The cross-regulation of G_i-protein by cholera toxin involves a phosphorylation by protein kinase A

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Pretreatment of alveolar macrophages with cholera toxin inhibits the release of arachidonic acid induced by the chemotactic peptide *N*-formylmethionyl-leucyl-phenylalanine. The results presented here show that cholera toxin might exert its inhibitory effect through the phosphorylation of G_iα by protein kinase A (PKA). (1) G_i-proteins from cells pretreated with cholera toxin showed parallel increases in their sensitivity to ADP-ribosylation by toxins *in vitro* and in G_iα phosphorylation. By contrast, the G_iα concentration was unchanged. (2) Cholera toxin pretreatment also decreased the functional activity of G_i, as assessed by the inhibition (80 %) of agonist-induced binding of guanosine-

5'-[γ-thio]triphosphate (GTP[γS]). (3) These effects of cholera toxin were blocked by a specific PKA inhibitor, *N*-(2-[methylamino]ethyl)-3-isoquinolinesulphonamide dihydrochloride (H8) and mimicked by a cyclic AMP (cAMP) analogue and a phosphatase inhibitor. (4) G_iα was also phosphorylated *in vitro* by the catalytic subunit of PKA. In contrast with other cell systems, the stimulation of protein kinase C seems to have no effect on the sensitivity of G_i to ADP-ribosylation or on its phosphorylation. Therefore, the phosphorylation of G_i-proteins by PKA seems to be the actual target of the negative control of arachidonic acid release via the cAMP-mediated pathway.

INTRODUCTION

Macrophages are stimulated to release arachidonic acid and produce eicosanoids by several agonists involved in inflammatory processes. One of them, the bacterial chemotactic peptide *N*-formylmethionyl-leucyl-phenylalanine (fMLP), binds to specific receptors that are, in turn, coupled to heterotrimeric G-proteins [1,2]. The fMLP receptors in macrophages and neutrophils are coupled to G_i-proteins [3,4]. Hence, the binding of fMLP leads to the activation of a range of effectors, such as phospholipase C, protein kinase C (PKC), and intracellular phospholipase A₂, and to the inhibition of adenylylcyclase [5,6].

The release of arachidonic acid by phospholipase A₂ is regulated negatively by stimulation of adenylylcyclase in several cell types. For example, in alveolar macrophages, an increase in cyclic AMP (cAMP) parallels a drop in arachidonic acid release induced by fMLP [7]. The way this cross-regulation operates is still unclear. G_i-proteins were phosphorylated following activation of protein kinase A (PKA) in hepatocytes [8] and in a macrophage-like cell line [9]. However, no direct phosphorylation of G_i by PKA could be demonstrated, although G_i-protein was phosphorylated by PKC *in vitro* [10].

Cholera toxin catalyses the ADP-ribosylation of G_s-proteins in whole cells. ADP-ribosylation occurs on an arginine residue that is highly conserved in several Gα subunits, including G_sα, G_iα and G_qα [11]. This covalent modification of G_sα inhibits its intrinsic GTPase activity, resulting in the sustained stimulation of adenylylcyclase in cholera toxin-treated cells. Cholera toxin also ADP-ribosylates G_iα subunits in isolated membranes incubated with an agonist but without GTP [12–14]. This ADP-ribosylation of G_i-proteins by cholera toxin seems to occur

preferentially on the G_i-protein in its heterotrimeric form that has been activated by an occupied receptor.

We have previously shown that pretreating alveolar macrophages with cholera toxin induces ADP-ribosylation of G_s-proteins. G_i-proteins from cholera toxin-treated cells appear to be more heavily labelled by ADP-ribosylation with pertussis toxin than are control cells [15]. Therefore, the aim of this study was to demonstrate that the target of the cross-regulation of arachidonic acid release by cholera toxin could be the G_i-protein itself. The present results show that the sustained stimulation of PKA by cholera toxin has no effect on the concentration of G_iα-protein, but increases its phosphorylation by PKA in both intact cells and isolated membranes. This phosphorylation parallels a decrease of functional interaction of G_i-protein with fMLP receptor.

