Zinc status and distribution of protein kinase C in rat platelets

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Platelet aggregation is impaired by zinc deficiency and in vitro zinc has been reported to affect subcellular distribution of protein kinase C (PKC), an enzyme required for platelet aggregation. In this study, the effects of zinc deprivation and in vitro calcium on phorbol ester-induced platelet aggregation and PKC distribution were investigated. Platelets were collected from rats fed a low zinc diet (<1 mg/kg) and controls that consumed a zinc adequate diet (100 mg/kg), ad libitum or pair-fed. Washed platelets were stimulated with phorbol myristate acetate (PMA, 160 nmol/L) and the rate of aggregation determined. Without added Ca^{2+} the rate of aggregation was not affected by zinc status. With added Ca^{2+} (1 mmol/L) the rate was decreased by zinc deficiency ($P < 0.05$). For PKC measurement, platelets from each animal were pretreated briefly with either 0 or 1 mmol/L Ca^{2+}, then suspended in a low Ca^{2+} buffer, and sonicated. Specific binding of phorbol dibutyrate (PDBu) to mixed membranes and cytosol was measured. Pretreatment with Ca^{2+} increased binding to membranes and decreased binding to cytosol. Overall, zinc deficiency decreased [^{3}H]PDBu binding to membranes approximately 10% ($P = 0.01$), but had no effect on cytosol binding. Only in the presence of in vitro Ca^{2+} did zinc deficiency decrease both PMA-induced aggregation and phorbol ester binding to mixed membranes. Zinc status had no effect on the distribution of phorbol ester binding, suggesting that low zinc status decreased availability of extracellular calcium and thus decreased membrane PKC binding affinity or the stability of PKC in the membranes.(J. Nutr. Biochem. 5:536–541, 1994.)

Keywords: rat; zinc status; calcium; platelet aggregation; phorbol ester binding; subcellular protein kinase C

Introduction

Zinc deficiency impairs hemostasis and platelet function as exhibited by prolonged saline bleeding time and impaired platelet aggregation in rats[1–3] and humans.[4] When washed platelets from zinc-deficient rats are stimulated with ADP or F^-, aggregation is impaired, and the uptake of external calcium is lower than in platelets from control rats.[5,6] Agonists such as ADP stimulate platelets by binding to a receptor, a process that induces a cellular signal. Transduction of the signal results in protein phosphorylation and ultimately in aggregation.

The in vitro addition of Zn^{2+}, along with the ionophore pyrithione, increased the binding of phorbol esters to intact B cells and platelets[7] and to B-cell homogenates[8] in a concentration-dependent manner. Zinc increased the number of sites on intact cells without changing binding affinity. It was suggested that zinc treatment induced translocation, i.e., changed the distribution of PKC from cytosol to membranes. Another laboratory has reported that in vitro zinc stimulates cytosolic PKC activity and its binding to the plasma membrane of T cells at the expense of cytosolic activity.[9] High concentrations (0.1 to 0.3 mmol/L) of zinc added in vitro caused aggregation of human platelets,[10,11] and it was suggested that zinc exerts its effect by activating PKC.[11] Phorbol esters cause aggregation of human platelets by stimulation of PKC, an enzyme whose catalysis of phosphorylation is presumably an essential step in the aggregation process.[12,13]

Protein kinase C is a family of serine and threonine kinases involved in signal transduction in a wide variety of eukaryotic cells.[14] The conventional PKC isozymes, α, β, and γ, in human platelets are zinc metalloenzymes whose activity is dependent on calcium, phospholipids, and a regulatory molecule, such as endogenous diacylglycerol (DG).[15–17] Phorbol esters, which are of exogenous origin and not so readily degraded as DG, also stimulate PKC activity and initiate platelet aggregation by substituting for DG.[13] Phorbol esters not only activate PKC but also regulate its binding to membranes.[18] The stability of PKC in membranes, whether measured by phorbol ester binding or by PKC activity, correlates with Ca^{2+} concentration, both in the presence and absence of phorbol esters.

