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Phosphorylation of the spliced variant forms of the recombinant stimulatory guanine-nucleotide-binding regulatory protein ($G_{s\alpha}$) by protein kinase C

Nigel J. PYNE,*‡ Michael FREISSMUTH† and Susan PALMER*

*Department of Physiology and Pharmacology, University of Strathclyde, Royal College, Glasgow G1 IXW, Scotland, U.K., and †Department of Pharmacology, University of Vienna, Vienna, Austria

Recombinant forms of $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}4}$ were shown to act as substrates for a purified preparation of brain protein kinase C. Both forms of $G_{s\alpha}$ were thermally denatured during the incubation such that phosphorylation was virtually complete (> 90%) after 30 min. The quantity of phosphate incorporated into approximately equivalent starting amounts of the two forms of $G_{s\alpha}$ (4.8 pmol of $G_{s\alpha\text{-}1}$ and 5.5 pmol of $G_{s\alpha\text{-}4}$) at maximal phosphorylation were 0.23 ± 0.08 pmol for $G_{s\alpha\text{-}1}$ and 0.56 ± 0.12 pmol for $G_{s\alpha\text{-}4}$. Since both forms of $G_{s\alpha}$ were thermally denatured to the same extent after 30 min, the increased phosphorylation state of $G_{s\alpha\text{-}4}$ provides evidence that $G_{s\alpha\text{-}4}$ contains an additional phosphorylation site. Bray and co-workers [Bray, Carter, Simmons, Guo, Puckett, Kamhollz, Spiegel & Nirenberg (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 8893–8897] proposed that an additional phosphorylation site may exist at the splice junction in $G_{s\alpha\text{-}4}$. The guanine-nucleotide-free form of $G_{s\alpha}$ appears to be the preferred substrate for phosphorylation. This interpretation is based upon the following observations. (i) Guanosine 5′-[β-thio]diphosphate at micromolar concentrations inhibits the susceptiblity of $G_{s\alpha}$ to phosphorylation; (ii) βγ-subunits, which inhibit GDP release from $G_{s\alpha}$–GDP at millimolar Mg^{2+} concentrations, also inhibit the susceptibility of $G_{s\alpha}$ to phosphorylation; and (iii) guanosine 5′[βγ-imido]triphosphate inhibits the susceptiblity of $G_{s\alpha}$ to act as a substrate for phosphorylation. These studies suggest that there is potential for cross-talk between receptors which trigger PtdIns(4,5)P_2 hydrolysis and subsequently protein kinase C activation, and receptors which stimulate adenylate cyclase via G_s.

INTRODUCTION

The hormonal regulation of adenylate cyclase is modulated by at least two guanine-nucleotide-binding regulatory proteins (G-proteins) (Gilman, 1987). These proteins function to transduce information from activated agonist–receptor complexes to effector systems. For stimulatory receptors linked to adenylate cyclase, the transducing protein is termed G_s, where for inhibitory receptors it is G_i. Both G-proteins are heterotrimers consisting of three non-identical subunits, α, β, and γ (Gilman, 1987; Birnbaumer, 1990). The α-subunits are structurally divergent peptides, whereas the βγ-subunits are shared by both G_i and G_s (Gilman, 1987).

The interaction of G_s with an appropriate agonist–receptor complex triggers the rapid exchange of GDP for GTP in the guanine-nucleotide-binding domain of the α-subunit, and a hypothetical dissociation of the activated α-subunit from the βγ-subunits. The GTP-bound α-subunit interacts directly with adenylate cyclase to stimulate its activity. The hydrolysis of bound GTP by an intrinsic GTPase switches the signalling mechanism off and allows reassociation of the α-subunit with the βγ-subunit complex.

Modulation of both G_s and G_i function by the phosphorylation of their respective α-subunits has been implicated in a number of cellular signalling processes. For instance, G_i has been shown to be a substrate for phosphorylation by protein kinase C (Katada *et al.*, 1985; Pyne *et al.*,1989; Bushfield *et al.*, 1990*a*,*b*, Issakani *et al.*, 1990), an event which leads to its inactivation and the subsequent uncoupling of receptor-mediated inhibition of adenylate cyclase (Katada *et al.*, 1985). No direct evidence for the phosphorylation of G_s by protein kinases has been found, although protein kinase C-catalysed attenuation of G_s function, i.e. attenuated guanosine 5′-[βγ-imido]triphosphate (Gpp[NH]p)-stimulated adenylate cyclase, has been shown in cells pretreated with phorbol esters (Murphy *et al.*, 1989).

