Zinc and thrombin increase phorbol ester binding to platelet membranes by a mechanism not involving translocation

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Addition of zinc to human platelets increases the binding of phorbol esters, and this was postulated to be the result of protein kinase C translocation. To test this hypothesis, washed rat platelets were treated with either a combination of 20 μmol/L zinc and 20 μmol/L pyrithione (a zinc ionophore), or 0.1 U/mL of thrombin before determination of phorbol dibutyrate binding to subcellular fractions. As described previously, phorbol ester binding to intact platelets was increased by treatment with zinc and pyrithione. To obtain cytosol and mixed membranes, platelets were sonicated in a buffer containing either no chelators or a combination of 1 mmol/L EDTA and 2.5 mmol/L EGTA. In the absence of chelators, both zinc and thrombin treatment increased specific binding to membranes approximately 12% but had no effect on cytosol binding. In their presence, most binding sites were in the cytosol. Both agonists increased membrane binding by 35% and decreased cytosol binding by 21%. The results suggested an increased affinity or stability of the phorbol ester binding sites associated with protein kinase C in membranes rather than the translocation of protein kinase C from cytosol to the membranes. To confirm that translocation was not responsible for the increase, membranes were treated with the agonists and phorbol dibutyrate binding measured after washing with and without chelators. Binding to the washed membranes was increased approximately 20% in all cases and was generally decreased by chelator treatment. Thus, both zinc and thrombin increased binding to platelet membranes by a process that did not involve translocation. (J. Nutr. Biochem. 5:542–546, 1994.)

Keywords: rat platelets; zinc; thrombin; protein kinase C; phorbol ester binding; translocation

Introduction

Zinc deficiency adversely affects platelet aggregation,[1–5] and high concentrations of zinc added in vitro stimulate aggregation.[6,7] The mechanism(s) by which dietary zinc deprivation and in vitro zinc addition affect aggregation is unknown, but it may involve the signal transduction pathway. Protein kinase C (PKC) could be a compromised component of the pathway in zinc deficiency in as much as it is a zinc metalloenzyme[8,9] and is essential for platelet stimulation and aggregation.[10,11] Platelets contain the conventional α, β, and γ isozymes of PKC, and the conserved C_1 region of the regulatory domain of β_1 binds four atoms of zinc.[8]

Supporting this hypothesis are several reports[12–15] showing that zinc increases the binding of phorbol esters to intact cells and cell homogenates. The results were interpreted to show that zinc activates PKC and causes its translocation from cytosol to membranes.[12,13] Others [16,17] have observed that zinc activates PKC and stimulates its binding to plasma membranes of T lymphocytes when they are treated with phorbol esters. The results were also interpreted to confirm earlier reports of translocation.[18–20] In an accompanying paper, we reported that zinc deficiency in rats decreases phorbol ester binding to platelet membranes but has no effect on the subcellular distribution of binding sites.[21]

The conventional PKC isozymes require calcium, phospholipid, and diacylglycerol (DG) or a DG equivalent such as phorbol ester, for enzymatic activity, but the activation process in a stimulated cell is not clearly understood. Phospholipid is present in membranes but must be added to cytosol to detect PKC activity or phorbol ester binding in that subcellular fraction. Phorbol esters bind to the DG site and can substitute for endogenously generated DG in the activation of PKC in intact cells.[22] Numerous authors have postulated that the activation of PKC in platelets[18] and other cells involves translocation of cytosolic PKC to the plasma

This research was supported by NRICG/USDA Grant No. 91-37200-6286.
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Received March 1, 1994; accepted June 13, 1994.

membrane. However, the authors,[19,20] who originally suggested the translocation mechanism pointed out other possibilities, such as stabilization of PKC in membranes, that would explain the results observed.

