Cyclic AMP agonists induce the phosphorylation of phospholipase C- τ and of a 76 kDa protein co-precipitated by anti-(phospholipase C- τ) monoclonal antibodies in BALB/c-3T3 cells

Relationship to inositol phosphate formation

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Previous studies have demonstrated enhanced phosphorylation of phospholipase C- τ (PLC- τ), a key regulatory enzyme in phosphoinositide metabolism, in cells treated with platelet-derived growth factor (PDGF) and epidermal growth factor, both of which act via specific receptor tyrosine kinases. Our studies on BALB/c-3T3 cells show that agents that promote cellular cyclic AMP accumulation also increase the phosphorylation, specifically the serine phosphorylation, of this enzyme. Increased phosphorylation of PLC- τ (2–3-fold) was evident within 5–10 min of addition of isobutylmethylxanthine (IBMX) and either cholera toxin or forskolin to cells, and persisted for at least 3 h. Treatment of cells with cyclic AMP agonists also enhanced, with similar kinetics, the phosphorylation of a 76 kDa protein co-precipitated by anti-PLC- τ monoclonal antibodies. Brief exposure of cells to cholera toxin/IBMX or forskolin/IBMX decreased inositol phosphate formation induced by the GTP-binding protein (G-protein) activator aluminium fluoride by approx. 50%, but was without effect on PDGF-stimulated inositol phosphate formation. These findings suggest that PLC- τ , and perhaps the 76 kDa co-precipitated protein, are substrates of cyclic AMP-dependent protein kinase in BALB/c-3T3 cells; however, the lack of effect of cyclic AMP elevation on PDGF-stimulated inositol phosphate formation indicates that the intrinsic activity of PLC- τ is unaltered by cyclic AMP-mediated phosphorylation.

INTRODUCTION

Phosphoinositide-specific phospholipase C (PLC) mediates the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) and the consequent formation of two second messengers, inositol 1,4,5-trisphosphate (IP₃), which releases calcium from intracellular stores, and diacylglycerol, which activates the serine/threonine kinase protein kinase C (reviewed in ref. [1]). Cleavage of PIP₂ occurs in response to a variety of hormones in a variety of cell types, and agents which modulate hormone-induced PIP₂ hydrolysis have been identified. The mechanisms by which various agonists influence PLC activity, however, remain unclear. An abundance of evidence suggests that hormone receptors communicate with PLC via GTP-binding proteins (G-proteins) (reviewed in ref. [2]). For example, in some systems, hormone-stimulated PIP₂ hydrolysis is ablated by pertussis toxin, an agent known to preclude receptor–G-protein interaction [3,4]. In addition, increased PIP₂ turnover has been observed in systems treated with non-hydrolysable GTP analogues [5,6] or by the G-protein activator aluminium fluoride [7,8].

Several isoenzymes of PLC have been purified and cloned [9]. These isoenzymes possess limited overall sequence similarity and thus may be regulated by different means. A mechanism of PLC activation alternative or supplemental to G-protein interaction is suggested by studies demonstrating tyrosine and serine phosphorylation of the 145 kDa PLC isoenzyme PLC- τ in cells

treated with epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), both of which interact with specific receptor tyrosine kinases [10–15]. Consistent with a cause–effect relationship, PLC- τ phosphorylation correlated in a time- and dose-dependent manner with IP₃ formation in EGF-treated A431 cells and PDGF-treated BALB/c-3T3 cells [10,13,14]. Thus, although definitive proof is lacking, phosphorylation of PLC- τ represents a potential means of regulating PLC- τ activity. It is noted that attempts to show altered activity of phosphorylated PLC- τ in assays *in vitro* have been hampered by the high intrinsic activity of purified PLC- τ that may, as proposed by Rhee *et al.* [9], reflect the absence of negative regulatory activities, normally operative *in vivo*, from the assay system. An involvement of accessory factors in the regulation of PLC- τ activity is supported by data showing the co-precipitation by anti-PLC- τ monoclonal antibodies of at least three, as yet unidentified, proteins [12,13,15].

