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Zinc deficiency in rats decreases thrombin-stimulated platelet aggregation by lowering protein kinase C activity secondary to impaired calcium uptake

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Aggregation of rat platelets, when stimulated by adenosine diphosphate (ADP) or fluoride, is impaired by zinc deficiency, and the defect is associated with a decreased uptake of external Ca^{2+}. Zinc deficiency also impairs the aggregatory response of platelets to phorbol myristate acetate (PMA), an activator of protein kinase C, but low zinc status decreases the PMA response only when calcium is added to the external medium. The purpose of this study was to determine the role of protein kinase C in rat platelet function and its relationship to the zinc deficiency pathology observed in platelets stimulated by thrombin (THR). The percent of maximal aggregation and the concentration of cytosolic-free Ca^{2+} were measured in washed platelets stimulated by THR and PMA. For the protein kinase C experiments platelets were obtained from rats fed a grain-based diet, and for the thrombin experiments they were from rats fed purified diets. In the latter experiments, immature male rats were fed for 2 weeks a low zinc diet (<1 mg/kg) ad libitum or a zinc adequate (100 mg/kg) diet either ad libitum or pair-fed. Zinc deficiency impaired the aggregation of platelets stimulated by 0.045 U/mL of THR by approximately 40%, and the external calcium uptake (0.03 U/mL of THR) was decreased by approximately 30%. Staurosporine, a protein kinase C inhibitor, decreased thrombin-induced aggregation in a concentration-dependent manner, but it had no effect on the external calcium uptake. While PMA had a synergistic effect with thrombin in the stimulation of platelet aggregation, it actually decreased the cytosolic-free calcium response to thrombin. It is concluded that zinc deficiency impairs thrombin-stimulated platelet aggregation and calcium uptake and that protein kinase C activity is essential for rat platelet aggregation. Protein kinase C does not stimulate calcium uptake and must act downstream of the calcium uptake defect. A model of rat platelet activation is presented depicting impaired Ca^{2+} uptake as the primary defect in zinc deficiency. (J. Nutr. Biochem. 6: 661–666, 1995.)

Keywords: rat; zinc status; platelet aggregation; cytosolic free calcium; thrombin; protein kinase C

Introduction

Bleeding time is prolonged in zinc-deficient rats,[1] and the defect is accompanied by impaired platelet aggregation.[2] The rate of aggregation of both platelet-rich plasma and washed platelets stimulated by several agonists is decreased.[2,3] The impairment is not due to lack of or defective receptors since impaired aggregation occurs when plasma membrane receptors are bypassed by the use of fluoride (F^-), an aggregating agent that stimulates a G-protein directly.[4] Impaired platelet aggregation, whether stimulated by ADP or F^-, is associated with decreased uptake of external calcium.[4,5] An increase in the concentration of cytosolic free calcium, $[Ca^{2+}]_i$, is essential for platelet aggregation.[6]

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Although thrombin is a commonly used stimulant of platelet aggregation in vitro, its effect on zinc-deficient rat platelets has not been tested. Its mechanisms of activation differs from that of adenosine diphosphate (ADP) in that it uses different signal transduction pathways.[7,8] Thrombin activates both phospholipase C (PLC) and phospholipase A_2, but it is equivocal whether ADP receptors are linked to phospholipase C.[9] While both agents induce calcium influx across the human platelet plasma membrane, the rate of influx produced by thrombin is slower than that induced by ADP.[10]

Recent results in this laboratory show that phorbol myristate acetate (PMA), a protein kinase C (PKC) activator, stimulates the aggregation of rat platelets and that the response is impaired by zinc deficiency.[11] The rate of PMA-stimulated aggregation was increased by the addition of calcium to the external medium of control platelets but not by its addition to platelets from zinc-deficient rats, i.e., aggregation was lower in platelets from zinc-deficient rats only when calcium was added to the medium. Low zinc status also significantly decreased the binding of phorbol dibutyrate to platelet membranes but only when the platelets were pretreated with Ca^{2+}, again suggesting that the primary effect of zinc deficiency is to decrease the availability of external Ca^{2+} for PKC activation.[11] PKC is a serine and threonine kinase that plays a key signal transduction role in human platelet aggregation.[12,13] It catalyzes the phosphorylation of a 40-kD protein whose function is unknown but appears to regulate dense granule release.[14,15] PKC is activated in vivo by Ca^{2+} and diacylglycerol but it can be activated exogenously by other compounds such as phorbol esters.[16] Phorbol esters initiate aggregation of human platelets. In contrast to the commonly used agonists, they suppress the rise in $[Ca^{2+}]_i$ induced by thrombin.[15]