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These in vitro studies suggest that the detrimental effect of low zinc status on platelet function could be the result of an altered subcellular distribution of PKC. The purpose of this study was to determine the effect of dietary zinc status on platelet aggregation induced by a phorbol ester and on the subcellular distribution of PKC in rat platelets. Phorbol ester binding was used as a measure of PKC concentration. Because of the important role of calcium in PKC binding to membranes, the effect of added calcium was investigated using a design that involved in vitro calcium treatment of platelets from rats of low and normal zinc status. In the presence, but not absence, of added calcium in the aggregation medium, platelets from zinc-deficient rats showed lower aggregatory response to a phorbol ester than controls. Membranes isolated from zinc-deficient platelets showed less binding of phorbol ester, but there was no effect of zinc status on the subcellular distribution of binding. Overall, zinc status had negligible effect on platelets without in vitro added calcium.

Methods and materials

Animal and diets

A total of 54 immature male rats of Wistar origin, weighing 140 to 170 g, were obtained from the departmental colony. During the 14-day experimental period, they were housed in suspended, stainless-steel, wire mesh cages kept in a room maintained at 22° C and with a 12-hr light-dark cycle. Deionized water was supplied ad libitum. One group consumed a low zinc basal diet, ad libitum (−ZnAL), one the basal diet supplemented with 100 mg/kg zinc, *ad libitum* (+ZnAL), and one the supplemented diet, pair-fed (+ZnPF). Each pair-fed rat was allotted daily the amount of food consumed the previous day by his pair mate. The basal diet was similar to the one described[19] except that it contained 0.4 rather than 0.2% methionine and 10% rather than 5% corn oil. This diet was based on EDTA-treated soybean protein and by analysis contained less than 1 mg zinc/kg. The control diet was the basal supplemented with 100 mg zinc/kg as $ZnCO_3$. The rats were fasted overnight before blood was collected on the last day of the experiment.

Materials

[20-^{3}H]4β-Phorbol-12,13-dibutyrate ([^{3}H]PDBu) was obtained from New England Nuclear (Boston, MA USA). Phorbol 12-myristate 13-acetate (PMA), phosphatidylserine, polyethylene glycol 8000, leupeptin, phenylmethylsulfonyl fluoride, dithiothreitol, sodium pyrithione, and rat fibrinogen were obtained from Sigma (St. Louis, MO USA). All other chemicals used were reagent grade. Glass-fiber filters (Whatman GF/B) were obtained from Brandel (Gaithersburg, MD USA). PMA was dissolved in dimethylsulfoxide (DMSO; 500 μmol/L) and diluted with buffer immediately before use.

Preparation of washed platelets

Experimental rats were anesthetized with ethyl ether and blood (4.5 mL) was collected from the abdominal aorta into a syringe containing 0.5 mL of 100 mmol/L sodium citrate (pH 7.4). Platelet-rich plasma (PRP) was prepared by centrifuging the citrated blood at 400*g* for 10 min at room temperature. The PRP was transferred to a polystyrene tube and centrifuged at 600*g* for 15 min to form a soft pellet. The supernate was completely removed and saved for zinc analysis. For aggregation studies the pellet was resuspended in a volume of modified Ardlie and Han[20] wash buffer, pH 6.5, equal to the supernate removed. The buffer contained 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; bovine serum albumin (BSA) (3.5 g/L) was added. After a 5-min rest, the platelet suspension was centrifuged at 400*g* for 10 min. The washed pellet was resuspended in the same buffer, except adjusted to pH 7.4 and used for aggregation measurements. For binding studies the BSA was omitted from the buffers used in the preparation of the platelets. The final suspension buffer contained either 0 or 1 mmol/L calcium as described below.

