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STUDIES ON STIMULUS-RESPONSE COUPLING IN HUMAN NEUTROPHILS

II. RELATIONSHIPS BETWEEN THE EFFECTS OF CHANGES OF EXTERNAL IONIC COMPOSITION ON THE PROPERTIES OF *N*-FORMYLMETHIONYLLEUCYLPHENYLALANINE RECEPTORS AND ON THE RESPIRATORY AND SECRETORY RESPONSES

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Studies were carried out on the mechanism responsible for the enhancement of the respiratory and secretory responses to *N*-formylmethionylleucylphenylalanine (fMet-Leu-Phe) exhibited by human neutrophils suspended in Na^+-free, high-K^+ buffered solution. The results demonstrate that: (a) the variation of Na^+ concentration in the suspending solution induces in human neutrophils a marked modification of the recognition apparatus for the chemotactic peptide fMet-Leu-Phe, the lack of or low concentration of this ion increasing the number of the receptors and their specific affinity for the ligand; (b) the greater respiratory burst and secretion induced by fMet-Leu-Phe in human neutrophils suspended in Na^+-free, high-K^+ medium are due to the increased formation of receptor-ligand complexes at the cell membrane; (c) the greater respiratory response is partially due also to a higher efficiency of these receptor-ligand complexes. The molecular mechanism by which Na^+ exerts a regulative role on the properties of the recognition apparatus for the chemotactic peptide and its possible significance are discussed.

Introduction

In the preceding paper [1], we have presented data showing that the extracellular concentration of the monovalent cations Na^+ and K^+ greatly influences the intensity of the response of human neutrophils to the chemotactic peptide *N*-formylmethionylleucylphenylalanine (fMet-Leu-Phe). Human neutrophils when suspended in buffered solutions lacking or containing low concentrations of Na^+ and a high concentration of K^+ exhibit both respiratory and secretory responses to fMet-Leu-Phe much higher than when suspended in buffered solutions containing physiological concentrations of Na^+ and K^+. This enhancement of the response is remarkable at low concentrations of the stimulant and disappears at the maximal stimulatory concentration of the peptide. Therefore, the change of external ionic composition does not influence the maximal responses, i.e., the oxygen consumption and secretory activity induced by maximal doses of fMet-Leu-Phe, but does influence the threshold of the response to the peptide.

This paper reports data showing that the variations of ionic composition of the suspending solutions induce in human neutrophils a marked modification of the properties of the recognition apparatus for fMet-Leu-Phe. The greater respiratory and secretory responses of cells suspended in Na^+

-free, high-K^+ buffer are dependent on the increase in the binding of fMet-Leu-Phe on the cell surface, due to an increase in the number and in the affinity of the specific receptors.

Materials and Methods

Reagents. fMet-Leu-Phe, ferricytochrome *c* type VI, catalase, cytochalasin B, choline hydrochloride gramicidin and valinomycin were purchased from Sigma; fMet-Leu-[^{3}H]-Phe from New England Nuclear. Superoxide dismutase was a gift from Dr. J.V. Bannister. Preliminary experiments have shown that the biological activity of the labeled peptide is indistinguishable from that of the unlabeled one. Stock solutions of fMet-Leu-Phe and cytochalasin B were made in dimethyl sulfoxide and kept frozen at −20°C.

Collection of cells. Leucyte suspensions containing 85–90% neutrophils were obtained as previously described [1].

Suspending buffered solutions. The solutions employed, buffered at pH 7.4 and containing 5 mM glucose, were as follows:

(1) Krebs-Ringer phosphate: [Na^+] 152 mM; [K^+] 5 mM; [Cl^-] 130 mM; [PO_4^{3-}] 16.6 mM; [Mg^{2+}] 1.22 mM.

(2) Na^+-free, high-K^+ Krebs-Ringer phosphate: [K^+] 157 mM; [Cl^-] 130 mM, [PO_4^{3-}] 16.6 mM; [Mg^{2+}] 1.22 mM.

