Resonance Energy Transfer between Guanine Nucleotide Binding Protein Subunits and Membrane Lipids†

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ABSTRACT: Resonance energy transfer was used to estimate the distances of closest approach between fluorescent labels on G protein subunits (α, β, and γ) and the phospholipid bilayer surface. Fluorescein-labeled α, β, and γ subunits were the energy transfer donors and hexadecylaminoeosin (HAE) in phospholipid vesicles was the acceptor. Bovine brain G protein (α_0, β, and γ subunits) were individually labeled with fluorescein as described in the accompanying paper [Kwon et al. (1993) *Biochemistry* (preceding paper in this issue)]. Fluorescein-labeled subunits were combined with the appropriate unlabeled G protein subunits and reconstituted into phospholipid vesicles. HAE quenched the fluorescein emission in a concentration-dependent manner which was dependent upon the G protein subunit labeled ($\gamma = \beta > \alpha_0$). From steady-state quenching data with hexadecylaminofluorescein (HAF) as a standard to determine the density of HAE in the phospholipid bilayer, the calculated distances between fluorescein-α_o, -β, and -γ and HAE are 46 ± 2, 38 ± 3, and 37 ± 2 Å, respectively. Energy transfer was identified as the means of fluorescence quenching by two methods: (1) addition of 0.1% Lubrol reversed the quenching of fluorescein-labeled G protein subunits by HAE and (2) sensitized HAE emission was observed in the presence of fluorescein-labeled G protein subunits. These results are the first physical measurements of the distances between sites on G protein subunits and the lipid bilayer. These data demonstrate that the label in the β and γ subunits is closer to the lipid bilayer than that in the α subunit.

Many cell surface receptors elicit an intracellular response by means of signal-transducing G proteins[1] located in the cytoplasmic leaflet of the cell membrane lipid bilayer [for review, see Freissmuth et al. (1989)]. G proteins are heterotrimers composed of α, β, and γ subunits. The α subunit, which specifically interacts with both cell surface receptors and effector proteins, binds and hydrolyzes GTP. The primary structures of the α subunits of G_i, G_0, and transducin are highly homologous (Birnbaumer, 1990). On the basis of the homology with the elongation factor Tu GTP binding site, a tertiary structure of the G protein α subunit has been proposed (Masters et al., 1986), although a region of membrane attachment was not defined.

There is limited information regarding the interaction of G protein and the plasma membrane. Detergent is necessary to release brain G_0/G_i from the membrane (Sternweis & Robishaw, 1984), although the α subunit does not require detergent to remain in solution (Sternweis, 1986). Tryptic cleavage of the amino-terminal 21 amino acids of G_i and G_0 α subunit prevents the interaction of α with the $\beta\gamma$ subunits (Neer et al., 1988) and causes the release of the remaining α subunit from the membrane (Eide et al., 1987). In addition, Eide et al. (1987) demonstrated that when membrane-bound G_0, G_{i1}, G_{i2} are activated by GTPγS, the α subunit does not dissociate from either brain or neutrophil membranes [however, see also Crouch (1991) and Ransnäs et al. (1989)].

The amino-terminal amino acid residue of G_0 and G_i (but not G_s) contains an amide-linked myristic acid (Buss et al., 1987). Possibly, this modification accounts for stable membrane association upon G protein activation by GTP (Jones et al., 1990; Mumby et al., 1990a). Although $\beta\gamma$ is required for the association of added α subunit with phospholipid vesicles (Sternweis, 1986), myristoylated α expressed in COS cells in excess of $\beta\gamma$ is associated with the cell membrane (Simonds et al., 1989). When a less hydrophobic lipid is incorporated into α instead of myristate, the localization of α in the cell is altered, such that membrane attachment is greatly diminished (Mumby et al., 1990a). In addition, the myristoylation of α alters the ability to recombine with $\beta\gamma$. The affinity of α for $\beta\gamma$ is greatly diminished when the α subunit is not myristoylated, as demonstrated by examining the ability of $\beta\gamma$ to slow the rate of GTPγS binding to α (Linder et al., 1991).

The $\beta\gamma$ subunits are not soluble in aqueous solution (Sternweis, 1986). The most likely explanation is that the γ subunit is modified by a geranylgeranyl moiety on the carboxymethylated C-terminal cysteine residue (Yamane et al., 1990; Mumby et al., 1990b). It is not known if the polyisoprenylation alters the affinity of $\beta\gamma$ for α. To date, no lipid modification of the β subunit has been reported.

