Biochemical Pharmacology, Vol. 29, pp. 3051–3057.

0006-2952/80/1115-3051 $02.00/0

THE INTERACTION OF FLUORIDE WITH RABBIT POLYMORPHONUCLEAR LEUKOCYTES: INDUCTION OF EXOCYTOSIS AND CYTOLYSIS

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(*Received* 20 *May* 1980; *accepted* 26 *June* 1980)

Abstract—Pretreatment of rabbit polymorphonuclear leukocytes (PMN's) with sodium fluoride, followed by removal of extracellular fluoride and addition of calcium, results in strong exocytosis. This is shown by the selective release of granule-associated components such as lysozyme and β-glucuronidase. The degree of exocytosis is dependent on temperature and fluoride concentration during preincubation. This effect is characterized by a pH-dependent lag time. Removal of fluoride and addition of calcium to PMN's preincubated for a period shorter than the lag time does not result in exocytosis. Fluoride-dependent exocytosis is a rapid process, being complete after 3 min incubation with calcium, and is dependent on the calcium concentration. The penetrating sulfhydryl reagents cytochalasin A and *N*-naphtyl maleimide, and the glycolysis inhibitors 2-deoxyglucose and iodoacetate, inhibit fluoride-dependent exocytosis. Fluoride-dependent exocytosis did not occur in human peripheral PMN's in contrast to rabbit (peritoneal) PMN's. Exocytosis may be due either to an interaction of fluoride with the inner side of the membrane or to a reaction with a specific membrane component. Apparently thereby calcium is enabled to cross the membrane and to act intracellularly. When calcium and fluoride are simultaneously present in the medium either exocytosis or cytolysis may occur. The hemolytic action of calcium and fluoride on erythrocytes suggests that here cytolysis is due to an interaction of calcium fluoride crystals with the cell.

Apart from its inhibitory effect on glycolysis, fluoride stimulates hexose monophosphate shunt activity and induces an increased oxygen consumption in polymorphonuclear leukocytes (PMN's) [1, 2]. Later studies have shown that in these cells fluoride is an effective stimulator of superoxide anion production and of chemiluminescence [3,–6]. In other cell types fluoride has been shown to release various cell constituents. In platelets, fluoride induces serotonin release, while lactate dehydrogenase (LDH) release is unaffected; in addition oxygen consumption was increased [7, 8]. Patkar *et al.* [9, 10] have found that in mast cells fluoride induces calcium-dependent histamine release. Other authors, however, reported the inhibition by fluoride of histamine release due to compound 48/80, a known secretogogue for mast cells [11].

With regard to PMN's, Selvaraj and Sbarra have reported [2] an interesting observation, namely, that granules disappear in fluoride-treated cells. Harvath *et al.* [5] and Curnutte *et al.* [6], however, found no degranulation in PMN's at 20 mM fluoride.

In the present study the ability of fluoride (in combination with calcium) to induce exocytosis or cytolysis in PMN's, depending on the experimental conditions, was considered. With regard to cytolysis, the action of fluoride and calcium on erythrocytes was also taken into account. For PMN's the release of the cytoplasmic enzyme LDH was measured as a degree of cytolysis. The release of the granule-associated enzymes lysozyme and β-glucuronidase was considered as a measure of exocytosis if under these circumstances LDH release was negligible.

MATERIALS AND METHODS

PMN's and erythrocytes. Rabbit polymorphonuclear leukocytes were obtained from the peritoneal cavity after stimulation with glycogen, as described earlier [12]. Human PMN's were isolated from peripheral blood and purified with a Ficoll-Hypaque gradient according to previously described methods [13]. Contaminating erythrocytes were lysed by exposure to 0.85% NH_4Cl for 3 min. The medium used in preincubation as well as in incubation consisted of 140 mM NaCl, 20 mM Tris–HCl pH 7.2, 5 mM KCl and 10 mM glucose. Human erythrocytes were obtained from fresh blood. The blood was centrifuged and the buffy coat cells discarded. The erythrocytes were washed three times with medium.