(3) Krebs-Ringer phosphate containing different concentrations of Na^+ and K^+.

(4) Krebs-Ringer phosphate where Na^+ was totally or partially substituted with choline.

Metabolic studies. O_2 consumption, O_2^- production, secretion of β-glucuronidase, of vitamin B-12-binding protein and release of lactate dehydrogenase were measured using the procedures and conditions described in the preceding paper [1].

f-Met-Leu-[^{3}H]Phe binding assay. fMet-Leu-[^{3}H]Phe binding to human neutrophils was measured by a rapid filtration technique as described in Refs. 2–4. Briefly, after the incubation of human neutrophils in the presence of fMet-Leu-[^{3}H]Phe, cells were filtered through Whatman (GF/C) glass fiber filters using a rapid filtration device (Millipore). Filters were then quickly washed with aliquots (3 × 5 ml) of ice-cold Krebs-Ringer phosphate. The filtration and washing procedure took less than 10 s. Filters were dried, placed in 5 ml of scintillation liquid and stored overnight before counting the radioactivity. Nonspecific binding was defined as the amount of binding not inhibited by a 1000-fold excess of unlabeled fMet-Leu-Phe and was usually from 10 to 20% of the total counts bound. Specific binding was defined as the total amount of fMet-Leu-[^{3}H]Phe bound minus the nonspecific binding.

Quantification of the receptors and of their affinity for fMet-Leu-Phe. $15 \cdot 10^6$ human neutrophils/ml were incubated in the appropriate buffered solutions containing 0.5 mM $CaCl_2$, 5 μg/ml cytochalasin B and 1 mM KCN for 5 min at 37°C. After the incubation the tubes were cooled in an ice bath and then the cells tested for binding. Triplicate aliquots of $3 \cdot 10^6$ neutrophils were incubated in the presence of different concentrations of fMet-Leu[^{3}H]Phe for 60 min in melting ice in a final volume of 1 ml of the same buffered solution used for the incubation at 37°C. At the end of the incubation period, samples were diluted with 5 ml of ice-cold Krebs-Ringer phosphate and then rapidly filtered and counted as described before. A Scatchard analysis was performed using specific binding data at varing concentrations of fMet-Leu-[^{3}H]Phe.

Time course and reversibility of fMet-Leu-[^{3}H]Phe binding. $15 \cdot 10^6$ neutrophils/ml were incubated with fMet-Leu[^{3}H]Phe in appropriate buffered solutions containing 5 μg/ml cytochalasin B, 0.5 mM $CaCl_2$ and 1 mM KCN at 37°C with continuous stirring, i.e., under the same conditions used for the measurement of O_2 consumption. In some experiments the incubation was performed in the oxygen electrode chamber in order to allow simultaneous measurement of the stimulation of O_2 consumption. At timed points, aliquots were collected and filtered as outlined above. The determination of nonspecific and specific binding was made in parallel.

The reversibility of binding was determined by adding a 500-fold excess of nonradioactive fMet-Leu-Phe after an appropriate time of incubation of the cells in the presence of 20 nM fMet-Leu-[^{3}H]Phe. Then sample aliquots were withdrawn, filtered and washed as above. The change in cell-associated radioactivity after the addition of excess nonradioactive fMet-Leu-Phe was used to

calculate the reversibly bound fMet-Leu-[^{3}H]Phe, i.e., the ligand-receptor complexes not yet internalized. The nonreversibly bound fMet-Leu-[^{3}H]Phe was the difference between the specific binding and the reversibly bound fMet-Leu-[^{3}H]Phe. The nonreversibly bound corresponds to the ligand-receptor complexes that have been internalized [4–7].

Results

Binding of fMet-Leu-[^{3}H]Phe to neutrophil receptor at 0°C

In the first group of experiments, the specific and unspecific binding of fMet-Leu-[^{3}H]Phe at 0°C to neutrophils, after a preincubation at 37°C for 3 min in high-K^+ Krebs-Ringer phosphate and in Krebs-Ringer phosphate was measured. These conditions, designed to minimize the internalization of the receptor-ligand complexes, allow a measure of the number of receptors, and their affinity for the specific ligand on the surface of leucocytes at the same moment when fMet-Leu-Phe is added to stimulate the metabolism of leucocytes as described in the preceding paper [1].

