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PROTAMINE SULFATE-INDUCED ENZYME SECRETION FROM RABBIT NEUTROPHILS

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Abstract—Protamine sulfate induces enzyme secretion from rabbit neutrophils. Enzyme secretion is mainly due to exocytosis but, depending on the experimental conditions, a small amount of cytolysis may occur. As compared with stimulation of neutrophil functions by other activators, protamine sulfate-induced enzyme release by exocytosis is a relatively slow process and is not accompanied by a marked activation of the metabolic burst. For optimal exocytosis, extracellular Ca^{2+} is required, but there is still some enzyme release in its absence, and other metal ions (Sr^{2+}, Ba^{2+}, Mg^{2+}) can partly mimic the effect of Ca^{2+}. Positive charges on protamine are of primary importance because the polyanion heparin completely inhibits protamine sulfate-induced enzyme release. Protamine linked to agarose beads is able to induce enzyme release; thus the induction of exocytosis is due to an interaction of the positive charges on protamine with the plasma membrane. Sialic acid residues on the membrane, however, seem not to play an important role in this process.

INTRODUCTION

Positively charged macromolecules play an important role in the defense reaction of the body against infection and in the inflammatory process. Cationic proteins are released by the neutrophil and the eosinophil during phagocytosis or upon stimulation with suitable activators. They cause a number of the symptoms of inflammation, and they possess bactericidal and tumoricidal properties (1–7). Furthermore these compounds may induce exocytosis in phagocytes and other secretory cells, causing the release of more inflammation-promoting substances (8–10).

Positively charged polyaminoacids, which are suitable model substances for the complex cationic proteins, likewise have bactericidal, antifungal, and tumoricidal properties (11). They induce enzyme secretion in mast cells and basophil leukocytes (9, 12) and lysis preceded by exocytosis in rabbit neutrophils (8).

Protamine sulfate is a cationic protein containing a high percentage of ar-

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ginine. Like a number of other polycations, it is an inhibitor of the growth of certain tumors but the systemic administration of protamine is limited by its toxicity (13–15). It has been shown to be capable of inducing degranulation in mast cells (16, 17). Erythrocytes were hemolyzed by protamine; hemolysis could be counteracted by both the isolated phospholipids and proteins of the erythrocyte membrane (18).

Some actions of protamine sulfate concerning neutrophil functions are described. Chemotaxis of neutrophils was inhibited by protamine sulfate (19). Phagocytosis by neutrophils and macrophages, on the other hand, was found to be significantly enhanced by protamine, as well as the killing of some types of bacteria (20, 21).

MATERIALS AND METHODS

Neutrophils. Neutrophils (polymorphonuclear leukocytes) were obtained from the peritoneal cavity of rabbits, as described previously (22). The medium used consisted of 140 mM NaCl, 5 mM KCl, 10 mM glucose, and 20 mM HEPES, pH 7.3. During the experiments, 1 mM Ca^{2+} was added unless otherwise indicated. During the experiments the cells, in a final concentration of 3×10^6 neutrophils/ml, were incubated for 30 min at 37°C. Then the cell suspension was centrifuged and the supernatant was analyzed.

Enzyme Release. The release of cytoplasmic enzyme lactate dehydrogenase (LDH) was considered as a measure for cell (plasma membrane) damage. The release of the granule-associated enzymes lysozyme and β-glucuronidase were measured to determine the involvement of granules in the release process. Lysozyme is present in both specific and azurophilic granules, whereas β-glucuronidase is present in the azurophilic granules only. The term exocytosis is used when there is a preferential release of granule-associated enzymes, e.g., when the percentage lysozyme release is higher than the percentage LDH release. LDH was determined by measuring the conversion of NADH into NAD^+ during the conversion of pyruvate into lactate. Lysozyme was determined by measuring the rate of lysis of *Micrococcus lysodeikticus*. Because protamine sulfate interacts with *M. lysodeikticus*, it was inactivated in the supernatant by addition of excess heparin. With high concentrations of protamine sulfate (more than 200 μg/ml), a precipitate was formed with heparin; this precipitate was removed by centrifugation and lysozyme determination was carried out in the supernatant. β-Glucuronidase was assayed by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-β-D-glucuronide, as described previously (22). 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 increase in nitroblue tetrazolium (NTB) reduction (23). For this purpose the medium was supplemented with 1 mM nitroblue tetrazolium chloride. After incubation with or without activator, 5 ml 0.5 M HCl was added to stop the reaction, the cells were centrifuged, and the residue was treated with 2 ml pyridine to dissolve the formazan that was formed during incubation. The absorbance of the pyridine solution was measured at 510 nm and, by comparison with chemically reduced NBT, converted into nanomoles NBT reduced in 30 min by 3×10^6 neutrophils.

