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MECHANISMS OF ACTION INVOLVED IN THE CHEMOATTRACTANT ACTIVITY OF THREE β-LACTAMIC ANTIBIOTICS UPON HUMAN NEUTROPHILS

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Abstract—The effects produced by three β-lactamic antibiotics (*N*-formimidoyl thienamycin or imipenem, cefmetazole and cefoxitin) *in vitro* on protein carboxylmethylation, cAMP and cGMP levels in human polymorphonuclear neutrophils were studied. These antibiotics (50 μg/mL) exhibited chemoattractant activity for phagocytic cells and produced a fast (0.5 min) and significant stimulation of protein carboxylmethylation. They also increased intraphagocytic cGMP levels although no changes in cAMP levels were observed. Since the involvement of the above-mentioned mechanisms in leukotaxis have been established, the stimulation of neutrophil chemotaxis by the three antibiotics studied could possibly be mediated by one or more of these mechanisns.

Chemotaxis, the directional migration of cells along a chemical gradient, is displayed by a variety of cells, including bacteria, cellular slime molds, leukocytes, tumour cells and neuronal cells [1]. This mobile response has been studied extensively in leukocytes and shown to be a receptor-mediated process [2] similar to that in bacteria [3]. Furthermore, studies using defined attractants have led to the discovery of those biochemical events which may be obligatory for chemotaxis in phagocytic cells [4–6].

For adequate chemotaxis, not only the presence of chemoattractants but also the existence of chemotactic modulators are necessary. These modulators are not chemotactically effective by themselves but are able to increase or decrease the cellular movement produced by the chemoattractants. In this sense, the intracellular cyclic nucleotides cAMP and cGMP play a role in chemotactical modulation. Estensen *et al.* observed that the levels of cyclic nucleotides regulated cellular movement [7]. In neutrophils the agents that increased the intracellular concentration of cAMP also brought about an inhibition of chemotaxis [8]. These authors suggest that cGMP plays a stimulatory role in the formation of the chemotactic response and cAMP a suppressive role, although both nucleotides appear to be necessary for both the activation and modulation of cellular movement.

Another process implicated in leukotaxis is protein carboxylmethylation [9]. O'Dea *et al.* (1978) demonstrated the presence of protein carboxylmethylase activity in membrane and cytoplasmic fractions of neutrophil leukocytes and synthetic peptides [10], which are similar to the bacterial attractants, stimulated a specific, rapid and transient protein carboxylmethylation in these cells. In addition, the incubation of leukocytes with compounds that inhibit methylation blocked the response to chemotactic stimulation [11].

For leukotaxis to take place the action of a "methyl-transferase", which incorporates methyl groups into phosphatidyl-ethanolamine, is also required. Schiffmann (1982) discovered that chemoattractants produced changes in the methylation of phospholipids [12]. Furthermore, an inhibition in Ca^{2+} flow, phospholipase A_2 and chemotaxis was observed in rabbit neutrophils incubated in the presence of f-Met-Leu-Phe and methyltransferase inhibitors [13]. These observations suggest that receptors for chemotaxis are also involved in the Ca^{2+} flow, phospholipid methylation and arachidonic acid metabolism and that phospholipid and protein methylation constitute the first steps in the chemotactic response.

Within the cefamycin groups, cefmetazole and cefoxitin are the most recently obtained semisynthetic derivatives of Cefamycin C, possessing an excellent antibacterial spectrum. The same can be said of imipenem, a β-lactamic antibiotic derived from thienamycin [14]. Preliminary results [15] demonstrate that 50 μg/mL of cefmetazole, cefoxitin and imipenem, stimulate *in vitro* the mobile capacity of neutrophils towards a chemotactic stimulus (casein). In this study, we are able to show that cefmetazole, cefoxitin and imipenem exert a chemically-attractive power over the above mentioned leukocytes. Since leukocyte chemotaxis is accompanied by an increase in protein methylation [16] and modifications in cyclic neucleotide levels [17], the influence of these antibiotics on these mechanisms and cellular

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mediators in neutrophils was subsequently studied with the aim of further understanding how imipenem, cefmetazole and cefoxitin exert their chemoattractant power.

MATERIALS AND METHODS

Preparation of neutrophil leukocytes. Cells were obtained from heparinized venous human blood of healthy volunteers. Blood samples were centrifuged at 300 *g* for 30 min in a density gradient using Mono-Poly-Resolving Medium (Flow), neutrophils washed in Hank's solution (Difco) and adjusted to 5×10^6 cells/mL of medium. The viability, determined using the trypan blue dye exclusion method, was 95 ± 6%.