Platelet aggregation

Aggregation of washed platelet was monitored in a Chronolog dual channel aggregometer (Chrono-Log Corp., Havertown, PA) as described.[21] After addition of 10 μg rat fibrinogen to a 0.5-mL aliquot containing 3×10^8 platelets/mL, the suspension was equilibrated to 37° C, during a period of 90 sec. Where indicated, 1 mmol/L $CaCl_2$ was added before addition of 160 nmol/L PMA. This concentration of PMA was chosen as the result of preliminary experiments involving platelets from stock rats stimulated with 10, 20, 40, 80, 160, 320, or 640 nmol/L PMA ($n = 5$ per treatment). The 160 nmol/L concentration produced maximal aggregation in a 3 to 4 min time frame, comparable to other agonists used. For comparison, thrombin at 0.045 U/mL with 1 mmol/L added calcium was used to induce aggregation; this was followed by 0.2 U/mL to elicit maximal response. The rate of aggregation was measured by the time required for aggregation to reach one half maximum ($T_{1/2max}$).[21] This index is inversely related to the rate of aggregation.

Separation of platelet membranes and cytosol

To determine the effect of short-term calcium treatment of intact platelets on the subsequent phorbol ester binding to membranes and cytosol, washed platelets from each animal were divided into two aliquots. To one aliquot, 1 mmol/L calcium was added, and to the other none was added before incubation at 37° C for 10 min. After sedimentation to remove most of the calcium, the platelets were resuspended in 0.8 mL of ice-cold buffer (25 mmol/L Tris-HCl, pH 7.4, containing 1 mmol/L dithiothreitol, 2 mmol/L phenylmethyl sulfonyl fluoride, and 10.5 μmol/L leupeptin). The suspensions were sonicated at 0° C using a Vibra Cell Model VC375 sonicator (Sonics and Materials, Inc, Danbury, CT USA), at power setting 4, for four 10-sec periods. Intact platelets were removed from the sonicate by centrifugation (12,000*g*) for 1 min, and the supernate centrifuged at 100,000*g* for 30 min at 4° C. The final supernate is referred to as cytosol and the pellet as mixed membranes. The cytosol contained approximately 1 mg protein per mL, and the mixed membranes, when suspended in 0.7 mL of buffer, contained approximately 1 mg protein per mL.

Binding assay

A filtration assay was used for the mixed membranes and a centrifugation assay for the cytosol because cytosolic proteins are not retained by the filter.

Mixed membranes. Phorbol ester binding to the membranes was determined by a modification of the method described by Csermely et al.,[9] using a constant amount of protein (30 to 100 μg in a given trial) per tube. The binding buffer contained 25 mmol/L Tris-HCl (pH 7.4), 2.5 mmol/L calcium chloride, 5 mmol/L magnesium chloride, and 10 nmol/L [^{3}H]PDBu (18.6 C_i or 688 GBq/mmol; NEN, DuPont, Wilmington, DE). After 30 min incubation at 37° C, the membranes were collected on a Whatman GF/B glass fiber filter by use of a cell harvester (Brandel, M-24R, Gaithersburg, MD USA). The tubes and filters were washed three times with 5

mL of ice-cold binding buffer. The filters were placed in vials with 5 mL of scintillation fluid (ScintiVerse BD, Fisher, Pittsburgh, PA, USA) and allowed to stand for a few hours before counting (Beckman model LS1701, Fullerton, CA, USA). Non-specific binding was measured by addition of 1 μmol/L PMA. Specific binding was defined as the difference between total binding and nonspecific binding. All measurements were made in duplicate.