The purpose of this study was to determine the role of PKC in rat platelet function and its relationship to the mechanism by which zinc deficiency affects platelet aggregation and calcium uptake by thrombin-stimulated platelets. The results show that thrombin-stimulated aggregation and external calcium uptake are impaired by zinc deficiency and that PKC activity is required for rat platelet aggregation. A schematic model of the mechanism involved in rat platelet aggregation is presented, showing that the basic defect in zinc deficiency is impaired calcium uptake, a condition that limits PKC activity.

Methods and materials

Materials

ADP, thrombin, rat fibrinogen, bovine serum albumin (BSA), staurosporine, dimethyl sulfoxide (DMSO), and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Co. (St. Louis, MO USA), and fura 2-AM from Molecular Probes (Eugene, OR USA); other reagent were reagent grade.

Animals and diets

For the zinc status experiments, immature (140 to 160 g) male rats of Wistar origin and from the departmental colony were housed individually in suspended stainless steel cages in a room maintained at 22°C and with a 12 hr light–dark cycle. Diet and deionized water were supplied ad libitum for a period of 2 weeks unless otherwise specified. The basal diet was the same as previously described[17] except that EDTA-treated soybean protein was used as the amino acid source, and 0.4% DL-methionine was added. Experimental blocks of three rats were started on a staggered time basis so that measurements were made on one rat from each dietary treatment group on the same day. One dietary treatment group consumed the basal low-zinc diet (<1 mg/kg) ad libitum (−ZNAL), one the control zinc-supplemented diet (100 mg/kg) ad libitum (+ZNAL), and one the control diet restricted to the intake of those fed the basal low zinc diet, i.e., pair-fed (+ZNPF). For the PKC studies, the rats were fed a grain-based diet. The number of rats per experiment is indicated in the respective tables and figures.

Preparation of washed platelets

Washed platelets were prepared as previously described.[3] Briefly, blood was drawn from the abdominal aorta into a 0.1 volume of sodium citrate in phosphate-buffered saline (PBS; pH 7.4) to provide a final citrate concentration of 10 mmol/L. Platelet-rich plasma was obtained by centrifugation at 400 *g* for 10 min. The platelets were then sedimented at 600 *g* for 15 min and washed with a buffer (HEPES 5.0, $MgCl_2$ 1.05, KCl 2.68, $NaHCO_3$ 11.9, NaCl 137, NaH_2PO_4 0.36, and glucose 5.5 mmol/L; BSA 3.5 g/L) at pH 6.5.[3] After 5 min, the platelets were pelleted at 400 *g* for 10 min and resuspended in the same buffer at pH 7.4.

Platelet aggregation

Aggregation of washed platelets was monitored[3] in a dual channel aggregometer (Model 340, Chrono-Log Corp., Haverton, PA USA). Aggregation was initiated by addition of 10 μg of rat fibrinogen (0.5 mL of platelet suspension) and 1 mmol/L $CaCl_2$ followed by thrombin (0.02 to 0.045 U/mL) or PMA (80 nmol/L). Maximal aggregation was then determined by further addition of 0.2 U/mL of thrombin. When used, staurosporine or PMA was added 1.5 min before the first addition of thrombin. The aggregatory response was expressed as the percent of maximum.

Cytosolic calcium

Fluorescence measurements for determination of cytosolic calcium concentration, $[Ca^{2+}]_i$, were carried out as previously described.[5] Washed platelets were loaded with fura 2-AM by the addition of 1 mmol/L to the medium,[3] collected by centrifugation at 600 *g* for 10 min, and resuspended in the pH 7.4 buffer described above. Fluorescence was measured in a Spex CM System dual-wavelength fluorometer (Spex Industries, Edison, NJ USA) using excitation at 340 and 380 nm and measuring emission at 505 nm. Internal release of calcium was determined as the increase in $[Ca^{2+}]_i$ in stimulated platelets that were suspended in the buffer containing 0.1 mmol/L of EGTA. External uptake was calculated as the difference between the total increase in $[Ca^{2+}]_i$ when the buffer contained 1 mmol/L calcium and the increase due to internal release. When used, staurosporine or PMA was added 1 min before the fluorescence scan.