Modulation of hormone-stimulated IP₃ formation by agents that promote cellular cyclic AMP accumulation has been observed in several systems [16–20]. As the actions of cyclic AMP are mediated by cyclic AMP-dependent protein kinase, and as PLC- τ activity may be altered by phosphorylation, we assessed the effect of cyclic AMP elevating agents on PLC- τ phosphorylation and on agonist-induced inositol phosphate formation in density-arrested BALB/c-3T3 cells. Our data show that cyclic AMP agonists rapidly increase the phosphorylation of PLC- τ and of a 76 kDa protein which is co-precipitated by anti-

Abbreviations used: PLC, phospholipase C; PIP₂, phosphatidylinositol 4,5-bisphosphate; IP₃, inositol 1,4,5-trisphosphate; G-protein, GTP-binding protein; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; IBMX, isobutylmethylxanthine; PMSF, phenylmethanesulphonyl fluoride.

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PLC- τ monoclonal antibodies. However, data demonstrating differential effects of cyclic AMP agonists on PDGF- versus aluminium fluoride-stimulated inositol phosphate formation suggest that the intrinsic activity of PLC- τ is unaffected by cyclic AMP-mediated phosphorylation. Our findings confirm and extend those of Kim *et al.* [21], who reported increased PLC- τ phosphorylation in rat glioma cells exposed to cyclic AMP-elevating agents and phosphorylation of purified PLC- τ by cyclic AMP-dependent protein kinase.

MATERIALS AND METHODS

Cell culture

Stock cultures of BALB/c-3T3 mouse fibroblasts (clone A31) were grown in Dulbecco-Vogt modified Eagle's medium supplemented with 4 mM-L-glutamine, 50 units of penicillin/ml and 50 μ g of streptomycin/ml in humidified CO₂/air (1:19). For all experimental procedures, cells were grown to confluency in serum-containing medium and were used 2–3 days after growth cessation. Cholera toxin, forskolin and isobutylmethylxanthine (IBMX) were used at final concentrations of 1 μ g/ml, 50 μ M and 100 μ M respectively.

Immunoprecipitation with anti-PLC- τ antiserum

Cells in 60 mm dishes were labelled either overnight in methionine-free medium containing 2% platelet-poor plasma and 80 μ Ci of [³⁵S]methionine/ml or for 3–4 h in phosphate-free medium containing 2% platelet-poor plasma and 200 μ Ci of [³²P]P_i/ml. Platelet-poor plasma at this concentration is not mitogenic for BALB/c-3T3 cells [22]. Following the appropriate treatment, cells were rinsed with phosphate-buffered saline (140 mM-NaCl/2.6 mM-KCl/8 mM-Na₂HPO₄/1.5 mM-KH₂PO₄, pH 7.4) and solubilized with Triton [1% Triton, 10% glycerol, 1 mM-phenylmethanesulphonyl fluoride (PMSF), 100 μ M-vanadate, 50 mM-NaF and 10 mM-Hepes, pH 7.4; TG buffer]. Solubilized material was precleared with Pansorbin (Calbiochem) precoated with anti-(mouse IgG), and precleared material was immunoprecipitated overnight with a mixture of monoclonal antibodies to PLC- τ [23] in the presence of anti-(mouse IgG)-precoated pansorbin. Samples were rinsed with radioimmune precipitation (RIPA) buffer (10 mM-Tris, pH 8.5, 0.15 M-NaCl, 1 mM-EDTA, 1% Triton, 1% deoxycholate, 0.1% SDS and 1 mM-PMSF), resuspended in Laemmli buffer [24] and boiled, and equal volumes applied to 7.5% polyacrylamide gels in the presence of SDS.

