Low Zinc Status in Rats Impairs Calcium Uptake and Aggregation of Platelets Stimulated by Fluoride (43626)

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Abstract. **Platelets from rats of low zinc status exhibit impaired aggregation in response to ADP stimulation. The abnormality has been traced to defective uptake of calcium from the external medium. This study was designed to determine the location of the molecular defect and whether or not the ADP receptor is involved. Washed platelets were collected from rats fed a low zinc diet (<1 mg/kg) and control groups that consumed a zinc-adequate diet (100 mg/kg), *ad libitum*- and pair-fed. Fluoride, a G-protein stimulant, was used to bypass the ADP receptor. F^- stimulated platelet aggregation and calcium uptake; both of these functions were impaired by zinc deficiency. At 10 m*M* F^-, the time to half maximal aggregation was increased from 1.8 min in platelets from control to 2.8 min in zinc deficient rats. At 8 m*M* F^-, the uptake of calcium was decreased from 170 to 85 n*M* cytosolic free calcium. At this concentration of F^- there was no release of internal calcium. The results show that the molecular defect in the zinc-deficient platelet is located in the aggregation pathway beyond the ADP receptor and suggest a point between, or including, a G-protein and the plasma membrane calcium channel.** [P.S.E.B.M. 1993, Vol 203]

Zinc deficiency in rats leads to a bleeding tendency (1, 2) that has been attributed to defective platelet function. In platelet-rich plasma (PRP), the secondary phase of platelet aggregation is seriously impaired, suggesting that part of the problem is related to the release reaction (3, 4). Washed platelets from rats of low zinc status also show decreased response to minimal doses of ADP compared to pair-fed and *ad libitum*-fed controls (4). The response of deficient PRP or washed platelets to a maximal or excess dose of ADP does not differ from that of controls showing that the defect is a relative, not an absolute, failure to respond.

The aggregation of rat platelets is more sensitive to the calcium concentration in the suspending medium than that of human platelets. In the presence of 10 m*M* citrate, rat PRP does not exhibit the biphasic aggregation typical of human PRP (5). Furthermore, addition of the calcium blocker verapamil to rat PRP impairs aggregation in a manner analogous to that of zinc deficiency (5). Calcium plays two essential roles in platelet aggregation, one external and one internal to the platelet plasma membrane. Calcium is required for fibrinogen binding and clumping of platelets. An increase in free cytosolic calcium concentration, $[Ca]_i$, is essential for the aggregation mechanism to proceed after ADP stimulation (6). The increase in cytosolic calcium concentration arises both by release from internal stores and by uptake from the external medium. When stimulated with ADP, washed platelets from zinc-deficient rats show no difference from controls in the release of internal calcium, but there is a significant decrease in the uptake of calcium from the medium (7).

Since the uptake of calcium plays a key role in rat platelet aggregation and the process is impaired by zinc deficiency, it is reasonable to assume that failure of calcium uptake is the basis of defective aggregation. A defect in one or more of several sites in the aggregation pathway(s) may be involved, e.g., in the ADP receptor. There is not a totally satisfactory method for evaluating ADP receptor number and function in viable platelets (8). For that reason another method, bypassing the

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receptor, has been used to determine whether or not the ADP receptor is defective in zinc deficiency. The function of many agonist-receptors is initiated via G-proteins, and platelets contain G-proteins that stimulate phospholipase C and inhibit adenylate cyclase (9). Platelet ADP receptors interact with and activate a G-protein(s) whose identity is unknown (10), but may well be involved in opening the calcium channel. Calcium influx into platelets is effected by a receptor-operated channel and its response to ADP is rapid, 10–20 msec (11).

Fluoride has long been recognized as a permeant G-protein stimulant of adenylate cyclase (12). In the presence of aluminum and magnesium, F^- is an activator of the trimeric regulatory G-proteins coupled to phospholipase C (13) as well as adenylate cyclase. There is compelling evidence that an aluminum-fluoride complex, AlF_4^-, is the active form of fluoride which mimics the γ-phosphate of GTP when bound to $G\alpha$ (14). In human platelets F^- stimulates aggregation, enhances cAMP formation, and elevates the concentrations of cytosolic calcium and inositol phosphates (15–18).

The purpose of this study was to determine the effect of zinc status of rats on the response of their platelets when stimulated by F^-. The rate and extent of aggregation of platelets and the concentration of cytosolic-free calcium in platelets from zinc-deficient and control rats were determined. Both responses to F^- were impaired in platelets from rats of low zinc status.