Statistical analysis

Data are presented as the mean ± the standard error of the mean (SEM). They were analyzed by ANOVA using Crunch software (Intractive statistical package, Crunch Software Corp., San Francisco, CA USA) followed by a post hoc *t*-test. *P* values less than 0.05 were considered significant.

Results

The nutritional status of the rats used in this study was evaluated by weight gain during the 2-week experimental period and by the plasma zinc concentration at the end of the period. As shown in *Table 1*, both the rate of weight gain and plasma zinc concentration were remarkably reduced in rats fed the low zinc basal diet, indicating distinct zinc deficiency.

Thrombin stimulated aggregation of rat platelets at a minimal concentration of approximately 0.03 U/mL, and the concentration range that produced submaximal aggregation was narrow. A concentration of 0.045 U/mL of thrombin gave 40 to 70% of maximum, depending upon the zinc status. As shown in *Figure 1*, platelets from zinc-deficient rats showed a lower aggregatory response to thrombin than those from controls. The percentage of maximal aggregation of platelets from zinc-deficient rats was 39% compared with 61% for pair-fed and 73% for ad libitum–fed controls.

As observed for other aggregatory stimulants,[2–4] thrombin increased the cytosolic free calcium concentration in rat platelets, but the rate of the increase was slower than that stimulated by ADP (data not shown). Thrombin at concentrations of 0.02 to 0.045 U/mL stimulated calcium uptake linearly. The effect of zinc status on the transient increase in $[Ca^{2+}]_i$ in response to various concentrations of thrombin is shown in *Table 2*. There was no dietary effect on internal calcium release at any thrombin concentration. However, the uptake of extracellular calcium by zinc-deficient platelets was impaired in response to both 0.02 and 0.03 U/mL of thrombin. At higher thrombin concentrations, the differences due to low zinc status decreased and became insignificant.

As reported earlier,[11] the phorbol ester PMA stimulated platelet aggregation at a concentration of 80 nmol/L, and there was a long lag time compared with the thrombin response. PMA and thrombin had a synergistic effect on aggregation (data not shown). To explore the role of PKC activity in the aggregation of rat platelets, graded levels of staurosporine, a PKC inhibitor, were added to platelet suspensions along with three different concentrations of thrombin. As shown in *Figure 2*, graded levels of staurosporine progressively inhibited aggregation, and at a concentration of 220 nmol/L totally blocked aggregation induced by 0.045 U/mL of thrombin. At thrombin concentrations of 0.1 and 0.2 U/mL, concentrations that normally induced maximal aggregation, the percentage of maximal aggregation was inhibited 98 and 90% by 440 and 880 nmol/L staurosporine, respectively.

To determine whether PKC plays a direct role in the

Table 1 Weight gain and plasma zinc concentration as indices of zinc status

Dietary group	Weight gain (g/day)	Plasma Zn (μmol/L)
−ZnAL	0.3 ± 0.1[a]	8.1 ± 0.3[a]
+ZnPF	1.9 ± 0.1[b]	17.0 ± 0.7[b]
+ZnAL	4.4 ± 0.2[c]	15.5 ± 0.3[b]

Means ± SEM. n = 20 for each group.
Within a column, values with different letters are significantly different at $P < 0.05$ as determined by ANOVA followed by a post hoc t-test.

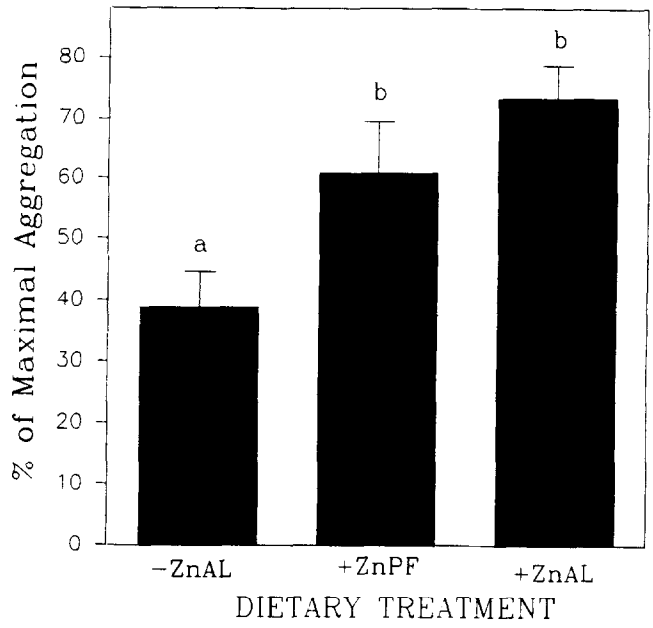

Figure 1 Zinc status and thrombin-stimulated (0.045 U/mL) platelet aggregation. The bars and extensions represent the mean ± SEM; n = 8 to 9. All of the values are expressed as a percentage of the maximal aggregation induced by 0.2 U/mL of thrombin. Different letters above the bars indicate statistically significant differences, $P < 0.05$.

