Synergy between zinc and phorbol ester in translocation of protein kinase C to cytoskeleton

P.D. Zalewski[1], I.J. Forbes[1], C. Giannakis[1], P.A. Cowled[1] and W.H. Betts[2]

[1]*Department of Medicine, University of Adelaide* and [2]*Rheumatology Unit, The Queen Elizabeth Hospital, Woodville, South Australia 5011, Australia*

Received 30 July 1990

Protein kinase C was measured in the cytoskeletal fraction of lymphocytes, platelets and HL60 cells, by specific binding of [^{3}H]phorbol dibutyrate and by immunoblotting with antibody to a consensus sequence in the regulatory domain of α-, β- and γ-isozymes of protein kinase C. Treatment of cells for 40 min with a combination of zinc (2–50 μM), zinc ionophore pyrithione and unlabelled phorbol dibutyrate (200 nM) caused up to a ten-fold increase in cytoskeletal protein kinase C and a corresponding decrease in other cellular compartments. Omission of any of the reagents resulted in much less or no translocation. These effects were inhibited by 1,10-phenanthroline, which chelates zinc, and were not seen with calcium. Increase in cytoskeletal protein kinase C persisted for several hours and appeared to involve attachment of the enzyme to actin microfilaments. We propose that zinc, like calcium, regulates the distribution of PKC in cells. However, unlike calcium which controls the binding of PKC to the lipid component on cell membranes, zinc controls the distribution of PKC to membrane cytoskeleton, possibly actin.

Zinc ionophore; Phorbol ester; Protein kinase C; Cytoskeleton

1. INTRODUCTION

The family of protein kinase C (PKC) enzymes plays a key role in normal cell activation and in the aberrant events leading to malignancy [1]. PKC is regulated by cofactor-dependent translocation from cytosol to cell membranes. Although the cofactors (e.g. calcium) involved in the translocation to the lipid component of cell membranes are well known, those involved in its translocation to membrane-associated cytoskeleton are not. The cytoskeleton is a major site of action of PKC, where its effects may be mediated by a distinct pool of the enzyme [2,3].

Previously we reported that zinc, in the presence of zinc ionophores such as pyrithione and diiodohydroxyquinoline, causes a large increase in the capacity of cells to bind a specific ligand of PKC, [^{3}H]phorbol dibutyrate ([^{3}H]PDBu) [4,5]. Here we demonstrate that this increase is associated with translocation of PKC from cytosol to a cellular cytoskeletal fraction.

Correspondence address: P.D. Zalewski, Department of Medicine, University of Adelaide, The Queen Elizabeth Hospital, Woodville, South Australia 5011, Australia

Abbreviations: Zn, zinc; Ca, calcium; NP40, Nonidet P-40; PDBu, phorbol dibutyrate; PKC, protein kinase C; PMSF, phenylmethylsulphonyl fluoride; Phe, 1,10-phenanthroline

2. MATERIALS AND METHODS

2.1. *Materials*

Zinc chloride (99% pure) was obtained from Merck; sodium pyrithione from Dr M. Whitehouse (Dept of Pathology, University of Adelaide, South Australia); [^{3}H]PDBu (13 Ci/mmol) from New England Nuclear; unlabelled PDBu, Nonidet P-40 (NP40), A23187, leupeptin, phenylmethylsulphonyl fluoride (PMSF), 1,10-phenanthroline (Phe) and desoxyribonuclease type I from Sigma; 1,2-*sn*-dioctanoylglycerol from Molecular Probes; sodium fluoride from BDH.