Materials and Methods

Animals and Diets. Male rats of the Wistar strain, produced in the departmental colony and weighing 150–200 g, were housed in suspended, stainless steel wire-mesh cages. They were kept in a room maintained at 22°C and maintained on a 12:12-hr light:dark cycle. All rats were fed the zinc-adequate control diet for 3 days, fasted overnight, then divided into three groups. One group was fed a low zinc diet *ad libitum* (-ZnAL), one an adequate zinc diet *ad libitum* (+ZnAL), and one the adequate zinc diet limited to the previous day's intake of a pair-mate (+ZnPF). The composition of the basal low zinc diet has been described (4). Egg white served as the source of protein, and on average the diet contained 0.3 ± 0.02 mg Zn/kg. The control diet was the same except that it was supplemented with 100 mg Zn/kg. Deionized water was supplied *ad libitum*. Following an overnight fast, platelets and plasma were collected from groups of three rats that had consumed the respective diets for 11 days. Experimental protocols were approved by the University of Missouri-Columbia Animal Care and Use Committee.

Platelet Collection and Washing. Rats were anesthetized with ethyl ether and approximately 5 ml of blood collected from the dorsal aorta into a syringe containing anticoagulant (100 m*M* citrate in phosphate-buffered saline, pH 7.4) in the ratio of 1 volume to 9 volumes of blood. The blood was transferred to a polystyrene tube and centrifuged at 200*g* for 10 min. The PRP was transferred with a plastic-tipped pipet to a polystyrene tube and centrifuged at 600*g* for 15 min to form a soft platelet pellet. Plasma was carefully and completely decanted and saved for zinc analysis. The pellet was gently resuspended, by use of a plastic-tipped pipet, in a volume of Ardlie and Han (19) wash buffer, pH 6.5, equal to that of the plasma removed. After a 5-min undisturbed rest, the platelets were centrifuged at 400*g* for 15 min. The pellet was resuspended in a similar buffer, pH 7.4, and the number of platelets determined by use of a Coulter counter, model B (Coulter Electronics, Hialeah, FL). All buffers were made as concentrates in volumetric glassware and immediately transferred to plastic containers for refrigerated storage. They were diluted 10-fold for daily use.

Cytosolic Free Calcium. For determination of cytosolic calcium, the platelet suspension was diluted to 10^8/ml in resuspending buffer, pH 7.4, and loaded with Fura-2 acetoxymethyl ester (Fura-2AM, Molecular Probes, Eugene, OR). A frozen stock solution of Fura-2AM in dimethyl sulfoxide was diluted daily with resuspending buffer (7). An aliquot of the aqueous Fura-2 solution was added to the resuspended platelets to provide a final concentration of 1 μM. The suspension was gently vortexed, covered with aluminum foil to protect against light, and incubated at 37°C for 30 min. The platelets were pelleted at 600*g* for 10 min and the supernatant discarded. They were resuspended in sufficient buffer to provide a concentration of 5×10^7 platelets/ml and stored in the dark at room temperature until fluoresence was measured. Samples from the three diet treatments were loaded and assayed at one time period. Measurements involving different concentrations of F^- were staggered so that the time from loading to assay was constant.

The methods of fluoresence measurement and calcium calculation were the same as previously described (7). Two ml of platelet suspension were placed in a thermostatted cuvette and stirred for 2 min to allow equilibration at 37°C. Aliquots (100 μl) of F^- solutions containing $AlCl_3$ were then added and incubated for 40 sec. The final F^- concentrations were 8 or 10 m*M* and Al^{3+} was constant at 10 μM. These concentrations of F^- are lower than required for optimal aggregation response, but were chosen because they lie on the linear portion of the F^--stimulated response curve. Although we observed no effect of Al^{3+} either alone or in the presence of F^-, it was added to all samples to ensure an adequate level for AlF-complex formation. Calcium flux was initiated by addition of 1 m*M* Ca^{2+}. F^- interfered with the determination of R_{max} and R_{min} so that these parameters, the basal free cytosolic calcium concentration, and the calcium flux without F^- were determined in another platelet aliquot. After incubation in the absence of F^- for 160 sec, 1 mM Ca^{2+} was added to measure basal calcium influx. This value was subtracted from total uptake to calculate the net F^--stimulated calcium uptake. R_{max} and R_{min} were determined in the same aliquot containing 1 mM Ca^{2+} by further addition of 5% Triton and EGTA buffered to pH 8 with Tris (7).