Although models of the relation of G_α and $\beta\gamma$ to the phospholipid bilayer have been proposed on the basis of subunit function and lipid modification (Buss et al., 1987; Sanford et

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[1] Abbreviations: G protein, guanine nucleotide binding protein; G_i, G protein that mediates inhibition of adenylyl cyclase; G_0, a G protein abundant in brain that can interact with receptors in vitro that are known to inhibit adenylyl cyclase; G_s, G protein that mediates stimulation of adenylyl cyclase; GTP, guanosine triphosphate; GTPγS, guanosine 5′-(3-*O*-thio)triphosphate; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; FITC, fluorescein 5-isothiocyanate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HAE, hexadecylaminoeosin; HAF, hexadecylaminofluorescein; K_{SV}, slope from the Stern–Volmer plot; PC, phosphatidylcholine; Q_D, quantum yield of resonance energy transfer donor; F_D, fluorescence of resonance energy transfer donor; F_{DA}, fluorescence of resonance energy transfer donor in the presence of acceptor; RET, resonance energy transfer.

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al., 1991; Spiegel et al., 1991), there is very little data to support or disprove these models. One goal of this paper was to assess the orientation of the G protein subunits in the phospholipid bilayer using resonance energy transfer (RET). RET can be used as a "spectroscopic ruler" to assess distances in biological macromolecules (Stryer, 1978). For example, fluorescence energy transfer was implemented to estimate the distance between the plasma membrane and the EGF binding site on the epidermal growth factor receptor (Carraway et al., 1990). We applied this approach to estimate the distance between the three G protein subunits and the phospholipid bilayer. The three subunits of G_o/G_i were each labeled with fluorescein and served as energy transfer donors. Fluorescein-labeled subunits were recombined with the appropriate unlabeled subunits and reconstituted into phospholipid vesicles. Hexadecylaminoeosin (HAE) in the vesicle lipid bilayer served as energy transfer acceptor. These results contribute to the understanding of G protein structure by demonstrating that the fluorophores incorporated in the $\beta\gamma$ subunits are closer to the lipid bilayer than that in the α subunit and the $\beta\gamma$ subunits may serve as a membrane anchor for the α subunit. A preliminary report on these findings has been presented (Remmers & Neubig, 1992).

EXPERIMENTAL PROCEDURES

Materials. Fluorescein, fluorescein 5-isothiocyanate (FITC), 5-(iodoacetamido)fluorescein, hexadecylaminoeosin (HAE), hexadecylaminofluorescein (HAF), and anti-fluorescein IgG were purchased from Molecular Probes, Inc. (Eugene, OR). Phosphatidylcholine (PC) from bovine brain was purchased from Serdary Research Laboratories, Inc. (Port Huron, MI).

Preparation of Fluorescein-Labeled G Protein Subunits. Fluorescein was incorporated into each of the three G protein subunits as described in the accompanying paper (Kwon et al., 1993). An additional preparation of fluorescein-labeled α subunit (F-α–Mono Q) was prepared using Mono Q anion-exchange chromatography and was also used in the resonance energy transfer experiments. Functional properties of fluorescein-labeled α subunit (F-α and F-α–Mono Q), as well as labeled β and γ, have been described (Kwon et al., 1993). The fluorescein:protein ratios for the two different preparations of α, α-Mono Q, β, and γ used in these experiments were 1.0 $\pm$ 0.2, 0.7 (n=1), 1.1 $\pm$ 0.1, and 1.4 $\pm$ 0.1, respectively (Kwon et al., 1993).

Preparation of Phospholipid Vesicles Containing G Protein. Bovine brain PC (3 mg) and cholic acid (3 mg) were sonicated in 0.5 mL of buffer containing 50 mM Na-Hepes and 1 mM EDTA, pH 8.0 (HE), until the solution was translucent.[2] Vesicles were prepared by gel filtration of the PC/cholate solution plus 0.3 mg of G protein (in cholate) through a Sephadex G-50 column equilibrated with HE + 1 mM DTT (HED). The molar ratio of G protein to lipid was approximately 1:1200. Turbid fractions (approximately 1 mL) were pooled and stored in aliquots in liquid nitrogen. Recovery of protein, GTPγS binding, and PC in vesicles was greater than 75%. Vesicles containing unlabeled G proteins were also prepared. Aliquots of the vesicle preparations gel-filtered through a 5-mL Ultrogel AcA-44 column showed that the fluorescein-labeled G proteins were associated with the PC vesicles, which appeared in the void volume (Rubenstein et al., 1991). Attempts to reconstitute G proteins which had been stored in 0.1% Lubrol instead of cholate did not result in complete partitioning into the PC vesicles.