2.2. *Cell preparations and assays*

Human lymphocytes and platelets were prepared as described in [4]. HL60 cells, grown in RPMI 1640 containing 10% foetal bovine serum, were used two days after subculture. Stimuli (e.g. zinc, fluoride, unlabelled PDBu, ionophores and chelators) were added to cells (2×10^7/ml) in serum free RPMI for 40 min at 37°C. Cells were washed twice in phosphate buffered saline and the detergent insoluble cytoskeleton and soluble fraction prepared by treatment for 15 min at 0°C with 25 mM Tris buffer (pH 8.0) containing 0.2% NP40, 30% sucrose, 25 mM KCl, 7.5 mM $MgCl_2$, 50 μg/ml leupeptin and 1 mM PMSF [6]. Insoluble cytoskeletal residues were washed and resuspended in 25 mM Tris buffer (pH 8.0) and ultrasonicated (Branson sonifier, setting 5 for 4×7 s). Binding of 20 nM [^{3}H]PDBu was assayed as in [4]. Soluble fractions were diluted to less than 0.01% NP40 before assaying [^{3}H]PDBu binding. Scatchard analysis of [^{3}H]PDBu binding in whole cells was performed as in [5]. Actin microfilaments were prepared from human platelets [7], and protein was assayed by the Bradford method. In some experiments, the NP40 soluble fractions and insoluble fractions from lymphocytes were collected and the proteins separated on 7.5% SDS-PAGE gels. PKC on these gels was then identified by immunoblotting with rabbit antibody to rat brain PKC (consensus sequence in α-, β- and γ-isozymes) [8] as in [9], using ^{125}I-labelled donkey anti-rabbit immunoglobulin (2000 Ci/mmol, Amersham) as second antibody. PKC activity of different

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

fractions was assayed both by ^{32}P-labelling of proteins identified on SDS-PAGE gels [10] and by phosphorylation of histone [11].

3. RESULTS AND DISCUSSION

Treatment of lymphocytes with 50 μM $ZnCl_2$ in the presence of 20 μM pyrithione increased cytoskeletal-bound PKC twofold (Fig. 1a). In contrast, neither $ZnCl_2$ pyrithione, nor $CaCl_2$ plus or minus A23187, had any effect on cytoskeleton-bound PKC. In similar experiments on cell lysates or sonicates, addition of $ZnCl_2$ without pyrithione also resulted in increased cytoskeleton-bound PKC (data not shown), confirming that in whole cells, pyrithione increases intracellular zinc by acting as a zinc ionophore.

PDBu (200 nM) alone did not cause translocation of PKC to cytoskeleton, but it greatly facilitated the action of zinc. In the presence of PDBu, 50 μM $ZnCl_2$ (plus pyrithione) caused a 10-fold increase in cytoskeletal PKC in lymphocytes (Fig. 1a). Effects of Zn were seen at concentrations as low as 2 μM (Fig. 1b). Similarly, 100 μg/ml 1,2-dioctanoylglycerol facilitated the effects of Zn (not shown). Synergy was also seen in platelets and HL-60 cells (not shown). Phe, a Zn chelator, inhibited both the stimulatory effect of zinc on PKC binding to cytoskeleton, and the facilitating effect of phorbol esters (Fig. 1a).

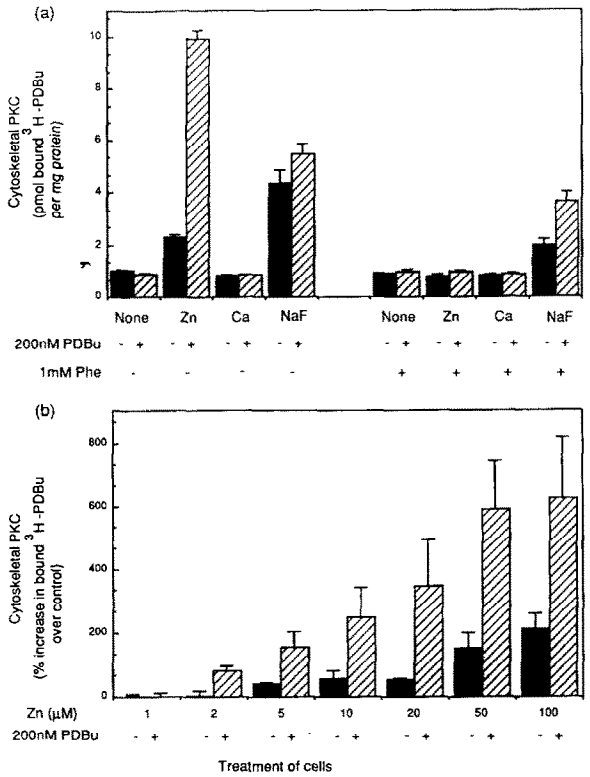

Fig. 1. Effect of Zn on [^{3}H]PDBu binding to cytoskeleton of human lymphocytes. (a) Lymphocytes were either untreated (None), or treated with Zn (50 μM $ZnCl_2$ plus 20 μM pyrithioine), Ca (600 μM $CaCl_2$ plus 1 μM A32187), or NaF (40 mM sodium fluoride), in the absence or presence of 200 nM PDBu (unlabelled) and 1,10-phenanthroline (Phe). (b) Synergy between Zn (1–100 μM $ZnCl_2$, 20 μM pyrithione) and 200 nM PDBu. Values (mean ± SE) pooled from 7 experiments each in triplicate.

