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CYCLOSPORIN A INHIBITS PROTEIN KINASE C ACTIVITY: A CONTRIBUTING MECHANISM IN THE DEVELOPMENT OF NEPHROTOXICITY?

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Summary. Cyclosporin A modifies many intracellular functions in a variety of different cells. This study investigated the potential interaction between cyclosporin A and protein kinase C, as a possible mechanism for the development of nephrotoxicity. The activity of protein kinase C, in the cytosol of renal epithelial cells, was shown to be significantly inhibited in a dose-dependent manner by CSA. Activation of protein kinase C by 12-O-tetradecanoylphorbol-13-acetate (phorbol ester) in rat mesangial cells in culture leads to an increase in PGE_2 release. Phorbol ester stimulated PGE_2 release was significantly inhibited by cyclosporin A. These results would suggest that intracellular site of action of cyclosporin A, in producing alterations in intracellular function and toxicity, may be at the level of protein kinase C.

Cyclosporin A (CSA) has been shown to modify different intracellular functions, including protein synthesis and DNA synthesis in renal tubular epithelial cells (1,2), and prostaglandin synthesis in mesangial cells (submitted for publication). CSA has previously been shown to modify the intracellular functions and cell proliferation in other cell lines, including lymphocytes (3), pancreatic islet cells (4) mesangial cells (5).

Due to the lipophilic nature of CSA, the drug rapidly enters cells via a partitioning process (6) and therefore, potentially, can interact with a variety of

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Abbreviations.
CSA cyclosporin A; CSD cyclosporin D; CSH cyclosporin H; AII angiotensin II; TPA 12-O-tetradecanoylphorbol-13-acetate

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intracellular regulatory pathways. Activation of the second-messenger system leads to changes in Ca^{2+} flux, activation of various protein kinases with subsequent phosphorylation of target molecules resulting changes in cell activation, synthesis and growth.

Protein kinase C is an ubiquitous enzyme, consisting of a large family of proteins with multiple subspecies that have individual enzymatic characteristics (7). It is present in many cells and has been shown to be present in renal epithelial and mesangial cells (7,8). Within the cell, protein kinase C is present predominantly as a cytosolic enzyme, with membrane translocation probably occurring concomitantly with enzyme activation (9). A variety of drugs, including the phenothiazines, have been shown to inhibit protein kinase C activity (10,11) and it has been suggested that alterations in protein kinase C activity may play an important role in the pharmacological actions of these drugs.

In view of CSA's lipophilic nature, it would seem probable that CSA might modify protein kinase C activity by altering the activation of the enzyme either directly, or indirectly, by way of binding to the phospholipids necessary for the activation of the enzyme, or altering the catalytic properties of the enzyme.

This study was designed to investigate how CSA may interact with protein kinase C to modify renal cellular function. Protein kinase C activity in cytosolic fractions, obtained from a renal tubular epithelial cell line (MDCK cells), were used to investigate a possible interaction between CSA and alterations in cellular protein kinase C activity.

In previous studies we have demonstrated that CSA attenuated mesangial cell PGE_2 release following angiotensin II (AII) stimulation. This interaction may be at the level of protein kinase C. The phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA) binds specifically with protein kinase C and activates the enzyme by inducing a translocation of the protein from the cytosol to phospholipid membranes, leading to a sustained increase in enzyme activity, including an increase in PGE_2 synthesis (5,12,13,14,15,16). TPA was used in combination with CSA, to examine the role of protein kinase C activation in mesangial cell PGE_2 release.

Methods

Cell Cultures. MDCK cells were grown in 225cm^2 flasks under standard conditions using an antibiotic free media. Upon reaching confluency, the cells from five to ten flasks were trypsinized, pooled and spun at 900 RPM for 15 minutes. The resultant cell pellet was then used to prepare cytosol fractions as outlined below.. Rat mesangial cells were obtained as cellular outgrowths from isolated glomeruli and their phenotype confirmed by previously published criteria (17,18). The mesangial cells were used between day eight and twelve following the first subculture for experimental studies.

Preparation of Cytosol Fractions (MDCK cells). The washed cell pellet was resuspended in 1-2ml of homogenisation buffer (buffer A) consisting of 20mM Tris, 0.25M sucrose, 2mM EDTA, 0.5mM EGTA, 50mM 2-mercaptoethanol, 2mM PMSF, 0.5% aprotin, 10 ug/ml leupeptin, 10 ug/ml trypsin inhibitor, pH 7.5 at 4^{o}C. All steps were carried out at 4^{o}C unless stated. The cells were disrupted using a Kontes micro ultrasonic cell disruptor (4 x 15 second bursts) and then ultra-centrifuged at 100,000 x g for 60 minutes (Sorvall OTD-65 ultracentrifuge). The cytosol was decanted, frozen and stored in liquid N_2, at -180^{o}C until assayed. Protein estimation was by the BioRad microprotein assay (19) using bovine serum albumin as the standard.