Neuraminidase Treatment. The neuraminidase treatment was carried out in a PIPES-buffer, with the same composition as the medium, but PIPES instead of HEPES, and pH 6.4 instead of 7.3. In 2 ml buffer, pH 6.4, 10^8 neutrophils were incubated with 10 units neuraminidase (Sigma type V) for 30 min at 37°C. Then the cells were centrifuged; in the supernatant the liberated sialic acid was estimated according to Warren (24). The cells were washed with medium (pH 7.3) and

resuspended in medium. Control cells were treated in the same way, but without neuraminidase. Total sialic acid of the neutrophils was determined after treating the cells for 1 h at 80°C with 0.05 M H_2SO_4.

Materials. Protamine sulfate (Salmine), from salmon, grade X, and protamine-agarose were obtained from Sigma Chemical Co. Protamine-agarose, binding 88 μg calf thymus DNA per milliliter packed gel, was washed with medium, and used as a stock suspension of 50% gel. Neuraminidase (from *Clostridium perfringens*; type V), nitroblue tetrazolium chloride, f-Met-Leu-Phe, and cytochalasin B were from Sigma Chemical Co.

RESULTS

Protamine sulfate causes a concentration dependent exocytosis in rabbit neutrophils (Figure 1). There is little LDH release, but the LDH release is significantly higher than that of control cells and increases with increasing protamine sulfate concentrations. There is a moderate glucuronidase release and a much stronger lysozyme release, which has a maximum with about 10^{-4} g protamine sulfate/ml.

The stronger release of lysozyme as compared with glucuronidase is also apparent from the time course of enzyme release (Figure 2). As compared with another activator of exocytosis, the chemotactic peptide f-Met-Leu-Phe in the presence of cytochalasin B, protamine sulfate-induced exocytosis is a relatively

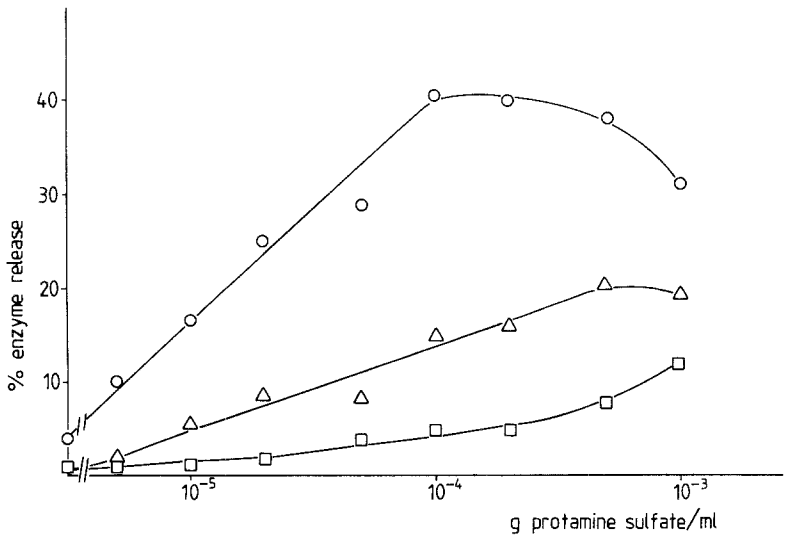

Fig 1. Enzyme release from rabbit neutrophils as a function of protamine sulfate concentration. Cells were exposed to the indicated amount of protamine sulfate for 30 min at 37°C, followed by centrifugation and analysis of the supernatant. The values given are the mean of six experiments. □, LDH release; ○, lysozyme release; △, β-glucuronidase release.

