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INHIBITION OF POLYMORPHONUCLEAR LEUKOCYTE FUNCTIONS BY CHLORTETRACYCLINE

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Abstract—Chortetracycline (CTC) inhibits chemotaxis, exocytosis and metabolic burst in rabbit polymorphonuclear leukocytes (PMNs), when these cells are activated in the absence of extracellular Ca^{2+}. In the presence of extracellular Ca^{2+} CTC has little or no inhibiting effect on these functions. The inhibiting effect of CTC in the absence of Ca^{2+} occurs at concentrations which are not cytolytic. The inhibiting effect of CTC can be reversed by washing the cells or by addition of Ca^{2+} or Mg^{2+} to CTC-pretreated cells. Inhibition of the metabolic burst by CTC depends on the activator used. When phorbolmyristate acetate is used to activate the cells, the metabolic burst is inhibited at lower CTC concentrations than with chemotactic peptide as an activator. With due observance of the chemical properties of CTC and literature data about the requirement of intracellular Ca^{2+} for neutrophil functions, the results obtained are consistent with the view that CTC interferes with neutrophil functions, by complexing intracellular Ca^{2+}, and that this inhibition is reversed when sufficient extracellular Ca^{2+} moves into the cell.

Chlortetracycline (CTC) forms a fluorescent complex with divalent metal ions such as Ca^{2+} and Mg^{2+}. The fluorescence of the complex is strongly dependent on the polarity of the medium [1, 2]. For this reason CTC is frequently used as a probe for membrane bound Ca^{2+} in several cell types. A decrease in CTC fluorescence indicates that Ca^{2+} is released from a membrane bound (hydrophobic) state into the (hydrophilic) cytosol. CTC fluorescence measurements have been used to observe binding and fluxes of calcium in erythrocyte ghosts [3], mast cells [4], pancreatic acinar [5, 6] and islet [7, 8] cells, platelets [9, 10] and polymorphonuclear leukocytes (PMNs) [11–13].

The PMN performs a number of functions upon cell activation: chemotaxis, phagocytosis, degranulation and the production of toxic oxygen metabolites. In all these functions Ca^{2+} is supposed to play an important role [14–16]. In a number of cases the Ca^{2+} is derived from the extracellular environment, and in other cases it comes from intracellular stores, such as the inner site of the plasma membrane [16–19]. It is generally believed that this Ca^{2+} plays a decisive role in one or more steps in the process of PMN activation, eventually resulting in chemotaxis, degranulation and the production of oxygen metabolites in the metabolic burst [19–21].

When CTC reacts with intracellular Ca^{2+} under the formation of a fluorescent complex, then that Ca^{2+} is no longer available for the activation of the PMN. In this way CTC may interfere with the process it is supposed to monitor. For this reason we have studied the modulating action of CTC on some PMN functions: chemotaxis, exocytosis and metabolic burst. The PMNs were activated by soluble agents, such as the chemotactic peptide formylmethionylleucylphenylalanine (FMLP), and phorbolmyristate acetate (PMA). Both agents are capable of inducing PMN activation in the presence of extracellular Ca^{2+}, but also in the absence of extracellular Ca^{2+} [18, 22], though then the magnitude of the response is lower in the case of FMLP as activator.

MATERIALS AND METHODS

PMNs. Polymorphonuclear leukocytes were obtained from rabbits, injected intraperitoneally with 200 ml isotonic saline containing 1.5 mg/ml glycogen. After 4 hr the exudate was collected by flushing the peritoneal cavity with isotonic saline containing citrate (0.4%, pH 7.4). The cells were centrifuged and washed with medium. The medium used consisted of 140 mM NaCl, 5 mM KCl, 10 mM glucose, 0.5% bovine serum albumin and 20 mM HEPES pH 7.3. The final cell suspension during the experiments contained 3×10^6 PMNs per ml.