MATERIALS AND METHODS

Materials

Male Hartley guinea-pigs (400–500 g) were purchased from Saint-Antoine, Pleudaniel, France. Fetal bovine serum, RPMI 1640 medium and phenylmethanesulphonyl fluoride (PMSF) were from Gibco-BRL. Fatty-acid-free BSA, leupeptin and alkaline phosphatase were from Boehringer. Polyacrylamide, bisacrylamide and SDS were from BioRad. Nitrocellulose (BA83) was from Schleicher and Schüll. [³²P]P_i (9000 Ci/mmol) and Hyperfilms MP were from Amersham. ¹²⁵I-Protein A (2 Ci/mmol), [³²P]NAD⁺ (30 Ci/mmol), [³⁵S]guanosine-5'-[γ-thio]triphosphate (GTP[γ³⁵S]) (1000 Ci/mmol) and AS7 antiserum were from Du Pont New England Nuclear. The anti-G-protein α-subunit anti-

Abbreviations used: Bt2 cAMP, dibutyryl cAMP; fMLP, *N*-formylmethionyl-leucyl-phenylalanine; G_i- and G_s-proteins, inhibitory and stimulatory G-proteins; H8, *N*-(2-[methylamino]ethyl)-3-isoquinolinesulphonamide dihydrochloride; PKA, protein kinase A; PKI, protein kinase inhibitor; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; cAMP, cyclic AMP.

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serum (internal peptide 40–54) was from Calbiochem. Pertussis toxin, cholera toxin and *N*-(2-[methylamino]ethyl)-3-isoquinolinesulphonamide dihydrochloride (H8) were from Seikagaku (Japan). All other reagents were from Sigma.

Macrophage culture, membrane preparation

Alveolar macrophages were harvested from anaesthetized guinea-pigs by broncho-alveolar lavage. The cells were allowed to adhere as previously described [16]. The adherent cells were washed and incubated with agonists [cholera toxin, dibutyryl (Bt2) cAMP, okadaic acid, H8, phorbol 12-myristate 13-acetate (PMA)] in RPMI medium at 37 °C. They were then washed twice with ice-cold saline and scraped off into buffer A (40 mM Tris/HCl containing 0.25 M sucrose and 0.1 mM PMSF, pH 7.4). The cells were disrupted by sonication and centrifuged at 30 000 *g* for 1 h. The pellet was resuspended in the same buffer and stored at –80 °C at a protein concentration of 0.5–1 mg/ml.

[³²P]ADP-ribosylation of membrane proteins by cholera toxin

The procedure for activating the toxin and incubating membranes with cholera toxin and [³²P]NAD⁺ is described in [15]. Briefly, 100 µg of membrane proteins were incubated for 10 min at 32 °C in a mixture of 0.1 mM PMSF/0.5 mg/ml aprotinin/0.5 mg/ml leupeptin/10 mM thymidine/5 mM MgCl₂ in 100 mM sodium phosphate buffer supplemented with 16 µg/ml activated cholera toxin/10 µM fMLP and a mixture of ³²P-labelled and unlabelled NAD⁺ (3 µM, 10⁷ c.p.m.).

G-proteins were analysed by immunoblotting [15] using AS7 antibody directed against the C-terminal decapeptide of G_iα₁ or G_iα₂ or an antibody directed against the internal peptide (40–54) of the G_iα₂ subunit [17].

Phosphorylation of G-proteins in intact cells

The cells were washed twice with 5 ml of buffer B (168 mM NaCl/1.4 mM CaCl₂/4.7 mM KCl/0.8 mM MgSO₄/25 mM NaHCO₃/10 mM glucose/10 mM Hepes, pH 7.2) and incubated with 100 µCi of [³²P]P_i per ml of buffer B supplemented with 1 % (w/v) BSA for 3 h. Cholera toxin, Bt2 cAMP, okadaic acid and H8 were added to the incubation medium as required. The cells were then washed twice with 5 ml of ice-cold buffer B and solubilized in 1 ml of buffer C (5 mM Hepes/1 % Triton X-100/0.1 % SDS/10 mM EDTA/100 mM NaH₂PO₄/10 mM NaF/10 mM glycerophosphate/10 µg/ml leupeptin/2 mM PMSF/10 µg/ml aprotinin) for 1 h at 4 °C. The non-solubilized material was removed by centrifugation at 14 000 *g* for 10 min.

