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LATERAL MOBILITY OF TETRAMETHYLRHODAMINE (TMR) LABELLED G PROTEIN α AND $\beta\gamma$ SUBUNITS IN NG 108-15 CELLS[*]

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Abstract—Multi-step signal transducing events, such as those mediated by G proteins, have been difficult to study in intact cells. We prepared fluorescently labelled G protein subunits, tetramethylrhodamine-α_O (TMR-α_O) and TMR-$\beta\gamma$, in order to study their subcellular distribution and lateral mobility. Heterotrimeric G proteins labelled in the α (TMR-$\alpha_O/\beta\gamma$) or β (TMR-$\beta\gamma/\alpha_O$) subunit were reconstituted into lipid vesicles and fused to NG-108-15 cells using polyethylene glycol (PEG). Vesicles fused completely to the cells as determined by dequenching of a fluorescent lipid probe, octadecyl rhodamine B. The orientation of G protein $\beta\gamma$ subunits after fusion followed the expected random distribution; the quenching of surface fluorescence with anti-fluorescein antibodies showed that about 50% of the label was accessible extracellularly. G proteins incorporated by the fusion method were able to couple to endogenous α_2 adrenergic receptors based on the restoration of high affinity agonist binding to pertussis toxin-treated cells. The subcellular localization of TMR-α_O and TMR-$\beta\gamma$ determined by differential centrifugation and confocal microscopy indicated that TMR-α_O was present in the plasma membrane and in intracellular membranes, whereas TMR-$\beta\gamma$ was mainly localized in the plasma membrane. The lateral mobility of TMR-α_O and TMR-$\beta\gamma$ measured using fluorescence recovery after photobleaching (FRAP) demonstrated low mobile fractions of 0.34 ± 0.03 and 0.16 ± 0.03, respectively. The translational diffusion coefficients of the mobile components were similar, 4.0×10^{-9} and 2.0×10^{-9} cm^2/s, for α and $\beta\gamma$ respectively. Neither activation of G_i-linked receptors nor cytoskeletal disruption with nocodozole or cytochalasin D changed the mobile fraction or diffusion coefficient of the α or $\beta\gamma$ subunits. The FRAP data combined with the localization of fluorescent subunits by confocal microscopy suggest that the $\beta\gamma$ subunits are highly constrained to localized regions of the plasma membrane while the α subunit may diffuse in intracellular regions to transmit signals from receptors to effector proteins.

Key words: G protein, fluorescence microscopy, membrane fluidity, neuroblastoma, signal transduction opioid receptors, cytoskeleton, diffusion.

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Abbreviations: G protein—guanine nucleotide binding protein; G_s—G protein that mediates stimulation of adenylyl cyclase; G_O—G protein abundant in brain; GTP—guanosine triphosphate; SDS—sodium dodecyl sulphate; EDTA— ethylenediamine tetraacetic acid; DTT—dithiothreitol; DMEM—Dulbecco's modified Eagle's medium; TMR— tetramethylrhodamine; R_{18}—octadecyl Rhodamine B chloride; PIC—paraiodoclonidine; PC—phosphatidylcholine; PS—phosphatidylserine; F-Ab—anti-fluorescein antibody; AchR—acetylcholine receptors; FRAP—fluorescence recovery after photobleaching; f_m—fraction mobile; D_T—translational diffusion coefficient; HE buffer—25mM Hepes, pH 8.0, 1mM EDTA; HEMN buffer—HE buffer supplemented with 2mM $MgCl_2$, 100mM NaCl; TED buffer—20mM Tris, pH 8.0, 1mM EDTA, 1mM DTT.

INTRODUCTION

A family of guanine nucleotide binding proteins (G proteins) containing three subunits, α, β and γ, is central in cellular signal transduction [1–3]. The currently accepted model of the mechanism of G protein activation in cells is that after receptor activation, GTP binds and the α and βγ subunits dissociate. Generally, the free α (or possibly βγ) subunit activates an appropriate effector protein [4, 5]. The deactivation of α subunit occurs when the GTPase of the α hydrolyses the bound GTP to GDP. The GDP-bound α subunit then reassociates with the βγ subunit and returns to an inactive state [4]. While this model of G protein activation is well established in solublized and reconstituted systems, there is much less information about G protein mechanisms in intact cells.

Whole cell studies will ultimately be required to assess the physiologic sequence of events, but it has been difficult to study such multi-step signal transducing events in whole cell systems. Although purified G protein, receptor, and effector are known to be sufficient to reconstitute hormonal activation of an effector in phospholipid vesicles, other components may be important for the modulation of the signal transduction in intact cell systems [1, 6, 7]. Even the subcellular distribution of G proteins is not clear [8–12].

The role of lateral mobility of G protein subunits or effector proteins has long been assumed in current thinking about G protein mechanisms. Tolkovsky and Levitzki [13] first hypothesized that adenylyl cyclase activation by the β-adrenergic receptor proceeds by a collision-coupling mechanism. At the time of their original proposal, Tolkovsky and Levitzki did not recognize the existence of G proteins as messengers between receptors and adenylyl cyclase. They later suggested that the G protein and cyclase behaved as one unit [14]. The functional coupling of receptor from one cell to the adenylyl cyclase from another, provided additional experimental support for this mechanism [15]. Rodbell [16] and Chabre [17] have proposed that G protein α subunits act as cytoplasmic shuttles between receptors and effectors. In this case, the requirement for the lateral diffusion of the receptor and the effector becomes minimal since G protein α subunits travel between the receptor and the effector. These models make certain predictions about the mobility of the G protein subunits in the cell membrane. The ability to reincorporate fluorescently labelled subunits into cells allows us to test some of these hypotheses.

In this paper, we describe the first measurements of the lateral mobility of G protein subunits in intact cells. The strikingly low mobility and restricted distribution of the βγ subunit are at odds with current models of G protein function and suggest that a new role of cell membrane organization must be considered in G protein mechanisms.

