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SHORT COMMUNICATION

Agonist-induced Release of Splice Variants of the α Subunit of the Stimulatory G-Protein from Rat Cardiac Membranes

Klaus Witte, Anke Schnecko and Björn Lemmer*

INSTITUTE OF PHARMACOLOGY & TOXICOLOGY, FACULTY OF CLINICAL MEDICINE MANNHEIM, UNIVERSITY OF HEIDELBERG, D-68169 MANNHEIM, GERMANY

ABSTRACT. It was the aim of the present study to evaluate whether, in physiological cardiac tissue, long and short splice variants of $G_s\alpha$ (the alpha subunit of the stimulatory G-protein) differ in their susceptibility to guanine nucleotide-mediated activation. As a measure of $G_s\alpha$ activation, we determined the proportion of $G_s\alpha$ subunits which were released from the plasma membrane upon stimulation. Membrane preparations from heart ventricles of Wistar rats were incubated with increasing concentrations of the non-hydrolyzable GTP analogue guanylyl-imidodiphosphate (GppNHp, 0–100 μmol/L) in the absence or presence of the β-adrenoceptor agonist isoprenaline (1 μmol/L). The 45 and 52 kDa forms of $G_s\alpha$ ($G_s\alpha$-S and -L, respectively) were measured in the supernatant of the incubation mixture by immunoblotting and densitometry. The increase in cyclic AMP induced by GppNHp was measured in the same supernatant. In the absence of isoprenaline, GppNHp increased cyclic AMP formation and the concentration-dependent release of $G_s\alpha$-L (Friedman test, $P < 0.05$), whereas the amount of soluble $G_s\alpha$-S was not affected. After addition of isoprenaline, the redistribution of $G_s\alpha$-S into the soluble fraction could be stimulated by GppNHp in a concentration-dependent manner ($P < 0.05$). Kinetic experiments revealed that activation of $G_s\alpha$-S by GppNHp was rather slow, but could be markedly enhanced by isoprenaline. Thus, it is likely that the different susceptibilities of $G_s\alpha$-S and -L towards GppNHp reflects differences in the rate of spontaneous GDP release. BIOCHEM PHARMACOL **57**;5:539–543, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. $G_s\alpha$ isoforms; rat heart ventricle; adenylyl cyclase; signal transduction; immunoblotting; subunit release

The α subunit of the stimulatory G-protein is known to exist in different splice variants with molecular masses of 45 and 52 kDa, both of which are expressed in human cardiac tissue [1]. It has been suggested that $G_s\alpha$-S† is more efficiently coupled to stimulation of adenylyl cyclase [2], but this observation was made in a clonal hamster cell line (HIT T-15) after numerous passages in cell culture. It is still unknown whether isoform-specific differences in effector coupling of $G_s\alpha$ subunits exist in physiological cardiac tissue. The present study, therefore, aimed to investigate the functional contribution of both $G_s\alpha$ splice variants in rat cardiac membranes by using the approach of agonist-induced G-protein elimination [3]. The α subunits of heterotrimeric G-proteins are hydrophilic proteins, which are attached to the plasma membrane by palmitoyl residues and by binding to hydrophobic G-protein βγ subunits. It has been shown that a substantial proportion of cardiac $G_s\alpha$ can be found in the soluble fraction of a high-speed centrifugation [4]. Activation of the G_s-protein by non-hydrolyzable GTP analogues and by agonists at G_s-coupled receptors is known to increase the release of $G_s\alpha$ from the plasma membrane [5, 6]. It has been hypothesized that the redistribution of $G_s\alpha$ into the cytosol represents a mechanism involved in heterologous desensitization. However, reconstitution assays of cytosolic $G_s\alpha$ into G_s-deficient cyc^- S49 cells demonstrated that soluble α_s subunits were able to restore fluoride-stimulated adenylyl cyclase activity [7] and may, therefore, be able to interact with membrane-bound effectors and/or intracellular organelles under physiological conditions.

Most studies on the dissociation of $G_s\alpha$ have been performed in cell lines of non-cardiac origin. In the present study, we, therefore, addressed the question as to whether activation of the G_s-protein in rat cardiac membranes by a guanine nucleotide and by the β-adrenoceptor agonist isoprenaline would result in a concentration-dependent release of both splice variants of $G_s\alpha$. In addition, we assessed the correlation between $G_s\alpha$-release and the stimulation of adenylyl cyclase in the same incubation assay.

