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COMPARISON OF DIFFERENT TUMOUR PROMOTERS AND BRYOSTATIN 1 ON PROTEIN KINASE C ACTIVATION AND DOWN-REGULATION IN RAT RENAL MESANGIAL CELLS

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Abstract—The effects of a series of protein kinase C (PKC) activators with different spectra of biological activities and reportedly different patterns of PKC isoenzyme activation were examined in renal mesangial cells. Treatment of mesangial cells with the tumor promoters phorbol 12-myristate 13-acetate (PMA), debromoaplysiatoxin, dihydroteleocidin and thymeleatoxin, as well as with the marine natural product bryostatin 1, caused translocation and at least partial down-regulation of the PKC-α,-δ and -ε isoenzymes as assessed by immunoblot analysis. Bryostatin 1 mediates a faster depletion of PKC-α isoform than any of the other PKC activators. Thymeleatoxin, which has been reported to selectively activate PKC-α,-β and -γ, but not PKC-δ or -ε isoenzymes *in vitro*, turned out to exert the most potent effect on PKC-δ and -ε in mesangial cells and down-regulated these isotypes within 8–24 hr. None of the compounds tested affected cellular distribution or amount of PKC-ζ in mesangial cells. Thus, all of the PKC activators tested are able to translocate and down-regulate three of the four PKC isoenzymes present in mesangial cells although with different kinetics. All PKC activators stimulated a phospholipase A_2-mediated arachidonic acid release, a phospholipase D-mediated phosphatidylcholine hydrolysis, a comparable small proliferative response and an inhibition of phospholipase C-mediated inositol trisphosphate generation. These results suggest: (i) that the PKC activators investigated in this study do not display any type of isotype-specificity that could be used to selectively activate or down-regulate PKC isoenzymes in intact cell-systems; (ii) that thymeleatoxin has a different isoenzyme selectivity in intact cells as compared to *in vitro* enzyme inhibition data; and (iii) PKC-ζ is resistent to all PKC activators investigated in this study.

Key words: protein kinase C; mesangial cells; bryostatin 1; tumour promoters, proliferation; phospholipase A_2

PKC§ is a serine-threonine kinase that is crucially involved in many signalling cascades and regulates diverse cellular processes such as cell growth and differentiation, secretion and gene expression [1, 2]. Molecular cloning and sequence analysis of PKC indicated that the enzyme exists as a family of at least ten different isoforms, designated α, β_1, β_2, γ, ε, ζ, η, Θ and ι, all having closely related structures but differing in their individual enzymological properties. Different tissue distribution and subcellular localization have been found for the PKC isoenzymes, suggesting specific roles for each isoform in cell regulation [1, 2]. Nevertheless it has proven difficult to appoint specific functional cell responses to activation of individual PKC isotypes.

In glomerular mesangial cells, PKC contributes to four major cellular functions: (i) it mediates hormone-stimulated phospholipase A_2 activation and subsequent arachidonic acid release and prostaglandin synthesis [3–5]; (ii) it acts as a negative feedback regulator of the inositol lipid signalling pathway [6, 7]; (iii) it activates a phosphatidylcholine-hydrolysing phospholipase D [7–10]; and (iv) it triggers mesangial cell proliferation [11]. By using specific antibodies we observed that mesangial cells express four PKC isoenzymes, PKC-α, -δ, -ε and ζ. No PKC-β, -γ and -η isoforms were detected [12–15]. In addition, we sought to determine the down-regulation kinetics of mesangial cell PKC isoenzymes in order to find a possible correlation with the time courses of removal of the specific cellular functions.

The experimental data suggested that PKC-α is the most likely candidate for triggering feedback inhibition of hormone-stimulated phosphoinositide hydrolysis [12, 13, 16], whereas PKC-ε regulates phospholipase A_2 [12, 13] and phospholipase D [9, 10] activity in mesangial cells. Isoenzyme-specific activators or inhibitors will be required to confirm this tentative appointment of PKC isoenzymes to cellular targets unequivocally. The search for specific inhibitors of PKC has been a difficult one. Most of the available inhibitors lack selectivity for PKC over other protein kinases, and little is known about their isoenzyme specificity [17]. Recently, derivatives of staurosporine have been described which, *in vitro*, show increased specificity for PKC inhibition [18, 19].

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§ Abbreviations: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; DMEM, Dulbecco's modified Eagle's medium; $InsP_3$, inositol trisphosphate.

Surprisingly, staurosporine and some structurally related compounds, although nonspecific in terms of differentiating PKC from other protein kinases, may serve as tools to differentiate between Ca^{2+}-dependent (PKC-α, -β, -γ) and Ca^{2+}-independent (PKC-δ, -ε, ζ and η) PKC isoenzymes *in vitro* [20, 21] and in intact cellular systems [16].

An alternative approach is to use isoenzyme-specific activators of PKC. Phorbol diesters such as PMA are widely used activators of PKC that are able to stimulate all isoenzymes except the ζ-isotype [14, 22, 23]. Recently, Ryves *et al.* [24] examined the abilities of six phorbol esters to activate PKC-isotypes α, β_1, γ, δ and ε *in vitro*. Among the compounds tested, thymeleatoxin, an analogue of mezerein, selectively activated PKC-α, -β_1 and γ, but failed to activate PKC-δ or -ε. In addition, 12-deoxyphorbol-13-*O*-phenylacetate-20-acetate appeared to exclusively activate PKC-β_1. In this study, we examined a series of PKC activators for their isoenzyme-selectivity with respect to translocation and down-regulation in rat renal mesangial cells by immunoblotting as well as by measuring three short-term functional cell responses (activation of phospholipase A_2 and phospholipase D, inhibition of phospholipase C) and one long-term response (cell proliferation), in which PKC has been implicated.

MATERIALS AND METHODS

Chemicals. Angiotensin II and PMA were purchased from Calbiochem (Lucerne, Switzerland); myo-[2-^{3}H]inositol and [methyl-^{3}H]choline-chloride (specific activity 54 Ci/mmol) were purchased from Amersham International (Dübendorf, Switzerland); [^{3}H]arachidonic acid (specific activity 240 Ci/mmol) and [methyl-^{3}H]thymidine (specific radioactivity 6.7 Ci/mmol) were from NEN DuPont de Nemours, (Regensdorf, Switzerland); bryostatin 1 was kindly provided by Aston Molecules Ltd, (Birmingham, U.K.); debromoaplysiatoxin and dihydroteleocidin were a generous gift from Dr Fujiki, Tokyo, Japan; thymeleatoxin was purchased from LC Services Corp. (MA, U.S.A.); all cell culture nutrients were from Gibco BRL, (Basel, Switzerland); all other chemicals used were either from Merck Darmstadt, (Germany), or Fluka, (Buchs, Switzerland).