[2] The predominant fatty acyl groups in bovine brain PC are palmitate (C16:0) and oleate (C18:1) (Sigma Chemical Co., Technical Services).

Phospholipid Assay. In order to ensure similar acceptor densities upon addition of HAE, the phospholipid concentration was determined for vesicles containing G proteins. Lipids were extracted using a procedure modified from Radin (1969). Aliquots of vesicles (20 and 40 μL) were vortexed with 680 μL of chloroform/methanol (2:1) to extract the lipids. Upon addition of 150 μL of 0.73% NaCl and mixing, two phases formed. The upper aqueous phase was aspirated, and methanol (150 μL) was added to make the residual aqueous phase miscible. The solvent was evaporated with a stream of N_2 in a heat block set at 60 °C. Phospholipid quantitation was performed by spectrophotometric quantitation of Prussian blue complex formed with lipid organic phosphorus (Raheja et al., 1973). Bovine brain PC was used as standard.

Resonance Energy Transfer from Fluorescein-Labeled α, β, and γ Subunits to HAE. The goal was to assess the relative distances of labeled α, β, and γ from the phospholipid bilayer. Fluorescein-α, -β, and -γ subunits were recombined with unlabeled subunits and reconstituted into PC vesicles. HAE served as resonance energy transfer (RET) acceptor, while fluorescein was the RET donor.

Fluorescence measurements were made using a PTI Alphascan fluorometer with a water-cooled 150-W arc lamp. Excitation and emission wavelengths of 460 and 515 nm were used (4-nm slit width). All measurements were made at 30 °C and corrected for variations in lamp output by division of the fluorescence emission by the reference cell quantum counter value at each wavelength. The sample chamber was fit with an adapter with 3.75-mm windows that fit a 5- × 5-mm quartz fluorometer cell. G protein vesicles were added to 0.495 mL of HED (final concentration 50 μM PC, ~50 nM G protein). Following equilibration, initial fluorescence levels were measured. Five subsequent 1-μL additions of HAE (in ethanol) to give a final concentration of 50–500 nM were made, and following a 2-min equilibration, fluorescence emission was determined. Holowka and Baird (1983) found that approximately 90% of added HAE and greater than 95% of added HAF was associated with the plasma membrane vesicles at equilibrium. The fluorescence was corrected for background, ethanol addition/dilution, and HAE fluorescence emission at 515 nm. The corrections for background and dilution were less than 5% while the correction for HAE emission at 515 nm ranged from 1% to 20% depending on the fluorescence of the donor protein and the concentration of HAE added. The quenching experiments were performed on two different preparations of labeled G protein subunits for F-α, F-β, and F-γ and one preparation of F-α–Mono Q.

R_0, the distance (in angstroms) where energy transfer efficiency is 50%, was calculated from the expression $R_0 = (9.79 \times 10^3)(J\kappa^2 Q_D n^{-4})^{1/6}$. κ^2, the orientation factor, was assumed to be 2/3 (see Discussion), and n, the refractive index, is that of water (1.33). Quantum yields of the fluorescence donors (Q_D) and acceptor (Q_A) were determined as described by Cantley and Hammes (1975) by comparison with sodium fluorescein in 0.1 N NaOH (Q = 0.92) (Weber & Teale, 1957). In the steady-state fluorescence experiments, it was assumed that the changes in donor fluorescence intensity were identical to changes in quantum yield. The overlap integral, J, where $J = \Sigma F_D(\lambda)\epsilon_A(\lambda)\lambda^4\Delta\lambda/\Sigma F_D(\lambda)\Delta\lambda$, was calculated with summation carried out over 5-nm step size using a program written by Joe Mersol, Department of Physics and Electrical Engineering, University of Michigan.

Quenching of hexadecylaminofluorescein (HAF) fluorescence by added HAE was used to assess acceptor density in the PC vesicles as described by Holowka and Baird (1983).

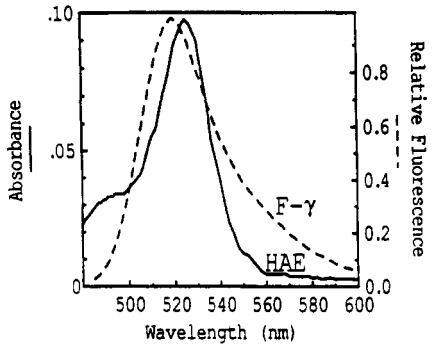

FIGURE 1: Spectral overlap of fluorescein-γ emission and the absorption of HAE in PC vesicles. The corrected emission spectrum of fluorescein-γ (F-γ) (ex 460 nm) is shown with the absorption spectrum of 1 μM HAE (HAE) in 100 μM PC vesicles. HAE was added to PC vesicles containing unlabeled G protein. Appropriate background spectra obtained with vesicles containing unlabeled G protein were subtracted from each. All samples were diluted in HED buffer.