Fig. 1 shows the data of a representative experiment on the specific and unspecific binding of fMet-Leu-[^{3}H]Phe as a function of its concentration. It can be seen that the specific binding of the ligand is higher in human neutrophils suspended in high-K^+ Krebs-Ringer phosphate than in Krebs-Ringer phosphate, while the unspecific binding is similar. The Scatchard analysis of the data of this experiment (Fig. 2) shows that the number of receptors for neutrophil (R_N) is higher and the apparent dissociation constant (K_D) is lower in neutrophils suspended in high-K^+ Krebs-Ringer phosphate. These results were confirmed in eight independent experiments (average values: R_N 26.175 ± 2.640 S.E. and K_D (nM) 16.8 ± 2.0 S.E. in human neutrophils suspended in Krebs-Ringer phosphate; R_N 44.731 ± 3.502 S.E. and K_D (nM) 6.2 ± 0.7 in human neutrophils suspended in high-K^+ Krebs-Ringer phosphate. These findings demonstrate that the suspension of human neutrophils for 3 min at 37°C in high-K^+ Krebs-Ringer phosphate induces an increase both

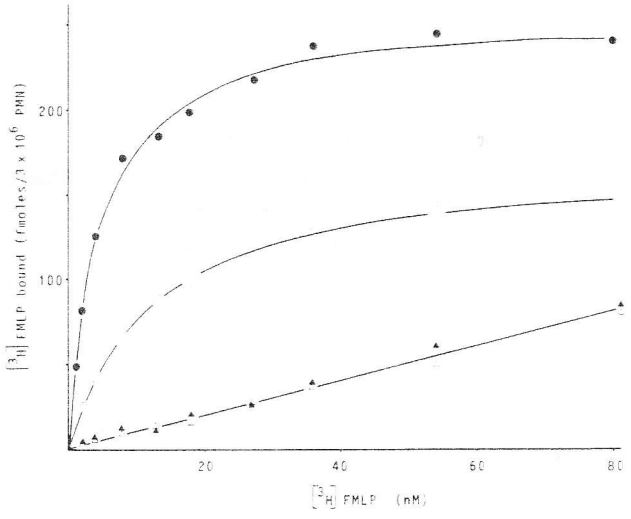

Fig. 1. Binding to human neutrophils (PMN) suspended in Krebs-Ringer phosphate and high-K^+ Krebs-Ringer phosphate as a function of increasing fMet-Leu-[^{3}H]Phe ([^{3}H] FMLP) concentration. Specific binding in Krebs-Ringer phosphate (○ ——— ○) and high-K^+ Krebs-Ringer phosphate (● ——— ●). Unspecific binding in Krebs-Ringer phosphate (△ ——— △) and high-K^+ Krebs-Ringer phosphate (▲ ——— ▲). For conditions see Materials and Methods.