Chemotaxis. Cell migration was measured with the Boyden chamber technique as described by Boyden [23]. The two compartments of the chamber were separated by a cellulose acetate Millipore filter (pore size 3 μm). As a chemotactic agent FMLP, 10^{-9} M was used. PMNs were preincubated with or without CTC for 15 min at room temperature, after which they were placed in the upper compartment of the Boyden chamber. The Boyden chambers were incubated for 60 min at 37° after which the filters with PMNs were fixed in ethanol and stained with Mayer's hemalum solution. Cell migration was determined microscopically according to the leading front technique [24]. The assays were carried out in triplicate and the migration distance of the PMNs was determined at three different filter sites.

Three types of experiments were carried out: locomotion (no FMLP); stimulated locomotion (FMLP in both upper and lower compartment of Boyden chamber); and chemotaxis (FMLP in lower com-

partment of the Boyden chamber). In the migration distance given for chemotaxis no correction has been made for (stimulated) locomotion. When experiments were carried out in the absence of extracellular divalent cations, the divalent cation complexing agent EDTA was included in the medium to eliminate Ca^{2+} which is adherent to or liberated by the PMN.

Exocytosis. Exocytosis was measured as the release of the granule-associated enzyme lysozyme, in the absence of significant release of the cytoplasmic enzyme lactate dehydrogenase (LDH) [25]. 3×10^6 PMNs were preincubated with or without CTC for 15 min at room temperature. Then the exocytosis-inducing agent (5×10^{-6} M cytochalasin B + 10^{-8} M FMLP) was added, followed by incubating at 37° for 20 min. After centrifugation the supernatant was analyzed for the presence of enzyme activity. Lysozyme was measured by determining the rate of lysis of *Micrococcus lysodeiktius*. The release of LDH was determined as a measure for cell damage. LDH was determined by measuring the conversion of NADH into NAD^+ during the conversion of pyruvate into lactate. Enzyme release was expressed as a percentage of a maximum value, obtained by treating the cells with 0.2% Triton X-100.

Metabolic burst. The metabolic burst was measured as an increased NBT reduction [26]. NBT reduction was determined by including 0.04% N(itro) B(lue) T(etrazolium)chloride and 1 mM KCN into the mixture with PMNs. Preincubation with or without CTC was carried out for 15 min at room temperature. Then the activating agent was added. As an activating agent either 5×10^{-6} M Cytochalasin B + 10^{-8} M FMLP, or PMA (100 ng/ml) was used. After incubation for 20 min at 37° the cells were centrifuged. The supernatant was analysed with respect to the release of lysozyme. To the residue 5 ml 0.5 M HCl was added to stop the reaction and to extract the non-reacted NBT. Then the mixture was centrifuged; to the residue 2 ml pyridine was added to dissolve the formazan, and the solution was placed in a boiling waterbath for 10 min. After cooling to room temperature the absorbance of the pyridine solution was measured at 510 nm. To quantify the results of the NBT reduction, a known quantity of NBT was reduced by ascorbic acid as described by Müller *et al.* [27].

The formazan was extracted with pyridine and under comparable conditions the extinction of this solution was compared with the extinction of the formazan formed during our experiments. The results obtained were expressed as nmoles NBT reduced per 3×10^6 cells per 20 min.

Chemicals. The chemotactic peptide FMLP, phorbolmyristate acetate, chlortetracycline HCl and nitrobluetetrazolium chloride were obtained from Sigma Chemical Co.; Cytochalasin B was from the Aldrich Chemical Co.

RESULTS

The modulating effects of CTC were compared for PMN functions, determined in the absence of extracellular Ca^{2+}, and the same function determined in experiments with extracellular Ca^{2+} present. In the absence of extracellular Ca^{2+}, 1 mM EDTA was added to remove cell-adherent Ca^{2+} ions. In the presence of EDTA chemotaxis towards FMLP is the same or slightly less than in the presence of 2 mM Ca^{2+}. The metabolic burst, and especially exocytosis, induced by cytochalasin B (CB) + FMLP, are more dependent on extracellular Ca^{2+}. Nevertheless these processes proceed sufficiently in the absence of divalent cations to demonstrate modulating effects of CTC.