In this case the distance of closest approach between donor and acceptor is negligible (Wolber & Hudson, 1979). The HAE density (C, the number of molecules of HAE/R_0^2) for each concentration of HAE was determined by entering the F/F_0 values for HAF quenching in the following equation: $F/F_0 = 0.6463e^{-4.7497C} + 0.3537e^{-2.0618C}$ and reading the C value using the standard curve function of InPlot (Graph Pad Software, San Diego, CA). The corrected fluorescence intensity data for the G protein subunits were plotted as a function of acceptor (HAE) density with the analytical solutions of the distance of closest approach/R_0 curves from Wolber and Hudson (1979).

Polarized Fluorescence Measurements. Steady-state fluorescence polarization of donor and acceptor fluorophores was determined by calculating anisotropy: $r = (I_{90} - I_0G)/I_{90} + 2I_0G)$, where I_{90} and I_0 are the fluorescence intensity when excitation and emission polarizers are parallel and perpendicular, respectively (excitation polarizer is vertical). Differences in the efficiency of transmitting vertically and horizontally polarized light was corrected for by determining G, the ratio of I_0/I_{90} using horizontally rather than vertically polarized excitation light.

Quenching of Fluorescein Fluorescence by Iodide. The ability of KI to quench fluorescein-labeled G protein subunits and fluorescein–glycine was assessed. Sequential additions of freshly prepared 3 M KI were made to fluorescein-labeled G protein containing vesicles (50 μM PC, ~50 nM G protein) in HE buffer (490-nm excitation, 520-nm emission). The ability of KI to quench fluorescein–glycine (~50 nM) was assessed in the presence of unlabeled G protein PC vesicles. To correct for dilution/salt effects on fluorescence measurements, 3 M KCl was added to samples as described above for KI. The corrected data (0–10% correction) were graphed in terms of a modified Stern–Volmer equation, $F_0/F - 1 = K_{SV}$[KI]. F_0 is the fluorescence emission in the absence of quencher and F is the emission in the presence of KI. K_{SV} is the Stern–Volmer quench constant.

RESULTS

Resonance Energy Transfer from Fluorescein-G Protein or HAF to HAE in Vesicles. To determine the distance between G protein subunit labels and the lipid bilayer by RET, fluorescein-labeled subunits were recombined with the appropriate unlabeled subunits to form heterotrimer with label in one subunit. Figure 1 shows the fluorescence emission spectrum of G_0/G_i labeled in the γ subunit (F-γ) and the HAE absorbance spectrum in PC vesicles. The emission spectra of all of the labeled subunits were similar. On the basis of the spectral overlap, the distance of 50% energy transfer, R_0, was calculated to be 54.9–60.8 Å, indicating that fluorescein and HAE represent a suitable donor–acceptor pair (Table I). An average value of 56.7 Å was used for all donors in calculations of distances from RET measurements. The anisotropy values for all probes were 0.18–0.20, indicating that there is somewhat limited orientational freedom of the donor and acceptor fluorophores. The minimal and maximal measured anisotropy values of fluorescein in HED buffer and in frozen glycerol were 0.02 and 0.39, respectively (data not shown).

Table I: Spectral Parameters of Fluorescein–Donor and HAE–Acceptor Pairs[a]

energy transfer donor	fluorescence anisotropy (r)	quantum yield (Q_D)	R_0 (Å)
HAF	0.20	0.48	56.6
fluorescein-α	0.19	0.68	60.8
fluorescein-β	0.18	0.37	54.9
fluorescein-γ	0.20	0.37	54.8
average			56.7

[a] All polarized fluorescence measurements were made with fluorophores in PC vesicles. HAE is the RET acceptor molecule with a fluorescence anisotropy value of 0.16. The HAE and HAF concentrations were 200 nM. The refractive index, n, was assumed to be 1.33 and the orientation factor, κ^2, was assumed to be $^2/_3$ in the calculations of R_0 (see Discussion).

