Stable Association between Gαq and Phospholipase Cβ1 in Living Cells

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Signal transduction through Gαq involves stimulation of phospholipase Cβ (PLCβ) that results in increased intracellular Ca2+ and activation of protein kinase C. We have measured complex formation between Gαq and PLCβ in vitro and in living PC12 and HEK293 cells by fluorescence resonance energy transfer. In vitro measurements show that PLCβ1 will bind to Gαq(guanosine 5’-3-O-(thio)triphosphate) and also to Gαq(GDP), and the latter association has a different protein-protein orientation. In cells, image analysis of fluorescent-tagged proteins shows that Gαq is localized almost entirely to the plasma membrane, whereas PLCβ1 has a significant cytosolic population. By using fluorescence resonance energy transfer, we found that these proteins are pre-associated in the unstimulated state in PC12 and HEK293 cells. By determining the cellular levels of the two proteins in transfected versus non-transfected cells, we found that under our conditions overexpression should not significantly promote complex formation. Gαq-PLCβ complexes are observed in both single cell measurements and measurements of a large (i.e. 10^6) cell suspension. The high level (~40%) of FRET is surprising considering that Gαq is more highly expressed than PLCβ1 and that not all PLCβ1 is plasma membrane-localized. Our measurements suggest a model in which G proteins and effectors can exist in stable complexes prior to activation and that activation is achieved through changes in intermolecular interactions rather than diffusion and association. These pre-formed complexes in turn give rise to rapid, localized signals.

The Gαq family of G proteins transduces signals connected to agents such as angiotensin II, catecholamines, endothelin 1, and prostaglandin E2. In its activated GTP-bound state, Gαq will stimulate the catalytic activity of its main effector phospholipase Cβ (PLCβ).2 PLCβ enzymes catalyze the hydrolysis of the signaling lipid, phosphatidylinositol 4,5-bisphosphate, to generate two second messengers that result in an increase in intracellular Ca2+ and a host of proliferative and mitogenic changes in the cell (for review see Refs. 1 and 2). There are four forms of PLCβ (PLCβ1-4) that differ in their tissue distribution and their regulation by G protein subunits. Here we will focus on PLCβ1, which is strongly activated by Gαq subunits. PLCβ1 is widely distributed and is most highly expressed in neuronal tissue where it may participate in rapid intracellular Ca2+ signaling (2).

Activation of PLCβ1 by Gαq(GTP) is thought to occur through direct contact between the enzyme and the activated G protein subunit. This idea stems from the close correlation between the lateral association of PLCβ1 and Gβγ subunits and the concentration dependence of activation (3). However, this mechanism may differ from Gα subunits that undergo significant conformational changes upon activation unlike Gβγ subunits (4).

The rate of activation of PLCβ1 by Gαq(GTP) will depend on the rate of association and the rate of the conformational changes that lead to effector activation. If the two proteins are complexed prior to Gαq activation, the signal will no longer depend on the diffusion rates of the two proteins, and the rate of PLCβ1 activation would be greatly accelerated. There is now accumulating evidence that higher order complexes of signaling proteins exist in cells. Ross and co-workers (5) found that the rate of Gαq-PLCβ1 signaling was so rapid that dissociation of Gαq from the receptor was not probable, and indeed, association between seven transmembrane receptors and Gαq has been observed in cells (6). In Drosophila, a signaling complex involving receptor, a PLCβ homolog, a protein kinase C homolog, and a scaffold protein has been identified (7). More evidence for signaling complexes comes from RGS4-dependent Ca2+ oscillations in cells using a PLC-β agonist (8), suggesting that the G protein-coupled receptor, the G protein heterotrimer, and PLC could be localized in a signaling complex. Although these studies are suggestive, to date the physical association of a G protein subunit and its corresponding effector have not yet been reported in living cells.

Although preformed G protein-effector complexes would not only give rise to rapid signals as mentioned above, the localization of the signal would no longer depend on the localization of the two proteins but rather on the diffusion of the products generated. Most importantly, these signals would only be generated by a specific receptor type. For PLCβ1-Gαq, the second messengers generated are expected to have very rapid diffusion as compared with the proteins, thus further enhancing signal speed.

The ability of proteins to form complexes depends on their local concentration as well as their affinities. We have previ-

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1 The abbreviations used are: PLC, phospholipase C; FRET, fluorescence resonance energy transfer; PI(4,5)P2, 1,4-piperazinediethanesulfonic acid; CFP, cyan fluorescent protein; eCFP, enhanced CFP; YFP, yellow fluorescent protein; eYFP, enhanced YFP; GFP, green fluorescent protein; GTPγS, guanosine 5’-3-O-(thio)triphosphate; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; NGF, nerve growth factor; FRAP, fluorescence recovery after photobleaching; MEM, minimum Eagle’s medium.
Cellular Complexes of Phospholipase Cβ and Gαq

ously used fluorescence methods to quantify the affinities of PLCβ enzymes to Gαq, Gβγ, and other components in this pathway on model membranes using purified proteins (9, 10). As expected, the binding of PLCβ1 to Gαq(GTPγS) is extremely strong as compared with binding to deactivated Gαq. Although this reduction in affinity is significant, it is important to note that if the local cellular concentrations of PLCβ1 and Gαq are above this dissociation constant, then they would remain together in the activated as well as the deactivated states.

In this study, we have determined the ability of PLCβ1 and Gαq to form complexes in the basal and stimulated states in vitro and in two cell lines using fluorescence resonance energy transfer (FRET) between fluorescent-tagged proteins. FRET measurements are based on the probability of transfer of excited energy from a donor fluorophore to an acceptor. This probability depends on the electronic properties of the donor and acceptor as well as their intermolecular distance (11, 12). Because the amount of FRET depends on the 6th power of this distance, it is a sensitive measure of protein-protein associations. For the eCFP-eYFP donor-acceptor FRET pair, the distance at which 50% of the donor fluorescence is lost to transfer is 30 Å, making this pair useful to monitor protein association (13). By using this method, we find that PLCβ1 and Gαq are complexed even when Gαq is in the deactivated state, although the nature of the association differs. In cells, we find that these proteins are strongly complexed in the unstimulated state despite their low affinity. These pre-formed PLCβ1-Gαq(GTPγS) complexes allow for rapid signaling through changes in protein orientation during G protein turnover and delocalization of the signal through diffusion of the second messengers produced by activation. Based on the cellular concentration of the proteins and their affinities, our results suggest that co-localization must occur through unidentified factors.

