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Auranofin enhances phosphorylation of putative substrates of protein kinase C in human platelets

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The receptor-mediated turnover of inositol phospholipids is now well established as a transmembrane signalling system [1–3]. The two products of lipid hydrolysis by phospholipase C are 1,2-diacylglycerol and inositol phosphates which result in activation of protein kinase C (PK-C) and mobilization of intracellular Ca^{2+} respectively. In many cell types these two responses act synergistically to generate a particular biological response. For example platelet aggregation by thrombin can be mimicked by the Ca^{2+} ionophore A23187 in combination with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) which activates PK-C by acting as a diacylglycerol analog [1, 2].

Auranofin (2,3,4,6-tetra-*O*-acetyl-1-thio-beta-D-glucopyranosato-*S*-[triethylphosphine]gold AF) is a lipophilic anti-inflammatory and immunoregulatory gold I complex which is widely used in the treatment of arthritis [4]. Recent studies have shown that AF modulates leucocyte functions involving PK-C. For example low concentrations of AF (1 μM) enhance and higher doses (> 10 μM) inhibit cellular responses to phorbol esters such as superoxide production [5, 6]. In addition AF is a strong inhibitor of platelet aggregation [7].

In the present studies we have compared the effects of AF, TPA and thrombin on protein phosphorylation in human platelets. Unexpectedly, we found that AF stimulated the phosphorylation of proteins of MW 40 kDa and 20 kDa, apparently by activating PK-C.

Materials and methods

AF was a gift from Dr H. Allars (Smith Kline & French Australia Ltd.); TPA was from CRC Inc. (Eden Prairie MN), and sodium aurothiomalate from May & Baker Ltd (Dagenham, Essex).

Human blood platelet concentrates were obtained from a blood bank and washed platelets prepared as described [8]; platelets were suspended at a final concentration of 1–2 × 10^9/ml in platelet buffer containing glucose (5.5 mM), Tris (15 mM), NaCl (0.14 M) and BSA (0.35%) at pH 7.4. TPA and AF were added from stock solutions in DMSO resulting in a solvent concentration of 0.1%.

For phosphorylation experiments, platelets were incubated with carrier free ^{32}P-orthophosphate (0.5 mCi/ml) for 30 min at 25° and washed twice with platelet buffer. These conditions were chosen to optimise platelet viability. Finally, platelets were suspended at 2 × 10^9/ml in modified platelet buffer (BSA reduced to 0.1%) and 100 μl aliquots dispensed for treatments. Reactions were terminated with 50 μl of 6% SDS, 40% sucrose in 200 mM Tris–Cl, pH 6.8. Aliquots of solubilised platelets were resolved on 12.5% SDS-polyacrylamide gels (PAGE) as described [9]. The gels were then autoradiographed to identify phosphorylated proteins, and radioactivity associated with the 20 kDa and 40 kDa bands were determined by solubilising the excised bands with 1% ammonia in 30% hydrogen peroxide for 16 hr at 37° followed by scintillation counting. For peptide analyses the 40 kDa protein was excised from dried gels, trypsinised, dissolved in 1% trifluoroacetic acid and applied to a C-18 reverse phase HPLC column. After washing for 2 min with 0.1% trifluoroacetic acid, 0.05% triethylamide, the [^{32}P]-peptides were resolved with a linear gradient (0–10%) acetonitrile with a flow rate of 2 ml/min. Fractions were collected at 0.5 min intervals and radioactivity determined by counting Cerenkov radiation.