MATERIALS AND METHODS

Reagents

Fluorescein isothiocyanate, tetramethylrhodamine–iodoacetamide (TMR–IAA), and Octadecyl Rhodamine B chloride were obtained from Molecular Probes (Eugene, OR). Bovine brain phosphatidylcholine (A-30) and phosphatidylserine (A-37) were purchased from Serdary Research Laboratories (Ontario, Canada). Polyethylene glycol (M_r 8000), soybean phosphatidylcholine (P-5638), and cholic acid were obtained from Sigma Chemical Co. (St Louis, MO). The cholic acid was purified by the method of Ross and Schatz [29]. Purified pertussis toxin was a kind gift from Dr Nadine Cohen (Jamaica Plains, MA). [1251]-*p*-Iodoclonidine (2200 Ci/mmol) was obtained from Du Pont-New England Nuclear (Boston, MA). Oxymetazoline was from Schering. Lubrol PX (L-3753) was obtained from Sigma and deionized prior to use. NG-108-15 cells were obtained from Dr Marshall Nirenberg (NIH). Glass coverslips (25 mm diameter) were obtained from Fisher Scientific.

Preparation of phospholipid vesicles containing fluorescently labelled G protein subunits

Bovine brain G_o/G_i was purified and labelled with tetramethylrhodamine or fluorescein as reported previously [18]. Phospholipid vesicles containing fluorescently labelled G protein subunits were prepared by the method of Kim and Neubig [19]. Briefly, bovine brain phosphatidylcholine (PC) and phosphatidylserine (PS) (3 mg each) in 600 μl of $CHCl_3$ were dried under N_2 and incubated with 1 ml of a 0.6% solution of sodium cholate in 25mM HEPES, pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA and 100 mM NaCl (HEMN buffer) at 23°C for 5 min. The mixture was vortexed for 5 min and then sonicated at 4°C under N_2 in a sonicator bath (Bransonic 12) for 30–45 min until the mixture appeared translucent. Fluorescently labelled proteins, TMR-α_o/βγ (dye/protein: ~1) or TMP-βγ/α_o (dye/protein: ~0.6), in TED buffer (20 mM Tris, pH 8.0, 1 mM EDTA, 1 mM DTT) containing 0.5% sodium cholate were added to a final concentration of 0.6 mg protein/ml and a protein:lipid mass ratio of 1:10. The cholate was removed by chromatography through a 5-ml Sephadex G-50 column equilibrated in HEMN buffer. Turbid fractions were pooled, quick frozen, and stored at −70°C until use. PC/PS vesicles containing the fluorescent lipid analogue 3,3'-dioctadecylindocarbocyanine iodide (DiI) were prepared following the same procedure as described above. In some early experiments (where indicated in the figure legends), soybean PC (Sigma P-5638) was used instead of bovine brain PC and PS.

Vesicle–cell fusion using polyethylene glycol (PEG)

NG-108-15 cells were grown as monolayer cultures in Dulbecco's modified Eagle medium (DMEM) containing 10% foetal calf serum until confluency was achieved (2 or 3 days). NG-108–15 cells (~0.7×10^6) were banged off the flasks and resuspended in 25ml DMEM. Cells were transferred to a 50 ml conical tube and washed twice by centrifugation at 500 *g* for 3 min. Two ml of 50% polyethylene glycol (body wt) was layered over the cell pellet, followed by the addition of 150 μl of vesicles containing fluorescently labelled proteins (50 μg). The mixture (total volume = 2–3 ml) was vortexed and incubated in a cell culture incubator at 37°C for 5 min. After the 5 min incubation, 35 ml of DMEM was added to the mixture which was incubated another 30 min to allow cells to recover from PEG treatment. After incubation, cells were washed four times and resuspended in 10 ml DMEM. One milliliter aliquots were plated on round coverslips (25 mm diameter) placed in 30 mm petri dishes. Cells were kept at 4°C until use.

Preparation of NG-108–15 cell membranes

NG-108–15 cells were grown as described above. After reaching confluency, cells were incubated in serum-free medium with or without pertussis toxin (100 ng/ml) for 18 h prior to preparation of membranes. Cells were harvested, collected by centrifugation at 800 *g* for 5 min, and washed twice in 10 mM Tris-HCl, pH 7.5, 0.5% NaCl. Cells were homogenized with 10 strokes in a glass-Teflon homogenizer in hypotonic Tris buffer (5 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA). The homogenate was centrifuged at 1000 *g* for 5 min, and the supernatant was saved. The pellet was homogenized, centrifuged again and the resulting supernatants were combined. The supernatants were centrifuged for 30 min at 20 000 *g*, then the pellet was resuspended in TME buffer (20 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT), frozen in liquid nitrogen, and stored at −70°C until use. After thawing, membranes were homogenized (10 strokes) in a glass–Teflon homogenizer before use.

Differential centrifugation and quantitation of G protein subunits

NG-108–15 cells were fused with vesicles containing G_o/G_i or TMR-α_o/βγ as described above. Cells were washed four times by centrifugation at 800 *g* then homogenized with 10 strokes in a glass–Teflon homogenizer in hypotonic Tris buffer (5 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA). The homogenate was centrifuged at 20,000 *g* for 30 min and the supernatant was separated from the pellet. The pellet was washed twice with TME buffer. The protein content of both the supernatant and the resuspended pellet were determined [20] and 60 μg of protein was loaded onto a 10% polyacrylamide gel [21]. The resolved proteins were transferred to polyvinyldifluoride (Immobilon, Millipore). Immunoblot analysis was performed following the protocol of Welsh *et al.* [22]. Anti-α_o antibody (NEI-804, GC/2, Du Pont-New England Nuclear) and anti-β subunit antibody (NEI-804, SW/1, Du Pont-New England Nuclear) were used as primary and ^{125}I-labelled donkey anti-rabbit IgG (IM-134, Amersham) as secondary antibodies, all at a 1:1000 dilution. Analysis of radioactivity was done by autoradiography using Kodak RP-Xomat film at 70°C for 12 to 16 h. The relative amount of α_o or β subunit in each band was determined by densitometry. Density was linear with amount loaded in the range studied.