Immunoprecipitation

AS7 antiserum (10 µl) was added to 1 ml of labelled cell extract and left overnight at 4 °C. In some experiments, AS7 antiserum was preincubated with 100 µg of immunogenic peptide (KNNLKDCGLF) at room temperature for 2 h. Protein A–Sepharose (5 mg) was added for 2 h at 4 °C, then collected by centrifugation at 14 000 *g* for 2 min, washed six times by resuspension, resuspended in buffer D (1 % Triton X-100/0.1 % SDS/0.5 M NaCl/0.1 M NaF/50 mM NaH₂PO₄/50 mM Hepes) and centrifuged. The final pellet was boiled in Laemmli sample buffer for 3 min. The samples were then centrifuged and the supernatant was run on SDS/PAGE. The gels were stained by Coomassie Brilliant Blue, dried and exposed to Hyperfilm MP. The identity of the G_iα-protein was assessed by a parallel migration of a sample immunoprecipitated following the same technique, and further ADP-ribosylated by cholera toxin in the

presence of [³²P]NAD⁺ and fMLP according to the procedure described above. For an unknown reason, the G_iα had a higher apparent molecular mass (43 kDa versus 40 kDa) after immunoprecipitation.

GTP[γS] binding assay

Membrane proteins (0.5 µg) were incubated in 100 µl of a buffer containing 50 mM Tris/HCl (pH 7.4), 1 mM dithiothreitol, 1 mM EDTA, 5 mM MgCl₂, 150 mM NaCl, 0.5 µM GDP and [³⁵S]GTP (0.02–0.04 µCi/tube, corresponding to 0.25 nM) for 60 min at 32 °C. The reaction was stopped by rapid filtration through Whatman GF/C filters, followed immediately by two washes with 2.5 ml of 50 mM Tris buffer (pH 7.4) containing 5 mM MgCl₂. Radioactivity was determined by liquid scintillation counting of the filters. The specific binding was calculated by subtracting the amount of [³⁵S]GTP bound in the presence of 10 µM GTP, and expressed as fmol GTP[γS] bound/mg of protein.

Membranes from cholera toxin-treated and control cells (100 µg of protein) were incubated with 50 units of alkaline phosphatase in 1 ml of incubation buffer (0.1 M glycine/1 mM MgCl₂/0.1 mM ZnCl₂/0.1 mM PMSF, pH 8.5) for 1 h at 37 °C. The membranes were then centrifuged out, resuspended in buffer A, washed twice and finally resuspended in buffer A.

Cell-free phosphorylation

Membrane proteins (100 µg) were incubated for 30 min at 32 °C in 200 µl of 50 mM Tris buffer (pH 7.8) containing 60 mM KCl, 10 mM MgCl₂, 0.1 mM dithiothreitol, 0.5 mg/ml leupeptin, 0.1 mM ATP, 10 mM NaF and 10 µCi of [³²P]ATP. In some experiments, 40 units of PKA catalytic subunit were added to the incubation medium, with or without 25 µg of protein kinase inhibitor (PKI). The reaction was stopped by adding 0.5 ml of ice-cold 25 % trichloroacetic acid and 0.8 ml of 5 % trichloroacetic acid containing fatty acid-free serum albumin. The pellets were collected by centrifugation at 14 000 *g* for 5 min resuspended in 0.5 ml of 5 % trichloroacetic acid, washed three times and suspended in the immunoprecipitation buffer.

RESULTS

We had shown that pretreatment of guinea-pig alveolar macrophages with cholera toxin increases ADP-ribosylation of G_i-protein by cholera or pertussis toxin *in vitro* [15]. This effect of cholera toxin was mimicked by a cAMP analogue, suggesting that it was mediated by a cAMP-dependent mechanism (Figure 1, lane d versus lanes b and a). The increased labelling of G_i-protein was completely abolished in cells treated simultaneously with cholera toxin or a cAMP analogue and a specific PKA inhibitor, H8 (lane c versus lane b). The stimulation of PKC by PMA had no significant effect on ADP-ribosylation of G_i by cholera toxin (lane f versus lane a).

As the ADP-ribosylation of G-proteins by toxins is considered to be a stoichiometric process, the increased ADP-ribosylation shown in Figure 1 could reflect an increased amount of G_i-protein.