To lipid vesicles containing either labeled G protein or unlabeled G protein and HAF, sequential additions of HAE were made (Figure 2). The lipid concentration in each cuvette was identical so that the density of HAE could be determined by using the HAF quenching data. Figure 2A demonstrates that addition of Lubrol (0.1% final concentration) reverses the quenching of fluorescein-γ by HAE.[3] In addition, at maximal HAE concentrations (500 nM), the absorbance of HAE at 460 nm is less than 0.01 for a path length of 5 mm. Thus, the quenching is dependent upon vesicle integrity and is not due to trivial reabsorption of fluorescein fluorescence. Also, HAE quenching is not simply due to HAE binding to the protein because the quenching is reversed by detergent addition. To quantitate the quenching of fluorescein due to energy transfer, the contribution to the fluorescence signal from HAE alone in vesicles was subtracted from the fluorescence of the mixture. G_0/G_i containing a fluorescein label in the β or γ subunits was quenched to the greatest extent by HAE followed by that with fluorescein label in the α subunit (F-α or F-α–Mono Q) (Figure 2B). This indicates that the labels on the β and γ subunits are closer to the bilayer than the label in the α subunit.

Sensitized HAE emission is demonstrated in Figure 3. The mixture of F-γ and HAE showed a decrease in donor emission (515 nm) compared to the sum of the F-γ and HAE emission spectra. There was also a concomitant increase in HAE emission (545 nm) in the mixed sample. Sensitized HAE emission was observed for all three labeled subunits in vesicles.[4]

[3] The rise in fluorescence above the initial fluorescence intensity is due to small increases in the donor and acceptor quantum yields in the presence of 0.1% Lubrol (or absence of PC vesicles).

[4] In addition, fluorescence lifetime measurements showed that the average lifetimes of fluorescein label on the G protein subunits decreased when HAE was added to vesicles containing labeled protein. The decrease in fluorescein lifetimes was dependent upon the location of the label where the HAF lifetime decreased the most followed by fluorescein in $\gamma > \beta > \alpha$ ($n = 1$). The decrease in lifetimes provides further corroboration that the mechanism of quenching by HAE is resonance energy transfer.

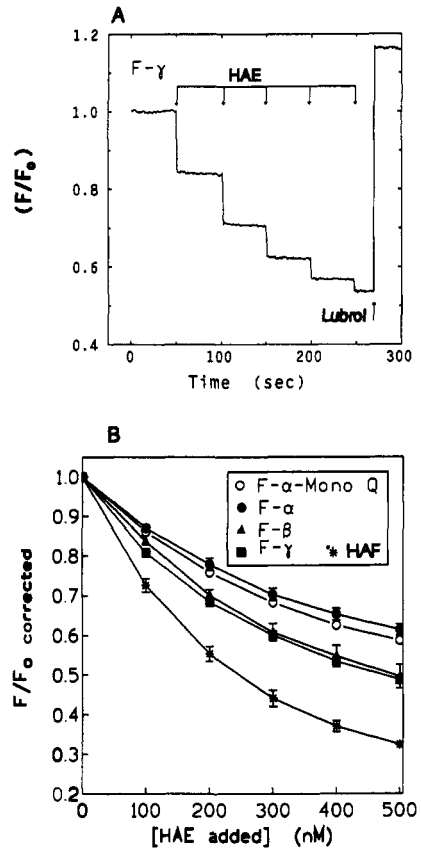

FIGURE 2: (A) Reversal of fluorescein quenching by HAE upon addition of detergent. Uncorrected fluorescein-γ emission (ex 460 nm, em 515 nm) is shown for vesicles titrated with 100–500 nM HAE. Following addition of 500 nM HAE, 5 μL of 10% Lubrol was added (0.1% final concentration) and the result is shown at time 270–300 s. (B) Quenching of fluorescein fluorescence by HAE in PC vesicles. Sequential additions of 100 nM HAE were made to G protein containing vesicles (50 μM PC, ~50 nM G protein) in HED. Following a 2-min equilibration, fluorescence at 515 nm was measured (ex 460 nm). This experiment was repeated 5 times and the mean F/F_0 and SEM are ploted for α(●), α-Mono Q(○), β(▲), γ(■), and HAF(*). F/F_0 is corrected for dilution, lipid vesicle background fluorescence, and HAE fluorescence.