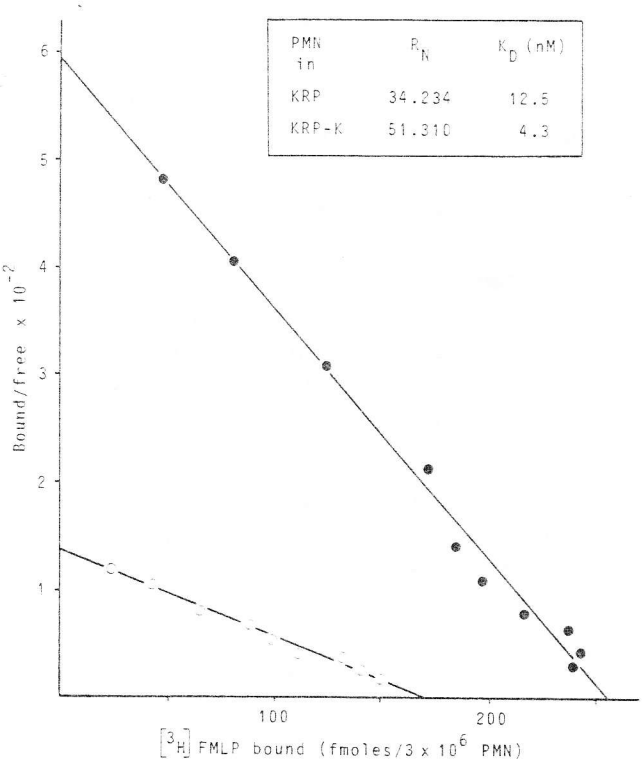

Fig. 2. Scatchard plot of specific binding of fMet-Leu-[^{3}H]Phe ([^{3}H] FMLP). The data are from the experiment of Fig. 1. Human neutrophils (PMN) in Krebs-Ringer phosphate (KRP) (○ ——— ○) and high-K^+ Krebs-Ringer phosphate (KRP-K) (● ——— ●). The inset reports the values of the number of receptors/cell (R_N) and of the dissociation constant (K_D).

in the number of surface receptors for fMet-Leu-Phe and, above all, in their affinity for the peptide.

Kinetics and reversibility of fMet-Leu-[^{3}H]Phe binding

Fig. 3 shows a representative experiment on the time course of the binding of fMet-Leu-[^{3}H]Phe (20 nM) to human neutrophils incubated at 37°C under the same conditions used for the measurement of the respiratory response. The association of the peptide to the cells is a complex phenomenon including, besides the binding, internalization of the receptor-ligand complexes [5–7], partitioning, digestion of the ligand and replacement of the receptors [8]. The results of the experiment of Fig. 3 show that the specific binding of the peptide was rapid and reached equilibrium within a few minutes. The binding of fMet-Leu-[^{3}H]Phe to human neutrophils suspended in high-K^+ Krebs-Ringer phosphate is much higher than that to cells suspended in Krebs-Ringer phosphate. The same figure reports also the measurement of the internalization of the receptor-fMet-Leu[^{3}H]Phe

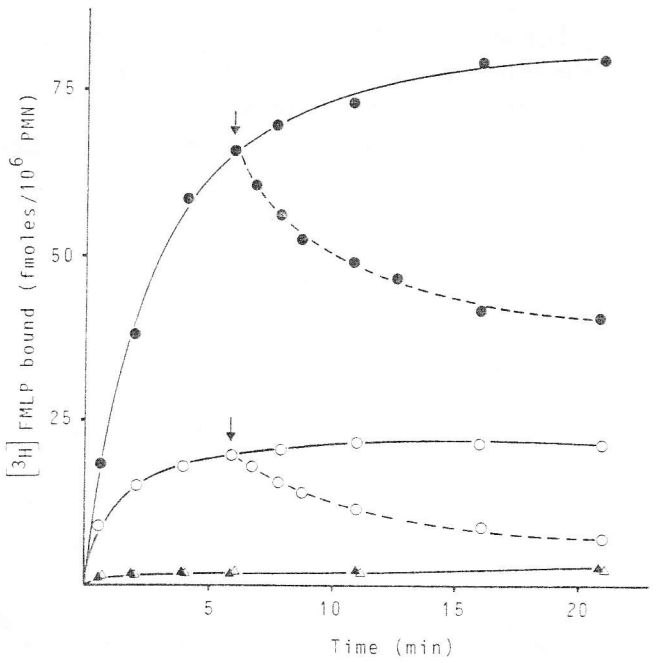

Fig. 3. Time course and reversibility of the binding of $2 \cdot 10^{-8}$ M fMet-Leu-[^{3}H]Phe ([^{3}H] FMLP) to human neutrophils (PMN) incubated at 37°C. Total binding in Krebs-Ringer phosphate (○——○) and in high-K^+ Krebs-Ringer phosphate (●——●). At the arrows a large excess of unlabeled fMet-Leu-Phe (10 μM) was added. Unspecific binding (△,▲).

complexes, performed by adding excess of nonradioactive peptide. It can be seen that the number of internalized receptor-ligand complexes is greater in human neutrophils suspended in high-K^+ Krebs-Ringer phosphate than in Krebs-Ringer phosphate.