Experiments in which immune complexes were treated with potato acid phosphatase (Boehringer-Mannheim) were performed essentially as described by Meisenhelder *et al.* [12]. Immunoprecipitates were rinsed sequentially with RIPA buffer, TG buffer and phosphatase buffer (20 mM-Mes, pH 5.5, 1 mM-MgCl₂, 4 μ g of soybean trypsin inhibitor/ml and 4 μ g of leupeptin/ml) and resuspended in 50 μ l of phosphatase buffer containing 0.2 units of potato acid phosphatase/ml in the presence or absence of 100 mM-sodium phosphate. Following a 1 h incubation at 37°C, immunoprecipitates were rinsed with RIPA buffer, resuspended in Laemmli buffer and boiled.

Phosphoamino acid analysis

³²P-labelled protein bands were visualized by autoradiography and eluted from the gel, and phosphoamino acid content was determined by two-dimensional electrophoresis at pH 1.9 and pH 3.5 on thin layer cellulose plates as previously described [25].

Immunoblots

Immunoprecipitated proteins were electrophoresed on SDS/polyacrylamide gels and transferred from the gels to

nitrocellulose using a Bio-Rad Transblot apparatus. The blot was rinsed for 1 h in blocking buffer (50 mM-Tris, pH 8.0, 2 mM-CaCl₂, 80 mM-NaCl, 5% non-fat dried milk and 0.2% Nonidet P-40) and incubated overnight in blocking buffer alone or with anti-PLC- τ antibodies. To detect proteins recognized by the antibodies, the blot was exposed for 1 h to ¹²⁵I-labelled goat anti-(mouse IgG). Following rinses in blocking buffer with and without the non-fat milk, the blot was dried and exposed to film.

Inositol phosphate assays

Cells were labelled overnight in inositol-free medium containing 2% platelet-poor plasma and 1 μ Ci of [³H]inositol/ml. All additions were made directly to this medium. LiCl was added to a final concentration of 20 mM as specified in the Figure legends. Inositol phosphates were extracted from cells with 10% (v/v) HClO₄; acid-soluble material was neutralized with KOH in the presence of 20 mM-Hepes, pH 7.4, and, following removal of the KClO₄ precipitate, was applied to 0.6 ml columns of Bio-Rad AG 1-X8 (formate form, 200–400 mesh). The columns were rinsed with water and 60 mM-ammonium formate/5 mM-sodium tetraborate; total inositol phosphates were eluted in 5 ml of 1.5 M-ammonium formate/0.1 M-formic acid. The amount of radioactivity incorporated in the eluted material was determined by liquid scintillation counting. The data represent the averages of duplicate plates.

Materials

PDGF (the BB isoform) was obtained from Amgen. Cholera toxin was purchased from Calbiochem, and forskolin and IBMX were from Sigma. Radioisotopes were from du Pont-New England Nuclear.

RESULTS

Effect of cyclic AMP agonists on PLC- τ phosphorylation

To determine whether cyclic AMP agonists induce the phosphorylation of PLC- τ in BALB/c-3T3 cells, cells prelabelled with ³²P were treated for various times with IBMX, a phosphodiesterase inhibitor, and either cholera toxin or forskolin, both of which activate adenylate cyclase. Cholera toxin and IBMX at the concentrations used maximally increased cyclic AMP levels by approx. 8-fold ([26]; results not shown). Following exposure to the cyclic AMP agonists, cells were solubilized and incubated with a mixture of monoclonal antibodies to PLC- τ [23]. Immunoprecipitated proteins were resolved on SDS/polyacrylamide gels. For comparative purposes, some cultures received PDGF-BB, which has been shown previously to stimulate PLC- τ phosphorylation on tyrosine and serine residues in BALB/c-3T3 cells [13].

