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ZINC INCREASES THE AFFINITY OF PHORBOL ESTER RECEPTOR IN T LYMPHOCYTES

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In the primary structure of the major phorbol ester receptor, protein kinase C the presence of putative metal (zinc) binding sites has been suggested. We have demonstrated earlier that zinc activates protein kinase C and contributes to its binding to plasma membranes in T lymphocytes. Here we report that zinc increases the phorbol ester binding affinity of cytosolic protein kinase C. The effect of zinc on the membrane-bound enzyme is much less pronounced. Our results raise the possibility that cytosolic protein kinase C is a mixture of isoenzymes with different sensitivity towards zinc ions.

Six years ago Castagna et al (1) have discovered that tumor promoter phorbol esters specifically and directly activate protein kinase C. After their initial studies numerous reports have demonstrated that indeed, the phorbol ester receptor is protein kinase C (2-4).

Defining the primary structure of protein kinase C Parker et al (5) have found cysteine-repeating sequences in the regulatory domain of the enzyme which often appears as a motif of Zn^{2+}- and DNA-binding domains (6,7). Examining the possible role of these "zinc-fingers" we have found that zinc can activate protein kinase C at nanomolar free concentration (8,9). Simultaneously Murakami et al (10) also demonstrated a zinc-induced activation of the enzyme. Besides the activation of protein kinase C zinc seems to contribute to the Ca^{2+}-, phorbol ester- and antigen-induced binding of the enzyme to plasma membranes in T lymphocytes (8,9).

In the present report as a next step in the elucidation of interactions between protein kinase C and zinc we examine the effect of zinc on binding of radioactive phorbol ester to its cytosolic and microsomal receptors in T lymphocytes.

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MATERIALS AND METHODS

Materials: bovine serum albumin, dithio-treitol, EDTA, EGTA, Hepes, phorbol dibutyrate, phosphatidyl serine and Triton X-100 were from Sigma. Cell culture media were Gibco products. N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN) was obtained from Calbiochem. Polyethylene glycol (M.w.: 6000) was purchased from Serva. $CaCl_2$, $MgCl_2$ and $ZnCl_2$ were ultrapure Merck chemicals. [20-^{3}H]phorbol-12,13-dibutyrate (^{3}H-pdbu), 18.9 Ci/mmol) was obtained from Du Pont-New England Nuclear.

Preparation of microsomes and cytosol of T lymphocytes: Rabbit (1 kg, male) thymocytes were prepared by the method of Kleiman et al (11) in Eagle's Minimal Essential Medium supplemented with 10 mM Hepes (pH 7.4). Cells were suspended in isolation buffer (consisting of 1 mM EGTA, 2 mM EDTA, 5 mM dithio-treitol and 20 mM Hepes, pH 7.4) 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 (12). Microsomes were suspended in isolation buffer at a protein concentration of 0.5-1.5 mg/ml. Protein concentration was determined by the method of Bradford (13).

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 (3) as described earlier (9). Measurements of [^{3}H]phorbol-dibutyrate binding were done in duplicates with a difference of less than 15 %. Data are expressed as a difference of [^{3}H]phorbol-dibutyrate binding in the absence and presence of 1 μM unlabeled phorbol-dibutyrate (specific binding).

RESULTS

Figure 1. shows the dependence of [^{3}H]phorbol-12,13-dibutyrate (^{3}H-pdbu) binding on the total concentration of zinc ions. Zinc increases the cytosolic binding of phorbol-dibutyrate to approximately 2-fold, while it affects only slightly the membrane binding of the phorbol ester. Zinc produces its maximal effect on the cytosolic phorbol ester receptor at a final concentration of 0.35-0.4 mM which corresponds to a free concentration of zinc in the range of 10^{-9} M. The approximate free zinc concentration was estimated after Bartfai (14) using the stability constants of EGTA, EDTA and dithio-treitol --given in refs. 14 and 15-- corrected to pH 7.4 with the corresponding α_H values. We have to note that this value can be regarded only as a very rough estimate, since even the estimation of free Zn^{2+} concentration is extremely difficult in a system which contains three chelators (EGTA, EDTA, dithio-treitol), three divalent cations (Ca^{2+}, Mg^{2+} and Zn^{2+}), endogenous chelators (proteins) and cations of the cytosol.

The aspecific binding of the cytosolic and membrane samples were 39 $\pm$ 6 and 58 $\pm$ 8 fmol respectively in the experiments of Figure 1. Zinc did not influence significantly the aspecific binding of [^{3}H]phorbol-dibutyrate throughout the whole concentration range tested. Without phosphatidyl serine zinc had no effect on ^{3}H-pdbu binding to membrane samples while it had only a very slight effect on the cytosolic binding of phorbol-dibutyrate. The effect of zinc was similar if we tested it at other protein concentrations (data not shown).