Experimental procedure. In a number of experiments the PMN's (3×10^6) were added to the medium described above with additional reagents as required and then incubated in a shaking waterbath for 30 min at 37°. The total volume was 1 ml. After incubation the cells were centrifuged at 500 *g* and the supernatant fraction was analysed. In those experiments where only exocytosis was studied the cells (3×10^6 PMN's) in a total volume of 1 ml, were preincubated for 20 min at 37° in the presence of fluoride. Then 2 ml medium was added, and the mixture was centrifuged. The supernatant fraction

was discarded, and then medium and Ca^{2+} (1 mM) was added to a total volume of 1 ml. After mixing the mixture was incubated for 30 min at 37°. In the experiments with erythrocytes the cells were added to a mixture containing all reagents, in a total volume of 5 ml, with 5×10^7 erythrocytes per ml. Incubation with erythrocytes was carried out for 2½ hr at 37°. Then the cells were centrifuged and hemolysis was assayed by measuring the absorption of the supernatant fraction at 540 nm.

Assays. Lysozyme was assayed by measuring the rate of lysis of *Micrococcus lysodeikticus*, at pH 6.2, according to the method of Shugar [14]. β-Glucuronidase was assayed by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-β-D-glucuronide. Lactate dehydrogenase (LDH) was assayed by measuring the conversion of NADH into NAD^+ during the conversion of pyruvate into lactate. Enzyme release was expressed as a percentage of the maximum value, obtained by treating PMN's with 0.2% Triton X-100.

RESULTS

Treatment of rabbit PMN's with fluoride in the absence of calcium does not result in significant enzyme release. In the presence of calcium the results of the interaction of fluoride with PMN's strongly depend on the experimental procedure. Three procedures will be distinguished:

(a) PMN's are preincubated with sodium fluoride, after which the mixture is diluted with medium, and centrifuged. The supernatant fraction is discarded and Ca^{2+}-containing medium is added, followed by incubation at 37°. This procedure appears to result in exocytosis. Modulation of exocytosis by varying the experimental conditions during preincubation with fluoride is treated in section A, while the effect of various conditions during incubation with calcium is treated in section B.

(b) Preincubation of PMN's with sodium fluoride, followed by addition of calcium to the medium in which fluoride is still present, and incubation at 37°.

(c) Incubation of PMN's with fluoride and calcium added simultaneously, without preincubation.

Procedures (b) and (c) may lead to cytolysis or exocytosis; the results of these experiments are treated in section D.

(A) *Exocytosis: variations during preincubation with fluoride*

Exocytosis is induced by addition of a calcium-containing medium to rabbit PMN's preincubated with sodium fluoride. The effect is dependent on the concentration of fluoride during the preincubation (Fig. 1). Low concentrations (below 5 mM) of fluoride do not result in exocytosis after addition of calcium. At about 10 mM fluoride the resulting exocytosis is maximal.

The temperature during preincubation has a strong influence on exocytosis afterwards. Preincubation with fluoride at 4° and 10°, followed by incubation with calcium at 37°, does not result in significant exocytosis. Maximal exocytosis occurs after preincubation with fluoride at 37°. At 45° exocytosis is completely blocked; we also observed this phenomenon in the absence of fluoride, with other secretogogues. The influence of the length of preincubation time (with fluoride) on exocytosis is depicted in Fig. 2. From this figure it appears that there is a lag time, after which exocytosis strongly increases. We have repeated this experiment — which was carried out at pH 7.2 — at other pH values and observed a strong dependence of the lag time on pH. The lag time is very short at a relative low pH and becomes longer as the pH increases (Figs. 2 and 3).

(B) *Exocytosis: variations during incubation with calcium*

Calcium-induced exocytosis in fluoride-treated PMN's is a time-dependent but rapid process. After removal of fluoride and addition of calcium, lysozyme and glucuronidase release are maximal after