In the presence of EDTA, CTC causes a concentration dependent inhibition of chemotaxis. CTC inhibition starts at about 2×10^{-5} M and is complete at 5×10^{-4} M (Fig. 1). The inhibiting effect of CTC applies to all types of locomotion and is not specific for chemotaxis: spontaneous locomotion, chemokinesis and chemotaxis are all inhibited (Table 1).

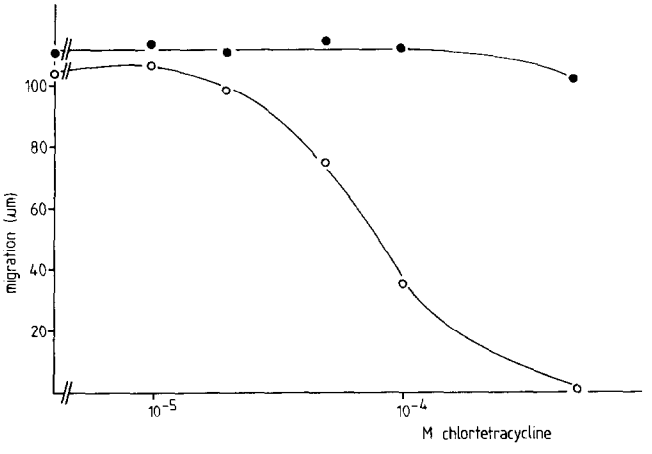

Fig. 1. Effect of chlortetracycline on FMLP-induced chemotaxis in rabbit PMNs. Cells were preincubated with CTC for 15 min at room temperature, and were then placed in the upper compartment of the Boyden chambers, followed by incubation for 1 hr. During incubation CTC remained present; both in the upper and lower compartment of the Boyden chamber. —○—, in the presence of 1 mM EDTA; —●—, in the presence of 2 mM Ca^{2+}.

Table 1. Effect of chlortetracycline on chemotaxis, (spontaneous) locomotion and stimulated locomotion

	Distance travelled (μm)		
	—	0.1 mM CTC	0.2 mM CTC
Locomotion	26 ± 3	19 ± 2	9 ± 3
Stimulated locomotion	95 ± 5	50 ± 4	25 ± 4
Chemotaxis	155 ± 2	90 ± 6	36 ± 3

PMNs were preincubated for 15 min at 22° with the CTC concentration indicated. Then the cells were placed in the upper compartment of the Boyden Chamber, followed by incubation for 1 hr. During incubation CTC remained present in the concentration given, in both compartments of the Boyden chamber.

Values given are the mean values of three experiments ± S.D.

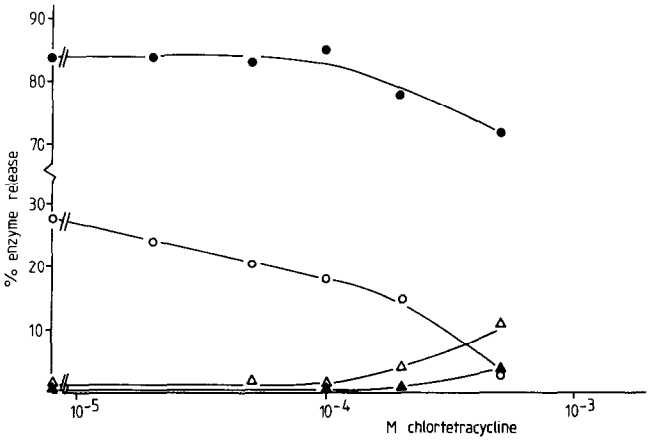

Fig. 2. Effect of CTC on cell integrity and on FMLP-induced exocytosis. Cells were preincubated with CTC for 15 min at room temperature. For determination of cell integrity cells were then incubated for an additional 20 min, now at 37°. For exocytosis CB/FMLP was added after preincubation, followed by incubation for 20 min at 37°. LDH release during exocytosis did not differ from that obtained in the absence of CB/FMLP. —△—, determination cell integrity: LDH release, 1 mM EDTA present; —▲—, determination cell integrity: LDH release, 2 mM Ca^{2+} present; —○—, exocytosis: lysozyme release, 1 mM EDTA present; —●—, exocytosis: lysozyme release, 2 mM Ca^{2+} present.