*Equilibrium binding of [^{125}I]-*p*-iodoclonidine*

Binding of the partial α_2 agonist [^{125}I]-*p*-iodoclonidine (PIC) was measured at 23–25°C as described by Gerhardt *et al.* [23]. Data were fit using the non linear least squares fitting routines of the computer program Inplot (Graph PAD Software, San Diego, CA).

Incorporation of octadecyl rhodamine B chloride (R_{18}) into phosphatidylcholine vesicles

Octadecyl Rhodamine B chloride in chloroform–methanol was dried under a stream of nitrogen gas and solubilized in ethanol. A total of 10 μl of this solution (0.12 mg) was mixed with sodium cholate solubilized soybean PC (3 mg) in HEMN buffer. The R_{18}-containing membrane fraction was recovered in the void volume of a G-50 column. Aliquots of the R_{18} containing vesicles were frozen in liquid nitrogen and stored at −70°C until use.

Determination of self-quenching of R_{18} fluorescence

Phospholipid vesicles were prepared containing 4 mole% of R_{18} with respect to total lipid. The vesicles were then fused to cells as described above. Fluorescence of R_{18}-containing vesicles or cells was determined before and after addition of 0.5% Lubrol PX. The percentage of fluorescence quenching was calculated after correcting for the change in fluorescence due to detergent and sample dilution. Four mole% R_{18} was sufficient to result in 90% quenching of the R_{18} fluorescence in the vesicles.

Orientation of fluorescent probes in lipid vesicles and in cells after fusion

PC/PS vesicles (3 mg) containing 150 μg of fluorescein labelled βγ subunits (F-βγ) and 10 μg of unlabelled α_o were prepared. Vesicles containing 3 μg of F-βγ/α_o were diluted into 600 μl of TEN buffer (20 mM Tris, pH 7.6, 1 mM EDTA, 150 mM NaCl) and fluorescence was determined before and after addition of increasing amounts of anti-fluorescein antibodies (F-Ab). Antibody quenching was similarly tested for the F-βγ/α_o which had been incorporated into cells.

Fluorescence measurements

R_{18} fluorescence was measured using a PTI Alpha scan fluorometer (λ_{EX}: 555 nm, λ_{EM}: 578 nm, slits 5 nm × 5 nm) in a 5 × 5 mm quartz cell. The sample chamber was equipped with a magnetic stirrer and the temperature was controlled at 25°C with a thermostated circulating water bath. The final sample volume was 600 μl for all measurements.

Confocal laser scanning microscopy

A Bio-Rad confocal laser scanning microscope (model MRC 600) was used to visualize the localization of TMR-α_o and TMR-βγ in NG-108–15 cells. Confocal sections (0.5–0.8 μm thickness) were obtained using an oil immersion Nikon 60 X/NA 1.4 objective and zoom settings between 3-5 X. Excitation was at 514 nm.

FRAP measurements

The fluorescence recovery after photobleaching (FRAP) measurements were performed using 63 × 1.4 Zeiss objective and the apparatus described previously [24]. NG-108–15 cells containing fluorescent G protein subunits were equilibrated for 5–10 min at 22°C in Hank's balanced salt solution before FRAP measurements. The argon beam (514 nm) was focused through an epi-illumination fluorescence microscope to a spot on the lower surface of the cell. A 50 ms pulse bleached the fluorophores and the fluorescence recovery was probed with the same focused beam attenuated by a factor of about 10,000. Probe-induced fluorescence was monitored by a Hitatchi photomultiplier (R 943-02). The bleach depth was maintained at approximately 40–60% in all experiments by use of appropriate neutral density filters. Because recovery from bleaching was incomplete, only one set of data was collected from a single spot on a cell. The beam was moved to another spot on the same cell or to a different cell before initiating another run. The distribution of fluorescence was not uniform on the cells. FRAP data were collected from cells with fluorescence that appeared most uniform and the brightest spots on the cells were avoided.

The sample bin size for data acquisition was 50 msec and one complete scan consisted of 100 prebleach points and 400 postbleach points. Fluorescence recovery was recorded for 20 sec. Recovery curves were fit using InPlot (GraphPad Software, San Diego CA) to Eqn 1 which is an approximation of the theoretical recovery curve [25]:

$$F(t) = F_O + F_r\, t/(t+\tau), \qquad (1)$$

where t is the time after bleach, $F(t)$ is the fluorescence as a function of t, F_O is the flourescence immediately after bleach, F_r is the amount of fluorescence recovery, and τ is the time for half-maximal recovery. The prebleach fluorescence F_P was determined by fitting the prebleach data to a constant. The fractional recovery of fluorescence was calculated from the expression $F_r/(F_p - F_0)$. Diffusion coefficients were calculated to be $D = (\gamma/4)w^2/\tau$ where γ is related to the bleaching depth and is approximately 1.2 under our experimental conditions [25], and w is the e^{-2} spot radius. The spot radius was measured to be 0.52 μm using this objective and optical system. There was no improvement in the fits if an additional recovery component was added.

Statistical analysis

Two-tailed paired t-tests were done using Statview (BrainPower Inc. Calabasas, CA) for comparison of fractional recovery and translational diffusion coefficients in the presence and absence of agonists. A P value of 0.05 was considered significant.

Miscellaneous

Protein was determined by the procedure of Schaffner and Weissmann [20] using bovine serum albumin as a standard. SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli [21] using 10% acrylamide gels. Proteins were stained with Coomassie Brilliant Blue.