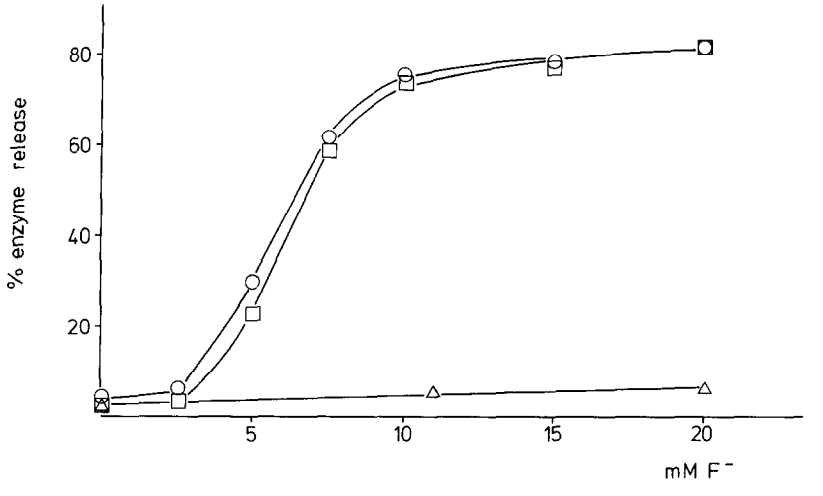

Fig. 1. Effect of fluoride concentration during preincubation on the subsequent exocytosis. Rabbit PMN's were preincubated with various concentrations of sodium fluoride. After 20 min preincubation at 37°, 2 ml medium was added and the mixture was centrifuged. Then the volume was brought to 1 ml with medium, containing 1 mM Ca^{2+}. After 30 min incubation at 37° the cells were centrifuged and the supernatant fraction was analysed. —○— lysozyme; —□— β-glucuronidase; —△— LDH. Each point is the mean value of four determinations.

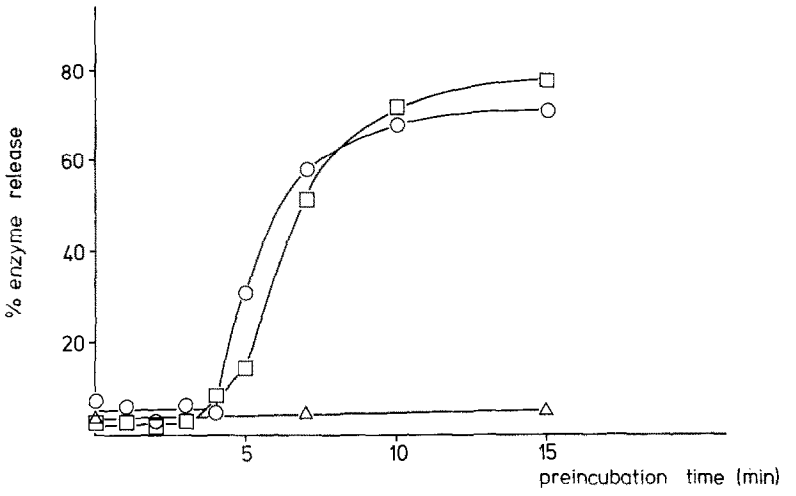

Fig. 2. Influence of the length of preincubation time with fluoride on the subsequent release of enzymes. Preincubation with 20 mM sodium fluoride was carried out for a variable time at 37°; incubation was carried out for 30 min at 37° (pH 7.2). Further experimental procedure was as described in the legend to Fig. 1. —○— lysozyme; —□— β-glucuronidase; —△— LDH. Each point is the mean value of four experiments.

about 3 min (Fig. 4). The dependence on calcium concentration during incubation is represented in Fig. 5. Up to 0.1 mM calcium no exocytosis occurs. At 1 mM calcium exocytosis is maximal and at higher concentrations calcium exocytosis diminishes. At the highest calcium concentration used (5 mM), LDH release occurs.

(C) *Exocytosis: β-glucuronidase release, inhibition, rabbit vs human PMN's*

A number of secretogogues induce the selective release of components of the specific granules, as is apparent from the release of lysozyme in the absence of the release of β-glucuronidase. In fluoride-dependent exocytosis, however, as is apparent from Figs. 1, 2 and 4–6, β-glucuronidase release equals lysozyme release.