Platelet Aggregation. Aggregation of washed platelets was monitored by use of a dual channel aggregometer (model 340, Chrono-Log Corp. Haverstown, PA) as previously described (4). To 0.5 ml of platelet suspension, containing 5 × 10^8 platelets/ml, was added 10 μg of rat fibrinogen (Fraction 1, citrated type IX, Sigma, St. Louis, MO) and the suspension stirred until equilibrated at 37°C. F^- solutions containing Al^{3+} were then added and stirred for 40 sec. Final F^- concentrations varied from 10–14 mM and Al^{3+} was constant at 10 μM. These concentrations of F^- lie on the linear portion of the aggregation response curve. To initiate aggregation a $CaCl_2$ solution was added to provide a final concentration of 1 mM. The order of addition is important because Ca^{2+} followed by F^- does not induce aggregation. The rate of aggregation, i.e., time to half-maximum aggregation ($T_{1/2\ max}$) was used as measure of platelet response.

Statistical Analysis. Data were treated by the analysis of variance (ANOVA) followed by the post-hoc *t* test (Interactive Statistical Package, Crunch Software Corp., San Francisco, CA).

Results

The growth rates and plasma zinc concentrations presented in Table I show that rats fed the low zinc diet had a low zinc status. Food intake was impaired so that the pair-fed group gained significantly less rapidly than the *ad libitum*-fed controls, but reduced food intake had no effect on the plasma zinc concentration.

Treatment of Fura-loaded platelets with F^- before addition of Ca^{2+} significantly increased the free cytosolic calcium concentration. Typical free calcium response curves, as determined by fluoresence, are shown in Figure 1. It should be noted that there was an immediate spike followed by a slow decline in concentration during the first 30 sec. This minimal decline was followed by a slow and steady rise in calcium over a period of a least 10 min. It appears that Ca^{2+} continued to leak into the platelets because addition of EGTA after the spike prevented the rise. The peak of the spike was the best defined response to F^- treatment and hence was employed as the response reference point. The difference in $[Ca]_i$ before and after Ca^{2+} addition was used as the measure of total calcium uptake due to F^-. The net uptake (Δ) due to F^- was the difference between cytosolic calcium concentration, with and without added F^-, after Ca^{2+} addition. It is notable that, in contrast to ADP which mobilizes internal as well as external calcium (7), F^- had no measurable effect on the release of internal calcium. The cytosolic free calcium concentration in the presence of EGTA was 119 nM (mean of five measurements) and, as illustrated by the bottom curve of Figure 1, the further addition of F^- had no measurable effect on cytosolic calcium concentration when there was no calcium in the external medium.

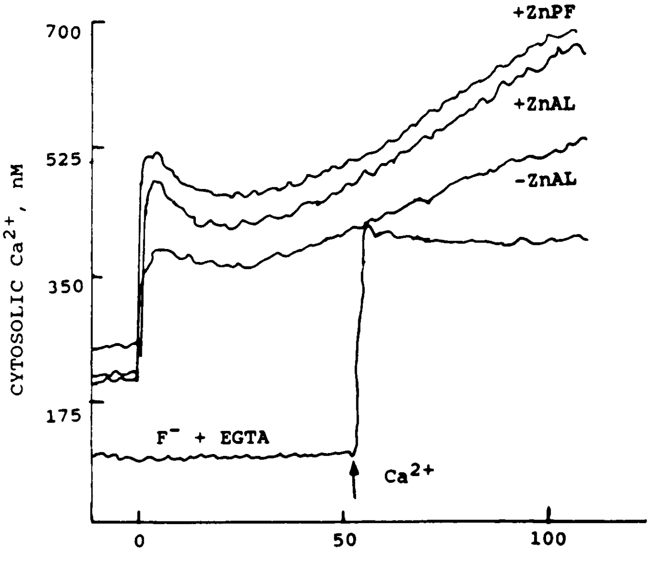

Figure 1. A representative trial showing the effect of zinc status on the concentration of free cytosolic calcium in platelets stimulated with fluoride. Washed platelets were stirred with 8 mM F^- for 40 sec before addition of 1 mM Ca^{2+} at time 0 for the three upper curves. The bottom curve shows the cytosolic calcium concentration in control platelets treated first with 0.1 mM EGTA, then with fluoride at Time 0 and with Ca^{2+} at 50 sec. There was no release of internal calcium by F^-. +ZnAL and +ZnPF refer to control rats fed *ad libitum* and pair-fed, respectively. -ZnAL refers to a rat fed the low-zinc basal diet.

