Signal transfer in the plant plasma membrane: phospholipase A2 is regulated via an inhibitory Gα protein and a cyclophilin

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The plasma membrane of the California poppy is known to harbour a PLA2 (phospholipase A2) that is associated with the Gα protein which facilitates its activation by a yeast glycoprotein, thereby eliciting the biosynthesis of phytoalexins. To understand the functional architecture of the protein complex, we titrated purified plasma membranes with the Gα protein (native or recombinant) and found that critical amounts of this subunit keep PLA2 in a low-activity state from which it is released either by elicitor plus GTP or by raising the Gα concentration, which probably causes oligomerization of Gα, as supported by FRET (fluorescence resonance energy transfer)-orientated fluorescence imaging and a semiquantitative split-ubiquitin assay. All effects of Gα were blocked by specific antibodies. A low-Gα mutant showed elevated PLA2 activity and lacked the GTP-dependent stimulation by elicitor, but regained this capability after pre-incubation with Gα.

The inhibition by Gα and the GTP-dependent stimulation of PLA2 were diminished by inhibitors of peptidylprolyl cis-trans isomerase. A cyclophilin was identified by sequence in the plasma membrane, and in immunoprecipitates with anti-Gα antibodies. We conclude that soluble and target-associated Gα interact at the plasma membrane to build complexes of varying architecture and signal amplification. Protein-folding activity is probably required to convey conformational transitions from Gα to its target PLA2.

Key words: California poppy (Eschscholzia californica), cyclophilin, phospholipase A2, plant G-proteins, plant plasma membrane, plant signalling, protein–protein interaction.

INTRODUCTION

Heterotrimeric G-proteins are conserved modules of signal transfer in all eukaryotic organisms. Although their peptide sequences and three-dimensional structures display some basic similarities between evolutionarily recent animal and plant cells [1], the mode of interaction with their targets obviously differs between the kingdoms. In animal cells, signal perception causes one or more out of several task-specific heterotrimers to release their subunits for interaction with target proteins, which is best documented for the large subunit Gα and its various isoforms [2–4]. A plant cell, which typically contains only one Gα, one Gβ and two Gδ subunits [5] (a third atypical Gα was found in Arabidopsis and rice [6,7]), obeys different strategies to allow a similar diversity of G-protein functions in the signal transfer [8–11]. A recent example that underlines the variability of plant G-protein signalling is the loss of the Gα-activating protein ROS in the evolution of eudicots and the acquisition of receptor-based activation mechanisms [12]. One important clue arises from the fact that a significant part of Gα, or the complete heterotrimer is embedded in large protein complexes at the plasma membrane [11,13,14], and some of these aggregates are known to contain target proteins [11,15]. This promoted the idea that task-specific combinations of G-proteins and their target enzymes might form a framework for the multitude of G-protein-triggered signal pathways in plants [11].

The inventory and architecture of such complexes are far from being understood, as is the mode of intrinsic interaction between Gα, other subunits and target enzymes. As an example, conformational changes triggered by GTP or GTPγS (guanosine 5′-[γ-thio]triphosphate; GTP[S]) are reported to cause the total dissociation of Gα-containing protein complexes in rice [13]. In contrast, in Arabidopsis and Eschscholzia, no or very limited liberation of Gα was found upon activation [11,14,16], implying that GTP-triggered conformational transitions are conveyed to the target without dissociation of the protein complex.

Actually, an increasing number of potential Gα-binding plant proteins has been deduced from the interaction of the recombining proteins expressed in yeast or plant protoplasts, or from co-immunoprecipitation experiments [17,18]. Confronting and complementing such studies with biochemical analyses of G-protein-containing complexes might allow new insights into the functional specificity of the signal process. Among the problems to be addressed is the stoichiometry of interaction, e.g. the number of G-protein subunits per target molecule required for regulatory impacts, and the structural and functional plasticity of such complexes, e.g. the ability of membrane-associated Gα to exchange or interact with the soluble protein components faced in its cytosolic environment [19].

In the last few years, the plasma membrane of Eschscholzia californica, the California poppy, has been established as an in vitro model system to study the control exerted by Gα over the target enzyme PLA2 (phospholipase A2; EC 3.1.1.4). The highest and most reproducible levels of PLA2 activity and its stimulation by GTP were obtained after...
solubilization of the plasma membrane with cholate. Among the solubilized proteins, co-immunoprecipitation and non-denaturing electrophoresis detected large detergent-resistant complexes that contain PLA₂ together with G₌a. The *in vitro* activity of the enzyme increased upon contact with yeast elictor plus GTP, thus reflecting its G₃a-dependent control [11]. In intact cells, the same elictor activates PLA₂ at the plasma membrane [20,21], and a product of this enzyme, lysophosphatidylcholine, acts as a second messenger for the induction of alkaloid biosynthesis [22,23]. Thus G₃a-controlled PLA₂ initiates a signal chain that induces the overproduction of phytoalexins [24]. This antimicrobial response is triggered very selectively, i.e. it is independent of the ubiquitous jasmonate-dependent hypersensitive cell death. The latter can be triggered in addition by exposing our strains to high elicitor concentrations [21,24]. Although the hypersensitive response might involve other PLA enzymes [25], there are no indications of their G-protein-dependent control [11,25]. The G₃a-dependent elicitor-responsive PLA₂ of *Eschscholzia* has now been cloned, sequenced and deposited in GenBank® (accession number JQ886492).

In the present study, we use recombinant G₃a of *Eschscholzia* to mimic the impact of soluble G₃a at the target enzyme PLA₂ of the isolated plasma membrane.

**EXPERIMENTAL**

**Plant material**

Cell suspensions of *Eschscholzia californica* Cham. were originally derived from hypocotyl discs via callus cultures [26]. For experiments, cultures were maintained in a 9–10 day growth cycle in Linsmaier–Skog medium supported with the hormones 2,4-D (2,4-dichlorophenoxyacetic acid) and α-naphthalene acetic acid (1 μM each). Cultivation was carried out on a gyrotary shaker (100 rev/min) at 24°C under continuous light (∼7 μmol·m⁻²·s⁻¹). Cells were used for experiments after 6 or 7 days of growth as described previously [22].

**Cell fractionation and preparation of plasma membrane**

*Eschscholzia* or *Arabidopsis* cell suspensions (80 g) were filtered, frozen in liquid nitrogen and fractionated as described previously [27]. The microsomal pellet was obtained after centrifugation at 100 000 g for 1 h (4°C; Beckman LE-80K, TI-70 angle rotor, 37 000 rev/min) and used to purify the plasma membrane by liquid two-phase partitioning [28]. Soluble and membrane fractions were also analysed by SDS/PAGE and subsequent Western blotting with IgG fractions purified from the polyclonal rabbit anti-G₃a antiserum at a dilution of 1:500 in TBS [Tris-buffered saline; 25 mM Tris/HCl (pH 7.5), 150 mM NaCl and 3 mM KCl].

The plasma membrane fraction was finally solubilized by mild shaking (2 h, 8°C) in TENC buffer (20 mM Tris/HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl and 0.5% sodium cholate). This detergent proved to be superior to a number of other compounds tested (including Lubrol and n-dodecyl-β-D-maltoside) with respect to the yield of solubilized PLA₂ activity and its stimulation by GTPγS. The supernatant obtained by centrifugation (100 000 g, 40 min, 4°C; Beckman LE-80K, TI-70 angle rotor, 37 000 rev/min) was adjusted to a protein content of 500 μg/ml and used in all experiments.