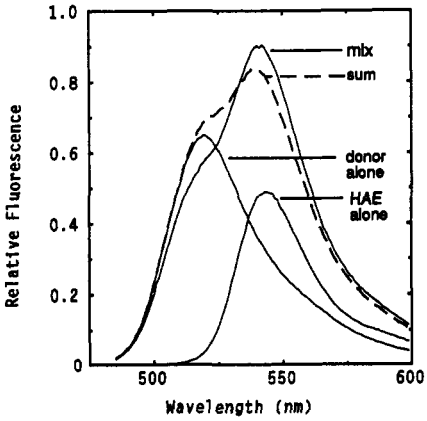

FIGURE 3: Emission spectra of fluorescein-γ labeled G protein (ex 460 nm) in PC vesicles alone, 100 nM HAE in vesicles, and F-γ vesicles with 100 nM HAE added. Also shown is the sum of the spectra for donor alone and acceptor alone (dashed line). The background spectrum (unlabeled G proteins in PC vesicles) was subtracted from each spectrum and accounted for less than 3% of the peak fluorescence.

These data indicate that the steady-state quenching of fluorescein fluorescence is due to RET.

Calculation of the Distance of Closest Approach. The quenching of HAF fluorescence in PC vesicles by HAE was used to determine the acceptor density in the vesicles by fitting the data to double-exponential decay curves where L/R_0 = 0 (Wolber & Hudson, 1979) as described by Holowka and Baird (1983) (see Experimental Procedures). Figure 4 depicts the quenching data for the three subunits expressed in terms of HAE density in the vesicles. Also plotted are the theoretical curves for the RET between a donor and a plane of acceptors (Wolber & Hudson, 1979) for values of L/R_0 in the same range as the experimental data. The curves that best fit the data were used to determine L, given R_0 (Table II). The five sets of data were plotted with the theoretical curves and L/R_0 values assigned to the nearest 0.05. The five determinations were then averaged and multipled by R_0 (56.7 Å) to determine the estimated distance of closest approach.

One potential artifact in these measurements would be the inaccessibility of HAE to the donor fluorophores in the inner leaflet of the vesicle bilayer. To determine if HAE had access to fluorophores in both the inner and outer leaflets of the PC vesicles, external fluorescence was quenched with anti-fluorescein IgG and HAE quenching of internal (i.e., antibody

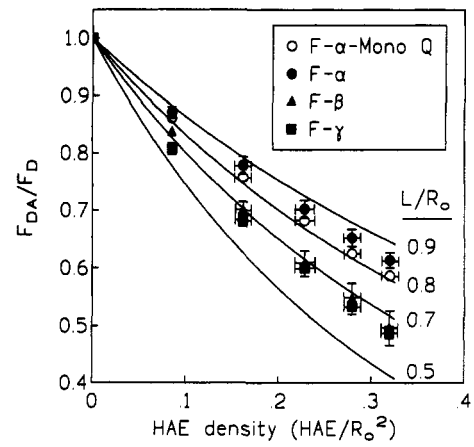

FIGURE 4: Energy transfer between fluorescein-labeled G protein subunits and HAE in PC vesicles. The efficiency of energy transfer, expressed as the ratio of donor fluorescence in the presence (F_{DA}) and absence (F_D) of acceptor, is plotted as a function of the density of acceptor at the membrane surface of the vesicles (see Experimental Procedures). Shown is F_{DA}/F_D for G protein labeled in the α [F-α-(●), F-α–Mono Q(○)] and β [F-β(▲)], and γ [F-γ(■)] subunits. L/R_0 is a ratio of the distance of closest approach between donor and acceptor and the distance at which the efficiency of energy transfer is 50%. The curves represent estimated theoretical quenching curves obtained for acceptor density ranging from 0 to 0.6 from an analytic solution to Förster energy transfer in two dimensions (Wolber & Hudson, 1979). The theoretical quench curves are for L/R_0 values ranging from 0.5 to 0.9. The data shown are the mean of five determinations and SEM. Estimated L/R_0 values for the subunits were determined for five sets of data, as described in Experimental Procedures, and the values were averaged to determine the distance estimates (Table II).

Table II: Estimated Distances between Fluorescein-Labeled G Protein Subunits and the Lipid Bilayer[a]

G protein subunit labeled	L/R_0	estimated distance of closest approach (Å)
α	0.85 ± 0.04	48 ± 2
α-Mono Q	0.82 ± 0.03	46 ± 2
β	0.67 ± 0.06	38 ± 3
γ	0.65 ± 0.05	37 ± 2

[a] HAE served as RET acceptor. The average R_0 for all four donors, 56.7 ± 1.4 Å, was used to estimate the distance of closest approach. The ratio of the estimated distance of closest approach (L/R_0) for HAF and HAE as donor and acceptor was assumed to be zero. The estimated distance of closest approach is the mean of five determinations, and the error listed is SEM.