MATERIALS AND METHODS

Reagents—eCFP-Gαq was derived from Gαq-GFP, as described previously (14), and was a generous gift from Dr. Catherine Berlot (Geisinger Clinic, Danville, PA) as were the constitutively active eCFP-Gαq (R183C) (eCFP-GαqRC) (15) and eCFP-Gβ1 (16). The eCFP construct was originally obtained from Clontech. eYFP-PLCβ1 was a generous gift from Loren Runnels (Department of Cell Biology, Rutgers University). This construct shows wild-type basal activity and activation by Gαq.

In Vitro FRET Studies—In vitro affinities between PLCβ1 and Gαq(GTPγS) or Gαq(GDP) were determined as described previously (10). Briefly, the proteins were expressed in SF9 cells through a baculovirus system and purified. GDP-bound Gαq was then labeled with an amine reactive probe, coumarin SE (Molecular Probes, Inc.), reconstituted on large unilamellar vesicles composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)/1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-1-serine (POPS)/1-palmityl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) (1:1:1), and the center of spectra mass and intensity was monitored as purified, and unlabeled PLCβ1 was incrementally added. Gαq was activated using the procedure of Ref. 17. This procedure results in at least 80% nucleotide exchange.

Trypsin Digestion—Samples of either PLCβ1 alone or in a 1:1 mixture with Gαq(GTPγS) or Gαq(GDP) were preincubated for 10 min on ice before the addition of trypsin. Proteolysis was allowed to proceed at 37 °C for either 5 or 20 min before addition of 10% SDS. Samples were then boiled for 3 min and subjected to SDS-PAGE. Bands were visualized using silver stain.

Cell Culture and Transfection—Rat pheochromocytoma cells (PC12), derived from the adrenal gland (ATCC, CRL-1721), were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% equine serum, 5% fetal bovine serum, and 100 mM sodium pyruvate and were incubated at 37 °C with 5% CO2. Nerve growth factor (NGF, Sigma) was added to a final concentration of 100 ng/ml to induce differentiation. Prior to transfection, cells were grown in T-25 flasks to 80–90% confluency.

Plasmids were introduced into cells by electroporation using a protocol adapted from Maniatis and co-workers (18). Briefly, the DMEM was aspirated, and the PC12 cells were harvested by adding 5 ml of fresh media and pipetting multiple times over the bottom of the flask. Cells were spun down for 5 min at 1500 × g and resuspended in 5 ml of phosphate-buffered saline (PBS). One ml of cells was removed and counted, and the cell suspension was pipetted into 0.4-cm Bio-Rad cuvettes and incubated on ice for 10 min. Then 10–30 μg of plasmid DNA was added to each cuvette and gently mixed. The electroporator (Bio-Rad Gene Pulser Xcell) was set to 0.25 kV with a capacitance of 500 microfarads. Cuvettes were placed in the shocking chamber and pulsed once. After the pulse, the cells were allowed to rest for 1–2 min, and 1 ml of DMEM was then added to each cuvette. Cells were placed in 15-ml conical tubes containing 3 ml of DMEM. Tubes were spun down at 1500 × g for 5 min, and cells were brought up in 1.5 ml of DMEM and plated onto LabTek chambers coated with 50 μg/ml fibronectin (Sigma). Three to four hours post-transfection, the wells were washed with 1 ml of PBS, and 1.5 ml of DMEM containing 100 ng/ml NGF was added. After 3 days of incubation with NGF, transfected cells were differentiated and used for imaging.

HEK cells were cultured in DMEM plus 10% FBS and 1% penicillin/streptomycin at 37 °C with 5% CO2. Plasmids were introduced to HEK cells as described except they were not pre-incubated on ice before the pulse, and the electroporator was set to 0.2 kV with a 960-microfarad capacitance.

Calcium Release—PC12 cells, in T-25 flasks, were washed two times with PBS. Cells were collected in Hanks’ buffered salt solution (HBSS, 15 mM Hepes (pH 7.67), HEPES 118 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 5 mM glucose, and 1 mg/ml BSA) and spun down at 1500 × g. Cells were counted and adjusted to a concentration of 1 × 107 cells/ml. Fura-2 AM (5 mM) (Sigma) was added to the cells, and cells were incubated in the dark at 37 °C for 40 min with rotation. After labeling, cells were spun down at 1500 × g and resuspended in HBSS. To make measurements, cells were diluted to 1.0 × 106 cells/ml in HBSS. One-ml cell suspensions were put into a cuvette with a stir bar and placed in the fluorometer. The samples were excited at 340 and 380 nm, and the emission was measured at
510 nm, and the ratios of the two excitations were recorded over time.

To measure calcium release upon stimulation, cells were stimulated with 1 μM acetylcholine or carbachol, and the ratio was measured again. To break open the cells, 10% Triton X-100 was added followed by calcium chelation with 2 mM EDTA to obtain the maximal and minimal amounts. To calculate internal calcium concentration, see Equation 1 (19),

\[ [Ca^{2+}]_{\text{free}}(\text{nm}) = \frac{(R - R_{\text{min}})(R_{\text{max}} - R)}{\left(\frac{F_{\text{max}}380}{F_{\text{min}}380}\right) \times 225} \]  

(Eq. 1)

where \( R \) is the measured ratio (fluorescence emitted at 340 and 380 nm); \( R_{\text{min}} \) and \( R_{\text{max}} \) are the ratios with EDTA and detergent; \( F_{\text{min}} \) is the fluorescence in the presence of EDTA (i.e. minimum calcium), and \( F_{\text{max}} \) is the fluorescence in the presence of detergent (i.e. maximum calcium). The \( K_d \) of Fura-2 AM is \( \approx 225 \text{ nm} \) (19).

**Cell Fractionation and Western Blot Analysis**—PC12 cells were transfected with protein expression vectors as described above. Five identical transfections were combined into a T-25 flask and differentiated for 3 days with NGF. Then PC12 cells were harvested from T-25 flasks and washed two times with PBS. After the second wash, the cells were brought up in PBS containing 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml aprotinin, placed on ice, and homogenized. Nuclei were removed by a low speed centrifugation at 750 \( \times \) g for 5 min at 4 °C. The supernatant was removed and spun at 28,000 rpm for 35 min at 4 °C. The supernatant or cytosolic fraction was removed, and the resulting pellet or membrane fraction was brought up in PBS containing 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml aprotinin. The protein concentration for the cytosolic and membrane fractions was assayed, and 4–15 μg was loaded into each well of an SDS-polyacrylamide gel. Proteins were then transferred to nitrocellulose; the membrane was blocked overnight and then probed for Gαq and PLCβ1 using a 1:200 dilution for primary antibody (Santa Cruz Biotechnology) and 1:2000 dilution for secondary antibody (Sigma). Western blots were developed using alkaline phosphatase reaction, and the amount of overexpressed protein per μg of protein loaded was calculated using a standard curve generated from purified Gαq and PLCβ1.