The metabolic inhibitors 2-deoxyglucose and iodoacetate and certain penetrating sulfhydryl reagents strongly inhibit fluoride-dependent exocytosis (Tables 1 and 2). Cytochalasin B has a small inhibiting effect, whereas colchicine is without effect on fluoride-dependent exocytosis. Because fluoride is known as an inhibitor of glycolysis and has been described as an inhibitor of several PMN functions, the effect on exocytosis of prolonged preincubation with fluoride was considered. When preincubation

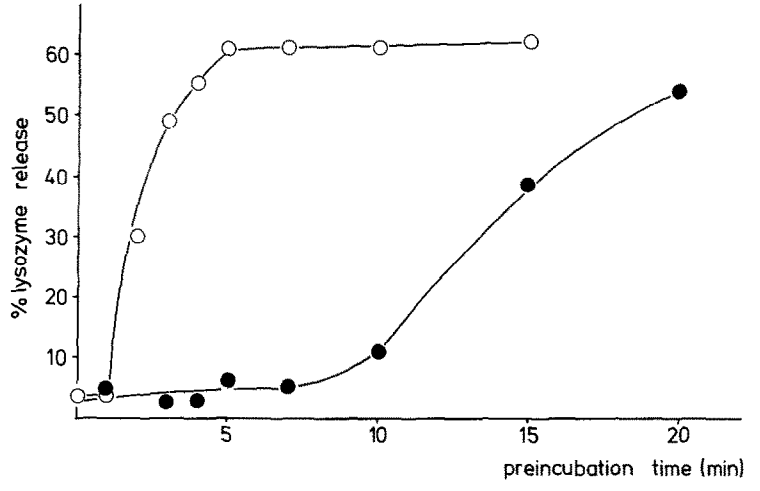

Fig. 3. Influence of the pH during preincubation with fluroide on the lag time of lysozyme release upon exposure to calcium. The experiment was the same as described in Fig. 2, except for the pH during preincubation. —○— pH 6.3; —●— pH 7.8. Composition of the buffer: 115 mM NaCl, 5 mM KCl, 40 mM Tris of given pH, 10 mM glucose. pH during incubation was 7.2. Each point is the mean value of four experiments.

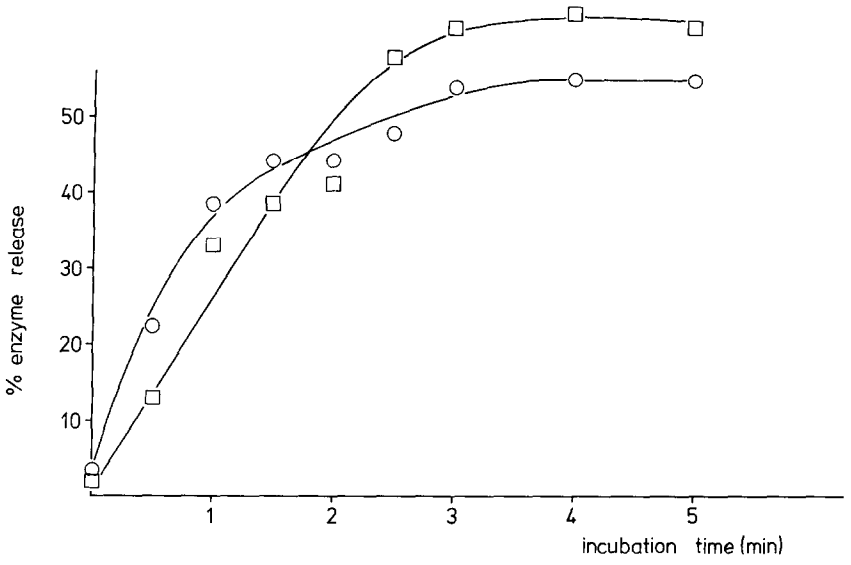

Fig. 4. Effect of incubation time on fluoride-dependent exocytosis. Preincubation with 20 mM sodium fluoride was carried out for 20 min at 37°. After removal of fluoride and addition of calcium (1 mM)-containing medium incubation was carried out for the time indicated. —○— lysozyme; —□— β-glucuronidase. Each point is the mean value of four experiments.

time exceeded 20 min, exocytosis gradually decreased. Exocytosis was absent when the preincubation time was increased to 80 min (Fig. 6).