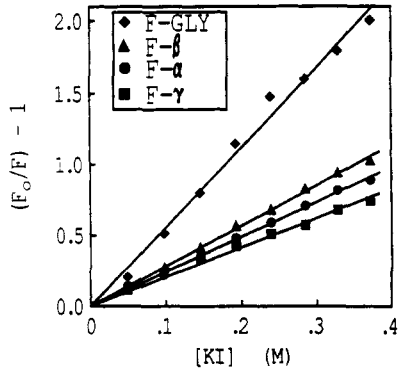

FIGURE 5: Stern–Volmer plot of the quenching of fluorescein by iodide. Fluorescein-labeled G proteins reconstituted into PC vesicles or fluorescein–glycine in the presence of PC vesicles were titrated with KI as described in Experimental Procedures. $F_0/F - 1$ from a representative experiment is plotted for fluorescein-labeled α(●), β(▲), and γ(■) and fluorescein–glycine (◆). Error bars represent standard deviation from two (fluorescein–glycine) or three determinations using two different preparations of fluorescein-labeled G protein subunits. The average K_{SV} values are listed in Results.

inaccessible) fluorescein was assessed. Following quenching of vesicle fluorescence by the antibody, HAE was added sequentially. The ability of HAE to quench the fluorescence inside the PC vesicles was similar to that observed for the quenching of total vesicle fluorescein fluorescence (data not shown). Therefore, compartmentation of fluorescein into acceptor-inaccessible domains does not appear to occur and thus does not alter the distance estimates.

Quenching of Fluorescein Fluorescence by Iodide. The location of the fluorescein label on the G protein subunits was investigated by determining the accessibility of label to quenching by potassium iodide, a water-soluble quencher. Stern–Volmer plots for KI quenching of free fluorescein (FITC reacted with glycine) and fluorescein-labeled G protein subunits are shown in Figure 5. The fluorescein-labeled G proteins were reconstituted in PC vesicles as in the previous experiments. Vesicles without labeled G protein were included in the experiment with fluorescein–glycine. The observed linearity of the Stern–Volmer plots suggests that environment of the probe on each protein subunit is homogeneous (i.e., no significant component of inaccessible fluorophore). The slopes obtained from the Stern–Volmer plots (K_{SV}) for fluorescein–glycine and fluorescein-labeled α, β, and γ subunits are 5.8 ± 0.3, 2.1 ± 0.3, 2.2 ± 0.5, and 1.9 ± 0.2 M^{-1}, respectively. There is no significant difference in Stern–Volmer quench constants for fluorescein-α, -β, and -γ. The decreased ability of KI to quench fluorescein bound to α, β, and γ subunits compared to free fluorescein demonstrates that the labels on the proteins are not completely accessible to the solvent. The reduced quenching efficiency of KI may, in part, arise from the reduced quantum yield of the fluorescein label on the proteins. The ability of KI to quench fluorescein-α, -β, and -γ indicates that the labeled amino acid residues are not completely buried in the hydrophobic domain of the PC vesicle bilayer.

DISCUSSION

Resonance energy transfer has been used as a tool to estimate distances between or within proteins as well as distances between proteins and lipid. Although models of G protein–membrane interaction have been proposed, these models have not been tested to date. Here we provide the first estimates of distances between sites on G protein subunits and the membrane surface. Although RET has been implemented to monitor activation of transducin by rhodopsin, the distances between the labeled proteins and the phospholipid vesicle bilayer were not assessed (Borochov-Neori & Montal, 1989).

The estimated distances of 46–49, 39, and 37 Å between membrane lipid headgroups and α, β, and γ, respectively, may not represent exact distances from the bilayer, yet the order observed where $\alpha > \beta = \gamma$ is highly consistent. Both HAF and HAE efficiently partition into the lipid bilayer. The fluorophore rings are probably anchored near the lipid–water interface yet partially buried in the hydrophobic region. A reduced quenching constant of KI is observed for HAF bound to low-density lipoprotein compared to aqueous HAF monomer (Sklar et al., 1980). The molar ratio of G protein to lipid in our bovine brain PC vesicles was approximately 1:1200 to reduce any possible G protein dimerization. Also, the reversibility of the fluorescein quenching by HAE upon disruption of the vesicle integrity with detergent, as well as the observed sensitized acceptor emission, lead us to conclude that we were observing Förster energy transfer and not other forms of fluorescein quenching.