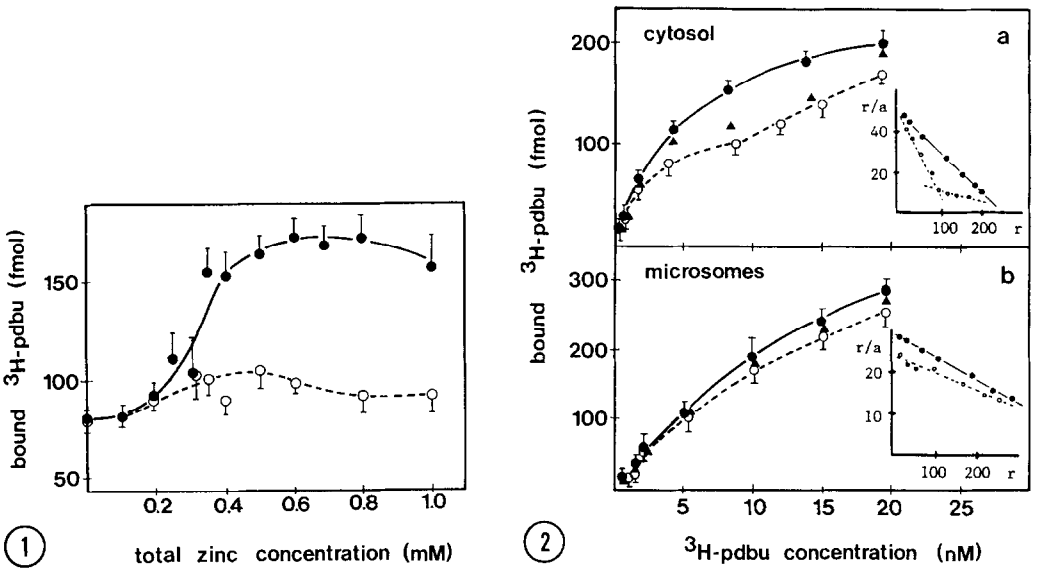

Figure 1. The effect of zinc on the binding of [^{3}H]phorbol-dibutyrate. Cytosolic (●—●) and microsomal (o - - - o) fractions of rabbit thymocytes were isolated and their [^{3}H]phorbol-dibutyrate (^{3}H-pdbu) binding was analysed as described in "Materials and Methods". The binding medium contained 10 nM [^{3}H]phorbol-dibutyrate, 50 μg of cytosolic and 100 μg of microsomal protein, respectively and $ZnCl_2$ at final concentrations indicated. Data are means ± SDs of three separate experiments.

Figure 2. Concentration dependency of [^{3}H]phorbol-dibutyrate (^{3}H-pdbu) binding in zinc depleted and supplemented cytosol (a) and microsomes (b). The cytosol and microsomes of rabbit thymocytes were isolated and binding of ^{3}H-pdbu was measured as described in "Materials and Methods". Binding medium contained 50 μg of cytosolic and 200 μg of microsomal protein, respectively; $ZnCl_2$ at final concentration of 0.7 mM (●—●), 100 μM TPEN (o - - - o) or no addition (▲). ^{3}H-pdbu was added at final concentrations indicated. The insets show the Scatchard analysis of the binding. Data are means ± SDs of three separate experiments.

Figure 2. shows the difference in the concentration dependency of [^{3}H]phorbol-dibutyrate binding in the presence (filled circles) and absence of zinc (open circles). The removal of endogenous zinc was enhanced by the addition of the hydrophobic chelator of heavy metals, N,N,N;N'-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN, 16). TPEN has 7 and 2 orders of magnitude higher affinity for zinc than EGTA and EDTA, respectively (15,17) and it can penetrate to hydrophobic environment as well. In this way TPEN may have a much higher efficacy in removal of zinc from its binding sites than EGTA or EDTA. In the zinc depleted cytosol the binding curve of phorbol-dibutyrate and its Scatchard analysis (see inset in Figure 2., panel a) suggests the presence of at least two binding sites for [^{3}H]phorbol-dibutyrate of different affinity for the phorbol ester. After the addition of zinc (filled circles) the binding of phorbol-dibutyrate can be characterised only

with one, "high-affinity" binding site. The binding constant of the high affinity site is in the range of 3-5 nM while the "low affinity" site of the zinc-depleted sample has a binding constant ≈ 27 nM. The high affinity binding in the zinc depleted cytosol corresponds to approximately 1/3 of the overall binding sites. Phorbol ester binding in the presence of only EGTA and EDTA is similar to that of the TPEN treated samples (see triangles).