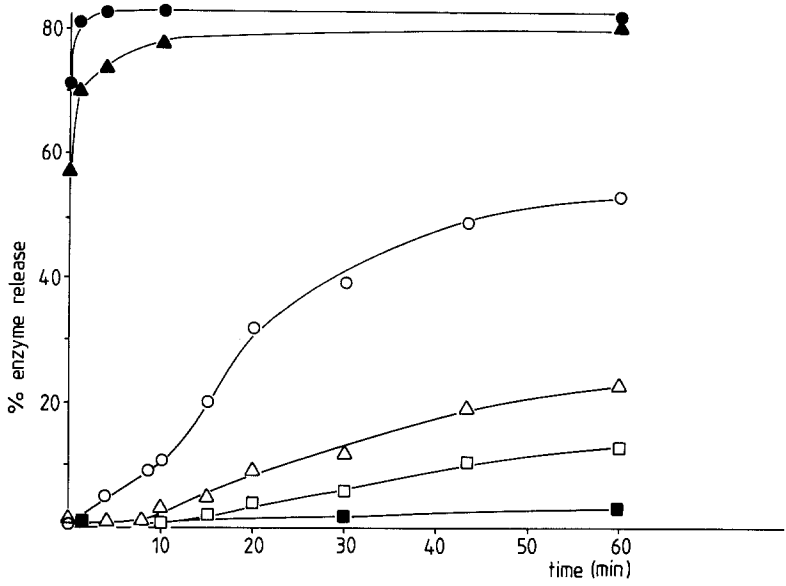

Fig. 2. Protamine sulfate-induced enzyme release from rabbit neutrophils as a function of time. As a comparison, f-Met-Leu-Phe + cytochalasin B-induced enzyme release is given. Neutrophils were exposed either to protamine sulfate (100 μg/ml or to f-Met-Leu-Phe (10^{-8} M) + cytochalasin B (5×10^{-6} M), for the indicated time in a shaking water bath at 37°C. Then the cells were centrifuged (1 min at 3000 rpm) and the supernatant was analyzed. The enzyme release at time 0 min thus represents the release that occurs during the handling of neutrophils to stop the reaction. The values given are the mean values of four experiments. □, LDH release, protamine sulfate; △, β-glucuronidase release, protamine sulfate; ○, lysozyme release, protamine sulfate; ■, LDH release, f-Met-Leu-Phe + cytochalasin B; ▲, glucuronidase release, f-Met-Leu-Phe + cytochalasin B; ●, lysozyme release, f-Met-Leu-Phe + cytochalasin B.

slow process. With f-Met-Leu-Phe exocytosis is nearly completed in the time required to stop the reaction by centrifugation (without incubation at 37°C). With protamine sulfate the strongest increase of lysozyme release occurs at about 15 min. Whereas lysozyme release levels off after incubation for 60 min, this is not the case for glucuronidase and LDH release. The time course of protamine sulfate-induced exocytosis strongly resembles the time course of polylysine-induced cytolysis in neutrophils (8).

Besides the difference in time course, there is another difference with other secretagogues: the extent of exocytosis, thus the maximal amount of lysozyme release, is considerably less for protamine sulfate as compared with other secretagogues. Whereas 30–40% lysozyme release is obtained with protamine sulfate, this is (with rabbit neutrophils) about 50–80% with ionophore A23187, f-Met-Leu-Phe + cytochalasin B, or fluoride (25).