RESULTS

Incorporation of fluorescently labelled G protein subunits into NG-108–15 cells

The PEG-mediated fusion of vesicles containing fluorescent G protein subunits with NG-108–15 cells resulted in approximately 8–10% of the fluorescence in the cell pellet under our standard conditions (i.e. 7×10^5 cells/25 ml). In all cases, the fluorescent subunits were added as heterotrimers in which either the α or β subunit was labelled. Thus, the only difference between the two G protein preparations was the site of labelling [18]. Photographs of labelled cells containing TMR-$\alpha_o/\beta\gamma$ or TMR-$\beta\gamma/\alpha_o$ and control cells are shown in Fig. 1. The autofluorescence of control cells (Fig. 1A) is substantially less than that of cells containing TMR-$\alpha_o/\beta\gamma$ (Fig. 1B) or TMR-$\beta\gamma/\alpha_o$ (Fig. 1C). Cells containing TMR-$\alpha_o/\beta\gamma$ show a more heterogeneous labelling pattern in both intensity and distribution than do cells containing TMR-$\beta\gamma/\alpha_o$. While the fluorescence of cells containing labelled β subunit is largely confined to a ring pattern around the periphery of the cells, the α-labelled cells show much more labelling throughout the cell. In both cases, there is a striking clumpiness to the labelling. This appeared to be somewhat more prominent for the cells with labelled β subunit.

Characterization of the incorporated fluorescent G proteins

While a "speckled" pattern of G protein immunofluorescence in cells has been reported previously [26], we were concerned that the bright spots could be due to clustered vesicles adhering to the cell surface or endocytosed vesicles. To rule out intact vesicles as the cause of the speckled fluorescence, we studied the self-quenching of the fluorescent lipid probe, octadecyl rhodamine B. This method has been commonly used to follow membrane fusion events. Soybean PC vesicles containing 4 mole% of R_{18} were fused to NG-108–15 cells using the same method used to incorporate G Protein subunits. The inset of Fig. 2 shows that the fluorescence of R_{18} in PC vesicles is very low and the fluorescence increases more than 10-fold in the presence of Lubrol. Thus, the R_{18} fluorescence is quenched more that 90% in the vesicles. When R_{18} vesicles were fused to NG-108–15 cells the amount of fluorescence recovered in the cell pellet increased almost linearly with cell number (Fig. 2). In the sample with no cells added, a small amount of fluorescent vesicles pelleted, but the fluorescence in those vesicles was still quenched as indicated by the increase in fluorescence upon addition of Lubrol. In contrast, all samples with cells showed minimal dequenching upon addition of Lubrol (<5–10%). This indicates that the vesicles are truly fusing with the cell membranes and becoming diluted in the cellular lipid pool. Therefore, simple sticking of vesicles on the cell surface or endocytosis of intact vesicles does not account for the clustered appearance.

We wanted to determine the orientation of the G proteins incorporated in the NG-108–15 cells. Since reconstitution of the G protein into vesicles is probably random, one would expect that half of the G protein would be facing the outer surface of the vesicle. Following fusion with cells, this same 50% would be expected to remain facing the outside of the cells. To assess this, we used commercially available anti-fluorescein antibodies (F-Ab) and G protein βγ subunits labelled with fluorescein-iodoacetamide. Figure 3A shows that about 80% of fluorescence in vesicles is quenched by F-Ab. The high percent quenching is probably due to leaky vesicles so that antibody can gain access to the inside of vesicles. After fusion to cells, only about 50% of the fluorescence is accessible to F-Ab (Fig. 3B). Thus, about 50% of exogenous G protein appears to be in the correct orientation (i.e. intracellular).

We examined the function of exogenous G proteins in NG-108–15 cells by testing their ability to couple to endogenous α_2 adrenergic receptors. High affinity binding of agonists to α_2 adrenergic receptors requires the presence of a G_o- or G_i-like G protein [19]. Equilibrium binding of [^{125}I]-*p*-iodoclonidine to membranes from NG-108–15 cells pretreated with pertussis toxin was markedly decreased from control (Fig. 4). Reconstitution with exogenous G proteins using the fusion method restores the high affinity binding of [^{125}I]PIC (Fig. 4). This indicates that the exogenous G protein can interact functionally with an endogenous receptor.

The amount of exogenous G protein $\beta\gamma$ subunits incorporated into NG-108–15 cells by the fusion method was quantitated by western blots with the SW1 anti-beta subunit antibody and [^{125}I] labelled secondary antibody. The incorporation of $\beta\gamma$ subunits in NG-108–15 cells was concentration-dependent and resulted in about a 2-fold increase over endogenous G proteins in NG-108–15 cells at the concentration of protein used in the FRAP studies (data not shown).

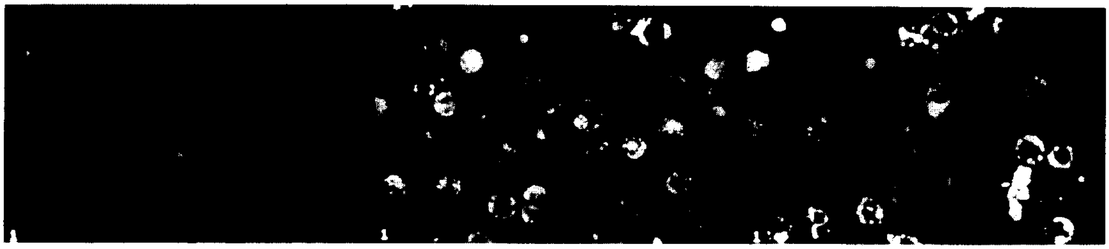
Subcellular distribution of fluorescently labelled G protein α and $\beta\gamma$ subunits

