Metabolic aspects of the secretion of stored compounds from blood platelets

The effect of NaF at different pH on nucleotide metabolism and function of washed platelets

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The purpose of this study was to investigate the response of human blood platelets to fluoride at different pH. The results were as follows. (1) Fluoride induced secretion faster and at a lower concentration when pH was lowered. (2) Platelets exposed to 2 mM-fluoride at 0°C at pH 5.3 underwent secretion when first pH and then temperature was raised, although no secretion was seen at 2 mM-fluoride concentration in the absence of the preincubation at low pH. (3) The concentration of [¹⁴C]ATP in platelets decreased steeply in response to fluoride before induction of secretion. Addition of antimycin blocked or partly inhibited secretion. Fluoride thus exerts an inhibitory effect on platelet glycolysis before induction of secretion. (4) Fluoride accumulated in the platelet pellet by a time course that preceded secretion. The accumulation was faster and greater at pH 6 than at 7.4. These four points are taken as indirect evidence that fluoride has to penetrate to the interior of the platelet to induce secretion. The activation takes place over a wide range of acid pH in contrast with induction of platelet function via the outside of the plasma membrane. In addition evidence is presented that the salvage pathway may under special circumstances play an important role in the re-synthesis of platelet adenine nucleotides.

NaF (10 mM) induces a slow secretion of adenine nucleotides, serotonin (5-hydroxytryptamine) and Ca²⁺ from washed human blood platelets at physiological pH without a marked increase in leakage of cytoplasmic material (Mürer, 1968, 1969; Mürer & Holme, 1970; Mürer *et al.*, 1974). The fluoride-induced secretion needs 15 min to reach completion at pH 7.4 and 37°C compared with 1 min for thrombin, but seems otherwise to fill the criteria for the 'platelet-release reaction' (Holmsen *et al.*, 1969), i.e. an energy-dependent extrusion from storage organelles directly to the external medium. The mode of action is so far unknown. It is not linked to fluoride's action as a metabolic inhibitor, as the combined effect of fluoride and an inhibitor of respiration blocks the secretion (Mürer, 1968; Mürer & Holme, 1970; Mürer *et al.*, 1974). A basic question has been whether fluoride acts on the exterior of the platelet or whether it must penetrate the plasma membrane to induce secretion. Vascular smooth muscle contracts at pH 7.4 when exposed to 10 mM-fluoride, whereas lower concentrations have little or no effect (Bergey, 1975). Fluoride (10 mM) excites ventral photoreceptors in *Limulus* (Fein & Corson, 1979), thus suggesting that the activation of

cellular processes by fluoride is a widespread and possibly general phenomenon. A lower pH decreases the threshold of fluoride's effect on the smooth-muscle system (Bergey, 1975). Oral bacteria increase their uptake of fluoride greatly when pH is lowered, which may be one explanation for the caries-preventive effect of fluoride (Whitford *et al.*, 1977; Eisenberg & Marquis, 1980). The main aim of the present study was to determine whether the slowness of the NaF-induced secretion was linked to the speed of penetration of NaF and whether this increased when pH was lowered. This was studied directly by correlating uptake of fluoride with the time of onset of secretion and also by investigating the effect of NaF on nucleotide metabolism in platelets. The direct effect on glycolysis and the effects secondary to platelet activation can be distinguished because of the time difference between initial decrease in metabolic [ATP] and initiation of secretion.

Experimental

[U-¹⁴C]Adenine (sp. radioactivity 281 Ci/mol) was obtained from Amersham Corporation,

Chicago, IL, U.S.A.; [^3H]serotonin binoxalate (sp. radioactivity 4.3 Ci/mmol) was from New England Nuclear, Boston, MA, U.S.A.; antimycin A, 2-deoxy-D-glucose, 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene from Sigma, St. Louis, MO, U.S.A. Imipramine was a gift from the Ciba-Geigy Corp., London, U.K., and Summit, NJ, U.S.A.

The experiments were performed with human platelets prelabelled with [^3H]serotonin and [^{14}C]adenine and treated as previously described (Mürer *et al.*, 1974, 1976). The incubation medium was made up with 0.13 M-NaCl and 10 mM-sodium citrate of the described pH. Fluoride was added to the flasks before the start of incubation or after preincubation (details in Figure legends). When incubation was initiated at a low pH and then continued at a higher pH, the latter pH adjustment was made with 0.4 M-Tris/HCl of pH 7.9. Tris (50 mM)/HCl was needed to neutralize 10 mM-citrate of pH 5.3 and 25 mM-Tris/HCl was needed to neutralize 10 mM-citrate of pH 6 for a final pH of 7.3–7.4. Incubation was stopped by cooling in an ice bath. The determination of ^{14}C -labelled nucleotides and [^3H]serotonin has previously been described (Mürer *et al.*, 1976).