Immunoblot analysis of G_iα-protein using an antibody specific for G_iα₂ and G_iα₃ showed that neither cholera toxin nor Bt2 cAMP pretreatment altered the G_iα content (Figure 2). Cells incubated with pertussis toxin for 3 h at a concentration that ADP-ribosylates G_i-proteins [15] resulted in an apparent increase in G_iα-protein in the cell membranes. When revealed by an antiserum identifying an epitope different from the C-terminal

Figure 1 Stimulation of G_i -protein ADP-ribosylation by cholera toxin or Bt2 cAMP pretreatment: effect of a PKA inhibitor (H8)

Membranes (100 μ g of protein) from control or pretreated cells were ADP-ribosylated by cholera toxin (CTX) in the presence of [32 P]NAD $^{+}$, 5 mM MgCl $_2$ and 10 μ M fMLP and in the absence of GTP. The samples were resolved by SDS/PAGE followed by autoradiography of the dried gel. Lanes: a, control cells; b, cells pretreated with cholera toxin (25 ng/ml for 3 h); c, cells pretreated with cholera toxin (25 ng/ml) and H8 (50 μ M) for 3 h; d, cells pretreated with Bt2 cAMP (100 μ M) and okadaic acid (0.1 μ M) for 3 h; e, cells pretreated with Bt2 cAMP (100 μ M), okadaic acid (0.1 μ M) and H8 (50 μ M) for 3 h; f, cells pretreated with PMA (0.1 μ M) for 3 h. Quantification of [32 P]NAD $^{+}$ radiolabelling of the 40 kDa protein by scanning the autoradiograms was as follows: lane a, 3; lane b, 10; lane c, 1.3; lane d, 8; lane e, 1.5; lane f, 3.6

region, the $G_i\alpha$ -protein level appeared to be identical in control and pertussis toxin-pretreated cells (results not shown).

The phosphorylation of G_i -proteins in cholera toxin-treated cells was assessed by the labelling of a protein immunoprecipitated with AS7 antiserum from 32 P-labelled cells (Figure 3, lane b versus lane a). The labelling of G_i -proteins was prevented by pretreating cells with H8 (lane c versus lane b). This effect of cholera toxin was also mimicked by pretreating cells with a cAMP analogue, and further increased by adding the phosphatase inhibitor okadaic acid (lane f versus lane d). In contrast, stimulation of PKC by PMA induced no phosphorylation of $G_i\alpha$ when compared with control cells (lane g versus lane a). Control immunoprecipitation experiments were performed with AS7 antiserum preincubated with the immunogenic peptide. This preincubation abolished the immunoprecipitation of the band corresponding to $G_i\alpha$ (Figure 4, lane b versus lane a).

$G_i\alpha$ was also phosphorylated *in vitro* by the purified catalytic subunit of PKA in the presence of labelled ATP (Figure 5, lane

Figure 2 Immunoblots of $G_i\alpha$ -proteins: effects of cholera toxin or Bt2 cAMP pretreatment on $G_i\alpha$ in alveolar macrophages

Membrane proteins (100 μ g) from control and pretreated cells were resolved by SDS/PAGE, transferred onto a nitrocellulose membrane and revealed with an AS7 antiserum specific for $G_i\alpha_1$ - and $G_i\alpha_2$ -subunits. Lanes: a, control cells; b, cells pretreated with cholera toxin (CTX) (25 ng/ml) for 3 h; c, cells pretreated with cholera toxin (25 ng/ml) for 24 h; d, cells pretreated with Bt2 cAMP (100 μ M) and okadaic acid (0.1 μ M) for 3 h; e, cells pretreated with pertussis toxin (PTX) (25 ng/ml) for 3 h. Quantification of the labelling was as follows: lane a, 13.52; lane b, 16.64; lane c, 13.28; lane d, 13.89; lane e, 21. Lanes a, b and e were also revealed by an anti- $G_i\alpha$ antiserum specific for a central region of the protein with the following results: lane a, 5.80; lane b, 5.46; lane e, 6.17 (results not shown).

b versus lane a). The labelling was blocked by a PKI (Figure 5, lane c).