Table I. Growth Rates and Plasma Zinc Concentrations as Indices of Zinc Status[a]

Diet and treatment	Gain, 11 days (*g*)	Plasma Zn (μM)
−ZnAL (9)	21 ± 2.1*	7.63 ± 0.69*
+ZnPF (9)	38 ± 1.7†	15.0 ± 0.70†
+ZnAL (8)	58 ± 1.6‡	14.7 ± 0.34†

[a] Means ± SE; number of animals per group shown is in parentheses and statistical significance ($P < 0.01$) within a parameter is indicated by different symbols.

As shown in Figure 2, over the 8–10 mM F^- concentration range, calcium uptake (Δ) increased with F^- concentration. Platelets from zinc-deficient rats took up significantly less calcium than controls and there was no food intake effect. At 8 mM F^-, calcium uptake

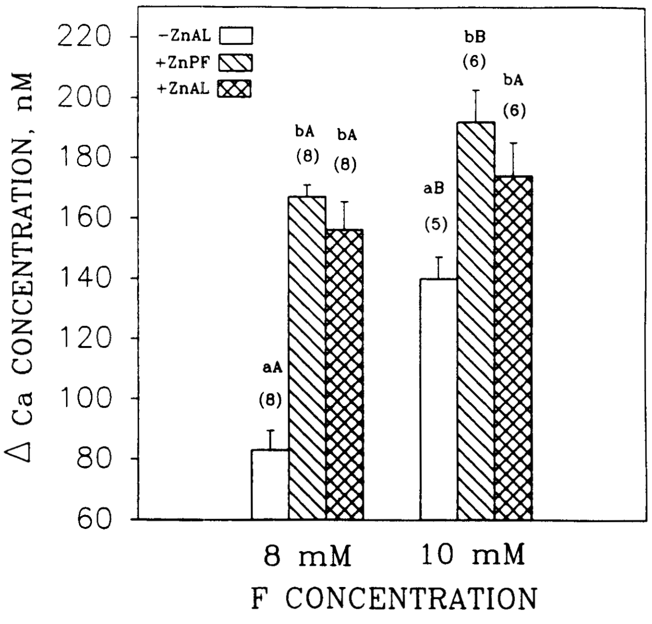

Figure 2. Zinc status and the uptake of external calcium induced by fluoride. The bars represent the means and the bar extensions the SE. The number of animals is indicated above the bars along with statistical significance. Different lower case letters designate $P < 0.05$ within a given fluoride level and upper case letters indicate statistically significant differences among fluoride levels.

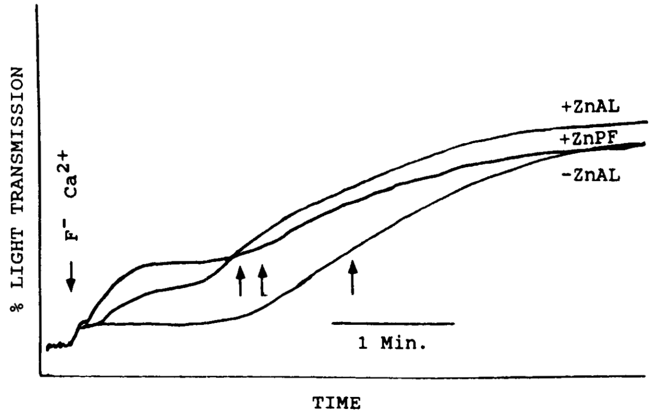

Figure 3. A representative trial showing the effect of zinc status on aggregation of platelets stimulated with fluoride. Washed platelets were stirred with fibrinogen and 12 m*M* F^- for 40 sec before addition of 1 m*M* Ca^{2+}. Arrows indicate the point of half maximum aggregation. Other symbols are the same as in Figure 1.

by platelets from rats fed the low zinc diet was less than 50% that of the pair-fed controls.

To relate the effect of F^- on cytosolic calcium to the process of aggregation, similar platelets were treated with F^- while they were stirred in an aggregometer. Typical tracings are shown in Figure 3. F^- stimulated aggregation, but the response was much slower than that observed with ADP and other commonly used agonists. There was a small initial aggregation-disaggregation phase followed by slowly initiated and irreversible aggregation. Platelets from zinc-deficient rats exhibited a slower rate of aggregation than controls as measured by $T_{1/2\ \mathrm{max}}$, but there was no significant difference in the maximal aggregation among treatment groups. Maximal aggregation induced by 10 and 12 m*M* F^- seldom exceeded 75% of that induced by a high concentration (20 μM) of ADP (7). Furthermore, F^- at these concentrations did not increase Ca^{2+} uptake to as great an extent as 0.8 m*M* of ADP, an agonist that causes release of calcium from internal stores as well as uptake from the external medium.