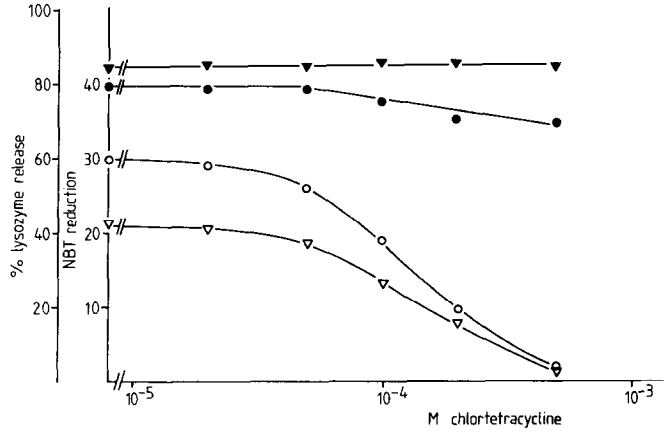

Fig. 3. Effect of CTC on FMLP-induced NBT-reduction and concomitant exocytosis. After 15 min preincubation (room temperature) with CTC the activating agent CB/FMLP was added, followed by incubation for 20 min at 37°. After centrifugation the residue was used for NBT reduction and in the supernatant lysozyme was determined to establish concomitant exocytosis. Only at the highest concentration CTC (0.5 mM) a slight increase of LDH release was observed. NBT reduction is expressed as nmoles NBT reduced per 20 min per 3×10^6 PMNs. —○—, NBT reduction, 1 mM EDTA present; —●—, NBT reduction, 2 mM Ca^{2+} present; —△—, lysozyme release, 1 mM EDTA present; —▲—, lysozyme release, 2 mM Ca^{2+} present.

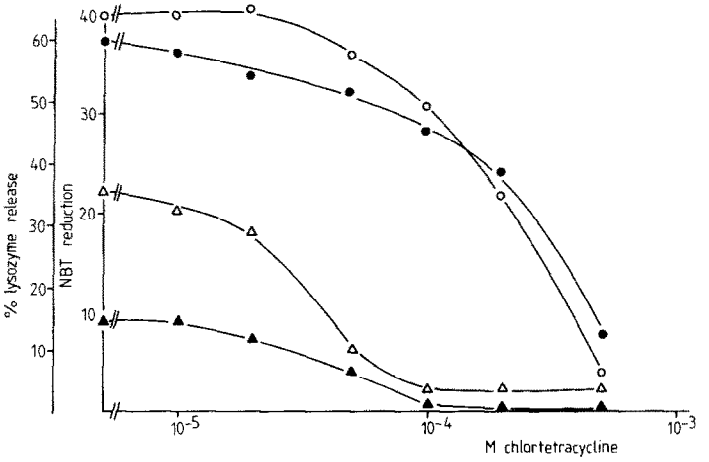

Fig. 4. Comparison between the inhibiting effect of CTC on FMLP-induced and PMA-induced NBT reduction. After 15 min preincubation with CTC at room temperature either CB/FMLP or PMA was added to induce the metabolic burst. After 20 min incubation at 37° NBT reduction was determined in the residue, and lysozyme was determined in the supernatant as a measure for concomitant exocytosis. The experiments were carried out in the presence of 1 mM EDTA. —○—, FMLP-induced NBT reduction; —●—, FMLP-induced lysozyme release during NBT reduction; —△—, PMA-induced NBT reduction; —▲—, PMA, induced lysozyme release during NBT reduction.

