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Linear gramicidin activates neutrophil functions and the activation is blocked by chemotactic peptide receptor antagonist

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The activation of functional responses in rabbit peritoneal neutrophils by gramicidin and the chemotactic peptide, *N*-formyl-methionyl-leucyl-phenylalanine methyl ester, was studied. Gramicidin activated superoxide generation, lysosomal enzyme release and a decrease in fluorescence of chlortetracycline-loaded cells, as for the chemotactic peptide. The maximum intensities of the responses by gramicidin were lower than that by chemotactic peptide. Responses by both these peptides could be inhibited by *t*-butyloxycarbonyl-methionyl-leucyl-phenylalanine, a chemotactic peptide receptor antagonist. Gramicidin gave responses at low doses comparable to that of the chemotactic peptide.

Neutrophil; Chemotactic peptide; Gramicidin; Chemotactic peptide receptor; Superoxide; Lysosomal enzyme

1. INTRODUCTION

N-Formylated peptides bind to receptor sites on the neutrophils and activate functional responses including chemotaxis, lysosomal enzyme release and superoxide anion generation [1–3]. Maximum activity was observed with *N*-formyl-methionyl-leucyl-phenylalanine [1]. Peptides having amino acids with aliphatic side chains, like valine and isoleucine, in place of methionine at position 1 have fairly high activity [3]. The *N*-protected tripeptide, BMLP is an antagonist of the chemotactic peptide receptor [3].

Gramicidin A, B and C are linear pentadecapeptide ethanolamide antibiotics having a formyl group at the amino-terminus valine or isoleucine [4–6]. Gramicidin forms trans-membrane channels for monovalent cations [7–9]. This property can result in depolarisation of membrane potential in biological membranes treated with gramicidin [10], including that of neutrophils [11,12].

This report presents data which show that gramicidin activates neutrophil functions and chemotactic peptide receptor antagonist inhibit that activation.

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Abbreviations: FMLP, formyl-methionyl-leucyl-phenylalanine methyl ester; BMLP, *t*-butyloxycarbonyl-methionyl-leucyl-phenylalanine

2. MATERIALS AND METHODS

2.1. *Materials*

Gramicidin from *Bacillus brevis* (Dubos), chlortetracycline HCl, cytochalasin B and all enzyme substrates were from Sigma. FMLP and BMLP were kindly provided by Professor P. Balaram. The commercial preparation of gramicidin, as a mixture of A, B and C, was used directly: the molecular mass of which was taken to be 1880 Da.

2.2. *Preparation of rabbit neutrophils*

Rabbit peritoneal exudate neutrophils were collected 4 h after injection of 0.1% glycogen in normal saline into the peritoneal cavity [13]. Cells were washed by centrifugation and suspended in buffer containing 17 mM Hepes, 122 mM NaCl, 5 mM KCl and 5 mM glucose. Ca^{2+} and Mg^{2+} were added as required in the assay.

2.3. *Measurement of superoxide anion generation*

Reduction of ferricytochrome *c* by superoxide anion was used to measure the generation of superoxide by neutrophils as described [14], with minor modifications. 1.5×10^6 cells in 2 ml of buffer containing 1 mM Ca^{2+}, 1 mM Mg^{2+} and 0.625 mg/ml of horse heart cytochrome *c* were stirred and in-

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cubated at 37°C in a Shimadzu UV-210A spectrophotometer. Cells were pretreated with 4 μg/ml of cytochalasin B for 4 min before addition of the test samples. Absorbance changes were measured at 550 nm.

2.4. *Assay of lysosomal enzyme release*

Lysosomal enzyme release assay was done at 37°C in 1 ml of buffer containing 1 × 10^6 cells, 1 mM Mg^{2+} and 1 mM Ca^{2+}. Cells were pretreated with cytochalasin B and test samples were added. Cells were separated by centrifugation and enzyme activities assayed in the supernatant. Lysozyme activity was measured by absorbance change in a suspension of *Micrococcus lysodeikticus* at 450 nm [15]. β-Glucuronidase was assayed by estimating the release of phenol-phthalein β-glucuronide at 550 nm [16]. Lactate dehydrogenase, a cytoplasmic enzyme, was assayed by measuring the change in absorbance at 340 nm in a solution containing NADH and pyruvate [17]. Total enzyme activities in the cells for all the three enzymes were measured after lysing the cells in 0.2% Triton X-100.