Preparation of antibiotics. Cefmetazol (Antibioticos S.A.), cefoxitin and imipenen (Merck, Sharp and Dohme Research Lab.) were prepared in Hank's solution (50 μg/mL) for each assay. Each experiment was accompanied by a control sample free from antibiotic.

Chemoattractant activity. The chemoattractant activity of the antibiotics was evaluated according to a modification of the original techniques described by Boyden [18] and Fontan *et al.* [19], using chambers with two compartments separated by a Millipore filter (3 μm pore diameter). The leukocyte suspensions were deposited in the upper compartment of Boyden chambers and the antibiotics in the lower compartment. Casein (Sigma) was used as a positive control *in vitro*, as described by Wilkinson [20] and an attractant-free medium used as a negative. The chambers were incubated for 3 hr at 37° with 100% relative humidity and in a 5% CO_2 atmosphere. Afterwards the filters were fixed and stained, and chemotactic indices, which represent the total number of neutrophils counted in 16 fields on the lower face of the filters, were calculated.

Protein carboxylmethylation. Protein carboxylmethylation was measured by a modification of the procedure previously described by Diliberto *et al.* [21]. f-Met-Leu-Phe (Sigma) a synthetic peptide similar to the natural bacterial attractants was utilized at 10^{-8} M, a concentration which produces a maximal stimulation of methylation [16]. After preincubation of the cell suspension (1×10^6 cells per tube) at 37° for 30 min with the antibiotics, peptide or medium, reactions were initiated by adding 10 μCi of [^{3}H]L-methionine (final concentration 12×10^{-7} M) in a final volume of 1.0 mL. The samples were incubated for 0.5, 1, 2.5 and 10 min and the reactions stopped by adding 0.5 mL of cold 20% trichloroacetic acid. After centrifugation for 10 min at 12,000 *g*, the precipitate esters formed from endogenous proteins were hydrolysed with 300 μL of 1.0 M borate buffer at pH 11.0 containing unlabelled methanol (2.6% v/v) as carrier. The level of carboxylmethylesters in the precipitate was measured taking the hydrolytic product, [^{3}H]methanol, according to Campillo and Ashcroft [22]. The 1.5 mL plastic tubes were placed in stoppered scintillation vials containing 1 mL methanol. The radioactive methanol in the plastic tubes was selectively recovered in the outer vials by allowing the vials to equilibrate at 37° overnight. The following morning and after 1 hr at 4° the plastic tubes were withdrawn from the phials and 5 mL of scintillation liquid was added to the latter. The radioactive methanol thus recovered was counted in a β-counter and the results were expressed as fmol of [^{3}H]methanol/10^6 cells. All samples were performed in duplicate.

cAMP levels. The cAMP levels in the leukocytes were determined by means of a commercial radioimmunoassay kit (CIS) which consisted of ^{125}I-cAMP (1.5 μCi/mL), rabbit anti-cAMP serum, standard cAMP, diluting agent (standard 0) and precipitating agent (isopropanol + polyethyleneglycol).

Samples containing 2×10^6 cells were incubated with antibiotic (50 μg/mL) or medium containing 0.25 μmol of 3-isobutyl-L-methylxanthine as phosphodiesterase inhibitor, in the case of controls at a final volume of 0.5 mL, for 30 min at 37°. Values of cAMP were compared with a non-incubated sample. Several assays were performed with f-met-leu-phe (10^{-8} M) in which the samples were treated in a similar manner.

Samples for radioimmunoassay were prepared by first adding 0.5 mL of 10% cold trichloroacetic acid. After vigorous shaking for 2 min, the samples were centrifuged for 10 min at 300 *g*, after which 600 μL of supernatant were collected. To the latter, 600 μL of acidified ethyl-ether with HCl 0.1 N were added before proceeding to another centrifugation in order to eliminate the top phase (ether); this process was repeated twice. After lyophilization the residue obtained was dissolved in 0.3 mL of diluting agent, leaving the samples ready for use with the kit. Once performed, the radioactivity of precipitate was measured in a γ-counter and the results expressed as fmoles of cAMP/10^6 cells.

cGMP levels. The study of intracellular levels of cGMP in PMNs was performed in the same way as cAMP, by using a radioimmunoassay kit (BTI) made up of ^{125}I-cGMP (0.14 μCi/mL), anti-cGMP serum, standard GMP, diluting agent (contains normal rabbit IgG), acetylant reagent (triethylamine + anhydric acetic acid), an acetate buffer and a non-specific binding reagent.