**Immunofluorescence**—For secondary immunofluorescence for endogenous expression of Gαq and PLCβ1, PC12 cells were plated and differentiated as described above. The cells were washed twice with PBS and fixed with 1.5 ml of 3% paraformaldehyde at room temperature for 10 min. The fixing solution was removed, and the cells were washed three times for 10 min each with MSM-PIPES Buffer (modified Shierds media; 18 mM MgSO4, 5 mM CaCl2, 40 mM KCl, 24 mM NaCl, 5 mM PIPES (pH 6.8), 0.5% Triton X-100, 0.5% Nonidet P-40). After washing, the cells were blocked in PBS containing 5% goat serum, 1% BSA, and 50 mM glycine for 15 min. Cells were incubated with the primary antibody (1:200 dilution) in PBS containing 0.5% BSA at 37 °C for 1 h and then washed three times with PBS for 10 min each. Cells were incubated with fluorescein isothiocyanate-conjugated secondary antibody (1:2000 dilution) in PBS containing 0.5% BSA at 37 °C for 1 h, and then washed three times with PBS for 10 min each. PBS was then added to wells, and the cells were imaged.

**Preparation of Membrane Fractions**—HEK293 cells (12.5 \( \times \) 106 per 150-mm dish) were transfected using DEAE-dextran (21) or using 62.5 μl of Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. 48 h after transfection, membranes were prepared as described (22). The amounts of plasmids used in the transfections are given in the legend to Fig. 7.

**Instrumentation**—Confocal images were taken at the University Microscopy Center on a Bio-Rad apparatus. Pixel analysis of confocal images was done using ImageJ (National Institutes of Health). All other images, time lapses, and z-stacks were taken on a Zeiss Axiovert 200M with an AxioCam MRm camera using Axiovision software. Fluorescence spectra were taken on a photon-counting spectrofluorometer, ISS-PC (ISS, Urbana, IL).

**Single Cell FRET Measurements**—FRET measurements were determined using the procedure of Devreotes and co-workers (24). Bleed through values were obtained by transfecting PC12 cells with 10 μg of free eCFP or free eYFP plasmid vectors and imaging under the appropriate filter sets (Chroma, Inc.). Cells expressing only CFP or YFP were then imaged under the CFP (Chroma 31044v2) or YFP (Chroma 41029) and FRET (Chroma 31052) filter sets to determine the FRET/CFP or FRET/YFP ratio. Averaging over 12 cells on our system, the bleed through values for CFP and YFP are 39 and 28%, respectively, using the background-corrected intensities calculated using ImageJ software from the National Institutes of Health. We generated a net FRET image by accounting for bleed through emission, see Equation 2,

\[ nF = \frac{l_{\text{FRET}} - l_{\text{YFP}}}{l_{\text{YFP}} + l_{\text{CFP}}} \times a - \frac{l_{\text{YFP}}}{l_{\text{YFP}} + l_{\text{CFP}}} \times b \]  

(Eq. 2)

where \( a \) and \( b \) equal the percentage of bleed through of YFP and CFP under the FRET filter set. However, to compare FRET values among cells with varying protein expression levels, the net FRET (nF) value can be normalized (N_{\text{FRET}}). From the entire intensity value of the image, one can calculate N_{\text{FRET}}. Normalized FRET (N_{\text{FRET}}) is given as shown in Equation 3,

\[ N_{\text{FRET}} = \frac{l_{\text{YFP}}}{l_{\text{YFP}} + l_{\text{CFP}}} \times \frac{a - l_{\text{YFP}}}{b} \]  

(Eq. 3)

where \( a \) and \( b \) equal the percentage of bleed through of CFP and YFP under the FRET. N_{\text{FRET}} was determined as described (23).

**Image Analysis**—Images were processed using ImageJ. Before any analysis, images were corrected for background as follows. First, the background was calculated from a 50 \( \times \) 50 pixel box at the top left corner of the raw image, and this intensity was subtracted from the entire image. Then uneven illumination was removed from the image by using three iterations of the Background Correction Plugin. This image was binary thresholded and then inverted to make the cell white (255) and the background black (0). Next, the image was divided by 255 to make the pixel values for white 1. Finally, the background subtracted image was multiplied by the thresh-
Cellular Complexes of Phospholipase Cβ and Gαq

A

![Graph showing FRET measurements of PLCβ1 to either 2 nM CM-Gαq(GDP) or CM-Gαq(GTPγS) reconstituted on 300 μM large, unilamellar vesicles composed of POPC/POPS/POPE (1:1:1), where n = 3. Binding was followed by the shift in the emission energy of the coumarin probe upon binding. Control studies substituted buffer for enzyme. The binding curves were fit to a bimolecular association constant using SigmaPlot, and the S.E. is shown. B, SDS-PAGE showing the extent of trypsin digestion at either 5 or 20 min of incubation of PLCβ1 when bound to Gαq(GDP) or Gαq(GTPγS).

olded image. These manipulations remove the background by making the background 0. Using the method of Xia and Yuechueng (23), nf images were created by multiplying the background corrected donor image by the donor correction factor, the background corrected acceptor image by the acceptor correction factor, and then subtracting each of these from the background corrected FRET image by the acceptor correction factor, making the background 0. Using the method of Xia and Yuechueng (23), nf images were created by multiplying the background corrected donor and acceptor images individually transfected with one of the fluorophores.

Thus, Equation 4 is given,

\[ \%FRET = 100 \times \frac{(R(s) - R(YFP))/(R(CFP) - R(YFP))}{R} \]  

where R(s) is the intensity ratio of the sample. Real time changes in FRET were also monitored by the change in the intensity ratio of the donor and acceptor (490/527 nm) when the donor is excited (440 nm).

FRAP—Fluorescence recovery after photobleaching (FRAP) measurements were carried out using an N2 laser (Spectra Physics) of 100-milliwatt power to photobleach a circular region of 2 μm in diameter on specific regions of the cells. The intensity of the photobleached portion was allowed to recover over time, and the recoveries were fit to 1 or 2 exponential curves.

RESULTS

In Vitro Binding of Purified PLCβ1 to Gαq in the Activated and Deactivated States—We have determined previously the affinity of PLCβ1 to activated Gαq laterally associating on model membrane surfaces (9) using fluorescence methods. Here, we have repeated this measurement and determined the decrease in affinity between the proteins when Gαq is in the excited state.
trypsin (Fig. 1B). Interestingly, we find that association of PLCβ1 to deactivated Gαq results in more protection to digestion as compared with isolated PLCβ1 or PLCβ1 bound to Gαq(GTPγS). Taken together, these studies quantify the binding between PLCβ1 and Gαq(GDP) and suggest that the proteins associate with an interface that differs from activated Gαq.

