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USE OF CYTOCHALASIN B TO DISTINGUISH BETWEEN EARLY AND LATE EVENTS IN NEUTROPHIL ACTIVATION

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Summary

Cytochalasin B greatly enhances secretion of β-glucuronidase and generation of superoxide on stimulation of rabbit peritoneal neutrophils with the soluble chemotactic factor *N*-formylmethionylleucylphenylalanine (f-Met-Leu-Phe). There are smaller changes due to cytochalasin B on binding of f-Met-Leu-[^{3}H]-Phe, stimulation of phosphatidylinositol turnover and the stimulated increase in the permeability of the cell membrane to Ca^{2+}. These latter changes are probably artefactual and arise as secondary consequences of cell stimulation. Our observations support the notion that changes in Ca^{2+} permeability of membranes and stimulation of phosphatidylinositol turnover reflect early stages in the sequence of events initiated by f-Met-Leu-Phe binding to its receptor and which lead to cell activation phenomena such as secretion and superoxide production.

Introduction

Activation of neutrophils following attachment of soluble agonists to surface receptors (e.g. complement peptides, bacterial factors) and by the ionophore A23187 can take a number of different forms [1]. When the cells are stimulated in the presence of cytochalasin B, the motile functions (chemotaxis and phagocytosis) are repressed [1] while lysosomal enzyme secretion [2,3] and the generation of superoxide (O_2^-) [4] are greatly enhanced. All these neutrophil functions are believed to result from the same ligand-receptor interaction [5,6]. In this paper we examine the possibility that the differential effects of

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

cytochalasin B on neutrophil functions could arise from an effect of the drug in the early stages of cellular activation following initial ligand-receptor interaction. The ligand that we have used is the synthetic tripeptide *N*-formylmethionylleucylphenylalanine (f-Met-Leu-Phe). This is believed to mimic natural chemotactic factors which are released by growing bacteria [1].

The activation of neutrophils (in common with stimulus-section coupling in many other cell types [7]) is thought to be mediated by an increase in cytoplasmic ionised free Ca^{2+} levels, occurring either because of an increased permeability of the plasma membrane to Ca^{2+}, or through release of Ca^{2+} from intracellular sources [8]. These changes can be monitored by observing $^{45}Ca^{2+}$ fluxes across the plasma membrane [9]. As in many other tissues in which Ca^{2+} has been implicated as the 'second messenger' there is also a ligand-directed increase in the rate of phosphatidylinositol turnover [10,11], often measured as an increased incorporation of [^{32}P]phosphate into phosphatidylinositol. The enhanced rate of phosphatidylinositol turnover is an early consequence of ligand-receptor interactions, and it has been suggested that it precedes and may regulate the change in Ca^{2+} permeability of cell membranes [12,13].

Here we show that cytochalasin B has only a small effect on the binding of f-Met-Leu-Phe to rabbit neutrophils and on the consequent receptor-directed phosphatidylinositol response and $^{45}Ca^{2+}$ movements. This is in contrast to the considerable effect of the drug on lysosomal enzyme secretion and O_2^- generation.

As far as we are aware, this is the first report of experiments in which measurements of receptor stimulated phosphatidylinositol responses in mammalian cells have been combined with measurements of agonist binding, Ca^{2+} fluxes and consequent cellular processes.

Materials and Methods

Rabbit peritoneal neutrophils were obtained 4—6 h after intraperitoneal injection of 250 ml sterile saline containing 1 mg · ml^{-1} glycogen [14]; these cells contained <5% of contaminating monocytes. The cells were centrifuged (5 min at 500 × *g*) and washed twice with a buffered salt solution (composition: 138 mM NaCl/5.4 mM KCl/0.9 mM $MgCl_2$/5.6 mM glucose/1.0 mg · ml^{-1} bovine serum albumin/0.8 mM phosphate/20 mM Hepes buffered at pH 7.4) and suspended at 10^7 cells · ml^{-1}. $CaCl_2$ was omitted from the medium at this stage in order to minimise aggregation of cells which sometimes occurred. The cells were incubated at 37°C for 20 min before addition of cytochalasin B (to 10 μg · ml^{-1}); reactions were initiated by addition of cells to an equal volume of buffer containing f-Met-Leu-Phe (to give final concentrations as indicated) and $CaCl_2$ (final concentration 1.8 mM). After 5 min at 37°C the reactions were terminated by centrifuging the cells.