Some investigators did not find degranulation with fluoride in their experiments [5, 6]. We compared the action of fluoride on rabbit peritoneal PMN's and on human peripheral PMN's (Table 3). It appeared that the origin of the PMN's determines the extent of the effect of fluoride. Whereas pretreatment with fluoride results in extensive exocytosis after pretreatment with calcium in rabbit PMN's, no exocytosis occurs with the same treatment in human PMN's.

(D) *Cytolysis vs. exocytosis*

If PMN's were added to a mixture containing sodium fluoride and calcium, cytolysis (release of LDH) was predominant (Table 4). If calcium was added to a mixture of PMN's and fluoride, exocytosis became prevalent (more lysozyme release as compared to LDH release). When fluoride was removed after preincubation of PMN's with fluoride, while subsequently a medium containing calcium was

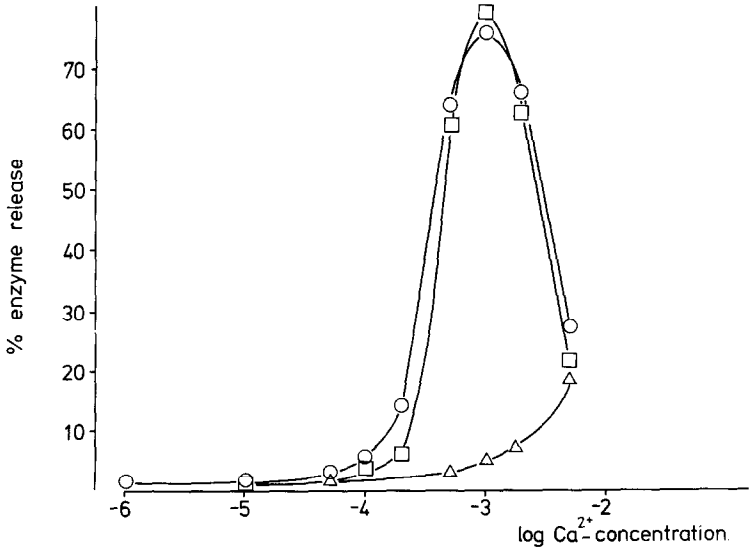

Fig. 5. Influence of calcium concentration during incubation. Preincubation with 20 mM sodium fluoride was carried out for 20 min at 37°. After removal of fluoride, and addition of medium containing a variable concentration of calcium, incubation was carried out for 30 min at 37°. —○— lysozyme; —□— β-glucuronidase; —△— LDH.

Table 1. Effect of sulfhydryl inhibitors, cytochalasin B and colchicine on fluoride-dependent exocytosis

Treatment	Lysozyme release (%)
Control	2 ± 1
F/Ca*	55 ± 4
F/Ca, 5μM cytochalasin A	3 ± 1
F/Ca, 3μM *N*-naphtylmaleimide	27 ± 7
F/Ca, 25 μM *N*-ethylmaleimide	16 ± 5
F/Ca, 25 μM dithiodipyridine	16 ± 5
F/Ca, 250 μM DTNB†	48 ± 6
F/Ca, cytochalasin B (5 μg/ml)	38 ± 5
F/Ca, 10^{-6} M colchicine	55 ± 3
F/Ca, 10^{-5} M colchicine	61 ± 5

* F/Ca: rabbit PMN's were preincubated for 20 min at 37° with 20 mM sodium fluoride and the reagents indicated, in a total volume of 1 ml. Then 2 ml medium was added, the mixture was centrifuged, and the supernatant fraction discarded. Then the cells were suspended in medium again containing the reagents indicated and 1 mM Ca^{2+}, in a total volume of 1 ml. This mixture was incubated at 37° for 30 min. The values given are the mean value of four experiments ± S.D.

† DTNB: 5,5'-dithiobis-(2-nitrobenzoic acid).

Table 2. Effect of inhibitors of glycolysis on fluoride-dependent exocytosis*

Treatment	Lysozyme release (%)
Control	7 ± 4
F/Ca	66 ± 4
F/Ca, 2 mM 2-deoxyglucose	58 ± 6
F/Ca, 10 mM 2-deoxyglucose	46 ± 4
F/Ca, 20 mM 2-deoxyglucose	12 ± 4
F/Ca, 0.1 mM iodoacetate	47 ± 3
F/Ca, 0.5 mM iodoacetate	37 ± 4
F/Ca, 1.0 mM iodoacetate	32 ± 3

* The experimental procedure is the same as described in Table 1; in the medium used, however, glucose was omitted.

added, only exocytosis occurs (Table 4, preceding sections).

