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Communication

Zinc Can Increase the Activity of Protein Kinase C and Contributes to Its Binding to Plasma Membranes in T Lymphocytes*

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In the primary structure of protein kinase C, the presence of a putative metal-binding site has been suggested (Parker, P. J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M. D., and Ullrich, A. (1986) *Science* 233, 853–859). In the present report, we demonstrate that the most abundant intracellular heavy metal, zinc, can increase the activity of cytosolic protein kinase C. Zinc reversibly binds the enzyme to plasma membranes, and it may contribute to the calcium-induced binding as well. The intracellular heavy metal chelator *N,N,N′,N′*-tetrakis(2-pyridylmethyl) ethylenediamine prevents the phorbol ester- and antigen-induced translocation of protein kinase C. This effect can be totally reversed by the concomitant addition of Zn^{2+}, while Fe^{2+} and Mn^{2+} are only partially counteractive. Our results suggest that zinc can activate protein kinase C and contributes to its binding to plasma membranes in T lymphocytes induced by Ca^{2+}, phorbol ester, or antigen.

The activation and translocation of protein kinase C are well established as key steps in the signal cascade, inducing the proliferation of T lymphocytes (1–3). Binding of protein kinase C to membranes requires an elevated level of intracellular Ca^{2+} concentration (Ca^{2+}-induced or "reversible" binding) or the presence of diacylglycerols which can be very effectively substituted by phorbol esters. These two factors may also act synergistically: Ca^{2+} may "prime" protein kinase C (and/or its putative docking protein, receptor) for its diacylglycerol- or phorbol ester-stabilized binding to membranes (4–8). However, the mechanism of the translocation of protein kinase C is far from being fully elucidated.

When defining the primary structure of protein kinase C, Parker *et al.* (9) suggested the existence of a putative metal-binding site in the enzyme. We have reported earlier that 12-*O*-tetradecanoylphorbol-13-acetate (TPA)[1] treatment of T lymphocytes results in the translocation of heavy metals (mainly zinc) from the nucleus and mitochondria to the cytosol and microsomes (10–12).[2] In our present report we demonstrate that zinc may be involved in both the activation of protein kinase C and its binding to microsomal membranes of T lymphocytes.

EXPERIMENTAL PROCEDURES

Materials—ATP, bovine serum albumin, CHAPS, Chelex 100, 1,2-diolein, DTT, EDTA, EGTA, Hepes, histone (type III-S), leupeptin, β-mercaptoethanol, Nonidet P-40, phorbol-12,13-dibutyrate, phosphatidylserine, sodium orthovanadate, TMB-8, TPA, and Triton X-100 were from Sigma. Cell culture media and its supplements were Gibco products. BMA 030 anti-CD3 antibody (13) was kindly provided by Dr. F. Seiler and Dr. R. Kurrle (Behringwerke AG, Marburg, Federal Republic of Germany). The antibody was originally referred to as BW 264/56 (13), but was later designated as BMA 030 which is the nomenclature used here. TPEN was obtained from Behring Diagnostics, and H-7 was from Seikagaku America Inc. (St. Petersburg, FL). Polyethylene glycol (M_r 6000) was purchased from Serva. $CaCl_2$, $FeSO_4$, $MgCl_2$, $MnCl_2$, and $ZnCl_2$ were ultrapure Merck chemicals. [γ-^{32}P]ATP (3000 Ci/mmol) and [20-^{3}H]phorbol-12,13-dibutyrate (18.9 Ci/mmol) were obtained from Amersham Corp. and Du Pont-New England Nuclear, respectively.

