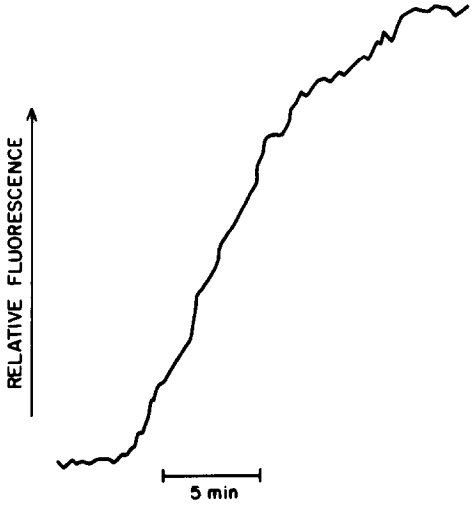

Figure 1. Time-course of quin-2/AM loading into rabbit neutrophils. Quin-2/AM (10 μM) was added to thermally equilibrated cell suspension (10^7 cell/ml) and the fluorescence intensity was monitored as described in the Methods Section. The excitation and emission wavelengths were 339 and 492 nm, respectively.

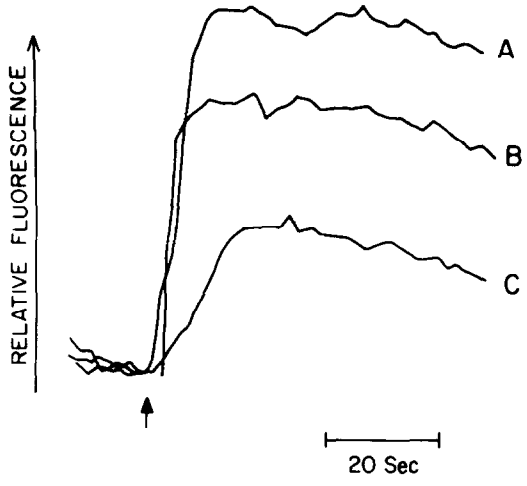

Figure 2. The effects of various chemotactic stimuli on the fluorescence of quin-2/AM loaded rabbit neutrophils. The cells (10^7 cell/ml) were prepared as described in the text. The arrow indicates the time of addition of the chemotactic factors. A, 10^{-9} M f-Met-Leu-Phe; B, 6×10^{-8} M leukotriene B_4; C, (3×10^{-9}) $C5_a$. In terms of biological response, the concentration of $C5_a$ used here is equivalent to 10^{-10} M f-Met-Leu-Phe.

The effects of the three most commonly used neutrophil stimuli, the synthetic peptide f-Met-Leu-Phe (10^{-9} M), the complement fragment $C5_a$ (3×10^{-9} M) and the dihydroxy metabolite of arachidonic acid leukotriene B_4 (6×10^{-8} M) on the fluorescence of cell-associated quin-2 were examined next. The concentrations of f-Met-Leu-Phe and leukotriene B_4 used produce a maximal secretory response, while that of $C5_a$ corresponds approximately to its ED_{50} as measured in the degranulation assay. As shown in figure 2, all three stimuli cause a rapid increase in the fluorescence of cell-associated quin-2. This response corresponds to a two to three fold increase in the concentration of cytoplasmic free calcium. In each instance, the maximal level of fluorescence is reached within about 15 seconds, the rise time of the fluorescence of the $C5_a$ stimulated cells being somewhat slower.

Chemotactic factor induced increases in cytoplasmic calcium can be detected with quin-2 not only in rabbit but also in human neutrophils (figure 3). In these