Although most activators of exocytosis also strongly activate the metabolic burst in neutrophils, this is not the case with protamine sulfate. As compared

Table 1. Stimulation of Rabbit Neutrophils with Different Activators[a]

Activator	Exocytosis enzyme release (%)		Metabolic burst NBT reduction
	LDH	Lysozyme	
	1 ± 0	4 ± 3	1.5 ± 0.1
f-Met-Leu-Phe + CB	1 ± 1	72 ± 5	32.0 ± 2.1
PMA	2 ± 1	21 ± 3	18.7 ± 2.5
Ionophore A23187	3 ± 2	52 ± 4	4.9 ± 0.3
Protamine sulfate	7 ± 2	34 ± 2	2.8 ± 0.5

[a] Activation of exocytosis and NBT reduction were carried out in separate experiments, with cells of the same animal and under comparable conditions. The values given are the mean value of four (exocytosis) or three (NBT reduction) experiments ±*SD*. NBT reduction is expressed as nmol nitroblue tetrazolium (NBT) reduced per 3×10^6 neutrophils per 30 min. Concentrations: f-Met-Leu-Phe: 10^{-8} M; CB: 5×10^{-6} M cytochalasin B; PMA: 100 ng phorbol myristate acetate/ml; ionophore A23187: 5×10^{-7} M; protamine sulfate: 100 μg/ml.

with f-Met-Leu-Phe + cytochalasin B, or with phorbol myristate acetate, activation of the metabolic burst by protamine sulfate is very poor. In a sense protamine sulfate resembles ionophore A23187, which also gives a poor activation of the metabolic burst under conditions in which exocytosis is activated strongly (Table 1).

Extracellular Ca^{2+} ions are required for optimal exocytosis by protamine sulfate (Table 2). In the absence of divalent cation, with EDTA present to remove adherent divalent cations, exocytosis is strongly reduced, although not abolished. In decreasing order of effectiveness Sr^{2+}, Ba^{2+}, and Mg^{2+} can partly mimic the role of Ca^{2+} in protamine sulfate-induced exocytosis. In the presence

Table 2. Role of Divalent Cations in Protamine Sulfate and f-Met-Leu-Phe + Cytochalasin B-Induced Enzyme Release[a]

Cation	Enzyme release (%)			
	Protamine Sulfate		CB + FMLP	
	LDH	Lysozyme	LDH	Lysozyme
—	5 ± 1	12 ± 2	0 ± 1	35 ± 5
1 mM EDTA	6 ± 1	11 ± 1	1 ± 0	22 ± 3
1 mM Ca^{2+}	6 ± 2	39 ± 3	1 ± 1	71 ± 7
1 mm Mg^{2+}	8 ± 2	19 ± 4	0 ± 0	31 ± 3
1 mM Sr^{2+}	11 ± 2	32 ± 4	2 ± 1	71 ± 4
1 mM Ba^{2+}	5 ± 4	20 ± 3	0 ± 0	16 ± 2

[a] Cells were added to Ca^{2+}-free medium, supplemented with the divalent cations as indicated and with either 100 μg protamine sulfate/ml, or 10^{-8} M f-Met-Leu-Phe (FMLP) + 5×10^{-6} M cytochalasin (CB). Values given are the mean of four experiments ±*SD*.