Preparation of T Lymphocytes and Their Subcellular Fractions—Mouse (CFLP, 6–8 weeks old, male) or rabbit (~1 kg, male) thymocytes were prepared by the method of Kleiman *et al.* (14). Human peripheral blood lymphocytes were obtained by centrifugation of heparinized buffy coat on a Ficoll density gradient. Cells were kept in RPMI 1640 medium supplemented with 10 mM Hepes (pH 7.4). The viability of the cells was never less than 95% as judged by trypan blue exclusion. Each experiment is shown in only one cell type but was usually performed in all three cell types: the results were not significantly different. After preincubation(s) specified in different experiments, cells were suspended in an isolation buffer (consisting of 1 mM EGTA, 2 mM EDTA, 5 mM DTT, and 20 mM Hepes (pH 7.4), if not otherwise indicated) at a cell density of 10^8 cells/ml. Disruption of cells and isolation of their cytosolic and microsomal fractions were done as described earlier (15). Since T lymphocytes contain a relatively small amount of endoplasmic reticulum (15), our microsomal preparation can be regarded as a crude plasma membrane fraction. In this way we use both terms for the designation of the membrane preparation. Microsomes were suspended in the isolation buffer at a protein concentration of 0.5–1.5 mg/ml. Protein concentration was determined simultaneously by the method of Lowry *et al.* (16) and Bradford (17), correcting the results for sulfhydryl compounds, Hepes, and detergents, respectively. Protein kinase C activity was determined immediately after isolation.

Measurement of Protein Kinase C Activity—Cytosolic protein kinase C activity was measured from aliquots of the cytosolic fraction without any further purification. Membrane-bound protein kinase C was solubilized from microsomes by incubation with either 0.5% (v/v) Triton X-100 or with 2 mM (2 mg/mg protein) CHAPS in isolation buffer for 20 min at 4 °C, setting the protein concentration to 1.0 to 0.5 mg/ml, respectively. In preliminary experiments we have found that the detergent CHAPS is much more suitable for the solubilization of protein kinase C than Triton X-100 or Nonidet P-40 for two reasons: 1) it does not decrease the activity of protein kinase C significantly, even at concentrations as high as 4–5 mM which was

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[1] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio)propanesulfonate; DTT, dithiothreitol; EGTA, [ethylenebis(oxyethylenenitrilo)tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; TMB-8, 8-(*N,N*-diethylamino)octyl-3,4,5-trimethoxybenzoate; TPEN, *N,N,N′,N′*-tetrakis(2-pyridylmethyl)ethylenediamine.

[2] P. Csermely and J. Somogyi, *J. Cell. Physiol.*, in press

proved by its removal by a Pierce Extracti-Gel D column (data not shown); therefore, we could eliminate the time-consuming DEAE ion-exchange chromatography from our protocol; 2) CHAPS does not significantly disturb the Bradford protein assay (18), in this way a much more exact protein determination could be achieved. After incubation with detergents, the nonsoluble material was removed by centrifugation (100,000 × *g*, 60 min, 4 °C). Protein kinase C activity was determined using a reaction medium containing 10 mM $MgCl_2$, 0.4 mM EGTA, 400 μg/ml histone, 400 mM Hepes (pH 7.4), and 60 μM [γ-^{32}P]ATP (100 cpm/pmol) with or without 2 mM $CaCl_2$, 200 μg/ml phosphatidylserine, and 20 μg/ml 1,2-diolein. (Lipids were dispersed in a double-concentrated Hepes/EGTA/Mg buffer by sonication). The reaction was started by mixing 100 μl of reaction medium with 4–6 μg of sample protein in 100 μl of isolation buffer. After an incubation of 3 min at 37 °C, unless otherwise indicated, the reaction was stopped by the addition of 200 μl of chilled, freshly made trichloroacetic acid (10%, w/v) containing 1 mM ATP and 15 mM K_2HPO_4. 100 μl of bovine serum albumin (2 mg/ml) was added as a carrier, and the precipitated proteins were collected in 0.45-μm Millipore filters. The precipitate was washed by 5 × 5 ml of chilled trichloroacetic acid (5%, w/v) containing 15 mM K_2HPO_4, the filters were dried, and the radioactivity was measured. The enzyme activity is expressed as the difference between the values measured in the presence and absence of phospholipids and Ca^{2+} in units of nanomoles of ^{32}P transferred per min/mg protein of the sample.