Quantifying the Endogenous and Overexpressed Gαq and PLCβ1 in Cells—To visualize complexes between PLCβ1 and Gαq in cells, we have overexpressed fluorescent-tagged Gαq and PLCβ1 and monitored association formation by fluorescence spectroscopy. Because overexpression could promote complex formation between the proteins, we quantified the amount of overexpressed eCFP-Gαq and eYFP-PLCβ1 in differentiated PC12 cells and compared these to endogenous levels found in cells transfected with empty vector by Western blot analysis (see “Materials and Methods”). This was accomplished by placing four different amounts of purified Gαq or PLCβ1 (7) to generate a calibration curve. The growth and morphology of the transfected cells were identical to untransfected cells, suggesting that at our levels of expression neither protein is disruptive to cell function. This observation correlates well with studies showing that PC12 clones that are stably expressing PLCβ1 have the same sensitivity to NGF stimulation, proliferation, and survival upon serum deprivation as compared with wild-type cells (27). We note that transfection of HEK293 cells with Gαq and PLCβ1 did not affect the growth or morphology of these cells.

Differentiated PC12 cells (see “Materials and Methods”) were harvested and ruptured, and the membrane and cytosolic fractions were isolated. We find that eCFP-Gαq levels in the membrane fractions were 0.33 ± 0.06 μg/mg total protein compared with 0.17 ± 0.02 μg/mg protein of endogenous protein. Thus overexpressed protein is ~2-fold higher than endogenous protein under our transfection conditions.

For eYFP-PLCβ1, the ratio of transfected to endogenous enzyme is roughly 3-fold (0.006 versus 0.002 μg/mg protein). Similarly, the cellular PLC activity is 10% higher in the transfected cells consistent with increased expression of functional PLCβ1 and also noting that other active PLC enzymes (e.g. PLCγ and PLCδ) are expressed in PC12 cells (2). This study shows that the overexpressed amounts of eCFP-Gαq and eYFP-PLCβ1 are proportional and not high enough to elicit abnormal cellular responses.

deactivated state. These measurements were carried out by covalently attaching the fluorescent probe coumarin to Gαq, reconstituting it on large unilamellar vesicles and monitoring the change in coumarin fluorescence intensity as PLCβ1 is added. We note that these studies were done at lipid concentrations exceeding the membrane binding constant of the proteins, and so only lateral associations between the membrane-bound proteins are viewed (25, 26). The binding curves were fit assuming a bimolecular association, although we cannot discount the possibility that different conformational states of Gαq contribute to the curve. The data in Fig. 1A show that PLCβ1 binds ~300-fold (K_{app} = 640 ± 68 nm versus 2.8 ± 0.7 nm) more weakly to deactived as opposed to activated Gαq(GTPγS). Thus, for complexes to form in the basal state, the proteins would have to be co-localized at high concentrations (i.e. above 500 nm).

Gα subunits undergo significant conformational changes upon nucleotide exchange, and it is likely that the protein-protein interface between PLCβ1 and Gαq differs in the activated and deactivated states. To determine whether this is the case, we formed complexes between PLCβ1 and Gαq(GDP) and PLCβ1 and Gαq(GTPγS) on membranes and subjected the complexes to protein digestion using...
Distribution of eCFP-Gαq and eYFP-PLCβ1 in Differentiated PC12 Cells—We characterized the cellular distribution of eCFP-Gαq and eYFP-PLCβ1 in transfected PC12 cells by confocal microscopy. Although undifferentiated PC12 cells show a fairly uniform morphology, NGF treatment results in terminal differentiation and production of neuronal-like processes (28). Initial studies were done using GFP-Gαq. We found that in every cell viewed (n > 30), GFP-Gαq was entirely localized on the plasma membrane with only trace amounts in the cytosol. None could be found in the nucleus. An example of a differentiated PC12 cell expressing GFP-Gαq is shown in Fig. 2A, which shows the top and side view of a cell. In Fig. 2B, we present the intensity distribution through a single point of the cell (red point in the top image of Fig. 2A). This analysis shows that all of the GFP-Gαq intensity is found on the top and bottom surfaces of the cell, which is consistent with plasma membrane localization. In this cell, most of the fluorescence intensity is concentrated on the cell bottom, and a smaller amount is on the apical side suggesting a larger number of signaling networks are localized on the basolateral side of the cell.

We assessed the amount of GFP-Gαq in the cytoplasm by pooling the intensities from 5.0 to 14.6 μm, which should correspond to the cytosol, and dividing by the pooled intensities from the bottom to top distance of 0 – 4.8 μm, which should correspond to the plasma membrane. This calculation gives an intensity ratio of the cytosolic/plasma membrane of 0.06.

The localization of eYFP-PLCβ1 was also viewed. Unlike GFP-Gαq, a larger percent of the protein was seen in the cytosol (Fig. 3, A and B). To ensure that the cytosolic population of eYFP-PLCβ1 was not caused by overexpression, we collected z-stack images of

![Distribution of eYFP-PLCβ1 in a PC12 cell](image)
endogenous PLCβ₁ and fixed PC12 cells stained with a PLCβ₁ monoclonal antibody (Fig. 4, A and B). The distribution of endogenous PLCβ₁ matched that of overexpressed eYFP-PLCβ₁. We verified that PC12 cells contain a significant population of cytosolic PLCβ₁ by Western blot analysis of the cytosolic and membrane fractions of nontransfected and transfected cells (data not shown). Comparing the pooled ratios of the histogram intensities from the cytosolic (2.4–6.0 μm) divided by plasma membrane populations (0–2.2 μm), we obtain a value of 0.48. This value is far higher than the ratio obtained for GFP-Gαq of 0.06. Similar results were obtained over a wide range of cells allowing us to conclude that differentiated PC12 cells have two populations of PLCβ₁ in the resting state, one localized on the plasma membrane and another in the cytosol. In contrast, Gαq is almost entirely localized on the plasma membrane.

**PLCβ₁ Is Associated with Gαq in Quiescent Cells**—We then determined the amount of eCFP-Gαq complexed with eYFP-PLCβ₁ in PC12 cells by FRET studies using fluorescence microscopy. These studies were carried out by co-transfecting eYFP-PLCβ₁ and eCFP-Gαq into differentiated PC12 cells and collecting z-stack images every 0.2 μm in three channels (CFP, YFP, and FRET). To quantify the amount of eYFP-PLCβ₁/eCFP-Gαq FRET over time, we collected images focusing on the bottom of the cell every 5 s for a total of 100 s and calculated the amount of FRET for each time point. A normalized FRET image was then generated (see “Materials and Methods”). In all cells, we find the highest FRET on the plasma membrane (e.g. Fig. 5) correlating well with the plasma membrane localization of Gαq. Calculating the cellular distribution and magnitude of the intensity of the FRET signal between eYFP-PLCβ₁ and eCFP-Gαq, we find the FRET to be constant over time suggesting a stable association in the basal state (see Fig. 5 and below). Additionally, a stable basal population was seen in parallel studies of HEK293 cells. Importantly, this basal population was also confirmed by co-immunoprecipitation studies (see supplemental material). Taken together, these results show that eYFP-PLCβ₁ and eCFP-Gαq are associated in cells even in the quiescent state.

