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Zinc increases phorbol ester receptors in intact B-cells, neutrophil polymorphs and platelets

I.J. Forbes, P.D. Zalewski, N.P. Hurst*, C. Giannakis and M.W. Whitehouse

*Department of Medicine, University of Adelaide, *Rheumatology Unit, Queen Elizabeth Hospital, Woodville, SA 5081* and *Department of Pathology, University of Adelaide, Adelaide, Australia*

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In the presence of pyrithione, which was used as a Zn^{2+} ionophore, Zn^{2+} (10–100 μM) increased phorbol ester binding by intact B-CLL cells in a dose-dependent fashion. Zn pyrithione increased 2-fold the number of phorbol ester receptors in B-cells (0.74 to 1.4 pmol/10^6 cells), neutrophil polymorphs (0.2 to 0.51 pmol/10^6 cells) and platelets (91 to 209 pmol/10^{10} cells). Fractionation of cells after treatment with Zn pyrithione showed that increased binding of PDBu occurred in the particulate fraction of cells and this was accompanied by loss of phorbol ester receptors from the cytosol. These data are compatible with a role for Zn in the subcellular distribution and activation of protein kinase C.

Zinc; Protein kinase C; (B-cell, Neutrophil, Platelet)

1. INTRODUCTION

The Ca^{2+}/phospholipid-dependent protein kinase, protein kinase C (PKC), is now known to represent a family of isoenzymes which are activated by 1,2-diacylglycerol or phorbol esters [1]. Sequence studies indicate a conserved cysteine-rich region [2] in the regulatory domain of PKC; the latter also contains the binding site for 1,2-diacylglycerol or phorbol esters. As this cysteine-rich region is homologous with the 'zinc fingers' found in some DNA-binding proteins, it may permit PKC to bind specific DNA sequences [2]. The relation of the cysteine-rich region to the phorbol ester-binding site is not known. Recent reports have indicated that Zn^{2+} modulates the catalytic activity of PKC [3] and alters the affinity of phorbol ester receptors in cytosol of T-cells [4]. In these experiments we have examined the effect of increases in intracellular Zn^{2+} on phorbol ester binding in intact B-CLL cells, neutrophil polymorphs and platelets using pyrithione as a Zn^{2+} ionophore.

Correspondence address: I.J. Forbes, Dept of Medicine, Queen Elizabeth Hospital, SA 5011, Australia

Abbreviations: CLL, chronic lymphatic leukaemia; PDBu, phorbol dibutyrate; PKC, protein kinase C; PBS, phosphate-buffered saline

2. MATERIALS AND METHODS

$^{65}ZnCl_2$ (spec. act. 1.667 Ci/g; Amersham Australia Pty); (4.4 mM) was diluted to 1 mM with distilled water. This was mixed 1:20 with unlabelled $ZnCl_2$ (1 mM) to provide a 1 mM stock solution. B-CLL cells were obtained from patients with CLL who were undergoing leukapheresis, washed and stored in liquid N_2 until ready for use. After removal of contaminating erythrocytes and neutrophils by Ficoll/Hypaque centrifugation, B-CLL cells were washed twice and resuspended in phosphate-buffered saline (PBS) (pH 7.4). Platelets were obtained from platelet-rich plasma, centrifuged, washed and resuspended in buffer as for CLL cells. PDBu-binding assays on cytosol were performed in buffer containing 4 mM $MgCl_2$, 5 mM $CaCl_2$, 1 mg/ml BSA, 25 mM Hepes, 25 mM Tris-HCl (pH 7.5). B-CLL cells or platelets were incubated with [^{3}H]PDBu with or without Zn^{2+} and/or pyrithione for 30 min prior to assay of [^{3}H]PDBu-binding (in triplicate) as described [5]; non-specific binding was assayed in the presence of a 50-fold molar excess of unlabelled phorbol ester. To prepare subcellular fractions, B-cells were suspended in Tris-HCl (pH 7.5) containing 0.1 mg/ml leupeptin and 1 mM EDTA and sonicated (six 5-s bursts). The sonicate was then centrifuged to provide a post 30 000 $\times$ g supernatant

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(soluble fraction) and pellet (particulate fraction). To measure ^{65}Zn uptake, cells (10^7 in 40 μl) were incubated with 10 or 100 μM ^{65}Zn (40 μl stock 1 mM solution), pyrithione (20 μl of required concentration) and buffer (300 μl) (as for PDBu-binding assay). Control cells were incubated without pyrithione. After 30 min, cells were washed once at 4°C with PBS containing 1 mM phenanthroline to chelate extracellular Zn, then 3 times with PBS containing 100 μM unlabelled Zn^{2+} in place of phenanthroline. The ^{65}Zn content of cell pellets was measured using an LKB γ-counter; cellular uptake of ^{65}Zn was calculated by subtracting counts measured in cells incubated without pyrithione from those incubated with pyrithione.