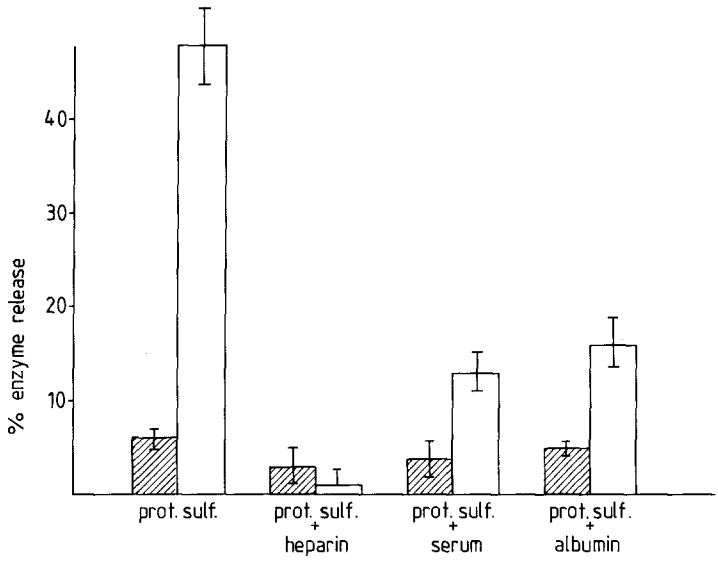

Fig. 3. Effect of heparin and proteins on protamine sulfate-induced enzyme release. Cells were added to a mixture containing reagents and 100 μg protamine sulfate/ml, and incubated for 30 min at 37°C; prot.sulf.: protamine sulfate, 100 μg/ml; heparin: 0.5 mg (or 70 USP K-1 units)/ml; serum: 5 mg rabbit serum (lyophilized)/ml; albumin: 5 mg rabbit albumin (lyophilized)/ml. Values given are the mean values of four experiments $\pm SD$. ▨: LDH release; ▭: lysozyme release.

of Sr^{2+}, however, there is significantly more LDH release upon exposure to protamine sulfate. The dependence on extracellular metal ions of protamine sulfate-induced exocytosis strongly resembles that of f-Met-Leu-Phe + cytochalasin B-induced exocytosis (Table 2).

Several substances interfere with the exocytosis-inducing effect of protamine sulfate. The negatively charged polymer heparin completely annihilates the effect of protamine sulfate. The same action, albeit somewhat less pronounced, was observed with serum and albumin (Figure 3). Removal of sialic acid from the plasma membrane of neutrophils by treatment with neuraminidase has little effect of protamine sulfate-induced enzyme release (Figure 4).

A number of reagents, known to interfere with neutrophil functions, inhibit to different degrees protamine sulfate-induced exocytosis. This applies to sulfhydryl reagents [1,5-difluoro-2,4-dinitrobenzene (26), ethacrynic acid (27), and *N*-ethylmaleimide], inhibitors of lipoxygenase [nordihydroquaiaretic acid (28)] and phospholipase A_2 [*p*-bromophenacylbromide (29)], and to inhibitors of glycolysis (iodoacetic acid and 2-deoxyglucose). Whereas lysozyme release is reduced by all these substances, LDH release is little changed or enhanced (Table 3). Protamine, coupled to agarose beads with a diameter of 50–100 μM, is still able to induce exocytosis (Figure 5). Both lysozyme release

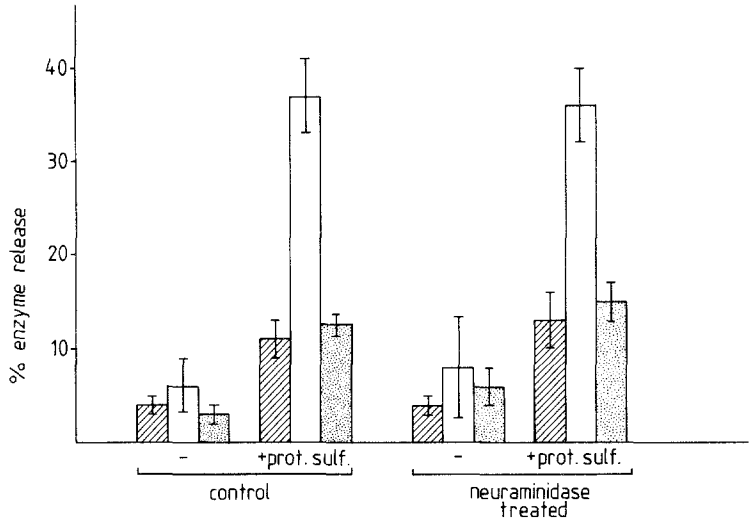

Fig. 4. Effect of pretreatment with neuraminidase on protamine sulfate-induced enzyme release. Neuraminidase-treated cells and control cells were obtained as described in Materials and Methods; prot.sulf.: 100 μg protamine sulfate/ml. Values given are the mean values of four experiments $\pm SD$. ▨ LDH release; □: lysozyme release; ▩ glucuronidase release. The total sialic acid content of the cells amounted to 76 nmol/10^8 cells; treatment with neuraminidase released 9 nmol/10^8 cells.