Cytosol. The binding assay for platelet cytosol was carried out in 1.5 mL microcentrifuge tubes containing the same binding buffer as used for membranes except that it contained in addition 300 μg/mL of phosphatidylserine. The protein and isotope concentration were the same as for membranes. The tubes were incubated at 37° C for 30 min. After incubation, the tubes were immediately placed in ice, and 50 μL of BSA solution (20 mg per mL of 25 mmol/L Tris-HCl, pH 7.4) was added, followed by 200 μL of 30% polyethylene glycol in the same buffer. The tubes were vortexed vigorously. After 15 min at 0° C, the tubes were centrifuged in an Eppendorf microcentrifuge (12,000*g*) for 15 min. The supernate was decanted, the tubes inverted on paper towels for 5 min, and the pellet superficially rinsed twice with 0.5 mL of ice-cold binding buffer. The tip of the tube containing precipitated protein was severed and transferred to a vial containing scintillation fluid. Radioactivity and specific binding were measured as for the membranes.

Protein assay

Protein concentration was determined by the method of Lowry et al.,[22] using BSA as the standard.

Plasma zinc analysis

Plasma was diluted four times with 1% HCl and zinc measured directly by flame atomic absorption spectrophotometry (Varian SpectrAA-30, Mulgrave, Australia).

Protocols

In addition to the two experiments described below, preliminary trials were performed to determine optimal conditions for aggregation. These trials involved rats fed a commercial grain-based diet.

Experiment 1. Platelets from 27 rats, 9 from each dietary treatment, were stimulated with 160 nmol/L PMA with and without the in vitro addition of 1 mmol/L Ca^{2+}. The in vitro calcium treatment was designed to determine whether addition of external (medium) calcium promotes aggregation stimulated by PMA, and if so, whether zinc status affects the response to external calcium.

Experiment 2. Platelets from 27 rats, 9 from each dietary treatment, were pretreated with 1 mmol/L Ca^{2+} before separation into two subcellular fractions, membranes and cytosol. Then PMA binding to the fractions was determined. The use of calcium pretreatment was based on the observation that zinc status affects the aggregation response to calcium added in vitro.

Data analysis

Data were analyzed statistically as split-plot analysis of variance (ANOVA) using the General Linear Models component of SAS (SAS Institute, Cary, NC USA). Data are expressed as the mean ± SEM.

Results

The nutritional status of rats fed the experimental diets was evaluated by weight gain during the experimental period and by the terminal plasma zinc concentration. These parameters are summarized in *Table 1*. Both criteria were significantly lower in rats fed the basal diet (−ZnAL) than in the ad libitum controls (+ZnAL), indicating explicit zinc deficiency. Although the pair-fed controls (+ZnPF) gained less than +ZnAL controls, they had normal plasma zinc concentrations. In agreement with an earlier report,[5] zinc status had no effect on platelet number in the plasma.

Figure 1 presents the results of a representative aggregation trial. PMA induced aggregation of rat platelets, with or without added calcium. Compared with the aggregation of control platelets induced by 0.045 U/mL thrombin, PMA-stimulated platelets exhibited a long lag time. PMA stimulated the aggregation of platelets from the +ZnAL rat without added calcium, but the rate was accelerated by 1 mmol/L Ca^{2+}. However, addition of Ca^{2+} had little effect on the aggregatory response of platelets from the −ZnAL rat. In the preliminary experiment involving rats fed a grain-based diet, PMA induced maximal aggregation at all concentrations tested even though the lag time was exaggerated (up to 30 min) at low concentrations.

The results of experiment 1, designed to determine the effect of zinc status on PMA-stimulated aggregation with and without in vitro calcium addition, are summarized in *Figure 2*. In the absence of added calcium, the rate of aggregation, i.e., the time to reach half maximal response ($T_{1/2\ max}$) was not affected by zinc status. Addition of calcium to control platelets increased the rate (decreased $T_{1/2\ max}$, P =

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

Dietary treatment	Weight gain	Plasma zinc
	g/d	μmol/L
−ZnAL	0.78 ± 0.10[a]	8.09 ± 0.33[a]
+ZnPF	1.67 ± 0.11[b]	15.28 ± 0.58[b]
+ZnAL	4.48 ± 0.23[c]	16.97 ± 0.50[b]

Means ± SEM. n = 18 for each group. Within a column values with different letters are significantly different at $P < 0.005$ as determined by ANOVA followed by post hoc t test.