Fluoride and calcium-induced cytolysis in PMN's is a complex phenomenon with a number of aspects which are difficult to interpret, e.g. the absence of lysozyme release during treatment of PMN's with calcium and fluoride, and the presence of lysozyme release with calcium, magnesium and fluoride (in rabbit PMN's). To obtain information about the combined effect of alkaline earth ions and fluoride on plasma membranes we performed some experiments with human erythrocytes (Table 5). It appears that Mg^{2+}, though ineffective when used with fluoride alone, strongly potentiates hemolysis induced by calcium and fluoride. Increase of calcium concentration also strongly influences hemolysis. On the other hand, negatively charged compounds such as pyruvate and especially the polymer poly-D-glutamic acid, inhibit hemolysis. Polyvinylpyridine-*N*-oxide has no influence on the lytic process.

DISCUSSION

When rabbit PMN's are pretreated with sodium fluoride, followed by removal of fluoride and addition of calcium, then a strong and rapid exocytosis occurs. There is considerable release of lysozyme and β-glucuronidase, in the absence of significant LDH release. Contrary to the selective release of lysozyme, induced by some other secretogogues, e.g. phorbol myristate acetate [15], β-glucuronidase and lysozyme are liberated in equal amounts. This suggests involvement of azurophilic as well as specific granules in fluoride-dependent exocytosis.

The interaction of fluoride with the cell membrane may enable extracellular calcium to trigger the process of exocytosis. It is possible that fluoride facilitates the passage of calcium across the membrane,

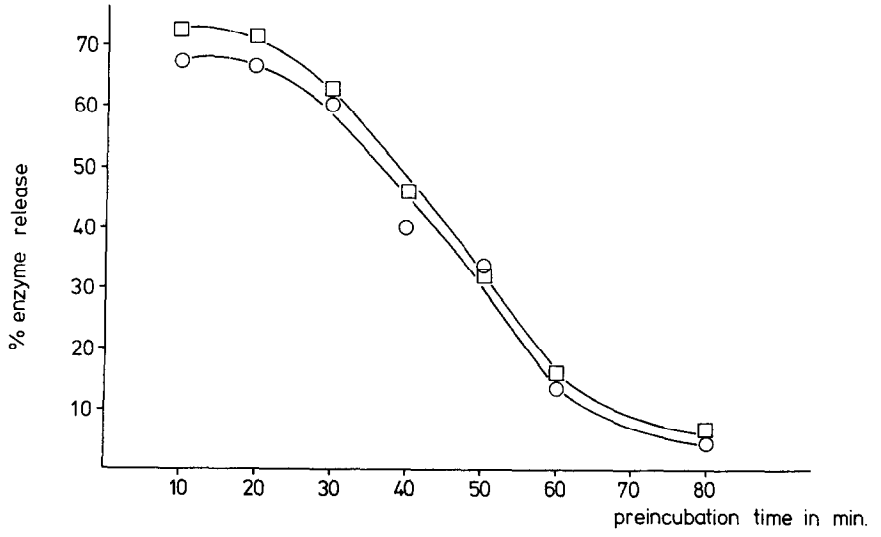

Fig. 6. Effect of prolonged preincubation with sodium fluoride on subsequent exocytosis. Preincubation with 20 mM fluoride was carried out during the time indicated, at 37°, followed by removal of fluoride and incubation with 1 mM calcium at 37° for 30 min. —○— lysozyme; —□— β-glucuronidase.