$G_{s\alpha}$ exists as at least four forms, and these are expressed as single polypeptide chains and are derived from the differential splicing of pre-mRNA transcribed from a single gene. Bray *et al.* (1986) have isolated the four different $G_{s\alpha}$ cDNAs ($G_{s\alpha\text{-}1}$ to $G_{s\alpha\text{-}4}$) from human brain and characterized their partial structures. $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}3}$ are identical, except that $G_{s\alpha\text{-}3}$ lacks a single stretch of 45 nucleotides corresponding to a sequence of 15 amino acids. $G_{s\alpha\text{-}2}$ and $G_{s\alpha\text{-}4}$ have three additional nucleotides (CAG) at the 5′ end of exon 4. $G_{s\alpha\text{-}4}$ also lacks the stretch of 15 amino acids. All forms display potential sites for phosphorylation by protein kinase C, although both $G_{s\alpha\text{-}2}$ and $G_{s\alpha\text{-}4}$ have an additional serine residue which forms a consensus sequence for an additional protein kinase C phosphorylation site. The alternative use of these splice sites may confer on $G_{s\alpha}$ differential regulatory properties. Expression of $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}4}$ in both COS-m6 cells and *Escherichia coli* yielded peptides with apparent molecular masses of 52 and 45 kDa respectively (Robishaw *et al.*, 1986; Graziano *et al.*, 1989).

In this paper, we have examined the possibility that both $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}4}$ are substrates for phosphorylation by protein kinase C. We have also assessed the effect by βγ-subunits and guanine nucleotides upon the ability of $G_{s\alpha}$ to be phosphorylated by protein kinase C. This approach has yielded information regarding the mechanism of phosphorylation and the potential for

Abbreviations used: GDP[S], guanosine 5′-[β-thio]diphosphate; Gpp[NH]p, guanosine 5′-[βγ-imido]triphosphate; GTP[S], guanosine 5′-[γ-thio]triphosphate; PMA, phorbol 12-myristate 13-acetate.

‡ To whom correspondence should be addressed.

cross-talk between the cyclic AMP signal cascade and protein kinase C.

EXPERIMENTAL

The CS1 antiserum ($G_{s\alpha}$-specific antibody) was a gift from Dr. G. Milligan (Department of Biochemistry, University of Glasgow). Protein kinase C was purchased from Lipidex Inc. (New York, NY, U.S.A.) and purified by them to homogeneity from rat brain according to Kitano *et al.* (1986). This preparation contains a mixture of isoenzymes. [γ-^{32}P]ATP was from Amersham, and guanosine 5'-[δ-[^{35}S]thio]triphosphate ([^{35}S]GTP[S]) was from Dupont. Chemicals were from Sigma and guanine nucleotides were from Boehringer Mannheim. Horseradish peroxidase-linked anti-(rabbit IgG) was from the Scottish Antibody Production Unit (Carluke, Scotland, U.K.).

Identification checks of $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}4}$ using immunoblotting with peptide-directed anti-$G_{s\alpha}$ antibodies

The recombinant spliced variants of $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}4}$ were subjected to SDS/PAGE in Laemmli (1970) buffer on 10%-acrylamide gels, and then subsequently transferred to nitrocellulose sheets. These were then blocked in 3% (w/v) gelatin in 20mM-Tris/HCl (pH 7.4)/0.5 M-NaCl and incubated at 37 °C for 1 h. The sheets were washed in distilled water and then incubated with CS1 antiserum (a peptide-directed antibody raised in rabbits to the *C*-terminal decapeptide RMHLRQYELL, of $G_{s\alpha}$) in 1% (w/v) gelatin/20 mM-Tris/HCl (pH 7.4)/0.5 M-NaCl and incubated at 30 °C for 12 h. The sheets were washed sequentially in 20 mM-Tris/HCl (pH 7.4)/0.5 M-NaCl/0.05% (v/v) Tween-20 and then 20 mM-Tris/HCl (pH 7.4)/0.5 M-NaCl, after which they were incubated in horseradish-peroxidase-linked anti-(rabbit IgG) (1:200 dilution) in 1% (w/v) gelatin/20 mM-Tris/HCl (pH 7.4)/0.5 M-NaCl for 1.5 h at 23 °C. The immunologically reactive peptide bands were then detected after sequential washing of the sheets as before, using 10 mM-Tris/HCl, pH 7.4, *O*-dianisidine (10 mg/ml) and hydrogen peroxide (0.75%, v/v).

Phosphorylation incubation procedure

Recombinant $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}4}$ (0.25 or 2.5 μg) were combined with an activation cocktail containing (final concentrations) 25 mM-Hepes, pH 7, 5 mM-$MgCl_2$, 0.75 mM-$CaCl_2$, 25 μM-ATP and [γ-^{32}P]ATP (5 μCi/assay). To this activation cocktail was added phosphatidylserine (50 μg/ml) which had been previously sonicated in 10 μM-Tris/HCl, pH 7.4. To initiate the reaction, purified protein kinase C from brain was added (specific activity 2.5 units/mg of protein kinase C, where 1 unit is 1 nmol of [^{32}P]phosphate/min incorporated into histone IIIS). In the incubation where the effect of $\beta\gamma$-subunits was assessed, a $\beta\gamma$-subunit storage buffer was included in the controls. In order to establish that the phosphorylation of $G_{s\alpha}$ was not due to the phosphorylation of a relatively small proportion of denatured $G_{s\alpha}$, boiled $G_{s\alpha}$ (0.25 μg) was included separately with the activation cocktail. All incubations were performed at 30 °C for 1 h. Time course experiments for the phosphorylation were also performed. To ensure that protein kinase C was not thermally unstable during the incubation, an additional 0.25 munit of protein kinase C was added after the first 60 min and an additional 30 min incubation was performed as before at 30 °C. Boiled protein kinase C was added as a control in these experiments.

