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PHLORETIN IS A POTENT INHIBITOR OF RABBIT NEUTROPHIL ACTIVATION BY CHEMOTACTIC FACTORS

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We have studied the effect of phloretin, a compound known to interfere with carrier mediated transport processes, on the functional responsiveness of rabbit neutrophils. Phloretin was found to inhibit neutrophil degranulation, aggregation and calcium uptake stimulated by the chemotactic peptide fMet-Leu-Phe. Part of these effects of phloretin may be accounted for by its interference with the binding of the synthetic peptide to its plasma membrane receptors. However, phloretin also inhibits the arachidonic acid and leukotriene B_4 induced calcium uptake. These results imply that phloretin affects a component of membrane structure which is central to the activation sequences available to the neutrophils. The results of the present experiments demonstrate that phloretin can interfere with stimulus-response coupling in soluble mediator ('hormone') responsive cells.

Introduction

The transport across the plasma membranes of hexose and of various other substances which utilize carrier mediated mechanisms is known to be inhibited by phloretin [1–4]. In addition, phloretin inhibits the movement across cell membranes of certain small hydrophilic solutes assumed to permeate predominantly via protein pathways while it enhances the transfer of some other substances which permeate via the lipid pathways [2,4]. This dual effect of phloretin is also reflected in its binding characteristics to red cell membrane [5,6]. In human red cell ghosts, phloretin binds with light affinity ($K_d = 1.5$ μM) to about $2.5 \cdot 10^6$ sites per cell; it also binds with low affinity ($K_d = 54$ μM) to a second ($5.5 \cdot 10^7$ sites per cell) set of sites [5]. The high and low affinity sites have been identified to represent phloretin binding to membrane protein and lipid, respectively. At present, the exact mechanism(s) of action of phloretin is not known. It is hypothesized that phloretin produces certain perturbations in membrane structure which affect both the protein and lipid components of the membrane composition. If this is the case then phloretin may change the binding characteristics of certain first messengers to their receptors and affect the excitation response coupling sequence in these cells.

We wish now to describe recent studies in which the effect of phloretin on stimulated polymorphonuclear leukocytes (neutrophils) functions and calcium metabolism have been studied. The results obtained demonstrate that phloretin, in addition to its interference with membrane transport, dramatically affects stimulus-response coupling. This observation could be extended to other cell types and other first messengers.

Abbreviations: Neutrophils, polymorphonuclear leukocytes; fMet-Leu-Phe, formylmethionyl-leucyl-phenylalanine; leukotriene B_4, 5(*S*), 12(*R*)-dihydroxy-6,8,10,14 (*cis-trans-trans-cis*)-eicosatetraenoic acid.

Materials and Methods

Rabbit peritoneal neutrophils obtained and handled as previously described [7] were used throughout these experiments. They were suspended at $1 \cdot 10^7$ cells/ml in magnesium and protein free modified Hanks' balanced salt solution buffered with 10 mM Hepes (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid), pH 7.4, containing 0.5 mM $CaCl_2$. A 20 min preincubation at 37°C preceded all further experimental manipulation.

Calcium transport was measured using the rapid sampling silicone oil method previously described in detail [8]. Uptake experiments measured the time course of the association of ^{45}Ca with the cells immediately following the addition of the radioisotope and the appropriate stimuli. This method was slightly modified for the measurement of the time course of the association of radiolabelled fMet-Leu-Phe to the cells [9]. The term association is used here to represent binding to the cell membrane and uptake into the cell cytoplasm. In order to obtain fast time course points, a known volume (0.8 ml) of the neutrophil suspension was layered on top of the silicone oil layer (density 1.05 g/cm^3) in 1.5 ml capacity microcentrifuge tubes. 0.2 ml of radioactive buffered solution containing the labelled fMet-Leu-Phe (New England Nuclear) was injected into the suspension on top of the oil and the sample was centrifuged after a preset time had elapsed [9]. This method enabled us to follow the early points in the time course of the binding.

Chemotactic factor (or fatty acids) and cytochalasin B (5 μg/ml) induced lysosomal enzyme release from rabbit peritoneal neutrophils was performed as previously described [13]. The release of lysozyme and of lactate dehydrogenase were routinely monitored. In all the experiments to be reported, the release of lactate dehydrogenase, a marker of cell death, was less than 5% of the total cell content and did not exceed the release observed in control, untreated cells.

