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CHLORPROMAZINE INHIBITS PHAGOCYTOSIS AND EXOCYTOSIS IN RABBIT POLYMORPHONUCLEAR LEUKOCYTES

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Abstract—Phagocytosis of zymosan particles and the concomitant release of lysosomal enzymes by exocytosis in polymorphonuclear leukocytes is inhibited by chlorpromazine. Cytochalasin B prevents particle uptake, but not enzyme release; the divalent cation ionophore A 23187 induces enzyme release in the absence of zymosan if Ca^{2+} is present. In both cases enzyme release is inhibited by chlorpromazine, thus demonstrating an effect of chlorpromazine on exocytosis independent from its effect on phagocytosis. The results are discussed in relation to the role of Ca^{2+} in exocytosis and the effect of chlorpromazine on membrane properties.

During phagocytosis of zymosan particles by polymorphonuclear leukocytes (PMN's) extracellular release of granule-associated enzymes is observed; there is no release of other cell constituents [1]. This selective release may be considered as a secretory process, which requires translocation of granules to the cell membrane, followed by fusion with the membrane. This enzyme release of PMN's strongly resembles secretory processes of other cell types. Phagocytosis and the translocation of granules depend on the functioning of a contractile system, consisting of microtubules and microfilaments [2–4]. In exocytosis membrane fusion is of crucial importance [5]. Both processes are incompletely understood. Apparently they require energy which is derived mainly from glycolysis [6, 7].

A number of investigators have suggested that the displacement of calcium ions from membranes is an essential step in cell fusion [8, 9]. Studying the effect of local anesthetics and phenothiazines on virus-induced cell fusion, Poste and Allison [5] found that these drugs significantly inhibited the fusion process. These drugs may inhibit cell fusion by occupying special sites within the plasma membrane.

Chlorpromazine is a phenothiazine with strong membrane-active properties [10]. It stabilizes erythrocyte membranes and inhibits membrane depolarization by the displacement of membrane bound calcium. This property is believed to be associated with the ability of chlorpromazine to inhibit virus-induced membrane fusion.

In this study the effect of chlorpromazine on the release of β-glucuronidase, lysozyme and lactate dehydrogenase (LDH) from rabbit PMN's is considered. LDH is a cytoplasmic enzyme and thus a marker for the integrity of the outer cell membrane. β-Glucuronidase is confined to the azurophilic granules (lysosomes) whereas lysozyme is present in both azurophilic and specific granules. Specific extracellular release of these two enzymes is a measure of exocytosis. Since the extracellular release of granule constituents is connected with a phagocytic stimulus, the influence of chlorpromazine on the uptake of zymosan particles is also considered here. In addition the influence of chlorpromazine on enzyme release is studied when a non-phagocytic stimulus (ionophore A 23187) is applied.

METHODS

Polymorphonuclear leukocytes were obtained from rabbits injected intraperitoneally (i.p.) with 200 ml of sterile isotonic saline, containing glycogen (1.5 mg/ml). The exudate was collected 6 hr later by flushing the peritoneal cavity with isotonic saline containing citrate (0.4%, pH 7.4). To the resulting suspension 10^{-3} M EDTA was added. The cells, consisting of about 99 per cent PMN's, were centrifuged and washed with medium. The medium used was Hank's solution, devoid of divalent cations. Zymosan (Sigma Chemical Co., St. Louis) was opsonized with fresh horse serum (5 mg per ml of serum) by incubation for 30 min at 37°. After washing, the particles were suspended in the medium. The final concentration of particles during incubation was 0.5 mg/ml. Ionophore A 23187 was a generous gift from Eli Lilly Labs. The ionophore was dissolved in dimethylsulfoxide to obtain a 0.01 M stock solution. Just before the experiment a dilution in medium was prepared: 10 μl 0.01 M ionophore solution was mixed with 2 ml medium. From this solution 10 μl was added to the incubation medium.

Aliquots of cell suspensions containing 5×10^6 PMN's were incubated with reagents in polypropylene tubes for 30 min at 37°, in a total volume of 1 ml. For this purpose 0.3 ml cell suspension was mixed with 0.7 ml medium, to which the chemicals described in the experiments were added in the concentration given before or in the legends of the figures. In the experiments with cytochalasin B the cells were preincubated with 5 μg cytochalasin B per ml for 30 min. For enzyme determinations the tubes were centrifuged at 500 *g* and the supernatant analyzed. For an evaluation of phagocytosis, 5×10^{-3} M EDTA was added at the end of incubation and then the uptake of zymosan

particles was quantitated by oil immersion microscopy. Cells with two or more zymosan particles were counted as phagocytic.