Binding of [^{3}H]Phorbol Dibutyrate—Binding of [20-^{3}H]phorbol-12,13-dibutyrate was measured by modification of the method of Leach *et al.* (19). Briefly, 20–100 μg of protein in 200 μl of isolation buffer was added to 200 μl of binding medium containing 40 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 nM [^{3}H]phorbol-12,13-dibutyrate. In preliminary experiments, we have found that the presence of bovine serum albumin (at a final concentration of 1–10 mg/ml) in the binding medium drastically reduces the specific binding in the case of both cytosolic and membrane samples, while the nonspecific binding (measured in the presence of 1 μM phorbol-12,13-dibutyrate) remains unaffected. Therefore, bovine serum albumin was omitted from the binding medium. Measurement of phorbol dibutyrate binding to cytosolic samples was done in the presence of 200 μg/ml phosphatidylserine. After an incubation of 30 min at 37 °C samples were cooled to 0 °C, and 50 μl of bovine serum albumin (20 mg/ml) and 200 μl of 30% (w/v) polyethylene glycol were added. Further steps of the binding assay were identical with those described by Leach *et al.* (19). Measurements of [^{3}H]phorbol dibutyrate binding were done in duplicate with a difference of less than 15%. Some samples were analyzed after freezing in liquid nitrogen and storing 1–4 weeks at −80 °C. Preliminary experiments indicated no significant difference in the phorbol ester binding capacity of fresh and frozen samples.

RESULTS AND DISCUSSION

Zinc Can Activate Protein Kinase C—As seen in Fig. 1, cytosolic protein kinase C is activated by the addition of Zn^{2+} while the activity of "basal" kinases (measured in the absence of calcium and phospholipids) does not change significantly. The activation of protein kinase C ranges between 30 and 90% in different preparations at optimal total zinc concentrations in the range of 0.7–1.0 mM. The activity of membrane-bound protein kinase C cannot be increased significantly by the addition of zinc. However, at zinc concentrations higher than 2.0 mM, zinc sharply inhibits the activity of both cytosolic and membrane-bound protein kinase C (data not shown). Under optimal conditions for the activation of protein kinase C, zinc has a free concentration in the range of 10^{-8} M, while the inhibition occurs at free Zn^{2+} concentrations in the micromolar range. The approximate free zinc concentrations were estimated after Bartfai (20) using the stability constants of EGTA, EDTA, and DTT (given in Refs. 20 and 21) corrected to pH 7.4 with the corresponding α_H values. We have to note that even the estimation of free Zn^{2+} concentrations is extremely difficult in a system that contains three chelators (EGTA, EDTA, DTT), three divalent cations (Ca^{2+}, Mg^{2+}, Zn^{2+}), endogenous chelators (proteins), and cations of the cytosolic fraction. In this way the values of free Zn^{2+} concentrations can be regarded only as very rough estimates.

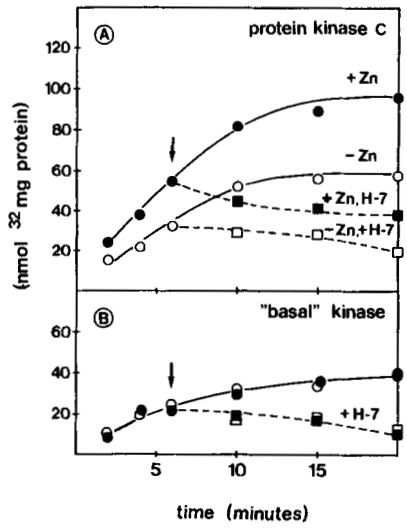

FIG. 1. **Activation of protein kinase C by zinc.** Cytosolic protein kinase C was isolated from rabbit thymocytes, and its activity was measured as described under "Experimental Procedures." In *panel A* the activity of protein kinase C and in *panel B* the activity of basal kinases are shown. *Open symbols* represent activities measured in the absence and *filled symbols* in the presence of zinc at a total concentration of 0.75 mM. At the *arrows* the protein kinase inhibitor H-7 was added to aliquots of the reaction mixtures, and protein kinase activities were measured at different time points in the absence (○—○, ●—●) and presence (□—□, ■—■) of H-7 at a final concentration of 100 μM. Data are representatives of four experiments with standard errors less than 15%.