The autoradiogram presented in Fig. 1 shows that PLC- τ was phosphorylated in untreated cells and that both cholera toxin/IBMX and forskolin/IBMX rapidly enhanced PLC- τ phosphorylation. Increased phosphorylation of PLC- τ was evident within 5–10 min of addition of the cyclic AMP agonists to cells (Fig. 1a) and persisted for at least 3 h (Fig. 1b). Treatment of cells with 100 ng of PDGF-BB/ml for 10 min increased PLC- τ phosphorylation to a greater extent (10.5-fold) than did treatment with the cyclic AMP agonists (maximum 3.5-fold; Figs. 1a and 1b); however, PLC- τ phosphorylation in the PDGF-BB-treated cells was substantially decreased at 60 min, presumably due to down-regulation of PDGF receptors (Fig. 1a). Thus, in comparison with PDGF, the cyclic AMP agonists produced a less dramatic but more sustained phosphorylation of PLC- τ . Similar to findings with rat glioma cells [21], PLC- τ was phosphorylated on serine in both control and cyclic AMP agonist-treated cells (Fig. 1c).

Fig. 1. Effect of cyclic AMP agonists on PLC- γ phosphorylation

(a) Cells prelabelled with ^{32}P received no addition (control; C), cholera toxin/IBMX (CT+IBMX; CI), forskolin/IBMX (FSK+IBMX; FI) or 100 ng of PDGF-BB/ml (BB) for the indicated times. Final concentrations of cholera toxin, forskolin and IBMX were 1 $\mu\text{g}/\text{ml}$, 50 μM and 100 μM respectively. Cells were solubilized and incubated overnight with or without (No Ab) antibodies to PLC- τ . Immunoprecipitated proteins were resolved on SDS/7.5%-polyacrylamide gels. (b) Cells prelabelled with ^{32}P received no addition (C), cholera toxin/IBMX (CI) or 100 ng of PDGF-BB/ml (BB) for the indicated times. Cell lysates were immunoprecipitated with anti-PLC- τ antibodies and immunoprecipitated proteins were resolved on a 7.5% gel. The positions of molecular mass standards are indicated. (c) Control and cholera toxin/IBMX-treated cells were solubilized, immunoprecipitated with anti-PLC- τ antibodies and electrophoresed on a 7.5% gel. The PLC- τ bands were visualized by autoradiography and eluted from the gel, and the phosphoamino acid content was determined by two-dimensional electrophoresis at pH 1.9 and 3.5 on thin layer cellulose plates. The arrow indicates the origin.

Effect of cyclic AMP agonists on co-precipitated phosphoproteins

Previous studies on BALB/c-3T3 cells showed that phosphoproteins of 83, 76 and 44 kDa co-precipitate with PLC- γ [13]. In addition to increasing the phosphorylation of PLC- γ , exposure of cells to cyclic AMP agonists also effected a change in the pattern of proteins co-precipitated by the anti-PLC- τ antibodies. In the experiment shown in Fig. 2(a), cells were prelabelled with [^{35}S]methionine or ^{32}P and treated with or without cholera toxin/IBMX for 30 min. In PLC- τ immunoprecipitates of untreated cells, a single band at 76 kDa was observed, whereas in immunoprecipitates of cholera toxin/IBMX-treated cells, a doublet at 76 kDa was seen (Fig. 2a). In the ^{32}P -labelled cells exposed to cholera toxin/IBMX, the upper band of the doublet was more prominent than the lower band; in other experiments, both bands were equally labelled (e.g. Fig. 1b).

To examine further the effect of cyclic AMP agonists on the 76 kDa co-precipitated phosphoprotein(s), cells prelabelled with [^{35}S]methionine were treated with cholera toxin/IBMX, forskolin/IBMX or PDGF-BB for various times or were left untreated, and solubilized cell lysates were immunoprecipitated with the anti-PLC- τ antibodies. As shown in Fig. 2(b), the 76 kDa doublet was apparent within 5 min of addition of either cholera toxin/IBMX or forskolin/IBMX to cells and throughout the remainder of the time course. Formation of the 76 kDa doublet was unaffected by the presence of the protein synthesis inhibitor cycloheximide. The doublet was not observed in immunoprecipitates of control or PDGF-treated cells or in lysates of cyclic AMP agonist-treated cells incubated with Pansorbin in the absence of antibodies.