It has been proposed that Zn is an intracellular messenger, which is mobilized in response to certain stimuli, including phorbol esters [12–14]. Fluoride, a potent activator of G proteins [15], caused an increase in lymphocyte cytoskeletal PKC, which was blocked by Phe (Fig. 1a), suggesting that Zn or another chelatable metal is involved.

Immunoblotting of lymphocyte proteins separated on SDS-PAGE gels (Fig. 2), demonstrated that Zn caused a change in distribution of 80 kDa PKC from NP40-soluble (lipid soluble and cytosolic) to NP40-insoluble (cytoskeletal) fractions. This treatment did not change total PKC (Fig. 2, lanes 2 and 3), suggesting that Zn does not increase the synthesis of PKC. This gel also demonstrates that there were lower molecular weight immunoreactive components in the soluble fraction that were unaffected by Zn. These may be proteolytic products of PKC. It is not possible to assay cytoskeletal PKC by the standard catalytic assay, because this assay measures PKC in a soluble form. However, the Ca- and phospholipid-dependent phosphorylation of histone in the NP40-soluble fraction was decreased by pretreatment of lymphocytes with Zn plus PDBu, and the incorporation of ^{32}P into several cytoskeletal proteins was increased (data not shown).

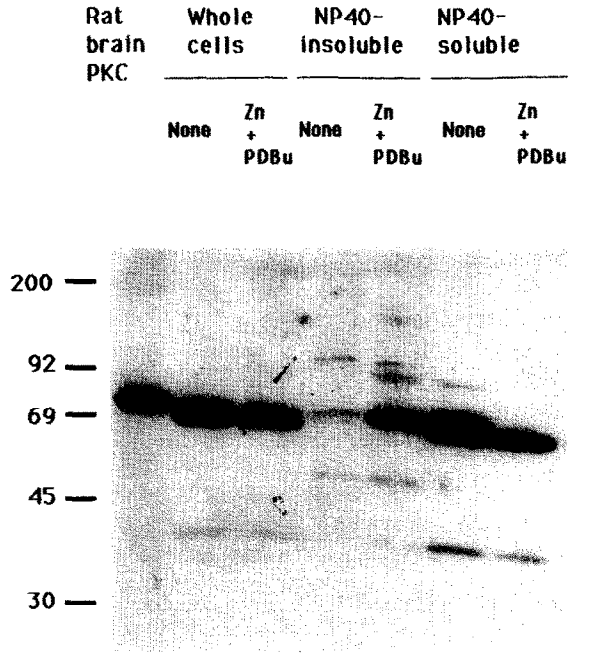

Fig. 2. Immunoblot of SDS-PAGE gels, showing translocation of 80 kDa PKC from detergent (NP40) soluble to cytoskeletal (NP40-insoluble) fraction of Zn-treated (50 μM $ZnCl_2$, 20 μM pyrithione and 200 nM PDBu) lymphocytes. Left lane contains pure rat brain PKC as marker.

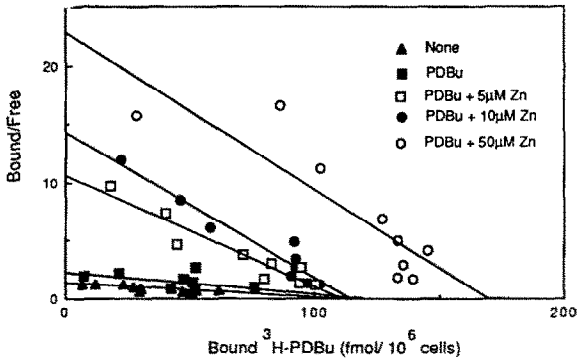

Fig. 3. Scatchard analysis of [^{3}H]PDBu binding to intact lymphocytes, untreated (None) or treated with 200 nM unlabelled PDBu and varying concentrations of Zn (0, 5, 10, or 50 μM $ZnCl_2$, 20 μM pyrithione). Unlabelled PDBu was removed by washing cells prior to assay for [^{3}H]PDBu binding.