Table 3. Comparison between the effect of fluoride on human peripheral PMN's and rabbit exudate PMN's*

Treatment	Rabbit PMN's			Human PMN's		
	LDH	Lysozyme	Glucuronidase	LDH	Lysozyme	Glucuronidase
Control	3 ± 2	3 ± 2	3 ± 1	3 ± 1	4 ± 4	6 ± 3
Ca^{2+}, Mg^{2+}, F^-†	43 ± 4	52 ± 6	39 ± 6	59 ± 4	21 ± 3	20 ± 4
F/Ca‡	5 ± 2	80 ± 6	59 ± 5	2 ± 1	4 ± 3	3 ± 1

* Concentrations used: F^-, 20 mM; Ca^{2+}, 1 mM; Mg^{2+}, 1 mM. The values given are the mean of four experiments ± S.D.

† Ca^{2+}, Mg^{2+} and F^- simultaneously present in the medium.

‡ Preincubation with fluoride for 20 min at 37°, followed by removal of fluoride and addition of calcium, and incubation for 30 min at 37°.

Table 4. Influence of the experimental procedure on the effect of fluoride on rabbit PMN's*

Treatment	Procedure A†			Procedure B‡			Procedure C§		
	LDH	lys	glucu	LDH	lys	glucu	LDH	lys	glucu
F	5 ± 2	2 ± 1	2 ± 1	6 ± 2	0 ± 3	2 ± 1	5 ± 2	1 ± 1	2 ± 1
F, Ca	5 ± 2	80 ± 6	59 ± 4	33 ± 4	11 ± 3	5 ± 3	31 ± 4	4 ± 2	4 ± 2
F, Ca, Mg	5 ± 1	78 ± 6	60 ± 7	17 ± 4	80 ± 8	45 ± 6	53 ± 5	57 ± 6	45 ± 6
F, 2 Ca	8 ± 2	69 ± 4	49 ± 2	22 ± 3	4 ± 3	4 ± 3	68 ± 3	15 ± 3	15 ± 3
F, 2 Mg	5 ± 1	0 ± 2	3 ± 1	7 ± 2	0 ± 2	2 ± 1	5 ± 2	0 ± 2	1 ± 1

* Abbreviations used: LDH, lactate dehydrogenase; lys, lysozyme; glucu, β-glucuronidase; F, 20 mM sodium fluoride; Ca, 1 mM Ca^{2+}; Mg, 1 mM Mg^{2+}; 2 Ca, 2 mM Ca^{2+}; 2 Mg, 2 mM Mg^{2+}.

† Procedure A: cells were preincubated with fluoride (20 min at 37°), then the fluoride-containing medium was removed and calcium containing medium added, followed by incubation for 30 min at 37°.

‡ Procedure B: after preincubation with fluoride for 20 min at 37°, calcium was added (no removal of fluoride), followed by incubation for 30 min at 37°.

† Procedure C: all chemicals were simultaneously present; incubation for 30 min at 37°. All values given are percentages of maximal enzyme release, and are the mean value of four experiments ± S.D.

Table 5. Cytolytic effect of fluoride and calcium on human erythrocytes

Treatment	Hemolysis (%)
	0 ± 0
1 mM Ca^{2+}, 20 mM F^-	16 ± 3
1 mM Ca^{2+}, 30 mM F^-	20 ± 3
2 mM Ca^{2+}, 20 mM F^-	43 ± 5
2 mM Ca^{2+}, 30 mM F^-	51 ± 4
2 mM Mg^{2+}, 30 mM F^-	0 ± 0
1 mM Ca^{2+}, 1 mM Mg^{2+}, 20 mM F^-	69 ± 4
1 mM Ca^{2+}, 1 mM Mg^{2+}, 30 mM F^-	79 ± 6
1 mM Ca^{2+}, 1 mM Mg^{2+}, 20 mM F^-, 5 mM pyruvate	35 ± 6
1 mM Ca^{2+}, 1 mM Mg^{2+}, 20 mM F^-, PGA (20 μg/ml)	26 ± 16
1 mM Ca^{2+}, 1 mM Mg^{2+}, 20 mM F^-, PVPNO (0.2%)	68 ± 16

* Erythrocytes were treated as described in Materials and Methods, with all reagents indicated present during incubation. Pretreatment with fluoride followed by removal of fluoride and exposure to Ca^{2+} or Ca^{2+} and Mg^{2+} did not give any lysis. The values given are the mean of six experiments, ± S.D. PGA: poly-D-glutamic acid; PVPNO: polyvinylpyridine-*N*-oxide.

while calcium induces intracellularly the sequence of events leading to exocytosis.

