Visualizing G protein signaling in living cells
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Citation for published version (APA):

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Chapter 7

Concluding remarks and Outlook
Chapter 2

First the gas pedal, then the brakes; primordial heterotrimeric G protein signaling in plants

Lipidation of GPα1 is highly comparable to its closest mammalian homolog Gαi1, whereas its GTPase and GDP exchange properties are entirely different (Willard and Siderovski, 2004). The plant Gα subunit combines a very low GTPase activity with a very high spontaneous release of GDP, a characteristic that is not found in any of the known mammalian heterotrimeric G proteins where instead of GAP activity, GDP release is the limiting step and in need of GEF activity provided by activated GPCRs. Plant heterotrimeric G protein subunits are judged to be representative of the ancient heterotrimeric G protein when compared with their mammalian counterparts (Temple and Jones, 2007).

The high rate of spontaneous GDP release may be caused by the divergent G-5 region. Iiri et al. (1994) described several natural mutations in this region in heterotrimeric Gα subunits that cause diseases in humans. As stated in Chapter 2, it may therefore very well be that the GPα1 wild-type protein is GTP-bound by default. Indeed, the rates determined in vitro predict that 99% of GPα1 is GTP-bound in ‘resting’ state (Temple and Jones, 2007). The discovery of a 7TM-RGS amalgam (termed RGS1) in Arabidopsis (Chen et al., 2003) led to the enticing idea that plant heterotrimeric G protein signaling may be backward as compared to mammalian signaling. Certainly, the RGS module may also keep the G protein in its GDP-bound form until the GPCR module becomes active, but this implies the occurrence of pre-coupling. Interestingly, unpublished FRET data suggest that GPα1 interacts with the GPCR-RGS amalgam upon recognition of a ligand, D-glucose, which supposedly leads to GTP-hydrolysis and concurrent loading of GPα1 with GDP. It is proposed that the GPCR-RGS amalgam split into two separate genes in the animal lineage after the divergence between plants and animals about 1.6 billion years ago, leading to the wealth in GPCR isoforms and RGS proteins present in contemporary mammals (Temple and Jones, 2007) (Chapter 1, Sections 1 and 5).

Taking these data into account, I conclude that there is no difference between the wild-type GPα1 and GPα1-Q222L presented in Chapter 2: both being primarily GTP loaded. Importantly, the fact that wild-type GPα1 is GTP-bound does not change our conclusions with respect to the integrity of the heterotrimer. However, the question that remains is: does the trimer change when GPα1 binds GDP? The Arabidopsis subunits could be efficiently modeled on the crystal structure of a mammalian heterotrimeric G protein, which suggests that the GTP-bound form is less apt at forming a trimer as compared to the GDP-bound form of GPα1. Therefore, dissociation upon binding of GDP is not expected. Yet, a conformational change could occur. An interesting experiment to be done is monitoring the effect of swapping the G-5 region for that of mammals. It is
expected that this will severely decrease the rate of spontaneous GDP release and thereby lead to GDP-bound \( \text{GP}\alpha_1 \). In addition, the consensus sequence R-[R/S/A]-[R/S/A/N]-E-[Y/F]-[Q/H]-L, as described in Section 3.3.1 (Chapter 1), is not conserved in plant \( \text{Go} \). Mutation of these residues in \( \text{Go} \) led to an increase in receptor-independent GDP release (Hinrichs et al., 2004). The lack of a salt bridge formed by the R and E residues may in part explain the observed high spontaneous GDP release in \( \text{GP}\alpha_1 \).

Application of this sequence in the plant \( \text{Go} \) subunit may also aid in the generation of its GDP-bound state. This would enable the study of the interaction between GDP-bound \( \text{GP}\alpha_1 \) and the \( \text{Go}\gamma \) dimer by means of FLIM and provide a direct comparison with the lifetime data presented in Chapter 2 for the GTP-bound forms.

