A Non-Ionic Vesicle Lipid Enhances Mastoparan-Stimulated GTPase Activity of Heterotrimeric G-Proteins

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Isolated heterotrimeric G-proteins exhibit full biological activity when reconstituted into liposomes. Here, we investigated the non-ionic surfactant macrogol-260-cetylstearylether (TA 6) as an efficient vehicle for the reconstitution of G-proteins. Reconstitution efficiency of G-proteins was recorded by GTPγS-binding. Their biological potency was measured as basal and mastoparan-stimulated GTPase-activity. G-proteins were fully active when associated with liposomes. On the other hand, G-proteins solubilized by TA 6 micelles or reconstituted into pure TA 6 vesicles exhibited impaired biological activity. However, vesicles containing different ratios of azolectin and non-ionic TA 6 showed about 50% higher reconstitution efficiency as compared to pure liposomes. In addition, basal and mastoparan-stimulated GTPase-activity in vesicles containing an axolectin / TA 6 ratio of 3:1 were 2.7 and 9.1 fold higher than in pure liposomes, respectively. These data emphasize that the composition of the lipid membranous environment significantly influences G-protein activity.

KEY WORDS: G-protein; Mastoparan; liposomes; Macrogol-260-cetylstearylether; reconstitution.

INTRODUCTION

Regulatory G-proteins are activated by ligand-bound transmembrane hormone receptors and modulate the activity of cellular effectors (1,2). Additionally, receptor independent activation of G-proteins by amphiphilic peptides like mastoparan has been shown (3). G-proteins consist of three subunits of which the α-subunit defines the heterotrimer. The intrinsic GTPase-activity is located on the α-subunit. Upon activation, the α-subunit exchanges GDP for GTP and dissociates from the βγ-dimer whereas β- and γ-subunits do not separate under native conditions. GTP-hydrolysis inactivates the G-protein. α-Subunits are hydrophilic whereas isolation of βγ-dimers or holoproteins requires detergent, most likely due to lipophilic posttranslational modifications such as isoprenylation located on the γ-subunit (4). Since G-proteins are assumed to be peripheral membrane constituents βγ-dimers function as membrane anchors. So far, four subfamilies were identified in mammals, entitled G_s, G_i, G_q and G_{12} (1). The G_i-subfamily consists of three G_i-subtypes, i.e., G_{i1}, G_{i2} and G_{i3}, and two G_o-subtypes, i.e., G_{o1} and G_{o2}. Purified G_i/G_o proteins exhibit impaired biological activity presumably due to the presence of detergents. Consistently, reconstitution of isolated G-proteins into liposomes and separation from detergents improved their biological activity (5). Hence, analysis of distinct signal transduction pathways became possible by incorporation of purified receptors, transducers, i.e., G-proteins, and effectors into liposomes (6). Previous studies emphasized the need of phospholipid vesicles to successfully reconstitute G-proteins (5). Generally, natural occurring phospholipids of various sources and poorly defined composition are used. Previously, we reported improved physicochemical properties of vesicles composed of the non-ionic surfactant macrogol-stearat 400 compared to phospholipids (7,8). These vesicles were successfully used as an efficient vehicle for DNA transfer into various mammalian cells. Here, we studied vesicles consisting of the chemically stable water-dispersible neutral lipid macrogol-260-cetylstearylether (TA 6) which should allow reconstitution of G-proteins.

MATERIALS AND METHODS

Materials

[^{32}P]Phosphoric acid was purchased from DuPont New England Nuclear (Bad Homburg, Germany). [γ-^{32}P]GTP was synthesized as described (9), and each preparation was used for less than three weeks. Phospholipids (azolectin) and the venom peptide mastoparan from *Vespula lewisii* were from Sigma (Deisenhofen, Germany). Macrogol-260-cetylstearylether (Marlowet® TA 6) which represents cetylstearyl moieties connected to polyoxyethylens with a median molecular mass of 260 was provided by Hüls AG (Marl, Germany). The sources of all other reagents used were described previously (10,11).

Gel Electrophoresis, Antibodies, and Immunoblot Analysis

Peptide antibodies AS 6, AS 11, AS 28, AS 36, and AS 266 were provided by Dr. Spicher and used as described (12,13). For detection of G-proteins, SDS-PAGE was employed and proteins were visualized by silver- or immunostaining (11).