All cells expressing eCFP-Gαq/eYFP-PLCβ₁ displayed a significant level of FRET with the mean value close to 40%. The transfection efficiency for eCFP-Gαq and eYFP-PLCβ₁ was 60–80% for both proteins, and we note that significantly decreasing the level of expression of either one of the proteins reduced the FRET to nonspecific values (i.e. less than 20% FRET, see below), correlating well with the properties of FRET (11).

To assess the significance of the maximal FRET values shown in Fig. 5, we can compare the average FRET observed for these proteins to the 55 ± 4% FRET observed for eCFP-Gαq and eYFP-PLCβ₁, the 50 ± 7% FRET observed for eCFP-Gαq and eYFP-bradykinin receptor when overexpressed in HEK293 cells. The

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3 F. Philip and S. Scarlata, submitted for publication.
Cellular Complexes of Phospholipase Cβ and Gαq
FRET from this latter complex decreases to a level below resolution (i.e., less than 20%) upon stimulation because of receptor internalization. Thus, the magnitude of the maximum FRET value correlates well with other complexes.

To assess the significance of the lower values of FRET, we measured the extent of FRET between two groups of noninteracting proteins. In the first, we co-expressed eCFP-Gαq and eYFP-PLCβ1, which should not interact (2). For these cells, the range of FRET was 8–18% with a mean value of 14% (n = 5). In a second series of controls, we co-expressed free CFP and free YFP at levels higher than the eCFP-Gαq/eYFP-PLCβ1 pair (i.e., ~80-fold). We obtain a FRET of 18 ± 2% (n = 8). These data give a nonspecific level of FRET that most likely reflects the limitations of the optics used in our studies and, in particular, our inability to completely eliminate bleed through fluorescence. These controls give us a lower limit on the extent of FRET from specific interactions. Thus, the much higher extent of FRET for the eCFP-Gαq/eYFP-PLCβ1 pair compared with these controls suggests specific complex formation.

To determine whether a basal population of eCFP-Gαq/eYFP-PLCβ complexes could be seen in other types of cells, we carried out the same study using HEK293 cells. Similar to that seen in PC12 cells, these cells showed a cytosolic population of eYFP-PLCβ and a plasma membrane population that is complexed with eCFP-Gαq in the unstimulated state. Thus, this same cellular localization is seen in other cell lines.

The measurements described above were performed on single cells. Even though many cells were viewed, there is a possibility that eYFP-PLCβ1 and eCFP-Gαq FRET in the basal state only occurs in limited cases. To determine the amount of FRET in a large number of cells, we co-transfected cells with eYFP-PLCβ1 and eCFP-Gαq and measured the degree of FRET for 10^6 cells in a spectrofluorometer. We found that the range of FRET for this large population of cells was identical to single cell measurements as described below (Fig. 5) verifying the single cell measurements that show that a significant fraction of eYFP-PLCβ1 and eCFP-Gαq are complexed in the basal state.

Cytoplasmic eYFP-PLCβ1 Is Not Recruited to the Membrane by eCFP-Gαq during Stimulation—As shown in Fig. 1, activation of Gαq increases its affinity to PLCβ1 by over 2 orders of magnitude. With this in mind, we measured changes in FRET with stimulation of PC12 cells expressing eCFP-Gαq and eYFP-PLCβ1 by the addition of either 1 μM carbachol or 1 μM acetylcholine. The results for four single cells are shown in Fig. 5. In all cells viewed and analyzed (n >12), we find that the amount of FRET does not significantly change upon the addition of a Gαq agonist. Moreover, as mentioned above, a stable co-localization of eYFP-PLCβ1/eCFP-Gαq was observed for eYFP-PLCβ1/eCFP-Gαq complexes in HEK293 cells by both FRET and by co-immunoprecipitation, suggesting that this behavior is not specific to PC12 cells. These results suggest that even though the affinity between eYFP-PLCβ1/eCFP-Gαq is expected to increase significantly upon activation, the cytoplasmic population of eYFP-PLCβ1 is not recruited to the plasma membrane surface by eCFP-Gαq.

To confirm the single cell studies above, we measured relative changes in FRET between eYFP-PLCβ1/eCFP-Gαq expressed in ~10^6 PC12 cells in a fluorometer as described above. Similar to the single cell studies, these results (Fig. 6A) also show that the number of eYFP-PLCβ1/eCFP-Gαq complexes does not significantly change with cell stimulation.

A trivial explanation of the constant FRET between eCFP-Gαq and eYFP-PLCβ1 with stimulation is that activation is not efficient. To determine whether this is the case, we measured the amount of FRET between eCFP-Gαq-RC and eYFP-PLCβ1. This point mutant produces a constitutively active Gαq (15), which is expected to be strongly associated with PLCβ1 in the unstimulated state, and we expect this association to change little with the addition of agonist. We find that both the basal and stimulated FRET values between eCFP-Gαq-RC and eYFP-PLCβ1 are similar to wild type (Fig. 6B) suggesting that association between the two proteins is not related to the stimulated state.

To ensure that the eCFP-Gαq/eYFP-PLCβ1 expressed in cells is part of a signaling complex coupled to a Gαq receptor, specifically the m1 and m5 muscarinic receptors in PC12 cells (29), and has the ability to increase intracellular calcium upon stimulation, we measured the amount of Ca^{2+} released upon the addition of 1 μM acetylcholine in 10^6 PC12 cells. The results, presented in Fig. 7, show that in the basal state all four cell groups have similar levels of internal Ca^{2+} as expected from its tight cellular regulation. Upon the addition of agonist, a similar robust increase in intracellular Ca^{2+} from nontransfected and singly transfected cells is observed. However, the double transfected cells gave a significantly higher amount of released Ca^{2+}. Considering that the Gαq-coupled m1 and m5 receptors constitute only 5% of the muscarinic receptors in these cells (29), this increase suggests that a large and significant population of the transfected proteins is functional and coupled to muscarinic receptors.