Table 3. Effect of Several Exocytosis Inhibitors on Protamine Sulfate-Induced Enzyme Release[a]

Inhibitor	Enzyme release (%)	
	LDH	Lysozyme
	6 ± 1	43 ± 2
1,5-Difluoro-2,4-dinitrobenzene (10 μM)	8 ± 3	6 ± 2
p-Bromophenacylbromide (10 μM)	12 ± 4	15 ± 4
Nordihydroguaiaretic acid (25 μM)	22 ± 1	23 ± 3
Ethacrynic acid (100 μM)	8 ± 2	10 ± 4
N-Ethylmaleimide (100 μM)	9 ± 3	14 ± 6
Iodoacetic acid (0.5 mM)	9 ± 3	13 ± 3
2-Deoxyglucose (10 mM)	11 ± 4	21 ± 6

[a] Cells were preincubated with the given reagents in the concentrations as indicated, for 10 min at 37°C. The protamine sulfate (100 μg/ml) was added, followed by incubation for 30 min at 37°C. Values given are the mean values of four experiments $\pm SD$. In the absence of protamine sulfate, the reagents do not cause cell damage (LDH release <5%).

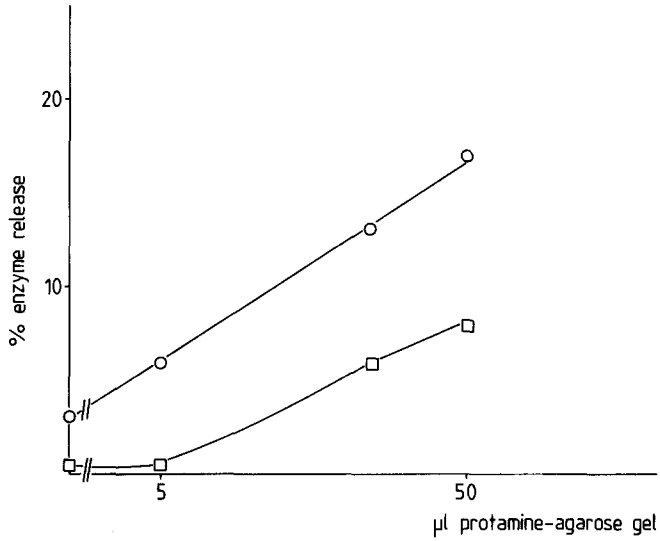

Fig. 5. Effect of agarose-linked protamine on enzyme release from neutrophils. Cells were incubated with the indicated amount of packed protamine-agarose gel (added to the mixture as the double amount of a 50% gel suspension) for 1 h at 37°C. Then the cells were centrifuged, and enzyme release in the supernatant was determined. Control cells (no protamine treatment) gave 1 ± 1% LDH and 4 ± 2% lysozyme release; 50 µl packed protamine-agarose gel gave 4 ± 1% LDH and 1 ± 2% lysozyme release if 500 µg heparin/ml was present; 50 µg protamine sulfate per ml gave a release of 14 ± 2% LDH and 39 ± 5% lysozyme under the conditions (incubation time: 1 h) of this experiment. The values given are the mean values of four experiments; □, LDH release; ○, lysozyme release.

and LDH release increase with increasing agarose-protamine concentration, but the percentage of lysozyme release is higher than that of LDH release. The enzyme release by agarose-coupled protamine is negligible if heparin is present in the medium.

DISCUSSION

The results show that protamine sulfate is able to induce exocytosis of both specific and azurophilic granules in rabbit neutrophils. Exocytosis of specific granules is more pronounced than that of azurophilic granules, as can be deduced from the higher percentage of lysozyme release as compared with glucuronidase release. Depending on the experimental conditions, a limited amount of cytolysis can be observed, as is evident from the percentage of LDH release.