Neutrophil aggregation was measured by following the change in optical transmission at 600 nm of a cell suspension containing $(2–5) \cdot 10^6$ cells/ml in a Zeiss PMQ111 spectrophotometer. Magnesium (as $MgCl_2$) was added 1–3 minutes before the various stimuli or inhibitor. This method is essentially an adaptation of that used to monitor platelet aggregation and has previously been used with human [10] and rabbit neutrophils [11].

The results presented in the next section are representative of at least three experiments, each carried in duplicates.

Phloretin and arachidonic acid were obtained from Sigma Chem. Co. (St. Louis, MO), formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe) from Peninsula Labs (San Carlos, CA) and cytochalasin B from Aldrich Chem. Co. (Milwaukee, WI). Leukotriene B_4 was generously provided by Dr. P. Borgeat, Département d'endocrinologie moleculaire, CHUL, St. Foy, Quebec, Canada. All other chemicals were reagent grade or better. Concentrated stock solutions (1000 times) of arachidonic acid and leukotriene B_4 were prepared in DMSO. When used, a known volume of the stock solution to give the desired final concentration was added to the cell suspension.

Results

Phloretin is a compound known to perturb cell membranes by interacting with the two major components (lipid and protein) of membrane structure. In an attempt to determine whether phloretin interferes with the sequence of excitation-response coupling, we have now examined the effects of phloretin on neutrophil degranulation, aggregation and stimulated ^{45}Ca movements.

The effects of phloretin (0.04–0.5 mM) on the fMet-Leu-Phe plus cytochalasin B induced lysosomal enzyme release are illustrated in Fig. 1. An inhibition (as evidenced by a shift to the right of the dose-response curve) of neutrophil degranulation is clear even at the lowest concentration of phloretin used in this experiment and is dose-dependent.

A complete dose-response curve of the inhibition by phloretin by the fMet-Leu-Phe plus cytochalasin B induced degranulation was also studied. The concentration of phloretin which depresses the chemotactic factor induced lysosomal enzyme release by 50% (IC_{50}) is about 10^{-4} M, a concentration similar to that which affects solute transfer across biological and synthetic membranes.

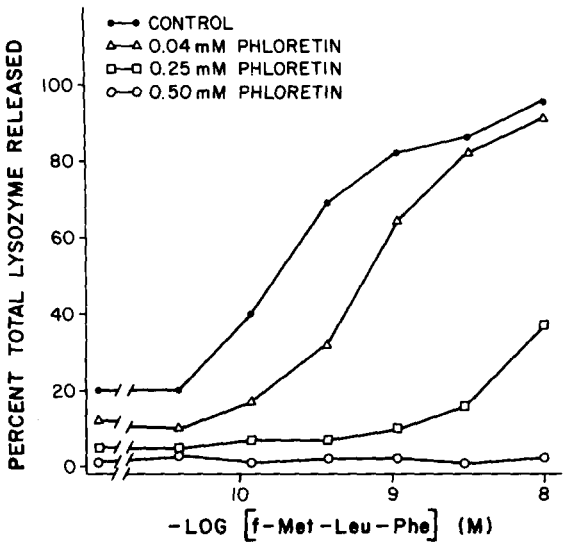

Fig. 1. Effect of phloretin on the cytochalasin B plus fMet-Leu-Phe induced degranulation of rabbit neutrophils. Lysosomal enzyme release in the presence or absence of the indicated concentrations of phloretin was measured as described in Methods. Phloretin was added 1 min before the cells were transferred to the prewarmed test tubes containing the desired concentrations of fMet-Leu-Phe and cytochalasin B. Means of duplicates differing by 10% or less. Representative of at least three experiments.

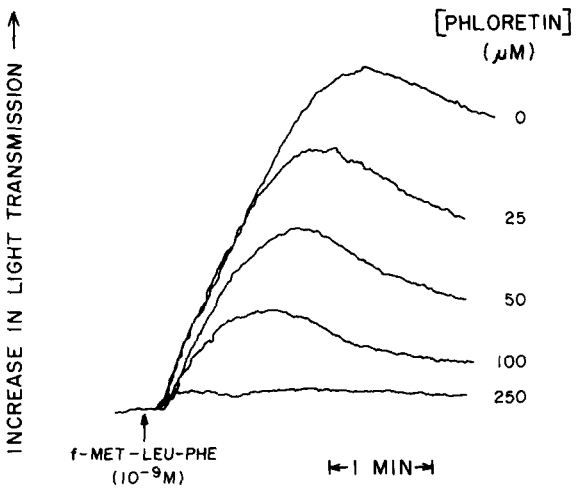

Fig. 2. Effect of phloretin on neutrophil aggregation induced by fMet-Leu-Phe. The experimental details are as in Materials and Methods. The cells were exposed to the indicated concentrations of phloretin for 1 min prior to the addition of the chemotactic peptide. These results are representative of three experiments.