Cell culture. Rat renal mesangial cells were cultured as described previously [3]. In a second step, single cells were cloned by limited dilution on 96-microwell plates. Clones with apparent mesangial cell morphology were used for further processing. The cells were grown in RPMI 1640 supplemented with 10% (v/v) fetal-calf serum, penicillin (100 units/mL), streptomycin (100 μg/mL) and bovine insulin (0.66 unit/mL). The cells exhibited the typical stellate morphology. Moreover, there was positive staining for the intermediate filaments desmin and vimentin, which are considered to be specific for myogenic cells, positive staining for the Thy 1.1 antigen, negative staining for factor VIII-related antigen and cytokeratin-excluded endothelial and epithelial contaminations, respectively. The generation of $InsP_3$ upon activation of the AT_1 receptor was used as a functional criterion for characterizing the cloned cell line [25]. For these experiments, passages 8–24 of mesangial cells were used.

Immunodetection of PKC isoenzymes. Confluent mesangial cells in 100-mm diameter dishes were washed with PBS and incubated in 15 mL DMEM containing 0.1 mg of fatty acid-free BSA/mL with or without PKC activators. After the indicated time periods, the medium was removed. The cells were washed with ice-cold PBS and scraped into 1.0 mL of ice-cold homogenization buffer (20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 2 mM dithiothreitol, 25 μg of leupeptin/mL, 1 mM phenylmethanesulphonyl fluoride, 10 mM benzamidine) with a rubber policeman. All subsequent steps were carried out at 4°. The cells were lysed with 3 × 10 sec bursts with a Branson B15 sonifier (setting 4.0) and centrifuged for 1 hr at 100,000 *g*. Supernatants were used as a source of cytosolic protein. Pellets were resonicated in 1 mL of the same buffer containing 1% (v/v) Triton X-100 and centrifuged for 1 hr at 100,000 *g*, yielding the solubilized particulate fractions. Protein concentration was determined by the method of Bradford [26]. The cell protein fractions were subjected to SDS–PAGE (8% acrylamide gel), and proteins were transferred on to nitrocellulose paper for 1 hr at 250 mA using a Bio-Rad Transblot apparatus. The blotting buffer used was 25 mM Tris–HCl (pH 7.4), 190 mM glycine in 20% (v/v) methanol. After transfer, nitrocellulose filters were washed extensively in distilled water and blocked in blocking buffer (50 mM Tris–HCl, pH 7.4, 200 mM NaCl, 0.2% (v/v) Triton X-100 with 3% (w/v) BSA and 10% (v/v) horse serum) for 1 hr at 25°. Filters were then incubated for 4 hr at 25° with monoclonal antibodies raised against the PKC-α, -β and -γ isoenzymes [27] or antiserum reactive with PKC-δ, -ε and -ζ isoenzymes [12, 14] (diluted in blocking buffer as indicated in the figure legend). The detailed characterization of the antibodies is described elsewhere [12, 14, 27]. After washing in buffer A (50 mM Tris–HCl, pH 7.4, 200 mM NaCl, 0.2% Triton X-100 (v/v); 4 × 5 min), the filters were incubated for 1 hr with horseradish-peroxidase-conjugated anti-mouse IgG antibodies (PKC-α, -β and -γ) or anti-rabbit IgG antibodies (PKC-δ, ε and -ζ), in blocking buffer. Thereafter, filters were washed again (4 × 5 min) in buffer A and finally, to determine colour reaction, were incubated in PBS containing 0.5 mg of 3,3′-diaminobenzidine/mL and 0.3% H_2O_2 for 10 min and then washed extensively in distilled water.

Determination of inositol phosphates. Confluent cells in 35 mm-diameter dishes were labelled for 72 hr with myo-[2-^{3}H]inositol (10 μCi/mL) in RPMI 1640 medium free of inositol, containing 2% dialysed fetal-calf serum. Thereafter, the medium was removed and the cells were rinsed several times to remove free [^{3}H]inositol and incubated for an additional 1 hr in fresh medium. Compounds (stock solutions of 10 mM in dimethylsulfoxide) or vehicle were added for the last 30 min of incubation. After this procedure, mesangial cells were incubated in 1 mL of DMEM with or without angiotensin II for the indicated time periods. Thereafter, the reaction was terminated by rapid aspiration of the medium and addition of 1 mL of 20% (w/v) trichloroacetic

acid. For extraction of inositol phosphates the dishes were put on ice for 1 hr. The trichloroacetic acid was then removed with diethyl ether and the final extract was neutralized and applied to anion-exchange columns containing 1 mL of Dowex 1-X8 (100–200 mesh, formate form; Serva, Heidelberg, Germany). Free inositol and the inositol phosphates were eluted sequentially as described previously [3]. By this method the isomers of $InsP_3$ coelute from the Dowex columns. However, previous HPLC separations have shown that at the early time points examined here, the hormone-stimulated increased in $InsP_3$ is mainly an increase of the $Ins(1,4,5)P_3$ isomer [28].

Determination of arachidonic acid release. Mesangial cells in 24-well plates were labelled for 24 hr with [^{3}H]arachidonic acid (1 μCi/mL; specific activity 240 Ci/mmol) in DMEM, containing 0.1 mg of fatty acid-free BSA/mL. Thereafter, the medium was sampled for remaining radioactivity and the cells were washed three times to remove all unincorporated [^{3}H]arachidonic acid. Approximately 80–90% of the added [^{3}H]arachidonic acid was incorporated by this procedure. The labelled cells were incubated in DMEM containing 1 mg of BSA/mL as a trap for the released [^{3}H]arachidonic acid. Cells were then stimulated with PKC activators or vehicle for the indicated time periods. Thereafter the medium was removed and centrifuged. Cells were dissolved in 0.5 M NaOH, and radioactivity was counted in the supernatants and cell extracts in a scintillation counter. The percentage of [^{3}H]arachidonic acid released from total incorporated radioactivity was calculated.

