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SHORT COMMUNICATIONS

Translocation of protein kinase C to a Triton-insoluble sub-cellular compartment induced by the lipophilic gold compound auranofin*

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The Ca^{2+}-activated, phospholipid-dependent protein kinase C (PKC)† phosphorylates substrates in cytosol, membrane, cytoskeleton and nucleus [1]. Diacylglycerol and phorbol esters in association with Ca^{2+} induce translocation of PKC from cytosol to membrane [2, 3]. Some of the translocated PKC is proteolytically cleaved and the catalytic fragment returns to the cytoplasm where it initiates further phosphorylation independently of calcium, phospholipid and diacylglycerol [4]. Another portion of PKC is translocated to a detergent-insoluble compartment [5], assumed to be the cytoskeleton and/or karyoskeleton.

Auranofin (AF), a lipophilic anti-rheumatic gold compound, mimics and synergizes with phorbol esters [6–8] in several cell models and increases both the affinity and apparent total number of PDBu receptors in human lymphocytes [9].

Here we show that AF markedly enhances the association of cytoplasmic phorbol receptors (PKC) with a detergent-insoluble subcellular fraction of B lymphocytes.

Materials and methods

AF (gift of Dr M. Whitehouse, Pathology Dept., University of Adelaide), A23187 and PDBu (Sigma) were stored in DMSO. CLL cells obtained by leukapheresis were stored in liquid nitrogen and cultured at $1–2 \times 10^7$/ml as described previously [9]. For subcellular fractionation, cells were resuspended in 25 mM Tris–HCl, pH 7.4, containing 1 mM EDTA and ultrasonicated (4×10 sec pulses, Heat Systems Ultrasonics), at 0°. Supernatant after 40,000 *g* centrifugation for 60 min was used as cytosol. The particulate fraction was sonicated in PBS for assay or further extracted with 0.2% Triton X-100 containing 1 mM EDTA and centrifuged at 40,000 *g* for 30 min to yield Triton-soluble and -insoluble material. Phorbol receptors were assayed by binding of ^{3}H-PDBu (New England Nuclear, 20 Ci/mmol) in cells, particulate and soluble fractions were determined as in [10, 11], the latter in the presence of phosphatidylserine (20 μg/ml) and Ca^{2+} (5 mM). Binding was <15% of total without these additions. PKC was assayed by incorporation of ^{32}P from ^{32}P-ATP into histone [4]. ^{32}P-incorporation into histone in replicates containing 1 mM EGTA and lacking phosphatidylserine and calcium, was subtracted from total phosphorylation to give PKC activity.

Results and discussion

AF causes a Ca^{2+}-dependent increase in affinity of phorbol receptors in CLL cells [9]. AF increased binding in cells pre-equilibrated for 40 min with ^{3}H-PDBu, and there was little or no subsequent time-dependent loss of this binding. This contrasts with the transient increase of binding in A23187-treated cells (Fig. 1). The apparent increase in total receptor numbers may be due to lack of time-dependent down-regulation of PDBu-binding capacity resulting from proteolytic cleavage of PKC [12], rather than to an actual increase in receptors, resulting possibly from resistance of PKC to degradation or a change in accessibility to protease. AF increases affinity of PDBu receptors without time-dependent loss of these receptors in neutrophils [manuscript in preparation].

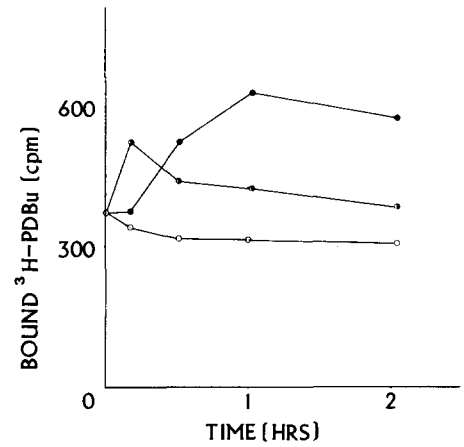

Fig. 1. Augmentation of binding of PDBu (cpm/10^6 cells) in intact CLL cells incubated with ^{3}H-PDBu (10 nM) for 40 min at 37° and then (at time 0) exposed to AF, 60 μM (●), A23187, 1 μM (◑) or control solvent dimethylsulphoxide, 0.1% v/v (○). Means of triplicates; SD did not exceed 10% of means.