The neutrophil suspension (2×10^6 cells/0.5 mL) was incubated during 15 or 30 min at 37° accompanied by shaking, with and without antibiotic (50 μL/mL) together with 0.25 μmol of 3-isobutyl-L-methylxanthine, a phosphodiesterase inhibitor. In all cases a sample was run without having been previously incubated (basal), in order to determine the start point for intracellular cGMP levels.

Before subjecting to radioimmunoassay, samples were extracted with 4.5 mL of 10% cold trichloroacetic acid. After homogenizing in a shaker, the suspensions were centrifuged at 300 *g*, allowing the supernatant to be collected. Trichloroacetic acid was eliminated by using 5 mL of ethyl-ether and the previously shaken samples were again centrifuged. Afterwards, the top phase (ether) was discarded while the ether dissolved in the lower phase was eliminated by heating the samples for 2 min at 50°. The treatment with ethyl-ether was repeated three times, leaving the samples ready for the radioimmunoassay. Finally, the results of these assays were expressed in fmoles of cGMP/10^6 cells.

Statistical analysis. All data are expressed as the

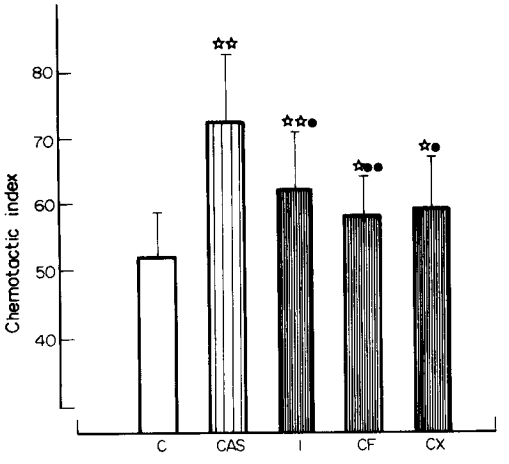

Fig. 1. Chemoattractant power of imipenem (I), cefmetazole (CF), cefoxitin (CX) and casein (CAS) on human neutrophils. The values are expressed as chemotactic index and represent the mean and the standard deviation of 10 experiments performed in duplicate. Statistical significance is expressed by: (☆) $P < 0.05$ and (☆☆) $P < 0.01$ with respect to the controls with medium (C). (●) $P < 0.05$ and (●●) $P < 0.01$ with respect to the casein values.

mean ± standard deviation of the number of experiments stated in the corresponding tables and figures. In the statistical study, the results were analysed by using the non-parametric tests: the Anova two-way test by addition of Friedman ranges (paired samples) and the Anova one-way test by addition of Kruskal–Wallis ranges (unpaired samples) for multiple comparisons.

RESULTS

Imipenem, cefmetazole and cefoxitin at 50 μg/mL exerted a chemically attractive power over neutrophils (Fig. 1), with a significant increase in the chemotactic indices ($P < 0.01$ imipenem, $P < 0.05$ cefmetazole and cefoxitin). However, the attracting ability of the antibiotics *in vitro* was not as strong as that exerted by casein, with statistically significant differences ($P < 0.05$ for imipenem and cefoxitin, $P < 0.01$ for cefmetazole) existing between the values of the latter and those obtained in the presence of any of the antibiotics studied.

Table 1 shows the protein carboxylmethylation produced by these antibiotics and the levels of significance of the samples containing antibiotics as compared to the control samples and those carrying the formylated peptide. The addition of [^{3}H]L-methionine to intact cells, previously incubated with the antibiotics or f-met-leu-phe (10^{-8} M), resulted in a rapid (0.5 min) increase ($P < 0.01$) in protein methylester formation compared to controls. With respect to the antibiotics, the values found at all the times studied may be considered as being constant and the only significant differences with respect to the controls were obtained at 1 min with imipenem. On the other hand, significantly decreased carboxylmethylation levels were obtained at all the incubation times in the samples containing any of the antibiotics in relation to the formylated peptide. In the absence of stimuli (controls) the cells showed a linear increase in protein carboxylmethylation with time. When neutrophil leukocytes were incubated in the presence of f-met-leu-phe, the carboxylmethylation levels decreased as the incubation time increased, although these were always significantly greater than the control values. Samples treated with antibiotics exhibited intermediate values ranging between those of the controls and the formylated peptide throughout all incubation times.

cAMP concentrations in human neutrophils for basal (0 time, after cellular extraction), control and with antibiotic (after 30 min of incubation with medium or with antibiotic, respectively) samples are shown in Fig. 2. cAMP levels in neutrophils were not different to the basal concentrations, neither after incubation for 30 min with culture medium (controls), nor after 30 min of incubation with 50 μg/mL of any of the antibiotics studied. The results obtained with 10^{-8} M of f-met-leu-phe following incubation were 220 ± 24 fmol/10^6 PMNs (mean ± SD of five experiments carried out in duplicate). In this case, no differences were observed between cAMP values in the presence of formylated peptide or antibiotic or in controls.