The functional coupling of fMLP receptors to G_i -proteins was measured by the specific binding of GTP[γ S] to isolated mem-

Figure 3 Pretreatment of alveolar macrophages with cholera toxin elicits the phosphorylation of $G_i\alpha_2$ by a PKA-mediated pathway

Alveolar macrophages were labelled with 32 P $_i$ for 3 h and with or without agonists. The cells were harvested and extracted with detergents. $G_i\alpha$ -proteins were immunoprecipitated with AS7 antiserum and analysed by SDS/PAGE and autoradiography. Lanes: a, no agonist; b, cholera toxin (CTX) (25 ng/ml); c, cholera toxin (25 ng/ml) and H8 (50 μ M); d, Bt2 cAMP (100 μ M); e, okadaic acid (0.1 μ M); f, Bt2 cAMP (100 μ M) and okadaic acid (0.1 μ M); g, PMA (1 μ M); h, unlabelled control cells. After immunoprecipitation, the extracts were ADP-ribosylated by cholera toxin in the presence of [32 P]NAD $^{+}$ and fMLP. Quantification of 32 P-labelling of $G_i\alpha$ by scanning the autoradiograms gave the following results: lane a, 0.25; lane b, 2.88; lane c, 0.58; lane d, 2.14; lane e, 0.22; lane f, 8.47; lane g, 0.11.

Figure 4 Immunoprecipitation of $G_i\alpha$ is prevented by preincubation of AS7 antiserum with the immunogenic peptide

Alveolar macrophages were harvested and extracted with detergents as described in Figure 3. $G_i\alpha$ -proteins were immunoprecipitated with AS7 antiserum (lane a) or with AS7 antiserum preincubated with 100 μ g of immunogenic peptide (KNNLKDCGLF) for 2 h (lane b). The samples were then analysed by SDS/PAGE, transferred onto a nitrocellulose membrane and revealed with AS7 antiserum.

Figure 5 Cell-free phosphorylation of $G_i\alpha_2$ by PKA

Membranes (100 μ g) were phosphorylated by purified PKA (40 units) for 30 min at 37 $^{\circ}$ C in the presence of [γ - 32 P]ATP. $G_i\alpha$ -proteins were immunoprecipitated with AS7 antiserum and analysed by SDS/PAGE and autoradiography. Lanes: a, control membranes (C); b, membranes incubated with PKA; c, membranes incubated with PKA and PKI (20 μ g). Quantification of 32 P-labelling was made by scanning the autoradiograms: lane a, 1.82; lane b, 0.15

Table 1 Functional inactivation of G_i -protein by cholera toxin (CTX) and Bt2 cAMP pretreatment of cells: effect of a PKA inhibitor (H8)

Membrane proteins (0.5 μ g) from control and pretreated cells were incubated with GTP[γ ³⁵S] at 32 °C for 60 min, with or without 10 μ M fMLP, and with or without 10 μ M GTP[γ S]. The samples were filtered and the supernatants counted by liquid scintillation. Results are the mean $\pm$ S.D. of three separate experiments performed in triplicate.

	Specific binding of GTP[γ S] induced by fMLP fmol/mg protein)
Control	2800 $\pm$ 160
CTX (3 h)	690 $\pm$ 240
CTX (24 h)	340 $\pm$ 300
CTX (3 h) + H ₈	2280 $\pm$ 530
Bt2 cAMP + okadaic acid	400 $\pm$ 310

branes after addition of fMLP. Pretreatment of cells with cholera toxin decreased the agonist-induced binding of GTP[γ S] to 25 % of the control value (Table 1). This inhibition was reversed by H8, and mimicked by a mixture of Bt2 cAMP and okadaic acid, showing that the phosphorylation of G_i by PKA altered its functional coupling to an occupied receptor. Lastly, incubating membranes from cholera toxin-pretreated cells with potato phosphatase reversed the inhibition of GTP[γ S] binding to 60 % of the control value (results not shown).