Transmembrane potential and binding of fMet-Leu-[^{3}H]Phe

Since the suspension of neutrophils in high-K^+ Krebs-Ringer phosphate causes both a depolarization [1] and an increase in the binding of fMet-Leu-[^{3}H]Phe to the specific receptors, the problem arises as to whether or not the decrease in the membrane potential per se is responsible for the change of the properties of the receptor for the peptide. It is known, in fact, that the transmembrane electrical field may control the configuration state and the functions of molecules of the cell membrane (electrometamorphosis) [9]. We have tried to answer this problem by investigating the binding of fMet-Leu-[^{3}H]Phe to human neutrophils previously depolarized with gramicidin and with ouabain, or to human neutrophils suspended in buffered solution where Na^+ was totally substituted with choline, i.e., under Na^+-free conditions, that does not change the transmembrane potential.

Fig. 4 shows that the treatments of human neutrophils with the depolarizing drugs gramicidin and ouabain do not modify the binding of fMet-Leu-[^{3}H]Phe, whilst the substitution of Na^+ with choline in the suspending medium causes an increase in binding of the peptide to the specific receptors. As shown in the preceding paper [1], the first condition does not change the responses to fMet-Leu-Phe, whilst the latter induces an increase in both the respiratory and secretory responses.

On the basis of these results, it can be concluded that the modification of the properties of the receptors for fMet-Leu-Phe on the surface of human neutrophils is not dependent on the decrease in membrane potential per se but on the variation of the ionic composition of the suspending solutions.

Binding of fMet-Leu-[^{3}H]-Phe as a function of different [Na^+] and [K^+].

Fig. 5 shows that by substituting Na^+ for K^+, the high binding of fMet-Leu-[^{3}H]Phe to neu-

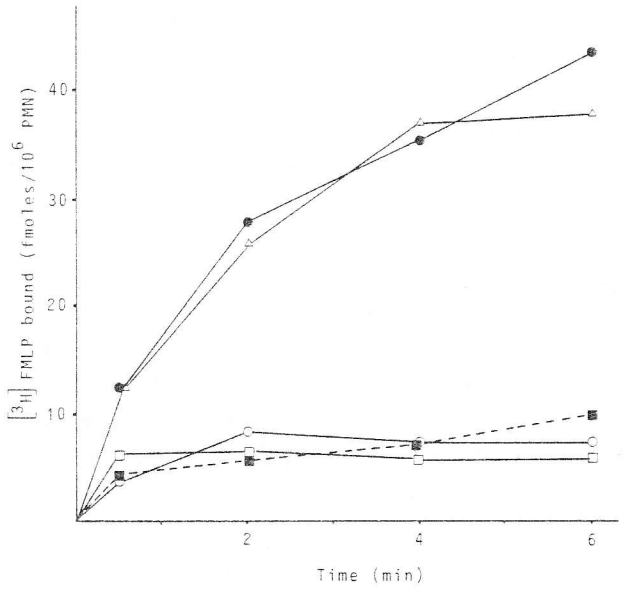

Fig. 4. Specific binding of $2 \cdot 10^{-8}$ M fMet-Leu-[^{3}H]Phe ([^{3}H] FMLP) to human neutrophils (PMN) suspended in Krebs-Ringer phosphate (○———○), in Krebs-Ringer phosphate plus gramicidin (1 μg/ml) (■———■) or plus ouabain (10^{-4} M) (□———□), in high-K^+ Krebs-Ringer phosphate (●———●) and in Krebs-Ringer phosphate where all Na^+ was substituted with choline (△———△). Temperature 37°C.

trophils progressively decreases and becomes similar to that to cells suspended in Krebs-Ringer phosphate when the concentration of Na^+ is increased to 40 mM. These results clearly indicate that the increase in binding of fMet-Leu-Phe to human neutrophils is directly related to changes in the properties of the receptors caused by the absence of Na^+.