Purification and Reconstitution of G-Proteins into Phospholipid Vesicles

G_i/G_o-proteins were purified from bovine brains as detailed elsewhere (11). Proteins were reconstituted into various lipid vesicles containing phospholipids, TA 6 or mixtures thereof. G-proteins were either incubated with preformed vesicles prepared by the evaporation method (10) or added to the solubilized lipid components. In the latter case (in-situ-preparation), lipids (1%, w/v) were solubilized together with a ten-fold excess of Na-cholate (w/v) in a buffer consisting of Hepes (20 mM, pH 8.0), NaCl (100 mM), $MgCl_2$ (2

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ABBREVIATIONS USED: GTPγS, guanosine 5′-(3-*O*-thio)triphosphate; liposome, vesicle consisting of phospholipids; M_r, relative molecular mass; SD, standard deviation; TA 6, macrogol-260-cetylstearylether; TEM, buffer containing 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 20 mM β-mercaptoethanol.

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mM), EDTA (1 mM) at 4°C. G-proteins (15 pmol) were mixed with 25 µl cholate-solubilized lipids to a final volume of 250 µl and kept on ice for 1 hour. Vesicles containing G-proteins were formed by passing the solution through an AcA 34 gel filtration column. In addition, G-proteins associated with preformed vesicles were separated from solubilized G-proteins by gel filtration, too. In order to calibrate the molecular sieve of the gel filtration column, the elution profiles of dextran blue (M_r: 2,000,000), of cytochrome C (M_r: 13,000) and of cholate-solubilized G-proteins were recorded.

Assays of GTPγS-Binding and GTPase-Activity

Quantification of G-proteins was performed by [^{35}S]GTPγS-binding (11). GTPase-activity was determined in a reaction mixture containing 50 µl of solubilized or reconstituted proteins, 0.5 µM [γ-^{32}P]GTP (0.1 µCi/tube), 0.5 µM GDP, 2 mM $MgCl_2$, 1 mM EDTA, 0.1 mM AppNHp, 0.2% BSA and the additions indicated in 50 mM triethanolamine/HCl, pH 7.4, at 25°C in a total volume of 100 µl. Low-K_m GTPase activity was calculated by subtracting high-K_m GTPase-activity determined at 50 µM GTP from total GTPase activity. High-K_m GTPase-activity was less than 10% of total radioactivity. Data are presented as turnover numbers (mol of hydrolyzed phosphate per mol of G-protein) per min.

Determination of Vesicle Radius

Particle size of concentrated vesicle preparations was measured by photon correlation spectroscopy (pcs) using a 4 mW laser beam set at 670 nm employing an Autosizer Lo-C (Malvern Instruments LTD., Malvern, UK) as described elsewhere (14).

RESULTS AND DISCUSSION

Characterization of Purified G_i/G_o-Proteins

Silver stained SDS-gels exhibited three proteins bands at 40, 39 and 35 kDa corresponding to α_i-, α_o- and β-subunits (Figure 1, A). Another band migrating close to the dye front represented γ-subunits with apparent molecular masses of 6-8 kDa (2). For identification of the subtype composition high resolution SDS-gels containing urea were employed (11,13). Specific antibodies showed three anti-$\alpha_{o\ common}$ and anti-$\alpha_{i\ common}$ immunoreactive bands in the 40 kDa range (see Figure 1, B). Further separation of this G_i/G_o-mixture by FPLC-chromatography on Mono Q-columns and analysis including identification by subtype-specific antibodies clarified that G_{o1} was the dominant subtype, whereas G_{o2}, another G_o-subtype termed G_{o3}, G_{i1} and G_{i2} were present in minor quantities. Only trace amounts of G_{i3} were detectable (unpublished work). In addition, two β-subtypes, i.e. β_1 and β_2, could be identified using subtype specific antibodies (see Figure 1, C). Interestingly, the apparent molecular masses of the β-subunits shifted from 35 to 40-42 kDa when urea containing SDS-gels were employed.

Reconstitution of Purified G-Proteins into Lipid Vesicles

Phospholipid liposomes prepared according to a published gel filtration technique (in-situ-preparation) were compared to preformed small phospholipid vesicles (5). We mixed G-proteins with cholate-solubilized soyabean phospholipids (azolectin) or preformed azolectin liposomes. Either preparation was passed through a gel filtration column which separated reconstituted from solubilized proteins (Figure 2). For cholate-solubilized azolectin, gel filtration was necessary in order to form G-protein-containing liposomes (5). Preformed vesicles had a larger size (101 ± 23 nm; mean value ± SD) than in situ-formed liposomes (48 ± 5 nm). Following either technique of vesicle preparation significant amounts of GTPγS-binding activity were found in the void volume, i.e. fractions 19 to 28 (see Figure 2). In contrast, cholate-solubilized G-proteins eluted in later frac-