2.5. *Measurement of chlortetracycline fluorescence response*

Fluorescence changes, in response to various stimulants in neutrophils loaded with chlortetracycline was measured as described elsewhere [18], with some modifications. Cells at 5 × 10^6/ml were incubated at 37°C for 30 min in buffer containing 100 μM chlortetracycline and 0.5 mM Ca^{2+} but no Mg^{2+}. Cells were pelleted by centrifugation, then washed once in buffer without Ca^{2+}, Mg^{2+} and chlortetracycline, and resuspended in the same buffer. These cells, suspended at 2.5 × 10^6/ml in buffer without Ca^{2+} and Mg^{2+}, were stirred and incubated at 37°C in a Perkin Elmer MPF-44A fluorescence spectrophotometer for the assay. The excitation and emission wavelengths were 380 nm and 560 nm, respectively.

3. RESULTS

Fig.1 shows superoxide anion generation by neutrophils in response to various stimuli. The response started after a delay of 20–30 s following the addition of samples. FMLP gave an optimum response at 10 nM. The 10 nM and 1 nM FMLP responses were inhibited to varying degrees by 10 μM BMLP. Gramicidin gave a maximum response at 10 nM and that response was much lower in intensity than that by FMLP. There was complete inhibition of the 10 nM gramicidin response by 10 μM BMLP and 1 μM gramicidin partially overcame that inhibition. Thus gramicidin gave a superoxide response at fairly low concentrations comparable to that of FMLP but BMLP inhibited the gramicidin response much more efficiently than it inhibited the FMLP response. The intensity of the responses by gramicidin were lower than that by FMLP.

Fig.2 shows the results of secretion of lysosomal

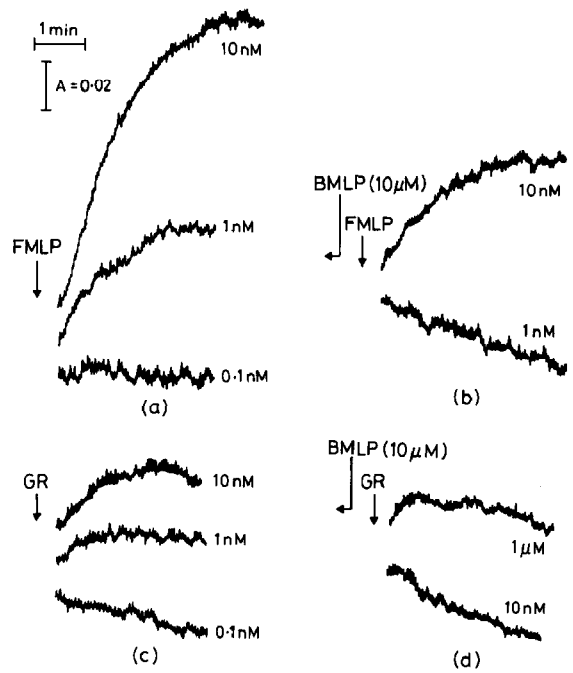

Fig.1. Superoxide generation by neutrophils in response to FMLP and gramicidin, and the inhibition by BMLP. (a,c) FMLP and gramicidin (GR) alone; (b,d) FMLP and gramicidin response of cells pretreated with 10 μM BMLP for 2 min.

enzymes by neutrophils in response to FMLP and gramicidin. The responses by gramicidin were of lower intensity than that by FMLP. 20 μM BMLP

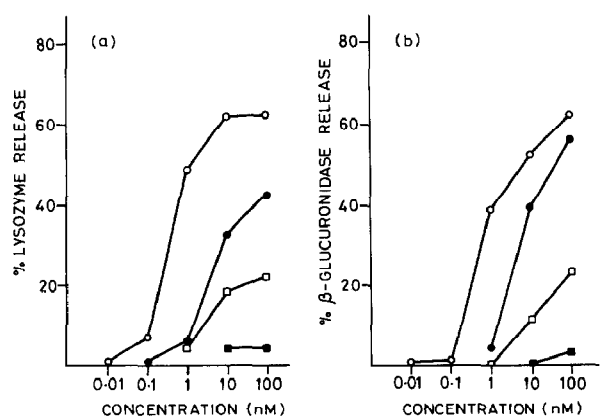

Fig.2. Lysosomal enzyme release by neutrophils in response to FMLP and gramicidin, and the inhibition by BMLP. Values expressed as percentage of total activity released from detergent lysed cells. Neutrophils were incubated with FMLP (○,●) or gramicidin (□, ■) in the presence (●, ■) or absence (○, □) of 20 μM BMLP.