Translocation of PKC to cytoskeleton, characterised by the increase in binding of [^{3}H]PDBu to intact cells, was due both to changes in binding affinity and, at higher concentrations of Zn, to the apparent total number of binding sites (Fig. 3). This suggests that cytoskeletal PKC has a higher affinity for [^{3}H]PDBu than lipid-soluble PKC. The very high [^{3}H]PDBu binding capacity of brain cells [16] may be due to their high content of Zn [17].

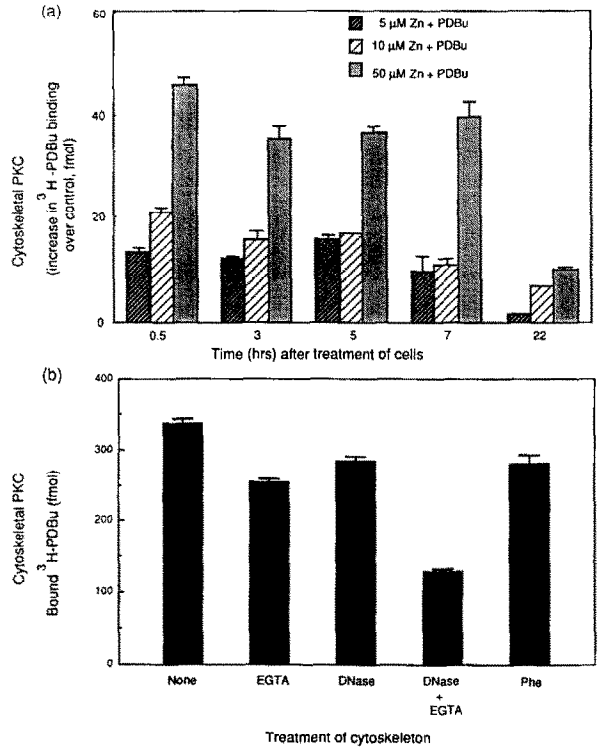

Fig. 4. (a) Persistence of cytoskeletal PKC after treatment of lymphocytes with Zn (0, 5, 10 or 50 μM $ZnCl_2$, 20 μM pyrithione and 200 nM PDBu) and, (b) effect of actin depolymerisation (100 μg/ml deoxyribonuclease plus 1 mM EGTA) or 1,10-phenanthroline (Phe) on [^{3}H]PDBu binding activity of cytoskeleton, isolated following treatment with Zn (50 μM $ZnCl_2$, 50 μM pyrithione, 200 nM PDBu). Values are mean ± SE (n = 3).

Fig. 4 shows that cellular [^{3}H]PDBu binding remained elevated for at least 7 h after treatment of cells with Zn plus PDBu, indicating that PKC translocated to the cytoskeleton remains bound for some time, particularly in comparison to Ca-dependent membrane-bound PKC which undergoes rapid turnover [1]. An example of where Zn may have a function in sustained cellular responses involving PKC is in the long-term potentiation of synaptic transmission in the hippocampus [18], where Zn, released into synapses of excited hippocampal neurons, reaches concentrations of up to 200 μM [14].

Previous immunocytochemical studies have shown that in fibroblasts, PKC is associated with actin filaments [3], and suggest that the cytoskeleton component to which PKC binds may be actin. This was tested using platelets since actin microfilaments are readily prepared from these cells [7]. Platelets were treated with 50 μM $ZnCl_2$ (plus 20 μM pyrithione and 200 nM PDBu) for 40 min, before isolation of actin microfilaments. These microfilaments had a 77% increase in [^{3}H]PDBu binding over microfilaments from control cells (Fig. 4b). Furthermore, treatment of the lymphocyte cytoskeleton with 100 μg/ml desoxyribonuclease I plus 1 mM EGTA, which de-polymerises actin [19], caused a 60% reduction in [^{3}H]PDBu binding (Fig. 4b). These results suggest that Zn causes PKC to translocate to polymerised actin components of the cytoskeleton. Phe treatment of the microfilaments did not reduce the [^{3}H]PDBu binding (Fig. 4b), suggesting that neither zinc nor other metals are required to maintain the PKC-actin complex.