Although eYFP-PLCβ1/eCFP-Gαq complexes may not dissociate upon stimulation, it is possible that their localization in the cell changes during activation. We tested this idea using the commercially available plasma membrane markers YFP-MEM and CFP-MEM (Clontech, Inc.). These markers show a transfection efficiency of ~100%. To follow protein localization, we co-transfected the CFP-MEM or YFP-MEM markers at a high enough level to give a significant and reproducible amount of FRET with eCFP-Gαq or eYFP-PLCβ1 (i.e., 46 and 41%, respectively).

Terminally differentiated PC12 cells were either co-transfected with CFP-MEM and eYFP-PLCβ1 or YFP-MEM and

**FIGURE 5.** Examples of normalized FRET images of four PC12 cells expressing eCFP-Gαq and eYFP-PLCβ1 after NGF treatment (see "Materials and Methods"). For each example, the 1st image corresponds to the basal FRET, and the 2nd image is 30 s after stimulation with 1 μM acetylcholine. These images were taken focusing on or close to the bottom of the cells, and the normalized FRET was analyzed using the method of Ref 23. Also shown are surface plots depicting the distribution of the FRET signal over time for each cell, where the y axis is the percent FRET, the x axis is the percent pixels in each FRET range, and the z axis is time. One can see the percentage of pixels within each FRET range does significantly change upon stimulation, indicating that there is not a redistribution of the signal.
Cellular Complexes of Phospholipase Cβ and Gαq

A

FRET Range Unstimulated Cells

FRET Range Stimulated Cells

FRET

0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%

Time (sec)

ACh stimulation

basal average (n=9)

stimulated average (n=4)

B

FRET in Unstimulated Cells

FRET in Stimulated Cells

FRET

0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%

Time (sec)

ACh Stimulation

basal average (n=8)

stimulated average (n=3)
eCFP-\(G_\alpha_q\) and changes in FRET with stimulation were measured for \(10^6\) cells in a fluorometer (see above) using the procedure described by Lohse and co-workers (30). After measuring a stable basal level of eYFP/eCFP emission for 5 min, the cells were stimulated, and the CFP and YFP emission intensity was monitored over 30 min. Both time and stimulation did not affect the intensity of the CFP and YFP emissions. These results (Fig. 8) indicate that the proteins do not move off the membrane upon stimulation with acetylcholine or carbachol.

A stable localization of the proteins during stimulation was also assessed by taking 2.4-\(\mu m\) slices through a cell starting at the bottom and measuring regions of interest that encompassed most of the plasma membrane and calculating its intensity through the cell stack before and after stimulation. This analysis, compiled for eight cells, also showed stable localization before and after stimulation (Fig. 9).

Studies of YFP-PLC\(\beta_1\)-eCFP-\(G_\alpha_q\) Complexes in Membrane Preparations—The FRET results of live cell studies performed above clearly showed eYFP-PLC\(\beta_1\)-eCFP-\(G_\alpha_q\) complexes in the quiescent state. The presence of these complexes suggests that some cellular factors, such as protein scaffolds, may stabilize eCFP-\(G_\alpha_q\)/eYFP-PLC\(\beta_1\) in the basal state. We extended the cellular studies using membrane preparations of cells expressing these proteins and related partners to better understand whether eYFP-PLC\(\beta_1\)-eCFP-\(G_\alpha_q\) complexes are stabilized by cytoskeletal or cytosolic proteins. We expressed pairs of \(G_\alpha_R\), \(G_\alpha_q\), PLC\(\beta_1\), G\(\beta_3\) \(\gamma_7\), and \(G_\alpha_q\) with either CFP or YFP tags in HEK293 cells by transient transfection and prepared membrane fractions (see “Materials and Methods”). We then determined the extent of eCFP/eYFP FRET in each membrane preparation by the ratio of donor and acceptor emission maximum intensities at 490:527 nm using an exciting wavelength of 440 nm as described (21). In an initial series of studies, the degree of FRET was measured as a function of the amount of cDNA used in the transfection. Similar to whole cell studies, our results show a systematic increase in the amount of FRET between eCFP-\(G_\alpha_q\) and eYFP-PLC\(\beta_1\) from 18 to 100% with increasing cDNA used for transfection. Taken together, these results show that in the absence of cytoskeletal and cytosolic components, eCFP-\(G_\alpha_q\) and eYFP-PLC\(\beta_1\) form stable and titratable complexes on membrane surfaces, and these complexes can be seen in the basal state even under low levels of transfection.

In Fig. 10 we show a comparison of different protein pairs in the basal and stimulated states at moderate levels of transfection. High levels of FRET are seen with the eCFP-\(G_\alpha_q\)/eYFP-PLC\(\beta_1\) probe pair, but comparably high levels are also seen for eCFP-\(G_\alpha_q\)/eCFP-PLC\(\beta_1\) pair. Stimulation of membranes containing the eCFP-\(G_\alpha_q\)/eYFP-PLC\(\beta_1\) pair does not cause a significant increase in the level of FRET in accordance with the results seen in living cells. This lack of change occurred for 18:21 samples regardless of the initial FRET value that ranged from 68 to 92% for identical transfection conditions. Treating the samples with SDS reduced the level of FRET to close to those obtained for a negative control using eCFP-\(G_\alpha_q\)/eYFP-\(G_\alpha_q\). Thus, the low FRET values for the SDS-treated samples are considered background noise because of the contribution of overlapping donor/acceptor emission when calculating FRET. The important point is that the relative FRET values between eYFP-PLC\(\beta_1\) and eCFP-\(G_\alpha_q\) and between eYFP-PLC\(\beta_1\) and eCFP-\(G_\alpha_q\)(RC) are high and similar to those obtained for eYFP-\(G_\alpha_q\) and eCFP-\(G_\beta_3\) \(\gamma_7\). This latter protein pair forms strong complexes in the basal state that weaken upon stimulation (i.e. isoproterenol and GTP\(\gamma_S\)) (7), and the data in Fig. 10 support this idea.