Preliminary characterization of the 76 kDa co-precipitated protein

To examine the relatedness of the 76 kDa protein to PLC- τ , PLC- τ immunoprecipitates of control and cyclic AMP agonist-treated cells were resolved by SDS/PAGE, transferred to nitrocellulose and immunoblotted with the anti-PLC- τ antibody mixture. Proteins reactive with the antibodies were visualized by exposure of the blot to ^{125}I -labelled anti-(mouse IgG). As shown in Fig. 3, PLC- τ was readily detected by the anti-PLC- τ antibodies in immunoblots of both control and treated cultures. Although the 76 kDa protein, and also the 83 kDa protein, were present on the blot, as verified by transfer of ^{32}P -labelled PLC- τ immunoprecipitates, they were not recognized by the antibodies. In contrast, a faint band was detected at 44 kDa. Longer exposure of the blot did not result in the appearance of a labelled band at 76 kDa or at 83 kDa (results not shown). This finding indicates that the 76 kDa protein (and also the 83 kDa protein) is neither a degradation product of PLC- τ nor a related protein containing an epitope reactive with the anti-PLC- τ antibodies. Previous studies suggest that the co-precipitated 44 kDa protein possesses an epitope recognized by the one of the antibodies in the anti-PLC- τ antibody mixture [12].

To determine whether the upper band of the doublet is a hyperphosphorylated form of the lower band, immunoprecipitates of [^{35}S]methionine-labelled untreated and cholera toxin/IBMX-treated cells were incubated with potato acid phosphatase in the presence or absence of the phosphatase inhibitor sodium phosphate. Control experiments on immunoprecipitates of ^{32}P -labelled cells verified the effective removal of phosphates from proteins by potato acid phosphatase and the inhibition of this action by 100 mM-sodium phosphate (Fig. 4). As shown in Fig. 4, potato acid phosphatase-treated immune complexes of cholera toxin/IBMX-treated cells contained only the lower band of

Fig. 2. Effect of cyclic AMP agonists on phosphorylation of the 76 kDa co-precipitated protein

(a) Cells prelabelled with [35 S]methionine (A) or 32 P (B) were treated with or without cholera toxin/IBMX for 30 min. Cell lysates were immunoprecipitated with anti-PLC- γ antibodies and electrophoresed on a 7.5% gel. C, control; CI, cholera toxin/IBMX. (b) Cells prelabelled with [35 S]methionine were treated with cholera toxin/IBMX (CT + IBMX; CI), forskolin/IBMX (FSK + IBMX) or 100 ng of PDGF-BB/ml (BB) or were left untreated (control) for the indicated times. Some cultures also received cycloheximide (CH) at 10 μ g/ml. Cells were solubilized and incubated with or without (no Ab) anti-PLC- γ antibodies, and samples were electrophoresed on 7.5% gels. The position of the 76 kDa protein is indicated.

Fig. 3. PLC- γ immunoblot

Control cells (C) and cells treated for 30 min with cholera toxin/IBMX (CI) or forskolin/IBMX (FI) were solubilized and immunoprecipitated with anti-PLC- γ antibodies. Immunoprecipitated proteins were resolved by SDS/PAGE and transferred to nitrocellulose. The blot was treated with or without anti-PLC- γ antibodies and exposed to 125 I-labelled anti-(mouse IgG) (Ab). To verify the presence of PLC- γ and the co-precipitated proteins on the blot, cells prelabelled with 32 P were treated as above, immunoprecipitated and transferred to nitrocellulose. The position of the 76 kDa protein is indicated.

Fig. 4. Phosphatase treatment of PLC- γ immunoprecipitates

Cells prelabelled with [35 S]methionine (a) or 32 P (b) were treated with cholera toxin (CT)/IBMX for 30 min and immunoprecipitated with anti-PLC- γ antibodies. The immunoprecipitates were washed and resuspended in phosphatase buffer alone (no addition, na) or buffer containing 100 mM-sodium phosphate (NaP), 0.2 units of potato acid phosphatase/ml (PAP) or both, and incubated for 1 h at 37 $^{\circ}$ C. Immunoprecipitated proteins were resolved by SDS/PAGE. The 76 kDa protein is indicated.