**Sticky fingers versus greasy handles**

\( \text{G}\gamma \) lipidation data in whole *Arabidopsis* plants were published in Plant Physiology (Zeng et al., 2007) just after our publication (Adjobo-Hermans et al., 2006) and they largely coincide with our data. Like us, they showed that \( \text{G}\beta\gamma \) localization to the plasma membrane is not dependent on \( \text{Go} \) and that dual lipidation of AGG2 is essential for membrane localization. In addition, they verified our hypothesis with regard to the possible role of the short charged sequence specific to AGG2 playing a role in subcellular localization. Swapping of this region into AGG1 leads to a subcellular localization comparable to that of AGG2, suggesting that this region promotes acylation of the cysteine proximal to the prenylated cysteine in the CaaX box of AGG1.

As to the localization of AGG1, they found this \( \text{G}\gamma \) subunit to partly co-localize with the Golgi-marker Man1. The lack of a complete overlap is explained by a preferred association with the trans-side of the Golgi, contrary to Man1 that is enriched at the cis-medial side of the Golgi stacks. Remarkably however, they found AG\( \beta_1 \) to be dispensable for plasma membrane targeting of AGG1, which is not consistent with our data. However, they did find AGG2 to be more abundant at the plasma membrane, whereas AGG1 is more enriched on Golgi vesicles. Possibly, a protein that aids in AGG1’s plasma membrane attachment is absent in cowpea protoplasts. The similarities in both studies demonstrate that the cowpea protoplast system is a reliable model system to study heterotrimeric G protein signaling in plants.

**Getting acquainted at the plasma membrane**

All mammalian heterotrimeric G proteins are thought to assemble before they reach the plasma membrane. However, experiments have not been conclusive, especially as to the locale where they first meet. A rationale for assembly prior to plasma membrane-targeting is the argument that \( \text{G}\beta\gamma \) is constitutively active in the absence of its \( \text{Go} \) partner. Therefore, assembly at for instance the Golgi would prevent spurious activation
of plasma membrane-localized effectors by G\(_{\beta\gamma}\). Strikingly, this mechanism does not seem applicable in yeast (Manahan et al., 2000), yet in this organism G\(_{\beta\gamma}\) is the main relaying signaling protein! Similarly, preassembly of the heterotrimer in plants does not seem a prerequisite to be able to attain the plasma membrane (Chapter 2). Likely, this is caused by the fact that G\(_{\gamma}\) subunits in these organisms are dually lipidated and therefore have a much higher initial affinity for the membrane, as compared to the singly prenylated G\(_{\gamma}\) subunits that are prominent in mammals. Consequently, the question arises as to how these G\(_{\beta\gamma}\) subunits are kept in the inactive state. Presumably, accessory proteins, such as phosducin, bind G\(_{\beta\gamma}\) dimers and effectively shield them from their downstream partners (Savage et al., 2000). Alternatively, G\(_{\beta\gamma}\) dimers may not be constitutively active in the absence of G\(_{\alpha}\) subunits. Several studies suggest a role for the lipid tail in inducing a conformational change in the G\(_{\beta\gamma}\) dimer, which is necessary for interaction with effectors (Loew et al., 1998; Myung and Garrison, 2000). Such conformational changes could not have been found in the crystal structures of mammalian heterotrimeric G proteins because non-lipidated trimers were used (Lambright et al., 1996; Wall et al., 1995).

**Functional selectivity forced upon G\(_{\beta\gamma}\) by G\(_{\gamma}\)**

A recent study reports on the effects of AGG1 and AGG2 deficiency (Trusov et al., 2007). Their gene expression patterns rarely overlap, which suggested the possibility of specific signaling properties of the two G\(_{\gamma}\) subunits. Indeed, AGG1 appeared specifically involved in resistance against fungal pathogens. In addition, AGG1 and AGG2 are involved in regulation of lateral root development, AGG1 controlling acropetal auxin transport and AGG2 controlling basipetal auxin transport. Since gravitropism is regulated by the latter, both AGG1- and AGG2-deficient mutant plants are less responsive to gravistimulation. The two G\(_{\gamma}\) subunits also play different roles in germination; AGG1 is primarily involved in abolishing the osmotic component of germination inhibition, while AGG2 plays a role in glucose signaling. The observed differences in localization between AGG1 and AGG2 (Chapter 2) may lie at the basis of these specific signaling properties.