Termination of all the incubations was by the addition of an equivalent volume of Laemmli buffer (100 μl) followed by boiling at 100 °C for 5 min. After cooling, the samples were subjected to SDS/PAGE on 10% acrylamide gels, after which the gels were fixed in 10% trichloroacetic acid for 1 h and dried prior to autoradiography in a Kodak X-omatic intensifying screen cassette. Routinely, autoradiography was performed for 10 h at −20 °C. In order to check whether GDP[S] and Gpp[NH]p could compete with ATP for protein kinase C, an identical procedure was adopted using histone IIIS (5 μg/assay) instead of $G_{s\alpha}$. The phosphorylation of histone IIIS allowed determination of the rate of [^{32}P]phosphate incorporation into this substrate under our assay conditions.

Quantification of the phosphate incorporated into $G_{s\alpha}$, protein kinase C and histone IIIS was achieved by Čerenkov counting of bands excised from the dried gels.

[^{35}S]GTP[S] binding

[^{35}S]GTP[S]binding was performed according to Graziano *et al.* (1989). In order to establish the amount of $G_{s\alpha}$ available for guanine nucleotide binding at the beginning and after 30 min of the phosphorylation incubation, $G_{s\alpha}$ was combined with the phosphorylation mixture in the absence of protein kinase C. Samples were withdrawn for zero time availability and the remainder was incubated at 30 °C for 30 min prior to sampling. This latter time point was chosen because phosphorylation is virtually maximal. In experiments where the effect of GDP[S] (10 μM) was investigated, the GDP[S] was diluted in the binding assay to 0.4 μM. In all cases, equilibrium binding was performed by incubating samples at 20 °C for 30 min.

$G_{s\alpha}$ preparation

The recombinant spliced variants $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}4}$ were expressed and purified from *E. coli* according to Graziano *et al.* (1989). Both forms of $G_{s\alpha}$ were stored in 50 mM-Hepes, pH 7.6, 1 mM-EDTA and 1 mM-dithiothreitol at −20 °C. $\beta\gamma$-Subunits were purified according to Graziano *et al.* (1989) and stored in 50 mM-Tris/HCl, pH 8, 100 mM-NaCl and 1% (w/v) cholate at −20 °C. In experiments where the effect of $\beta\gamma$-subunits was investigated, a 1:40 dilution of the $\beta\gamma$-subunits was achieved in the incubation.

Protein kinase C preparation

Protein kinase C was stored in 10 mM-Tris/HCl, pH 7.5, 0.5 mM-EGTA, 0.5 mM-EDTA, 10 mM-β-mercaptoethanol, 10% (v/v) glycerol, 0.05% (v/v) Triton X-100 and 400 mM-NaCl at −20 °C. This was diluted 1:10 in Hepes (pH 7) prior to use, and diluted 1:5 in the incubation.

RESULTS

Immunoblot analysis of the α-subunit of G_s

Purified preparations of $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}4}$ were resolved on an SDS/polyacrylamide gel and transferred to nitrocellulose for immunoblotting with CS1 antiserum. As shown in Fig. 1, an antibody produced to the *C*-terminal decapeptide of $G_{s\alpha}$ recognized and identified both $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}4}$. No other immunogenic peptides could be detected in the purified preparations.

Phosphorylation of $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}4}$ by protein kinase C

$G_{s\alpha\text{-}4}$ was phosphorylated by protein kinase C (Fig. 2). SDS/PAGE of the phosphorylated peptides revealed two ^{32}P-labelled radioactive bands, one of which has a molecular mass of 84 kDa and is the subunit of protein kinase C, whereas the other is $G_{s\alpha\text{-}4}$. Inclusion of phorbol 12-myristate 13-acetate (PMA; 80 nM) in a 1 h incubation increased the phosphorylation of $G_{s\alpha\text{-}4}$ by 60%, but only elicited a marginal increase (less than 10%) in the phosphorylation of $G_{s\alpha\text{-}1}$ (Table 1). No phosphorylation of $G_{s\alpha\text{-}4}$ was observed in the absence of protein kinase C (Fig. 2), and no phosphorylation of the peptide was seen in the

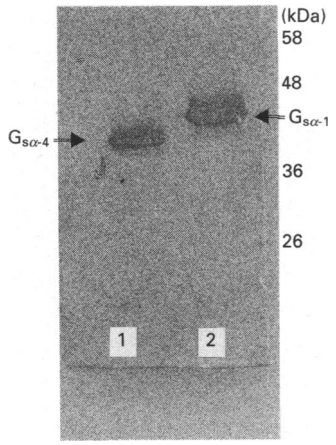

Fig. 1. Immunological analysis of $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}4}$

Western blotting of recombinant $G_{s\alpha}$ with CS1 antiserum: lane 1, an immunoreactive peptide corresponding to $G_{s\alpha\text{-}4}$; lane 2, an immunoreactive peptide corresponding to $G_{s\alpha\text{-}1}$. Molecular mass markers are shown. This experiment is typical of one performed three times.