Table 2 Effect of zinc status on the change in cytosolic calcium concentration in rat platelets stimulated by thrombin*

Thrombin level (U/mL)	Dietary group	Internal Ca release† (nmol/L)	External Ca uptake‡ (nmol/L)
0.020	−ZnAL	15.8 ± 3.5	214 ± 20.8[a]
	+ZnPF	14.6 ± 2.2	304 ± 21.6[b]
	+ZnAL	18.4 ± 5.0	324 ± 14.8[b]
0.030	−ZnAL	69.2 ± 10.2	390 ± 34.8[a]
	+ZnPF	47.0 ± 8.8	534 ± 31.8[b]
	+ZnAL	50.7 ± 8.7	535 ± 30.5[b]
0.045	−ZnAL	82.2 ± 17.5	694 ± 65.6
	+ZnPF	66.0 ± 12.6	772 ± 73.3
	+ZnAL	54.8 ± 6.4	878 ± 56.0
0.200	−ZnAL	119 ± 13.8	1301 ± 45.9
	+ZnPF	94.2 ± 13.7	1403 ± 117
	+ZnAL	110 ± 7.3	1532 ± 107

*Means ± SEM. n = 5 to 6 per group.
Within columns, values obtained with the same thrombin concentration that have different superscripts are significantly different at $P < 0.05$ as determined by ANOVA followed by a post hoc t-test.
†Internal calcium release is the increase in cytosolic-free calcium induced by stimulation of platelets in the buffer containing 0.1 mmol/L of EGTA.
‡External calcium uptake is the calculated difference between internal release and the increase in cytosolic-free calcium induced by the stimulation of platelets in the buffer containing 1 mmol/L of calcium.

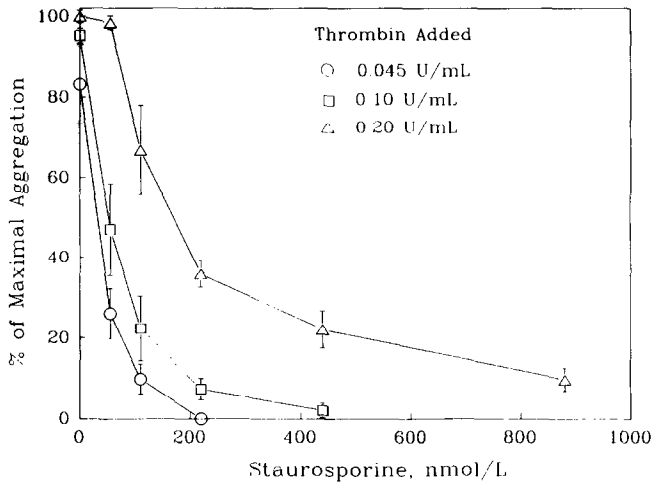

Figure 2 Inhibitory effect of graded levels of staurosporine on the percent of maximal platelet aggregation induced by three concentrations of thrombin. Staurosporine was added 90 sec prior to thrombin; n = 3 to 5.

calcium uptake process, the effect of staurosporine on cytosolic calcium concentration was determined in platelets stimulated with 0.045 U/mL of thrombin. The results are summarized in *Table 3*. Staurosporine, at concentrations that inhibited platelet aggregation, had no effect on the maximal increase in $[Ca^{2+}]_i$. Neither internal release nor uptake of external calcium was affected. However, the rate of decay of the peak levels of $[Ca^{2+}]_i$ in platelets stimulated with thrombin was less in the presence of staurosporine (data not shown).

Although PMA induced aggregation, it inhibited the transient increase in $[Ca^{2+}]_i$ observed when platelets were stimulated by 0.045 U/mL of thrombin alone. The cytosolic-free calcium data are presented in *Table 4*. Both the internal release of calcium and the uptake of external calcium induced by thrombin were decreased when PMA was present at concentrations of 80 and 160 nmol/L. In the absence of thrombin, PMA had no effect on the $[Ca^{2+}]_i$ (data not shown).