Fluoride-dependent exocytosis can be modulated in the same way as ionophore A23187-induced exocytosis in which calcium enters the cell. There is a strong inhibition by penetrating sulfhydryl reagents (cytochalasin A and *N*-naphtylmaleimide [16, 17]) and exocytosis is inhibited by the metabolic inhibitors 2-deoxyglucose and iodoacetate. These results are somewhat paradoxical because fluoride is itself an inhibitor of glycolysis. The situation resembles that noted in fluoride-induced superoxide production: this process is also inhibited by deoxyglucose [18]. The decrease of exocytosis after prolonged preincubation with fluoride could possibly result from its inhibitory effect on glycolysis.

A remarkable aspect of fluoride-dependent exocytosis is the occurrence of a critical preincubation period with fluoride. A similar phenomenon was also observed in the interaction of fluoride with platelets [7] and mast cells [10]. The length of the lag-time strongly depends on the pH: it becomes longer as the pH increases. Some time is required for fluoride to associate with the cell in a pH-dependent manner. Calcium has no effect in PMN's after a shorter preincubation period with fluoride than this critical lag-time. A possible method for fluoride to penetrate if for it to cross the membrane in non-ionized form — the K_a of HF is 3.5×10^{-4} [19] — and this process is favoured by a lowering of the pH.

Changes in cell function due to inorganic fluoride appear to be pH-dependent; for instance fluoride inhibits the mobility of bull spermatozoa at pH 6.5 three times as effectively as at pH 7.0. Since there is a rise in HF-concentration due to this pH-change the investigators suggested that fluoride enters mammalian cells by weak-acid equilibration [20]. In HeLa cells an intracellular/extracellular *F*-distribution ratio of 0.27–0.37 was found after 60 min in a fluoride-containing medium at the physiological pH, and a similar ratio has been suggested for PMN's [21, 22]. The required time-span for fluoride-incubation of PMN's and the influence of the pH both indicate that fluoride accumulates inside the cells and thereby make the cells sensitive to subsequently added extracellular calcium.

Low temperatures during fluoride preincubation prevent exocytosis. Restricted mobility of membrane components at low temperature may prevent the passage of fluoride across the membrane, and so this observation supports the penetration hypothesis. The alternative hypothesis, namely that fluoride interacts with specific membrane components in a pH- and temperature-dependent process, the resulting compound being responsible for the change in permeability for Ca^{2+}, appears less likely. Exocytosis can be induced by phagocytosis, by calcium in combination with an ionophore, and by phorbol myristate acetate. It appears that the combination of fluoride and calcium may also effectively induce exocytosis, and the observed phenomena may provide some clues as to the mechanisms underlying exocytosis.

In accordance with previously published results [5, 6], we found no exocytosis by fluoride and calcium in human peripheral PMN's. Apparently there are considerable differences between the structure and/or composition of rabbit peritoneal PMN's and human peripheral PMN's. A simultaneous presence of calcium and fluoride may cause either exocytosis or cytolysis in rabbit PMN's, depending on the experimental conditions. Observations on the cytolytic effect of the combination of calcium and fluoride on erythrocytes suggest that calcium (or magnesium) potentiates cytolysis, whereas negatively charged compounds such as pyruvate and poly-D-glutamic acid strongly inhibit cytolysis. Polyvinyl pyrinine-*N*-oxide, a strong hydrogen acceptor, is without effect. Cytolysis induced by calcium and fluoride thus strongly resembles calcium oxalate crystal-induced cytolysis, which we have studied earlier [23]. Though we did not obtain visible precipitates of calcium fluoride (in the absence of cells) under the conditions of our cytolysis experiments, the concentrations used (1 mM Ca^{2+}, 20 mM F^-) are far in excess of the solubility product of CaF_2 (3.4×10^{-11}) [19]. The results obtained may be explained tentatively on the assumption that the cells accelerate the formation of CaF_2 microcrystals, which cause membrane damage and lysis through ionic interactions with membrane components [23]. This damage may be caused either directly or after they have been phagocytized and taken up in phagolysosomes.

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