The measurements reported here are the first physical evidence that portions of the $\beta\gamma$ subunits are closer to the membrane than the α subunit. These data support the model of the α subunit tethered to the membrane via the N-terminus, which is myristoylated. Although α subunit produced by overexpression of myrisoylated α in COS cells does not require $\beta\gamma$ for association with the plasma membrane (Simonds et al., 1989; Mumby et al., 1990), the orientation of α in the membrane may be different in the presence of $\beta\gamma$. In addition, $\beta\gamma$ is not necessary for membrane attachment of α, yet it is necessary for receptor coupling (Florio & Sternweis, 1989). Because α may not be released from the cell membrane upon activation by GTPγS (Eide et al., 1987), and because α is unable to associate with the membrane or $\beta\gamma$ upon removal of its 2-kDa N-terminus (Eide et al., 1988; Neer et al., 1984),[5] we support a model for the membrane attachment of G protein subunits where α is anchored to the membrane by myristoylation and to $\beta\gamma$ by the N-terminus. It is likely that the association of α and $\beta\gamma$ is mediated by both protein–protein and lipid–lipid interactions. The estimated distance of α from the surface of the lipid bilayer (46–49 Å) taken with an estimated diameter of 60 Å for α (Huff et al., 1985) and an indication that α is globular (Northup et al., 1983) suggests that either the fluorescein label is located at a far point in the protein from the lipid bilayer or that the α subunit is tethered to the membrane and is not directly apposed to the membrane surface. The accessibility of iodide to the fluorescein labels on the G protein subunits suggest that the labels are not located in the hydrophobic core of the lipid bilayer.

Several factors limit the exact distance measurements by RET. The distances may be artificially high due to sequestration of PC around the G protein and thus exclusion of the RET acceptor HAE from the lipid annulus surrounding the G protein. Sources of error are also introduced by assumptions made in the calculation of R_0. The orientation factor κ^2 is assumed to be 2/3. The donor and acceptor fluorophores are assumed to be freely rotating during the donor fluorescence lifetime. The measured polarization values of donors and acceptor in PC vesicles indicate that the fluorophores are neither freely rotating nor completely immobilized (Table I).

[5] In preliminary experiments, the distances between fluorescein-α and -γ and HAE in PC vesicles were unaltered following incubation in 20 μM GTPγS and 10 mM $MgCl_2$ or 20 μM $AlCl_3$, 10 mM $MgCl_2$, and 10 mM NaF. These results suggest that, upon G protein activation, the distance of the α and $\beta\gamma$ subunits from the membrane is unaltered.

Based on our measured anisotropy values and reference to Figure 9 in Dale et al. (1979), the assumption of the value of κ^2 to be 2/3 may lead to a *maximal* error of 20% above or below the calculated R_0. Yet, when the likely possibility of two or three transition dipole moments (instead of a single moment) for donor and acceptor electronic transitions are taken into account, the maximal error for the measured polarization values is ±10% (Haas et al., 1978).[6] A smaller source of error in the distance estimates may be due to RET across the lipid bilayer. Since R_0 is relatively large and the distance between polar headgroups on either side of the lipid bilayer is approximately 50 Å (Rawn, 1983), quenching by RET acceptor on the other side of the lipid bilayer from that of the donor may occur. This source of error could lead to the slight overestimation of acceptor density and underestimation of the distance to the subunits as described (Carraway et al., 1990). These sources of error in estimating the absolute distance between donor and acceptor do not significantly alter the relative quenching of the different subunits by HAE.

If the protein was covalently labeled in more than one location on the protein, the estimated distance will be an average of these two distances. Or, if the two sites of labeling have large differences in the distance from the membrane, one would not expect the HAE quench data to parallel the theoretical quench curves. From the characterization of F-α, we know that the label is not in one site, so the quench data most likely reflect an average of the multiple sites labeled.

In summary, it appears that the location of the label on the α subunit is further away from the lipid bilayer than the labels on β and γ, while the difference in estimated distances between β and γ and the bilayer are not significant. To further refine the model of G protein membrane interaction, the specific amino acid residue(s) modified on each subunit will be determined. Future studies will also assess the molecular architecture of G protein subunit association as well as attempt to monitor the activation of G protein in signal transduction using RET.

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[6] The same rank order potency of donor quenching was observed when octadecylrhodamine B in PC vesicles served as an alternative RET acceptor. These data suggest that any possible error introduced by assuming $\kappa^2 = 2/3$ does not alter the observation that quenching by a RET acceptor is dependent upon the G protein subunit labeled where the distance between lipid bilayer and G protein subunit is greatest for α, followed by β and γ.