Calcium ionophores increase cellular binding of PDBu [3, 13] by promoting translocation of PKC to the particulate fraction [2, 3]. B-CLL cells were treated with unlabelled PDBu for 40 min in the presence or absence of AF, washed thoroughly, then assayed for binding of 10 nM ^{3}H-PDBu in the isolated particulate fraction. PDBu increased PDBu receptor activity of the particulate fraction in a concentration-dependent manner (Fig. 2). This effect was greatly increased in the presence of AF, although AF alone (60 μM) had little or no effect. The synergism was evident within 5 min (not shown).

While binding of ^{3}H-PDBu to the particulate fraction increased in cells treated with AF plus PDBu, calcium- and phospholipid-dependent histone kinase activity was almost completely lost from the cytosol (Fig. 3). However, little kinase or PDBu receptor activity could be extracted with Triton X-100 from the particulate fraction of cells treated with AF or with AF plus PDBu, relative to control cells or cells treated with PDBu alone. In contrast, Triton-insoluble residues of particulate fractions from cells treated with AF or AF plus PDBu were considerably enriched for PDBu receptor activity (up to 88%, Fig. 3). The content of PDBu

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† Abbreviations used: AF, auranofin; CLL, chronic lymphocytic leukaemia; DMSO, dimethyl sulphoxide; EDTA, ethylene diamine tetra acetic acid; EGTA, ethylene glycol-bis(beta-amino-ethyl ether) *N*, *N*, *N'*, *N'*-tetra acetic acid; kD, kilodalton; PBS, phosphate buffered saline; PDBu, phorbol dibutyrate; PKC, protein kinase C; PS, phosphatidylserine.

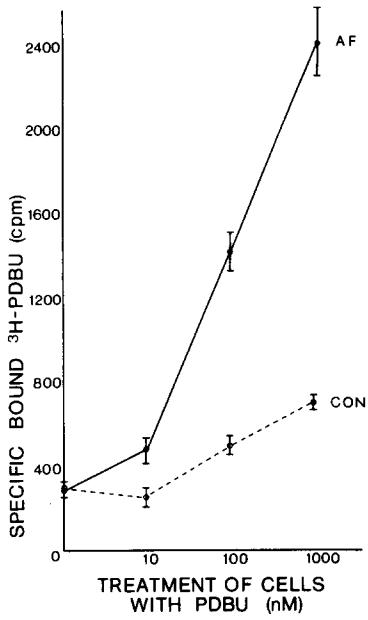

Fig. 2. Cells were treated for 40 min with varying concentrations of unlabelled PDBu in the absence (CON) or presence of AF (60 μM) and washed. Particulate membrane fractions were assayed for binding of 10 nM ^{3}H-PDBu. Means of triplicates, bars indicate SD.

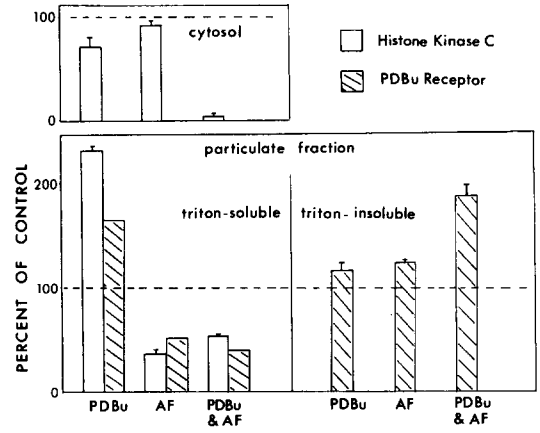

Fig. 3. B-CLL cells were treated with AF (60 μM), PDBu (200 nM), AF + PDBu or control solvent DMSO (0.1% v/v). After 40 min cytosol, Triton-soluble and -insoluble fractions were prepared and assayed for binding of ^{3}H-PDBu (10 nM) or histone kinase C activity. Activities in control cell fractions were taken as 100%. Bars indicate SD. ▨, PDBu-receptor; □, histone kinase C.

receptors recovered in the Triton-insoluble fraction increased progressively from 28% in control preparations to 43% in cells treated with AF 60 μM, and Triton-extractable receptors (Fig. 4) and histone kinase C activity (not shown) decreased with increasing concentrations of AF. It is not yet possible to determine how much histone kinase C activity is present in this Triton-insoluble fraction because the assay requires soluble PKC.

The detergent-insoluble portion of the particulate fraction consists largely of cytoskeletal and nuclear material [14]. PKC has been reported in both the cytoskeleton and nucleus of lymphocytes [5, 15]. Many cytoskeletal proteins are substrates for PKC [1] and some tyrosine kinases (which may be regulated by PKC) are located in the cytoskeleton [16]. Translocation of PKC to the cytoskeleton may mediate down-regulation by AF of EGF receptors in HeLa cells [6] and mouse erythrocyte receptors in CLL cells [manuscript submitted].