Determination of fluoride in the platelet pellet

A suspension (4 ml) of [^3H]serotonin-labelled washed platelets from 150 ml of blood was incubated with 10 mM-NaF at pH 6 or 7.4. Samples (1 ml) were removed from the incubation mixture and added to 9 ml of ice-cold 0.15 M-NaCl with 10 mM-Tris/HCl, pH 7.9. After 20 min centrifugation at 2000 g the platelets were washed once with ice-cold NaCl/Tris/HCl and finally suspended in 0.1 ml of 10% Triton X-100 and 1 ml of TISAB III buffer and mixed with a Pasteur pipette. In each sample the fluoride concentration was measured with a model 96-09 Orion combination fluoride electrode.

Determination of adenylate energy charge

The determination was done as described by Mills (1973).

Determination of protein

This was done by the biuret method (Gornall *et al.*, 1949) with human albumin (fraction V) from Sigma as standard.

Results and discussion

The present study investigates the response of washed human platelets to exposure to fluoride at different pH; specifically, to test the hypothesis that fluoride must penetrate the platelet plasma membrane to induce platelet secretion.

The increased effect of fluoride on platelet secretion and nucleotide metabolism at lower pH

NaF (10 mM) caused slow secretion from platelets and, after a 1 min lag, a drop in [^{14}C]ATP concentration that preceded secretion (Mürer *et al.*, 1974). At pH 6, a slow secretion was observed with 1 mM-NaF (Fig. 1a) and a fast secretion with 10 mM-NaF with a rate comparable with that induced by thrombin (Mürer, 1968) (Fig. 1b). A steep fall in [^{14}C]ATP concentration was accompanied by an increase in [^{14}C]ADP, [^{14}C]AMP and [^{14}C]IMP concentration in a wave that was more pronounced at the higher [NaF]. [Inosine] and [hypoxanthine] increased with the decrease in the three di- and mononucleotides lagging behind secretion at high [NaF] and preceding secretion at low [NaF].

At pH 5.3, fast secretion was induced with 2 mM-fluoride and a significant secretion could be obtained even at 0.3 mM-fluoride (Table 1). Slow secretion occurs without secretagogue at this pH, but does not influence the fast release induced by NaF. Both at pH 5.3 and 6 energy charge and platelet integrity is preserved (no increase in leakage is observed) (Mürer & Stewart, 1977).

The initial drop in metabolic [ATP] (reflecting inhibition of enolase) precedes secretion at each tested pH, indicating that glycolysis is inhibited before the induction of secretion. It is delayed for about 1 min at pH 7.4, but is immediate and steep for two different concentrations at pH 6 and at 5.3. At pH 6 the drop is much steeper with 10 mM- than with 1 mM-fluoride. This indicates that at the high pH the rate of penetration of fluoride is limiting, whereas at lower pH the external concentration determines the rate of increase in intracellular [fluoride].

Direct measurement of fluoride in platelets

When platelets were incubated with 10 mM-fluoride at pH 7.4 and then washed with about 100 vol. of buffered saline, about 7 μM -fluoride was found in the cold in the 1 ml suspension of platelets containing 6 mg of protein. The fluoride concentration in the lysate reached about 18.5 μM after 8 min at 37°C and stabilized at this value. Secretion of serotonin took place after 8 min incubation. Platelets exposed to 10 mM-NaF at pH 6.0 showed a fast increase in fluoride uptake, reaching about 38 μM in the platelet suspension after 3 min incubation, followed by a decrease in fluoride content. Secretion lagged behind the fluoride uptake, but reached a plateau at 3 min of about 90% secretion of the platelet store of pre-absorbed serotonin, again indicating that fluoride penetrates the platelet membrane before exerting its induction effect.