The variation of both the optimal concentration of zinc and the rate of activation of protein kinase C may reflect that the metal-binding site of the enzyme is differently saturated with endogenous heavy metals in different preparations. This possible diversity may be related to the existence of different, genetically defined isoforms of the enzyme, as reported by many laboratories (22, 23 and references therein). In their early report Nishizuka and his co-workers (24) did not find any effect of zinc on the activity of protein kinase C. However, in their early studies they investigated the proteolytic product of the enzyme (protein kinase M) which lacks the regulatory domain. Since the metal-binding site has been proposed to be part of the regulatory domain (9), this apparent discrepancy supports the role of the putative metal-binding site in the zinc-induced activation of protein kinase C.

The cause of the zinc-induced protein kinase C activation is neither the effect of the Cl^- anions, since it can be demonstrated by $ZnSO_4$ or zinc acetate as well, nor the effect of Ca^{2+} ions which are displaced by zinc in their EGTA-, EDTA-complexes, since no activation of protein kinase C occurs if we add equimolar excess of calcium instead of zinc (data not shown). The activation cannot be attributed to the activation of a Ca^{2+}-dependent protein kinase, since no activation occurs in the presence of both zinc and calcium (and in the absence of phospholipids) (not shown). Zinc does not influence the "basal" protein kinase activity (Fig. 1). This argues against the general role of proteolysis in the zinc-induced enzyme activation. The activation is a "real" activation of the kinase and not the inhibition of the corresponding phosphatases, since after the inhibition of protein kinase C by the protein kinase inhibitor, H-7 (25), the decline in the level of phosphorylation has a similar rate in the absence and presence of zinc (Fig. 1). However, zinc is known to be a better inhibitor of phosphotyrosine protein phosphatases than those of phosphoserine and phosphothreonine phosphatases (26). This raises

TABLE I

Binding of protein kinase C to plasma membranes induced by calcium and zinc

The cytosol and crude plasma membrane fraction of rabbit thymocytes were isolated as described under "Experimental Procedures." The isolation buffer contained 20 mM Hepes (pH 7.4), 10 mM β-mercaptoethanol, and the specific additions indicated. After isolation, protein kinase C activity was determined as described under "Experimental Procedures." Data are mean ± S.D. of three experiments.

Method of isolation (specific additions to the isolation buffer)	Protein kinase C activity	
	Cytosol	Plasma membranes
	nmol ^{32}P/min/mg protein	
EDTA (2 mM), EGTA (1 mM)	6.0 ± 0.5	1.5 ± 0.3
EDTA (2 mM), EGTA (1 mM), 50 μg/ml leupeptin	6.1 ± 0.4	1.4 ± 0.4
$CaCl_2$ (3.1 mM), EGTA (1 mM), EDTA (2 mM)	5.1 ± 0.7	2.0 ± 0.3
$CaCl_2$ (0.1 mM), 50 μg/ml leupeptin	2.0 ± 0.2	4.8 ± 0.8
"Ca-plasma membranes" + EGTA extraction[a]	4.6 ± 0.5	2.1 ± 0.4
$ZnCl_2$ (50 μM), 50 μg/ml leupeptin	2.5 ± 0.3	4.5 ± 0.7
"Zn-plasma membranes" + EGTA extraction[a]	4.8 ± 0.4	2.2 ± 0.3

[a] Plasma membranes isolated in the presence of 0.1 mM $CaCl_2$ or 50 μM $ZnCl_2$ were treated with EGTA and EDTA at final concentrations of 1.0 and 2.0 mM, respectively, in the presence of 20 mM Hepes (pH 7.4), and 10 mM β-mercaptoethanol for 60 min at 4 °C. After incubation, membrane vesicles were separated by centrifugation. "Cytosol" denotes the supernatant and "plasma membranes" the pellet, respectively, in these cases.