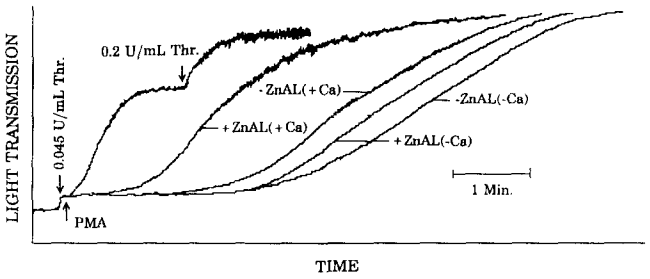


Figure 1 Representative tracings showing the aggregation of washed rat platelets stimulated by a phorbol ester. PMA (160 nmol/L) was added to the aggregation medium, with (+Ca) and without (−Ca) 1 mmol/L Ca^{2+} added and containing 20 μg/mL rat fibrinogen. Platelets were from a zinc deficient (−ZnAL) or ad libitum-fed control (+ZnAL) rat. Stimulation of control platelets with 0.045 U/mL thrombin (Thr) and 1 mmol/L Ca^{2+} are shown for comparison. Agonists added at the time point indicated by the arrows. The increase in light transmission indicates decreased turbidity as the platelets aggregate and clump together.

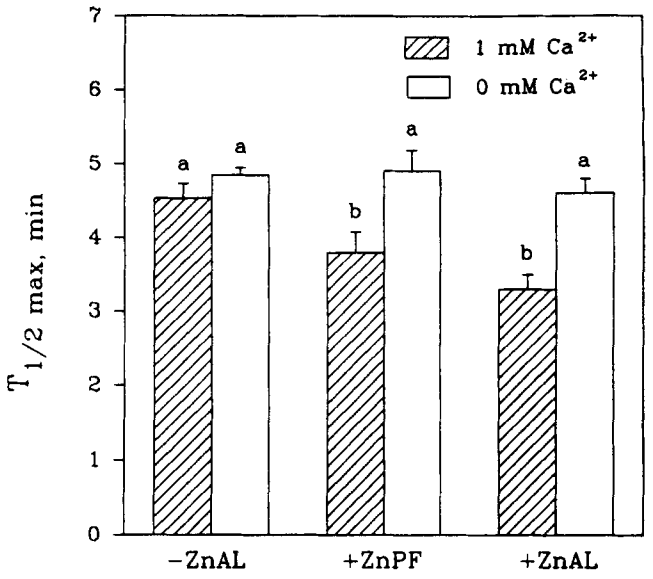


Figure 2 Effect of zinc status on the aggregation of platelets stimulated with phorbol ester (160 nmol/L PMA) in the presence and absence of 1 mmol/L calcium. Conditions and designations as in *Figure 1*. The bars represent the means and the extensions the SEM; $n = 8$. Means with different letters were statistically different, $P < 0.05$. There was a significant calcium × zinc status interaction, $P < 0.05$.

0.0016 for +ZnPF and $P = 0.0002$ for +ZnAL), but had no effect on platelets from zinc-deficient rats. Thus, with added calcium the rate of aggregation of platelets from the deficient rats was significantly less than controls; the $T_{1/2}$ for the −ZnAL group was greater ($P < 0.05$) than that of the +ZnAL and +ZnPF groups.