The levels of cGMP in human neutrophils at 0, 15 and 30 min of incubation with culture medium (controls) or with 50 μg/mL of imipenem, cefmetazole or cefoxitin are shown in Fig. 3. In the presence of any of the three antibiotics, statistically greater values were obtained with respect to those of the corresponding controls. Significant differences

Table 1. Protein carboxylmethylation in human neutrophil leukocytes

	[^{3}H]Methanol released (fmol/10^6 cells) after incubation periods (min)			
	0.5	1	2.5	10
Controls	17 ± 4	25 ± 10	27 ± 5	32 ± 6
f-Met-leu-phe	50 ± 12†	42 ± 11†	36 ± 3†	39 ± 7†
Imipenem	29 ± 2†§	33 ± 12*§	28 ± 3§	31 ± 7§
Cefmetazole	30 ± 5†§	29 ± 8§	26 ± 2§	30 ± 5§
Cefoxitin	28 ± 2†§	29 ± 11§	27 ± 2§	30 ± 13‡

Each value represents the mean ±SD of ten experiments performed in duplicate.
The statistical differences are shown as: * $P < 0.05$ and † $P < 0.01$ with respect to controls, ‡ $P < 0.05$ and § $P < 0.01$ with respect to f-met-leu-phe.

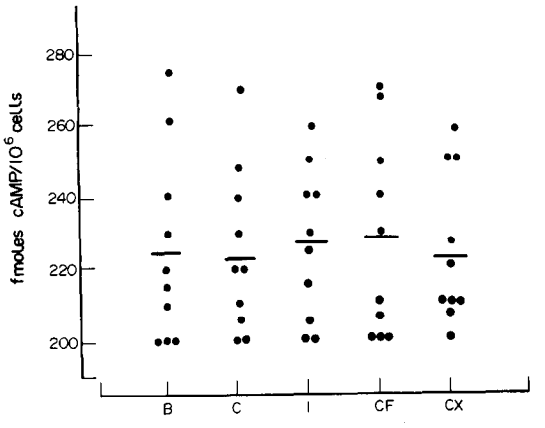

Fig. 2. Levels of cAMP (fmol/10^6 cells) in human neutrophils after their isolation (basal levels, B) and after 30 min of incubation in culture medium (controls, C) or with 50 μg/mL of imipenem (I), cefmetazole (CF) or cefoxitin (CX). Each point represents the mean value of an experiment performed in duplicate. The mean value obtained from all the points found in each of the cases studied is shown as a horizontal line.

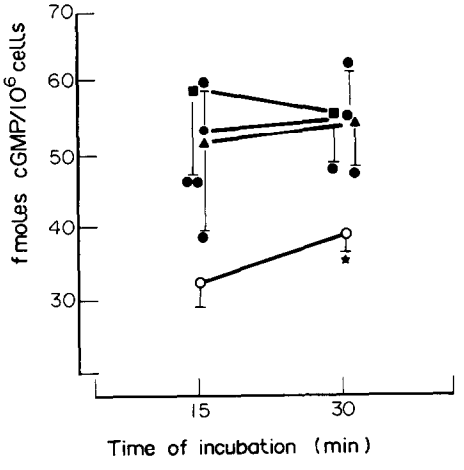

Fig. 3. Levels of cGMP (fmol/10^6 cells) in human neutrophils incubated for 15 or 30 min with culture medium (controls; ○——○, C) or with 50 μg/mL of imipenem (●——●, I), cefmetazole (□——□, CF) or cefoxitin (△——△, CX). Each point represents the mean value and standard deviation of 10 experiments performed in duplicate. (●) $P < 0.05$ and (●●) $P < 0.01$ with respect to the corresponding control value. (*) $P < 0.05$ with respect to the corresponding control value after 15 min.