Whether or not Zn interacts directly with PKC is not known. However, the cysteine-rich motifs in the regulatory domain of PKC are similar to the Zn-binding 'finger motifs' of steroid receptors, and are essential for [^{3}H]PDBu binding [20]. This type of motif also appears to be involved in other types of Zn-dependent protein-protein interactions [21].

We propose that membrane PKC exists in at least two states, one in association with phospholipid and Ca, and the other with phospholipid, Zn and cytoskeletal protein, possibly polymerised actin. The kinetics of the formation of the cytoskeletal-PKC complex and its effects on PKC specificity and activity are now being investigated in our laboratory.

Acknowledgements: This research was supported by the National Health and Medical Research Council of Australia, the Anti-Cancer Foundation of South Australia, and a major research grant from the University of Adelaide.

REFERENCES

[1] Nishizuka, Y. (1988) Nature 334, 661-665.
[2] Papadopolous, Y. and Hall, P.F. (1989) J. Cell Biol. 108, 553-567.

[3] Jaken, S., Leach, K. and Klauck, T. (1989) J. Cell Biol. 109, 697-704.
[4] Forbes, I.J., Zalewski, P.D., Hurst, N.P., Giannakis, C. and Whitehouse, M.W. (1989) FEBS Lett. 247, 445-447.
[5] Forbes, I.J., Zalewski, P.D., Giannakis, C., Petkoff, H.S. and Cowled, P.A (1990) Biochim. Biophys. Acta (in press).
[6] Chen, Z.Z., Coggeshall, L.M. and Cambier, J.C. (1987) J. Immunol. 136, 2360-2364.
[7] Rosenberg, S., Stracher, A. and Lucas, R.C. (1981) J. Cell Biol. 91, 201-211.
[8] Testori, A., Hill, C.S.T., Fournier, A., Burgoyne, L.A. and Murray, A.W. (1988) Biochem. Biophys. Res. Commun. 156, 222-227.
[9] Fournier, A., Hardy, S.J., Clark, R.J. and Murray, A.W. (1989) Biochem. Biophys. Res. Commun. 161, 556-561.
[10] Hurst, N.P., Gorjatschko, L., Betts, W.H., Zalewski, P.D. and Forbes, I.J. (1989) Rheumatol. Int. 8, 245-250.
[11] Tapley, P.M. and Murray, A.W. (1984) Biochem. Biophys. Res. Commun. 122, 158-164.
[12] Grummt, F., Weinmann-Dorsch, C., Schneider-Schaulies, J. and Lux, A. (1986) Exp. Cell Res. 163, 191-200.
[13] Csermely, P., Gueth, S. and Somogyi, J. (1987) Carcinogenesis 8, 1663-1666.
[14] Assaf, S.Y. and Chung, S.-H. (1984) Nature 308, 734-736.
[15] Strnad, C.F. and Wong, K. (1985) Biochem. Biophys. Res. Commun. 133, 161-167.
[16] Blumberg, P.M., Jaken, S., Konig, B., Sharkey, N.A., Leach, K.L., Jeng, A.Y. and Yen, H. (1984) Biochem. Pharmacol. 33, 933-940.
[17] Danscher, G., Shipley, M.T. and Andersen, P. (1976) Brain Res. 85, 522-526.
[18] Akers, R.F., Lovinger, D.M., Collery, P.A., Linden, D.J. and Touttenberg, A. (1986) Science 231, 587-589.
[19] Woda, B.A. and Woodin, M.B. (1984) J. Immunol. 133, 2767-2772.
[20] Ono, Y., Fujii, T., Igarishi, K., Kuno, T., Tanaka, C., Kikkawa, U. and Nishizuka, Y. (1989) Proc. Natl. Acad. Sci. USA 86, 4868-4871.
[21] Frankel, A.D. and Pabo, C.O. (1988) Cell 53, 675-676.