* Corresponding author: Dr. Klaus Witte, Institute of Pharmacology & Toxicology, Faculty of Clinical Medicine Mannheim, University of Heidelberg, Maybachstr. 14-16, D-68169 Mannheim, Germany. Tel. +49-621-330030; FAX +49-621-3300333.

† *Abbreviations:* GppNHp, guanylyl-imidodiphosphate; $G_s\alpha$-L, 52 kDa splice variant of $G_s\alpha$; $G_s\alpha$-S, 45 kDa splice variant of $G_s\alpha$; TBS-T, Tris-buffered saline plus Tween; and $G_s\alpha$, the alpha subunit of the stimulatory G-protein.

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MATERIALS AND METHODS

Preparation of Cardiac Membranes

Male Wistar rats (N = 10, 150–250 g body weight) were obtained from Charles River Wiga. Animals were killed by decapitation, heart ventricles were dissected quickly (within 1 min after decapitation), freed from fat and connective tissue, dried on filter paper, immediately frozen in liquid nitrogen, and stored at −60° until the biochemical assays were performed. Single ventricles were weighed and immediately homogenized in ice-cold assay buffer (Tris 50 mmol/L, $MgCl_2$ 1 mmol/L, pH 7.4 at 37°) using an Ultra-Turrax homogenizer (IKA Labortechnik) at 20,000 rpm. After a 10-min centrifugation at 25,000*g*, pellets were resuspended in 8–10 μL assay buffer per mg of tissue. Protein content of the membrane preparation was determined by the method of Lowry *et al.* [8] with minor modifications.

Incubation of Cardiac Membranes

Cardiac membranes were incubated for 8 min at 37° in assay buffer containing 3-isobutyl-7-methyl-xanthine (1 mmol/L), ATP (0.5 mmol/L), phosphocreatine (10 mmol/L), and creatine phosphokinase (0.1 mg/mL), with or without addition of GppNHp (1–100 μmol/L) and isoprenaline (1 μmol/L). The reaction was stopped by placing the tubes in an ice-cold water bath followed by 30-min centrifugation at 10,000*g* and 4°. Amounts of $G_s\alpha$ were determined in the supernatant, representing the cytosolic fraction (soluble $G_s\alpha$), and in the original membrane preparation prior to incubation, representing total $G_s\alpha$ content (membrane $G_s\alpha$). Soluble $G_s\alpha$ was expressed as percent of the total amount of membrane $G_s\alpha$. The concentration of cyclic AMP in supernatant was measured after heat denaturation (120°) of proteins using a commercially available radioassay kit (TRK 432, Amersham Pharmacia Biotech). In order to evaluate the kinetics of $G_s\alpha$ release, an additional experimental series was performed in which membranes were incubated for up to 16 min in assay buffer containing GppNHp (100 μmol/L) with or without addition of isoprenaline (1 μmol/L). Aliquots were taken in 4-min intervals and processed as described above.

Determination of $G_s\alpha$

The amount of $G_s\alpha$ in soluble and membrane fractions was determined by Western Blot according to the methods of Laemmli [9] and Towbin *et al.* [10], respectively, with minor modifications. Membranes were sonicated in sample buffer (Tris 125 mM, pH 6.8, SDS 1%, glycerol 10%, dithiothreitol 0.5%, bromphenol blue 0.01%), and concentrated sample buffer was added to supernatants of the incubation assay (soluble $G_s\alpha$), yielding the same final concentrations of buffer components. Samples were boiled for 5 min, and aliquots (20–30 μg protein) were subjected to SDS-polyacrylamide gel electrophoresis with 4% and 10% acrylamide in the stacking and running gel, respectively. After transfer to nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech) in blotting buffer (Tris 16 mM, glycine 120 mM, SDS 0.025%, methanol 20%), blots were incubated in TBS-T buffer (Tris 10 mM, pH 7.4, NaCl 0.9%, Tween-20 0.1%) with 3% bovine serum albumin for blocking of non-specific binding sites. Subsequently, blots were washed three times with TBS-T, and immunodetection was carried out with a highly specific antibody for $G_s\alpha$ (RM/1, NEN Life Science Products) diluted 1:5000 with TBS-T. The membranes were then thoroughly washed three times for 10 min each with TBS-T and incubated for 60 min at room temperature with a horseradish peroxidase-labeled anti-rabbit immunoglobulin G (NA 934, Amersham Pharmacia Biotech) diluted 1:5000 in TBS-T. Immunoreactivity was detected with the ECL-western blotting analysis system (RPN 2106, Amersham Pharmacia Biotech) according to the manufacturer's instructions. Intensities of the 45 and 52 kDa bands were quantified by transmission densitometry.