Determination of phosphatidylcholine hydrolysis. PKC activator-stimulated phosphatidylcholine hydrolysis was measured in cells prelabelled for 40 hr with [methyl-^{3}H]choline-chloride. Prelabelled confluent cells were washed several times with medium to remove unincorporated label. The cells were incubated for a further 1 hr. Cells were subsequently stimulated with the different PKC activators (100 nM each) or vehicle for 20 min and incubations were terminated by collecting the medium. Media were separated by ion-exchange chromatography on Dowex 50-WH$^+$ columns exactly as described by Cook and Wakelam [29]. Separation of aqueous extracts was cross-checked by a TLC procedure modified from that described by Pritchard and Vance [30] on Silica gel G plates, developed with 0.5% NaCl/methanol/concentrated NH_4OH (10:10:1, by vol.).

Determination of cell proliferation. Confluent mesangial cells in 24-well-plates were made quiescent by incubating for 3 days in serum-free DMEM supplemented with 0.1 mg/mL of fatty acid-free BSA, and then incubated for 24 hr with 2 μCi [methyl-^{3}H]thymidine/mL in the presence or absence of the different compounds. To stop the incubation, the medium was withdrawn and the cells washed twice with PBS and incubated for 30 min at 4° with 5% (w/v) trichloroacetic acid. Cells were then washed twice with 5% (w/v) trichloroacetic acid and incubated for 30 min at 37° in 1 M NaOH to solubilize the DNA, and the radioactivity, incorporated in the DNA, was counted in a β-scintillation counter.

RESULTS

PKC activators translocate and down-regulate PKC isoenzymes

When mesangial cells were treated with the tumor promoter PMA (100 nM), the classical PKC activator, we observed a translocation of PKC-α and -ε isoenzymes from the cytosol to the particulate fraction within 5 min (Fig. 1, lanes 1 and 2). Due to the high levels of PKC-δ present in the particulate fraction already under basal conditions, we were not able to detect a significant increase in the membrane despite the fact that PKC-δ disappeared from the cytosol. Moreover, long-term treatment with PMA caused a differential down-regulation of the PKC subtypes. After 24 hr, mesangial cells were completely depleted of PKC-α, whereas PKC-δ was only partially down-regulated (by approximately 70%) within 24 hr, (Fig. 1, lane 5). In contrast, PKC-ε was only slightly affected by PMA treatment and thus seemed to be quite resistant to a 100 nM dose of the phorbol ester (Fig. 1, lane 5). PKC-ζ gave only a weak staining in this experiment, but we have previously shown that PKC-ζ is completely refractory to PMA treatment and neither translocates nor down-regulates [14, 15], probably due to the lack of a second zinc-finger binding domain [23].

Exposure of mesangial cells to debromoaplysiatoxin (100 nM) showed the very same translocation and down-regulation kinetics for the different PKC isoenzymes as observed with PMA (Fig. 2). However, debromoaplysiatoxin seemed to be somewhat less potent than PMA, as α was only partially down-regulated after 24 hr treatment (Fig. 2, lane 5).

Dehydroteleocidin (100 nM) displayed a more potent profile when compared to PMA and completely down regulated PKC-α and -δ isoenzymes (Fig. 3). Even PKC-ε staining was reduced by approximately 50% after a 24 hr treatment with dehydroteleocidin (Fig. 3, lane 5).

When cells were exposed to thymeleatoxin (100 nM), PKC-α showed a complete translocation from the cytosol to the membrane within 5 min and became almost completely degraded after 8 hr of thymeleatoxin treatment (Fig. 4, lane 6). PKC-δ translocated more slowly to the membrane. After 2 hr (Fig. 4, lane 4) it could still be detected in the cytosol and 8 hr incubation was required (lane 6) for full translocation of PKC-δ. Due to the high levels of PKC-δ present in the particulate fraction already under basal conditions we were not able to detect a significant increase in the membrane despite the fact that PKC-δ disappeared from the cytosol. Down-regulation of PKC-δ occurred with the same kinetics as PKC-α, whereas PKC-ε again seemed to be very resistant to activation and degradation by thymeleatoxin. It translocated even slower than PKC-δ. At least 24 hr (lane 7) are required for a complete translocation to the membrane compartment, and down-regulation was only partial (by approximately 80%) after 24 hr of thymeleatoxin treatment. PKC-ζ was clearly not affected by thymeleatoxin (Fig. 4).

The last compound examined was the anti-tumor drug bryostatin 1. Exposure of mesangial cells to