In the presence of extracellular Ca^{2+} there is no significant inhibition of chemotaxis by CTC (Fig. 1).

Under the conditions of our experiments CTC does not damage the cells significantly. Only at the highest CTC concentration used (5×10^{-4} M) a slight release of LDH occurs in the absence of Ca^{2+}. The release of the cytoplasmic enzyme LDH can be considered as a measure for cell damage. In the presence of Ca^{2+} no release of LDH occurs (Fig. 2).

CTC inhibits CB/FMLP-induced exocytosis in the presence of EDTA. In the presence of extracellular Ca^{2+} there is some inhibition, but this effect is less pronounced than in the absence of Ca^{2+} (Fig. 2).

CTC inhibits NBT reduction and concomitant enzyme release by exocytosis induced by CB/FMLP in the absence of extracellular Ca^{2+}. Both NBT reduction and concomitant lysozyme release are inhibited at the same concentration of CTC, albeit at somewhat lower concentrations than with CB/FMLP-induced exocytosis. In the presence of extracellular Ca^{2+}, the inhibition is small (Fig. 3). The inhibiting effect of CTC on the metabolic burst appears to be strongly dependent on the activator. NBT reduction by PMA-activated cells is inhibited at a lower CTC concentration than NBT reduction (and concomitant enzyme release) by CB/FMLP-activated PMNs (Fig. 4).

Inhibition of NBT reduction by CTC is to some degree dependent on the preincubation time: inhibition is more pronounced with longer preincubation times than with (very) short preincubation times (Fig. 5). A large part of the inhibiting effect is obtained in the first 5 min of preincubation. When the preincubation times are longer than 15 min, little additional inhibiting effect is observed.

The inhibiting effect of CTC on chemotaxis and NBT reduction is largely reversible. Preincubation of cells with CTC, followed by removal of CTC and addition of new medium annihilates the inhibiting effect of CTC (Tables 2 and 3). The reversal is not quite complete: the same amount of CTC, added after preincubation, has a stronger effect in CTC-pretreated cells than in control cells (Table 3).

Addition of extracellular Ca^{2+} to CTC-pretreated PMNs annihilates the inhibiting effect of CTC on NBT reduction, both with CB/FMLP and PMA as activator. This effect is not specific for Ca^{2+}: the same annihilation of CTC inhibition can be observed after addition of extracellular Mg^{2+} to CTC-pretreated cells (Table 4).

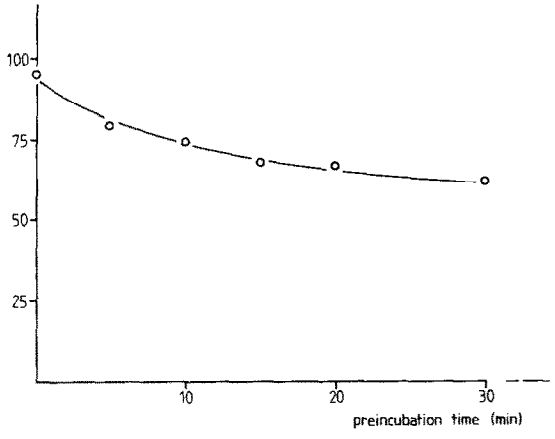

Fig. 5. Effect of preincubation time on CTC inhibition of FMLP-induced NBT-reduction. After a variable preincubation time a 37° CB/FMLP was added, followed by incubation for 20 min at 37°. The concentration of CTC was 10^{-4} M. The NBT reduction is expressed as a percentage of NBT reduction in the absence of CTC.