With regard to its ability to induce enzyme release, protamine sulfate fits in the general picture of polycationic molecules. Polycations may induce exocytosis or cytolysis, depending on cell type and experimental conditions (8, 9,

11). An example is polylysine, which induces exocytosis in mast cells and basophils (9, 11), and cytolysis preceded by exocytosis in rabbit neutrophils (8); under certain conditions cytolysis could be shifted in the direction of exocytosis or vice versa. To a lesser degree the same can be observed with the effect of protamine sulfate on neutrophils: a slight enhancement of lysis sometimes occurs, when exocytosis is inhibited (Table 3). Whereas polyarginine induced mainly cytolysis in neutrophils (8), protamine sulfate—whose positive charge is mainly due to the high amount of arginine residues—primarily induces exocytosis under comparable conditions. These results support the previoulsy proposed hypothesis that exocytosis and cytolysis have certain traits in common.

Protamine, polylysine, and polyarginine induce hemolysis in erythrocytes (18, 30). Maximal hemolysis occurred at a certain optimal polycation concentration; higher concentrations gave a lower percentage of hemolysis. It was suggested that the lowering of hemolysis at polycation overdosage was due to cell-surface reorganization (30). We observed a comparable phenomenon with polyarginine-induced LDH release and with polylysine-induced lysozyme release in neutrophils; here too an optimal polycation concentration existed for maximal enzyme release. With protamine sulfate an optimal concentration also exists for maximal lysozyme release. It is possible that a reorganization of membrane structures at high protamine sulfate concentrations results in a diminished response, but apparently cytolysis, and exocytosis of specific granules are affected differently.

The hydrophilic character of polycations make it unlikely that these substances can easily penetrate into the cell. The experiment with agarose-coupled protamine confirms that protamine exerts its effects by an interaction with the outer surface of the plasma membrane, because the particles are too large to be phagocytized and thus the protamine cannot get into the cell. Apparently there are structures present whose perturbations by polycations results in the initiation of events leading to exocytosis (or cytolysis). That positive charges of protamine sulfate are indeed crucial for its effect is demonstrated by the fact that the negatively charged polymer heparin completely annihilates the effect of protamine sulfate. The inhibition of serum on protamine sulfate-induced exocytosis—which may be relevant for in vivo actions of protamine sulfate—may also be due to shielding of positive charges by the negatively charged albumin.

Because of the predominant role of positive charges for the induction of exocytosis by protamine sulfate, it seemed reasonable to expect that negatively charged sialic acid on the plasma membrane could be a primary target. The experiments with neuraminidase-treated cells do not support this supposition: it seems that sialic acid residues on the cell surface play a limited, if any, roll in protamine sulfate-induced enzyme release. It remains to be determined whether proteins or the lipid bilayer of the plasma membrane are primarily involved in polycation-induced enzyme release.

Concerning the requirement of extracellular Ca^{2+}, two classes of activators

can be distinguished. On one hand, there are some activators, such as ionophore A23187 and fluoride (31), which are completely dependent on extracellular Ca^{2+}. In the presence of other, chemically similar, metal ions such as Sr^{2+}, no exocytosis takes place (31). Protamine sulfate- or f-Met-Leu-Phe-induced exocytosis, on the other hand, are optimal in the presence of extracellular Ca^{2+}, but there is still some exocytosis in its absence. Furthermore, other alkaline earth metal ions, such as Sr^{2+}, are able to replace Ca^{2+}.

There are two main features which make protamine sulfate an exceptional activator of neutrophil function. The first feature is the lack of activity of protamine sulfate to induce a metabolic burst in neutrophils. Most activators of exocytosis also give a strong activation of the metabolic burst (32, 33). This is, however, not the case with protamine sulfate which is only a very poor activator of the metabolic burst. In a sense, it resembles ionophore A23187. The latter compound, however, may give a strong activation of the metabolic burst in rabbit neutrophils, but then higher concentrations of A23187 are necessary than required for optimal exocytosis (34). Another difference with other activators has to do with the time course of enzyme release. Most soluble activators of exocytosis, such as f-Met-Leu-Phe, give a rapid exocytosis. Protamine sulfate-induced exocytosis however, is a relatively slow process, which may provide certain advantages in the study of the mechanism of exocytosis.

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