**Chapter 3**

**Disqualifying the dissociation dogma**

The results, presented in Chapter 2 and Chapter 3, suggest that, with respect to AtGP\(_{\alpha1}\) and HsGu\(_{\alpha}\), the dissociation dogma is not applicable. As proposed in Chapter 3, G\(_{\beta\gamma}\) may stay attached to G\(_{\alpha}\) through a sustained interaction with the N-terminus of the G\(_{\alpha}\) subunit. This hypothesis is based on the fact that mutation of the G\(_{\beta}\) residues N88/K89 that interact with \(\alpha N\) has the most severe effect on the interaction with G\(_{\alpha}\) subunits (Li et
al., 1998). Conversely, mutation of these residues has little effect on the activation of PLCβ2/3 and regulation of ACI/II. Thus far, PTX-mediated ADP ribosylation of the Gα subunit has been used as a measure of the efficiency of heterotrimer formation, since this process is enhanced by the Gβγ dimer. However, relatively high concentrations of the subunits (> 1μM) are required and the exact mechanism remains unclear (Hou et al., 2000). Using this assay, the constitutively active Gαo(Q205L) was found to be a good substrate for ADP ribosylation in the presence of Gβγ, again suggesting that Gβγ does not dissociate from active Gα subunits at all times.

The discrepancies observed between the crystal structures and the data obtained in living cells may be explained by the absence of lipid modifications in the crystallized proteins. In addition, all structural studies have been done in aqueous media, whereas the physiologically relevant environment is the inner leaflet of the plasma membrane. Interestingly, a lack of dissociation implies that it is the heterotrimer that signals. In addition, this may increase signaling specificity and speed by reducing diffusion distances and maintaining optimal stoichiometry. The sustained interaction of Gβ with αN may enable enough space for effectors to interact with both Gα and Gβγ. However, depending on the effector species, cell signaling may also be abrogated for Gα or Gβγ or both Gα and Gβγ. Importantly, Wedegaertner and co-workers found Gβγ to be essential for activation of PLCβ via Gαq, which is suggestive of a signaling function for the heterotrimer (Evanko et al., 2005). Also, Ross and co-workers reported Gβγ to have a much higher affinity for active Gα than was previously supposed (Tang et al., 2006). They found Gβγ to compete with PLCβ for Gαq-GTP, thereby inhibiting PLCβ-GAP activity. The N-terminus of PLCβ was not required for this competitive behavior, suggesting that the effect of Gβγ is on the level of Gαq. Differentially changing the affinity of Gαq for either Gβγ or PLCβ could give more insight into this mechanism.

**Functionality & efficiency**

The development of a functional fluorescent protein-tagged Gαq has proven to be a daunting task and stresses that testing the functionality of FP-tagged proteins is of utmost importance. In addition, the FRET pair that was subsequently prepared had to be improved, since FRET did not change upon activation of the G proteins. Apparently, removal of 8 residues from the C-terminal tail of CFP forces the fluorophore in an orientation that is more apt at detecting subtle changes in the orientation between the CFP inserted into Gαq and the YFP fused to Gi2. This indicates that FRET pairs can be optimized by changing the relative orientation of the fluorophores, as shown formerly for the calcium FRET sensor cameleon (Miyawaki et al., 2003; Truong et al., 2001).
The FRET pair we prepared to measure Gq activation is the first heterotrimeric G protein that shows recovery of the activation response in the continued presence of agonist. The FRET pairs that have been developed to monitor Gi/o and Gs activation do not recover, not even when the agonist is removed. Only upon addition of an antagonist does the FRET ratio increase to basal level. It is not known why Gq is deactivated faster as compared to the published heterotrimeric G proteins from yeast and Dictyostelium and the human family members. It might be that the HeLa cell line used by us contains a high amount of RGS proteins that accelerate GTP hydrolysis. Alternatively, this fast recovery may be specific for the Gq heterotrimer, since the hydrolysis of its GTP is also accelerated by its effector, PLCβ. This would mean that signaling via Gq is strictly controlled, which is probably necessary in order to prevent a large, long-lasting, decrease in PtdIns(4,5)P₂ and a large increase in intracellular calcium, both of which can induce apoptosis (Berridge et al., 2000; Halstead et al., 2005). In agreement, stable transduction of the constitutively active Gα₉(Q209L) into vascular smooth muscle cells leads to apoptosis (Peavy et al., 2005). Similarly, overexpression of Gα₉(Q209L) in cardiomyocytes causes apoptosis (Adams et al., 1998).