Results and discussion

As shown in Fig. 1, TPA stimulated the phosphorylation of proteins of about 40 kDa and 20 kDa in human platelets. A similar result was obtained in five separate experiments. This has been reported previously and evidence presented that in the presence of the phorbol ester both phosphorylations are mediated by PK-C [10, 11]. Phosphorylation of both proteins is also enhanced by thrombin and in this case the 40 kDa protein is phosphorylated by PK-C and the 20 kDa protein by calmodulin-dependent myosin light chain kinase [10, 11]. An unexpected result summarised in Figs 2 and 3 was that AF also stimulated the phosphorylation of both the 40 kDa and the 20 kDa proteins. AF did not stimulate the incorporation of label into the large number of additional proteins phosphorylated during the preincubation with $^{32}P_i$. This point establishes that the increases in phosphorylation were specific and not due to a generalised auranofin-induced increase in the specific activity of the ATP pool. The AF-induced phosphorylation was dose-dependent (Fig. 3) and showed a lag of about 10 min, possibly reflecting the time required for uptake of the drug or its conversion into an active form. At suboptimal concentrations of TPA (10^{-8}M), AF stimulated the phosphorylation of both proteins, but no evidence for synergism was observed (Fig. 2). Aurothiomalate, a parenterally administered anti-rheumatic gold-containing drug did not stimulate protein phosphorylation (data not shown).

HPLC analysis of the tryptic peptides derived from the 40 kDa proteins phosphorylated in the presence of TPA, thrombin and AF was also carried out (Fig. 4). All three agents generated an almost identical pattern of phosphorylation. This result was reproducible in a separate exper-

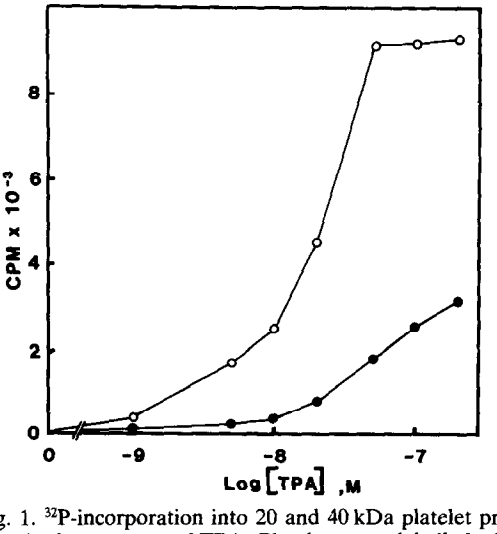

Fig. 1. ^{32}P-incorporation into 20 and 40 kDa platelet proteins in the presence of TPA. Platelets were labelled with ^{32}P as described in Materials and Methods followed by a 10 min exposure at 37° to the indicated concentrations of TPA. After SDS–PAGE, radioactivity associated with the 20 and 40 kDa bands was determined. Each point is a single determination (20 kDa protein, ●; 40 kDa protein, ○).

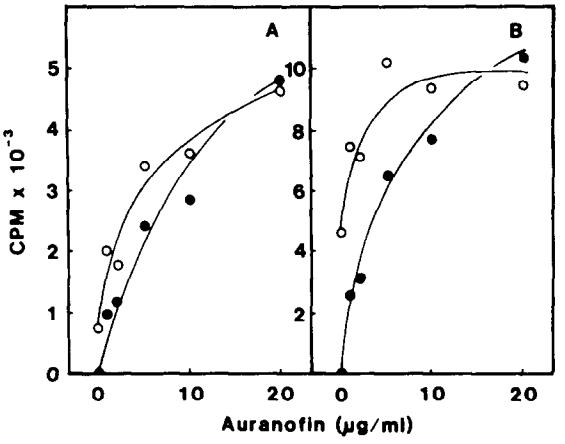

Fig. 2. ^{32}P-incorporation into 40 kDa and 20 kDa platelet proteins in the presence of TPA and AF. ^{32}P-labelled platelets were incubated with various concentrations of AF for 50 min at 37°. TPA, 10^{-8}M (○) or DMSO (●) was added and incubation continued for a further 10 min before resolving on SDS-PAGE as described in Materials and Methods. Panel A, 20 kDa protein; panel B, 40 kDa protein.