β-Glucuronidase was assayed by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-β-*D*-glucuronide. An aliquot (0.2 ml) of supernatant was incubated with 0.3 ml of the substrate (0.01 M) and 1.5 ml 0.2 M acetate buffer (pH 4.5) for 16 hr at 37°. Then 0.5 ml 1N NaOH was added and the extinction at 405 nm was measured. Lysozyme activity was determined by measuring the rate of lysis of *Micrococcus lysodeikticus* at pH 6.2, according to the method of Shugar [11]. Lactate dehydrogenase (LDH) was assayed by measuring the conversion of NADH in NAD^+ during the reaction of pyruvate to lactate. The enzyme activities were expressed as a percentage of total enzyme activity. These were determined after disruption of the cells with 0.2% Triton X-100.

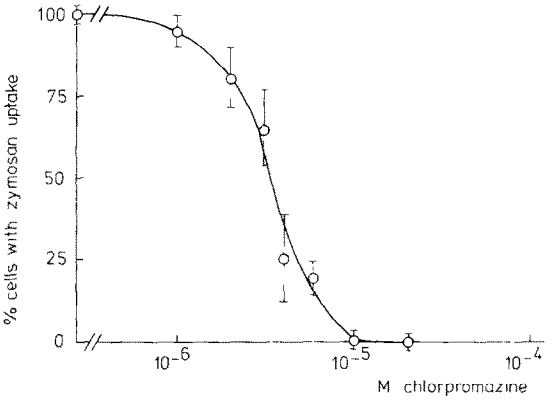

Fig. 1. Inhibition of zymosan phagocytosis by PMN's at different chlorpromazine concentrations.
In the incubations both Ca^{2+} (1 mM) and Mg^{2+} (1 mM) were present. Each point is the mean value of three separate determinations

RESULTS

The effect of chlorpromazine on phagocytosis and lysosomal enzyme release is represented in Figs. 1 and 2. Both the uptake of zymosan particles and the release of granule-associated enzymes is inhibited in a concentration dependent way. At 3×10^{-5} M chlorpromazine the inhibition of enzyme release is maximal. At higher concentrations the cell membrane is disrupted, as is evident from leakage of the cytoplasmic enzyme LDH. There is already considerable leakage of LDH when the release of glucuronidase and lysozyme is maximally inhibited.

For an optimal phagocytosis of zymosan particles and concomitant enzyme release both calcium and magnesium cations appear to be necessary. If only one of these divalent cations is present, there is little phagocytosis accompanied by enzyme release. This enzyme release (Fig. 3) is inhibited by chlorpromazine when calcium is present. In the presence of magnesium as the only divalent cation chlorpromazine is less effective; here a considerable individual variation was observed with rabbit PMN's and an inhibitory effect was sometimes observed and sometimes not.

Cytochalasin B completely inhibits the ingestion of zymosan particles, but not the attachment of zymosan to the cells. The release of granule-associated enzymes is not diminished [12]. In Fig. 4 the effect of chlorpromazine on enzyme release from cytochalasin B pretreated cells is depicted: there is an inhibition of enzyme release. As with magnesium, here too an individual variation was observed. In two of four series with zymosan + cytochalasin B there was a statistically clear inhibition of enzyme release by chlorpromazine