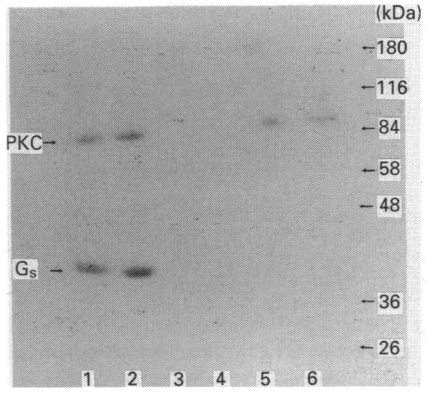

Fig. 2. Phosphorylation of $G_{s\alpha\text{-}4}$ by protein kinase C

An autoradiogram of SDS/PAGE showing phosphorylation of $G_{s\alpha\text{-}4}$ by protein kinase C. Additions to the activation cocktail were: lane 1, $G_{s\alpha\text{-}4}$ (2.5 μg) and protein kinase C (0.75 munit); lane 2, $G_{s\alpha\text{-}4}$ (2.5 μg), protein kinase C (0.75 munit) and PMA (80 nM); lane 3, $G_{s\alpha\text{-}4}$ (2.5 μg) and no protein kinase C; lane 4, $G_{s\alpha\text{-}4}$ (2.5 μg), PMA (80 nM) and no protein kinase C; lane 5, protein kinase C (0.75 munit) and no $G_{s\alpha\text{-}4}$; lane 6, protein kinase C (0.75 munit), PMA (80 nM) and no $G_{s\alpha\text{-}4}$. The locations of phosphorylated peptides corresponding to $G_{s\alpha\text{-}4}$ and protein kinase C are shown. Molecular mass markers are shown. This is a typical result from an experiment performed three times.

incubations of protein kinase C alone (Fig. 2). Similar results were observed for $G_{s\alpha\text{-}1}$. The protein kinase C inhibitor staurosporine (1 μM) completely inhibited the phosphorylation by protein kinase C of both forms of the $G_{s\alpha}$ subunits (Table 1).

Time course of phosphorylation of $G_{s\alpha}$

The phosphorylation of $G_{s\alpha\text{-}4}$ was time-dependent and was 90% complete within 30 min (Fig. 3). Indeed, a further addition of protein kinase C at 1 h did not result in further phosphorylation of $G_{s\alpha\text{-}4}$ (Fig. 4), suggesting that the phosphorylation had reached completion. Similar results were obtained from $G_{s\alpha\text{-}1}$ (results not shown). During the time course of phosphorylation the $G_{s\alpha}$ subunits appear to be extremely thermally labile, as revealed by a substantial loss in the ability of both $G_{s\alpha}$ subunits to bind [^{35}S]GTP[S] after a 30 min incubation in a protein kinase C-free

Table 1. Effect of PMA, Gpp[NH]p, GDP[S] and $\beta\gamma$-subunits on the apparent phosphorylation of $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}4}$ by protein kinase C

Results are from at least three experiments (means ± S.D.). No change in ^{32}P labelling of protein kinase C (84 kDa) was detected. The effect was determined at 1 h time points, using 0.25 munit of protein kinase C and 0.25 μg of $G_{s\alpha}$. −denotes inhibition; + denotes increase in phosphorylation.

Treatment	Change in apparent extent of phosphorylation (%)
$G_{s\alpha\text{-}4}$ + staurosporine (1 μM)	−95 ± 2
$G_{s\alpha\text{-}1}$ + staurosporine (1 μM)	−98 ± 4
$G_{s\alpha\text{-}1}$ + PMA (80 nM)	+8 ± 20
$G_{s\alpha\text{-}4}$ + PMA (80 nM)	+60 ± 12
$G_{s\alpha\text{-}1}$ + Gpp[NH]p (100 μM)	−69 ± 6
$G_{s\alpha\text{-}4}$ + Gpp[NH]p (100 μM)	−61 ± 20
$G_{s\alpha\text{-}1}$ + $\beta\gamma$ (5 μg)	−50 ± 10
$G_{s\alpha\text{-}4}$ + $\beta\gamma$ (5 μg)	−57 ± 6
$G_{s\alpha\text{-}4}$ + GDP[S] (10 μM)	−54 ± 1
$G_{s\alpha\text{-}4}$ + GDP[S] (100 μM)	−96 ± 1
$G_{s\alpha\text{-}4}$ + GDP[S] (10 μM) + $\beta\gamma$ (5 μg)	−86 ± 1
$G_{s\alpha\text{-}1}$ + GDP[S] (10 μM)	−52 ± 4

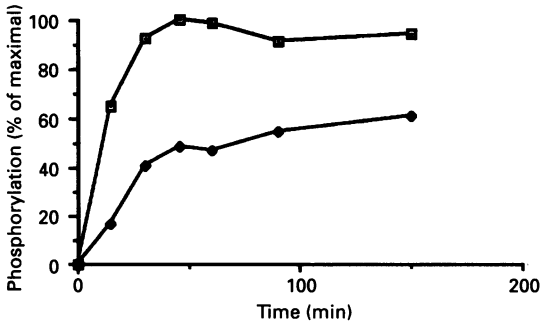

Fig. 3. Time course of phosphorylation of $G_{s\alpha\text{-}4}$ by protein kinase C