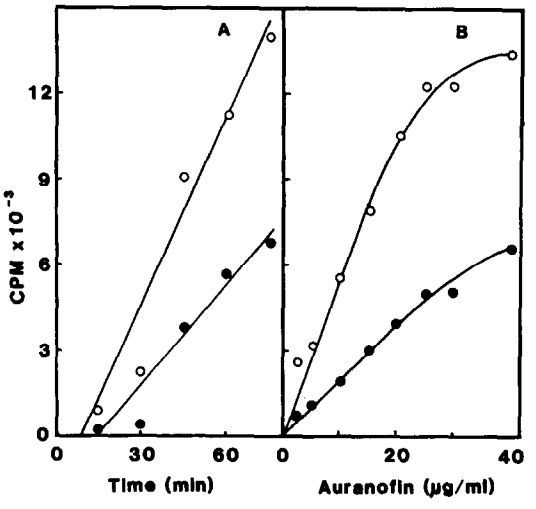

Fig. 3. AF stimulation of ^{32}P into platelet 20 kDa and 40 kDa proteins. ^{32}P labelled platelets were incubated for various times with 40 μg/ml AF at 37° (panel A) or various concentrations of AF for 60 min at 37° (panel B). Proteins were resolved on SDS–PAGE as described in Materials and Methods (20 kDa protein, ●; 40 kDa protein, ○).

iment. Given the previous evidence that both TPA and thrombin-stimulated phosphorylation of the 40 kDa protein involves PK-C, this result strongly suggests that treatment of platelets with AF results in a net increase in phosphorylation by this enzyme. A preliminary tryptic peptide analysis of the 20 kDa protein phosphorylated in the presence of AF indicates that PK-C is also responsible for this phosphorylation (data not shown).

The simplest explanation of the above results is that AF treatment of platelets results in direct activation of PK-C. In this respect AF would then resemble the range of tumour promoting agents including phorbol diesters, mezerein and teleocidin [1, 2] which probably function by acting as structural analogs of 1,2-diacylglycerol, a natural regulator of

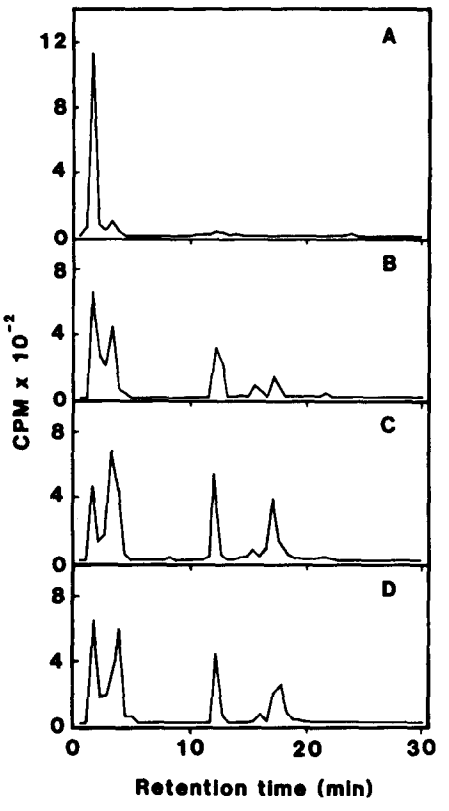

Fig. 4. HPLC profiles of 40 kDa tryptic peptides from platelets treated for 60 min with DMSO (A) or AF (B), for 10 min with TPA (C) and for 1 min with thrombin (D). Peptides were resolved as described in Materials and Methods.

PK-C. It does not, however, seem likely that AF functions in a similar way, as preliminary experiments indicate that the drug does not enhance the cytosolic-membrane translocation of PK-C in platelets (data not shown). In other experiments we have shown that AF shares with phorbol esters the ability to inhibit binding of EGF to HeLa cells [13] and to enhance phosphorylation of the EGF receptor (data not shown). These results are consistent with the proposal that AF stimulates PK-C activity. Other possible explanations of the data are that AF inhibits a protein phosphatase specific for PK-C phosphorylated proteins or that AF stimulates the hydrolysis of inositol phospholipids with the resultant production of diacylglycerol and activation of PK-C.

Despite the ability of AF to stimulate PK-C-mediated phosphorylation in human platelets, the drug does not lead to platelet aggregation, either alone or in the presence of the Ca^{2+} ionophore A23187 (data not shown). Aggregation is initiated by other agents which activate PK-C (e.g. phorbol esters) in combination with the ionophore [1, 2]. In fact AF strongly inhibits platelet aggregation induced by ADP or collagen [7] or by thrombin (data not shown). It therefore seems likely that AF has multiple effects on cells, possibly resulting from thiol exchange reactions or from non-specific membrane perturbation [12].