The subcellular distribution of fluorescently labelled G protein subunits was studied using confocal microscopy and differential centrifugation. Confocal sections of cells containing TMR-α_o/$\beta\gamma$ and TMR-$\beta\gamma$/α_o are shown in Fig. 5A and B, respectively. The fluorescent labelling patterns for α- and β-subunit were significantly different. The cells fused with vesicles containing TMR-α_o/$\beta\gamma$ showed fluorescence at the cell periphery but clearly had some uniform, diffuse labelling throughout the intracellular region (Fig. 5A). In contrast, cells fused with TMR-$\beta\gamma$/α_o showed virtually all of the fluorescence at the cell periphery (Fig. 5B). As suggested by the ring-enhancement of the fluorescence in the standard fluorescence photographs in Fig. 1, the β subunit seems to stay in the plasma membrane where it is initially deposited by the fusion technique. In contrast the α subunit (despite being introduced as a heterotrimer) is less tightly confined to the plasma membrane. This difference is not simply due to a higher autofluorescence background for cells containing the TMR-α_o/$\beta\gamma$ since the cells with fluorescent α generally were brighter overall than those with labelled β. Figure 5C shows confocal sections of cells fused with vesicles containing the hydrophobic lipid probe DiI. As expected, the DiI is almost entirely localized to the plasma membrane. These results suggest that the $\alpha\beta\gamma$ is organized in clusters in NG-108–15 cells as previously reported for A431 cells [26]. Also, exogenous TMR-$\beta\gamma$ is highly localized in the plasma membrane while TMR-α_o appears in both the plasma membrane and the intracellular region.

The identity of the intracellular TMR-α_o with respect to its association with membranes was further studied by differential centrifugation. Figure 6 shows the distribution of α_o in the supernatants and the pellets after homogenization and centrifugation at 20,000 **g** for 30 min. The cells fused with G_o/G_i and TMR-α_o/$\beta\gamma$ show a 50% increase in the amount of α_o subunits in the pellet fractions but only a constant 5% or so of total α subunit in the supernatant. Thus the exogenous G protein (labelled or unlabelled) appears to be largely membrane-bound as is the endogenous G protein.

Lateral mobility of fluorescently labelled G protein α and $\beta\gamma$ subunits

The recovery after photobleaching also showed a striking difference between the fluorescent $\beta\gamma$ and α subunits (Fig. 7). The TMR-$\beta\gamma$ always showed a much lower recovery than the α subunit. The mobile fractions (f_m) were determined in five experiments with 20–30 determinations in each experiment. For TMR-$\beta\gamma$ the (f_m) was 0.16 ± 0.03 and for TMR-α_o it was 0.34 ± 0.03 (Table 1). The fractional recovery for TMR-$\beta\gamma$ was significantly lower than that of TMR-α_o (paired τ test $P<0.01$). The diffusion coefficients of the mobile fractions for the two proteins, however, were not significantly different, $4.0 \pm 1.3 \times 10^{-9}$ and $2.2 \pm 0.7 \times 10^{-9}$ cm^{2}s^{-1} for TMR-α_o and TMR-$\beta\gamma$, respectively ($P>0.3$). This is at the high end of the range of diffusion coefficients usually reported for membrane proteins [27] and even approach the diffusion coefficients seen for lipids (see Discussion). There were no significant changes in either the diffusion coefficent or mobile fraction for TMR-α and



Autofluorescence TMR-α_0 TMR-$\beta\gamma$

Fig. 1. Photographs of cells fused with vesicles containing lipid alone, TMR-$\alpha_0/\beta\gamma$, or TMR-$\beta\gamma/\alpha_0$. NG-108–15 cells were fused with control lipid, TMR-$\alpha_0/\beta\gamma$, or TMR-$\beta\gamma/\alpha_0$ as described in Materials and Methods. Total fluorescence from cells was viewed using the BioRad confocal laser scanning microscope. Left—autofluorescence; Middle—cells containing TMR-$\alpha_0/\beta\gamma$; Right—cells containing TMR-$\beta\gamma/\alpha_0$.

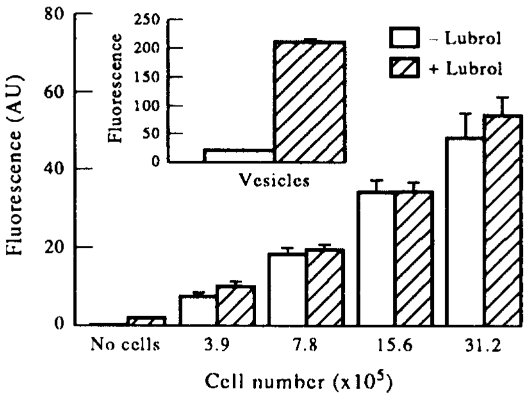


Fig. 2. Extent of PEG-mediated fusion determined from the loss of self-quenching of the vesicle-bound lipid probe Octadecyl Rhodamine B chloride (R_{18}). Soybean PC vesicles containing 4 mole% of R_{18} were prepared. R_{18} containing vesicles were fused to various number of cells (0.4, 0.8, 1.6, 3.2 × 10^6) using PEG (see Materials and Methods). Cells were washed four times after fusion and resuspended in 600μl of Tris buffer. Fluorescence (EX 555 and EM 578) was measured prior to (open bars) and after (hatched bars) addition of Lubrol PX (0.5% v/v, final concentration). The inset shows dequenching of rhodamine fluorescence in the PC vesicles containing 4 mole% of R_{18} indicated by the 10-fold increase in fluorescence in the presence of 0.5% Lubrol PX. After fusing vesicles with cells, the increase in fluorescence in the pellet (open bars) was nearly linear with cell number but there was no significant increase after addition of Lubrol (solid bars). Data shown are means ± SD of triplicate determinations from one experiment which was representative of three experiments.

TMR-βγ, upon stimulation by a mixture of three agonists to stimulate G_i-linked receptors in these cells (epinephrine for α_2 adrenergic, carbamylcholine for m4 muscarinic, and DPDPE for δ opiate). Since cytoskeletal associations have been proposed we also wanted to see if the markedly low mobile fraction of the TMR-βγ subunit in the NG-108–15 cells was due to binding to actin or tubulin. Cells containing TMR-βγ/α_o were treated with cytochalasin D or nocodozole for 4 h but there was no change in the lateral mobility of TMR-βγ after drug treatment. Neither the mobile fraction nor the D_T values were changed significantly (data not shown).