$G_{s\alpha\text{-}4}$ was incubated in the presence of protein kinase C and [γ-^{32}P]ATP as described in the Experimental section for various lengths of time, using $G_{s\alpha\text{-}4}$ (0.25 μg) and protein kinase C (0.25 munit). Time courses were performed in the presence (●) and absence (□) of GDP[S](10 μM). Results are expressed as percentages of maximal incorporation of [^{32}P]phosphate into $G_{s\alpha\text{-}4}$ in the absence of GDP[S]. Data are from a representative experiment, performed three times.

phosphorylation reaction (Table 2). For both forms of $G_{s\alpha}$ the amounts of functionally active $G_{s\alpha}$ remaining after a 30 min incubation were identical. The thermal lability of $G_{s\alpha}$ probably leads to partial denaturation, and the maximal phosphorylation observed occurs because denatured $G_{s\alpha}$ is not a substrate for phosphorylation by protein kinase C (Fig. 4). We suggest that the lability of $G_{s\alpha}$ is due to thermal denaturation rather than proteolysis catalysed by low amounts of a potential proteinase contaminant, since the amount of $G_{s\alpha}$ remaining after a phosphorylation incubation was the same as the amount of starting material when measured by immunoblot analysis with CS1 antisera (results not shown). We can confirm that the apparent maximal phosphorylation was due to depletion of available $G_{s\alpha}$, since the addition of further $G_{s\alpha}$ after 1 h resulted in further incorporation of [^{32}P]phosphate into the newly added $G_{s\alpha}$ (results not shown). This result indicates that, although the specific activity of protein kinase C used in these incubations is low, it is not limiting.

The quantity of phosphate incorporated into equivalent starting amounts (4.8 pmol of $G_{s\alpha\text{-}1}$ and 5.5 pmol of $G_{s\alpha\text{-}4}$) of the two

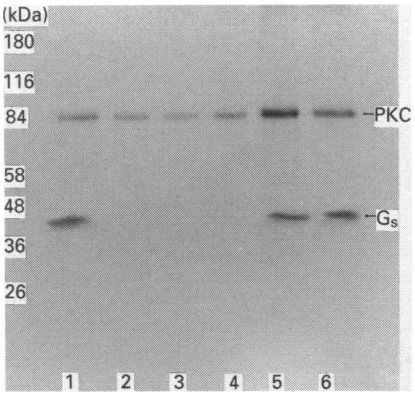

Fig. 4. Phosphorylation of $G_{s\alpha\text{-}4}$: comparison with denatured $G_{s\alpha\text{-}4}$

An autoradiogram of SDS/PAGE showing phosphorylation of $G_{s\alpha\text{-}4}$ by protein kinase C. Additions to the activation cocktail were: lane 1, $G_{s\alpha\text{-}4}$ (0.25 μg) and protein kinase C (0.25 munit); lane 2, $G_{s\alpha\text{-}4}$ (0.25 μg), protein kinase C (0.25 munit) and Gpp[NH]p (100 μM); lane 3, denatured $G_{s\alpha\text{-}4}$ (0.25 μg) and protein kinase C (0.25 munit); lane 4, denatured $G_{s\alpha\text{-}4}$ (0.25 μg), protein kinase C (0.25 munit) and Gpp[NH]p (100 μM); lane 5, $G_{s\alpha\text{-}4}$ (0.25 μg) and protein kinase C (0.25 munits), and at 60 min an additional 0.25 munit of protein kinase C as described in the Experimental section; lane 6, $G_{s\alpha\text{-}4}$ (0.25 μg) and protein kinase C (0.25 munit), and at 60 min an additional 0.25 munit of boiled protein kinase C. The arrows denote the locations of phosphorylated peptides corresponding to $G_{s\alpha\text{-}4}$ and protein kinase C. Molecular mass markers are shown. This is a typical result from an experiment performed three times.

Table 2. [^{35}S]GTP[S] binding to $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}4}$: effect of incubation in phosphorylation mixture

Experiments were performed as described in the Experimental section. Data are represented as percentages of $G_{s\alpha}$ remaining after incubation. This was determined from equilibrium binding (means ± S.D., $n = 3$); 5.5 pmol of $G_{s\alpha\text{-}4}$ or $G_{s\alpha\text{-}1}$ and 1 μM-[^{35}S]GTP[S] (0.5 μCi/assay) were added to each incubation.

Sample	$G_{s\alpha}$ remaining (%)
$G_{s\alpha-4}$, no incubation	100
$G_{s\alpha-4}$, 30 min, 30 °C	5.6±07
$G_{s\alpha-4}$, 30 min, 30 °C, +GDP[S] (10 μM)	68±3
$G_{s\alpha-1}$, no incubation	100
$G_{s\alpha-1}$, 30 min, 30 °C	6±1
$G_{s\alpha-1}$, 30 min, 30 °C, +GDP[S] (10 μM)	50±4

forms of $G_{s\alpha}$ at maximal phosphorylation was 0.23±0.08 pmol for $G_{s\alpha\text{-}1}$ and 0.56±0.12 pmol for $G_{s\alpha\text{-}4}$.