Partial Purification of Cytosolic Protein Kinase C. The cytosolic fraction was further purified by the use of DEAE ion exchange column chromatography (12,15,16). The cytosol in a final volume of 5ml (total protein of 0.87 mg/ml) was applied to the column. After adsorption, the column was washed with buffer B (buffer A minus proteases) and eluted with a linear gradient of 20ml of buffer B and 20ml buffer B containing 0.15M NaCl. Fractions of maximal enzyme activity were combined for subsequent assay.

Protein Kinase C Assay. Calcium/phospholipid-dependent protein kinase activity was determined by measuring the incorporation of ^{32}P from [g-^{32}P]ATP into histone in the presence, or absence, of Ca^{2+} and phosphatidylserine (12,15). The total reaction volume contained 200 ul of buffer C consisting of 25mM Tris (pH 7.5), 0.25M sucrose, 6.25mM $MgCl_2$, 12.5mM [^{32}P]ATP (2uCi per 200ul aliquot, Amersham UK.), and 50 ug histone (lysine-rich subgroup, Sigma [type V-S]), 10ul Ca^{2+} (concentrations as stated in experiments), 20ul of cytosol and 20ul containing 24ug phosphatidylserine (Sigma) with or without 0.8ug diolein. 10ul CSA (0.02 ug/ml to 20 ug/ml) was also added to the reaction volume where indicated. The blanks contained 20ul 20mM Tris-saline (replacing the phospholipids) and 10ul 2mM EDTA/0.5mM EGTA (in place of Ca^{2+}). The phospholipids were added to the reaction mixture immediately before initiation of the reaction by the addition of the tissue fraction. In all experiments, a final cytosol protein concentration of 70 to 90ug/250ul was used. The assay was incubated at 30^{o}C for three minutes and the reaction terminated by the addition of 0.5ml 20% trichloroacetic acid plus 50mg bovine albumin. The mixture was vortexed, then centrifuged at 2,500 RPM for 30 minutes, the supernatant aspirated and the pellet dissolved in 100ul 1M NaOH. The sample was suspended in 10ml Pico-fluor 30 (Packard) and the radioactivity counted (Beckman LS 2800 liquid scintillation spectrometer). Protein kinase C activity was expressed as the percentage increase in activity compared to the blank. In each assay, the value obtained was the mean of five to eight reactions per individual sample, (at each concentration of CSA and/or Ca^{2+}). The final value used is the mean of three experiments using different cytosol extracts.

Cyclosporin A and TPA Stimulated Mesangial Cell PGE_2 Release. After an one hour pre-incubation period with RPMI media only, the mesangial cells were incubated with CSA plus TPA 0.1uM for 5 minutes, 0.5ml media aspirated and replaced with the same media for a further 5 to 15 minutes. The media was then aspirated, snap-frozen and stored at -80^{o}C until assayed. The data is the mean $\pm$ SEM of two experiments, N=20 for each concentration of CSA. For all mesangial cell experiments, the Ca^{2+} concentration was kept constant at 4mM (the concentration of Ca^{2+} in RPMI 1640 cell culture media) to prevent variations in Ca^{2+} concentrations, from adding a further variable to the results. CSA (Sandoz) was prepared from stock solutions with a final

ethanol concentration of 0.1%. TPA (Sigma) was dissolved in ethanol with a final ethanol concentration of 0.1% .

PGE_2 concentrations were determined by RIA (New England Nuclear, Du Pont USA.) after appropriate dilutions. There was no cross-reactivity evident in the culture media containing TPA, or CSA with the PGE_2 assay. PGE_2 release is expressed as pg/ug protein.

Results

Protein Kinase C Activity in MDCK Cell Fractions. Initial experiments were carried out using the unpurified cellular fractions. The addition of CSA to the assay inhibited cytosolic protein kinase C activity (Fig 1), which was not affected by changing the enzyme affinity for phospholipids and calcium, by the addition of diolein.

The partially purified enzyme activity, when assayed in the presence of CSA, demonstrated a greater inhibition of protein kinase C activity (Fig 2), than that seen in the crude cytosolic fraction (Fig 1). This confirms, that in this experimental model, CSA inhibits the action of protein kinase C, as measured by the incorporation of ^{32}P into histone.

Cyclosporin A and Phorbol Ester Stimulated PGE_2 Release. There was no significant alteration in PGE_2 release when the mesangial cells were