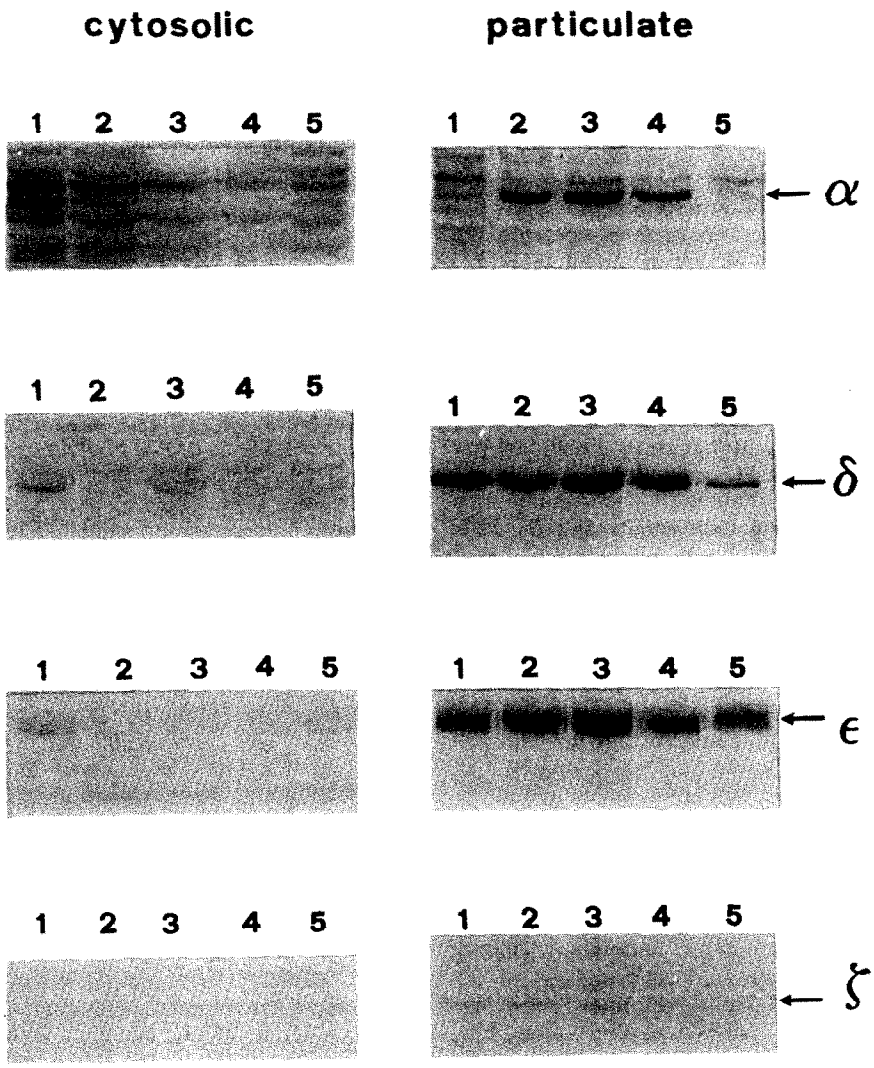

Fig. 1. Immunoblot analysis of PKC isoenzymes in mesangial cells treated with PMA. Cells were treated with vehicle for 5 min (lane 1) or PMA (100 nM) for 5 min (lane 2), 1 hr (lane 3), 4 hr (lane 4) or 24 hr (lane 5), and the cytosolic and particulate fractions were prepared. Samples (100 μg of protein) were applied to SDS–PAGE, and western-blot analysis was performed using a monoclonal antibody against PKC-α at a dilution of 1:100 or antiserum against PKC-δ, -ε or -ζ at a dilution of 1:1000. Bands were detected with horseradish peroxidase. The arrows indicate the respective PKC isoenzymes.

bryostatin 1 (100 nM) resulted in the complete removal of PKC-α from mesangial cells after 4 hr of bryostatin treatment (Fig. 5, lane 4). This was faster than with PMA. In contrast, PKC-δ was only degraded to approximately 50% after 24 hr exposure to bryostatin 1, whereas PKC-ε was reduced to about 20% of the control levels (Fig. 5). Thus PKC-δ turned out to be more resistant, whereas PKC-ε was less resistant to down-regulation by bryostatin 1 as compared to the other PKC activators (Figs 1–4). Again, PKC-ζ was not affected by bryostatin 1 (Fig. 5).

PKC activators stimulate phospholipase A_2 and arachidonic acid release

Figure 6 shows that all PKC activators induced a moderate increase in arachidonic acid release from prelabelled mesangial cells. Unstimulated cells released 1.12 ± 0.21% of the total incorporated [^{3}H]arachidonic acid (mean ± SD, N = 4). The addition of PMA (100 nM) enhanced arachidonic acid release by 55% and thus turned out to be the most potent trigger of phospholipase A_2 activation of the PKC activators investigated. For comparison, the Ca^{2+} mobilizing peptide hormone angiotensin II (100 nM) increased arachidonic acid release by 101% (Fig. 6). Conversely, the biologically inactive phorbol ester 4 α-phorbol 12,13-didecanoate did not increase arachidonic acid release from mesangial cells (Fig. 6).

PKC activators attenuate angiotensin II-stimulated $InsP_3$ formation

Stimulation of mesangial cells prelabelled with

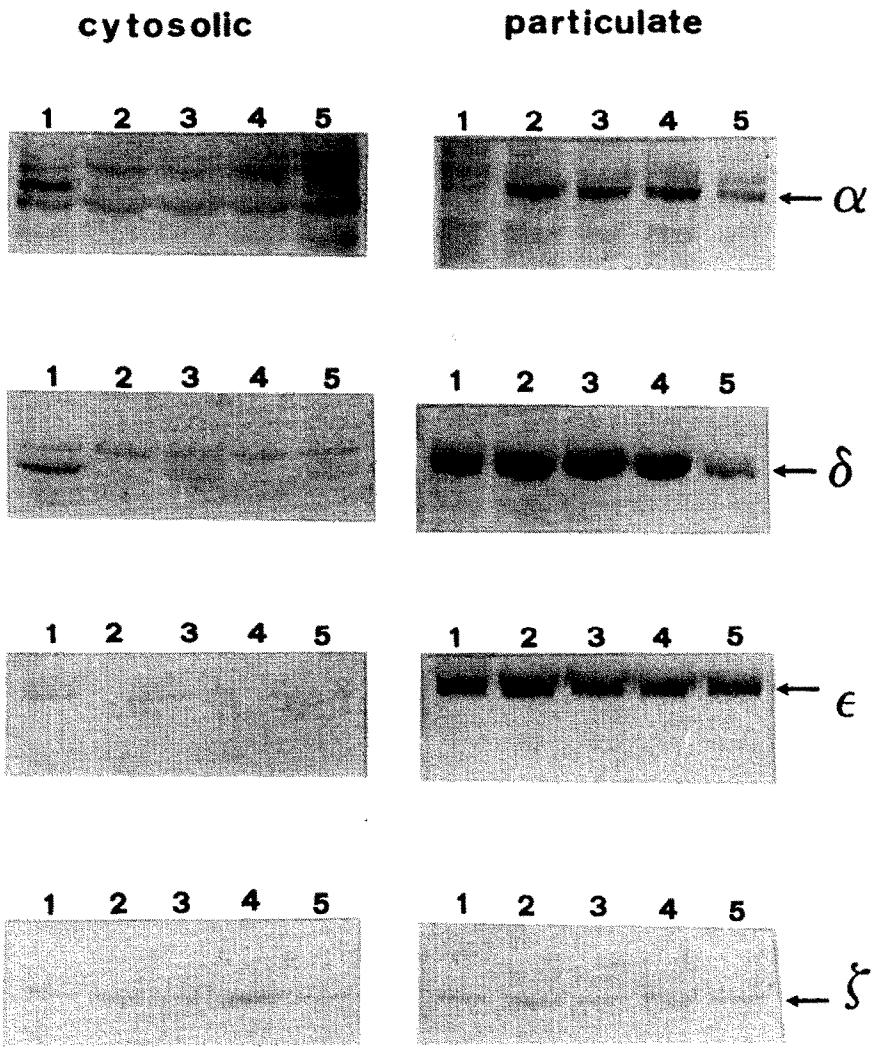

Fig. 2. Immunoblot analysis of PKC isoenzymes in mesangial cells treated with debromoaplysiatoxin. Cells were treated with vehicle for 5 min (lane 1) or debromoaplysiatoxin (100 nM) for 5 min (lane 2), 1 hr (lane 3), 4 hr (lane 4) or 24 hr (lane 5), and the cytosolic and particulate fractions were prepared. Samples (100 μg of protein) were applied to SDS–PAGE, and western-blot analysis was performed using a monoclonal antibody against PKC-α at a dilution of 1:100 or antiserum against PKC-δ, -ε or -ζ at a dilution of 1:1000. Bands were detected with horseradish peroxidase. The arrows indicate the respective PKC isoenzymes.