Rates of phosphorylation of $G_{s\alpha\text{-}4}$ and histone IIIS

The rate of phosphorylation of $G_{s\alpha\text{-}4}$ was 0.2 nmol/min per mg of protein kinase C, and this compared with a rate of phosphorylation of histone IIIS of 2.5 nmol/min per mg of protein kinase C (Table 3) under the assay conditions described in the Experimental section.

Effect of guanine nucleotides on $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}4}$ phosphorylation states

The recombinant spliced variants $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}4}$ were purified in the inactive GDP-bound state. GDP[S] (10 μM) was an inhibitor of $G_{s\alpha\text{-}4}$ and $G_{s\alpha\text{-}1}$ phosphorylation (54% and 52% respectively) by protein kinase C (Fig. 5, Table 1). GDP[S] inhibited phosphorylation at micromolar concentrations; no effect was observed with nanomolar GDP[S] concentrations (results not shown). The apparent inhibition of the protein kinase C-catalysed phosphorylation of $G_{s\alpha\text{-}4}$ by GDP[S] was due to a decrease in the initial rate at which $G_{s\alpha\text{-}4}$ was phosphorylated (Fig. 3). The maximal amount of $G_{s\alpha\text{-}4}$ that was phosphorylated was decreased, and maximal phosphorylation was achieved between 30 and 60 min (Fig. 3). This was not due to increased thermal lability of $G_{s\alpha}$ in the presence of GDP[S], since this guanine nucleotide actually stabilizes $G_{s\alpha}$ as indicated by enhanced [^{35}S]GTP[S] binding to $G_{s\alpha}$ (Table 2). Increasing the GDP[S] concentration to 100 μM completely inhibited phosphorylation of $G_{s\alpha\text{-}4}$ (Table 1).

Inclusion of a 20-fold excess of $\beta\gamma$-subunits produced 50% and 57% decreases in the ability of $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}4}$ respectively to act as a substrate for protein kinase C (Fig. 5, Table 1). The addition of both $\beta\gamma$-subunits and GDP[S] (10 μM) almost completely abolished phosphorylation of $G_{s\alpha\text{-}4}$. The $\beta\gamma$-subunits were not themselves substrates for phosphorylation by protein kinase C (Fig. 5).

Addition of Gpp[NH]p (100 μM), a non-hydrolysable analogue of GTP, to the activation cocktail reduced the protein kinase C-catalysed phosphorylation of both $G_{s\alpha}$ subunits (Fig. 6, Table 1).

Table 3. Phosphorylation of histone IIIS by protein kinase C

Incubations were for 20 min at 30 °C using protein kinase C (0.25 munit) and were performed under conditions of linear rates, where less than 2.5% of the substrate was used. Results are means ± S.D. from three separate experiments.

	^{32}P incorporation (d.p.m.)
Histone (5 μg)+PKC	23193±1803
Histone (5 μg)+PKC+GDP[S] (10 μM)	22040±100
Histone (5 μg)+PKC+Gpp[NH]p (100 μM)	26200±300

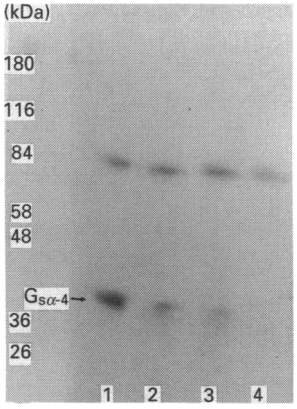

Fig. 5. Effect of $\beta\gamma$-subunits and GDP[S] on $G_{s\alpha\text{-}4}$ phosphorylation

Autoradiogram of an SDS/PAGE showing the effect of $\beta\gamma$-subunits and GDP[S] (10 μM) upon protein kinase C-mediated phosphorylation of $G_{s\alpha\text{-}4}$. Additions to the activation cocktail were: lane 1, $G_{s\alpha\text{-}4}$ (0.25 μg) and protein kinase C (0.25 munit); lane 2, $G_{s\alpha\text{-}4}$ (0.25 μg), protein kinase C (0.25 munit) and $\beta\gamma$-subunits (5 μg); lane 3, $G_{s\alpha\text{-}4}$ (0.25 μg), protein kinase C (0.25 munit) and GDP[S]; lane 4, $G_{s\alpha\text{-}4}$ (0.25 μg), protein kinase C (0.25 munit), $\beta\gamma$-subunits (5 μg) and GDP[S]. The arrow denotes the location of phosphorylated peptides corresponding to $G_{s\alpha\text{-}4}$. Molecular mass markers are shown. This is a typical result from an experiment performed three times.

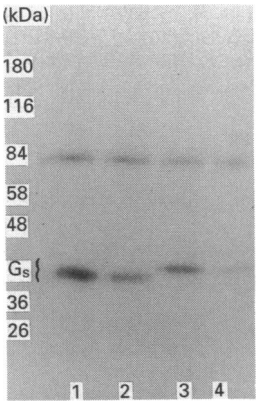

Fig. 6. Effect of Gpp[NH]p on $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}4}$ phosphorylation states

Autoradiogram of SDS/PAGE showing the effect of Gpp[NH]p (100 μM) on phosphorylation of $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}4}$ by protein kinase C. Additions to the activation cocktail were: lane 1, $G_{s\alpha\text{-}4}$ (0.25 μg) and protein kinase C (0.25 munit); lane 2, $G_{s\alpha\text{-}4}$ (0.25 μg), protein kinase C (0.25 munit) and Gpp[NH]p; lane 3, $G_{s\alpha\text{-}1}$ (0.25 μg) and protein kinase C (0.25 munit); lane 4, $G_{s\alpha\text{-}1}$ (0.25 μg), protein kinase C (0.25 munit) and Gpp[NH]p. The brackets denote the positions of $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}4}$. Molecular mass markers are shown. This is a typical result from an experiment performed three times.