The concentration of non-dissociated HF as a percentage of added NaF increases 10-fold when pH is decreased from 7 to 6 ($\text{p}K_{\text{HF}} = 3.17$; Holleman & Wiberg, 1976). This increases the passive transport

Fig. 1. Changes in concentrations of intracellular ATP metabolites and extracellular serotonin in response to incubation with fluoride

Platelets were prelabelled with [^{14}C]adenine and [^3H]serotonin, isolated and washed as described in the Materials and methods section. A buffered platelet suspension was incubated a total of 20 min and NaF was added after 0, 5, 10, 13, 15, 17 or 19 min incubation to give a total incubation time with NaF of 20, 15, 10, 7, 5, 3 and 1 min respectively. The zero point represents platelets

of fluoride through the platelet plasma membrane and might therefore explain the above results.

Preloading with NaF at low pH in the cold

Incubation at 37°C is not needed for the uptake of NaF at lower pH. Platelets incubated for 10 min at pH 7.6 and 37°C with 2 mM-fluoride showed little increase in extracellular serotonin (7.2% against 3–4% by incubation in the absence of secretagogue). After 60 min with 2 mM-fluoride in ice-bath conditions at low pH followed by incubation at 37°C and pH 7.6 we observed a decrease in metabolic [ATP], a transient increase in [IMP] and finally an increase in [inosine] and [hypoxanthine] and an accompanying drop in metabolic [IMP] after 10 min incubation. Extracellular serotonin increased to 29.3% after 1 min and 50% after 10 min incubation (Table 2). Thus sufficient fluoride was accumulated to give an immediate impulse to secretion to at least 20% of the platelets. There was no significant increase in leakage of cytoplasmic material, so that the increase in extracellular serotonin represents true secretion.

When platelets were exposed to 1 mM-fluoride for 15 min at 0°C and pH 5.3, the subsequent incubation at pH 7.6 and 37°C resulted in an initial drop in adenine nucleotides accompanied by an increase in [inosine] and [hypoxanthine]. Between 3 and 10 min incubation there was a rise in [^{14}C]adenine nucleotides, which between 3 and 6 min corresponded with a fall in [^{14}C]IMP concentration and between 6 and 10 min with a decrease in [inosine] and [hypoxanthine] (Fig. 2).

This indicates that IMP can serve as an efficient source for metabolic ATP in the platelet, presumably via the succinate intermediate. Rivard *et al.* (1975) found that hypoxanthine can serve as a substrate for the synthesis of adenine nucleotides in platelets incubated for 1–4 h, but we observed an effect after only 3 min incubation. They suggested that the synthesis took place via the hypoxanthine–guanine phosphoribosyltransferase reaction,

incubated for 20 min without added fluoride. The Figure represents the amount of radioactivity in the different ATP metabolites in the whole platelet suspension after incubation as a percentage of total [^{14}C] radioactivity and radioactivity of serotonin in the supernatant after removal of the platelets as a percentage of [^3H] radioactivity in the whole platelet suspension. Symbols: $\circ$, [^{14}C]ATP; $\blacktriangle$, [^{14}C]ADP; $\triangle$, [^{14}C]AMP; $\square$, [^{14}C]IMP; $\bullet$, [^{14}C]inosine + hypoxanthine; $\blacksquare$, [^3H]serotonin. In (a), the suspension was incubated at pH 6 (buffered with 10 mM-sodium citrate) with 1 mM-NaF. In (b), the suspension was incubated at pH 6 with 10 mM-NaF. (b) represents the mean for two experiments, (a) represents the mean for four experiments.

Table 1. *Effect of incubation at 37°C of a platelet suspension at two different pH values in succession*

Experiments were performed as outlined in the legend to Fig. 1. Two sets of platelet suspensions were incubated for 10 min at 37°C with different concentrations of NaF. The initial pH was 5.3. The second set contained 0.5 ml of 0.265 M-NaCl less than first set. The first set was cooled and the content of supernatant [³H]serotonin and the distribution of ¹⁴C radioactivity among ATP metabolites was determined. To the second set was added 0.5 ml of 0.4 M-Tris/HCl, pH 7.9, to give a final pH value of 7.3–7.4 and the incubation was continued for 5 min, followed by cooling and removal of samples. ATP metabolites are expressed as the percentages of ¹⁴C in the total incubation mixture; serotonin is given as a percentage of the total [³H]serotonin found extracellularly. Results are means for two experiments.