**Assay of PLA₂ activity**

PLA₂ activity was quantified by monitoring the fluorescence emission from the artificial substrate BPC (bis-BODIPY®-FL-C11 phosphatidylcholine) (Molecular Probes) as described in [11,29]. A typical assay contained per well of a 96-well microtitre plate (Microfluor II; Greiner) 170 μl of substrate solution (0.4 μM BPC) followed by 30 μl of effectors (see below). After 10 min of equilibration (mild shaking at room temperature, 22°C), 20 μl of solubilized plasma membrane preparation (containing 10 μg of protein) were added and the fluorescence was recorded at λex 485 nm/λem 528 nm over 10 or 60 s (dependent on the rate of fluorescence development) in a FLX800 microplate fluorescence reader (BioTek). To eliminate non-enzymatic generation of fluorescence, a control experiment was performed with the enzyme preparation replaced by TENC buffer. Initial rates of hydrolysis were converted into enzyme activities by using calibration assays with bee venom PLA₂ and a series of substrate concentrations.

The final concentrations of effectors in the 220 μl reaction mixture were as follows: G₃a, 45–1350 nM as indicated; cyclosporin A, 1 μM; GTP, 5 μM; yeast elictor, 1 μg/ml (see below); and antibodies (IgG or scFv), 68 nM. These concentrations were optimized for each compound or antibody in a test series and yielded either saturating or optimum effects. In short-time incubations as used in the present study, GTP and GTPγS displayed similar effects on PLA₂. Yeast elictor is a glycoprotein fraction prepared from baker’s yeast according to [30] and further purified by ultrafiltration (30 kDa molecular mass cut-off), FPLC (anion exchange and size exclusion) and SDS/PAGE. The active fraction comprises glycoproteins of 30–42 kDa that contain approximately 40% mannose [20]. Dosage refers to the dry weight of the crude preparation.

**Preparation of recombinant G₃a and G₅γ proteins**

The ORF (open reading frame) of EcGPA1 (GenBank® accession number HQ830348) encoding the G₅γ protein was amplified from a cDNA library of *Eschscholzia* [23] and expressed in the pET-23(+) vector. Similarly, the G₁ coding gene from *Arabidopsis thaliana* (AT3G63420.1) was cloned into the same vector. Expression was performed using standard procedures. Briefly, competent cells of *Escherichia coli* BL21(DE3) were transformed with these plasmids by the heat-shock procedure (42°C) and grown on ampicillin selection agar. Colonies were transformed to liquid LB (Luria–Bertani) medium, grown for 24 h at 35°C and 1 mM IPTG (isopropyl β-D-thiogalactopyranoside) was added to stimulate protein production. After 3 h the bacterial pellet was harvested by centrifugation (20 min, 4°C; Sorvall GSA rotor, 4000 rev/min) and lysed using lysozyme and ultrasonic treatment. In the 30 000 g supernatant, the C-terminally His₆-tagged protein was trapped on 50% Ni-NTA (Ni²⁺-nitrilotriacetic)–agarose (Ni Sepharose™ High Performance, GE Healthcare), purified by FPLC (AKTA, GE Healthcare) and finally eluted with buffer containing 250 mM imidazole.

The recombinant G₅γ protein contained <1% impurities, most probably from the bacteria used for cloning, as determined by SDS/PAGE. To confirm that the bacterial impurities did not mimic or change the observed effects of G₅γ, test experiments were performed in the absence of G₅γ but with the contaminating proteins, obtained by the same expression and purification procedure using an empty pET-23(+) vector. Addition of this test extract together with G₅γ did not significantly change its effects on PLA₂.

**Preparation of native G₅γ by immunoabsorption**

G₅γ was isolated from the soluble *Eschscholzia* proteins (100 000 g supernatant, obtained according to [27]). An immunomatrix was...
prepared by binding and crosslinking the IgGs from the polyclonal anti-Gα antiserum (see above) to Protein A-Sepharose 4 Fast Flow (GE Healthcare). After incubation with the Gα-containing protein fraction, the bound Gα was eluted at alkaline pH. The whole procedure followed essentially a protocol published by I.J. Delgado (http://www.ivaan.com/protocols/126.html).

The Gα was further purified by SDS/PAGE, the 42 kDa band was excised from the gel and the protein was renatured in the presence of 5 mM CaCl2, 5 M cysteine and 50 mM ammonium acetate, pH 7.0, according to [31]. Gel extraction was facilitated by a freeze–thaw cycle, followed by dialysis and ultrafiltration, as detailed in [11].

**Antibodies**

To raise antisera against recombinant Gα, two rabbits were each immunized with 1 mg of Gα and Freund’s complete adjuvant and boosted with 500 μg of Gα and Freund’s incomplete adjuvant after 4 weeks, 5 weeks and 6 weeks respectively. The anti-Gα sera were affinity-purified via Gα-Sepharose columns made from CNBr-activated Sepharose by loading Gα following the manufacturer’s protocol (GE Healthcare). The resulting IgG fraction was used in PLA2 assays.

Isolation of specific scFv against Gα was performed as described previously [32]. Briefly, the phage display libraries Tomlinson A and B [33] were screened against Gα produced in pET-23a [23]. After four rounds of selection one monoclonal scFv (anti-Gα scFv2) was isolated and characterized by sequencing and indirect ELISA as described previously [32]. Briefly, antigens were adsorbed to Maxisorb plates at neutral pH overnight, the wells blocked and specific scFv, anti-c-Myc antibody and anti-mouse alkaline phosphatase conjugate were added sequentially after washing between steps. Alkaline phosphatase activity was measured using p-nitrophenylphosphate. A selectivity test of anti-Gα scFv2 is exemplified in Supplementary Figure S1 at http://www.biochemj.org/bj/450/bj4500497add.htm by showing an indirect ELISA experiment with Gα, Gα1 and Gα2 as antigens. Anti-Gα scFv2 binds to Gα with a dissociation constant of 1.3×10⁻⁷ M as determined by competitive ELISA (results not shown).

Anti-cyclophilin antibodies, raised against human cyclophilin A, were purchased from NovusBio, NB300-553. The recombinant human cyclophilin A used to raise this IgG fraction shows 71% sequence identity with the cyclophilin isolated from *Eschscholzia* (see Figure 10B).

All animal work was performed according to national guidelines described in the German Animal Welfare Act AWA (Deutsches Tierschutzgesetz). Immunization studies were carried out by an authorized employee under licence according to Section 8b from the district veterinary office Aschersleben-Stassfurt.

**Co-immunoprecipitation of plasma membrane proteins**

IgG fractions from antisera raised in rabbits against Gα (see above), or against human cyclophilin A were immobilized on a Protein A–Sepharose immunomatrix (Fast Flow 4; GE Healthcare) and incubated with the solubilized plasma membrane as described in [11].

**The mating-based split-ubiquitin assay**

The assay was performed in principle as described previously [34]. Briefly, the gene encoding Gα and the gene of a potential interactor protein were fused to the C-terminal or the N-terminal half respectively of the ubiquitin gene and expressed in yeast strains of different mating type. During mating, interactions between the membrane-bound fusion proteins are expected to create a ubiquitin-like structure by interacting the N-terminal part of ubiquitin (Nub) with the C-terminal part of ubiquitin (Cub) that harbours the PLV transcription factor (Cub-PLV). This structure activates ubiquitin-specific proteases, thus causing the release of PLV, which can be quantified by the expression of the yeast reporter genes lacZ, HIS3 and ADE2.