For $G_{s\alpha\text{-}1}$, Gpp[NH]p induced an apparent 69 % reduction in the susceptibility of the α-subunit to act as a substrate for protein kinase C-catalysed phosphorylation, and for $G_{s\alpha\text{-}4}$ the apparent reduction was 61 %.

The effect of Gpp[NH]p and GDP[S] upon phosphorylation of $G_{s\alpha}$ by protein kinase C is not due to competition of these guanine nucleotides with ATP for the catalytic site of protein kinase C, since they did not inhibit protein kinase C-catalysed phosphorylation of histone IIIS (Table 3) or the autophosphorylation of protein kinase C (Figs. 5 and 6).

DISCUSSION

The difference between $G_{s\alpha\text{-}4}$ and $G_{s\alpha\text{-}1}$ is respectively the lack (short) and inclusion (long) of a stretch of 15 amino acids. In addition, $G_{s\alpha\text{-}4}$ and $G_{s\alpha\text{-}1}$ respectively contain and lack a serine residue at the splice junction. The biological significance of the diversity is unknown. All forms of $G_{s\alpha}$ appear to be capable of activating adenylate cyclase and Ca^{2+} channels to equal extents (Mattera *et al.*, 1989; Graziano *et al.*, 1989). In addition, both interact with β_1- and β_2-adrenoceptors with similar affinities (Freissmuth *et al.*, 1991). The only difference that has been noted so far is an approx. 2.5-fold faster rate of GDP release from $G_{s\alpha\text{-}1}$ compared with $G_{s\alpha\text{-}4}$. The purified α_s-subunits bind GTP[S] stoichiometrically, contain intrinsic GTPase, display $\beta\gamma$-subunit inhibition of GTPase activity and reconstitute agonist-stimulated adenylate cyclase in cyc$^-$ membranes. In this respect, $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}4}$ are very similar to native $G_{s\alpha}$.

We have observed that the addition of protein kinase C to both $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}4}$ catalyses the phosphorylation of these polypeptides. Phosphorylation is virtually complete within a 30 min incubation. However, the $G_{s\alpha}$ subunits are extremely thermally labile during the time course, and maximal phosphorylation occurs because only a small proportion of $G_{s\alpha}$ is available as a substrate, and the ratio of free GDP to guanine-nucleotide-free $G_{s\alpha}$ is likely to increase, thus decreasing the lifetime of the guanine-nucleotide-free $G_{s\alpha}$. The amount of phosphate incorporated into $G_{s\alpha\text{-}4}$ was approx. 2-fold greater than for $G_{s\alpha\text{-}1}$. Since both spliced variant forms of $G_{s\alpha}$ were thermally denatured to yield equivalent amounts of GTP[S] binding under the incubation conditions, this result indicates that $G_{s\alpha\text{-}4}$ may contain an additional, unique, phosphorylation site. In this respect, Bray *et al.* (1986) have proposed that $G_{s\alpha\text{-}4}$ contains an additional phosphorylation site at the splice junction. Our results are, therefore, in accord with this proposal, although a definitive answer to our suggestion will only be achieved from peptide mapping and sequencing of the phosphorylation sites. The differential effect of PMA upon the phosphorylation of the $G_{s\alpha}$ subunits is difficult to interpret. Clearly, since both forms of $G_{s\alpha}$ are equally thermally labile, the increased phosphorylation state of $G_{s\alpha\text{-}4}$ is not likely to be due to an enhanced rate of phosphorylation of a site common to $G_{s\alpha\text{-}1}$. One possible explanation is that activated protein kinase C phosphorylates the potential unique site in $G_{s\alpha\text{-}4}$ at a faster rate, and presumably this potential site is more accessible.

The lability of $G_{s\alpha}$ and its effect upon the kinetics of phosphorylation are similar to the phosphorylation *in vitro* of $G_{i\alpha}$ by protein kinase C (Katada *et al.*, 1985), where $G_{i\alpha}$ is also extremely labile.