Most investigations of subcellular PKC distribution have involved separation of cell components in the presence of calcium chelators and measurement of PKC activity. The chelators were added originally to protect PKC against proteolysis by calcium-dependent proteases and were essential for measurement of PKC activity. When cells are extracted in the presence of calcium chelators, most of the PKC is recovered in the cytosol. In the absence of chelators and with free calcium present, most of the PKC is found in the membrane fraction.[23] This has been demonstrated in human platelets stimulated by PMA or thrombin[18,24] and in B-cells and neutrophils stimulated by zinc.[12,16]

Though the translocation theory has been questioned,[25] there is little or no experimental evidence against it. This paper presents data that support another explanation of "translocation." Platelets from rats fed a commercial diet were stimulated with zinc or thrombin and sonicated in the presence and absence of calcium chelators. Treatment of both intact platelets and washed mixed membranes with the stimulants increased phorbol ester binding to the membranes. The results suggest that the stimulants either increased binding affinity or stabilized a phorbol ester-PKC-membrane complex in the membranes but did not cause translocation of PKC. The results agree with those made with rats fed a purified diet containing low and adequate zinc concentrations.[21]

Methods and materials

Source of platelets

Male rats of Wistar origin were obtained from a closed colony maintained in the Department of Biochemistry. They were fed a grain-based diet, and blood was collected in 0.1. volume of 100 mmol/L sodium citrate, pH 7.4. Washed platelets were prepared as in the accompanying paper[21] and were suspended finally in modified Ardlie and Han buffer,[21] pH 7.4, without bovine serum albumin (BSA).

Separation of membranes and cytosol

After treatment of the washed platelets with an agonist as described below, they were collected by a 1-min spin in an Eppendorf microcentrifuge (12,000*g*). The pellets was quickly resuspended in 0.8 mL of ice-cold isolation buffer (25 mmol/L Tris-HCL, pH 7.4, 1 mmol/L dithiothreitol, 2 mmol/L PMSF, 10.5 μmol/L leupeptin) with or without added chelators (a combination of 1 mmol/L EDTA and 2.5 mmol/L EGTA). The suspension was sonicated with four 10-sec bursts with a Vibra Cell sonicator (Model VC375, Sonics and Materials, Inc, Danbury, CT USA) using a microtip at power setting of 4. After sonication, intact platelets were removed by a 1-min spin in the microcentrifuge, and the supernate centrifuged at 100,000*g* for 30 min at 4° C. The final supernate, designated cytosol, contained approximately 1 mg protein per mL, and the particulate fraction, designated mixed membranes, contained about 1 mg protein per mL when suspended in 0.7 mL of buffer.

Preparation of washed mixed membranes

To determine the effect of agonists on membranes in the absence of cytosol, washed mixed membranes were prepared. After the 100,000*g* centrifugation, the cytosolic fraction was decanted and 0.5 mL of 25 mmol/L Tris-HCl buffer, pH 7.4, was added. The tubes were gently shaken, the buffer decanted, and the superficially rinsed pellet sonicated in 0.7 mL of the Tris buffer. The membranes were collected by centrifugation at 100,000*g* for 30 min at 4° C. The supernate was decanted, the pellet resuspended, and sonicated again. The pellet at this stage was designated washed mixed membranes.

Binding assay

Phorbol ester binding was performed as previously reported[16,21] and is described briefly below:

Mixed membranes. 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 (New England Nuclear, DuPont, Wilmington, DE USA). 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).

Intact platelets. The procedure for [^{3}H]PDBu binding to intact platelets was similar to that for the mixed membranes except that sodium pyrithione (Sigma Chemical, St. Louis, MO USA) and zinc chloride were added directly to the binding buffer. In the case of mixed membranes, these agents were present only prior to separation of membranes and cytosol.

Cytosol. The assay was carried out in 1.5 mL microcentrifuge tubes containing the same binding buffer used for membranes and intact platelets except that it contained in addition 300 μg/mL of phosphatidylserine. 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. After 15 min of 0° C, the tubes were centrifuged in an Eppendorf microcentrifuge (12,000*g*), and precipitated protein transferred to a vial containing scintillation fluid.