In our experiments, the ORF of Gα from *E. californica* was fused by *in vivo* recombination cloning to the C-terminal part of ubiquitin (Gα-Cub) followed by PLV. In the same way, this ORF was also fused to the N-terminal part of the ubiquitin-13 mutant (NubG).

The haploid yeast strains THY.AP4 (harbouring the Cub-fused Gα proteins) and THY.AP5 (harbouring the the Nub-fused test protein) were mated and allowed to generate diploid yeast cells on medium lacking histidine and adenine. Growth of diploids in SC medium and in addition the expression of LacZ and β-galactosidase [assayed with X-Gal (5-bromo-4-chloroindol-3-yl β-d-galactopyranoside)], was taken as an indicator of interaction between the Nub- and Cub-fused proteins [34]. In order to detect false-positive effects of test proteins, which may not arise from an interaction with the bait, e.g. direct influences at the yeast reporter genes and growth (if any), negative control experiments were performed by mating the THY.AP5 strain (with the Nub-fused test protein) to a THY.AP4 strain that did not contain the Gα gene in the Cub-vector. No colony growth was detectable in such experiments.

The expression of Gα encoded in the Cub-PLV vector can be controlled via a methionine-sensitive promoter and thus the Gα available for binding can be reduced to definable levels. In our hands, methionine concentrations between 0.15 mM and 5 mM caused the Gα content in the diploid clones to decrease from approximately 90% to 15% [18]. This feature was used to favour the formation and growth of clones with higher affinity interactors and thus to rank the interactors by their binding strength. To measure growth rates at different methionine concentrations, diploid clones obtained after mating on solid medium were picked and each subcultured in liquid medium with no methionine. After another 2 days of growth, all cell suspensions were adjusted to an attenuance of 0.1 and 3 μl aliquots were spotted on to solid medium with the same nutrient composition but different methionine concentration. After 7 days of incubation at 30°C digital images of the colonies were recorded. The increase in colony size was compared between cultures at the specified methionine concentration and without methionine, and normalized to the increase displayed by the same clone on a methionine-free agar plate.

**Purification, MS-based sequencing and PCR-based cloning of cyclophilin**

The immunoprecipitate obtained from the solubilized plasma membrane with the anti-Gα antibody (see above) was separated by SDS/PAGE. The cyclophilin band (approximately 19 kDa) was identified by comparison with a Western blot detected with the anti-cyclophilin antibody. The band was excised from the gel, digested with trypsin and the fragments identified by MS/MS (tandem MS)-based sequencing, as detailed with Gα in [11]. On the basis of two peptide sequences, a forward primer (5’-AAAGTTTTTCTTGTATATGCTTGGTGGG-3’) and an antisense reverse primer (5’-AGTTTTACCAGG-CCATGAAAGTTT-3’) were synthesized based on *Eschscholzia* codon usage (http://www.kazusa.or.jp), which amplified a 390 bp
Table 1 Primers for RT–PCR of Gα-fused fluorescent proteins

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5′→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gα1 for (5′→22)</td>
<td>TTGTCCTCAAATGGGCTCTACACATGACAGAC</td>
</tr>
<tr>
<td>Gα2 for (970–979)</td>
<td>GCCTGAATTTGCTAAGAAGGAAATTCG</td>
</tr>
<tr>
<td>CFP rev (3′-end)</td>
<td>TTGTGCTACAGCTACTCACAGCTCACAC</td>
</tr>
<tr>
<td>YFP rev (3′-end)</td>
<td>TTGTGCTACAGCTACTCACAGCTCACAC</td>
</tr>
<tr>
<td>eGFP rev (3′-end)</td>
<td>TTGTGCTACAGCTACTCACAGCTCACAC</td>
</tr>
</tbody>
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fragment from a cDNA template of Eschscholzia californica. PCR conditions were: 5 min at 94°C followed by 35 cycles with 20 s at 94°C, 1 min at 50°C and 1 min at 72°C, finalized by one cycle at 72°C for 10 min. The full-length ORF was completed from the above fragment by genome walking (Genome Walker™ Universal Kit; Clontech) according to the manufacturer’s protocol. Genomic DNA as required for genome walking was isolated from the Eschscholzia cell culture, digested with EcoRV and adaptor-ligated. The gene-specific primers were as follows: forward, 5′-GTCACAAGGTGGAGATTCTTACAGCCGGAAC-3′; and antisense reverse, 5′-AGATCCATTGTGCTGGACCAGCGGTTAGC-3′. Two overlapping fragments were obtained, from which the full-length cDNA was obtained by PCR (see above) with primers from non-coding regions, 5′ (upstream) 5′-CCCCGATATAAACCTATCCAGAA-3′ and 3′ (downstream) 5′-GAGGGAGAGATGGGTGAATTGGGGATGT-3′. The ORF of the cyclophilin gene was cloned into the pDRIVE vector (Qiagen) and expressed in E. coli.

Construction of vectors for plant transformation

The 3′-end of the ORF of Gα of Eschscholzia (GenBank® accession number HQ830348) was fused to the 5′-end of the ORFs of eGFP (enhanced GFP; green fluorescent protein), CFP (cyan fluorescent protein) or YFP (yellow fluorescent protein). A spacer of three alanine triplets (5′-GCTGCGGC-3′) was placed between the two genes. ORFs encoding eGFP, CFP or YFP were used as references.

The plasmid DNAs were amplified by PCR with gene-specific primers, which introduced flanking Bsal restriction sites and were finally cloned into the binary vector pICH56022 (Icon Genetics) and expressed in E. coli. The biolistic bombardment resulted in a mixture of paromomycin-resistant (i.e. stably transformed) and -sensitive cells. The former initiated regenerative growth 2–4 days after bombardment and often generated multicellular aggregates. The latter included cells that clearly suffered under the influence of the antibiotic treatment as indicated by the expression of fluorescent CFP and/or YFP but irregular cell shapes. The microscopic analysis was done with newly formed cells that were clearly distinguishable from the detrimental cells.

Verification of transformation by RT (reverse transcription)–PCR

At 2 days after biolistic treatment, 100 mg [fw [fresh weight]] samples of the transformed cultures were harvested by vacuum filtration and stored at −80°C. RNA was isolated by the NucleoSpin RNA Plant isolation kit (Macherey-Nagel). cDNA synthesis was performed with the Qiagen RevertAid™ M H Minus First Strand cDNA Synthesis kit using an oligo(dT)18 primer, according to the manufacturer’s instructions. An aliquot (2 μl) of the resulting cDNA mixture was used as a template for PCR, which was performed in an Eppendorf Mastercycler gradient PCR machine: 2 min at 94°C; 1 min at 55°C, 1 min at 72°C, 30 s at 94°C (35 repeats); and 10 min at 72°C. The primers used are listed in Table 1. The resulting PCR products were separated (Supplementary Figure S2 at http://www.biochemj.org/bj/450/bj4500497add.htm) and identified by DNA sequencing.