were observed after 15 min with imipenem and cefoxitin ($P < 0.01$) and after 30 min with all three antibiotics ($P < 0.05$) and after 15 min with cefmetazole ($P < 0.01$). Furthermore, no differences were detected between the cGMP levels obtained after 15 and 30 min of incubation with each of the three antibiotics whereas in controls the cGMP levels after 30 min were significantly greater ($P < 0.05$) than at 15 min. Control and antibiotic samples demonstrated a significant increase in cGMP levels in neutrophils compared to controls after 15 and 30 min of incubation ($P < 0.05$ in control and $P < 0.01$ in antibiotic samples). The values obtained after incubating neutrophils with f-met-leu-phe (10^{-8} M) for 15 and 30 min were 55 ± 2 and $69 \pm 8/10^6$ PMNs respectively (mean ± SD of five experiments carried out in duplicate). These results differed very significantly ($P < 0.01$) from their corresponding control values (31 ± 3 and 39 ± 2 fmol/10^6 cells, respectively). However, although they were slightly higher than those found in the presence of imipenem, cefmetazole and cefoxitin, no statistical differences were obtained.

DISCUSSION

In previous studies, imipenem, cefmetazole and cefoxitin stimulated neutrophil migration towards a casein gradient, a potent attractant *in vitro* [15]. In this work we observed that 50 μg/mL of these antibiotics also possessed a capacity for human neutrophil attraction. The study of those mechanisms directly involved in the union "stimulus–response" of neutrophils is thus of great interest in order to identify through which metabolic pathways imipenem, cefmetazole and cefoxitin exert their chemotactic capacity. Consequently, in this study, in addition to assessing the chemoattractant ability of neutrophils we also undertook the study of protein carboxylmethylation and the measurement of intracellular cyclic nucleotides in the presence of these three antibiotics.

The formation and hydrolysis of methylesterified proteins play a very important role in the signal process in bacterial chemotaxis [23]. Protein methylesterase has been demonstrated in a variety of cell types including neutrophil leukocytes as well as its participation in leukocyte chemotaxis [9, 10]. Furthermore, a turnover of protein methylesters is involved in leukotaxis, which includes both formation and hydrolysis of protein methylesters and which increases in chemotactically stimulated cells [9]. The rapidity of these reactions is in accordance with the time of appearance of the earliest events in the transduction of the chemotactic signal to produce directed migration [12]. In an extensive study of the protein carboxylmethylation reaction with f-met-leu-phe, Venkatasubramanian *et al.* [9] found that over a longer incubation period, 30 min, the apparent rate of carboxylmethylation in the presence of a stimulated gradient of attractant is decreased. However, after short incubation periods, 0.5 and 1.0 min, there is a transient and variable increase in this methylation reaction in the presence of attractants [10].

In our studies with imipenem, cefmetazole and cefoxitin we observed a rapid increase (0.5 min) in the carboxylmethylation of proteins when the neutrophils were incubated simultaneously with the antibiotics and [^{3}H]methyl-methionine. Using *S*-adenosyl-L-methionine as the methyl donor, the enzyme carboxylmethylase catalyses the formation of protein methylesters which undergo rapid, spontaneous hydrolysis at a physiological pH to liberate methanol [12, 24]. Considering that the carboxylmethylation of proteins in neutrophils is a process mediated by a specific chemotactic receptor [12] and

not dependent on *de novo* protein synthesis [9], this suggests that the interaction of antibiotics with a receptor, in a similar way to other types of attractant, presumably generates a signal which ultimately results in directed motion of the cell. Such mobile behaviour in phagocytic leukocytes is of critical importance in the defence of the host against invading organisms. The observation that the antibiotics studied (50 μg/mL) demonstrate an attractant power and that at 0.5 min they produce significantly different levels in carboxylmethylation with respect to the controls, in the presence of the formylated peptide, further suggests that imipenen, cefmetazole and cefoxitin may act in a similar way to the peptide. This action produced by interaction with specific membrane receptors causes carboxylmethylation stimulation in a short period of time, and generates the signal that leads to phagocytic locomotion.

In addition to the methylation of proteins, other mechanisms involved in the phagocytic response to chemotactic stimuli include the participation of the Ca^{2+} ion, as well as the intracellular cyclic nucleotides cAMP and cGMP [17, 25, 26]. Substances which increase the intracellular cAMP level suppress the response to the stimulus if there is no concurrent increase in cGMP levels [27, 28]. On the other hand, substances that increase the intracellular cGMP also increase the chemotactic response and this does not always need to be accompanied by a reduction in cAMP levels [8, 29].