Elucidation of the mechanism for the AF stimulation of PK-C mediated phsophorylation in intact platelets may provide insights into the factors which can modulate the activity of this key regulatory enzyme, and contribute to an understanding of the pharmacological effects of AF.

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Enzymic reduction of *N*-hydroxyamphetamine: the role of electron transfer system containing cytochrome b_5

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Methamphetamine (MP) has been known to be transformed to amphetamine (AP) by *N*-demethylation in mammals [1–4], which proceeds through either *N*-hydroxylation or *C*-hydroxylation pathways. We previously demonstrated that MP was mainly *N*-demethylated by the former pathway in guinea-pigs [4], and suggested further that *N*-hydroxy-MP formed was dehydrogenated to *N*-[(1-methyl-2-phenyl)ethyl]methanimine *N*-oxide (nitrone) which immediately decomposed to formaldehyde and *N*-hydroxy-AP [5]. Furthermore resulting *N*-hydroxy-AP was assumed to be reduced to AP. Kadlubar *et al.* found hydroxylamine reductase system in hog liver microsomes [6] and showed by monitoring the reduction of *N*-methyl-*N*-benzyl-hydroxylamine that the purified system consisted of cytochrome (cyt.) b_5, NADH-cyt. b_5 reductase and unknown SH-protein [7]. Although they have demonstrated also the participation of this hydroxylamine reductase in the reduction of *N*-hydroxy-AP in hog liver microsomes [6], the extent of contribution of this system to the reaction in microsomes has not been evaluated. In the present study, a key component of this system, cyt. b_5 was purified from guinea-pig liver microsomes, and its participation in the reduction of *N*-hydroxy-AP was evaluated by use of anti-cyt. b_5 serum from rabbits.

Materials and methods

Chemicals. The neutral oxalate of *N*-hydroxy-AP was synthesized by the method of Coutts *et al.* [8]. NADH and Freund's complete adjuvant were purchased from Kyowa Hakko Industries, (Tokyo) and Difco Lab. (Detroit) respectively. All other reagents used were from the sources described earlier [4, 5] or of the highest quality commercially available.

Purification of guinea-pig liver cyt. b_5. Cyt. b_5 was purified from the liver microsomes from Hartley guinea pigs according to the method of Spatts and Strittmatter [9]. Purity and molecular weight of this enzyme were determined by use of SDS-polyacryamide gel electrophoresis on 15% acrylamide gels in the presence of 0.1% SDS by the method of Laemmli [10].

Determination of N-hydroxy-AP reductase. Incubation mixture consisted of 5.0 μmol of *N*-hydroxy-AP, 10 μmol of NADH and NADPH, 2–3 mg of liver microsomes and 0.1 M phosphate buffer (pH 6.3) to make a final volume of 6.0 ml. After the incubation for 30 min at 37°, AP formed was determined as a trifluoroacetyl derivative by GLC [3].

Preparation of anti-cyt. b_5 serum. Immunization of the rabbit with purified cyt. b_5, and preparation of anti-serum and nonimmune serum were performed similarly as described elsewhere [11] with some modifications. About 1 mg of cyt. b_5 suspended in Freund's complete adjuvant (1.0 ml) was injected into the foot pads of a rabbit. The rabbit was boosted twice three and four weeks later by s.c. injections of the same amount of the antigen suspension at the back. The anti-serum (about 20 ml) was obtained after a week of the last booster injection. Nonimmune serum was obtained from a nonimmune rabbit. The purified cyt. b_5 formed precipitates only with the serum from the immunized rabbit by Ouchterlony double diffusion method [12].

Results and discussion

N-Hydroxy-AP was effectively reduced to AP with guinea-pig liver microsomes, requiring preferably NADH as a cofactor (Table 1), at optimum pH of 5.0–6.3. These characteristics closely resemble those of hog liver hydroxylamine reductase system containing cyt. b_5 [6]. We therefore attempted to isolate cytochrome b_5 from guinea-pig liver microsomes for preparing anti-cyt. b_5 serum. The purification steps for cyt. b_5 are shown in Table 2. The prep-