Confocal laser-scanning microscopy

Confocal microscopy was done with the LEICA TCS SP laser-scanning unit mounted on a DCM RE fluorescence microscope equipped with a 63-fold apochromate objective. Fluorescence was excited at 488 nm (Argon-Ion laser) and detected in the range between 496 and 530 nm. The detection pinhole was set at 1.0. In parallel with the laser-excited fluorescence emission, a (non-confocal) transmission image of each specimen was scanned. All images are averaged from six subsequently scanned frames of 1025 pixels×1025 pixels or 512 pixels×512 pixels. Intensities were visualized by a standard lookup table consisting of different shades of green (dark at low and bright at high intensity).

The red fluorescence of benzophenanthridine alkaloids, which is partially emitted in the transformed cells, was detected in a parallel precautionary scan at 570–670 nm. Cells with detectable fluorescence in this wavelength area were discarded from analysis.
Fluorescence microscopy and image-processing for FRET (fluorescence resonance energy transfer) analysis

Transformed cell suspensions were examined with a Nikon Optiphot fluorescence microscope equipped with a Sony 3-chip CCD (charge-coupled-device) camera. Images were obtained successively by using three fluorescence filters: For YFP FRET, \( \lambda_{\text{ex}} = 380–425 \text{ nm}, \lambda_{\text{em}} = 520 \text{ nm BP} \) (bandpass filter); for CFP, \( \lambda_{\text{ex}} = 380–425 \text{ nm}, \lambda_{\text{em}} = 50 \text{ nm BP} \); and for the YFP reference, \( \lambda_{\text{ex}} = 482–500 \text{ nm, } \lambda_{\text{em}} = 520–560 \text{ nm} \), followed by a transmitted light image. The digitalized images were converted into greyscale and ratioed with the Optimas 6.2 software: (i) the acceptor image (obtained via the YFP-FRET filter) was divided by the donor image (obtained via the CFP filter) and amplified by 100; (ii) the resulting quotient image was again divided by the donor image and amplified by 100; and (iii) the new image was divided by the YFP reference image (obtained via the YFP reference filter) and amplified by 30. The final ratio image was processed with Adobe Photoshop CS5, firstly by color coding with a spectral lookup table and secondly aligned with the transmitted light image. The procedure is exemplified in Figure 5. Apart from the linear amplifications, the final ratio image contains the original image informations converted as: donor/acceptor\(^2\) per YFP (reference).

When fluorescence intensities were to be compared between different channels (e.g. in Figure 4), the emission in the FRET-YFP channel was corrected for the ‘spillover’ of CFP-derived emission. For this purpose, a correction factor was established by dividing the fluorescence intensities in the FRET-YFP channel (\( \lambda_{\text{ex}} = 380–425 \text{ nm}, \lambda_{\text{em}} = 520 \text{ nm BP} \)) of cells transformed with \( G_{\alpha} \)-CFP alone with cells that expressed both fusion proteins. A total of 12 areas of cytoplasmic regions (31 pixels \( \times \) 31 pixels) with maximum intensity were averaged in each cell strain. When measured under identical conditions, the greyscale images in \( G_{\alpha} \)-CFP cells displayed a mean intensity of 36% compared with \( G_{\alpha} \)-CFP + \( G_{\alpha} \)-YFP cells. This value was used as an average for the CFP-derived ‘spillover’.

RESULTS

Intracellular distribution of \( G_{\alpha} \)

The isolated plasma membrane preparation of \( Eschscholzia \) used in our previous and current studies contains approximately 12 \( \mu \text{g} \) (286 pmol) of \( G_{\alpha} \) per mg of protein. This amount represents only approximately 0.5% of the total cellular \( G_{\alpha} \) (cell homogenate excluding mitochondria), of which >50% is not membrane-bound, i.e. detectable in the 100 000 g supernatant. This distribution contrasts with data obtained from \( Arabidopsis \) cell cultures: as seen in Figure 1, the \( G_{\alpha} \) of \( Arabidopsis \) is located almost completely in the microsomal pellet and in the isolated plasma membrane, whereas only a small percentage is detectable in the soluble fraction. This finding is in line with earlier cell fractionation studies in meristematic cells of \( Arabidopsis \) and cauliflower, where Weiss et al. [37] found most, if not all, \( G_{\alpha} \) associated with the plasma membrane and ER (endoplasmic reticulum) membranes. Binding of \( G_{\alpha} \) to the plasma membrane and the ER has now been confirmed by the visualization of \( G_{\alpha} \)-GFP hybrids in protoplasts (e.g. [16]) or by electron microscopy studies in immunogold-labelled tissues [38]. In order to substantiate the apparent species difference, a localization study of \( G_{\alpha} \) was performed in \( Eschscholzia \) cells by confocal microscopy. As seen in Figure 2, a \( G_{\alpha} \)-eGFP hybrid protein spreads over all detectable cytoplasmic areas and does not report a substantial accumulation at the plasma membrane.

Confocal images were scanned as described in the Experimental section 3 days after biolistic transformation of cell cultures with a vector encoding the \( G_{\alpha} \)-eGFP fusion protein. The images align the confocal plane, coded in green, with the transmission image (non-confocal) of the same specimen. The bars represent 50 \( \mu \text{m} \) (Figure 1) or 10 \( \mu \text{m} \) (Figure 2). Vascular (v), cytoplasmic (c) and nuclear (n) regions are marked. (A and B) Rapidly growing cells (A) or aggregates (B) which in this stage have no dominant vacuoles. (C and D) Adult cells with the typical large central vacuole. Note that the fluorescence of the fusion protein fills all cytoplasmic regions, including the plasma strands connecting to the nuclear region. No distinct accumulation of \( G_{\alpha} \)-eGFP at the cellular surface was detectable.

This result is in line with the data of the cell fractionation study described above. It appears therefore that substantial amounts of soluble \( G_{\alpha} \) are present together with the membrane-bound species, at least in some plants. For instance, in growing wheat tissue cultures the larger part of \( G_{\alpha} \) was recovered in a 120 000 g supernatant and is thus considered a soluble protein [39], similar to our experience with \( Eschscholzia \). More comparative experiments are justified to reveal whether the localization of \( G_{\alpha} \) changed during species evolution (actually, the monocot wheat and the early eudicot \( Eschscholzia \) most probably share a pattern that differs from the late eudicots \( Arabidopsis \) and \( Brassica \)) or whether the reported differences represent expression profiles of different cell types and growth stages.

The present study is based on the assumption that a small fraction of \( G_{\alpha} \), tightly bound to the plasma membrane faces an excess of soluble or weakly bound \( G_{\alpha} \), at the cytoplasmic surface. We address the question whether and how the interplay of both
The activity of PLA₂ was measured in the presence of the indicated concentrations of Gₐₐ, which was added 10 min prior to the solubilization of the plasma membrane (see the Experimental section). Where indicated, antibodies were added together with Gₐₐ. Numbers next to Gₐₐ are final concentrations in mM. The results are means ± S.D. of five independent measurements, normalized to the PLA₂ activity of the untreated plasma membrane (5 pkat/mg of protein) which is set to 100%. The experiment was repeated with seven different plasma membrane preparations, which all yielded similar results. Data labelled with * are significantly different (95%) from the untreated plasma membrane (100%).