Fig. 5 also shows that the decrease in binding of fMet-Leu-[^{3}H] and the depression of the respiratory response to the peptide induced by the substitution of K^+ with Na^+ in the suspending solution are not strictly parallel. When 40 mM K^+ is substituted with 40 mM Na^+, the binding of fMet-Leu-[^{3}H]Phe becomes similar to that to human neutrophils suspended in Krebs-Ringer phosphate, whilst the respiratory response decreases but remains still greater than that of neutrophils suspended in Krebs-Ringer phosphate. This finding indicates that only part of the higher respiratory response of human neutrophils in high-K^+ Krebs-Ringer phosphate is due to the greater binding of the stimulant to the specific receptors.

The data presented in Fig. 6 compare the depression of the secretory response of fMet-Leu-Phe with that of the binding of fMet-Leu-[^{3}H]Phe as a function of the progressive substitution of K^+ with Na^+. It can be seen that the effect of the substitution of K^+ with Na^+ on the secretion of β-glucuronidase and vitamin B-12-binding protein is similar to that on the binding of fMet-Leu-[^{3}H]Phe. No differences are detectable between the binding and the secretory response of human neutrophils suspended in Krebs-Ringer phosphate and in high-K^+ Krebs-Ringer phosphate where 40

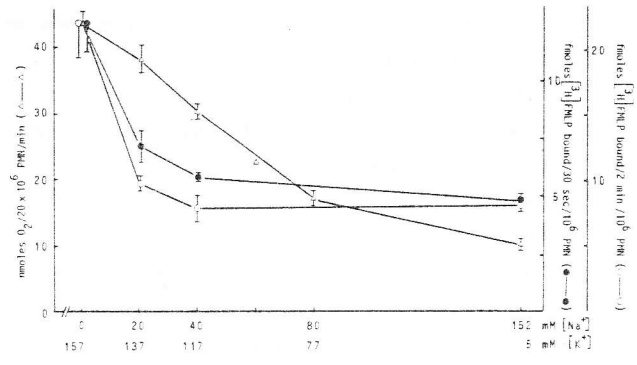

Fig. 5. Respiratory response to $2 \cdot 10^{-8}$ M fMet-Leu-Phe and binding of $2 \cdot 10^{-8}$ M fMet-Leu-[^{3}H]Phe ([^{3}H] FMLP) to human neutrophils (PMN) suspended in buffered solutions containing different concentrations of K^+ and Na^+. Temperature 37°C. O_2 consumption was measured in parallel experiments and under the same conditions. Data are means ± S.E. of seven experiments for binding and of six experiments for O_2 consumption.

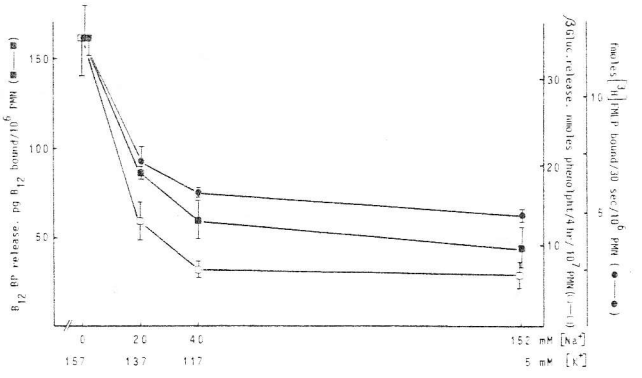

Fig. 6. Comparison between the decrease in secretory response to fMet-Leu-Phe and of the binding of $2 \cdot 10^{-8}$ M fMet-Leu-[^{3}H]Phe ([^{3}H] FMLP) in human neutrophils (PMN) suspended in buffered solutions containing different concentrations of K^+ and Na^+. The values are the means ± S.E. of three experiments for binding and of three and four experiments for the secretion of vitamin B-12-binding protein (B_{12}BP) and β-glucuronidase, respectively.