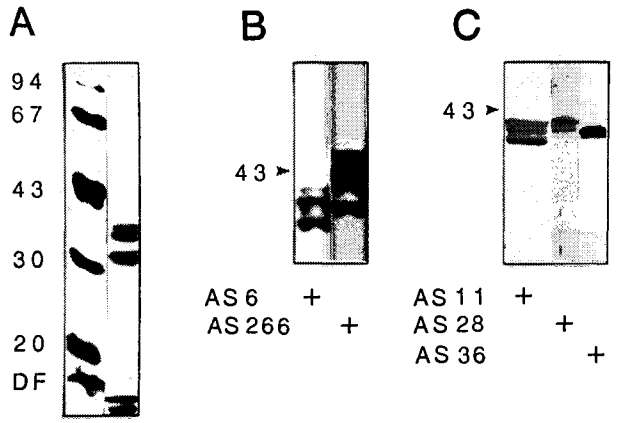

Fig. 1. Identification of purified G-proteins. (A) Purified G_i/G_o proteins (right lane) were resolved on a SDS-gel containing 10% acrylamide and silver stained. Marker proteins are shown on the left lane. DF: dye front (B) and (C) Proteins were loaded on SDS-polyacrylamide-gels containing 6 M urea and 9% acrylamide. After blotting, nitrocellulose filters were incubated with either (B) AS 6 (anti-$\alpha_{o\ common}$ serum, left lane) and AS 266 (anti-$\alpha_{i\ common}$ antibodies, right lane) or (C) AS 11 (anti-$\beta_{1/2}$ serum, left lane), AS 28 (anti-β_1 serum, middle lane) and AS 36 (anti-β_2 serum, right lane). The molecular masses (kDa) of marker proteins are indicated.

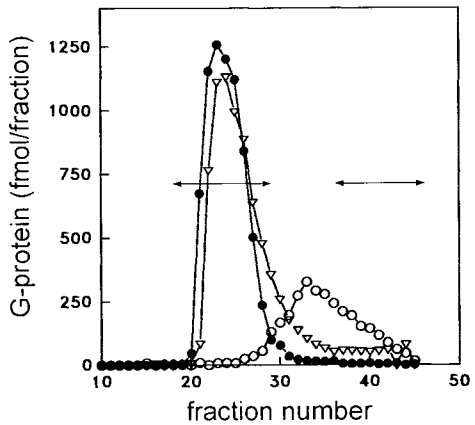

Fig. 2. Elution profile of G-proteins on an AcA 34 gel filtration column. Fifteen pmoles of cholate solubilized G_i/G_o-proteins (○), G_i/G_o-proteins mixed with solubilized azolectin (▽) or G_i/G_o-proteins incubated with preformed liposomes (●) were passed over an AcA 34 gel filtration column. Eluates were collected in fractions and analyzed for [^{35}S]GTPγS-binding. Fractions containing dextran blue (left) or cytochrome C (right) are indicated by horizontal lines.

tions, i.e. fractions 29 to 45, as one single peak. Thus, preformed and in-situ formed liposomes could reconstitute G-proteins with similar efficiency of 45% and 43%, respectively. Preformed vesicles showed one sharp peak of GTPγS-binding activity. In contrast, following the in situ-preparation technique significant GTPγS-binding activity (13%) eluted after the liposome containing fractions, too. In order to test the biological activity of cholate-solubilized or reconstituted G-proteins, we measured basal and mastoparan stimulated GTPase-activity. The maximal effective concentration of mastoparan on GTPase-activity of G_i/G_o-proteins was found at 100 μM (Figure 3) where GTPγS-binding activity was enhanced by 40-60% above basal (not shown). At this concentration mastoparan stimulated GTP hydrolysis of solubilized G_i/G_o-proteins by only 1.6 fold above basal (mean basal turnover number: 0.086 min^{-1}). A similar low stimulatory effect of mastoparan (100 μM) was seen when G-proteins were mixed with solubilized azolectin not forming vesicles (stimulation factor 1.8). In contrast, when in situ-prepared or preformed azolectin vesicle preparations loaded with G-proteins were tested mastoparan stimulated the GTPase-activity by 3- to 3.3-fold above basal (mean basal turnover number: 0.086 - 0.129 min^{-1}). Interestingly, the same stimulatory effect of mastoparan was seen with G-protein-loaded preformed liposomes which were not separated from detergents carried over with G-protein storage buffer. From these data we conclude that G-proteins become fully active when associated to a phospholipid bilayer.