Interactions with Gₐₐ is inhibitory to PLA₂

In the isolated and solubilized plasma membrane of Eschscholzia the activity of PLA₂ was assayed as the rate of hydrolysis of a fluorigenic substrate, BPC, according to an optimized protocol [11,20,23,29]. Recombinant Gₐₐ, encoded by Eschscholzia cDNA and produced in E. coli, inhibits PLA₂ activity if added in concentrations between 20 and 100 nM (Figure 3, columns 2–4). Typically, 45 nM Gₐₐ caused a reduction in activity of approximately 66%. This inhibition disappeared if the concentration of recombinant Gₐₐ was raised, and above a saturating level (approximately 900 nM), the enzyme activity was even stimulated (140–145%, Figure 3, columns 6–8). Neither the inhibition nor the activation caused by recombinant Gₐₐ was due to its His₆-tag or potential bacterial contaminants, as in test experiments native Gₐₐ isolated from the soluble proteins of Eschscholzia cell cultures (see the Experimental section), displayed the same effect as the recombinant protein (see columns 2/3 and 6/7 in Figure 3). Furthermore, the effects of native Gₐₐ on PLA₂ activity did not change if it was added together with extracts prepared in a similar manner from untransformed bacteria or from those transformed with the empty vector (results not shown).

The concentration-dependence in Figure 3 suggests that the mode of interaction between PLA₂ and external Gₐₐ changes above a critical concentration of this protein. Gₐₐ molecules might then either access additional activating binding site(s) at the enzyme or lose their affinity by self-interaction, thus competing with the target. As the latter idea appears more plausible, we tested the ability of Gₐₐ to dimerize or oligomerize by two alternative procedures: (i) by fluorescence microscopy to search for FRET-like interactions between different Gₐₐ–GFP hybrids in the Eschscholzia cell; or (ii) by the mating-based split-ubiquitin assay with the Gₐₐ of Eschscholzia expressed in yeast cells.

FRET caused by interaction of fluorescently labelled Gₐₐ molecules in Eschscholzia cells

In this approach, cultured cells were biolistically transformed with two DNA vectors, each encoding a fusion protein of Gₐₐ with either CFP or YFP. Control cell lines were established similarly by transferring the CFP or the YFP gene alone, or a mixture of both. After 2–4 days of regeneration, the transformed cultures expressed the desired mRNAs (Supplementary Figure S1) and most cells emitted fluorescence typical of YFP and CFP.

In cells expressing both Gₐₐ–CFP and Gₐₐ–YFP, the excitation of CFP with a wavelength of ~400 nm caused a substantial increase in fluorescence in the YFP channel, i.e. at >520 nm. This effect persisted after correcting for the average ‘spillover’ of CFP (donor) fluorescence into the YFP (acceptor) channel, and was not found in cells that expressed Gₐₐ–YFP alone (Figure 4). This finding was taken as a first hint for energy transfer between Gₐₐ–CFP and Gₐₐ–YFP, but could not easily be quantified in individual cells as the proportions between CFP- and YFP-fusion proteins appeared to differ significantly from cell to cell, which would require extensive individual correction measurements.

We thus established an alternative procedure to visualize potential interactions between the Gₐₐ-moieties by comparing fluorescence properties between cells that expressed both hybrid proteins (Gₐₐ–CFP plus Gₐₐ–YFP) and others that contained the pair of unsubstituted CFP plus YFP. Three images from each cell were obtained and processed by ratioing acceptor emission (Fₐₓₐₐ–CFP) to donor emission (Fₐₓ₋CFP). The resulting map (Fₐₓ₋CFP/Fₐₓₐₐ–CFP) was

![Figure 3](image3.png)

**Figure 3** Effect of recombinant and native Gₐₐ on the PLA₂ activity of the plasma membrane

![Figure 4](image4.png)

**Figure 4** Fluorescence emission of cells containing the Gₐₐ–YFP fusion protein alone or together with Gₐₐ–CFP
Figure 5  An example of the work flow towards FRET ratio images

(A, B and C) Fluorescent images obtained by the YFP-FRET, CFP and YFP filter respectively. (D) The transmitted light image showing the cell shape. (E) The ratio map, obtained by dividing the YFP-FRET image by the CFP image (twice) and the result divided by the YFP image, as described in the Experimental section. The colour code is shown in Figure 6. The map is overlayed by the cell shape. (F) The same fluorescence images processed according to the FRET algorithm of Gordon et al. [40] by the Zeiss AxioVision SE64 software. This procedure uses a lookup table with different colours. For comparison, average ratio values are notified for selected encircled areas. In our hands, the ratios obtained by the described procedure emphasized the differences between central and peripheral regions somewhat more than the Gordon method, most probably because we used a more thorough correction for different cellular amounts of the acceptor (YFP or Go-YFP).

Figure 6  Comparison of ratio images between cells expressing CFP plus YFP unmodified or fused to Go

Cell cultures transformed with Go-CFP plus Go-YFP (top panel) or with CFP plus YFP (bottom panel) were examined by fluorescence microscopy (see the Experimental section). As exemplified in Figure 5, images obtained through the FRET filter, the CFP filter or the YFP filter were related to give ratios of acceptor/donor that were further related to the amount of YFP in each cell. The ratio images were colour-coded and compared on a scale from 1–100. Note that significant differences between the two cell strains exist in the peripheral cytoplasmic regions. The maximum ratios in these areas, averaged over a 31 pixel×31 pixel square are as follows: top panel (Go-CFP plus Go-YFP strain), 62.6±7; bottom panel (CFP plus YFP strain), 37±8.

divided by an image of the specific YFP emission in order to correct for different concentrations of the acceptor in individual cells (Figure 5 and see the Experimental section). The ratio maps obtained by this procedure are comparable with those produced by the FRET algorithm of Gordon et al. [40] and a commercial software (Zeiss AxioVision SE64), but in our hands yielded more intracellular details (Figure 5).

The results collected in Figure 6 suggest that the simultaneous presence of both fusion proteins (Go-CFP plus Go-YFP) causes higher acceptor/donor ratios than the unsubstituted fluorescent proteins (CFP plus YFP) in a substantial majority of cells. The ratio maps differ mainly in the peripheral cytoplasmic areas, whereas central regions including the nucleus display lower ratios and more similarities between Go-containing and Go-free fluorescent cell lines.

Taken together, the fluorescence imaging data support a FRET-like energy transfer between the Go-coupled fusion proteins that is probably caused by interaction of Go-moieties.
its interaction with itself and with other G subunits. The results of hybrid proteins with ubiquitin moieties in yeast cells and studied or C-terminal ubiquitin (Nub13 or Cub respectively). The interaction of each protein with Gαdiploid colonies on SC medium (see the Experimental section). (A) containing Gα(Gαcub). Colonies growing with 2 mM or 5 mM methionine contain approximately 34 % or normalized to the maximum growth rate of the same clone, observed at 0 mM methionine, and rates. The results are growth rates averaged between cultures at 2 mM and 5 mM methionine, Note that diploids expressing Gα instead of Nub13 and thus do not require fused proteins to force their assembly with Cub. The mbSUS (mating-based split-ubiquitin system) was demonstrated by the split-ubiquitin assay. The cDNAs of Gα, Gβ, and Gγ were expressed in haploid yeast as fusion proteins with N-terminal or C-terminal ubiquitin (Nub13 or Cub respectively). The interaction of each protein with Gα (Eschscholzia) was force by mating the haploid strains and quantified via the growth of the diploid colonies on SC medium (see the Experimental section). (A) Diploid colonies obtained by the interaction of Nub-fusion proteins containing Gα, Gβ, or Gγ with Cub-fusion proteins containing Gα of either Eschscholzia or Arabidopsis (for comparison). No culture was obtained in the absence of Nub-fused test proteins (first row). (B) Influence of methionine on the growth rates. The results are growth rates averaged between cultures at 2 mM and 5 mM methionine, normalized to the maximum growth rate of the same clone, observed at 0 mM methionine, and set to 100 %. Colonies growing with 2 mM or 5 mM methionine contain approximately 34 % or 16 % respectively of the Gα content of those growing without added methionine [18]. The ‘control’ column shows the growth of diploids obtained with haploid strains that carry a non-mutated Nub instead of Nub13 and thus do not require fused proteins to force their assembly with Cub. Note that diploids expressing Gα plus Gβ or Gγ plus Gα show similar growth rates that are not severely depressed (less than 20 %) by increasing methionine concentrations, indicating the ability to interact even at low Gα concentrations. The interactors A14G02600 (an ML01 protein) and A1G66410 (a calmodulin) provide examples of a strong and a weakly interacting protein respectively found under similar conditions. The experiment was repeated twice and always yielded the same ranking of interactors. A.t., Arabidopsis thaliana; E.c., E. californica.