We have examined next the effect of phloretin on another neutrophil function, namely aggregation. These experiments were conducted to extend the results just described concerning degranulation to other functional responses of the cells and in particular to one that can be measured in the absence of cytochalasin B. As shown in Fig. 2, phloretin in a dose-dependent fashion, inhibits the fMet-Leu-Phe induced neutrophil aggregation. The concentrations of phloretin required to inhibit stimulated aggregation are very similar to those required to affect degranulation.

We have next utilized the real time monitoring capability of the aggregation assay to examine the time course of the effect of phloretin on stimulated neutrophil functions. In these experiments, neutrophils were preincubated for various times (0–60 s) with two concentrations of phloretin (50 and 500 μM) and the subsequent responses to fMet-Leu-Phe determined. The results of these studies showed that no significant differences in the effectiveness of phloretin as an inhibitor of fMet-Leu-Phe stimulated aggregation could be detected between the different conditions tested. The effects of phloretin were thus experimentally instantaneous.

The effects of phloretin were reversible. Cells preincubated with $5 \cdot 10^{-4}$ M phloretin, washed and resuspended in fresh media respond to fMet-Leu-Phe just as well as control untreated cells that had gone through the same experimental manipulations. In addition, both cell populations were equally sensitive to further inhibition by phloretin.

The biological activities of fMet-Leu-Phe are thought to be mediated by the ability of the chemotactic peptide to increase the neutrophils' cytoplasmic calcium levels [12]. The effect of phloretin (0.1 and 0.5 mM) on the fMet-Leu-Phe induced increase in calcium uptake is illustrated in Fig. 3. As shown in this figure, phloretin greatly inhibits the calcium uptake response to the chemotactic factor. Concentrations of phloretin equal to or greater than 10^{-5} M were found to be required for this inhibitory effect of phloretin. On the other hand, the basal, unstimulated rate of ^{45}Ca uptake is slightly ($< 15\%$) increased by 10^{-3} M phloretin but not by any lower concentrations (results not shown).

In order to define, at least in part, the site of

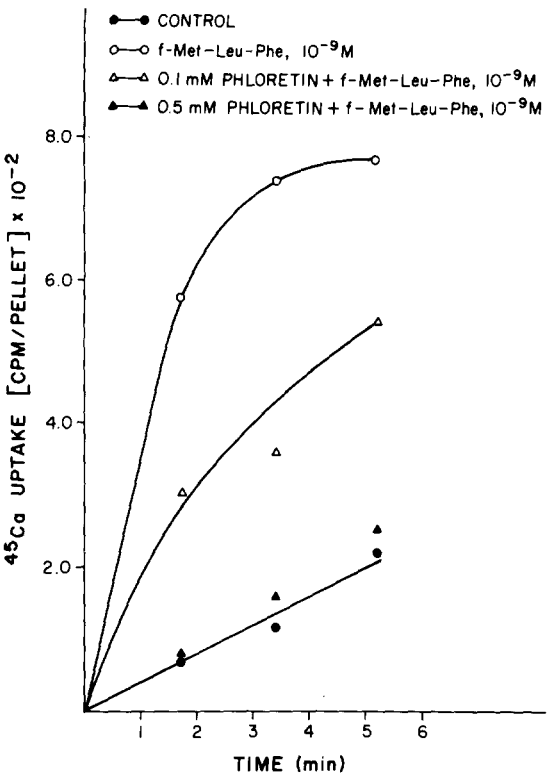

Fig. 3. Effect of phloretin on the fMet-Leu-Phe induced calcium uptake response. Phloretin was added 1 min prior to ^{45}Ca and fMet-Leu-Phe. At the concentrations used in these experiments phloretin was without effect on the basal, unstimulated rate of ^{45}Ca uptake. Calcium transport was measured as described in Methods. Means of duplicates differing by 20% or less. Representative of at least three experiments.