Table 2. Reversal of CTC inhibition of chemotaxis

Present during preinc.	Added after preinc.	Distance travelled (μm)
1 mM EDTA	1 mM EDTA	128 ± 6
1 mM EDTA	2 mM Ca^{2+}	121 ± 4
1 mM EDTA, 0.2 mM CTC	1 mM EDTA	133 ± 6
1 mM EDTA, 0.2 mM CTC	2 mM Ca^{2+}	128 ± 5
Controls (no removal of agents after preinc.)		
1 mM EDTA		124 ± 4
1 mM EDTA, 0.2 mM CTC		16 ± 2

Cells were preincubated with the agents indicated during 15 min at 22°. The controls were then submitted to chemotaxis. In the other experiments the cells were centrifuged and the supernatant removed. Fresh medium was added, containing the agents as indicated, and then the cells were submitted to chemotaxis. The results given are the mean values of three experiments ± S.D.

Table 3. Reversal of CTC inhibition of CB/FMLP-induced NBT reduction

Present during preinc.	Added after preinc.	nmoles NBT reduced/3 × 10^6 PMNs
1 mM EDTA		35.1 ± 0.6
1 mM EDTA	0.2 mM CTC	19.2 ± 0.1
1 mM EDTA, 0.2 mM CTC		34.9 ± 0.5
1 mM EDTA, 0.2 mM CTC	0.2 mM CTC	14.0 ± 0.5
1 mM EDTA	0.4 mM CTC	7.2 ± 0.9

PMNs were preincubated with the agents indicated during 15 min at 22°. Then the cells were centrifuged, and fresh medium containing the agents indicated was added. After addition of CB/FMLP the cells were incubated for 20 min at 37°.

The values given are the mean values of three experiments.

Table 4. The effect of Ca^{2+} and Mg^{2+} on CTC inhibition of NBT reduction

Present during: Preincubation	Incubation	nmoles NBT reduced/3 × 10^6 PMNs
—	1 mM EDTA, CB/FMLP	41.1 ± 1.6
0.2 mM CTC	1 mM EDTA, CB/FMLP	21.5 ± 0.7
—	1 mM Ca^{2+}, CB/FMLP	55.0 ± 3.7
0.2 mM CTC	2 mM Ca^{2+}, CB/FMLP	47.5 ± 2.4
—	2 mM Mg^{2+}, CB/FMLP	46.2 ± 3.6
0.2 mM CTC	2 mM Mg^{2+}, CB/FMLP	45.7 ± 1.6
—	1 mM EDTA, PMA	23.1 ± 0.4
0.1 mM CTC	1 mM EDTA, PMA	5.6 ± 0.8
—	2 mM Ca^{2+}, PMA	23.4 ± 0.2
0.1 mM CTC	2 mM Ca^{2+}, PMA	20.7 ± 1.2
—	2 mM Mg^{2+}, PMA	28.8 ± 0.6
0.1 mM CTC	2 mM Mg^{2+}, PMA	24.0 ± 2.0

PMNs were preincubated with 0.2 mM or 0.1 mM CTC for 15 min at 22°. Then EDTA, Ca^{2+} of Mg^{2+} was added, as well as the activating agent. Incubation was carried out for 20 min at 37° and NBT reduction was determined. The results given are the mean values of three experiments.

DISCUSSION

The results clearly show that CTC inhibits all PMN functions in the absence of extracellular Ca^{2+}. For the activation of the PMN Ca^{2+} ions, either from extracellular of intracellular sources, are supposed to play an important role [13–18]. In the absence of extracellular Ca^{2+} the PMN is dependent on intracellular Ca^{2+}. Smolen *et al.* found, that intracellular, but not extracellular Ca^{2+} plays an obligatory role in the response of the PMN to a variety of stimuli [19, 20]. In this regard the chemotactic peptide FMLP is extensively studied. Petroski *et al.* [28, 29] concluded from their experiments, that activation with FMLP causes a graded displacement of prebound Ca^{2+} and an increase of membrane permeability to Ca^{2+}. Whereas FMLP stimulated PMNs both in the absence and presence of extracellular Ca^{2+}, the stimulation in the absence of extracellular Ca^{2+} was blocked when the cells were depleted of

intracellular Ca^{2+} by pretreatment with the calcium ionophore A23187 [21, 30].