TABLE I

RATIO BETWEEN THE STIMULATION OF O_2 CONSUMPTION AND THE BINDING OF $2 \cdot 10^{-8}$ M FMet-Leu[^{3}H]Phe ([^{3}H] FMLP)

Data represent the mean ± S.E. of the number of experiments given in parentheses.

Conditions of incubation			O_2 (nmol O_2/min per $2 \cdot 10^7$ neutrophils)	[^{3}H]FMLP bound (fmol [^{3}H]FMLP bound/30 s per $2 \cdot 10^7$ neutrophils)	O_2/[^{3}H] FMLP (nmol O_2: fmol [^{3}H]FMLP bound/$2 \cdot 10^7$ neutrophils)
[K^+] (mM)	[Na^+] (mM)	[Ch^+] (mM)			
7	147	0 (4)	7.3 ± 1.3	67.8 ± 11.9	0.108
154	0	0 (4)	52.1 ± 6.9	193.8 ± 31.6	0.269
7	0	147 (2)	34.6	168	0.206

mM K^+ are substituted with 40 mM Na^+. This finding indicates that the higher secretory response of neutrophils in Na^+-free solution is totally dependent on the greater binding of the stimulant to the specific receptors.

Efficiency of the binding of fMet-Leu-[^{3}H]Phe

The results reported in Fig. 5 indicate that the higher respiratory response in human neutrophils suspended in Na^+-free or in low-Na^+ buffered solutions is not totally accounted for by the increase in binding of fMet-Leu-Phe. A more accurate analysis of the efficiency of binding of the peptide in the stimulation of the respiratory response was carried out.

The binding efficiency has been calculated by the ratio O_2 consumed/fmol fMet-Leu-[^{3}H]Phe bound to human neutrophils suspended in solutions with different ionic composition in the presence of submaximal concentrations of fMet-Leu-Phe (20 nM) (see Table I). It can be seen that in human neutrophils suspended in Na^+-free solutions, i.e., in high-K^+ Krebs-Ringer phosphate and in Krebs-Ringer phosphate containing choline, the ratio is higher than that in human neutrophils suspended in Krebs-Ringer phosphate containing physiological concentrations of K^+ and of Na^+. This means that the partial occupation of the same number of receptors triggers a greater activation of the respiratory apparatus in human neutrophils suspended under Na^+-free conditions. These results indicate that the absence of Na^+ in the suspending solution induces, besides an increase in binding of the peptide, a higher efficiency of the ligand-receptor complexes in triggering of the respiratory response. An higher efficiency was also shown not to occur with regard to the secretory response.

Discussion

The main finding of the present study is that changes of ionic composition of the suspending solutions induce a marked modification of the surface recognition apparatus for the chemotactic peptide fMet-Leu-Phe in human neutrophils. When the cells are suspended in Na^+-free, high-K^+ medium both the number of available receptors and their affinity for the peptide increase.

In an attempt to clarify the mechanism responsible for these results, we have looked for a possible relationship between the modification of the properties of the receptors and the collapse of transmembrane potential that occurs when neutrophils are suspended in Na^+-free, high-K^+ medium [1,10]. The rationale of this possible relationships is based on the notion that an important function of transmembrane potential is to maintain the membrane proteins in a certain configuration, a role called electromorphostasis, and that changes of the transmembrane electrical field could result in new configuration states of membrane proteins, with consequent functional changes [9]. The results presented make clear that modification of the receptors of the neutrophil surface is not dependent on the state of depolarization but on the change of Na^+ in the suspending solutions. It is known that ions may play a regulative role on the recognition apparatus of other cells. It has been demonstrated that Na^+ decreases the affinity

of the platelet receptors for epinephrine [11] and of brain opiate receptors for the agonist [12–14].