For measurements of calcium uptake, $^{45}Ca^{2+}$ was used (approx. 4 · 10^6 cpm · μmol^{-1}). At the conclusion of the reaction the cells (final volume 0.2 ml) were centrifuged at approx. 12 000 × *g* for 1 min through 0.18 ml Ficoll 400 (15% w/v containing 1 mg · ml^{-1} bovine serum albumin and adjusted to 300 mosM with NaCl) using a Beckman microcentrifuge (model 152). Ficoll was used in place of the silicone oil previously described [9,15] since it was found to cause a large decrease in the amount of extracellular medium associated with the cell

pellets (data not shown). The pellets were sampled for scintillation counting by freezing the tubes at −60°C and cutting off the tips containing the pellets using a pair of garden secateurs.

Calcium efflux was measured by prelabelling the cells with $^{45}CaCl_2$ at approx. 10 $\mu Ci \cdot ml^{-1}$ as described by Naccache et al. [9]. Washed cells were incubated with f-Met-Leu-Phe and the cell pellets sampled for scintillation counting by centrifugation through Ficoll as described above.

Binding of f-Met-Leu-Phe to cells was measured by the above technique of centrifugation through Ficoll, using f-Met-Leu-[^{3}H]Phe at approx. 10^4 cpm · $pmol^{-1}$. Non-specific binding was measured by saturating the binding sites with unlabelled f-Met-Leu-Phe ($5 \cdot 10^{-6}$ M) in the presence of f-Met-Leu-[^{3}H]Phe and this value was subtracted from the value for total binding.

The phosphatidylinositol response was measured after preincubating the cells for 20 min at 37°C in a medium similar to that described above but with phosphate replaced by carrier-free [^{32}P]phosphate at approx. 100 $\mu Ci \cdot ml^{-1}$. Reactions were carried out in glass tubes in a total volume of 0.4 ml. Incorporation of radioactivity into phospholipids was allowed to continue for a further 20 min and the reaction was stopped by cooling the tubes in an ice bath and centrifuging the cells at $1500 \times g$ for 5 min at 4°C. After sampling the supernatant for secreted β-glucuronidase, the lipids were extracted with chloroform/methanol and the phospholipids were separated by chromatography on formaldehyde-treated papers as previously described [16]. The phosphatidylinositol spots were cut from the paper for counting of radioactivity.

β-Glucuronidase (a marker lysosomal enzyme) was measured in 50-μl samples of the supernatants using the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide [17]. Enzyme activity released is expressed as a percentage of the total activity in cells released by lysis with Triton X-100 (0.2%).

Superoxide production was measured [4] by adding 0.2 ml cells to 0.2 ml buffer containing f-Met-Leu-Phe (final concentration as indicated) and cytochrome *c* (reduced form, 2.6 $mg \cdot ml^{-1}$) in the presence or absence of superoxide dismutase (60 units · ml^{-1}). Reactions were quenched by the addition of *N*-ethylmaleimide (1.25 $mg \cdot ml^{-1}$) and O_2^- was determined from the change in absorbance of cytochrome *c* at 550 nm which was abolished by superoxide dismutase.

Results presented are from typical experiments which were carried out on at least three occasions. Experimental points are means of duplicate determinations. The greatest discrepancies between duplicates in the experiments reported were as follows: Fig. 1a, 10 fmol/10^6 cells; Fig. 1b, 50 cpm; Fig. 1c, 0.4 of control; Fig. 1d, 0.05 of control; Fig. 1e, 2% of total; Fig. 1f, 0.5 nmol/10^6 cells.

f-Met-Leu-Phe was a gift from Dr. Derek Hudson, Royal Postgraduate Medical School, London. Cytochalasin B was obtained from Aldrich Chemical Co., Dorset (U.K.). $^{45}CaCl_2$ and [^{32}P]phosphate were obtained from the Radiochemical Centre, Amersham (U.K.). *N*-Formylmethionylleucyl[*ring*-2,6-$^3H_2$]-phenylalanine was obtained from New England Nuclear, Dreieich (F.R.G.).