Certainly, protein expression levels in single cells have to be larger than 0.1-1 μM to enable identification of transfected cells and to obtain a sufficiently good signal-to-noise ratio. Such concentrations may not be representative of physiological amounts and can interfere with normal signaling (Teruel and Meyer, 2000). Decreased expression of the G proteins can be achieved by transducing MEF cells devoid of Gα₁₁ subunits with Gα₉H₁-CFP, Gβ₁ and YFP-Gγ₂ and focus on clones with low expression levels.

**What effects do accessory proteins/drugs have on the activation of Gq?**

Now that we can monitor Gq activation, the kinetics and spatial distribution of this signal can be investigated and the dose response of downstream signaling events can be determined. In addition, this FRET pair enables examining the effects of accessory proteins on Gα₉-Gβγ interaction. For instance, GRK2 has been shown to interact with Gα₉ via its RGS homology (RH) domain and with Gβγ through its PH domain. Recently, this complex has been crystallized and shows that Gα₉ and Gβγ are physically separated in the presence of GRK2 (Tesmer et al., 2005). Ratiometric FRET imaging and FLIM will be helpful in elucidating whether this separation actually occurs at the plasma membrane of living cells. Furthermore, the effect of NHERF proteins, of which NHERF1 has been published to interact with Gα₉, can be tested. NHERF1 is able to inhibit PLCβ signaling by virtue of this interaction. Competition experiments can be performed between NHERF1 and (the CT domain of) PLCβ in order to determine their relative affinity for Gα₉. In addition, the effect of using Gα₉ mutants that are no longer able to interact with RGS proteins can be tested.
Heterotrimeric G protein activation is a very early signaling event and is less subject to amplification or regulation, as compared to the general approach of sampling second messenger production in pharmacology (Seifert et al., 1999). Therefore, the FRET pair described in Chapter 3 provides a means to screen the effects other proteins or drugs have at the level of the G protein itself. Certainly, ligand/receptor screening is also highly sensitive since it does not entail an amplification step, but insight into the specific G protein involved cannot be ascertained.