Fig. 5. Inositol phosphate assays of cells treated with cyclic AMP agonists and aluminium fluoride

Cells labelled with [3 H]inositol received 10 μ g of cycloheximide/ml for 15 min. Cells were then either not pretreated or pretreated with cholera toxin/IBMX (a) or forskolin/IBMX (b) for the indicated times. Cells then received either no addition (■) or NaF + AlCl₃ (▣) and were harvested 30 min later for determination of total inositol phosphates. All cultures received LiCl 15 min prior to addition of NaF + AlCl₃. Final concentrations of NaF and AlCl₃ were 10 mM and 10 μ M respectively.

the 76 kDa doublet whereas both bands were evident in immunoprecipitates not exposed to the phosphatase. Loss of the upper band on potato acid phosphatase treatment was partially blocked by 100 mM-sodium phosphate (Fig. 4) and totally blocked by 200 mM-sodium phosphate (results not shown). These findings indicate, therefore, that the upper band of the doublet is generated by phosphorylation of the lower band.

Short-term effect of cyclic AMP agonists on inositol phosphate formation

We have previously shown that long-term exposure (2–4 h) of BALB/c-3T3 cells to cyclic AMP agonists potentiated inositol phosphate formation in response to EGF, PDGF and the G-protein agonist aluminium fluoride; this action of cyclic AMP-elevating agents required protein synthesis ([20]; N. E. Olashaw & W. J. Pledger, unpublished work). Cyclic AMP agonist-induced phosphorylation of PLC- τ , however, was a rapid event, unaffected by cycloheximide (Fig. 1b; results not shown). To determine whether cyclic AMP-mediated phosphorylation of PLC- γ correlated with rapid changes in inositol phosphate formation, cells labelled with [3 H]inositol were pretreated with either cholera toxin/IBMX or forskolin/IBMX for 0 (i.e. added at the same time as aluminium fluoride) to 45 min in the presence of cycloheximide; aluminium fluoride was added and cells were harvested 30 min later for determination of total inositol phosphates. As shown in Fig. 5, cholera toxin/IBMX and forskolin/IBMX decreased aluminium fluoride-stimulated inositol phosphate formation by approx. 50% at all time points examined. Decreases in basal levels of inositol phosphates were sometimes (Fig. 6), although not always (Fig. 5), observed. Although F⁻ has been shown to inhibit the activity of serine phosphatases, aluminium fluoride did not affect PLC- γ phosphorylation in either control or cholera toxin/IBMX-treated cells (results not shown).

Short-term exposure of cells to cholera toxin/IBMX and forskolin/IBMX did not, however, affect PDGF-induced formation of inositol phosphates (Fig. 6). In the experiment shown in Fig. 6, cells were pretreated with cyclic AMP agonists

Fig. 6. Inositol phosphate assays of cells treated with cyclic AMP agonists and PDGF

Cells labelled with [3 H]inositol were pretreated with (▣) or without (□) forskolin/IBMX (a) or cholera toxin/IBMX (b) for 15 min in the presence of LiCl and 10 μ g of cycloheximide/ml. Cells then received either no addition (control), NaF + AlCl₃ (AF) or various concentrations of PDGF-BB for 30 min. Total inositol phosphates were measured.

for 15 min prior to addition of aluminium fluoride or various concentrations of PDGF-BB. Although a 50% decrease in aluminium fluoride-induced inositol phosphate formation was again apparent, PDGF-stimulated inositol phosphate formation was either unaffected or only slightly decreased. Thus phosphorylation of PLC- τ in response to elevated cyclic AMP apparently does not affect the activity of the enzyme *per se*.