The effect of zinc status and in vitro calcium on the subcellular distribution of phorbol ester binding (experiment 2) is shown in *Table 2*. As demonstrated by the statistical analysis (ANOVA), calcium pretreatment significantly increased binding to membranes and decreased cytosol binding. Overall, zinc deficiency decreased binding to mixed membranes but had no effect on cytosol binding. With calcium pretreatment, binding to mixed membranes from −ZnAL rats was less than binding to both pair-fed and ad libitum control membranes. Without added calcium, binding to −ZnAL membranes differed only from those of pair-fed controls. In the presence of added calcium, cytosolic protein from zinc-deficient platelets bound more phorbol dibutyrate than that from ad libitum, but not pair-fed, controls. The higher cytosol binding of deficient rats was apparently due to their reduced food intake rather than to a specific zinc effect.

Discussion

Consistent with the observations made with human platelets, the phorbol ester PMA stimulated the aggregation of rat platelets. PMA-induced aggregation differed from that induced by thrombin and ADP in two respects. At low concentrations of PMA the aggregation exhibited a long lag period, but eventually reached maximum, i.e., all the platelets aggregated regardless of PMA concentration. Aggregation occurred without the addition of calcium to the medium, although the rate of control platelet aggregation was increased by additional calcium.

Calcium is required for platelet aggregation in at least two mechanisms, the binding of fibrinogen to its receptor and the activation of PKC. Presumably, there was sufficient free calcium in the platelet and/or medium for activation of PKC in the presence of phorbol ester. However, the calcium concentration in the basal medium was limiting, and the addition of calcium stimulated the rate of aggregation of normal platelets. It appears that low-zinc status made external calcium less available for PKC activation and the aggregation process. Clearly, PMA, an exogenous PKC stimulant, is more stable and possibly more potent than diacylglycerol, the normal endogenous activator of PKC. The high stability and potency of PMA allows it to stimulate platelets in the presence of extremely low concentrations of free calcium. Thus, the action of PMA in platelet activation imitates that of the endogenous activator and no doubt minimizes the

Table 2 Effect of zinc status on [^{3}H]PDBu binding to platelet mixed membranes and cytosol fractions

Dietary treatment	Mixed membrane binding	Cytosol binding	Membrane/cytosol ratio
	nmoles/mg protein		
A. No added Ca			
−ZnAL	34.2 ± 1.9^a	13.9 ± 1.3^a	2.6 ± 0.1^a
+ZnPF	38.2 ± 1.3^b	13.0 ± 1.2^a	3.1 ± 0.3^a
+ZnAL	36.0 ± 2.4^{ab}	12.5 ± 0.7^a	3.0 ± 0.2^a
B. 1 mmol/L Ca* added			
−ZnAL	36.5 ± 2.4^a	10.2 ± 0.9^a	3.8 ± 0.3^a
+ZnPF	40.2 ± 1.9^b	10.0 ± 0.8^{ab}	4.2 ± 0.3^{ab}
+ZnAL	40.9 ± 1.2^b	9.4 ± 0.7^b	4.6 ± 0.4^b
	ANOVA, *P* values		
Diet	0.013	0.2062	0.012
Ca	0.001	0.0001	0.0001
Diet*Ca	NS	NS	NS

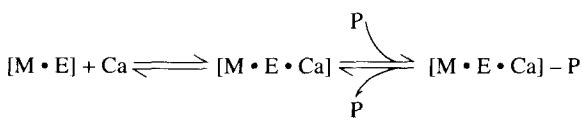
Means ± SEM; $n = 9$ for all groups.
Means within a column and with the same calcium treatment without a common letter are significantly different.
*Calcium chloride (1 mmol/L) was added to the platelet suspension and incubated for 10 min at 37° C before sonication and separation.

effect of internal calcium concentration because of its persistent effect on PKC.