DISCUSSION

Cholera and pertussis toxins can enter mammalian cells and catalyse the ADP-ribosylation of their substrates in the cell membrane. The ADP-ribosylated G-proteins are not able to incorporate ADP-ribose when the isolated membranes from toxin-treated cells are further incubated with the same toxin in the presence of [³²P]NAD⁺. Cholera toxin treatment of alveolar macrophages reduces the labelling of $G_{i\alpha}$ in membranes incubated *in vitro* with cholera toxin and labelled NAD⁺ [15]. The addition of an agonist binding to a G_i -coupled receptor seems to induce the ADP-ribosylation of G_i by cholera toxin in intact HL60 cells [18]. Under our experimental conditions, i.e. in the absence of agonist, no ADP-ribosylation of G_i was detected when the cells were incubated with cholera toxin. The labelling of membranes incubated with cholera or pertussis toxin was not inhibited. There was also a considerable increase in the labelling of G_i -proteins in membranes from cholera toxin-pretreated cells (Figure 1, lane b versus lane a). This rules out any possibility of a direct ADP-ribosylation of G_i -proteins by cholera toxin in whole cells. In addition, the effect of cholera toxin was mimicked by a cAMP analogue (Figure 1, lane e) and inhibited by H8, which is a specific, potent inhibitor of PKA in this cell type [19] (Figure 1, lanes c and e). The modification of G_i -protein induced by cholera toxin pretreatment seems therefore to be mediated by the increased intracellular cAMP resulting from the sustained stimulation of adenylcyclase by activated G_s . Such persistent activation of adenylcyclase has been shown to increase the concentration of $G_{i\alpha}$ in mouse lymphoma cells via a PKA-mediated pathway [20]. This is not the case in alveolar macrophages, where stimulation of adenylcyclase by cholera toxin for 3 h or even 24 h produces no increase in $G_{i\alpha}$ (Figure 2). Lastly, the direct stimulation of PKA by a mixture of cAMP analogue and a phosphatase inhibitor also had no effect on the G_i level.

$G_{i\alpha}$ could also be modified by its phosphorylation by PKA, since more ³²P was incorporated into $G_{i\alpha}$ in cells pretreated with cholera toxin than in control cells (Figure 3). This effect was mimicked by Bt2 cAMP and okadaic acid and was blocked by H8. G_i -protein has been shown to be phosphorylated by a cAMP-mediated pathway in hepatocytes [8]. $G_{i\alpha}$ also seems to be phosphorylated in response to PKC stimulation by PMA in many cell systems [8,21–23], but this is not the case in alveolar macrophages. Pretreatment of cells with PMA for 3 h does not induce a significant increase in ADP-ribosylation or any phosphorylation of $G_{i\alpha}$. The stimulation of PKC by PMA is biphasic, with a transient stimulation after a short incubation, followed by down-regulation of PKC, which is maximal after 3 h in alveolar macrophages [19]. Shorter incubations with PMA were therefore tested (1 h and 30 min) and gave the same negative result.

In contrast to other results, obtained with membranes from human brain [24] and rat or chicken hepatocytes [8,25], the phosphorylation of $G_{i\alpha}$ by PKA was reproduced in a cell-free system by incubating macrophage membranes with purified PKA and labelled ATP (Figure 5) and was inhibited by a PKI. However, this result does not rule out the phosphorylation of $G_{i\alpha}$ by an unidentified protein kinase activated by PKA. This phosphorylation might lead to the $G_{i\alpha}$ reacting less well with the occupied receptor, as assessed by the decreased agonist-induced binding of GTP[γ S] to membranes from cholera toxin-treated cells. This decrease was blocked by H8 and phosphatase treatment and reproduced by cAMP analogue and okadaic acid.

The release of arachidonic acid by fMLP is a pertussis toxin-sensitive event mediated by a G_i -protein. The stimulation of arachidonic acid release from macrophages is negatively regulated by cAMP. The increase in cellular cAMP by a receptor-mediated pathway, by stimulation of G_s -protein, by addition of cAMP analogues or by phosphodiesterase inhibitors strongly inhibits the release of arachidonic acid induced by fMLP [7,15].

The results presented here indicate that the cross-regulation of arachidonic acid release by cAMP might occur via a phosphorylation of $G_{i\alpha_2}$ by PKA. This phosphorylation catalysed by a cAMP-dependent protein kinase results in G_i being more readily ADP-ribosylated by toxins. In contrast, stimulation of PKC, does not seem to regulate G_i -protein function in alveolar macrophages.

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