Statistical Analysis

Concentration–response curves were fitted to the data using PHARMFIT [11]. Curve parameters were compared statistically by Student's *t*-test. Concentration dependency of the effects of GppNHp was tested by the non-parametric Friedman test. The kinetics of the reaction in the absence or presence of isoprenaline was compared statistically using a bivariate ANOVA. The program package BiAS was used [12]. Data are shown as means ± SD unless otherwise indicated.

RESULTS

Addition of GppNHp to rat cardiac membranes resulted in a concentration-dependent ($P < 0.01$) increase in cyclic AMP formation from 6.1 ± 1.1 pmol/mg/min under basal conditions up to 11.6 ± 2.6 pmol/mg/min in the presence of GppNHp (100 μmol/L). Addition of isoprenaline (1 μmol/L) led to a significant ($P < 0.05$) increase in the pD_2 value [$-\log(EC_{50})$] of GppNHp from 5.4 ± 0.2 to 5.9 ± 0.3, indicating a greater potency of the guanine nucleotide in the presence of an agonist-bound receptor (Fig. 1). Splice variants of $G_s\alpha$ were differently affected by stimulation, 52 kDa $G_s\alpha$ being more susceptible to GppNHp-induced dissociation than the 45 kDa splice variant (Fig. 1). The amount of soluble 52 kDa $G_s\alpha$-L was increased by GppNHp concentration dependently ($P < 0.05$) from 10.7 ± 6.2% (without GppNHp) up to 19.9 ± 4.5% (GppNHp: 100 μmol/L). Addition of isoprenaline induced a small leftward shift of the concentration–response curve which, however, did not reach statistical significance (Fig. 1). The release of 45 kDa $G_s\alpha$-S could not be stimulated by GppNHp alone (Friedman test, not significant), being 17.5 ± 5.1% and 17.0 ± 3.1% under basal (without GppNHp) and stimulated conditions (GppNHp: 100 μmol/L), respectively. In