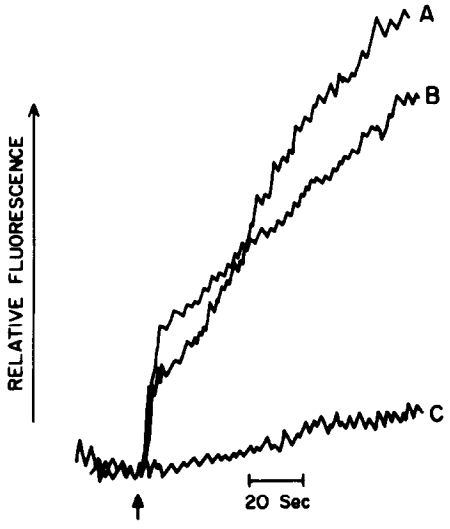

Figure 3. The effects of chemotactic stimuli on the fluorescence of quin-2 loaded human neutrophils. A, 10^{-7} M f-Met-Leu-Phe; B, 6 x 10^{-8} M leukotriene B_4; C, 10^{-9} M f-Met-Leu-Phe. This is a single experiment representative of at least two separate experiments.

cells f-Met-Leu-Phe and leukotriene B_4 cause a rapid rise followed by a slower and a sustained increase in the fluorescence of cell-associated quin-2.

The effect of f-Met-Leu-Phe on the fluorescence of cell-associated quin-2 is dose-dependent (figure 4). Increases in fluorescence are detectable at concentrations of f-Met-Leu-Phe as small as 10^{-11} M and appear to plateau around 10^{-8} M. The ED_{50} for f-Met-Leu-Phe is 8 x 10^{-11} M which is very close to the value of 7 x 10^{-11} for chemotaxis (14).

It is generally hypothesized that chemotactic factors increase the level of intracellular exchangeable calcium by mobilizing calcium from internal stores, by displacement of previously bound calcium, and from the extracellular medium by increasing the plasma membrane permeability to Ca^{2+}. In order to examine the role of extracellular Ca^{2+} in the increase of the cytoplasmic levels of free Ca^{2+}, we have examined the effect of the addition of EGTA on the fluorescence changes caused by f-Met-Leu-Phe and the results are summarized in figure 5. As illustrated in figure 5, f-Met-Leu-Phe causes smaller increases in quin-2 fluorescence in cells resuspended in media lacking calcium and to which EGTA (2 mM)

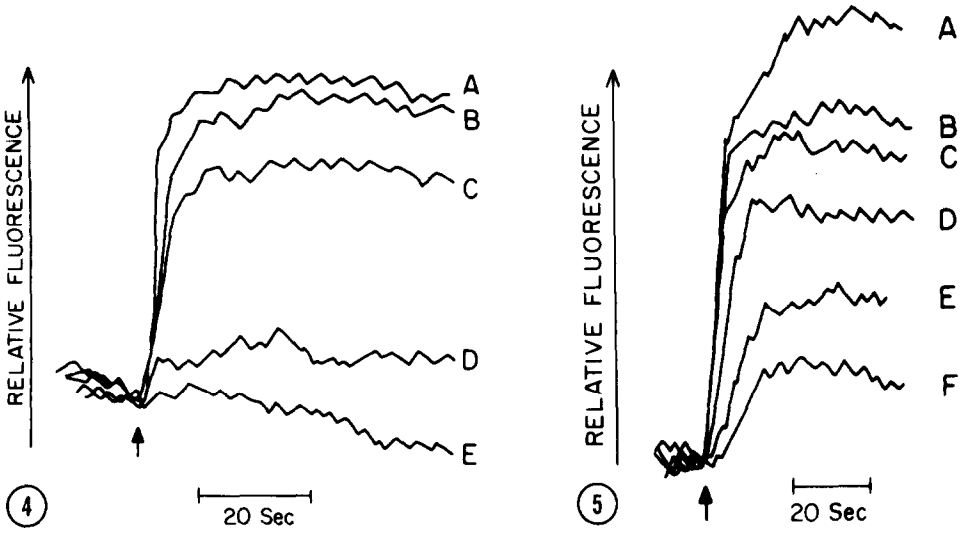

Figure 4. Dose-response curves for the f-Met-Leu-Phe-induced increase of the fluorescence of cell-associated quin-2. The cells (10^7 cell/ml) were prepared as described in the text. The concentrations of f-Met-Leu-Phe were as follows: A, 10^{-8} M; B, 10^{-9} M; C, 10^{-10} M; D, 5 x 10^{-11} M; E, 10^{-11} M. The arrow indicates the time of addition of f-Met-Leu-Phe. This is a single experiment representative of at least three separate experiments.