Results

Fig. 1(a—f) shows the effect of f-Met-Leu-Phe concentration on the six cell processes that we have measured: ligand binding, phosphatidylinositol turnover, $^{45}Ca^{2+}$ influx, $^{45}Ca^{2+}$ efflux, β-glucuronidase secretion and O_2^- generation. With the exception of ligand binding, all of these processes occur at concentration of f-Met-Leu-Phe which are less than 10^{-8} M (in the presence of cyto-

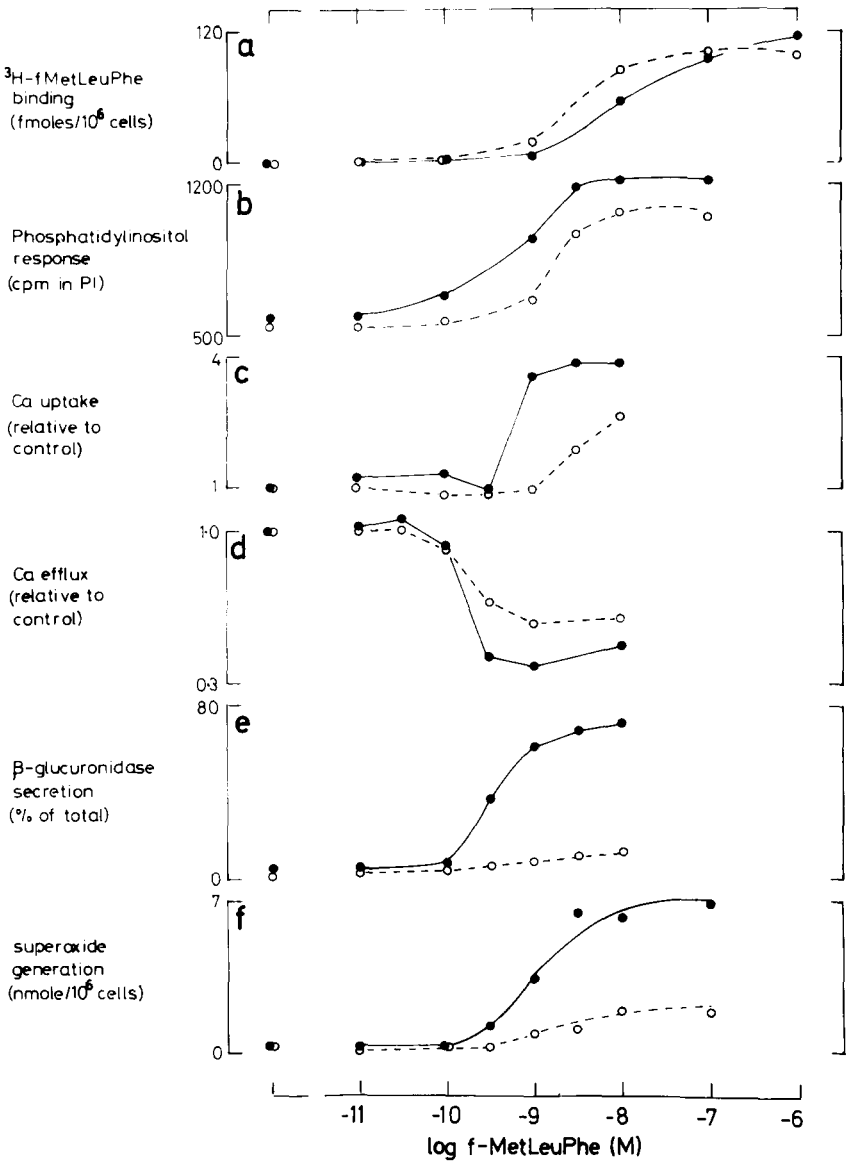

Fig. 1. Concentration dependence on f-Met-Leu-Phe in the presence (●———●) and absence (○- - - - -○) of cytochalasin B (5 μg · ml^{-1}) of (a) binding to neutrophils of [^{3}H]f-Met-Leu-Phe; (b) incorporation of [^{32}P]phosphate into phosphatidylinositol; (c) uptake of $^{45}Ca^{2+}$; (d) efflux of $^{45}Ca^{2+}$ from prelabelled cells; (e) secretion of β-glucuronidase into the extracellular medium; (f) generation of O_2^-.

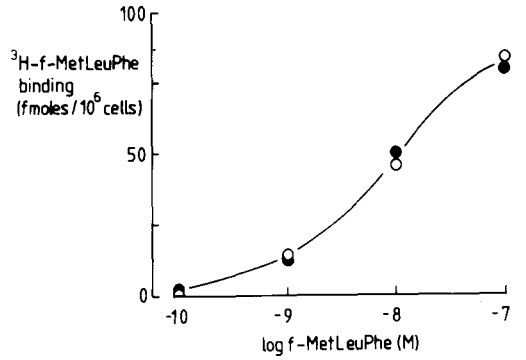

Fig. 2. Binding of f-Met-Leu-[^{3}H]Phe in the presence (•) and absence (○) of cytochalasin B (5 $\mu g \cdot ml^{-1}$) to neutrophils which had been metabolically inhibited by preincubation for 20 min in a glucose-free buffer containing antimycin A (5 μM) and 2-deoxy-D-glucose (5.6 mM). Secretion of β-glucuronidase was less than 2% at 10^{-7} M f-Met-Leu-Phe in the presence of cytochalasin B. This figure should be compared with the data presented in Fig. 1a for uninhibited cells.