All these data suggest that in the absence of extracellular Ca^{2+}, the PMN uses Ca^{2+} from intracellular stores for the activation process. The liberation of this Ca^{2+} occurs upon stimulation with a suitable activating agent, such as FMLP. CTC forms a complex with Ca^{2+}, which is the base of its fluorescence properties, because the Ca–CTC complex has a polarity dependent fluorescence. However, by complexing intracellular Ca^{2+} CTC reduces the amount of available Ca^{2+} for triggering the series of processes, leading to the activation of the function measured, and therewith inhibits that function.

The reversal of CTC inhibition by washing, after pretreatment with CTC, and before addition of the activator, suggests that CTC complexes intracellular Ca^{2+} after it has been released by the activator, or that sufficient Ca^{2+} remains in the pools to give a complete response after removal of CTC. The reversal of CTC inhibition by washing further indicates that CTC moves rather rapidly across the PMN plasma membrane. This is supported by the finding that a large part of the inhibiting effect of CTC is obtained after a few minutes of preincubation, and that longer preincubation times only give a small increase of that effect.

In the presence of extracellular Ca^{2+}, or upon the addition of extracellular Ca^{2+} to CTC-pretreated cells, there is little or no inhibition of PMN functions by CTC. Because the Ca–CTC complex moves more easily across the membrane than free CTC [31], it means that Ca–CTC does not inhibit PMN functions. Therefore the conclusion seems justified that inhibition by CTC—in the absence of extracellular Ca^{2+}—depends on the Ca-complexing ability of CTC. The reversal of inhibition upon addition of extracellular Ca^{2+} to CTC-pretreated cells can be either a consequence of the ability of CTC to remove sufficiently rapidly into and and out of the cells, or an increased influx of extracellular Ca^{2+} upon addition of the stimulus, which alters the plasma membrane permeability to Ca^{2+}. An increase of Ca^{2+} permeability of the plasma membrane has been observed with FMLP [29], whereas stimulation with PMN induces a release of cell calcium [32]. If the latter possibility is playing a role then it is possible that the activator also changes the permeability of the membrane for Mg^{2+}, because Ca^{2+} and Mg^{2+} are equally effective in reducing the inhibition by CTC.

CTC inhibition of PMN functions apparently depends on the stimulus which is used to activate the PMN: PMA-activated cells are inhibited at lower concentrations than CB/FMLP-activated cells. This probably reflects differences in the mobilization of intracellular Ca^{2+}, but though it is known that differences exist—PMA activation is independent of extracellular Ca^{2+} [22], whereas FMLP activation depends on both extracellular and intracellular Ca^{2+} [19]—little is known about the nature of these differences. It may be that the differences in sensitivity may be explained on the basis that PMA activates the cell without a rise in intracellular Ca^{2+} [33].

Dependent on the conditions, especially with regard to the presence or absence of extracellular divalent cation, CTC may thus interfere with the PMN functions it is supposed to monitor. Interference with cell functions by CTC has also been observed in other cell types. CTC inhibits thrombin-induced secretion from platelets [34]. In mast cells CTC induces exocytosis when Ca^{2+} is present in the surrounding medium [35]. Under the conditions of our experiments however, CTC–Ca does not induce exocytosis in rabbit PMNs (results not shown).

CTC is frequently used in PMNs as well as in other cell types, for monitoring intracellular Ca^{2+} and for demonstration of the involvement of intracellular Ca^{2+} in cell functions. In most cases these experiments were performed in the presence of extracellular Ca^{2+} and no inhibition was observed. Nevertheless the results in this paper indicate that utmost care has to be taken in the interpretation of experiments where movements of intracellular Ca^{2+} are measured with CTC.

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