3. RESULTS AND DISCUSSION

Zn^{2+} (0–100 μM) in the presence of pyrithione increased specific binding of [^{3}H]PDBu to B-CLL cells in a concentration-dependent manner (fig.1A). Pyrithione in the absence of added Zn^{2+} also stimulated a small increase in binding of [^{3}H]PDBu; this effect is probably due to the presence of Zn^{2+} ($\approx$2 μM) in the incubation medium. The time course of the increase in [^{3}H]PDBu binding was closely paralleled by increases in cellular Zn content (fig.1B). The Zn^{2+} chelator phenanthroline caused slight inhibition of PDBu binding in control preparations, completely inhibited the enhancing effect of Zn^{2+} and pyrithione on PDBu binding when added shortly after Zn^{2+}, and partially inhibited the increase in binding if added after addition of [^{3}H]PDBu (table 1). Thus, the increase in phorbol ester binding induced by Zn^{2+} is largely reversible.

Scatchard analysis showed that the increase in binding of [^{3}H]PDBu to B-cells was due to an increase in the total number of phorbol ester receptors with little or no change in affinity; similar results were obtained with platelets and neutrophil polymorphs (fig.2A–C). Fractionation of B-cells showed that treatment with Zn^{2+} caused translocation of phorbol receptors from the soluble to the particulate (membrane) fraction of cells (table 2), but the mechanism for the increase in numbers of PDBu receptors was not clear.

The apparent increase in receptor numbers observed in intact cells could be due to more rapid degradation of PDBu receptors in control preparations compared with Zn^{2+} treated cells during the 40 min elapsing during the experiment. However, there was no loss of [^{3}H]PDBu binding in control cells during the course of the experiments.

In contrast to rises in free cytosolic Ca^{2+} which

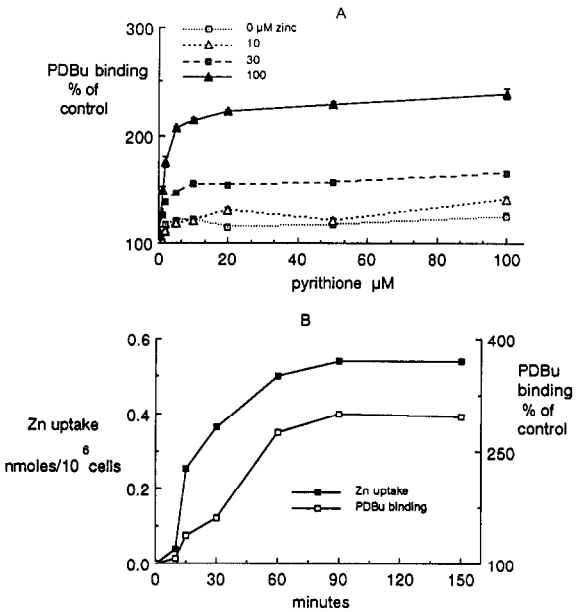

Fig.1. (A) Dose dependence of the effect of increasing Zn^{2+} and pyrithione concentrations on [^{3}H]PDBu binding in B-CLL cells. (B) Time course of ^{65}Zn uptake by B-cells and increases in PDBu binding after treatment of cells with Zn (50 μM) plus pyrithione (5 μM).

cause an increase in affinity of phorbol receptors with little alteration in total numbers [6], a rise in cellular total Zn^{2+} concentration caused a large increase in the apparent numbers of PDBu receptors in B-CLL cells, neutrophils and platelets but only small increases in affinity. Similar changes also occurred in homogenates of B-CLL cells treated directly with Zn^{2+} (not shown). It is of interest that

Table 1

Effect of phenanthroline on the increase in PDBu binding in B-cells induced by Zn pyrithione