chalasin B). Saturation of the receptors with f-Met-Leu-Phe occurs as its concentration is raised to 10^{-7} M at which point approx. 10^5 molecules are bound per cell, in agreement with the findings of others [18,19]. It would thus seem that at least half of the receptors for f-Met-Leu-Phe on the neutrophil cell surface are redundant to the extent that they play no part in the regulation of phospholipid metabolism, mobilisation of Ca^{2+} and later consequential responses.

We found that cytochalasin B caused small (but non-negligible) alterations in the binding of f-Met-Leu-Phe, the metabolism of phosphatidylinositol and $^{45}Ca^{2+}$ efflux (Fig. 1, a, b and d). The effects of cytochalasin B on $^{45}Ca^{2+}$ influx, β-glucuronidase secretion and O_2^- generation were very much larger (Fig. 1, c, e and f). We have evidence that the apparent cytochalasin B-dependent change in ligand binding is an artefact arising from cell stimulation due to the ligand itself. When measurements were made on cells which had been metabolically inhibited to prevent secretion (20 min preincubation with 5 μM antimycin A in a buffered salt solution with glucose replaced by 2-deoxy-D-glucose) the binding of f-Met-Leu-Phe became insensitive to the presence of cytochalasin B. In these circumstances, binding measurements are not complicated by cell stimulation events, and both binding curves resemble that shown in Fig. 1a for the presence of cytochalasin B (Fig. 2).

Addition of f-Met-Leu-Phe stimulates a large increase in the rate of phosphatidylinositol turnover (Fig. 1b): we obtained qualitatively similar results with the use of both [^{32}P]phosphate and [^{3}H]inositol. It is widely accepted that the primary ligand induced stimulus to increased phosphatidylinositol turnover is breakdown of preexisting phosphatidylinositol [12] and we have preliminary evidence [30] that the breakdown event in neutrophils is largely independent of cytochalasin B. The increase in labelling that we measure here is the consequence of the resultant resynthesis of phosphatidylinositol [12]. In agreement with the finding of others [20], we find that addition of cytochalasin B produces a slight enhancement in the basal rate of phosphatidylinositol labelling, and this might be related to the very small increase in enzyme release which

also occurs when cytochalasin B is added to neutrophils (Fig. 1, b and e). However, these effects are small compared to those induced by f-Met-Leu-Phe.

Figs. 1c and 1d show the effect of cytochalasin B on f-Met-Leu-Phe induced influx and efflux of $^{45}Ca^{2+}$. While there is a considerable enhancement of influx due to cytochalasin B, as well as a shift in the concentration-effect curve, its effect on efflux is much less marked.

Discussion

We have compared the relationship between ligand binding to receptors, and the consequent metabolism of phosphatidylinositol, $^{45}Ca^{2+}$ movements and expressions of cellular activity in rabbit neutrophils. The five induced functions due to receptor stimulation that we have measured show slight differences with respect to their dependence on the concentration of f-Met-Leu-Phe. However, there is good evidence that there exists only a single class of receptors for this agonist [5,6]. The maximal cell responses are all observed at concentrations of f-Met-Leu-Phe when binding to receptors is at a small fraction of saturation. This situation is analagous to the 'spare receptors' which have been described for adrenergic and muscarinic systems [13,21] where maximal biological activity occurs when less than 1% of the receptors are occupied; it has been suggested that the phenomenon has a biological importance in allowing a discriminatory action of a ligand on different tissues possessing the same receptor [21]. In neutrophils, phosphatidylinositol turnover also becomes maximal when receptor occupation is sub-maximal: this contrasts with the situation of cholinergic muscarinic receptors in which phosphatidylinositol turnover and receptor occupation appear to be tightly coupled [13].

Measurements of ligand binding were complicated since the amount of f-Met-Leu-Phe associated with the cells in the absence of cytochalasin B was altered as a consequence of cell stimulation. This might be due to internalisation of cell membrane together with receptors and bound ligand as recently demonstrated for similar receptors on human neutrophils [19]. Cytochalasin B is known to inhibit stimulated endocytotic processes [22,23]. For this reason it is unclear whether the effect of cytochalasin B on stimulated phosphatidylinositol turnover (Fig. 1b) is due to altered receptor function, or to an alteration in the number of exposed receptors accessible to the ligand. A small increase in ligand binding might be expected from microscopic observations which show that neutrophils become more rounded and less invaginated in the presence of cytochalasin B [24].