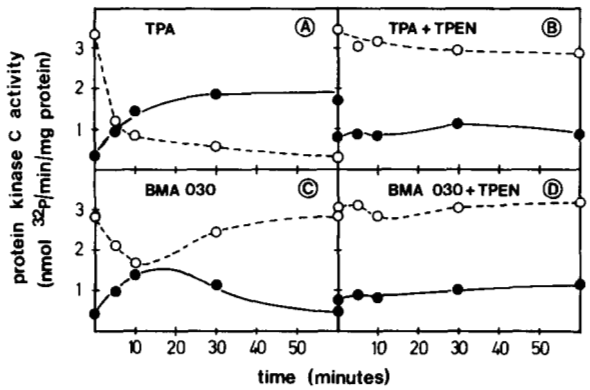

FIG. 2. **The effect of the intracellular heavy metal chelator TPEN on the translocation of protein kinase C.** Human peripheral blood lymphocytes were isolated as described under "Experimental Procedures." Cells were preincubated at 37 °C with TPEN (*panels B and D*) at a final concentration of 50 μM or with its solvent dimethyl sulfoxide (0.5% v/v; *panels A and C*) for 20 min, and the incubation was continued with TPA (*panels A and B*) or with the anti-CD3 antibody BMA 030 (*panels C and D*) at final concentrations of 20 nM and 20 ng/ml, respectively. (The concentration of BMA 030 was selected as the optimum for its mitogenic activity, data not shown.) The incubation was terminated at time points indicated by rapid cooling to 0 °C and centrifugation of the cells. Cytosol and crude plasma membrane fractions were isolated, and their protein kinase C activity was measured as described under "Experimental Procedures." *Open* and *closed circles* represent protein kinase C activities of the cytosol and plasma membranes, respectively. Data are representatives of three experiments.

the possibility that zinc develops a heretofore hidden induction of protein kinase C activity via tyrosine phosphorylation. Our results demonstrate no activation of the cytosolic enzyme after preincubation with 60 μM ATP in the absence or presence of 100 μM sodium vanadate, another known inhibitor of phosphotyrosine protein phosphatases (27) (data not shown). This argues against the role of tyrosine phosphorylation in the zinc-induced activation of cytosolic protein kinase C.

Zinc Induces the Reversible Binding of Protein Kinase C to Microsomes—The results of Table I show that zinc induces the binding of protein kinase C to plasma membranes similarly to the reversible binding induced by calcium which was observed by several groups (5–7, 28). The endogenous calcium content of cytosolic and microsomal fractions can contribute to the zinc-induced binding of protein kinase C. However, we got very similar results after the samples had been treated with the chelating resin, Chelex 100, to remove endogenous calcium (data not shown). This strengthens the conclusion that zinc can induce the binding of protein kinase C to microsomes. The zinc-induced binding is also reversible in the sense that protein kinase C activity can be recovered in the supernatant after EGTA treatment. Interestingly, calcium alone (under conditions when heavy metals are chelated) can induce only a partial translocation of protein kinase C activity to plasma membranes. This suggests that zinc which is usually present in solutions as a contaminant at concentrations as high as 0.5–1 μM (as determined by plasma emission spectroscopy (12)) may contribute to the "Ca^{2+}-induced" binding of protein kinase C and thus plays a general role in the reversible binding of the enzyme to membranes.

The Chelation of Heavy Metals Can Abolish the TPA- and Antigen-induced Translocation of Protein Kinase C: the Effect Can Be Reversed by the Addition of Zinc—Fig. 2 shows that in agreement with earlier reports (see Refs. 1, 3 and references therein), TPA causes a sustained translocation of protein kinase C while the antibody directed against the T cell receptor induces a transient change in the localization of the enzyme activity. Fig. 2 also shows that the intracellular heavy metal chelator TPEN, re-introduced by Arslan *et al.* (29), can abolish the TPA- and antigen-induced translocation of protein kinase C in T lymphocytes. The reason for this inhibition is not the chelation of intracellular calcium ions by TPEN because TPEN causes no decrease in the intracellular calcium concentration (29)[2] and the calcium antagonist TMB-8 is not able to prevent the translocation of protein kinase C (Table II).