Reconstitution of Purified G-Proteins into Macrogol-260-Cetylstearylether-Containing Lipid Vesicles

Employing the non-ionic surfactant TA 6 and the in-situ-reconstitution technique large vesicles with a mean diameter of >1000 nm were formed as detected by polarizing microscopy. The vesicle-containing fractions carried 42% of the applied G-proteins which showed a mean basal GTPase activity of 0.090 ± 0.016 min^{-1} (Figures 4 & 5, panels 5). In addition, significant GTPγS-binding activity was detectable in later fractions where solubilized G-proteins eluted. Therefore, it is likely that one part of TA 6 formed vesicles whereas another part remained as micelles which solubilize G-proteins. This conclusion is in line with the fact that TA 6 represents a mixture of polyoxyethylens of different chain length connected to cetylstearyl moieties. Hence, the lipophilic entities of TA 6 form vesicles whereas the more hydrophilic entities, i.e. those with longer polyoxyetylen chains, are water-soluble and form micelles (13). Although bound to TA 6 vesicles G-proteins had a GTPase-activity which was only weakly stimulated by mastoparan to 0.169 ± 0.017 min^{-1}, i.e. 1.9-fold (see Figure 5, panel 5). This was slightly but significantly more than that obtained with cholate-solubilized or TA 6-solubilized G-proteins. Thus, a non-ionic lipophilic membranous environment appears to be

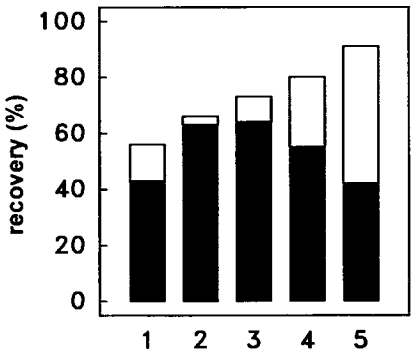

Fig. 4. Reconstitution efficacy of different G_i/G_o-containing vesicle preparations. G_i/G_o-proteins (15 pmoles) were reconstituted into lipid vesicles containing different ratios of azolectin and TA 6 by using the in-situ technique and gel filtration: 1: azolectin; 2: azolectin/TA 6 3:1; 3: azolectin/TA 6 1:1; 4: azolectin/TA 6 1:3; 5: TA 6. The recoveries of G-proteins from the vesicle-containing fractions 19-28 (filled bars) and from fractions 19-45 (total bars) are shown. Quantification was performed by [^{35}S]GTPγS-binding to G-proteins.

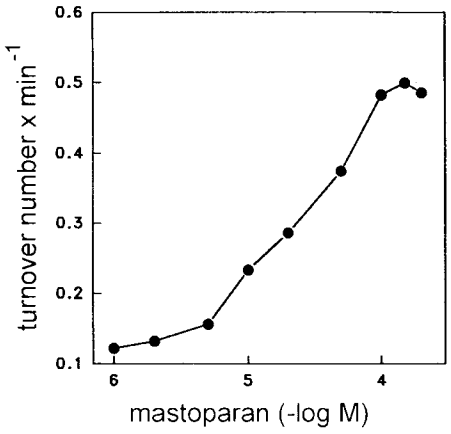

Fig. 3. Concentration-response curve of the effect of mastoparan on GTP hydrolysis by reconstituted G_i/G_o-proteins. GTP hydrolysis by G_i/G_o-proteins was determined in the presence of mastoparan at the indicated concentrations. Data shown are the means of assay triplicates. Mean basal GTP hydrolysis (control) was 0.09 (mol inorganic phosphate / mol G-protein) × min^{-1}. Amount of G-proteins was calculated from [^{35}S]GTPγS-binding to reconstituted vesicles.