Ideally, it should be possible to monitor an increase in membrane permeability to Ca^{2+} by making measurements of either $^{45}Ca^{2+}$ influx or $^{45}Ca^{2+}$ efflux. These should show a similar response because it is the rate of entry of Ca^{2+} into the cell cytoplasm which dictates the rate of Ca^{2+} exit due to the activation of plasma membrane Ca^{2+} pumps. In our experiments, the two processes show different responses to cytochalasin B and therefore one of them must be monitoring something other than a simple membrane permeability change. We suspect that it is the influx measurement which is misleading. It has been previously argued that the predominant reason for $^{45}Ca^{2+}$ uptake is the equilibration of radioactivity between medium and Ca^{2+} bound to secretory granules which

become exposed to the extracellular medium as a consequence of exocytotic secretion [7,25]. Thus, a significant part of the observed $^{45}Ca^{2+}$ uptake in the presence of cytochalasin B reflects not an early stage in cell activation, but a final consequence of the secretory process. The situation in the absence of cytochalasin B may also be complicated if stimulated endocytosis occurs, as discussed above in relation to ligand binding. By and large, measurements of $^{45}Ca^{2+}$ uptake in these and other cell types are likely to be misleading.

A more meaningful approach is to measure stimulated $^{45}Ca^{2+}$ efflux although there are still some problems of interpretation. The effect of cytochalasin B on f-Met-Leu-Phe induced $^{45}Ca^{2+}$ efflux is one of magnitude only. Cytochalasin B has no effect on the concentration range of f-Met-Leu-Phe over which the change occurs. We have shown elsewhere [26] that the flavonoid quercetin, which depresses secretion (in the presence of cytochalasin B) without altering receptor occupation, does not alter $^{45}Ca^{2+}$ efflux so that this measurement must reflect an early consequence of receptor binding rather than a late consequence of secretion. It is likely that the extra radioactivity which is released in the presence of cytochalasin B reflects $^{45}Ca^{2+}$ associated with the secretory granules.

In the absence of cytochalasin B, there is little secretion and the $^{45}Ca^{2+}$ which is released reflects that which is mobilised by the activated receptors. It is likely that this $^{45}Ca^{2+}$ is derived primarily from a cytoplasmic pool together with other rapidly exchangeable sources within the cell. Release of $^{45}Ca^{2+}$ from cells maximises at concentrations of f-Met-Leu-Phe 3—10 times lower than does phosphatidylinositol turnover. It is possible that the $^{45}Ca^{2+}$ available for efflux becomes exhausted at these lower concentrations so that while commencement of efflux (at approx. 10^{-10} M f-Met-Leu-Phe) reflects changes in membrane permeability, saturation of the process (approx. 10^{-9} M) is dictated by other factors.

The requirement for slightly higher concentrations of f-Met-Leu-Phe to stimlate O_2^- generation compared with that needed to elicit β-glucuronidase secretion was consistently observed. The ionophore A23187 also stimulates both cell functions, but unlike the agonist-induced processes, this is strictly Ca^{2+}-dependent. As O_2^- generation also requires a slightly higher concentration of the ionophore (unpublished results) we conclude that the triggering concentration of intracellular Ca^{2+} is slightly higher for O_2^- generation.

Cytochalasin B is known to modulate both endocytotic and exocytotic functions of different cell types [27], probably through actions on intracellular contractile microfilaments [28]. Another example of a system in which cytochalasin B actually potentiates secretion is the pancreatic β cell [29]. Here it enhances early and late phase insulin secretion stimulated by both glucose and leucine; it has no effect by itself. While the effects of cytochalasin B on β-glucuronidase secretion and O_2^- generation by neutrophils are large it is clear from our results that it has little or no effect on receptor function. The measured effects on f-Met-Leu-Phe binding and phosphatidylinositol turnover are small and probably explicable in terms of endocytosis and altered exposure of receptor sites. The effect of cytochalasin B on the concentration range of f-Met-Leu-Phe which stimulates $^{45}Ca^{2+}$ efflux was small. This evidence is consistent with the idea that phosphatidylinositol turnover and increase in membrane

permeability to Ca^{2+} are early receptor directed events, and that the cytochalasin B sensitive step lies between these and the final measured responses.

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