[^{3}H]inositol by angiotensin II (100 nM) for 20 sec caused marked increases in the formation of inositol monophosphate (+45%), inositol bisphosphate (+740%) and inositol trisphosphate (+180%), consistent with previously published data [3, 12]. Preincubation with the different PKC activators (100 nM each) for 15 min had no significant effect on basal $InsP_3$ values, but potently attenuated angiotensin II-stimulated generation of $InsP_3$ (Fig. 7). In contrast, the biologically inactive phorbol ester 4α-phorbol 12,13-didecanoate had no effect on angiotensin II-induced $InsP_3$ generation (Fig. 7).

PKC activators stimulate phospholipase D and choline release

In order to monitor phospholipase D activity, mesangial cells were labelled with [^{3}H]choline and phosphatidylcholine hydrolysis was studied by analysis of the hydrophilic degradation products [9, 10]. Stimulation of the cells with the different PKC activators (100 nM each) caused a marked increase in choline production as shown in Fig. 8. The most potent compound was bryostatin 1 that caused a 152% increase in choline production. For comparison, the peptide agonist angiotensin II (100 nM) augmented choline release by 56%, whereas the biologically inactive phorbol ester did not stimulate phospholipase D activity in the cells (Fig. 8).

PKC activators stimulate mesangial cell proliferation

The mitogenic potential of PKC activators was

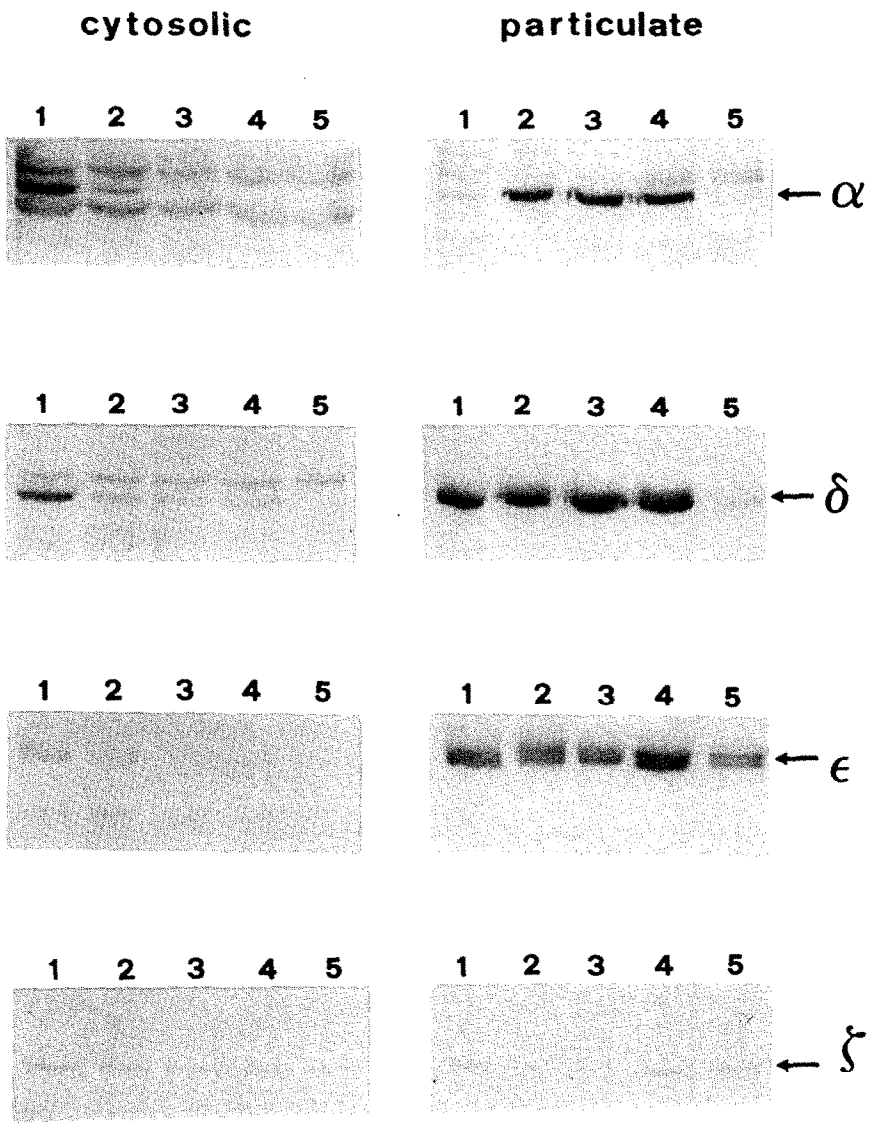

Fig. 3. Immunoblot analysis of PKC isoenzymes in mesangial cells treated with dihydrotelcocidin. Cells were treated with vehicle for 5 min (lane 1) or dihydroteleocidin (100 nM) for 5 min (lane 2), 1 hr (lane 3), 4 hr (lane 4) or 24 hr (lane 5), and the cytosolic and particulate fractions were prepared. Samples (100 μg of protein) were applied to SDS–PAGE, and western-blot analysis was performed using a monoclonal antibody against PKC-α at a dilution of 1:100 or antiserum against PKC-δ, -ε or -ζ at a dilution of 1:1000. Bands were detected with horseradish peroxidase. The arrows indicate the respective PKC isoenzymes.

studied with mesangial cells grown in serum-free medium for 3 days. Thereafter cells were exposed to the different compounds and DNA synthetic capacity was monitored by measuring [^{3}H]thymidine incorporation into DNA. When the PKC activators were added under these conditions, a moderate but significant increase in [^{3}H]thymidine uptake was observed after 24 hr (Table 1). Bryostatin 1, often used as an anti-tumor drug, showed the highest mitogenic activity with an 81% increase in [^{3}H]-thymidine incorporation.