Protein assay

Protein was measured by the method of Lowry et al. using BSA as the standard and the isolation buffer as the blank.[26]

Experimental protocols

Three binding experiments were performed that involved intact platelets treated with zinc, mixed membranes, and cytosol isolated from platelets treated with zinc or thrombin (Sigma Chemical, St. Louis, MO USA) and washed membranes treated directly with zinc or thrombin. Except for binding to intact platelets, all zinc treatments were in combination with 20 μmol/L pyrithione, the zinc ionophore. In the results, "n" refers to the number of independent experiments, i.e., platelet pools assayed.

Experiment 1

Washed platelets from individual rats (3 to 6 for each treatment) were used. Except when added alone, sodium pyrithione (20 μmol/L) was added 3 min before the addition of graded levels (0 to 100 μmol/L) of zinc chloride. [^{3}H]PDBu binding was performed as described.

Experiment 2

Five pools of washed platelets (four rats/pool) were used to determine the effects of zinc or thrombin treatment, respectively, on

[^{3}H]PDBu binding to membranes and cytosol. For the zinc treatment, pyrithione was added to intact platelets, as described above, followed by 20 μmol/L zinc chloride. For thrombin (Sigma Chemical) treatment, 0.1 U/mL thrombin was added. Tubes were shaken gently and allowed to stand for 1 min before collection, sonication, and separation of cell fractions as described above. Note that the procedure for isolation of membranes and cytosol removed the calcium-containing buffer before sonication, and that sonication was performed in the presence and absence of chelators.

Experiment 3

Four different pools of washed platelets (four rats/pool) were used to determine the stimulatory effect of zinc and thrombin on [^{3}H]PDBu binding to isolated washed membranes. For zinc and thrombin treatment of washed mixed membranes, a suspension of mixed membranes in 25 mmol/L Tris-HCl buffer containing 1 mmol/L Ca^{2+} and approximately 1.5 mg of protein was divided into four 0.5-mL aliquots. Two aliquots were used to determine the effect of zinc (± 20 μmol/L) with and without chelator treatment and two for the effect of thrombin (± 0.1 U/mL) with and without chelator treatment. After addition of the agonists, the tubes were allowed to stand for 10 min at room temperature and the membranes collected by centrifugation at 100,000*g* for 30 min. The supernate was decanted and the membrane pellet sonicated in the Tris-HCl buffer with or without the addition of 2.5 mmol/L EGTA and 1 mmol/L EDTA. The membranes were collected again by centrifugation, suspended in 0.7 mL of buffer, and sonicated as before.

Statistical analysis

Data from experiment 1 were analyzed by one-way analysis of variance, using the CRUNCH statistical program for the IBM PC (Interactive Statistical Package, Crunch Software Corp., San Francisco, CA USA), with three to six replications (rats) per treatment. Differences between treatment means were assessed by post-hoc *t* test, with $P < 0.05$ considered significant. Data for experiments 2 and 3 were analyzed as two-way factorial designs with replication, using the GLM procedure of SAS (SAS Institute, Cary, NC USA). Data for thrombin and zinc treatments for each experiment were analyzed separately. Analysis included main effects of replicate (platelet pool), agonist (thrombin or zinc), and chelators, as well as the agonist-chelator interaction. Differences between means were assessed using the Least Squares Means component of the GLM procedure, with significance set at $P < 0.05$.

Results

The effect of added Zn^{2+} and pyrithione on [^{3}H]PDBu binding to intact platelets (experiment 1) is shown in *Figure 1*. In agreement with the results of Forbes et al.,[13] both Zn^{2+} alone and pyrithione alone enhanced the binding of [^{3}H]PDBu to intact platelets. The combination of Zn^{2+} (10 μmol/L) and pyrithione (20 μmol/L) increased binding threefold to fourfold. In the presence of pyrithione, there was little difference between 3 μmol/L and 30 μmol/L zinc. The small increase of phorbol ester binding due to pyrithione alone was probably the result of slight zinc contamination of reagents.