Chapter 4 and 5

**PLCβ activation mechanism**

The activation mechanism of PLCβ isozymes seems to involve the XY linker that occludes the active site in the crystal structure of PLCβ2 (Jezyk et al., 2006). Removal of this linker led to constitutive activity in PLCβ2 (Zhang and Neer, 2001). Displacement of this XY linker is therefore thought to enable activation of the full-length enzyme. Plasma membrane interaction may fulfill this role since the negatively charged XY linker and the negatively charged plasma membrane are thought to repel each other, inducing a movement of the XY linker, thereby opening up the catalytic cleft. However, PLCβ1a and PLCβ4a are already located at the plasma membrane. The fact that they are not constitutively active suggests that their catalytic site may not be efficiently oriented towards the plasma membrane. A change in orientation may then be required in order to position the substrate binding site correctly under the plasma membrane in order to reach and hydrolyze PtdIns(4,5)P2. It may be GTP-bound Gαq alone that mediates this structural rearrangement. Alternatively, such a change in orientation may require the heterotrimer, as suggested by our results (Chapter 3) and those of Evanko et al. (2005). Figure 20 (Chapter 1) shows a model of the activation of PLCβ2 by Gβγ. This mechanism requires an interaction between Gβγ and the PH domain of PLCβ2, in addition to an interaction with the XY barrel. Importantly, PLCβ1 and PLCβ4 are not activated by Gβγ dimers. Moreover, the inhibitory effect of Gβγ on the GAP activity of PLCβ1 towards Gαq was shown to be independent on the PH domain of PLCβ. Therefore, we hypothesize that the Gαq subunit in the heterotrimer holds on to the full-length protein by interacting with the CT domain, enabling Gβγ to bind the XY barrel and work as a lever to bring the catalytic site closer to the plasma membrane. If the XY binding model is correct, overexpression of a peptide encoding the Gβγ interaction interface should lead to a decrease in PLCβ activity. In addition, if Gβγ is crucial in the activation of PLCβ via Gαq, mutations that reduce the affinity of Gαq for Gβγ are expected to have a profound impact on PLCβ activity. Unfortunately, the constitutively active Gαq-Q209L mutant that has always been used as a protein defective in Gβγ interaction turned out to have a much
higher affinity for Gi\_\gamma than initially supposed (Tang et al., 2006). Likely, G\_\alpha\_q subunits with mutations in their N-terminus will provide the best candidates with compromised Gi\_\gamma binding. However, as shown in Chapter 3 and by Evanko and colleagues, such mutants do no longer localize sufficiently to the plasma membrane (Evanko et al., 2001).

Importantly, the PLC\_\beta\, dimer appears to offer the optimal interface for activation by G\_\alpha\_q. Probably, this is caused by a decrease in the concentration of G\_\alpha\_q needed to convert to full activity upon dimerization of PLC\_\beta, which has been described by Ross and co-workers (Paulssen et al., 1996). In addition, weak binding to the plasma membrane can be enhanced by oligomerization (Carlton and Cullen, 2005). Interestingly, Filtz and co-workers reported differences among the PLC\_\beta isozymes in their aptitude to form dimers, which may have effects on plasma membrane binding, GAP activity and activation by G\_\alpha\_q (Zhang et al., 2006). In order to investigate the dimerization of PLC\_\beta isozymes and its regulation upon activation by G\_\alpha\_q, ratiometric FRET imaging can be applied.

G\_\alpha\_q interaction & PtdIns(4,5)P\_2 binding

The CT domain of PLC\_\beta\, clearly plays two roles: 1) it is needed for activation by GTP-bound G\_\alpha\_q; 2) it causes the full-length PLC\_\beta\, to tether to the plasma membrane by means of PtdIns(4,5)P\_2 and possibly also other phosphoinositides or acidic phospholipids such as PS or PA (Boguslavsky et al., 1994; Ross et al., 2006). Therefore, PLC\_\beta\, is tethered to the plasma membrane by its own substrate and, as described in Chapter 5, upon hydrolysis of this substrate it can no longer attach to the plasma membrane. Likely, this translocation from the membrane upon activation of G\_\alpha\_q constitutes a negative feedback control with respect to PtdIns(4,5)P\_2 hydrolysis and intracellular calcium mobilization. Such a control mechanism may be crucial, since a cell can go into apoptosis when the intracellular Ca\^{2+} concentration is elevated for continuous time spans. Additionally, decreasing the level of PtdIns(4,5)P\_2 produces a dramatic release of the cytoskeleton from the membrane and has been shown to trigger apoptosis as well (Raucher et al., 2000). The initial binding of PLC\_\beta\, to the plasma membrane might be regulated by the so-called pipmodulins, e.g. MARCKS (myristoylated alanine-rich C kinase substrate), gelsolin and CapG. The last two proteins are members of the actin filament severing and capping protein family and they inhibit PLC\_\beta and PLC\_\gamma activity by sequestration of PtdIns(4,5)P\_2. These proteins are also regulated during signaling and are therefore expected to fluctuate with respect to their cytosolic and membrane distribution (Sun et al., 1997). It will be interesting to determine the effect of these pipmodulins on the translocation behavior of PLC\_\beta isozymes.