FRAP Studies of Complex Diffusion—If eYFP-PLC\(\beta_1\)-eCFP-\(G_\alpha_q\) complexes were also stably associated with other proteins in higher order complexes, their cellular diffusion would be limited. To determine whether this is the case, we used FRAP to measure the diffusion rates of eCFP-\(G_\alpha_q\) and eYFP-PLC\(\beta_1\). This was accomplished by laser pulsing a 2-\(\mu m^2\) spot on the bottom of the cell to bleach the fluorophores and taking images every 0.5–2 s, depending on the sample. Images were analyzed by determining the change in intensity with time of the region that was bleached versus a region of the cell far from the bleach. For simplicity, we fit the data to 1–3 exponential decay curves to determine the number of decay mechanisms and their corresponding time constants. Free eYFP diffusing in the cell body

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**Cellular Complexes of Phospholipase C\(\beta_1\) and \(G_\alpha_q\)**

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**FIGURE 7. Internal Ca\(^{2+}\) levels of PC12 cells in the basal, stimulated, and recovery states (200 s after stimulation) for four groups of transfected cells labeled with Fura-2AM as follows: empty vector, G\(\alpha_q\)-eCFP, PLC\(\beta_1\)-eYFP, and co-transfected G\(\alpha_q\)-eCFP and PLC\(\beta_1\)-eYFP. Asterisks indicate significant difference (p < 0.05) between groups or between different states within each individual group. The only significant difference between groups resulted from comparing the stimulated state of empty vector and the co-transfected cells. Error bars indicate S.E.**

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**FIGURE 6.** Analysis of time lapse FRET images of PC12 cells expressing eCFP-\(G_\alpha_q\) and eYFP-PLC\(\beta_1\). The variation of the percentage of FRET for stimulated and unstimulated cells is shown in the insets where stimulation was carried out by the addition of 1 \(\mu m\) acetylcholine (\(ACh\)) after obtaining a flat baseline for 30 s. Error bars indicate S.E. for each time point. B, identical study as in A, except that cells expressed the constitutively active eCFP-\(G_\alpha_q\)-RC, rather than wild type.
fits best to a single exponential with a time constant of 0.5 ± 0.05 s \((n = 5)\) correlating well with previous work \((31)\). For simplicity, we will give comparative values of these rates in PC12 cells as follows: 76.2% of eYFP-PLC\(_\beta_1\) is mobile and diffuses with a single exponential rate that is 40-fold slower than free YFP \((n = 7)\). 63.5% of eCFP-G\(\alpha_q\) is mobile and shows two diffusion rates that are 10- and 40-fold slower than free YFP \((n = 5)\). These results show that the proteins have restricted diffusion; both proteins have a significant immobile population and a slower similar diffusion that is on the order of those reported for membrane-bound cellular proteins \((31)\).

**DISCUSSION**

Signal transduction through heterotrimeric G proteins in part involves transient association of activated \(G_\alpha\) subunits to specific effector(s) that in turn results in a series of coordinated events in the cell. In this study, we find a significant population of \(G_\alpha_q\) subunits and its main effector, PLC\(_\beta_1\), are pre-associated in the basal state in two cell lines, PC12 and HEK293. This pre-association is unexpected given the weak affinity between the two proteins. These pre-formed PLC\(_\beta_1\)-\(G_\alpha_q\) complexes will shorten the time scale of the signal transduced by these proteins and also will direct the signal along a specific pathway as discussed below.

We measured the association of \(G_\alpha_q\) and PLC\(_\beta_1\) by overexpressing fluorescent-tagged proteins. First, we determined whether overexpression of the protein would promote complex formation by comparing the level of expressed protein to endogenous levels. We find that our level of expression is only 2–3-fold higher than that of the endogenous protein which, as detailed below, is not expected to significantly promote complex formation. Western blot analysis of the protein levels found in a known number of cells allows us to roughly estimate the endogenous cellular concentrations. We find that these approximate concentrations are low in the cell \((\text{e.g.} \, 20 \text{ fM for } G_\alpha_q \text{ and } 3 \text{ fM for PLC\(_\beta_1\)}\). Moreover, these values are far lower than the dissociation constants estimated for these proteins \((\text{Fig. 1})\) even taking into account that their association may be slightly promoted by the tendency of the GFP analog tags to dimerize \((K_d \text{ GFP is } \approx 100 \mu\text{M } (32))\) and considering that attachment to the membrane may promote association more than the confinement to 300 \(\mu\text{M}\) lipid vesicles using for the in vitro measurements \((9)\). Given the magnitude of these affinities, increasing the levels of the proteins 2–3-fold is not expected to alter the results obtained here.

We find that the cellular concentration of \(G_\alpha_q\) far exceeds PLC\(_\beta_1\). Considering that \(G_\alpha_q\) interacts with other proteins such as receptor, \(G_\beta\) subunits, and a second effector phosphatidylinositol 3-kinase \((33)\), it is probable that this “excess” \(G_\alpha_q\) is bound to other partners. It is also possible that \(G_\alpha_q\) has

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**FIGURE 8.** A, emission signal and ratio from cells co-transfected with eCFP-\(G_\alpha_q\) and eYFP-PLC\(_\beta_1\). The spike in the emission signal indicates when the stimulus was added. B, emission signal and ratio from cells co-transfected with eCFP-\(G_\alpha_q\) and MEM-YFP. C, emission signal and ratio from cells co-transfected with eYFP-PLC\(_\beta_1\) and MEM-CFP. Cells in A were stimulated at the 120-s time point, and cells in B and C were stimulated at the 300-s time point.
nonoverlapping interacting sites between these partners allowing for multiple interactions.

Not only are the cellular concentrations of Gαq and PLCβ1 estimated to be much lower than their Kd values, but we also find their cellular localization does not completely overlap. Although Gαq appears to be almost entirely localized to the plasma membrane, we find a significant amount of PLCβ1 localized in cytosol as well as the plasma membrane in both of the cell lines studied here, i.e. differentiated PC12 cells and HEK293 cells. This distribution is supported by immunofluorescence of the endogenous enzyme using monoclonal antibodies and by cell fractionation. We note that cytosolic PLCβ1 has also been observed by cell fractionation in COS cells (34). Interestingly, previous localization studies of the closely related eGFP-PLCβ2 have been carried out by overexpression of the protein in HEK293 cells (35). These studies suggest that eGFP-PLCβ2, which binds Gαq(GTP) 20-fold more weakly than PLCβ1 (9), is cytosolic until cell stimulation where it then moves to the plasma membrane presumably because of the release of activated G protein subunits.

To understand the basis of the cytosolic and plasma membrane populations of PLCβ1, we note that its cellular localization should be a function of its intrinsic membrane binding constant and the presence of protein partners. We have characterized the membrane binding properties of purified PLCβ1 to lipid bilayers of varying composition (25). These studies showed that PLCβ1 binds strongly and fairly nonspecifically to membranes with a mem-

![Graph](image_url)
brane partition coefficient of ~50 μM. Based on these membrane affinities, we expect almost all PLCβ1 to be bound to cellular membranes. Localization of PLCβ1 because of membrane interactions rather than specific interactions with Gαq would be expected to give rise to both cytosolic and plasma membrane populations. This idea also correlates well with the low cellular concentrations of the two proteins.