DISCUSSION

PKC is a rapidly growing family of isoenzymes with at present, at least 10 members [31]. This panel of discrete isoforms of PKC shows distinct enzymological properties, differential tissue distribution and diverse intracellular localization, suggesting that each of these isoenzymes may have specific cellular functions. However, our knowledge about specific roles of PKC isotypes is limited and indirect. Part of the problem, of course, is the lack of specific inhibitors or activators of the different PKC isoenzymes. Most of the presently available inhibitors are nonspecific, even in terms of differentiating PKC from other protein kinases [17]. Recently, progress has been made and compounds such as UCN01 [32], CGP 41251 [18] and Ro 31-8425 [33] display increased selectivity for PKC over

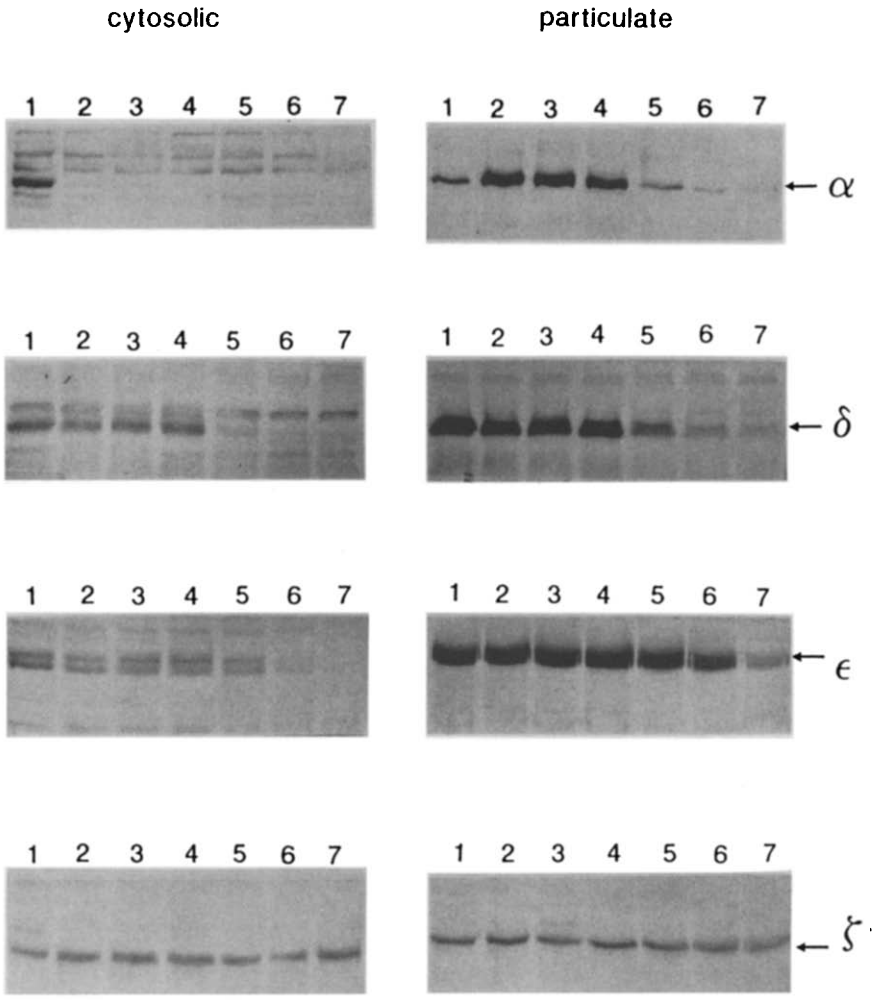

Fig. 4. Immunoblot analysis of PKC isoenzymes in mesangial cells treated with thymeleatoxin. Cells were treated with vehicle for 5 min (lane 1) or thymeleatoxin (100 nM) for 5 min (lane 2), 1 hr (lane 3), 2 hr (lane 4), 4 hr (lane 5), 8 hr (lane 6) or 24 hr (lane 7) and the cytosolic and particulate fractions were prepared. Samples (100 μg of protein) were applied to SDS–PAGE, and western-blot analysis was performed using a monoclonal antibody against PKC-α at a dilution of 1:100 or antiserum against PKC-δ, -ε or -ζ at a dilution of 1:1000. Bands were detected with horseradish peroxidase. The arrows indicate the respective PKC isoenzymes.

other kinases. However, none of these drugs have been reported to display specificity for a certain isoform of PKC.

Alternatively, isoenzyme-specific activators of PKC could be useful in delineating the functions of isozymes. The phorbol ester PMA is the most commonly used activator of PKC and exhibits a complete range of biological activities, whilst other chemically related compounds display a more restricted pattern of activities [34]. However, PMA activates and down-regulates all PKC isoforms with the exception of PKC-ζ [1, 2, 14, 22, 23], thus excluding its use for targeting a specific isoform of PKC. In mesangial cells, PMA translocates and at least partially depletes PKC-α, -δ and -ε isoenzymes, whilst leaving PKC-ζ unaffected (Fig. 1). Teleocidin and aplysiatoxin, which are structurally different from PMA, were reported to be potent tumor promoters in the mouse skin carcinogenesis model and to compete with PMA for the phorbol ester binding site [35]. We used two derivatives of these compounds, debromoaplysiatoxin and dihydroteleocidin, both potent tumor promoters [35]. Like PMA, debromoaplysiatoxin and dihydroteleocidin translocate and down-regulate PKC-α, -δ and -ε isotypes and leave PKC-ζ unchanged (Figs 2 and 3). To the best of our knowledge, this is the first report on the action of this class of compounds on PKC isoenzymes in a cellular system. However, the uniform activation and depletion of a whole panel of PKC isoenzymes by dihydroteleocidin and debromoaplysiatoxin do not allow us to address the role of a specific PKC isoform.

A recent report by Ryves *et al.* [24] indicated that some progress has been made in terms of the isotype-specific activation of PKC. These authors observed