The mechanisms underlying the effect of ions on the properties of the receptors of neutrophils are unknown. It is likely that the change of Na^+ concentration in the membrane induces conformational modification of membrane molecules, resulting in a variation both of the number of exposed receptors and of the affinity state of all the receptors. Alternatively, the changes of Na^+ concentration in the membrane could regulate the exposure of new receptors with very high affinity for the peptide. The existence of receptors for chemotactic peptides in different states of affinity has been recently demonstrated in human and rabbit neutrophils [15,16]. However, the last hypothesis seems to be unlikely, since Scatchard analysis of the data of binding of fMet-Leu-Phe demonstrates that a single population of receptors is present in human neutrophils suspended both in Krebs-Ringer phosphate and in high-K^+ Krebs-Ringer phosphate. Whichever interpretation is correct, the data presented in this paper suggest that Na^+ plays a regulative role in the properties of surface receptors and, as a consequence, in the various functions of neutrophils. Since modifications of the ionic composition of extracellular milieu are common events inside and near the inflammatory site and in the vicinity of damaged cells in various pathological processes, it is likely that the ionic regulation of recognition apparatus represents one of the physiological mechanism for the modulation of movement, secretion and respiratory activity of the inflammatory cells.

The experiments reported in this paper have been designed to investigate the mechanism responsible for the increased rate of the responses to fMet-Leu-Phe of neutrophils suspended in Na^+-free buffered solutions. The data demonstrate that the phenomenon can be directly correlated with the greater binding of the peptide to the specific receptors. However, the relationships between the increase in binding and in the secretory and the respiratory response are different. The higher secretory response to fMet-Leu-Phe of human neutrophils suspended in Na^+-free medium with respect that of those suspended in physiological concentration of Na^+, is totally dependent on the higher binding of the stimulant. This is demonstrated by the data of Fig. 6. In contrast, the higher respiratory response of human neutrophils under Na^+-free conditions is only partially due to a greater binding of the stimulant. In fact, as shown in Fig. 5, the binding of fMet-Leu-Phe to human neutrophils suspended in high-K^+ Krebs-Ringer phosphate where 40 mM K^+ has been substituted with 40 mM Na^+ is similar to that to human neutrophils suspended in Krebs-Ringer phosphate, whilst the respiratory response remains still markedly higher. In other words, the same number of fMet-Leu-Phe-receptor complexes at the cell surface induces a higher respiratory response in human neutrophils suspended in low-Na^+, high-K^+ medium.

This higher efficiency of the ligand-receptor complexes is also shown by comparing the ratio O_2 consumed/fmol fMet-Leu-Phe bound at 30 s and at 2 min in neutrophils suspended in high-K^+ Krebs-Ringer phosphate and in Krebs-Ringer phosphate in the presence of 20 nM fMet-Leu-Phe. This fact could be due either to the absence of Na^+ or to the concentration of K^+ being higher than that of the physiological one. The higher efficiency of the binding is demonstrable also when neutrophils are suspended in Krebs-Ringer phosphate containing choline, i.e., at low concentration of K^+, indicating that the phenomenon is related to the absence, or to the low concentration, of Na^+.

A higher efficiency of binding is not detectable at high concentrations of fMet-Leu-Phe because under this condition the activation mechanisms and the respiratory enzyme are expressed as the maximal activity in human neutrophils suspended in the presence and absence of Na^+.

Thus, the data so far presented show that the increased rate of respiratory response is due both to the higher binding of the stimulant and to the higher efficiency of the ligand-receptor complexes. The mechanism underlying the last phenomenon is at present unknown. It is likely that one or more of the multiple events specifically linking the recognition steps at the level of the surface receptors with the target system, i.e., with the NADPH oxidase, are involved. The possibility cannot be ruled out that the actual event responsible for a lowering in the threshold of activation is a molecular modification of the NADPH oxidase itself (or

of the oxidase system). Whichever is the mechanism, it represents another physiological way by which the concentration of Na^+ in the tissues may regulate the respiratory function of phagocytes that, through the formation of very active intermediates of O_2 reduction, is relevant in the defense against invading organisms.

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