Discussion

In agreement with earlier studies[2–4] that used ADP and F^- as agonists, zinc deficiency in rats had a detrimental effect on the aggregation response of platelets stimulated with thrombin. Also in agreement is the observation that zinc deficiency decreases the uptake of external calcium resulting from stimulation with thrombin. Since a sharp transient increase in cytosolic-free calcium is required to initiate aggregation, the impaired cytosolic calcium response provides strong evidence that the basic defect in platelets from zinc-deficient rats resides in the function of a plasma membrane calcium channel. The effect of low zinc status on calcium uptake was observed at a lower concentration (0.03 U/mL) of thrombin than the concentration (0.045 U/mL) used to induce aggregation, suggesting different sensitivities of the two systems. The platelets used for measurement of cytosolic-free calcium were loaded with fura-2 and consequently had been mechanically manipulated more and for a longer time than those used for aggregation. Fibrinogen is added during the aggregation but not during the calcium uptake assay. These factors may have made the fura-loaded platelets more sensitive. In preliminary experiments we observed that the aggregation response of fura-loaded platelets to thrombin stimulation is significantly greater ($P < 0.05$, n = 6) than that of an unloaded aliquot of the same platelets. It is possible that the chelation of cytosolic calcium by internalized fura depletes the internal calcium store and thereby potentiates the opening of calcium channels. Calcium depletion of a lymphocyte cell line causes a messenger, termed calcium influx factor (CIF), to be released from intracellular organelles.[18] When applied to other cells CIF causes calcium influx. Because of the differences in the two platelet systems used in this study—fura-loaded and not-loaded—differences in the concentration of thrombin required to elicit a linear response is to be expected.

Aggregation of rat platelets is more sensitive to the external calcium concentration than is the aggregation of human platelets[19] because rat platelets release less internal calcium when stimulated.[20] Rat platelets have a smaller internal calcium pool and depend largely upon the uptake of external calcium to initiate aggregation. The ratio of internal release to external calcium uptake in the rat platelets stimulated with 0.045 U/mL of thrombin was approximately 0.09; this is appreciably less than the ratio (0.37) observed in human platelets.[21] Only external uptake is affected by zinc deficiency in the rat.

The increase in $[Ca^{2+}]_i$ induced by ADP is more rapid

Table 3 Effect of staurosporine on the change in cytosolic calcium concentration of platelets stimulated by thrombin

Treatment	Internal Ca release (nmol/L)	External Ca uptake (nmol/L)
Control (vehicle)	82.5 ± 20.0	763.0 ± 21.6
Staurosporine (nmol/L)		
55	76.0 ± 17.6	718.5 ± 45.5
220	73.3 ± 15.1	688.8 ± 46.4
880	81.8 ± 15.1	785.7 ± 53.5

Mean ± SEM. n = 6 for each group. The thrombin concentration was 0.045 U/mL. The DMSO concentration in the vehicle (the solvent for staurosporine) was 0.5% for all trials. The platelets were from rats fed a grain-based diet. No statistically significant differences were observed.

Table 4 Effect of phorbol myristate acetate (PMA) on the change in cytosolic calcium concentration of platelets stimulated by thrombin

Treatment	Internal Ca release (nmol/L)	External Ca uptake (nmol/L)
Control (vehicle)	69.3 ± 9.7[a]	767 ± 55.2[a]
PMA (nmol/L)		
80	21.3 ± 4.3[b]	274 ± 30.6[b]
160	8.0 ± 2.3[b]	131 ± 34[b]

Mean ± SEM. n = 3 for all values. Thrombin concentration was 0.045 U/mL. Within a column values with different letters are significantly different at $P < 0.05$ as determined by ANOVA followed by a post hoc t-test. Platelets were from rats fed a grain-based diet.