With added calcium the rate of the phorbol ester-stimulated aggregation of platelets from rats of low zinc status was significantly lower than that of controls. This difference could be the result of disparate rates of passive uptake of external calcium. Addition of calcium to a suspension of rat platelets increases the cytosolic free calcium concentration slightly although there is no agonist stimulation. To test the hypothesis that lower passive calcium uptake was responsible for the difference in aggregation, the increase in free calcium concentration was measured in Fura-2 loaded platelets after addition of 1 mmol/L calcium.[6] The hypothesis was not confirmed because platelets from all donors, regardless of zinc status, took up the same amount of calcium, 130 ± 13 nmol/L (n = 12; unpublished data). While the mechanism by which calcium concentration in the medium affects PMA-stimulated platelet aggregation is unclear, calcium probably serves to increase PKC binding and activity in the plasma membrane without increasing the concentration of calcium detectable by Fura-2. Calcium is known to stabilize membrane PKC in vitro.[23] As discussed below and in the companion paper,[24] both calcium and zinc increase phorbol ester binding to platelet mixed membranes. The positive effect of calcium treatment on binding is consistent with the effect it had on phorbol ester-induced aggregation.

As was shown in *Table 2*, brief exposure of intact platelets to calcium before the sonication and extraction procedure had a significant effect on binding of phorbol dibutyrate. Zinc deficiency significantly decreased membrane binding compared with controls, but the effect was minor compared with the effect of adding zinc to intact platelets.[7,24] The effect of zinc status on cytosol binding was negligible. Only when compared with ad libitum-fed control rats was cytosol binding greater in zinc deficiency. Any effect of low zinc status on the binding of phorbol ester to cytosolic protein was related to decreased food intake rather than to a specific zinc effect. In summary, zinc deficiency decreased mixed membrane binding of phorbol esters but not the subcellular distribution of binding.

Whether zinc and calcium act independently in increasing membrane binding of phorbol ester is not clear, but there was no statistically significant interaction. The binding data are consistent with the aggregation results in that low zinc status decreased membrane binding and then most significantly when calcium was added. The present data agree with previous results[18,23] showing that calcium stabilizes a PKC complex in membranes and that phorbol esters interact with the complex to form a more stable complex; one that requires removal of calcium with a chelator to cause dissociation. This concept is illustrated below.



where M is membrane, E is PKC, and P is phorbol ester. According to this scheme, calcium promotes the formation of a membrane-PKC complex that is readily dissociated. Phorbol ester binds to the complex with high affinity when the calcium concentration is high. The mechanism(s) by which zinc increases the stability of the membrane-PKC complex, and thereby phorbol dibutyrate binding, is unknown. Zinc may change the conformation of one or more components of the [M·E·Ca] complex and thus increase its stability. Alternatively, zinc may have a direct and independent effect on the phorbol ester binding affinity of the complex. As used here, the term "stabilize" refers to increased phorbol ester binding, resulting from either increased binding affinity or a decrease in loss of binding sites.

It should be noted that, in contrast to most studies of PKC subcellular distribution, calcium chelators were not present during the sonication and separation procedure used in this study. As shown in the accompanying paper,[24] the presence of calcium chelators has a dramatic effect on the subcellular distribution of phorbol ester binding and thus on the phenomenon commonly attributed to translocation of PKC. Chelators extract calcium and thereby destabilize the PKC-membrane complex. As a result, the phorbol ester bound to PKC appears in the soluble or cytosolic fraction. Any factor, such as zinc, that stabilizes the membrane complex leads to an apparent change in distribution that has been referred to as translocation.[24]

From these results it is evident that the impaired platelet aggregation observed in zinc-deficient rats is not due to a change in the subcellular distribution of PKC. In agreement with earlier observations,[5,6] the biochemical defect in phorbol ester-mediated aggregation appears to be related to the uptake of calcium by the intact platelet or the access of calcium to membrane-bound PKC. Adequate zinc status stabilizes the association of PKC with membranes, as shown here, and maintains the function of calcium channels,[5] supporting the hypothesis that zinc has a primary role in the function of the plasma membrane.[25] Zinc status has a negligible effect on PKC location within the platelet and on PKC activity without in vitro addition of calcium, suggesting that the primary effect of zinc relates to membrane transport of calcium.

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