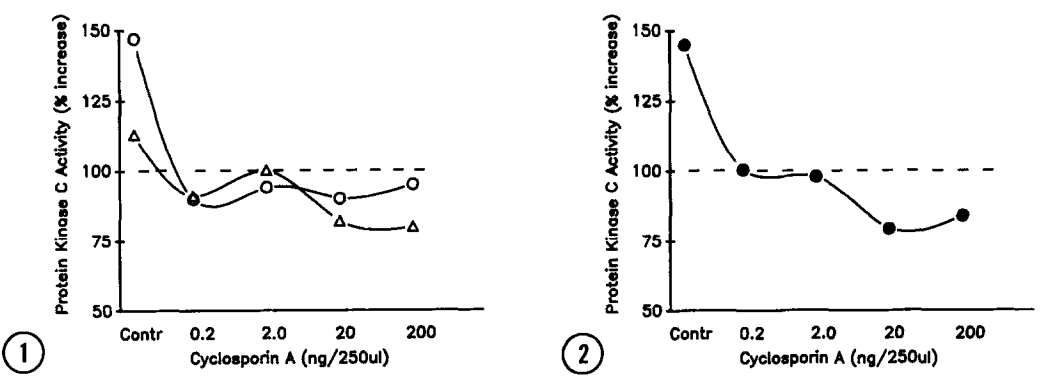

Figure 1. Cyclosporin A Inhibition of Protein Kinase C Activity. Protein kinase C activity in the cytosolic fractions of MDCK cells, was measured as the increase in radioactivity (^{32}P) by the phosphorylation of histone.
(o—o) Enzyme activity in the presence of phosphatidylserine (24ug) and Ca^{2+} (0.5mM). (Δ—Δ) Enzyme activity in the presence of phosphatidylserine (24ug) plus diolein (0.8ug) and Ca^{2+} (0.1uM).

Figure 2. Cyclosporin A Inhibition of Partially Purified Protein Kinase C Activity. Protein kinase C was purified using ion-exchange chromatography. Enzyme activity was measured following activation by phosphatidylserine (24ug) plus diolein (0.8ug) and Ca^{2+} (0.1uM).

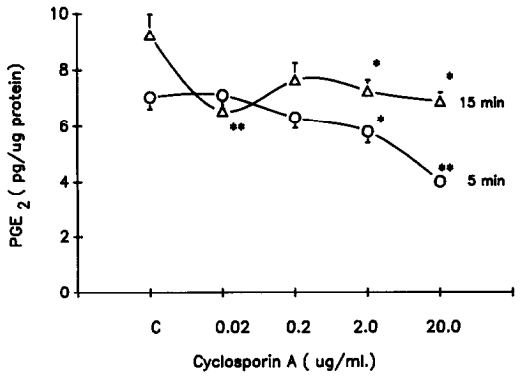

Figure 3. Cyclosporin A Inhibition of Phorbol Ester (TPA) Activated PGE_2 Release from Mesangial Cells. Following a 5 minute pre-incubation with CSA, TPA (0.1uM) was added for a further 5 to 15 minute incubation. PGE_2 release was measured by RIA. (o–o) PGE_2 release at 5 minutes. (Δ–Δ) PGE_2 release at 15 minutes. * $p < 0.05$ ** $p < 0.01$ compared to control.

incubated with CSA only. Incubation of the mesangial cells with TPA alone produced an increased release in PGE_2. When CSA was added to TPA, there was a reduction in PGE_2 release (Fig 3) evident at 5 and 15 minutes.

Discussion

This study has demonstrated clearly that CSA produced an inhibition of protein kinase C activity *in vitro*, most evident when partially purified enzyme fractions were used in the assay (Fig 2). Therefore, CSA-induced inhibition of protein kinase C phosphorylation would have the potential to modify numerous intracellular processes including protein synthesis, DNA formation and cell growth. This would contribute to the renal cytotoxic effects that are seen with CSA. It was also demonstrated that CSA produced a reduction in TPA-induced PGE_2 release from mesangial cells. This would suggest that CSA is inhibiting protein kinase C activity *in vivo* in mesangial cells in culture. Protein kinase C is important in modulating the phosphoinositol cascade and subsequent activation of prostaglandin synthesis (20,21). Therefore, the CSA induced alterations in renal hemodynamics may be mediated at an intracellular level by the inhibition of protein kinase C. This will need to be confirmed by future experiments.

Cyclosporins, CSA,CSD and CSH, have all been shown to signficantly inhibit various biological effects of TPA in mouse skin, including protein synthesis, DNA synthesis and tumour promotion (22). It was shown that these

cyclosporins inhibited TPA-induced and Ca^{2+}/calmodulin-dependent phosphorylations *in vitro* and that these actions appear to be independent of CSA's immunosuppressive action (20). These studies thus add further evidence that CSA is modulating the activity of protein kinase C.

In summary, the present study has demonstrated that CSA significantly inhibits, *in vitro*, the activation of protein kinase C from renal tubular epithelial cells. The inhibition of protein kinase C, preventing phosphorylation of enzymes regulating protein and DNA synthesis, may in part, explain the inhibition of cell protein and DNA synthesis and inhibition of cell growth demonstrated in renal tubular epithelial cells in culture (1,2). The inhibition or modulation of protein kinase C activity in mesangial cells by CSA, appears to play a major role in the CSA induced inhibition of AII stimulated PGE_2 release. Further work is now underway in this laboratory, to try and delineate more clearly the interaction between protein kinase C regulated phosphorylation, intracellular Ca^{2+} flux, and CSA.

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