Gα effector activation has been thought to occur by the large increase in effector affinity that occurs upon GDP/GTP exchange. Our studies using FRET strongly suggest that Gαq and PLCβ1 are pre-associated even in the basal state. This observation is seen for the proteins expressed in PC12 cells as well as HEK293 cells and is supported by co-immunoprecipitation studies. Although FRET is only related to the distance between two probes and cannot assess whether two proteins are in close proximity or interacting, we note that the distance at which 50% of excited light from the donor is lost to transfer (R0) is on the order of the size of the proteins used here (i.e. 30 Å (13) and the FRET dependence goes as the 6th power of the distance (i.e. E = 1/(1 + (R/R0)^6)) assuming free rotation of the probes. If we crudely estimate the approximate dimensions of Gαq and PLCβ1 based on the crystal structures of Gαq and the catalytic domain of PLCβ1 (4, 36), then proteins of areas 150 × 50 and 50 × 50 Å will be laterally associating on the membrane surface (as shown in Fig. 5, FRET is confined to the plasma membrane). Although we do not yet know the orientation of the two proteins when they are complexed, we do know the placement of the fluorescent tags (i.e. on the N terminus of PLCβ1 and toward the middle Gαq (14)). The observation of an average FRET of ~0.5 in the unstimulated state suggests that the probe separation must be ~35 Å, supporting the idea that the proteins are physically adjacent.

Although the high degree of FRET between eCFP-Gαq/eYFP-PLCβ1 in the basal state may be due to intrinsic factors that co-localize the proteins, it is more likely that scaffold proteins exist. We attempted to address this question by measuring FRET between eCFP-Gαq/eYFP-PLCβ1 in membrane preparations, but FRET results similar to the whole cells were obtained. Thus, either factors that promote self-assembly are retained in these preparations or the proteins have the ability to self-scaffold. This idea stems from previous studies showing that several of these proteins have strong binding sites for their functional partners and secondary weaker sites for other related proteins in their signaling domain (10).

The function of the cytosolic population of PLCβ1 is unclear. It is possible that this population serves as an exchange factor for the plasma membrane population that is complexed with Gαq allowing for rapid delocalization of the signal. It is possible that the turnover rate of PLCβ1 in this cell line is high, and the cytosolic population represents the nascent protein. Alternatively, the cytosolic population of PLCβ1 may serve as a reservoir in the case of rapid and high levels of Gαq-related agonists that may release previously unavailable Gαq. However, our studies that show this cytosolic population of PLCβ1 is stable upon stimulation argues against this idea. Based on the much stronger affinity of PLCβ1 to activated Gαq, it was surprising that the cytosolic population of PLCβ1 remained intact. In any case, the presence of a stable cytosolic PLCβ1 population points to a specific function, such as the regulation of phosphoinositide levels of internal membranes. Studies are underway to discriminate between these possibilities.

Both single cell and cell suspension studies show a high degree of eCFP-Gαq/eYFP-PLCβ1 FRET, which is significantly above values obtained for noninteracting proteins. This high value of FRET is surprising because not all cellular PLCβ1 is localized on the plasma membrane and because the cellular concentration of Gαq is ~3-fold higher than PLCβ1. Thus, the overall FRET value of ~40% may correlate with complete association of the plasma membrane population of eYFP-PLCβ1, and may additionally correlate to higher order donor-acceptor complexes. The strong driving force for complex formation is unclear, because the two proteins exist at low cellular concentrations relative to their dissociation constants, and because the affinity between unactivated versus activated Gαq and PLCβ1 is considerably weaker even when the two are confined to the membrane surfaces. Thus, there must be other factors, such as multiple interactions with other localized partners, that allow the local concentrations of the two proteins to be above their apparent dissociation constant and cause the proteins to remain bound.
Stable Gaq-PLCβ1 complexes are also supported by FRAP studies in which we bleached spots on the plasma membrane. Analysis of these results showed that both proteins have a large immobile population (i.e. ~24% for eYFP-PLCβ1 and 36% for eCFP-Gαq) and that the rate of eYFP-PLCβ1 recovery matches one of the two fitted rates of eCFP-Gαq recovery. Although there are multiple interpretations of these results, the simplest one that is supported by our FREt studies is that there are stable Gaq-PLCβ complexes that are immobile and also stable but slowly diffusing complexes. The faster rate observed for Gαq may correspond to protein that is not complexed with PLCβ. The presence of a large percentage of slowly diffusing and immobile populations suggests that Gαq and PLCβ signals in part may not be spatially delocalized by protein diffusion but rather by the rapid diffusion of the inositol 1,4,5-trisphosphate and diacylglycerol second messengers generated upon activation of phospholipase C.

Surprisingly, we do not see significant changes in the amount of eYFP-PLCβ1-eCFP-Gαq FREt in living cells upon stimulation despite the large increase in affinity between the proteins. As mentioned above, this observation implies that activated Gαq does not recruit a significant population of cytosolic PLCβ1 to the plasma membrane even though the concentration of Gαq exceeds PLCβ1. The stable cytosolic population of PLCβ1 was confirmed by distribution analysis. Thus, either plasma membrane Gαq or cytosolic PLCβ1 is inaccessible to their partner through strong interactions with other cellular components. It is important to note that recruitment to activated Gαq is diminished by the ability of PLCβ1 to stimulate the GTPase activity of Gαq subunits (37); therefore, the duration of Gαq signaling is greatly shortened allowing for a strong, short lived signal. Enhancement of the GTPase activity of Gαq by PLCβ1 will eliminate the strong driving force for association because of activation and maintain the cytosolic level of PLCβ1.

If Gαq and PLCβ are pre-associated in the basal state, then enzyme activation must occur through changes in Gαq-PLCβ molecular interactions presumably induced by the Gαq conformational changes that occur during GDP/GTP exchange. In Fig. 11, we present a model of this activation process. This model is supported by our results showing that proteolysis of PLCβ differs when it is complexed to activated versus deactivated Gαq. It is noteworthy that activation of PLCβ1 by Gβγ subunits appears to involve small, low energy conformational changes that can be reversed by subtle changes in the protein-protein interface (3, 38). Thus, it is likely that activation by Gαq involves similar movements produced by effective interaction between only the GTP-bound and not the GDP-bound Gα subunit.

It is notable that FREt has been used to study the interactions between G protein subunits during activation. Some studies suggest heterotrimer dissociation (24), and others suggest changes in orientation between stably associated Gα-Gβγ (30). It is unclear whether these conflicting reports are because of experimental differences, including differences in the level of protein expression. However, our results showing a stable Gαq and PLCβ1 population under basal conditions favor these latter studies and suggest a model of receptor-G protein-effector complexes that may self-scaffold because of multiple interactions. Previous measurements of RGS4 interactions with both Gβγ and PLCβ1 as well as Gαq support this idea (10), whereas further studies suggest a very specific nature of these multiple interactions (26). It would be interesting to see whether these PLCβ1-Gαq complexes are in turn associated with other proteins, in particular signaling pathways in order to give rise to the rapid and specific signals needed in neural signaling.

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