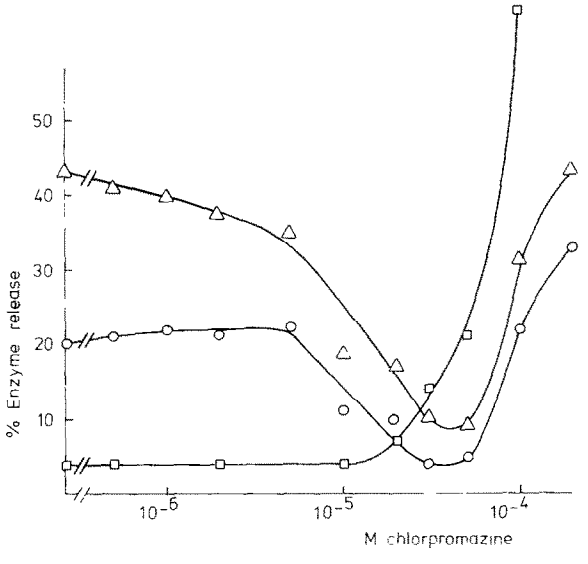

Fig. 2. Effect of chlorpromazine on enzyme release from rabbit PMN's, with zymosan as enzyme-release-inducing agent
–□–: LDH; –○–: β-Glucuronidase; –△–: lysozyme. In all experiments both Ca^{2+} (1 mM) and Mg^{2+} (1 mM) were present. Each point is the average of four determinations.

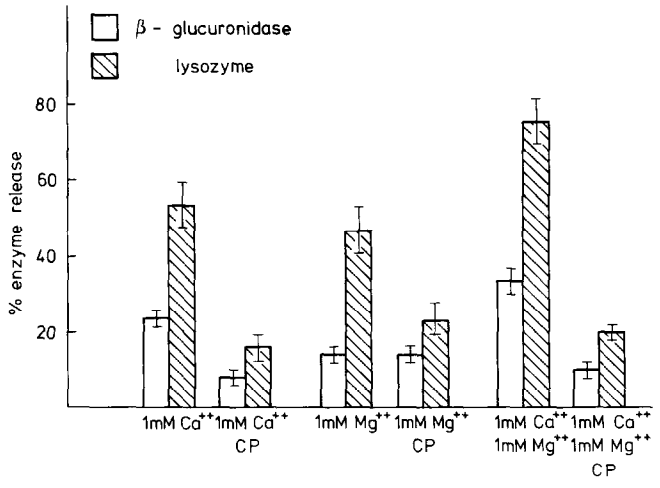

Fig. 3. Effect of chlorpromazine (2×10^{-5} M) on zymosan-induced enzyme release in the presence of either Ca^{2+} (1 mM) or Mg^{2+} (1 mM) or Ca^{2+} + Mg^{2+} (each 1 mM), CP = chlorpromazine.
The results represented are the mean value of six experiments on cells of one animal.

($P < 0.001$), in other series the effect was not evident.

The divalent cation ionophore A 23187 has been described as an effective non-phagocytic stimulus for the specific release of granule-associated enzymes from leukocytes [12, 13]. Under the conditions of our experiments with rabbit PMN's a release of glucuronidase and lysozyme was observed. In the absence of calcium, with 10^{-3} M EDTA, or with magnesium as the only divalent cation, there was no effect of the ionophore. As can be seen in Fig. 4, the chlorpromazine inhibits the ionophore-induced enzyme release. This inhibiting effect is very pronounced at an ionophore concentration of 5×10^{-7} M, and is much smaller at 10^{-6} M. The presence of a high calcium concentration (10 mM) partially reverses the inhibitory effect of chlorpromazine (Table 1).

With horse blood PMN's, isolated according the method of Tsan [14] the variation as described in the experiments with magnesium and cytochalasin B did not exist. In the presence of magnesium as only divalent cation chlorpromazine did not inhibit lysozyme release from horse leukocytes; chlorpromazine always inhibited lysozyme release induced by zymosan + cytochalasin B. With horse leukocytes there was also a clear inhibition of enzyme release by chlorpromazine with ionophore A 23187 in a concentration of 10^{-6} M.

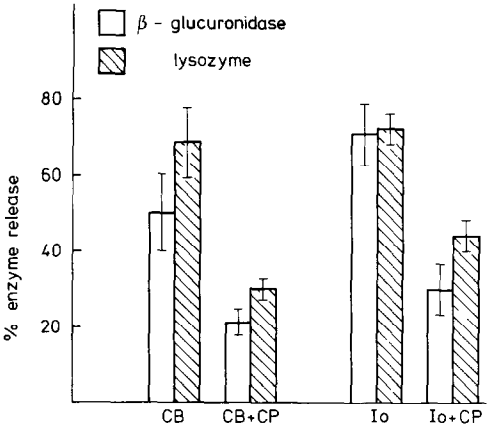

Fig. 4. Inhibition of lysosomal enzyme release from rabbit PMN's with zymosan + cytochalasin B (CB), or ionophore A 23187 as secretogogue.
The results are the mean of six experiments on cells of one animal. Chlorpromazine (CP) concentration: 2×10^{-5} M. Ionophore (Io) concentration: 5×10^{-7} M. In the experiments with cytochalasin B Ca^{2+} (1 mM) and Mg^{2+} (1 mM) were present; in the experiments with ionophore Ca^{2+} (1 mM) was present.