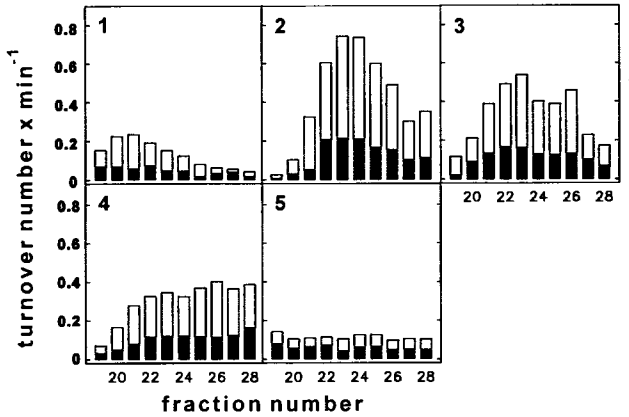

Fig. 5. GTP hydrolysis of G-proteins associated with different lipid vesicles. G_i/G_o-proteins (15 pmoles) were reconstituted in lipid vesicles containing different ratios of azolectin and TA 6 by using the in-situ technique and gel filtration: 1: azolectin; 2: azolectin/TA 6 3:1; 3: azolectin/TA 6 1:1; 4: azolectin/TA 6 1:3; 5: TA 6. Shown are turnover numbers of basal (filled bars) and mastoparan stimulated (100 μM; total bars) GTP hydrolysis of vesicle-containing fractions 19-28. Amount of G-proteins were determined by [^{35}S]GTPγS-binding.

sufficient to bind G-proteins to the vesicles but allowed only a moderate stimulation of the GTPase-activity by mastoparan.

We therefore studied different mixtures of ionic phospholipids and non-ionic TA 6 for reconstitution efficiency and GTPase-activity using the in-situ-reconstitution technique. All tested preparations formed vesicles with diameters of 220 ± 51 nm (azolectin / TA 6 ratio of 1:3), 128 ± 24 nm (1:1) and 29 ± 6 nm (3:1). As depicted in Figure 4, liposomes containing an azolectin / TA 6 mixture of 3:1 and 1:1 (w/w) exhibited the highest recovery of G-proteins (63% and 64%, respectively) compared to pure azolectin (43%) or pure TA 6 vesicles (42%). Thus, TA 6-supplemented phospholipid vesicles increased the uptake of G-proteins by almost 50% compared to pure azolectin liposomes. Furthermore, the mean basal GTPase-activity of reconstituted G-proteins per fraction varied significantly between the different preparations (see Figure 5). Whereas G-proteins associated with pure azolectin or TA 6 vesicles exhibited a basal GTPase activity of 0.086 ± 0.017 min^{-1} or 0.090 ± 0.016 min^{-1}, respectively, G-proteins bound to mixtures thereof had a significantly enhanced basal GTPase-activity ranging from 0.178 ± 0.025 min^{-1} to 0.239 ± 0.060 min^{-1}. Hence, liposomes with an azolectin / TA 6 ratio of 3:1 enhanced the basal GTPase-activity of reconstituted G-proteins by 2.7-fold. In addition, mastoparan stimulated GTPase-activity of G-proteins was dependent on the nature of the vesicles, too. Again, a maximum effect was seen with a preparation containing an azolectin / TA 6 ratio of 3:1 where mastoparan stimulated GTP hydrolysis to 0.780 ± 0.231 min^{-1}. This represents a 3.3-fold increase compared to the basal activity of the same preparation and a 9.1-fold increase compared to the basal GTPase-activity of G-proteins reconstituted to pure azolectin vesicles. Interestingly, the mean vesicle diameter correlated inversely with the biological activity.

CONCLUSIONS

In the present study, we analyzed the reconstitution efficiency of preformed and in-situ-formed liposomes and vesicles. We found that G-proteins are fully active when bound to but not necessarily integrated into liposomes. This is in accordance with the physiological model of G-proteins being peripheral membranous components located at the inner surface of the plasma membrane (2). Next, we tested whether the non-ionic surfactant TA 6 alone or in combination with phospholipids forms vesicles and studied its ability to reconstitute G-proteins. G-proteins solubilized by TA 6 micelles or reconstituted into pure TA 6 vesicles exhibited only impaired biological activity as determined by GTPase-activity. In contrast, vesicles containing different ratios of azolectin and TA 6 showed (i) increased reconstitution efficiency up to 50% and (ii) enhanced basal and mastoparan-stimulated GTP hydrolysis up to 2.7- and 9.1-fold compared to pure azolectin vesicles. From these data we suppose that TA 6 molecules integrated into phospholipid vesicles enhance G-protein activity. To our knowledge this is the first study recognizing the importance of non-ionic vesicle constituents for G-protein activity. At this point we do not know the mechanism, but it is tempting to speculate that TA 6 molecules may interact with covalently linked lipid modifications of the α- and/or γ-subunit since these posttranslational G-protein modifications were previously shown to be important for G-protein function (4,15,16). This study points to a modulating role of the lipid membranous environment of basal and mastoparan-stimulated G-protein activity.

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