We were concerned that the low mobility of G protein subunits (especially the β subunit) could be due to an artifact of the method of incorporation. Consequently we measured the lateral motion of a substance known to be mobile in cell membranes, the lipid fluorescent probe, 3,3'-dioctadecylindo-carbocyanine iodide (DiI). DiI was reconstituted into phospholipid vesicles and incorporated into NG-108–15 cells using the same method used for the G proteins. A representative FRAP curve is shown in Fig. 7D. The fractional recovery was essentially complete (f_m 1.0) and the diffusion coefficient (4.0 × 10^{-9} cm^2 s^{-1}) for DiI was within the published range for mouse neuroblastoma cells [28]. The complete recovery of fluo-

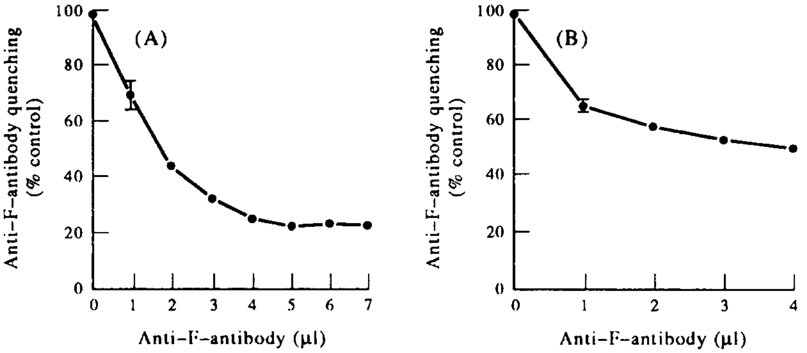


Fig. 3. Orientation of fluorescent probes in lipid vesicles and in cells after fusion. PC/PS vesicles containing of fluorescein labelled G protein βγ (F-βγ) and unlabelled α_o were prepared as described in Materials and Methods. (A) Vesicles containing 0.3 μg of F-βγ were diluted into 600 μl of Tris buffer and fluorescence was determined prior to and after addition of increasing amounts of anti-fluorescein antibodies (F-Ab). (B) Vesicles containing F-βγ/α_o were fused to NG-108–15 cells using polyethylene glycol. Cells were washed after fusion and resuspended in 600 μl of Tris buffer. Fluorescence was measured prior to and after addition of various amount of F-Ab. Data are from one experiment and are representative of three different experiments.

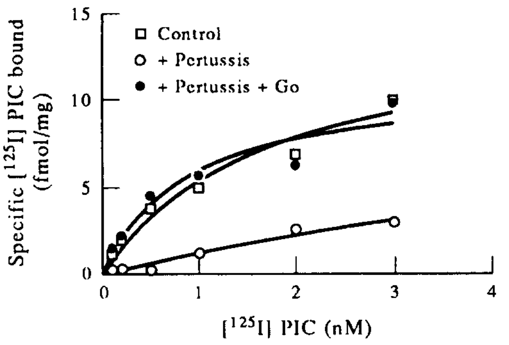


Fig. 4. Reconstitution of [^{125}I]-*p*-iodoclonidine high affinity binding by fusion of G protein vesicles to pertussis-toxin treated NG-108–15 cells. NG-108–15 cells were treated with (circles) or without (squares) 100 ng/ml pertussis toxin in serum-free medium for 18 h prior to membrane preparation. Cells were fused with PEG plus soybean PC lipid vesicles containing 1 mg of BSA (open symbols) or PEG plus soybean PC lipid vesicles containing 1mg G_o/G_i (filled circle). Binding of the α_2 agonist [^{125}I]-*p*-iodoclonidine (PIC) was measured at 23–25°C. Membranes (40 µg/tube) were added to tubes containing [^{125}I]PIC (0.1–0.5 nM), vortexed, and allowed to incubate at room temperature for 1 h. Bound and free ligand were separated on Whatman GF/C filters by vacuum filtration using a Brandel filtration apparatus. Filters with [^{125}I]PIC were dried and counted in 4 ml of Scintiverse liquid scintillation cocktail. Nonspecific binding was measured in the presence of 10^{-5} M oxymetazoline as described [23]. Data are means of triplicate determinations from one experiment which is representative of three experiments.

rescence of DiI (Fig. 7D) after photobleaching in conjunction with the dequenching data from Fig. 2 clearly shows that the low mobility of the TMR-α_o and TMR-$\beta\gamma$ is not due to sticking of unfused vesicles to the cell surface. Mixing of the lipids from the vesicles into the cellular lipid pool must be occurring. Finally, we wanted to be sure that the low mobility of the $\beta\gamma$ subunit was not due to complete immobility of the extracellularly oriented half of the $\beta\gamma$ subunits. To test this we used fluorescein-labelled $\beta\gamma$ subunits for which the extracellular fluorescence was quenched with anti-fluorescein antibodies. The f_m was essentially the same (0.15–0.2) indicating that intracellularly localized $\beta\gamma$ subunits show the same low mobility as the total population (data not shown).

DISCUSSION

In this report, we describe the first measurements of the lateral mobility of G protein subunits in intact cells. This was made possible by combining the PEG fusion method and fluorescent G protein subunits [18]. In addition, we obtain new information about the handling of G protein subunits in intact cells.

The most striking observation was the very low mobile fraction of TMR-$\beta\gamma$. It is also interesting that the mobile fraction of the α subunit is significantly greater than that of the $\beta\gamma$ subunit even though both α and $\beta\gamma$ were introduced as heterotrimers. The different mobile fractions of TMR-α_o and TMR-$\beta\gamma$ are consistent with the distinct but overlapping cellular distributions of the two proteins as shown in our confocal microscopy results (Fig. 5) and in the literature ([29] and see [6] for review).