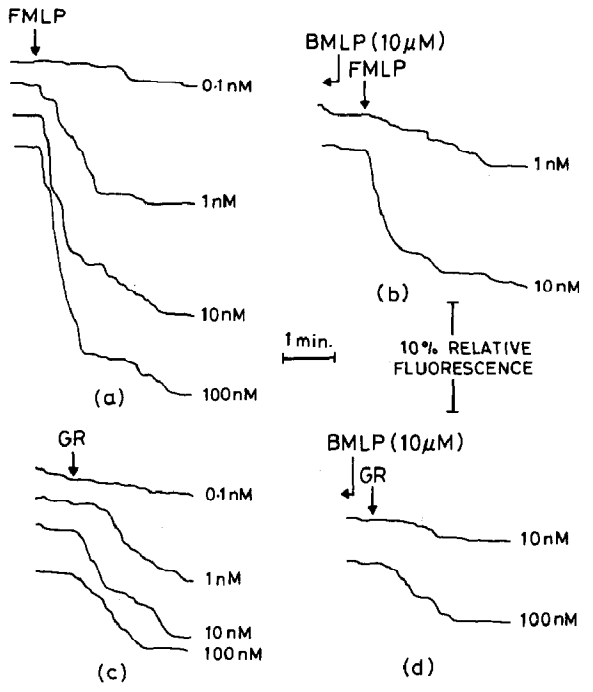

Fig.3. Fluorescence decrease in chlortetracycline-loaded neutrophils in response to FMLP and gramicidin. (a,c) FMLP and gramicidin (GR) alone; (b,d) FMLP and gramicidin response, respectively, of cells pretreated with 10 μM BMLP for 2 min.

inhibited the responses by gramicidin much more efficiently than the responses by FMLP. The lactate dehydrogenase activities in all assays were close to that of the blank (less than 4% of total activity).

Fluorescence decrease in response to FMLP and gramicidin were studied in chlortetracycline-loaded neutrophils (fig.3). The fluorescence response started within 4 s of the addition of FMLP. But there was a delay of about 30 s in the onset of responses by 1 nM gramicidin. Gramicidin gave a maximum response which was of lower intensity than the maximum response of FMLP. Dose-related responses were similar with both the peptides, 10 nM giving an optimum response and no response at 0.1 nM. In the presence of 10 μM BMLP, 1 nM FMLP and 10 nM gramicidin did not give a response. Inhibition by BMLP of the gramicidin response was much better than the inhibition of the FMLP response.

4. DISCUSSION

Experiments reported in this work clearly show that linear gramicidin activates functional responses in neutrophils. The presence of formyl group at the amino-terminus valine/isoleucine of gramicidin [4–6] suggests a structure compatible with chemotactic peptide receptor binding. Another possible mode of activation is by the ability of gramicidin to depolarise membrane potential in cells, including neutrophils [10–12]. The observation that all the three responses described for gramicidin in neutrophils can be inhibited by chemotactic peptide receptor antagonist, suggests that gramicidin acts through this receptor and not by membrane depolarisation.

Although gramicidin and FMLP gave responses at similar concentrations, there were three differences noted in their responses. (i) The maximum intensity of the response by gramicidin is much lower than that by FMLP. (ii) BMLP appears to inhibit gramicidin response more efficiently than FMLP response. (iii) There was a greater delay in the onset of responses by 1 nM gramicidin and by 100 nM gramicidin in the presence of 10 μM BMLP (fig.3c and d). This delay was absent in the case of FMLP. It is not possible to explain these observations with the available data. The alternating D- and L-amino acids, the larger size of gramicidin compared to FMLP and their relatively high lipid solubility may be contributing factors. The higher molecular mass of gramicidin and their tendency to dimerize in lipid bilayers [19] to form NH_2-terminal to NH_2-terminal dimers [20] might restrict their mobility and the accessibility of formyl groups in membranes. These may affect the ability of gramicidin to interact with the chemotactic peptide receptors.

Decrease in chlortetracycline fluorescence measures release of membrane bound calcium inside cells [18,21–23]. Presence or absence of 1 mM Ca^{2+} or 1 mM EGTA in the absence of added Ca^{2+} in the extracellular medium do not produce any significant changes in the fluorescence response (not shown). The assay reported here was done in the absence of extracellular Ca^{2+}. Gramicidin has ionophoretic activity only for monovalent cations [7–9] and is not a Ca^{2+} ionophore [8]. The response by gramicidin can be completely blocked by BMLP. Hence fluorescence response is not due

to the ionophoretic activity of gramicidin but due to flux of Ca^{2+} in the intracellular pools, mediated through the membrane receptor.

Tyrothricin, an antibiotic preparation from *Bacillus brevis*, contains about 20% gramicidin and 80% tyrocidin. It has been used therapeutically as a local antibiotic on infected surface ulcers and wounds. It is toxic when given parenterally and on fresh wounds [24,25]. On the basis of the above results, one of the potential toxicities of gramicidin may be due to activation of neutrophil functions resulting in inflammation at the site of application.

Chemotactic peptide receptors have been demonstrated in other cell types like macrophages and spermatozoa [26,27], in addition to neutrophils. Hence caution should be taken in interpreting the results in such cells when gramicidin is used for depolarisation of membrane potential by its channel properties.

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