**Gα-interaction studies with the split-ubiquitin system**

The mbSUS (mating-based split-ubiquitin system) was established to detect plant membrane protein interactions [34]. This and similar two-hybrid assays proved successful in the characterization of protein complexes with a variety of membrane-integral and membrane-associated proteins [41–44]. For the present study, we expressed Gα of Eschscholzia as a part of hybrid proteins with ubiquitin moieties in yeast cells and studied its interaction with itself and with other G subunits. The results presented in Figure 7(A) indicate that two Gα molecules can form dimers with high affinity. As the mbSUS allows the ranking of interactions by their binding strength (via a methionine-controlled promoter [18]), it could be shown that the self-interaction between two Gα molecules is in the same order as the well-known interaction between Gα and Gα [1,14], whereas the binding in the Gα-Gα couple was much weaker. Figure 7(B) shows the influence of increased methionine concentrations that cause a decrease in available Gα. The assay does not discriminate between dimers and higher oligomers, and therefore it cannot be excluded that higher oligomers might be formed as well.

Thus the split-ubiquitin studies support the findings of a tight interaction between two Gα molecules obtained from the above-mentioned FRET experiments. This is especially interesting as it suggests that the dimerization of Gα molecules occurs not only in the cells of their origin but also in the heterologous yeast cell system. Currently, the self-interaction of Gα appears the most likely reason why its inhibitory power is lost at increasing concentrations. The stimulation of PLA1 by saturating amounts of Gα might reflect the involvement in the dimerization process of the inhibitory Gα present in the original plasma membrane preparation.

The functional specificity of the interaction and stimulation between Gα and PLA2

The observed impacts on PLA1 activity involved immunologically targetable sites at the Gα protein, as shown by the effects of antibodies raised against the Gα protein of Eschscholzia. As seen in Figure 3 (columns 10, 11, 13 and 14), both the inhibition by low and the stimulation by high Gα concentrations are relieved by polyclonal antisera or monoclonal single-chain (scFv) antibodies. Both types of antibodies did not affect the activity of PLA2 if added in the absence of external Gα. Further data support their selective binding to Gα: (i) the polyclonal antisemur detected only the expected 43 kDa band among the plasma membrane proteins separated by SDS/PAGE; and (ii) the anti-Gα scFv antibody preparation was pre-selected by a four-step process of repeated screening and screening for Gα-binding molecules in a relevant phage-display library (see the Experimental section) and did not detect the His6-tag of the recombinant protein (Supplementary Figure S1). Thus it appears that binding of antibody to the Gα protein impedes both its interaction with PLA2 and its self-interaction.

It is known that the association of Gα and PLA2 allows activation of the enzyme by yeast glycoprotein elicitor in the presence of GTP/GTPγS, but not GDP/GDPβS (guanosine 5’- [β-thio]diphosphate) [11]. In the present study, this stimulation attained the same level irrespective of whether inhibitory concentrations of external Gα were present or not (Figure 8, columns 1–3 and 5–10). Although GTP and GTPγS act similarly, GDβS had no stimulatory effects under any of the conditions tested (Figure 8, columns 1, 5 and 6). It is thus likely that elicitor plus GTP act at the Gα molecules bound (or accessible) to PLA2, thus releasing the enzyme from an inhibited state. Two further experiments support this conclusion.

First, the increase caused by elicitor plus GTP is significantly smaller if Gα is present at high stimulating concentrations (Figure 8, compare columns 1–3 with 11–13, the high conversion rates proved not to be limited by substrate supply). This is consistent with the tendency of Gα to undergo dimerization, as would shift the binding equilibrium away from the PLA2-associated form of the Gα protein, which confers the stimulation by GTP plus elicitor.

Second, in the antisense-Gα strain TG11, whose plasma membrane contains only approximately 15 % of the wild-type Gα content [23], the specific activity of PLA1 at the outset was...
The activity of PLA2 was measured in the solubilized plasma membrane (see the Experimental section). The indicated effectors were added 10 min prior to the membrane preparation. Numbers next to Gα are final concentrations in nM. Final concentrations of the effectors GTP, GTPγS, or GDPγS, 5 μM: yeast elicitor, 1 μg/ml; cyclosporin A, 1 μM. Antibodies, if indicated, were present at 68 nM. Results are means ± S.D. of five independent experiments, normalized to the PLA2 activity of the untreated plasma membrane (5.0 pkat/mg of protein) which is set to 100 %. Data marked by * significantly differ (95 %) from the first column of each box. Data with ** significantly differ (95 %) from GTP plus elicitor. The experiment was repeated with five different plasma membrane preparations which all yielded similar results. GTP, plasma membrane; rGα, recombinant Gα. In previous test experiments, the anti-Gα-αG fraction, the anti-Gβ-βFv antibodies and the anti-cyclophilin IgG fraction showed no intrinsic PLA2 activity at the concentration used (results not shown).

*Figures 8 and 9.*

**Figure 8.** Influence of low-molecular-mass effectors and antibodies on the interaction of Gα and PLA2.

**Figure 9.** Effect of external Gα, GTP and elicitor on the activity of PLA2 in the mutant TG11.

In an experiment comparable with that shown in Figure 3, the activity of PLA2 was measured in the solubilized plasma membrane of the mutant TG11. The Gα content of this preparation is 15 % compared with the wild-type [23]. Numbers next to Gα indicate the final concentration in nM. Data with * are significantly different (95 %) from the untreated plasma membrane (first column). The results are means ± S.D. of five independent measurements, normalized to the PLA2 activity of the wild-type plasma membrane, which is set to 100 %. In comparison, the PLA2 activity of TG11 is approximately 120 %. elf, yeast elicitor; PM_TG11, mutant plasma membrane; rGα, recombinant Gα; gGα, native Gα. The experiment was repeated with four different plasma membrane preparations, which all yielded similar results.

**DISCUSSION**

The relevance of our experimental data for the understanding of the PLA2-Gα complex rests on the assumption that the added Gα protein is functionally intact and equivalent to the native protein associated with the plasma membrane of *Escherichia coli* cells. This is supported by the following facts: first, the recombinant Gα and the native Gα are products of the same gene, but produced by entirely different procedures (a bacterial His6-tagged product, purified by Ni2+-affinity chromatography, or a soluble protein of the plant cell homogenate, purified by immune absorption) and are thus unlikely to contain similar contaminants. Nonetheless, either protein caused the same inhibitory or stimulatory effects on PLA2 if added to the plasma membrane. Secondly, either protein reconstitutes the low-Gα mutant TG11 with respect to the GTP-dependent activation of PLA2. Thirdly, potential bacterial contaminants of the recombinant Gα preparation had no influence on PLA2 activity and the impact of Gα, as tested with extracts from non-Gα-expressing bacteria.