Controversy exists as to the involvement of the N-terminal cysteines of G\_\alpha\_q in the attachment to the plasma membrane of the heterotrimer and in PLC\_\beta activation.
Localization of G\textsubscript{\alpha} to the plasma membrane is needed for activation of PLC\textsubscript{\beta}, but it does not seem to matter which lipid tail targets the G\textsubscript{\alpha} subunit to this locale. However, activity appears decreased by a tag with a lower affinity for the membrane (i.e. a myristoyl lipid tail). Apparently, the CT domain does not induce repalmitoylation of G\textsubscript{\alpha} in order to increase its residence time at the plasma membrane. The mechanism that the CT domain does apply remains to be determined, but it might increase the residence time of active G\textsubscript{\alpha} at the plasma membrane by bringing it close to a scaffolding protein, thereby enabling an interaction between the two proteins. The affinity of CT\textsubscript{\beta\textsubscript{1a}} for active G\textsubscript{\alpha} appears to be the highest one, when compared to CT\textsubscript{\beta\textsubscript{3}} and CT\textsubscript{\beta\textsubscript{4a}}. This particular ranking is also observed when the ability to redirect constitutively active G\textsubscript{\alpha} to the plasma membrane is ascertained. In addition, the effects of the CT domains on G protein activation (Chapter 3) positively correlate with the data presented in Chapter 4. Strikingly, the affinity for G\textsubscript{\alpha} does not correlate with the residence time at the plasma membrane, since CT\textsubscript{\beta\textsubscript{4a}} displays similarly high plasma membrane fluorescence as compared to CT\textsubscript{\beta\textsubscript{1a}}, whereas CT\textsubscript{\beta\textsubscript{3}} is much less prominent at the plasma membrane. The lower affinity of CT\textsubscript{\beta\textsubscript{4a}} for active G\textsubscript{\alpha} may be increased by means of a scaffolding protein that brings the two together at the plasma membrane. For instance, NorpA, which is Drosophila’s homolog of PLC\textsubscript{\beta\textsubscript{4}}, only functions properly (both as a GAP and a PtdIns(4,5)P\textsubscript{2} hydrolase) in the presence of its scaffold InaD (Cook et al., 2000), stressing the importance of multiprotein structures. Correct stoichiometry also appeared crucial in this signaling pathway and may be disturbed in the experiments described in Chapter 5, where PLC\textsubscript{\beta\textsubscript{1a}} translocates to the cytosol upon stimulation of the G\textsubscript{\alpha} pathway. Overexpression of this enzyme possibly distorts the ratio between scaffold and substrate (PLC\textsubscript{\beta\textsubscript{1a}}) and may trigger movement into the cytosol of those enzymes that are not tethered through a PDZ domain. In future studies, the relevance of the PDZ domain-binding motif in this translocation should be ascertained by means of a PLC\textsubscript{\beta\textsubscript{1a}} PDZ domain-binding motif mutant.

Having a signaling complex
The dependence of the full-length PLC\textsubscript{\beta\textsubscript{3}} on scaffolding proteins in order to reach the plasma membrane is very pronounced. This affinity may be regulated by coincidence detection of PtdIns(4,5)P\textsubscript{2} by the CT domain and interaction with scaffolding proteins by means of its C-terminal PDZ domain-binding motif. GPCR-PLC\textsubscript{\beta} signalosome formation by NHERF family members prolongs calcium signaling. It remains to be determined whether a similar increase in potency occurs for the other scaffolding partners identified, e.g. Shank, ZO-1 and Par. Importantly, these proteins likely induce compartmentalization of their protein partners, leading to spatial diversification of signaling. The formation of
signaling complexes suggests that a trade-off exists between specificity/spatial heterogeneity and the amplification of a response, especially when a complex comprises both effectors and regulators (Katz and Clemens, 2001). Interestingly, the loss of spatial structure can have a profound impact on signaling (Nakagawa and Sheng, 2000).