Figure 5. Effects of f-Met-Leu-Phe on the fluorescence of quin-2-loaded neutrophils in the presence and absence of extracellular calcium. The cells (10^7 cell/ml) were prepared as described in the text and then resuspended in buffered Hanks' solution containing either 1.6 mM Ca^{2+} or in Hanks' solution in which calcium is replaced by 2 mM EGTA. Tracings A, B and E contain 1.6 mM calcium and tracings C, D and E contain 2 mM EGTA. The f-Met-Leu-Phe concentrations are as follows: A and C, 5 x 10^{-9} M; B and D, 5 x 10^{-10} M; and E and F, 10^{-10} M. The arrow indicates the time of addition. The results are those of a single experiment representative of at least three separate experiments.

was added than in cells resuspended in the presence of 1.6 mM calcium. These results, therefore, demonstrate that the elevation in the level of intracellular free calcium detected by quin-2 upon stimulation is derived from both internal and external sources.

The results presented in the present communication provide the first direct experimental evidence that the chemotactic stimuli, f-Met-Leu-Phe, $C5_a$ and leukotriene B_4, cause a rapid elevation in the concentration of intracellular free calcium in both rabbit and human neutrophils, and that this elevation comes from both release from internal stores and a net uptake from the extracellular medium. Therefore, they lend considerable and needed support to the hypothesis that the neutrophil's second messenger function is carried out by calcium ions.

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REFERENCES

1. Romeo, D., Zabucchi, G. and Soranzo, M.R. (1975). Calcium Transport in Contraction and Secretion (ed. E. Carafoli, F. Clementi, W. Drabikowski and A. Margeth). Elsevier Publishing Company, Inc., 195.
2. Gomperts, B. (1976). Receptors and Recognition, (ed. P. Cuatrecasas and M.F. Greaves), vol. 2. John Wiley and Sons Inc., 43.
3. Gallin, J.I., Gallin, E.K., Malech, H.L. and Cramer, E.B. (1978). Leukocyte Chemotaxis: Methods, Physiology and Clinical Implications, (ed. J.I. Gallin and P.G. Quie), Raven Press, 123.
4. Weissmann, G., Korchak, H.M., Perez, H.D., Smolen, J.E., Goldstein, I.M. and Hoffstein, S.T. (1979). Advance in Inflammation Research, (ed. G. Weissmann, S.M. Samuelsson and R. Paoletti), vol. 1. Raven Press, 95.
5. Sha'afi, R.I. and Naccache, P.H. (1981). Advances in Inflammation Research, (ed. G. Weissmann), vol. 2. Raven Press, 115.
6. Becker, E.L., Naccache, P.H., Showell, H.J. and Walenga, R.W. (1981). Lymphokines (eds. E. Pike and M. Lands), vol. 4. Academic Press, 194.
7. Wilkinson, P.C. (1982). Chemotaxis and Inflammation; second edition. Churchill Livinstone
8. Tsien, R.Y., Pozzan, T. and Rink, T.J. (1982). J. Cell. Biol. 94:325.
9. Tsien, R.Y. (1980). Biochemistry 19:2396.
10. Pozzan, T., Arslan, P., Tsien, R.Y. and Rink, T.J. (1982). J. Cell. Biol. 94:335.
11. Showell, H.J., Williams, D., Becker, E.L., Naccache, P.H. and Sha'afi, R.I. (1979). J. Reticuloendo. Soc. 75:138.
12. English, D. and Anderson, B.R. (1974). J. Immunol. Meth. 5:249
13. Borgeat, P. and Samuelsson, B. (1979). J. Biol. Chem. 754:7865.
14. Showell, H.J., Freer, R.J., Zigmond, S.H., Schiffmann, E., Aswanikumar, S., Corcoran, B. and Becker, E.L. (1976). J. Exp. Med. 143:1154.