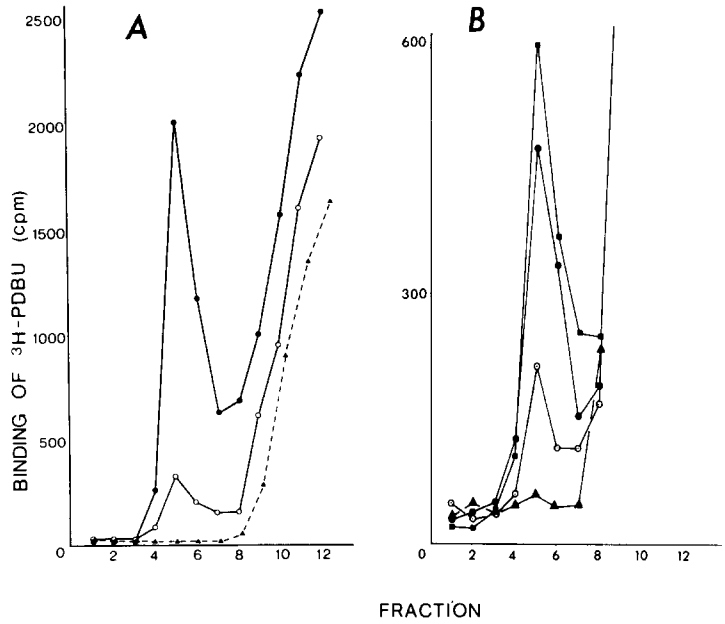

Fig. 4. Triton-soluble material was assayed for PDBu receptors by column filtration. (A) Calcium and phospholipid-dependence of binding; ●, Triton soluble material, PS and Ca^{2+}; ○, Triton-soluble material alone; ▲, PS and Ca^{2+} alone. (B) Triton-soluble materials from cells treated with AF at 0 (■), 6 (●), 24 (○) or (▲) 60 μM.

AF may induce translocation of PKC by elevating cAMP [17], by analogy with induction of translocation of PKC to nuclear fractions by cAMP in mouse lymphocytes [18]. Alternatively, AF and metals which are known to interact with thiols [19], may act via the cysteine-rich site in the regulatory domain of PKC [20]. Metals have an affinity for the cytoskeleton [21], a region rich in sulphydryls.

In summary, AF, which modulates PKC-dependent events *in vitro*, synergizes with the phorbol ester PDBu, a specific ligand of PKC, in causing translocation of PKC from cytosol to the particulate subcellular fraction. This translocation is accompanied by increased affinity of binding of phorbol ester. Translocation induced in the presence of AF, unlike that induced by phorbol ester alone, results in attachment of PKC to a detergent-insoluble compartment. In this state PKC is not subject to normal down-regulation. Studies are now needed to show whether translocation of PKC to particular subcellular compartments is mediated by particular intracellular messengers.

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Azidopine photoaffinity labeling of multidrug resistance-associated glycoproteins

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The problem of developing effective cancer chemotherapeutic regimens in humans in the presence of drug-resistant tumor cells is being approached by studying model systems in tissue culture. Mammalian cells selected for resistance to drugs often display the multidrug resistance (MDR*) phenotype which includes (1) cross-resistance to unrelated drugs, (2) a net decrease in drug accumulation, and (3) the overexpression of a MDR-associated glycoprotein (MDRG), also known as P-glycoprotein, that is found in the plasma membrane [1]. Calcium antagonists such as verapamil can partially reverse MDR by increasing intracellular drug levels [2–8]. Verapamil specifically inhibits the binding of vinblastine and its photoactive analog to the MDRG, thereby suggesting that it interacts with the MDRG [9–12].

MDR cell lines derived from the murine cell, J774.2, have been selected in our laboratory for resistance to colchicine (CLC), vinblastine (VBL) or taxol (TAX) [13, 14]. The MDRGs observed by SDS–PAGE in the CLC- and VBL-resistant cells have an approximate M_r of 130–150 kDa, whereas the MDRG from the TAX-resistant cells migrates as a doublet in a similar molecular weight range [15, 16].

The arylazide 1,4-dihydropyridine, azidopine, is a calcium antagonist which photolabels calcium channels [17]. [^{3}H]Azidopine has the advantages of being relatively stable in the absence of UV light and capable of forming covalent bonds via nitrene intermediates upon UV irradiation. In

* Abbreviations: MDR, multidrug resistance; MDRG, multidrug resistance glycoprotein; CLC, colchicine; VBL, vinblastine; TAX, taxol; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; and TPCK, L-1-tosylamide-2-phenylethylchloromethyl ketone.