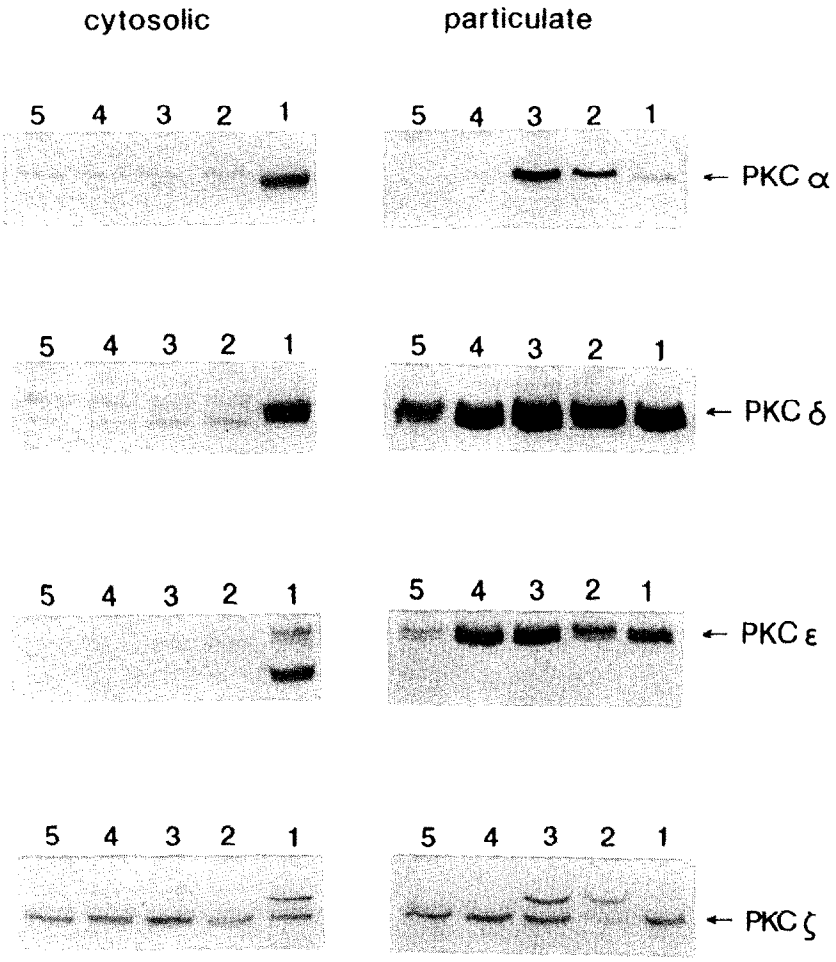

Fig. 5. Immunoblot analysis of PKC isoenzymes in mesangial cells treated with bryostatin 1. Cells were treated with vehicle for 5 min (lane 1) or bryostatin 1 (100 nM) for 5 min (lane 2), 1 hr (lane 3), 4 hr (lane 4) or 24 hr (lane 5), and the cytosolic and particulate fractions were prepared. Samples (100 μg of protein) were applied to SDS–PAGE, and western-blot analysis was performed using a monoclonal antibody against PKC-α at a dilution of 1:100 or antiserum against PKC-δ, -ε or -ζ at a dilution of 1:1000. Bands were detected with horseradish peroxidase. The arrows indicate the respective PKC isoenzymes.

a selective activation of PKC-β_1 by 12-deoxyphorbol-13-*O*-phenylacetate-20-acetate and of PKC-α, -β_1 and -γ by thymeleatoxin, an analog of mezerein isolated from Thymelea hirsuta [24, 36]. Ryves and colleagues used PKC-α, -β_1 and -γ purified from bovine brain and recombinant bovine PKC-δ and murine PKC-ε to evaluate the compounds. Thymeleatoxin activated PKC-α, -β_1 and -γ with ED_{50}'s of 100 nM or less but was inactive on PKC-δ and -ε up to concentrations of 1 μM, thus making thymeleatoxin a very selective activator for the Ca^{2+}-dependent PKC isotypes. We tested thymeleatoxin on renal mesangial cells to see whether it selectively activates the Ca^{2+}-dependent PKC-α, by assaying translocation and down-regulation of the different isoenzymes present in this cell type. Surprisingly, thymeleatoxin not only activated and depleted PKC-α but also the Ca^{2+}-independent PKC-δ and -ε isoforms (Fig. 4). Actually, thymeleatoxin turned out to be the most potent compound in triggering PKC-δ and -ε activation in mesangial cells (investigated in this study). These data clearly demonstrate that in an intact cell system the isoenzyme selectivity of thymeleatoxin seen *in vitro* is completely lost and that this compound is therefore not suitable to selectively down-regulate Ca^{2+}-dependent, conventional PKC isoenzymes. An activation of multiple PKC isotypes, including PKC-δ and -ε, by 12-deoxyphorbol-13-*O*-phenylacetate-20-acetate and thymeleatoxin, has recently also been observed in PC-12 cells [37]. In summary, these results suggest that thymeleatoxin displays no more isoenzyme selectivity than the phorbol ester PMA and like PMA, does not affect the distribution and amount of PKC-ζ (Fig. 4). Furthermore, our observations indicate that results from *in vitro* enzyme assays must be interpreted with caution and cannot simply be transferred to the situation of intact cell systems.

The macrocyclic lactone bryostatin 1 isolated from

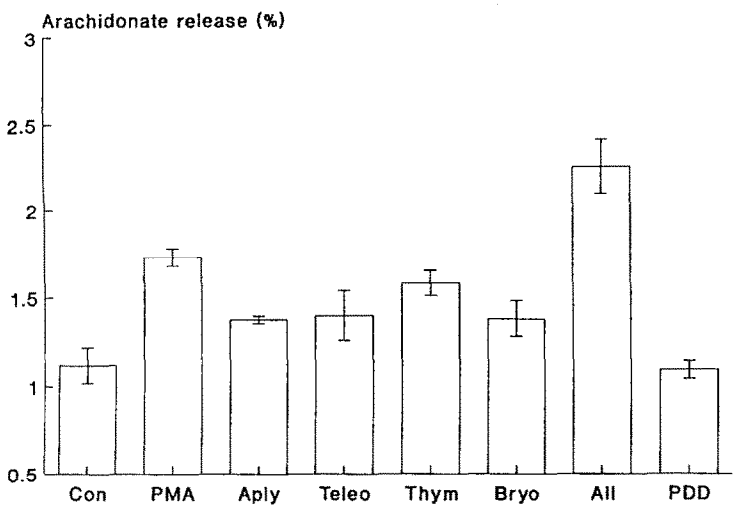

Fig. 6. Effects of different PKC activators and angiotensin II on arachidonate release from mesangial cells. Cells were labelled with [^{3}H]arachidonic acid and stimulated with vehicle (con), PMA (100 nM), debromoaplysiatoxin (Aply, 100 nM), dihydroteleocidin (Teleo, 100 nM), thymeleatoxin (Thym, 100 nM), bryostatin 1 (Bryo, 100 nM), angiotensin II (AII, 100 nM) or 4 α-phorbol 12,13-didecanoate (PDD, 100 nM) for 1 hr and arachidonic acid release was determined as described in the Materials and Methods section. Results are expressed as % release of the total incorporated [^{3}H]arachidonic acid and are means ± SEM, N = 4.