The confocal images also show a discrete, punctate labelling pattern with bright spots in or near the plasma membrane suggesting that the majority of TMR-$\beta\gamma$ is either associated with anchoring proteins or forms micro-aggregates. The concordance of the data for distribution and mobility strengthens the conclusion that $\beta\gamma$ subunits do not distribute freely but are strongly immobilized once introduced into the cells. This is a striking contrast to the C_{18}-rhodamine, DiI and the α subunits. Similar observations of non-uniform distributions of endogenous $\beta\gamma$ subunits indicate that this is not just an artifact of the method of introduction of $\beta\gamma$ into the cells. Immunofluorescence localization of G protein β subunits in human A431 cells also showed punctate staining of the β subunit [26]. Overexpressed $\beta\gamma$ subunits show a "clumpy" pattern on the inner leaflet of the plasma membranes of COS cells [30]. This distribution was not seen in the absence of γ subunit prenylation [30]. The recently described role for protein prenylation in protein-protein interactions [31] raises the possibility that the $\beta\gamma$ subunits might be anchored to proteins in the plasma membrane or cytoskeleton via the γ subunit prenyl groups. The lack of effect of modulators of actin and tubulin polymerization is puzzling.

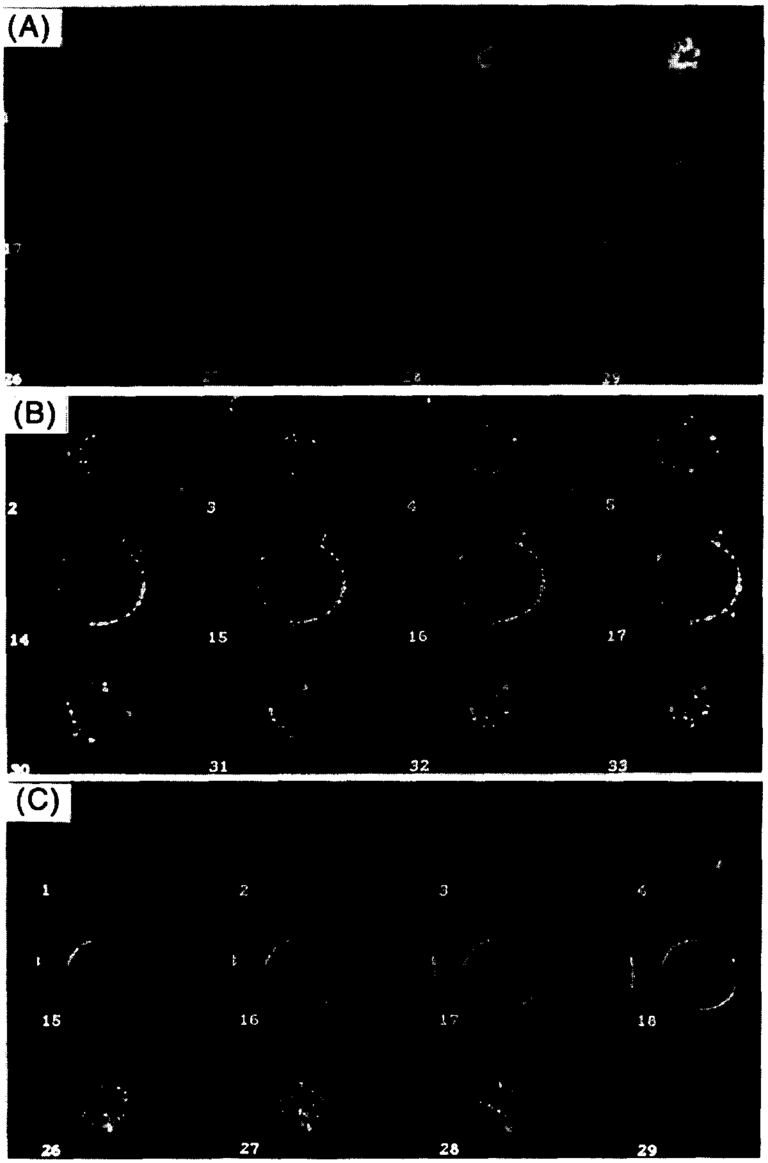

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Fig. 5. Confocal images of cells containing TMR-$\alpha_0/\beta\gamma$, TMR-$\beta\gamma/\alpha_0$ and DiI. Cells were fused with vesicles containing TMR-$\alpha_0/\beta\gamma$, TMR-$\beta\gamma/\alpha_0$ and DiI as described in the Materials and Methods section. Four consecutive 0.8 μm confocal section images from the bottom, middle, and top of cells containing fluorescently labelled proteins and DiI are shown. (A) TMR-$\alpha_0/\beta\gamma$, (B) TMR-$\beta\gamma/\alpha_0$, (C) DiI.

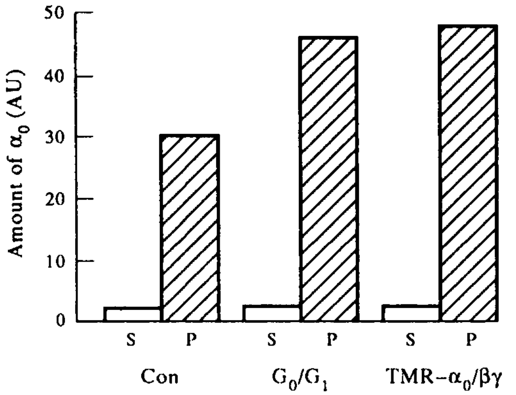

Fig. 6. Differential centrifugation of endogenous α_o exogenous α_o and TMR-α_o. Cells fused with lipid vesicles alone (control), vesicles containing G_o/G_i or vesicles containing TMR-α_o/βγ were homogenized and centrifuged at 20,000 *g* for 30 min. The supernatants were separated from the pellets. Sixty microgrammes of protein from either the supernatant or pellet was loaded onto a 10% acrylamide gel and transferred to Immobilon. Immunoblot analysis was performed using anti-α_o-antibody (GC/2) as the primary and ^{125}I-labelled goat anti-rabbit IgG as the secondary antibody, both at a 1:1000 dilution. Autoradiography was done using Kodak RP-XOMAT film and the amounts of α_o in each band were quantitated by densitometry. Data shown are representative of five different experiments.