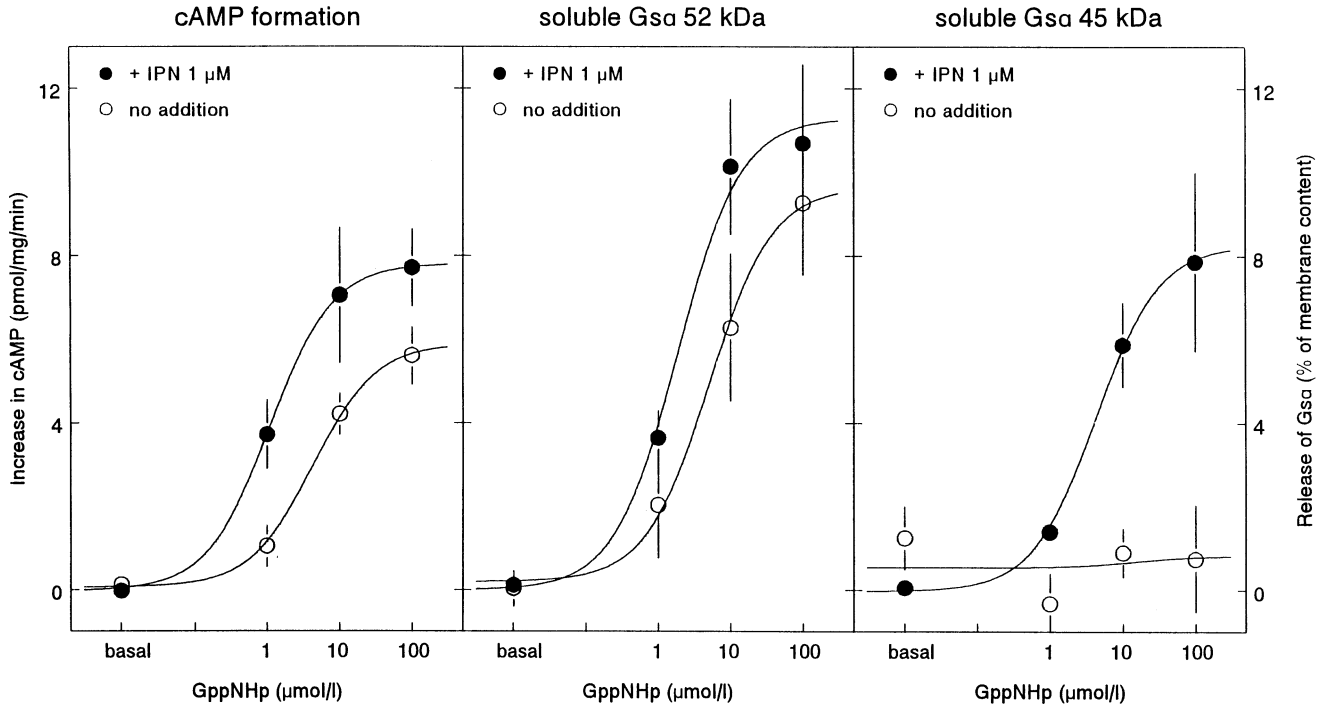

FIG. 1. Effects of GppNHp in the absence (open circles) and presence (closed circles) of isoprenaline (1 µmol/L) on cyclic AMP (cAMP) formation (left), release of 52 kDa $G_s\alpha$-L (middle), and 45 kDa $G_s\alpha$-S (right) in membrane preparations from rat heart ventricles. Formation of cAMP and the amount of soluble $G_s\alpha$ isoforms were measured in the same assay after 8-min incubation of membranes at 37° with the indicated concentrations of the non-hydrolyzable GTP analogue GppNHp. Mean values ± SEM of 4–5 ventricles.

the presence of isoprenaline, however, GppNHp induced a concentration-dependent ($P < 0.05$) increase in soluble $G_s\alpha$-S from 17.1 ± 5.1% up to 24.9 ± 9.2%.

The kinetics of $G_s\alpha$ release by GppNHp with or without addition of isoprenaline is shown in Fig. 2. In the presence of GppNHp, the amount of soluble $G_s\alpha$-L was steadily increased for up to 12 min, reaching a plateau thereafter. Addition of isoprenaline had no significant effect on the kinetics of this reaction (ANOVA, not significant). In contrast, $G_s\alpha$-S showed a slow rate of dissociation with GppNHp alone, which was significantly enhanced by addition of isoprenaline (ANOVA, $P < 0.01$).

DISCUSSION

In the present study, we have used a simple and direct assay for the simultaneous determination of agonist-induced $G_s\alpha$-release and stimulation of adenylyl cyclase activity. We observed that the long and short splice variants of $G_s\alpha$ differed in their susceptibility towards GppNHp-induced dissociation from the membrane-bound G-protein. Release of $G_s\alpha$-S by GppNHp was much slower than that of $G_s\alpha$-L, but could be enhanced in the presence of the β-adrenoceptor agonist isoprenaline.

The different susceptibility of $G_s\alpha$ splice variants towards guanine nucleotide-induced dissociation in rat cardiac membranes confirms recently published observations on the properties of β_2-adrenoceptor–$G_s\alpha$ fusion proteins [13]. In that study, the β_2-adrenoceptor was fused with the long and short splice variant of $G_s\alpha$ and expressed in Sf9 cells. Interestingly, fusion of the β_2-adrenoceptor with $G_s\alpha$-L but not with $G_s\alpha$-S resulted in a constitutively active receptor protein [13], which could be due to greater spontaneous release of GDP from $G_s\alpha$-L. Indeed, the dissociation rate of GDP was found to be markedly slower from 45 kDa $G_s\alpha$ than from the 52 kDa variant [14]. The present study demonstrates that the difference between the splice variants of $G_s\alpha$ is not only observed in artificial cell lines, but also occurs in mammalian cardiac tissue. In our kinetic experiments, the GppNHp-induced membrane release of $G_s\alpha$-S could, indeed, be enhanced by stimulation of the β-adrenoceptor with isoprenaline, which is known to promote the dissociation of GDP from the G-protein.