We propose that the guanine-nucleotide-free form of $G_{s\alpha}$ is the substrate for protein kinase C-catalysed phosphorylation. This interpretation is based upon the following observations. Micromolar concentrations of GDP[S] inhibit the phosphorylation of $G_{s\alpha}$ by protein kinase C. The purified forms of $G_{s\alpha}$ exist in the preparation in a GDP-bound conformation, due to an intrinsic GTPase activity (Cerione *et al.*, 1985; Brandt & Ross, 1985). In solution, the GDP present in the guanine-nucleotide-binding domain of the α-subunit will dissociate (Gilman, 1987; Graziano *et al.*, 1989). The addition of high concentrations of GDP[S] will increase the rate of formation of the guanine-nucleotide-bound $G_{s\alpha}$ (Braun *et al.*, 1981; Gilman 1987; Cerione *et al.*, 1985; Brandt & Ross, 1985) and, at micromolar concentrations, the association rate of the guanine-nucleotide-binding equilibrium will increase by three to four orders of magnitude. Under these conditions, the lifetime of guanine-nucleotide-free $G_{s\alpha}$ will be severely reduced, and less will be available for phosphorylation by protein kinase C at any moment in time. Thus the apparent rate of phosphorylation is reduced and will progressively diminish to a negligible rate dependent upon the kinetics of GDP[S] binding. We propose that this phenomenon accounts for the reduced extent of phosphorylation of $G_{s\alpha}$ that is measured after 1 h. Thus both the rate and extent of phosphorylation are being decreased in the presence of GDP[S].

It is also important to note that the actual inhibition of phosphorylation in the presence of GDP[S] is likely to be appreciably greater than apparently measured, since GDP[S] stabilizes $G_{s\alpha}$.

Gpp[NH]p was also capable of reducing the susceptibility of $G_{s\alpha}$ to phosphorylation. Gpp[NH]p stabilizes $G_{s\alpha}$ by occupying it in a quasi-irreversible reaction in the presence of millimolar Mg^{2+} concentrations (Gilman, 1987). Thus the lifetime of the guanine-nucleotide-free $G_{s\alpha}$ is reduced, since Gpp[NH]p (100 μM) will accelerate the rate of removal of guanine-nucleotide-free $G_{s\alpha}$ and inhibit its formation. $\beta\gamma$-Subunits inhibit the susceptibility of both $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}4}$ to phosphorylation by protein kinase C. $\beta\gamma$-Subunits inhibit the rate of release of GDP from the guanine-nucleotide-binding domain at millimolar [Mg^{2+}] by reducing the rate constant of dissociation of GDP and therefore stabilizing $G_{s\alpha}$ by allowing occupancy with guanine nucleotide (Brandt & Ross, 1985; Gilman, 1987; Graziano *et al.*, 1989). $\beta\gamma$-Subunits, by virtue of their ability to inhibit GDP release (Freissmuth & Gilman, 1989), will also lower the availability of guanine-nucleotide-free $G_{s\alpha}$ for phosphorlation by protein kinase C.

There is at least one potential phosphorylation site present in the guanine-nucleotide-binding domain of $G_{s\alpha\text{-}1}$. For $G_{s\alpha-1}$ and

$G_{s\alpha\text{-}4}$ this site is at Ser-286 and Ser-272 respectively. The sequence arrangement around this phosphorylation site in $G_{s\alpha\text{-}4}$ is Arg-Trp-Leu-Arg-Thr-Ile-*Ser*-Val; this lies between the Asn-Lys-Gln-Asp sequence necessary for guanine nucleotide binding and Arg-268, which appears to be important for effector recognition (Itoh & Gilman, 1991), since site-directed mutagenesis of this residue can partially ablate the interaction of G_s with adenylate cyclase. The polypeptide that contains this phosphorylation site is in a cytoplasmic loop, which would be accessible to phophorylation. Furthermore, this serine residue is conserved in $G_{i\alpha}$, which is also a substrate for protein kinase C (Katada *et al.*, 1985). For $G_{s\alpha\text{-}4}$, it was proposed by Bray *et al.* (1986) that there is a phosphorylation site for protein kinase C in the splice junction at Ser-72; the sequence arrangement is His-Val-Asn-Gly-Phe-Asn-Gly-Asp-*Ser*-Glu. This latter site is a potential candidate for phosphorylation, since this region is the only structural difference between the two forms. If both sites are phosphorylated, then our results suggest that each is under the control of guanine-nucleotide occupancy.

In summary, $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}4}$ are substrates for phosphorylation by protein kinase C. The differences in the extent of phosphorylation may reflect genuine differences in the number of sites phosphorylated. The preferential substrate may be the guanine-nucleotide-free form of $G_{s\alpha}$. During the activation of $G_{s\alpha}$ by an agonist–receptor complex, the rate of GDP–GTP exchange is increased (Gilman, 1987), resulting in the formation of nucleotide-free G_s, the lifetime of which will be short. In membranes, agonist-bound receptors promote GDP release even in the presence of $\beta\gamma$-subunits and we speculate that, in cells, the nucleotide-free $\alpha\beta\gamma$ complex may serve as a substrate for protein kinase C. Our studies suggest a potential for cross-talk between cyclic AMP signalling and PtdIns(4,5)P_2 signalling in cells, which supports previous studies describing perturbations of adenylate cyclase regulation by protein kinase C (Cronin *et al.*, 1986; Dobson *et al.*, 1990; Houslay, 1991). Further studies are required to isolate phosphorylated G_s from cells and to establish the effect of phosphorylation upon the regulation of adenylate cyclase. We also tentatively propose the existence of different phosphorylation sites in the two forms of $G_{s\alpha}$. This is an important question, and further studies will require an increase in the stability of $G_{s\alpha}$ prior to cyanogen bromide digestion and peptide mapping of these sites.

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