In experiment 2, after stimulation with zinc and pyrithione, intact platelets were sonicated in the presence or absence of calcium chelators, and the mixed membranes separated from the cytosol. As shown in *Figure 2*, the combination of 2.5 mmol/L EGTA and 1 mmol/L EDTA had a major effect

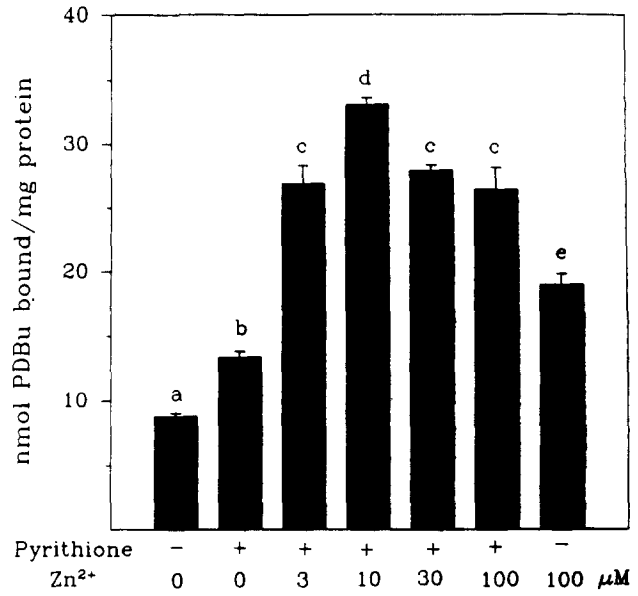

Figure 1 Effect of Zn^{2+} and pyrithione on specific binding of phorbol dibutyrate (PDBu) to intact platelets. Zinc and pyrithione alone and graded concentrations of Zn^{2+} (0 to 100 μmol/L) in combination with 20 μmol/L sodium pyrithione were added to platelet suspension before binding of [^{3}H]PDBu was determined. Bars represent means; the number of trials, n, equals 3 to 6. SEM is indicated by the bar extensions. Bars with different superscripts represent significantly different means ($P < 0.05$).

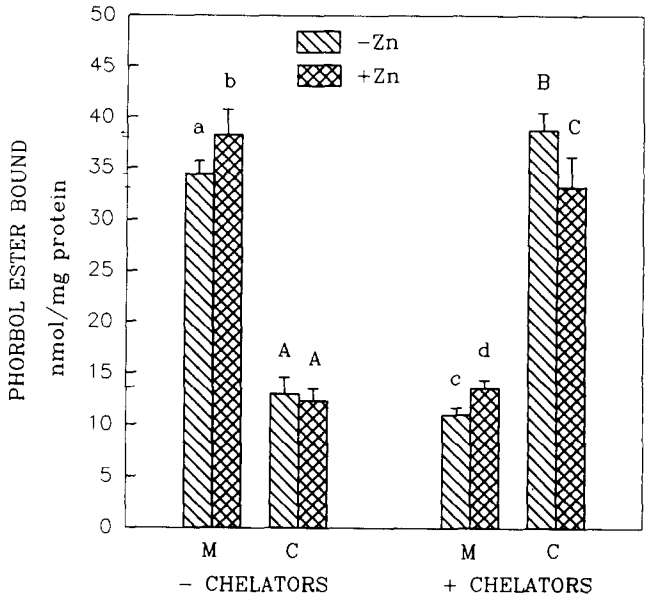

Figure 2 Effect of zinc plus pyrithione on the binding of phorbol dibutyrate to platelet mixed membranes (M) and cytosol (C). Zinc (20 μmol/L and pyrithione 20 μmol/L) were added to intact platelets before separation of components in the presence (+) and absence (−) of the chelators, EDTA and EGTA. n equals 5. Other symbols and conditions are the same as in *Figure 1*.

on PDBu binding, reducing membrane binding approximately threefold and increasing cytosolic binding by a similar factor. The addition of chelators reduced the membranes to cytosol binding ratio from approximately 3 to 0.3. Without chelators, zinc caused a significant increase (12%) in PDBu binding to mixed membranes but had no effect on the cytosol binding. In the presence of chelators, zinc treatment also significantly increased membrane binding (23%) but decreased cytosol binding (47%). Thus, compared with control platelets, in vitro zinc increased PDBu binding to platelet mixed membrane regardless of chelators, but the effect on cytosol binding depended on the free calcium concentration during the separation procedure.