Although the changes in cyclic nucleotide levels in the presence of different substances are considered to be rapid (5–30 min), maximal increases are to be observed after 30 min with cAMP [27, 28] and in the case of cGMP between 10 and 30 min [8]. For this reason we chose the incubation times for human neutrophils of 30 min for the study of cAMP levels and 15 and 30 min for cGMP concentrations.

Our results show that no changes are produced, either in controls or with any of the antibiotics studied, in intracellular cAMP levels after 30 min of incubation with respect to basal values, obtained after cellular extraction. Likewise, no changes with 10^{-8} M of f-met-leu-phe at the same incubation times were detected. On the contrary, both in the presence of medium and with any of the antibiotics at the incubation times tested (15 and 30 min), an increase in cGMP levels in neutrophils occurred with respect to basal values. The same phenomenon also occurred with f-met-leu-phe (10^{-8} M) both at 15 and 30 min of incubation. Although, in the controls, differences were observed between the values obtained at 15 and 30 min, in the case of the antibiotics as well as formylated peptide no such differences were detected. Possibly the antibiotics increase the cGMP levels to a maximum during the first 15 min of incubation time so as no greater increase is possible. The control and formylated peptide values are consistent with the findings of other workers who have obtained similar results in relation to cAMP levels after 30 min of incubation and in the cGMP levels after 15 and 30 min [8, 27, 28].

A possible explanation for the fact that significantly greater cGMP values are obtained in controls after 30 min of incubation as compared both to corresponding values found after 15 min and basal levels, may lie in the possible oxidative stress undergone by the cells when removed from their natural medium and placed in a foreign environment. This would give rise to an increase in intracellular levels of free Ca^{2+}, thus regulating directly cGMP formation [30]. This may be affected by the presence of calcium chloride in the incubation medium. Moreover, it is known that during cell stress an increase in the release of chemoattractive substances takes place such that phagocytes become activated both *in vivo* and *in vitro* [31, 32], a phenomenon that is accompanied by an increase in intracellular Ca^{2+} levels [33].

On the other hand, no differences in cAMP and cGMP levels in the human neutrophils were observed between the values obtained with 50 μg/mL of imipenem, cefmetazole and cefoxitin and those with 10^{-8} M of a typical chemoattractant such as the peptide f-met-leu-phe. This again demonstrates the chemoattractant power inherent of imipenem, cefmetazole and cefoxitin at the dose studied. Moreover, this fact corroborates the suggestion that chemotactic factors in general induce stimulation in intraphagocytic levels of cGMP to concentrations similar to those which trigger the beginning of chemotactic responses. Furthermore, it is also consistent with the hypothesis that most chemoattractant agents which increase intracellular levels of cGMP do not modify cAMP levels. This may be due to the fact that both cGMP and cAMP are necessary at a given concentration to initiate and modulate the mechanism of cell movement [8, 29].

Ca^{2+} can modify, either directly or indirectly, the levels of intracellular cyclic nucleotides, this ion being essential for the chemotactic response as it stimulates guanilcyclase and thus favours the formation of cGMP from GTP [25]. In turn, Ca^{2+} activates specific phosphodiesterase which transforms cAMP into 5'-AMP at the same time as it inhibits adenylcyclase. Preliminary data (Rodriguez *et al.*, unpublished observations) obtained using Fura 2-AM, a fluorescent marker, indicates that in control neutrophils no modification occurs in intracellular Ca^{2+} levels during phagocyte incubation in culture medium. However, immediately after the addition of antibiotics, values were significantly increased (82 ± 9 nM in controls and 188 ± 5 nM or 165 ± 4 nM with cefmetazole or cefoxitin, respectively). Although these results are not conclusive, the present data indicates a rapid increase in intracellular Ca^{2+} levels in the presence of antibiotic.

There is evidence to show that when phagocytes are stimulated, a rapid increase in free Ca^{2+} intracellular levels occurs which results from both extracellular Ca^{2+} and membrane bound Ca^{2+}. This leads to the activation of the chemotactic response in addition to the respiratory burst which accompanies the phagocytic process [34, 35]. Recently, an increase in the levels of acetosyl-methylester of Fura 2 joined to intracellular Ca^{2+} was observed in stimulated macrophages indicating an intracellular increase of this ion [36]. Therefore, the results obtained concerning the activation of neutrophils by 50 μg/mL of imipenem, cefmetazole and cefoxitin may be accounted for to a large extent by an increase in intracellular levels of Ca^{2+}.

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