**Regulation of phosphoinositide signaling**

Until recently, only cytosolic Ca\(^{2+}\) concentrations could be measured at the level of single cells in order to analyze the characteristics of Gq-mediated signaling. However, the profile of the calcium response is tuned by many different regulatory pathways. For instance by calcium induced calcium release, changes in the sensitivity and conductance of IP\(_3\)Rs and calcium buffering mechanisms. At present, more upstream molecules can also be visualized (e.g. heterotrimeric G protein activation, Chapter 3). The relevance of examining several components of a signaling pathway has been clearly shown by Jalink and co-workers (van der Wal et al., 2001). This group found that stimulation of different GPCRs caused similar transient increases in calcium, while decreases in PtdIns(4,5)P\(_2\) showed highly variable profiles. Importantly, also PtdIns(4,5)P\(_2\) levels and all the other components involved in phosphoinositide signaling are regulated by many different enzymes. In order to obtain a complete picture of this signaling pathway one should ideally analyze all of the components.

The duration (short/long; immediate/delayed) and nature (continuous/oscillatory) of a signal output depends on the interplay (hysteresis/positive/negative feedback) of the reactions involved. An extra dimension of regulation is imposed by the morphology of the cell and by compartmentalization of the enzymes by for instance scaffolding proteins, signal sequences or membrane lipid content (Cockcroft, 2001; Xu et al., 2003). The regulation of the localization of PLC\(_{\beta1a}\) by its substrate PtdIns(4,5)P\(_2\) introduces a new phenomenon and it remains to be ascertained whether this behavior by itself may be sufficient to induce oscillations in both PtdIns(4,5)P\(_2\) and calcium.

**Outlook**

The panoply of functions displayed by proteins belonging to the Gq-signaling pathway (Chapter 1) seems enormous. Similar complex networks can be devised for other signaling cascades. One should bear in mind that results obtained in immortal cell cultures do not always translate directly to physiological properties of an intact organism (Dumont et al., 2002). For instance, similar pathways may have different or even opposite outputs in different cell types, depending on the complement of signaling proteins present and their expression levels. In general, data on the expression patterns and levels of signaling proteins is scarce, as is information on the regulation of these patterns and levels during embryogenesis and growth.
The research described in this thesis does not end here. Certainly, many unanswered questions remain and, as stated above, this field of research should move to more physiologically relevant cell/tissue systems. Ultimately, we would like to study signaling in differentiated cells and eventually in tissues and intact organisms by applying multiparameter imaging to analyze crosstalk between different signal transduction pathways, since cells are permanently co-stimulated by various agonists under physiological conditions. Synergistic/antagonistic interactions may profoundly modulate the outcome and play an important role in the occurrence of side effects upon application of certain drugs. We will start out studying Gq-PLC\(\beta\) signaling in neurons in collaboration with Dr. Verhage and co-workers from the Free University. At present we are examining crosstalk between the Gq pathway and cAMP signaling in collaboration with Dr. Jalink and co-workers from the Netherlands Cancer Institute. In addition, we are developing a FRET sensor to monitor the activation of the H\(_1\) receptor using CFP and the FlAsH probe (Hoffmann et al., 2005), in collaboration with Dr. Hoffmann from the University of Würzburg. This sensor will enable us to ascertain the pharmacological characteristics of this important receptor and help us to correlate GPCR activation, heterotrimeric G protein activation, PLC\(\beta\) activation, calcium mobilization and annexin array formation at the plasma membrane. Another part of our research will involve the effects of scaffolding proteins on PLC\(\beta\) signaling, using a cell line that is devoid of endogenous PLC\(\beta\) isozymes. Clearly, there will be no dull moment during the coming year in which I will continue my research at Molecular Cytology in this thrilling field of biology!

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