	[^{3}H]PDBu binding (cpm) (±SE)	
Control (no Zn pyrithione)	3977 (±71)	
Control + phenanthroline	3260 (±121)	
	A	B
Zn pyrithione	7407 (±115)	6996 (±105)
Zn pyrithione + phenanthroline	3879 (±146)	4702 (±46)

Cells were incubated with either buffer (control) or Zn (50 μM) plus pyrithione (20 μM) for 30 min before addition of [^{3}H]PDBu (20 nM) and incubation for a further 30 min. Phenanthroline (1 mM) was added either (A) 10 min after the beginning of incubation or (B) 10 min after addition of [^{3}H]PDBu (n=4)

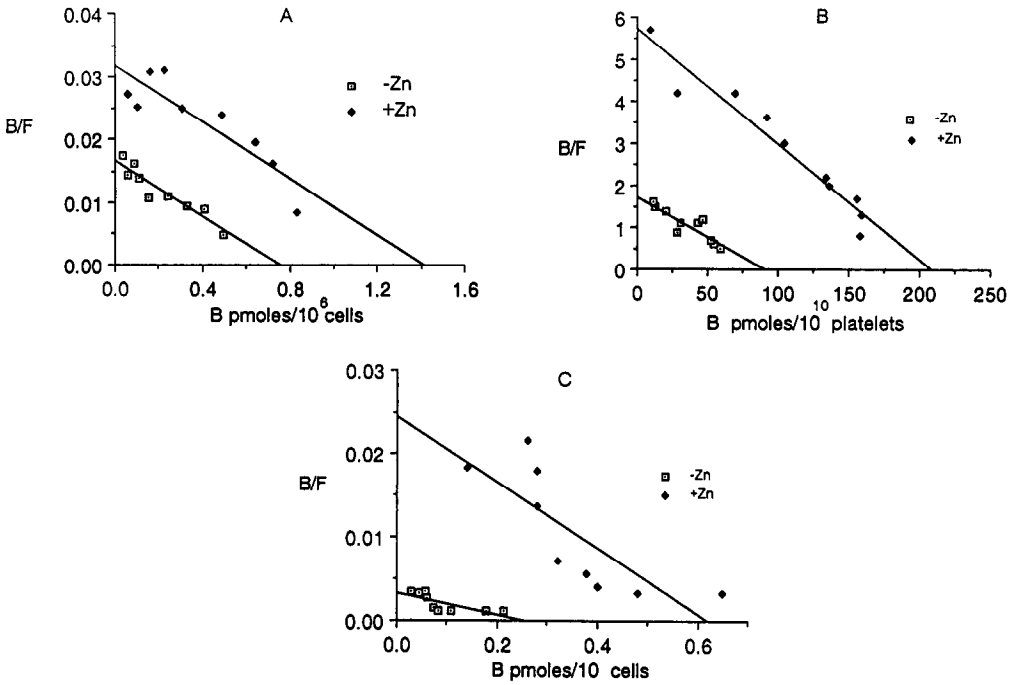

Fig.2. Scatchard plots of [^{3}H]PDBu binding in the presence or absence of zinc pyrithione (50 μM Zn). (A) B-CLL cells, (B) platelets, (C) neutrophil polymorphs.

Csermely et al. [4] have shown that increases in Zn^{2+} concentration increase the affinity but not the number of PDBu receptors in T-cell cytosol and, to a lesser extent, T-cell microsomes.

The physiological significance of the effect of Zn^{2+} on phorbol ester receptors remains unclear; furthermore, the free Zn^{2+} concentrations in either the cytosolic or intact cell preparations cannot be determined with any certainty [4]. The availability of Zn^{2+} chromophores to estimate intracellular free Zn^{2+} would help to resolve these questions. These reservations apart, our data provide evidence that increases in cytosolic Zn^{2+} play a role in regulating the activity and subcellular distribution of PKC, and shed some light on the possible significance of the redistribution of Zn^{2+} which occurs between whole organs in disease states [7] and between cell organelles during cell activation by phorbol esters [8].

Table 2

PDBu-binding activity of the soluble and particulate fractions of CLL cells after treatment with Zn pyrithione

	[^{3}H]PDBu binding (cpm) (±SE)	
Treatment	Soluble	Particulate
Control	5659 (±62)	4384 (±10)
Zinc pyrithione	3159 (±46)	6346 (±84)

After incubation of B-cells with either buffer (control) or Zn^{2+} (50 μM) plus pyrithione (20 μM), cells were sonicated and subcellular fractions prepared as described ($n = 3$)

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