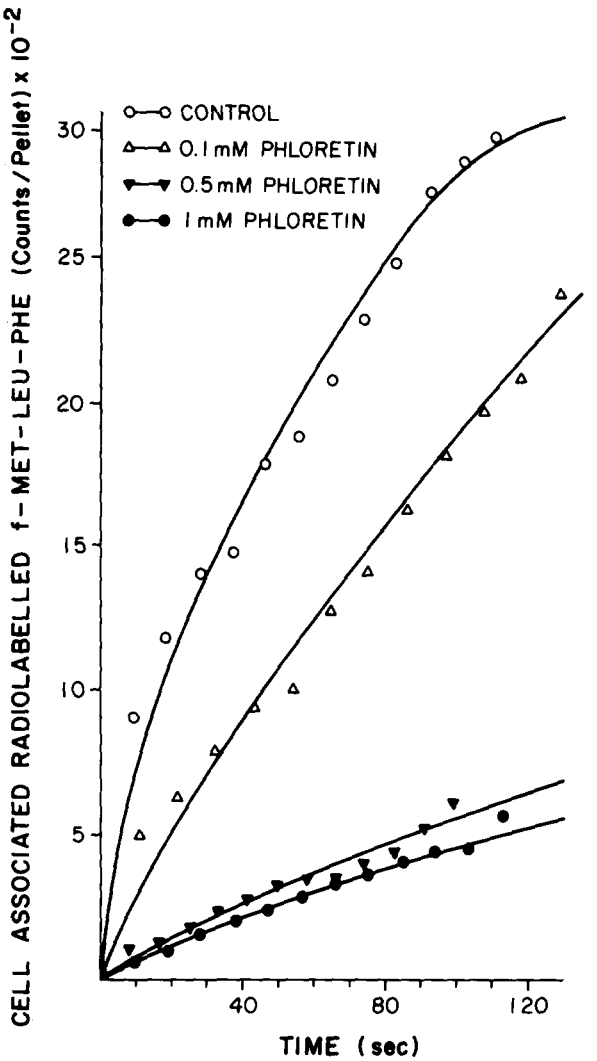

Fig. 4. Effect of phloretin on the time course of the association of fMet-Leu-Phe with rabbit neutrophils. In these experiments conducted at 37°C, phloretin at the indicated concentrations was added 1 min before ^{3}H-fMet-Leu-Phe (10^{-9} M). The experimental details are described in Methods ($P < 0.001$ between control and either of the three phloretin samples, Student's t-test).

action of phloretin, we have investigated its effects on the association of fMet-Leu-Phe with its plasma membrane receptors, the first step in the excitation-activation coupling sequence. As shown in Fig. 4 the association of ^{3}H-fMet-Leu-Phe with the neutrophils (chosen as an initial index of binding) is inhibited by phloretin (0.1–1.0 mM). The effects of phloretin on the equilibrium binding characteristics of ^{3}H-fMet-Leu-Phe have not been studied yet as the required experimental conditions for binding (4°C at long incubation times) differ from those used during the functional assays and therefore do not directly reflect on the physiological status of the receptors during the above experimental manipulations.

We were interested next to determine whether the effects of phloretin were limited to interaction with the fMet-Leu-Phe receptors. To this end, we have first utilized the biologically active fatty acids, arachidonic acid and leukotriene B_4. Although the mechanisms by which these two compounds activate neutrophils are still essentially unknown and may involve separate sets of receptor or specific binding sites, it is thought that they enter the excitation-response squence at a step distinct

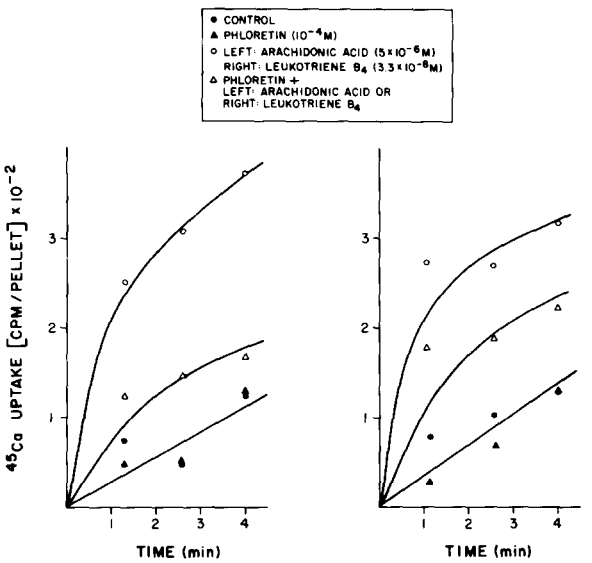

Fig. 5. Effect of phloretin on the arachidonic acid and leukotriene B_4 induced calcium uptake response in rabbit neutrophils. Phloretin was added 1 min prior to ^{45}Ca and the fatty acids. Experimental conditions are as described in Methods.

from the fMet-Leu-Phe receptor. Their effects on neutrophil activation may be mediated by their ability to raise the intracellular level of exchangeable calcium [13,14]. Phloretin, as shown in Fig. 5, inhibits the ability of the two fatty acids to increase the initial rate of uptake of ^{45}Ca in rabbit neutrophils.