Incubation	[NaF] (mM)	ATP	ADP + AMP	IMP	Inosine + hypoxanthine	Serotonin in supernatant	Adenylate energy charge
10 min at pH 5.3	0	36.5	43.5	6.1	13.5	6.1	0.601
+ 5 min at pH 7.3		56.9	19.4	4.7	18.4	9.4	0.847
10 min at pH 5.3	0.1	18.0	45.1	10.7	25.6	7.9	0.440
+ 5 min at pH 7.3		40.9	16.3	4.9	37.7	12.9	0.826
10 min at pH 5.3	0.3	6.1	39.4	14.1	39.4	23.2	0.298
+ 5 min at pH 7.3		34.9	15.5	6.4	42.8	32.0	0.808

Table 2. *Effect of exposure of platelets to 2 mM-fluoride at 0°C at pH 5.3 on subsequent incubation at pH 7.4 at 37°C*

The platelets were prepared as described in the Experimental section and incubated in saline buffered with 10 mM-sodium citrate, pH 5.3, either with or without NaF. After incubation in an ice bath the pH of the incubation mixture was increased to pH 7.3–7.4 by the addition of 50 mM-Tris/HCl, pH 7.9, whereafter portions were taken out and incubated for 1 and 10 min followed by cooling. Samples were collected after incubation at pH 5.3, after adjustment of pH to 7.6 and after 1 and 10 min incubation at 37°C at this pH. Results are means for four experiments.

Incubation time at pH 5.3 and 0°C (min)	60	60	60	60	0
pH increased to 7.6	No	Yes	Yes	Yes	Yes
Incubation time at 37°C (min)	—	0	1	10	10
¹⁴ C (% of total in incubation mixture)					
ATP	31.4	39.7	32.2	16.9	41.0
ADP	36.1	27.5	16.6	13.1	26.0
IMP	3.7	11.4	35.5	10.8	3.2
AMP	26.2	18.4	6.5	4.7	6.6
Inosine + hypoxanthine	2.4	2.6	9.1	54.2	24.2
Radioactivity in supernatant (%)					
¹⁴ C-labelled nucleotides	7.6	8.6	9.0	7.6	5.8
[³ H]Serotonin	2.7	9.9	29.3	50.0	7.2

since the synthesis was absent in platelets from patients with the Lesch–Nyhan syndrome, who lack this enzyme (Van Lancker, 1976). Jerushalmy *et al.* (1973) have found phosphoribosyl pyrophosphate synthetase and purine phosphoribosyltransferase activity in human and rabbit platelet lysates, indicating that enzymes are available for the initial part of the salvage pathway for the conversion of hypoxanthine into IMP. The presence of adenylosuccinate lyase has not been demonstrated (Holmsen *et al.*, 1977), but a relatively high concentration of GTP (about 5% of total nucleotide radioactivity) indicates that such a pathway for AMP production might be quite active (Mürer, 1969).

Effect of low pH on platelet viability

Platelets that have been incubated at pH 5.3 and

0°C without NaF show no increase in extracellular serotonin at pH 7.4 and 37°C. Given the lack of leakage of nucleotides after exposure to pH 5.3 and 6 (Mürer & Stewart, 1977) the platelet plasma membrane seems not to have been extensively damaged by the lower pH. This is thus not likely to be a cause of the increased penetration of fluoride.

The decrease in metabolic [ATP] and in adenylate energy charge after incubation with fluoride at pH 5.3 can be reversed by incubation at 37°C and pH 7.6, whether platelets are pre-incubated with fluoride at 37°C (Table 1) or are preloaded with fluoride at 0°C. Such a reversion after exposure to non-physiological conditions could be utilized for storage of platelets. The value of the adenylate energy charge as a parameter for functionality outside the physiological pH seems questionable, but its reversibility may be of great importance in deter-

Fig. 2. Evidence for the 'salvage pathway' The Figure presents evidence that a resynthesis of ATP has taken place in the platelet incubation mixture and that the radioactivity label came partly from IMP and partly (after 6 min) from inosine and hypoxanthine. The experiment was performed as described in Table 2. Samples were collected at 0, 1, 3 and 10 min of incubation at pH 7.6 and 37°C. . . . , [¹⁴C]ATP; - · - · - , [¹⁴C]IMP; —, ¹⁴C-labelled adenine nucleotides; ----, [¹⁴C]inosine + [¹⁴C]-hypoxanthine. Results are means for two experiments.

mining how far the lowering of pH has irreversibly damaged the platelets.