The most plausible interpretation of our results leads to the following five conclusions. They are followed by the main results supporting them. Figure 12 summarizes our interpretation of the flexible architecture and PLA2 activities of protein complexes in the form of a hypothetical model.
Figure 10  The DNA sequence coding for the cyclophilin identified in the plasma membrane of *Eschscholzia* and sequence alignment of selected plant cyclophilins (NCBI BLASTp)

(A) The gene was identified by 3′ and 5′ genome walking using genomic DNA. The primer sequences (see the Experimental section) and the start and stop codon (ATG and TGA) are underlined. Shadowed sequences mark the primers used for PCR, which amplified the ORF that was cloned in *E. coli* (see the Experimental section). The sequence data of the *Eschscholzia* cyclophilin have been deposited in GenBank® under the accession number JQ886493. (B) The amino acid similarity or identity between the plant sequences is >90% or >70%, and 79% or 71% between human and *Eschscholzia*. Non-identical amino acids are shadowed.

Soluble Gα interacts with the plasma membrane complex of PLA2 and Gα at the cytoplasmic side, thereby changing its structure and functionality

Gα is a soluble protein of the cytoplasm in *Eschscholzia* as shown by cell fractionation and confocal imaging of a Gα-GFP hybrid protein (Figures 1 and 2). Addition of increasing amounts of Gα, recombinant or native, changed the PLA2 activity of the solubilized plasma membrane in an unique manner (Figure 3). The Gα-deprived mutant TG11 reacted to the addition of Gα much like the wild-type, indicating that
The activity of PLA₂ was measured in the isolated and solubilized plasma membrane in the presence and absence of 45 nM Gₐ. Inhibitors were present at 1 μM. The results are the means ± S.D. of five independent measurements, normalized to the PLA₂ activity of the untreated plasma membrane (5 pkat/mg of protein) which is set to 100%. The experiment was repeated with seven different plasma membrane preparations which yielded similar results. Data marked with * significantly differ (95 %) from rGₐ, as cyp, IgG fraction of anti-human-cyclophilin antiserum; CsA, cyclosporin A; PM, plasma membrane; rGₐ, recombinant Gₐ; TG11, plasma membrane of low-Gₐ mutant.

The scheme presents the most plausible interpretation of present and previous data [11,27,41]. The tight binding to PLA₂ of low (native) Gₐ prevents any stimulation. The activation of PLA₂ by elicitor in the presence of GTP [11] probably reflects the relief of the enzyme from a pre-existing inhibited state.

With increasing Gₐ concentrations, this protein undergoes self-interaction, thereby competing with its binding to PLA₂. Both the FRET-based analysis and the yeast split-ubiquitin assay indicated a high affinity of Gₐ for di- or oligo-merization (Figures 4, 6 and 7). Although the actual outputs of both assays are only qualitative or semiquantitative in nature, it is remarkable that products of the same Gₐ gene display the same tendency in the *Eschscholzia* cell and in yeast. The di- or oligo-merization at increasing concentrations most likely explains why the effect of added Gₐ changes from inhibition to stimulation of PLA₂ (Figure 3). The stimulation of PLA₂ by elicitor plus GTP was higher at 45 nM Gₐ than at 900 nM Gₐ, which is consistent with the loss of PLA₂-bound Gₐ at high concentrations. The latter conditions allow the highest specific activity of PLA₂ to be measured in the solubilized plasma membrane (approximately 190 %, Figure 8). Under such conditions, most of the inhibitory Gₐ molecules are expected to be inaccessible to the enzyme due to di- or oligo-merization, with the remainder maximally stimulated by elicitor plus GTP.

The interaction between PLA₂ and Gₐ involves the catalytic activity of a cyclophilin.

A plant-type PPIase co-precipitated with Gₐ and the cognate gene could be cloned (Figure 10). Also, an anti-cyclophilin antibody caused a significant inhibition of PLA₂ activity (Figure 11). The basal level of PLA₂ activity, its increase caused by elicitor plus GTP, the inhibitory effect of low Gₐ and the stimulation caused by high Gₐ concentrations were all attenuated by cyclosporin A (Figures 8 and 11).

The effect of cyclosporin A is related to the endogenous Gₐ content: the low-Gₐ mutant TG11 is less strongly inhibited than the untreated wild-type (Figure 11, TG11). Taken together, our data show that PLA₂ is regulated by Gₐ via controlled inhibition. In the absence of elicitor, the Gₐ contained in the plasma membrane complex keeps the enzyme in a low activity state. This inhibition can be intensified by increasing the native Gₐ content (approximately 20 nM under assay conditions) up to 3-fold, i.e. through addition of approximately 45 nM of the native or recombinant protein.

The tight binding to PLA₂ of low (native) Gₐ concentrations is well documented, e.g. by various immunoprecipitations and native protein electrophoresis that revealed detergent-resistant

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Figure 11 Influence of cyclosporin A and anti-cyclophilin antibodies on PLA₂ activity

Figure 12 Hypothetical scheme of the complex interaction of Gₐ and PLA₂

The impact of Gₐ on PLA₂ is inhibitory in nature

Low Gₐ concentrations inhibited the enzyme activity under a variety of conditions (Figures 3 and 8). In the absence of extra Gₐ, the low-Gₐ mutant TG11 displayed a higher specific activity of PLA₂ than the wild-type (Figure 9). The loss of Gₐ (85 %) is stronger than the gain of enzyme activity (20 %), which is consistent with earlier findings that not all Gₐ molecules of the plasma membrane are part of the complex [11] and could also reflect a different architecture of the mutant plasma membrane complex.
complexes of approximately 130 and 170 kDa which did not dissociate upon addition of GTP plus elicitor [11]. The elicitor signal causes a release from inhibition, most probably via the known conformational activation of $G_{\alpha}$ that requires GTP. Although not the focus of the present study, we assume that this elicitor effect is mediated via a GPCR (G-protein-coupled receptor), as a seven-transmembrane protein (subfamily MLO1) was found among the interactors of $G_{\alpha}$ in the split-ubiquitin assay (Figure 7B and C. Massalski and W. Roos, unpublished work). Classically, plant G-proteins are thought to be coupled to seven-transmembrane receptors [47] that share limited homology with animal GPCRs [48] and homologues of this type are present in an actual interactome of $G_{\alpha}$ of Arabidopsis [17].

A novel mechanism that tends to shift $G_{\alpha}$ away from the PL$A_2$-inhibiting state is the self-interaction of this protein at high local concentrations. The competition of oligomerization with the binding to PL$A_2$ probably gives rise to complexes of different G$\alpha$/PL$A_2$ stoichiometry. Such complexes would also display different influences of GTP at the enzyme activity level. As a consequence, a broad range of PL$A_2$ activities can be expected, each depending on the actual $G_{\alpha}$ content and GTP level. Not only would their basal PL$A_2$ activity be different, but also the final amplification of the elicitor signal would differ significantly (Figure 12).