As shown in Figure 4, the rate of aggregation increased, i.e., $T_{1/2\ \mathrm{max}}$ decreased, with increasing concentrations of F^- over the range, 10–14 m*M*. At 10 m*M* F^-, platelets from zinc-deficient rats had a significantly slower rate of aggregation than controls. At 12 m*M* F^-, the deficient platelets differed only from the *ad libitum* controls and at 14 m*M* there were no differences among the treatment groups. The reason for the difference between pair-fed and *ad libitum* controls at the 12 m*M* F^- concentration is not clear, but the slow aggregation of the pair-fed group appears anomalous.

Discussion

In confirmation of work with human platelets (17), this study shows that rat platelets respond to F^- to induce aggregation and calcium uptake. Furthermore, platelets from zinc-deficient rats did not respond to F^- as well as those from controls. This is analogous to the decreased aggregation response and calcium uptake of platelets from deficient rats to ADP stimulation.

Since F^- stimulates G-proteins directly and bypasses plasma membrane receptors, it may be concluded that the defect in the aggregation mechanism

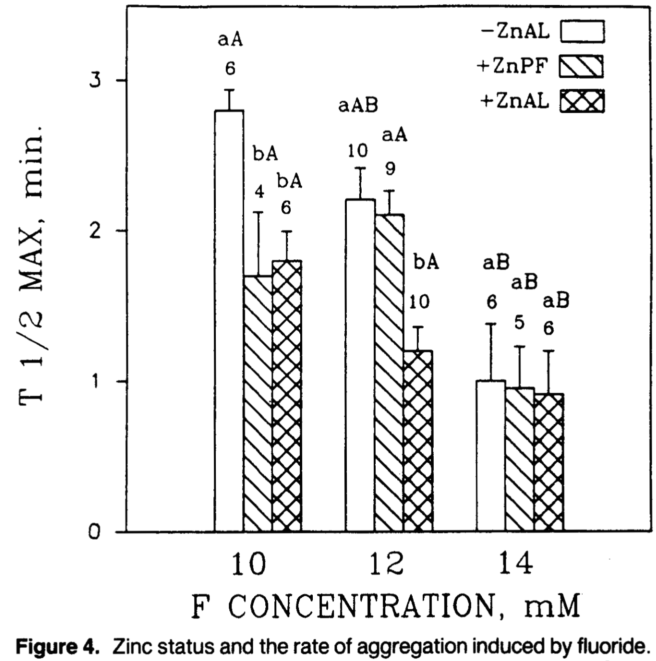

Figure 4. Zinc status and the rate of aggregation induced by fluoride. $T_{1/2\ \mathrm{max}}$ is the time required to attain half maximal aggregation. Other designations are the same as those in Figure 2.

lies downstream of receptors. The ADP receptor is not the site of the defect. The slower aggregation response to F^-, compared to ADP, suggests that at the concentrations used here, F^- either acts by a different mechanism or does not activate all of the pathways stimulated by ADP. In human platelets, ADP activates both phospholipase C (17) and the G-protein that inhibits adenylate cyclase (15). At high concentrations, F^- activated both pathways in human platelets, but it stimulated only phospholipase C at the concentration used in this study (17). ADP increases the concentration of rat platelet cytosolic free calcium, and the process is adversely affected by zinc deficiency. However, only the external uptake component is influenced by zinc status. As shown here, F^- also increases the cytosolic calcium concentration in rat platelets, but this agent causes uptake of external calcium only. At the concentrations studied there was no measurable internal calcium release. Significantly, from the standpoint of possible pathway defects, platelets from zinc-deficient rats also fail to respond normally to F^-. Thus, the defect relates to external calcium uptake, and it lies downstream of a G-protein concerned with opening the calcium channel. Probably the same G-protein is stimulated by F^- and ADP, but at low concentrations F^- does not appreciably stimulate the inhibitory G-protein that leads to a decrease in cAMP concentration, as ADP does. An alternative explanation of the results is that platelets from zinc-deficient rats do not take up F^- normally.

The present results show that the defect in platelet aggregation that occurs in zinc-deficient rats relates to the uptake of external calcium and is probably not the result of a defective agonist receptor. It is postulated that it is the result of a defective plasma membrane calcium channel per se or to a compromised component of the signal transduction pathway.

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