Table 1. Inhibition of lysozyme release by chlorpromazine (2×10^{-5} M) in the presence of ionophore A 23187 (5×10^{-7} M)) and different concentrations of calcium

	% lysozyme release *
Ionophore, 1 mM Ca^{2+}	72 ± 4
Ionophore, 10 mM Ca^{2+}	73 ± 5
Ionophore, 1 mM Ca^{2+}, chlorpromazine	44 ± 3
Ionophore, 10 mM Ca^{2+}, chlorpromazine	60 ± 6

* Mean value of six experiments, with S. D.

DISCUSSION

An inhibitory effect of chlorpromazine on leukocyte function has been described before. The results described in this paper resemble the effect of chlorpromazine on phagocytosis of *Staphylococcus epidermidis* [16] and the inhibitory effect of local anesthetics on lysosmal enzyme release [17]. Phagocytosis of zymosan and the concomitant enzyme release by exocytosis are closely coupled. The observed decrease in enzyme release could therefore be due to an effect on phagocytosis. With cytochalasin B + zymosan and ionophore A 23187 enzyme release occurs by exocytosis in the absence of phagocytosis [12, 13, 15]; chlorpromazine inhibits this enzyme release. Thus chlorpromazine appears to have two effects: inhibition of phagocytosis and inhibition of the exocytosis of granule constituents.

In the specific release of granule constituents several processes are involved. Glycolysis is supposed to provide the main source of metabolic energy, required for the process [6, 7]. Contractile systems and membrane fusion play an important role. The inhibition of enzyme release by chlorpromazine may be due to interference with one or more of these processes. Lahrichi *et al.* [18] studied the influence of chlorpromazine on the metabolism of leukocytes. They found that chlorpromazine inhibits glycolysis but only at high concentrations.

Though a role of Mg^{2+} in the uptake process cannot be denied, probably for the attachment phase [19], the presence of extracellular Ca^{2+} is of crucial importance for both the uptake of zymosan and the release of enzymes. The release of granule constituents from PMN's, especially with regard to the action of the ionophore A23187, bears a striking resemblance to histamine secretion from mast cells [20]. The divalent cation ionophore A23187 is known to facilitate a membrane flux of calcium. The addition of calcium ions has been shown to induce exocytosis, resulting in histamine release from mast cells and lysosomal enzyme release from PMN's.

The plasma membrane may be the main target for chlorpromazine action. Chlorpromazine has a high affinity for both the proteins and the lipids of the membrane [21, 22]. Many of its biological effects are due to interactions with membrane constituents. There is abundant evidence in the literature that chlorpromazine competes with calcium for membrane binding sites; chlorpromazine also inhibits calcium movement across the membrane [23, 26]. Our results are consistent with the hypothesis that chlorpromazine inhibits phagocytosis and enzyme release by interfering with certain membrane functions. As a consequence, chlorpromazine might create a situation in which calcium is not available at sites where it is required for phagocytosis or exocytosis. This interference may be due to a displacement of calcium from the membrane or to a change in the membrane permeability for calcium.

In agreement with the hypothesis is the finding of Kvarstein [27] that chlorpromazine has only a small inhibitory effect on latex phagocytosis in concentrations where we find a complete inhibition of zymosan uptake and phagocytosis. Extracellular calcium is crucial for the uptake of zymosan, but latex uptake is possible in the absence of calcium ions and divalent ions only have a modifying influence [28]. The observed variability of chlorpromazine inhibition may reflect differences in membrane composition and in permeability for calcium ions. A comparable variation in the response of leukocytes from different donors has been observed by other investigators [23].

Poste and Allison [5] have suggested that chlorpromazine inhibits cell fusion by removal of calcium from certain membrane sites. Chlorpromazine may inhibit exocytosis by a similar mechanism. Calcium ions also play a role in several (for exocytosis) important processes like microtubule assembly and cyclic nucleotide regulation [30, 31]. Therefore a correct evaluation of the effect of chlorpromazine on the fusion process remains to be determined.

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