As shown in Table II, TPEN loses its ability to prevent the translocation of protein kinase C if it is administered together with zinc, forming a Zn-TPEN complex. This suggests that the reason for the inhibition is not the effect of TPEN as a chemical compound but the effective intracellular chelation of heavy metals (most probably zinc) during the activation of T lymphocytes. The selectivity for zinc is further supported by the fact that two out of the four most abundant intracellular heavy metals (in the order of decreasing total concentration: zinc, copper ≈ iron, and manganese (11, 12)), iron and manganese, can only partially reverse the effect of TPEN (Table II). Fe- and Mn-TPEN *alone* did not cause any effect in the distribution and activity of protein kinase C (data not shown). Copper was not examined since it has a 5 magnitudes higher affinity for TPEN than zinc (30). In this way copper displaces zinc from its complex with TPEN, making it impossible to discriminate between their effects.

The translocation of Ca^{2+}- and phospholipid-dependent histone kinase activity cannot be regarded as an unambiguous sign of the translocation of protein kinase C as a protein because of possible changes in the substrate specificity of the enzyme (31). Therefore, we have investigated the distribution of [^{3}H]phorbol dibutyrate receptors simultaneously. Our results in Table II show that the distribution of phorbol dibutyrate receptor is corresponding to the distribution of protein kinase C activity in different samples. These results suggest that the changes in the distribution of protein kinase C

TABLE II

The effect of different agents on the TPA- and antigen-induced translocation of protein kinase C

Human peripheral blood lymphocytes and their cytosolic and crude plasma membrane fractions were isolated, and the protein kinase C activity and [^{3}H]phorbol dibutyrate-binding capacity were analyzed as described under "Experimental Procedures." Treatment of the cells with different agents was performed as described in the legend of Fig. 2, with the exception that the time of incubation with TPA or the anti-CD3 antibody BMA 030 was 10 min at 37 °C. Zn-TPEN, Fe-TPEN, and Mn-TPEN were produced by mixing equimolar amounts of TPEN and $ZnCl_2$, $FeSO_4$, and $MnCl_2$, respectively. The complexes were administered to get the same final concentration for TPEN (50 μM) as in the treatment with TPEN alone. Cells were treated with TMB-8 at a final concentration of 100 μM for 20 min at 37 °C. Data are mean ± S.D. of three experiments.

Treatment	Protein kinase C activity		[^{3}H]Phorbol dibutyrate binding	
	Cytosol	Plasma membranes	Cytosol	Plasma membranes
	nmol ^{32}P/min/mg protein		*fmol/mg protein*	
Control	3.4 ± 0.6	0.7 ± 0.3	1200 ± 120	150 ± 30
+ TPA	0.4 ± 0.2	1.8 ± 0.4	80 ± 10	560 ± 60
+ BMA 030 antibody	1.6 ± 0.3	1.5 ± 0.2	700 ± 110	510 ± 90
+ TPEN	3.5 ± 0.4	0.6 ± 0.2	1010 ± 30	180 ± 40
+ TPEN, TPA	2.9 ± 0.5	0.6 ± 0.1	950 ± 50	230 ± 20
+ Zn-TPEN, TPA	0.3 ± 0.1	1.5 ± 0.2	ND[a]	470 ± 50
+ TPEN, BMA 030	3.1 ± 0.4	0.8 ± 0.2	1130 ± 70	ND[a]
+ Zn-TPEN, BMA 030	1.7 ± 0.4	1.6 ± 0.3	760 ± 80	360 ± 60
+ Fe-TPEN, TPA	1.8 ± 0.2	0.8 ± 0.2	750 ± 20	150 ± 40
+ Mn-TPEN, TPA	1.5 ± 0.7	1.1 ± 0.3	580 ± 20	250 ± 20
+ TMB-8	3.6 ± 0.3	0.8 ± 0.3	1350 ± 140	200 ± 20
+ TMB-8, TPA	0.3 ± 0.05	1.6 ± 0.3	40 ± 20	570 ± 40
+ TMB-8, BMA 030	1.7 ± 0.3	1.6 ± 0.2	800 ± 50	540 ± 20

[a] ND, not detectable.

activity reflect real changes in the distribution of the enzyme in our system.

Addendum—While this manuscript was being reviewed, Murakami *et al.* (32) published similar results regarding the biphasic activation and inactivation of protein kinase C by Zn^{2+}.

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