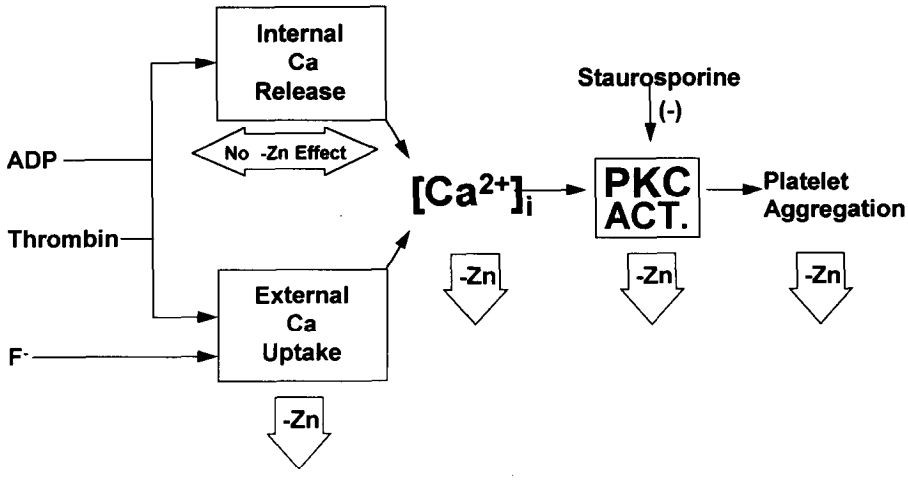

Figure 3 Model of rat platelet aggregation depicting the effect of zinc deficiency. PKC plays a key role in aggregation, and its in vivo activity is dependent upon increases in calcium and diacyglycerol. In platelets from zinc-deficient rats, PKC activity is decreased secondary to impaired calcium uptake. The vertical hollow arrows with [-Zn] indicate decreased concentration, activity, or function in platelets from zinc-deficient rats.

than that induced by thrombin in both rat and human platelets,[22] suggesting that ADP-evoked calcium influx involves a different transduction system than that used by thrombin. Thrombin activates PLC, an enzyme that catalyzes the formation of diacylglycerol (DG) and inositol trisphosphate (IP_3). DG activates PKC, and IP_3 stimulates the release of calcium from internal stores. Since the increase in $[Ca^{2+}]_i$ in response to all agonists tested is impaired by zinc deficiency, it is likely that a calcium channel, rather than a transduction mechanism, is defective. Nevertheless, the basic biochemical defect involved, the mechanism by which calcium uptake is impaired, remains unknown.

Exposure of fibrinogen binding sites is regulated by protein phosphorylation catalyzed by PKC, a zinc metalloenzyme.[23] The glycoprotein IIb–IIIa complex at the binding site is not only involved with fibrinogen binding but is also associated with calcium uptake.[24] PKC activation requires both Ca^{2+} and DG or a DG analog such as phorbol esters. PKC activation of human platelets leads to shape change, secretion, as well as the exposure of fibrinogen binding sites.[23] These are all components of the platelet aggregation process but they do not depend upon an increase in cytosolic-free calcium.[25]

PKC is essential for platelet aggregation but it does not increase $[Ca^{2+}]_i$. In rat platelets PMA by itself had no effect on $[Ca^{2+}]_i$; it reduced the transient calcium increase produced by thrombin. The PKC inhibitor, staurosporine, at concentrations that inhibited platelet aggregation (55 to 880 nmol/L), had no effect on the peak value of $[Ca^{2+}]_i$ that resulted from thrombin stimulation. However, staurosporine slowed the rate at which $[Ca^{2+}]_i$ returned to the basal level, confirming the observation made with human platelets using a structural analog of staurosporine.[26] While staurosporine inhibits the aggregation of human platelets,[27] its effect on $[Ca^{2+}]_i$ in thrombin-stimulated platelets is controversial.[28,29] Differences in published results may have arisen because a single concentration of staurosporine was used and the concentration differed between studies.

Based on this study and our previous results,[2–5,11] a model that depicts the role of zinc in rat platelet aggregation is presented in *Figure 3*. The three agonists, ADP, F^-, and thrombin, stimulate platelet aggregation by a mechanism involving an increase in the cytosolic calcium concentration. The increase in $[Ca^{2+}]_i$ is impaired by zinc deficiency, leading to decreased PKC activity and subsequently to impaired platelet aggregation. Uptake of external calcium is subnormal but release of calcium from internal stores is unaffected by zinc deficiency. PKC activity is essential for platelet aggregation and its in vivo activation requires both calcium and DG in addition to the phospholipid in or released from membranes. Stimulation of platelet aggregation by the three agonists is a calcium-dependent process that is impaired by zinc deficiency.

How zinc deficiency affects the calcium channel in platelets is unknown. Whether GPIIb–IIIa serves as a calcium channel[24,30] and whether a voltage-gated calcium channel[31] exists in platelets remain moot. Based on our observations with platelets and the fact that zinc deficiency decreases voltage-gated calcium channel calcium uptake by brain synaptosomes as well,[32] we speculate that the defect in platelets is due to the decreased activity of a voltage-gated calcium channel, though the existence of such a channel in platelets is unproven.[22,31]

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