Similar to the data of Kim *et al.* [21], phosphorylation of PLC- τ did not affect PLC- τ activity *in vitro*, as assayed in PLC- τ immunoprecipitates of control and cyclic AMP-agonist-treated cells by the method of Wahl *et al.* [14]. Values for control and cholera toxin/IBMX-treated cells were 100.7 and 100.6 pmol of IP₃/min respectively. However, as discussed by Wahl *et al.* [13], alterations in PLC activity may be undetectable in immune complexes due to the removal or inactivation of putative negative regulatory factors. It is noted that PLC activity in PLC- τ immunoprecipitates of PDGF-treated cells was also comparable with that of immunoprecipitates of control cells (106.4 pmol of IP₃/min).

DISCUSSION

The data presented here demonstrate enhanced phosphorylation of PLC- τ in BALB/c-3T3 cells treated with cyclic AMP-elevating agents. PLC- τ phosphorylation in cells exposed to cholera toxin/IBMX or forskolin/IBMX was rapid and sustained and occurred on serine residues (Fig. 1). These findings confirm and extend a previous report [21] showing serine phosphorylation of PLC- τ in rat glioma cells exposed to cyclic AMP agonists and of purified PLC- τ incubated with cyclic AMP-dependent protein kinase. Although our studies did not address the stoichiometry of cyclic AMP-mediated PLC- τ phosphorylation, Kim *et al.* [21] reported that purified PLC- τ was phosphorylated to a stoi-

chiometry of 0.87 mol of phosphate/mol of PLC- τ by cyclic AMP-dependent protein kinase.

PLC- τ , like the recently cloned PLC IV [27], differs from the PLC- α , - β , and - δ in that it shares sequence identity with regions contained within the non-catalytic domain of the *src* family of non-receptor tyrosine kinases [28,29]. These regions, which are also present in the *crk* oncogene product [30] and GTPase-activating protein [31], are thought to modulate enzymic and/or functional activity via interactions with specific cellular components [32,33]. Previous studies using monoclonal antibodies to PLC- τ have shown that several proteins co-precipitate with PLC- τ ; whether these proteins bind to the regions that are similar to the *src* proteins is, at present, unknown. Proteins co-precipitated by the PLC- τ antibodies include the EGF and PDGF receptors, which associate with PLC- τ in a ligand-dependent manner [10,12,34] and three phosphoproteins observed in immunoprecipitates of both treated and untreated cells [12,13,15]. In our studies, we found that cyclic AMP agonists, in addition to effecting an increase in the phosphorylation of PLC- τ , also produced a change in the pattern of proteins co-precipitated by anti-PLC- τ antibodies from [35 S]methionine- or 32 P-labelled cells. In immunoprecipitates of untreated and PDGF-treated cells a single band was observed at 76 kDa, whereas in immunoprecipitates of cyclic AMP agonist-treated cells a doublet was seen at 76 kDa (Fig. 2a). Similar to increased PLC- τ phosphorylation (Fig. 1a), formation of the 76 kDa doublet was evident within 5–10 min of addition of the cyclic AMP agonists to cells and for periods of up to 3 h (Fig. 2b; results not shown). Combined immunoprecipitation/immunoblotting experiments (Fig. 3) suggest that the 76 kDa protein is unrelated to PLC- τ ; thus its presence in PLC- τ immune complexes may reflect its association with PLC- τ in intact cells.

Additional experiments in which immune complexes of cyclic AMP agonist-treated cells were exposed to potato acid phosphatase indicate that the upper band of the 76 kDa doublet is generated by phosphorylation of the lower band. As shown in Fig. 4, the upper band of the doublet was not present in phosphatase-treated immunoprecipitates of cholera toxin/IBMX-treated cells; in contrast, both bands were observed in immunoprecipitates either not exposed to potato acid phosphatase or receiving the phosphatase in the presence of sodium phosphate (Fig. 4; results not shown). Whether the 76 kDa co-precipitated phosphoprotein is a direct substrate of cyclic AMP-dependent kinase is not known at present; however, the rapidity with which it is phosphorylated is consistent with this possibility. Nonetheless, to our knowledge, this is the first demonstration of a modification of a protein that may physically interact with PLC- τ *in vivo*.