As shown in *Figure 3,* thrombin stimulation of platelets had an effect similar to that of zinc. Thrombin increased membrane binding of [^{3}H]PHBu regardless of the presence of chelators and decreased cytosolic binding only when the calcium chelators were present. These results, as well as those with zinc, were not consonant with the concept of translocation.

To confirm that the effects of the stimulants involved membrane stabilization of PKC rather than its translocation from the cytosol to the membranes, binding to washed membranes was determined (experiment 3). As shown in *Figure 4,* treatment of washed mixed membranes with both zinc and thrombin increased PDBu binding regardless of the subsequent extraction with EGTA and EDTA. In general, there was less PDBu bound by membranes that were extracted with chelators, confirming earlier observations[27,28] that calcium has a major stabilizing effect on phorbol ester binding by membranes.

Discussion

The results of experiment 1 show that in vitro zinc stimulates the binding of a phorbol ester to intact rat platelets confirming

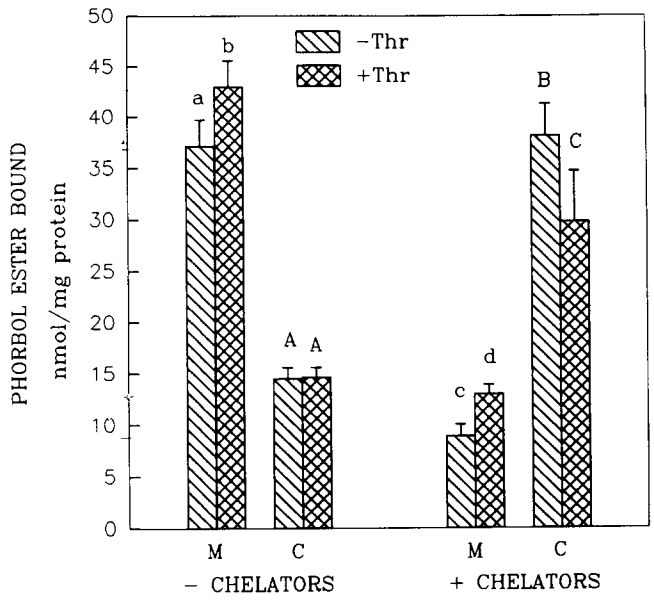

Figure 3 Effect of thrombin on the binding of phorbol dibutyrate to platelet mixed membranes (M) and cytosol (C). Thrombin (0.1 unit/mL) added to intact platelets before separation of components. n equals 5. Other symbols and conditions are the same as in *Figure 1.*

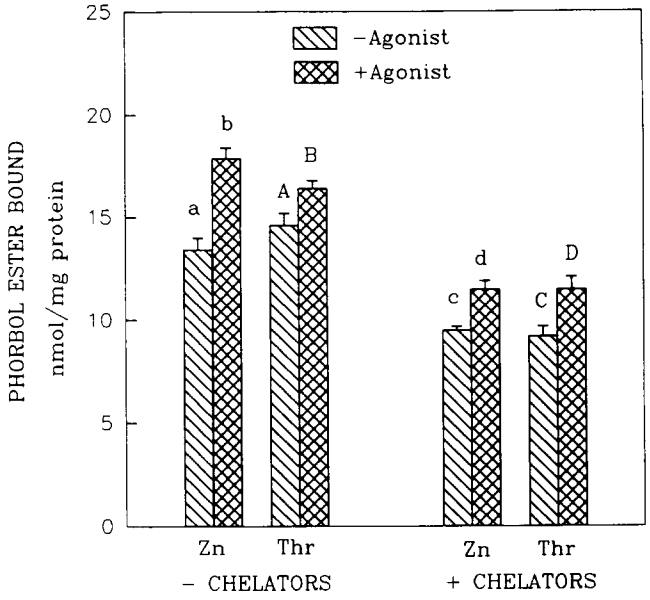

Figure 4 Effect of zinc and thrombin on the binding of phorbol dibutyrate to washed platelet membranes. Zinc and pyrithione (20 μmol/L each) and thrombin (Thr, 0.1 U/mL) were added to the washed membranes before the second sonication. They were then extracted with and without chelators. n equals 4. Other symbols and conditions are the same as in *Figure 1.*