Metabolic effects of inhibitors

The addition of antimycin, which together with fluoride would block energy metabolism (Mürer, 1968) inhibited secretion strongly at pH 6, but was less effective at pH 5.3 (Table 3). At both pH values an immediate steep drop in metabolic [ATP] and accumulation of [AMP] and [IMP] was observed. At pH 6 the production of inosine and hypoxanthine was greatly decreased when antimycin was added in addition to fluoride (Table 3). Although secretion is initiated at a stage where 50% or more of metabolic ATP is depleted and continues when only 15% is left, energy must be needed for some stage of the reaction at pH 6 and 7.4. The apparent lack of inhibitory effect at pH 5.3 may reflect properties of the inhibitor itself, or indicate that secretion at this pH is not energy-requiring. Other studies (E. H. Mürer & K. Davenport, unpublished work) show too that when fluoride is accumulated in a resting platelet at pH 5.3 and 0°C, the secretion at pH 7.6 and 37°C is little affected by antimycin.

Statistical treatment of results in Table 3

The significance of the different results at pH 5.3 shown in Table 3 was determined with the aid of the Student's *t* test (Texas Instrument Manual for Calculator TI 59). The differences between the values for ATP in all four columns were highly significant (*P* < 0.53%), and for AMP very significant (*P* < 1.4%), whereas for IMP only the first column was significantly different from the others (*P* < 2.1%). For serotonin the numbers in columns 1 and 3 were significantly different from those in

Table 3. The effect of antimycin on fluoride-treated platelets at pH 6 and 5.3 Antimycin was added in 5 µl of ethanol. Experiments were performed as described in the legend to Fig. 1. Results are means for five experiments. Concentrations of NaF used were: at pH 5.3, 2 mM; at pH 6, 10 mM.

Incubation time (min)	At pH 5.3				At pH 6.0			
	1	5	1	5	1	5	1	5
Antimycin (2.5 µg/ml)	—	—	+	+	0	0	+	+
¹⁴ C (% of total)								
ATP	70.7	12.8	29.1	4.1	60.7	6.2	20.6	3.9
AMP	4.0	41.2	25.0	57.0	5.5	10.7	25.0	25.2
IMP	3.5	13.6	12.9	17.7	7.5	41.0	27.7	50.3
Inosine + hypoxanthine	2.2	10.6	3.0	10.7	7.8	34.1	6.6	14.6
Adenylate energy charge	0.855	0.311	0.527	0.129	0.882	0.410	0.446	0.201
Radioactivity in supernatant (%)								
[³ H]Serotonin	11.9	63.2	11.4	52.2	26.8	84.0	12.0	18.1
¹⁴ C-labelled nucleotides	5.2	4.2	5.9	5.8	5.2	4.9	6.3	5.1

columns 2 and 4 ($P = 0.5$ and 2%), whereas the difference between columns 2 and 4 was not significant ($P = 10.1\%$), suggesting that antimycin does not significantly decrease fluoride-induced secretion at pH 5.3. In contrast at pH 6 the decrease from column 2 to column 4 was highly significant ($P = 0.2\%$), showing a significant inhibition by antimycin at this pH.

On the mechanism of induction of secretion by fluoride

The cause of the induction of secretion by fluoride is obscure. It seems evident from the present study that fluoride acts from the inside of the platelets and not via surface sites, in contrast with thrombin, ADP, collagen and other physiological inducers of platelet secretion. The effects of the latter are all blocked below pH 6. A comparison of the NaF-induced secretion with the secretion induced by ionophore A23187 is tempting, since this inducer has also been shown to have a full secretagogue effect at pH 5.3 (Mürer & Stewart, 1977). Fluoride activates adenylate cyclase in platelet homogenates (Zieve & Greenough, 1969), but an enhanced cyclic AMP concentration has been shown to inhibit rather than to activate platelet function (Haslam, 1975). However, fluoride is not believed to activate adenylate cyclase in intact cells (Vigdahl *et al.*, 1969). In leucocytes (Curnutte & Babior, 1975) and in platelets (Marcus *et al.*, 1977) fluoride causes production of superoxide, which may activate platelets in synergism with other inducers, but such secondary activation is unlikely to be the main mechanism of fluoride-induced secretion. One might postulate that fluoride could act via an activation of the production of thromboxane A_2 ; but no malonaldehyde production (E. H. Mürer & M. H. Fukami, unpublished work) or O_2 burst (Mürer, 1968) accompanies fluoride-induced secretion, which argues against this suggestion.

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54, II-195) and were referred to in a review article [Mürer (1976) *Fluoride* **9**, 173–184].

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