Phloretin also inhibits, in a dose-dependent manner, neutrophils degranulation induced by arachidonic acid or leukotriene B_4 and cytochalasin B (Fig. 6). The responses to both fatty acids appear to be equally sensitive to phloretin at concentrations similar to those required to inhibit fMet-Leu-Phe plus cytochalasin B induced degranulation (Fig. 1).

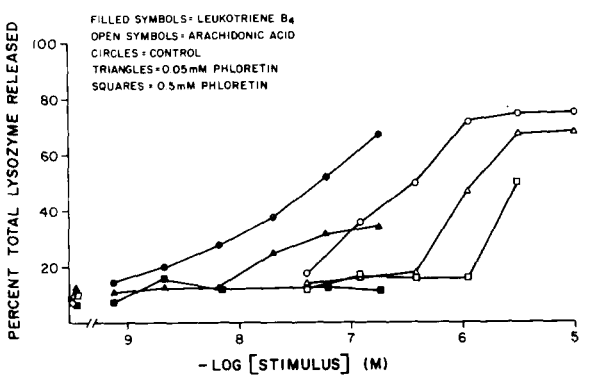

Fig. 6. Effect of phloretin on the arachidonic acid and the leukotriene B_4 induced degranulation of rabbit neutrophils. Experimental conditions are as described in Materials and Methods. Phloretin was added 3 min prior to transferring the cells to tubes containing cytochalasin B and the indicated stimuli concentrations.

In contrast to the findings with fMet-Leu-Phe (Fig. 4), phloretin at concentrations as high as 1 mM does not affect the rate of association of arachidonic acid with the neutrophils ($P < 0.9$ for control versus 0.1 mM phloretin; $P < 0.7$ for control versus 1 mM phloretin, Student's t-test). The lack of availability of radiolabelled leukotriene B_4 precluded performing a similar experiment with the dihydroxy metabolite of arachidonic acid.

Discussion

In the present study we have examined the effects of phloretin, a compound known to interact with both the lipid and protein components of membranes, on the activation of neutrophils by three different stimuli: the synthetic chemotactic peptide fMet-Leu-Phe and the two neutrophil active fatty acids, arachidonic acid and its dihydroxy metabolite leukotriene B_4 [15] which are thought to mediate in part the activities of the former. The results presented in the preceding section demonstrate that phoretin interferes with the excitation response coupling sequence in rabbit neutrophils.

Phloretin inhibits fMet-Leu-Phe stimulated degranulation, aggregation and calcium influx with an IC_{50} of about 0.1 mM. These effects of phloretin can be explained in part by its interference with the binding of the peptide to its plasma membrane receptors, the initial event in the activation of the neutrophils. Niedel et al. [16] and Goetzl et al. [17] have recently described the initial characterization of the fMet-Leu-Phe receptor from human neutrophils. The binding activity was found to reside with proteins of apparent molecular weight 40000 to 94000. Phloretin has been found to bind to the proteins of the red cell membranes with an affinity constant of $2 \cdot 10^{-6}$ M and to the lipids of these cells with an affinity constant of $60 \cdot 10^{-6}$ M. The IC_{50} of phloretin's action on the neutrophils (10^{-4} M) is thus much closer to its affinity constant for the red cell lipids than to the

proteins. It is conceivable therefore that phloretin affects the binding of fMet-Leu-Phe not by interacting directly with the receptor protein but by modifying its lipid environment and thereby the physical characteristics of the binding site(s).

This interpretation is consistent with the findings that phloretin inhibits the arachidonic acid and leukotriene B_4 induced degranulation and calcium uptake responses in addition to interfering with the fMet-Leu-Phe induced responses. The definition of the mechanism of action of phloretin will therefore require an investigation of its physical effects on the structure of the plasma membrane as well as its possible modulation of the activities of several enzyme systems thought to be involved in stimulus activation sequences (e.g. kinases and methyltransferases).

The results described in this communication demonstrate that phloretin, in addition to its ability to interfere with carrier-mediated transport mechanism, can also interrupt the sequence of events leading from receptor occupancy to neutrophil activation. The preliminary data available so far suggest that it is phloretin's interactions with membrane lipids that are responsible for its observed biological effects. Phloretin therefore may provide a new tool for the study of the lipid environment of functional domains of the plasma membranes of hormonally responsive cells.

Acknowledgement

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