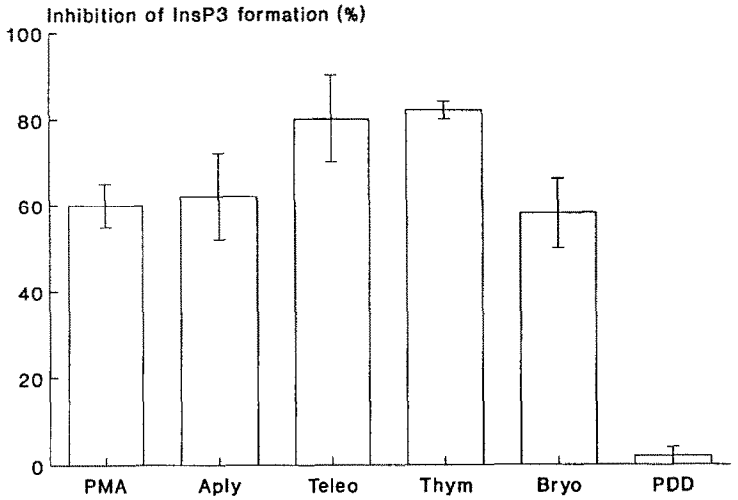

Fig. 7. Effects of different PKC activators on angiotensin II-stimulated inositoltrisphophate ($InsP_3$) formation in mesangial cells. Cells were labelled with myo-[^{3}H]inositol and treated with PMA (100 nM) debromoaplysiatoxin (Aply, 100 nM), dihydroteleocidin (Teleo, 100 nM), thymeleatoxin (Thym, 100 nM), bryostatin 1 (Bryo, 100 nM) or 4 α-phorbol 12,13-didecanoate (PDD, 100 nM) for 30 min and then exposed to angiotensin II (100 nM) for 20 sec. $InsP_3$ formation was determined as described in the Materials and Methods section. Results are expressed as % inhibition of angiotensin II-stimulated $InsP_3$ formation in control cells not treated with PKC activators and are means ± SEM, N = 4.

the marine bryozoan Bugula neritina is a potent activator of PKC [38]. However, bryostatin 1 has been found to have effects both agonistic and antagonistic to those of PMA [39, 40]. These divergent actions of bryostatin 1 and PMA have been explained by a possible isoenzyme-specific action of bryostatin 1 [41, 42]. Hocevar and Fields [42] observed that bryostatin 1 induced selective translocation of PKC-β to the nuclear membrane of a myeloid cell line. In contrast, Kennedy *et al.* [41] reported an extremely rapid depletion of PKC-α in a human breast cancer cell line upon bryostatin 1 treatment. Similarly, in mesangial cells bryostatin 1 caused a rapid down-regulation of PKC-α that was much faster than after PMA treatment (Fig. 5). PKC-δ and -ε were translocated but only partially

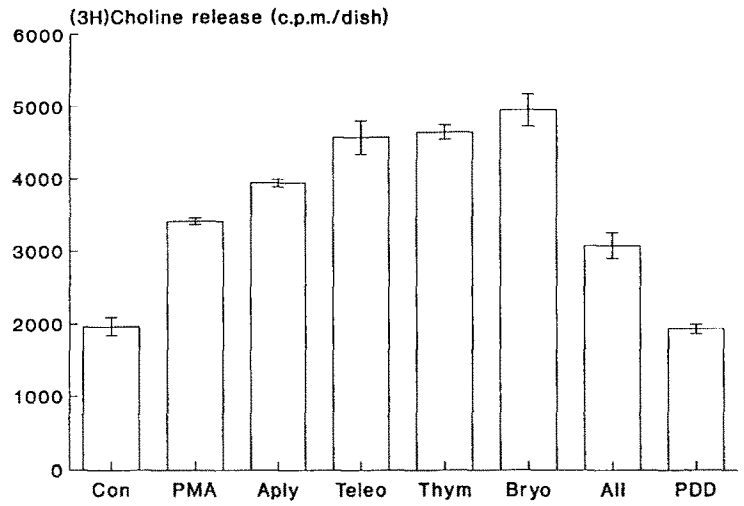

Fig. 8. Effects of different PKC activators and angiotensin II on choline formation by mesangial cells. Cells were labelled with [^{3}H]choline and stimulated with vehicle (con), PMA (100 nM), debromoaplysiatoxin (Aply, 100 nM), dihydroteleocidin (Teleo, 100 nM), thymeleatoxin (Thym, 100 nM), bryostatin 1 (Bryo, 100 nM), angiotensin II (AII, 100 nM) or 4 α-phorbol 12,13-didecanoate (PDD, 100 nM) for 20 min and choline formation was determined in the culture supernatant as described in the Materials and Methods. Results are means ± SEM, N = 4.

Table 1. Effects of different PKC activators on mesangial cell proliferation

Compounds	[^{3}H]thymidine uptake (cpm/well)
Control	3099 ± 119
PMA (100 nM)	3836 ± 108*
Debromoaplysiatoxin (100 nM)	4903 ± 101†
Dihydroteleocidin (100 nM)	4488 ± 236†
Thymeleatoxin (100 nM)	3983 ± 118*
Bryostatin 1 (100 nM)	5632 ± 218‡
4 α-phorbol 12,13-didecanoate (100 nM)	3012 ± 122

Data are expressed as means ± SEM of four experiments. Statistical analysis was performed by one way analysis of variance (ANOVA) and for multiple comparisons corrected according to Bonferroni. *: $P < 0.05$, †: $P < 0.01$ and ‡: $P < 0.001$ (vs control)

down-regulated by bryostatin 1 and, again, PKC-ζ was not affected by the drug. These data suggest that bryostatin 1, like all the other PKC activators tested, shows little selectivity for the limited number of PKC isoenzymes present in mesangial cells.

This lack of selectivity is also reflected in the uniform activation of a number of cellular functions, thought to be mediated by PKC in mesangial cells, by all PKC activators tested (Figs 6–8, Table 1). They all stimulated a phospholipase A_2-mediated release of arachidonic acid (Fig. 6) and a phospholipase D-induced production of choline (Fig. 8), two cellular responses that are most probably mediated by PKC-ε [9, 10, 12]. Moreover, all activators potently attenuated angiotensin II-stimulated $InsP_3$ generation, an important negative feedback mechanism thought to be exerted by PKC-α [12, 13, 16]. Furthermore, all compounds tested increased thymidine incorporation in mesangial cells, a measure of cell proliferation.

In summary, we conclude from our data that PKC activators are not suitable as tools to probe the role of particular PKC isotypes in mesangial cells and probably other cell functional responses. Furthermore, the isoenzyme selectivity of PKC activators observed in *in vitro* enzyme assays is not necessarily transferable to intact cell systems, as shown here for thymeleatoxin. Moreover, PKC-ζ is completely resistant to the whole panel of PKC activators used in this report. Additional work is

needed to improve the repertoire of PKC activators and inhibitors and to develop isoenzyme specific tools.

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