In the absence of isoprenaline, the increase in adenylyl cyclase activity by GppNHp was reflected by membrane release of $G_s\alpha$-L, whereas $G_s\alpha$-S was not affected. This could indicate a better functional coupling of $G_s\alpha$-L and the adenylyl cyclase. This assumption is in contrast to a previous observation in hamster pancreatic β cells (HIT T-15), in which the smaller $G_s\alpha$ subunit had greater functional efficacy [2]. Differences between species and tissues used may be responsible for this discrepancy. Alternatively, $G_s\alpha$ in the supernatant fraction may represent a desensitized form of the G-protein, as suggested by Kvapil *et al.* [15]. In their study, the authors observed that isoprenaline treatment of S49 lymphoma cells induced an unequal

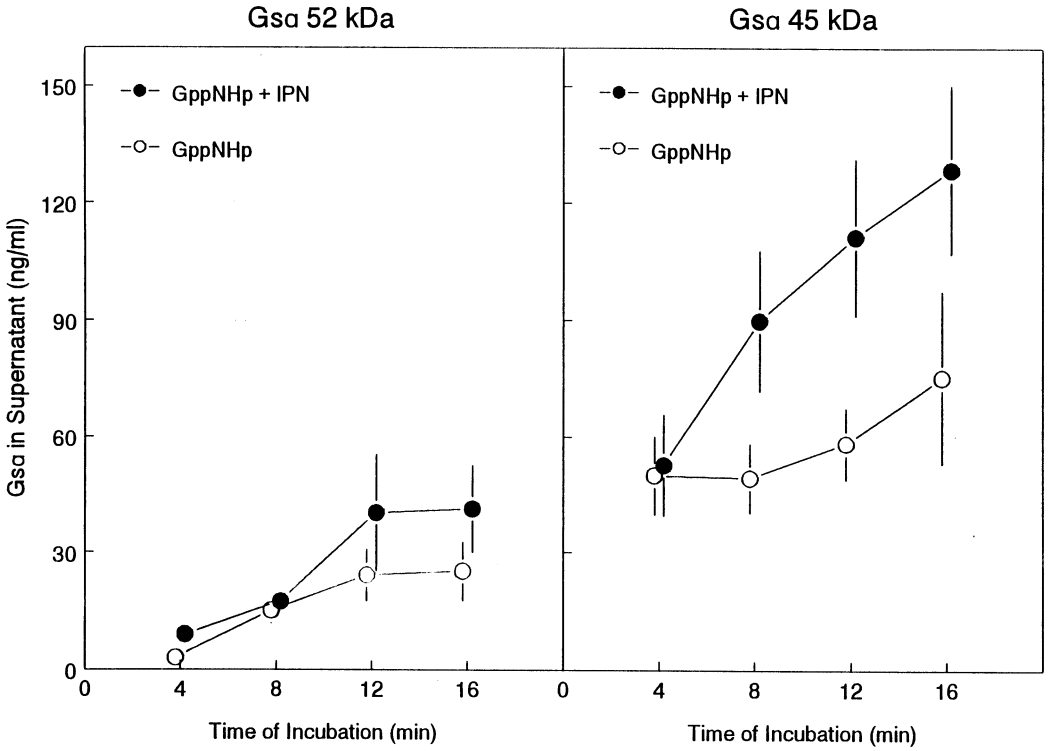

FIG. 2. Release of 52 kDa $G_s\alpha$-L (left) and 45 kDa $G_s\alpha$-S (right) in the presence of GppNHp (100 μmol/L) with or without addition of isoprenaline (IPN: 1 μmol/L) in membrane preparations from rat heart ventricles. Membranes were incubated at 37° for up to 16 min. Mean values ± SEM of 4–5 ventricles.

redistribution of $G_s\alpha$ isoforms between plasma membrane and a light-density membrane fraction, with $G_s\alpha$-L being increased and $G_s\alpha$-S being decreased in the light-density fraction after 10 and 60 min of stimulation [15]. Thus, the definite functional consequences of agonist-induced redistribution of G-protein subunits remain to be elucidated in future studies.

In conclusion, our study in rat cardiac tissue demonstrates that the GppNHp-induced release of $G_s\alpha$-S from the plasma membrane is much slower than that of $G_s\alpha$-L. Its marked enhancement by receptor activation indicates that the spontaneous rate of dissociation of GDP from the $G_s\alpha$ subunit must differ between the splice variants.

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