the observations of Forbes et al.[12,13] made on human platelets and other cells. However, the mechanism by which zinc increases phorbol ester binding to membranes and its relationship to the subcellular distribution of PKC remains moot. Clearly, the binding of PDBu to mixed membranes after stimulation of intact platelets by both zinc and thrombin was increased, but there was not a comparable decrease in cytosol binding when the platelets were sonicated in the presence of free calcium, i.e., without the addition of calcium chelators. This observation does not support the concept that the stimulants caused PKC to move from cytosol to membranes. A decreased ratio of cytosol to membrane PKC when cells are treated with various agonists has been observed repeatedly.[12,16,24] The phenomenon has been explained as translocation of PKC from cytosol to membranes, although it is recognized that there are other explanations.[19,20] In most studies, relatively high concentrations of calcium chelators were employed during the separation of membrane and cytosol so as to protect against proteolysis of the PKC by calpain and other Ca^{2+}-dependent neutral proteases.[29] While proteolysis may have occurred in the present studies in which the subcellular separation was done in the presence of calcium, the results remain valid in as much as the regulatory domain remained intact and allowed phorbol ester binding. Although the free calcium concentration during sonication is unknown, it was negligible in the presence of the chelators.

Phorbol esters bind to the regulatory domain of PKC, and subcellular binding depends on PKC location. Calcium concentration has a major effect on the location of PKC in membranes and thus on the binding of phorbol esters and the PKC activity.[27,28] Calcium promotes the formation of a PKC-membrane complex that is readily reversible, and phorbol esters stabilize the complex so that it dissociates only

when calcium is removed by chelators. As shown here, in the presence of calcium most of the binding sites are in the membranes, and with low calcium most are in the cytosol. The membrane to cytosol binding ratio was changed 10-fold, confirming many earlier observations.[13–18] It is significant that treatment of platelets with either zinc or thrombin had an additional effect on phorbol ester binding, increasing membrane binding regardless of calcium concentration. These results suggest that the agonists either increased the affinity of the membrane binding sites, presumably membrane-associated PKC, or stabilized binding sites in the membrane. Only with the extremely low free calcium concentration, produced by chelators, did zinc and thrombin decrease cytosol binding in concert with increased membrane binding of phorbol dibutyrate.

A critical test of the translocation hypothesis was the use of washed mixed membranes. Stimulation of these membranes in the absence of cytosol gave results analogous to those obtained with intact platelets. The results show that both zinc- and thrombin-treated mixed membranes bound more PDBu than untreated membranes. In agreement with the intact platelet experiment, calcium chelators removed less binding activity from the stimulated membranes than from untreated controls.

The mechanism by which zinc and thrombin increase phorbol ester binding to membranes is unclear. One possibility is that they change the conformation of the membrane-PKC complex so as to increase its binding affinity for phorbol esters. Another possibility is that they increase the stability of the complex, thus reducing the loss of membrane binding sites during the isolation procedure. Stabilization would likely involve the step that can be reversed only by detergents.[28]

Calcium effects conformational change in PKC so as to activate the enzyme.[30] Zinc and thrombin appear to form or promote more stable complexes involving calcium and PKC in rat platelet membranes. They may accommodate the formation of the calcium-PKC complex or independently induce conformational change that increases membrane binding of PKC. It is notable that zinc deficiency decreases phorbol ester binding to membranes,[21] while addition of zinc in vitro increases it. In the presence of adequate free calcium, neither zinc nor thrombin affects platelet cytosol binding of phorbol esters.

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