Zinc has a much slighter effect on the phorbol-dibutyrate binding of microsomal samples (Figure 2., panel b). Microsomal samples show only a "low affinity" binding. Their binding constants are ≈21 and ≈28 nM in the presence or in the absence of zinc, respectively. The difference in the binding constant of high affinity cytosolic and microsomal phorbol ester receptors is in good agreement with the results of several other laboratories (4,18). The overall increase in the binding constant of microsomal phorbol--dibutyrate binding compared to the high affinity binding sites of the cytosol may reflect the reduced accessibility of phorbol ester binding sites, a possible occupation of the binding sites by endogenous diacyl-glycerols or simply the "dilution" of hydrophobic phorbol ester by the membrane lipids.

DISCUSSION

Our present report demonstrates that zinc markedly increases the binding affinity of cytosolic phorbol ester receptors while it has only a slight effect on the phorbol-dibutyrate binding of microsomal samples. The effect of zinc can not be a simple aspecific aggregation of the polyethylene glycol precipitated proteins since zinc does not increase the aspecific binding of phorbol-dibutyrate and it has markedly different effects on cytosolic and microsomal samples.

Calcium is known to increase the binding affinity of phorbol ester to its receptor (17,19,20). Zinc having a higher affinity to EDTA and EGTA than calcium excludes Ca^{2+} from its complexes with these chelators. However, the effects of zinc were specific since the addition of equimolar $CaCl_2$ did not cause a significant increase in the binding of ^{3}H-pdbu (data not shown). The free Ca^{2+} concentration in our binding medium was approx. 110 μM while Sando at Young (18) have demonstrated that ^{3}H-pdbu binding has a saturation above a free Ca^{2+} concentration of 10 μM. The effects of zinc are different from those of Ca^{2+}. Calcium induces a marked increase in the affinity of ^{3}H-pdbu binding of particulate fractions (19,20). On the contrary, zinc has only a slight effect on the microsomal phorbol ester binding.

If endogenous zinc is depleted from the cytosol by the addition of N,N,N',N'--tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN) the cytosolic phorbol-

-dibutyrate binding can be characterised by multiple (at least two) binding sites with different affinities. Our results are similar to the data of Ashendel (4) who also demonstrated the presence of a high and a low affinity binding site of ^{3}H-tetradecanoyl-phorbol-acetate in the cytosol. This phenomena may reflect the presence of isoenzymes of protein kinase C which were discovered just recently (21-23). Taken these data together it can be supposed that cytosol contains various isoforms of phorbol ester receptor/ /protein kinase C which are different in respect of their zinc content. The addition of zinc may "prime" the "low affinity" cytosolic phorbol ester receptor and "convert" it to a "high affinity" one. Further experiments are needed to clarify whether TPEN-treated cytosolic protein kinase C represents a partially or totaly zinc-depleted enzyme. If TPEN deplets only a part of the zinc content of protein kinase C the existence of phorbol ester receptors with even lower affinity may be anticipated.

Zinc has a much smaller effect on the affinity of membrane-bound phorbol ester receptors. Our recent studies suggested that zinc is necessary for the binding of phorbol ester receptors to microsomal membranes (9). Membrane-bound protein kinase C may be saturated with zinc --as a possible preequisite of membrane binding-- and therefore additional zinc causes only a slight increase in its binding affinity towards phorbol esters. The zinc binding site(s) of protein kinase C may be buried after membrane binding and/or the affinity of these sites may be higher than that of TPEN. This may explain the slight difference between phorbol ester binding of TPEN- and zinc-treated microsomal preparations. However, to strengthen this hypothesis numerous further evidences are needed. The investigation of interrelationships between zinc and the isoforms of protein kinase C is in progress in our laboratory.

In recent studies we demonstrated that phorbol ester treatment causes an intracellular translocation of zinc from the nucleus and mitochondria to the cytosol and microsomes of T lymphocytes (24,25). The phorbol ester-induced increase in the zinc conentration of the cytosol was supported further by the observation that phorbol ester (or diacyl glycerol) treatment impairs the Ca^{2+}-transporting ability of Ca-ionophores most probably by enhancing the formation of stable Zn-ionophore complexes (26). This effect can be generalised: zinc can substitute Ca^{2+} in a number of Ca^{2+}-binding sites (27). In this way zinc may be involved in the phorbol ester (protein kinase C)-induced attenuation of Ca^{2+} signals.

On the other hand the intracellular translocation of zinc after phorbol ester treatment activates protein kinase C, supports its binding to plasma

membranes (9) and enhances the affinity of cytosolic protein kinase C towards phorbol-dibutyrate. These effects may take place at a later period of T lymphocyte activation (approximately after 1.5 hours, see ref. 26). Thus zinc may be involved in the prolonged activation of protein kinase C under conditions when the initial activators of the enzyme (mostly: diacyl glycerols) have been already metabolised. In this way the zinc-induced increase in phorbol ester binding affinity may have a physiological importance in the activation of T lymphocytes.

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