From the biological point of view, the results of the present study support the idea that soluble $G_{\alpha}$ in the neighbouring cytoplasm can modulate the activity of the target PL$A_2$ at the plasma membrane via exchange and equilibration with the bound G-protein. Although, in the cell culture used in the present study, the cellular content of $G_{\alpha}$ did not undergo dramatic changes (M. Heinze and W. Roos, unpublished work), differences in the $G_{\alpha}$ levels associated with growth and cellular development [49,50] are worthwhile for investigation in future studies. In either case, local imbalances in soluble $G_{\alpha}$ probably influence the architecture and effectiveness of the PL$A_2$-containing complex(es).

Cyclophilins, which have not yet been reported as components of the $G_{\alpha}$-containing complexes, may now be considered as intrinsic catalysts or mediators of the interaction between $G_{\alpha}$ and its neighbouring targets. The involvement of these chaperone-like proteins, as suggested from the results of the present study, could motivate new experiments that aim at understanding the conformation-based signal transfer within a $G_{\alpha}$-target complex.

It also remains an aim of further experiments to determine whether or not the control of PL$A_2$ at the plasma membrane involves the $G_{\alpha}$ complex or its components. In vitro studies compatible with that shown in the present study are hampered by the lack of supply of $G_{\alpha}$, as the recombinant overproduction of this plant plasma membrane-associated protein has not yet proved successful (U. Conrad, unpublished work). The $G_{\alpha}$ subunit of Arabidopsis is available via recombinant expression in bacteria, and preliminary data show a significant influence of this protein on the interaction between $G_{\alpha}$ and PL$A_2$. (Supplementary Figure S3 at http://www.biochem.org/bj/450/bj4500497add.htm). This might serve as a first indication that $G_{\alpha}$, whose interaction with $G_{\alpha}$ is weak but still detectable (Figure 7B), can modulate the interaction between $G_{\alpha}$ and its target. However, to obtain biologically relevant data, experiments that include the simultaneous presence of the $G_{\alpha}$ and $G_{\alpha}$ subunit of Escherichia coli is indispensable. Thus, although the regulatory effect of the $G_{\alpha}$ subunit clearly dominates the PL$A_2$-dependent signal path in Escherichia coli (the present study and [23]), modulating influences of the other subunits cannot be excluded. In Arabidopsis, the use of mutants either lacking or with reduced amounts of a distinct G-subunit, has yielded evidence that distinct signal paths only require $G_{\alpha}$, whereas others require the $G_{\beta\gamma}$ complex or the heterotrimer [8,10,51,52]. To date, the interaction of $G_{\alpha}$ and PL$A_2$ as documented by the present study is not reflected in genome-wide interactome studies in Arabidopsis: one of the published sequences in [17] shows detectable, but very low, similarity to the PL$A_2$ of Escherichia coli. Such discrepancies might be resolved in the near future by completing the coverage of this and related genomes and performing a more thorough comparison with other species. Although in Arabidopsis $G_{\alpha}$ is almost completely located at the plasma membrane and ER (see above), the co-existence of monomeric and complex-bound $G_{\alpha}$ has been reported [14].

Thus the present data may open new research avenues to understand the diversity of available and new $G_{\alpha}$ interactors. The interplay of bound and soluble $G_{\alpha}$ can create membrane-associated complexes of varying architecture and efficiency. They constitute a hitherto underestimated level of tuning in G-protein-controlled signalling which contributes much to its flexibility and adaptability.

**Author contribution**

Michael Heinze did the assays of PL$A_2$ activity, plasma membrane preparations, and established and characterized all of the transgenic Escherichia coli strains. Madeleine Herre, supervised by Michael Heinze and Werner Roos, contributed with PL$A_2$ assays in the G-protein-transfected plasma membrane. Werner Roos and Michael Heinze did the FRET analysis of $G_{\alpha}$ interaction. Carolin Massalski did all the mSUS appropach experiments and supplied plasma membrane samples of Arabidopsis. Udo Conrad and Isabella Herrmann produced and characterized recombinant $G_{\alpha}$ and $G_{\beta\gamma}$, and anti-$G_{\alpha}$ scFv antibodies and antisera. Werner Roos co-ordinated the study and wrote the paper.

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**References**


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SUPPLEMENTARY ONLINE DATA

Signal transfer in the plant plasma membrane: phospholipase A\textsubscript{2} is regulated via an inhibitory G\textsubscript{\alpha} protein and a cyclophilin

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Figure S1 Specific binding of anti-G\textsubscript{\alpha} scFv2 to G\textsubscript{\alpha}

The graph shows the activities of alkaline phosphatase (means ± S.D., n = 3) obtained using the indirect ELISA assay (see the Experimental section of the main text). Background binding to BSA was subtracted. The three antigens were produced in bacteria using the same vector (pET23a). Thus the G\textsubscript{\gamma} protein preparations contain the same tags and the same bacterial impurities (if any) as G\textsubscript{\alpha}. Note that these non-G\textsubscript{\alpha} epitopes (including G\textsubscript{\gamma}) are not detected by the used scFv.

Figure S2 Expression of genes encoding G\textsubscript{\alpha}–CFP, G\textsubscript{\alpha}–YFP and G\textsubscript{\alpha}–eGFP fusion proteins in \textit{Eschscholzia} cells

RT–PCR was performed with mRNA extracted from transformed cells 4 days after biolistic gene transfer. Samples were transcribed into cDNA and used as templates for PCR with specific primers as indicated. The expression of the G\textsubscript{\alpha}–CFP and G\textsubscript{\alpha}–YFP fusion genes was tested by amplifying the full-length ORF and a shorter sequence, which contains the 3′-part of the gene encoding G\textsubscript{\alpha} plus a fluorophore gene. Lane 1, DNA molecular markers with values in bp (Fermentas SM1331). All samples except four contained template mRNA from cells transformed with G\textsubscript{\alpha}–CFP plus G\textsubscript{\alpha}–YFP. Lane 2, primers G\textsubscript{\alpha}1 for/CFP rev; lane 3, primers G\textsubscript{\alpha}1 for/YFP rev; lane 5, primers G\textsubscript{\alpha}2 for/CFP rev; lane 6, primers G\textsubscript{\alpha}2 for/YFP rev. Lane 4, mRNA of cells transformed with G\textsubscript{\alpha}–eGFP, primers G\textsubscript{\alpha}1 for/eGFP rev. Each band shown in the Figure corresponded to the calculated product size. Primers are given in Table 1 of the main text.

Figure S3 A G\textsubscript{\gamma} protein attenuates the inhibitory effect of G\textsubscript{\alpha} at PLA\textsubscript{2}

Using the same experimental design as in Figure 3 of the main text, 900 nM of recombinant G\textsubscript{\gamma} from \textit{Arabidopsis} was added to the plasma membrane either alone or together with inhibitory concentrations of G\textsubscript{\alpha} and the resulting PLA\textsubscript{2} activity was measured as described in the Experimental section of the main text. Data are means ± S.D., n = 3. In a parallel experiment, G\textsubscript{\gamma} displayed similar effects (results not shown). rG\textsubscript{\alpha}, recombinant G\textsubscript{\alpha}; PM, plasma membrane.

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The nucleotide sequence data of the \textit{Eschscholzia} cyclophilin reported will appear in GenBank®, EMBL, DDBJ and GSDB Nucleotide Sequence Databases under the accession number JQ886493.