Previous results from our laboratory showed that extended exposure (2–4 h) of BALB/c-3T3 cells to cyclic AMP agonists potentiated PDGF-, EGF- and aluminium fluoride-stimulated inositol phosphate formation, and also increased basal levels of inositol phosphates ([20]; N. E. Olashaw & W. J. Pledger, unpublished work). These actions of cyclic AMP were abolished by cycloheximide. The data presented here demonstrate that cyclic AMP agonists also have a rapid, inhibitory and protein-synthesis-independent effect on aluminium fluoride-stimulated inositol phosphate production in these cells. Inositol phosphate formation in response to aluminium fluoride was reduced by approx. 50% in cells co-treated or briefly pretreated with cholera toxin/IBMX or forskolin/IBMX in the presence of cycloheximide (Fig. 5). Inhibition of aluminium fluoride-stimulated inositol phosphate formation occurred within 30 min of addition of cyclic AMP agonists to cells, as did the increase in PLC- τ phosphorylation and the formation of the 76 kDa doublet (Figs. 1a, 2b and 5). Similarly, in rat glioma cells, cyclic AMP-elevating

agents increased PLC- γ phosphorylation and decreased inositol phosphate production induced by noradrenaline, an agent thought to interact with PLC via G-proteins [35].

Previous studies have demonstrated a differential effect of phorbol esters on PDGF-stimulated compared with aluminium fluoride-stimulated inositol phosphate formation [4]. Our data show that brief exposure of BALB/c-3T3 cells to cyclic AMP agonists also differentially affects the capacity of these agents to effect this response; thus PDGF and aluminium fluoride may increase inositol phosphate production by different means. Whereas cholera toxin/IBMX and forskolin/IBMX inhibited aluminium fluoride-stimulated inositol phosphate formation by approx. 50%, PDGF-induced inositol phosphate formation in the treated cells was unaffected or only marginally diminished (Fig. 6). The lack of effect of cyclic AMP agonists on PDGF-stimulated inositol phosphate formation indicates that the intrinsic activity of PLC- τ is unaffected by its phosphorylation occurring in response to elevated cyclic AMP. It is possible that cyclic AMP-mediated phosphorylation of PLC- τ , while not affecting PLC- τ activity *per se*, inhibits its capacity to interact with G-proteins. Whether G-proteins mediate PDGF-stimulated PIP₂ hydrolysis is unclear; the GTP analogue guanosine 5'-[γ -thio]triphosphate has been reported to increase [36], decrease [37] and have no effect on [38] PDGF-stimulated inositol phosphate formation in permeabilized cells.

The possibility that cyclic AMP inhibits aluminium fluoride-stimulated inositol phosphate formation via actions on G-proteins or PLC isoenzymes other than PLC- τ cannot at present be excluded. It is noted that purified PLC- β and PLC- δ are not substrates of cyclic AMP-dependent protein kinase [21]. Our preliminary studies show that cyclic AMP agonists also increase PLC- τ phosphorylation by approx. 3-fold in Swiss 3T3, NIH 3T3 and rat 1 cells (N. E. Olashaw, unpublished work). However, whereas these agonists virtually abolished aluminium fluoride-stimulated inositol phosphate formation in rat 1 cells, they did not affect this response in either Swiss 3T3 or NIH 3T3 cells. These observations suggest that phosphorylation of PLC- τ in response to elevated cyclic AMP is, at best, insufficient for cyclic AMP-mediated inhibition of aluminium fluoride-induced inositol phosphate formation. However, it is possible that different G-proteins interact with PLC- τ in different cell lines and that phosphorylation of PLC- τ in response to cyclic AMP-elevating agents precludes its association with some, but not all, G-proteins. Thus, although treatment of BALB/c-3T3 cells with cyclic AMP agonists clearly enhances the phosphorylation of PLC- τ , the repercussions of this effect remain to be elucidated.

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