Table 1. Fractional mobility and translational diffusion coefficients for TMR-α_o and TMR-βγ

	TMR-α		TMR-βγ	
Expt. Number	f_m	D_T (10^{-9} cm^2/s)	f_m	D_T (10^{-9} cm^2/s)
1	0.382	9.0	0.195	1.0
2	0.359	4.0	0.238	4.4
3	0.324	2.4	0.103	2.6
4	0.250	2.2	0.180	0.6
5	0.371	2.2	0.105	2.4
Mean	0.337	4.0	0.164	2.2
± S.E.	± 0.024	± 1.3	± 0.026	± 0.66

Twenty to thirty FRAP curves were obtained for TMR-α_o and TMR-βγ on each day (as in Fig. 7), averaged, and fitted to the hyperbolic equation as described in Methods. f_m and D_T were calculated and the data from 5 different experiments are shown. The fractional mobility of TMR-βγ is significantly less than that of TMR-α ($P < 0.01$) but the diffusion coefficients are not significantly different.

However, recent suggestion that βγ subunits bind to the pleckstrin homology domain in the β adrenergic receptor kinase [32, 33] raises the intriguing speculation that non-erythroid spectrin might anchor βγ subunits via the pleckstrin homology domain near the carboxy terminal end of β spectrin [6].

The interpretation of our FRAP results depends on the validity of the method used to introduce the proteins. Because of this we examined several possible artifacts which could account for our results and were able to rule out those causes. As discussed below, we controlled for low mobility due to: (1) sticking of vesicles to the surface of the cells rather than true incorporation, (2) endocytosis of intact vesicles, (3) extracellularly oriented proteins having a very low mobility, and (4) introduction of protein into a non-functional compartment of the cells.

The PEG-mediated fusion method has been used frequently for functional incorporation of membrane proteins involved in signal transduction [34–42], but the fate of such reconstituted proteins has not been evaluated. Thus it was essential to be sure that the incorporation of fluorescently labelled G protein subunits into NG-108–15 cells was biologically meaningful. Prior to using the fusion method we attempted to incorporate TMR-labelled βγ subunits into NG-108–15 cells by microinjection. This was unsuccessful due to the requirement of detergents and due to clogging of micropipette tips. With the fusion method, we were concerned that the "speckled" pattern could be an artifact of vesicles sticking to the cell surface without true incorporation or endocytosis of intact vesicles. Two pieces of data rule out these artifacts. First, the relief of self-quenching of R_{18} fluorescence shows that there is a very large decrease in surface density of the lipid fluorophore upon fusion. Our vesicles contain 4 mole % of R_{18} which resulted in 90% self-quenching. Hoekstra *et al.* [43] reported that R_{18} self-quenching is proportional to its surface density up to concentrations of about 9 mole %. Thus, the nearly

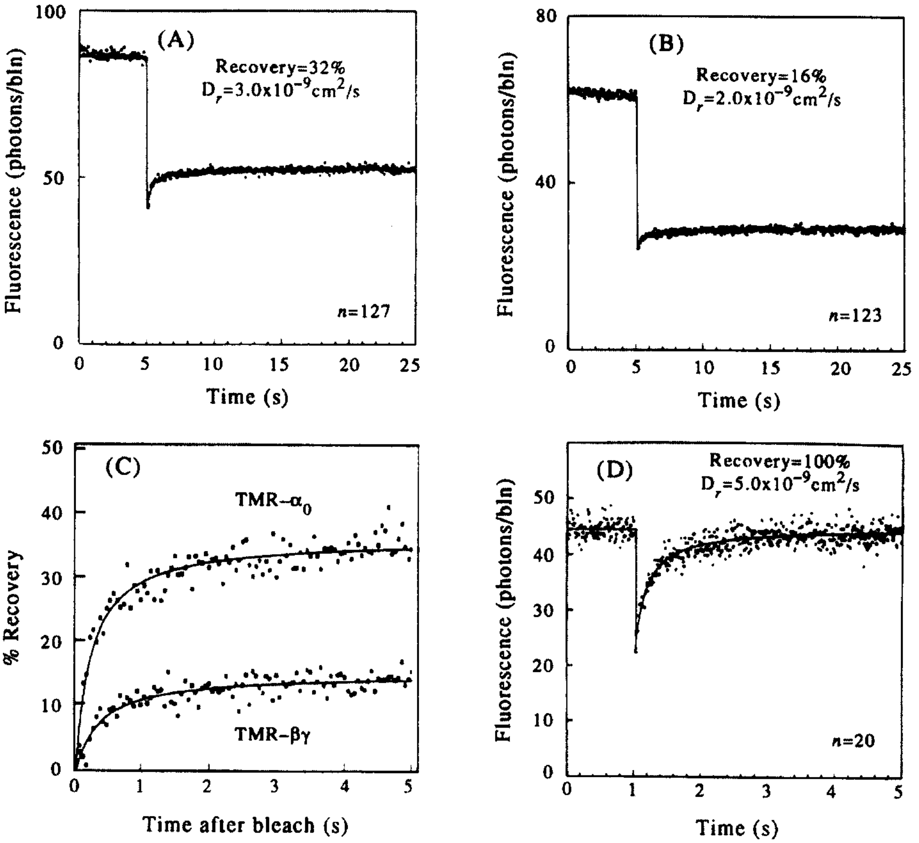

Fig. 7. Lateral mobility of TMR-α_0 and TMR-$\beta\gamma$ in NG-108–15 cells. NG-108–15 cells were fused with vesicles containing TMR-$\alpha_0/\beta\gamma$, TMR-$\beta\gamma/\alpha_0$ or DiI using the PEG-mediated fusion method. The lateral diffusion coefficients and the fractional recoveries were measured by the fluorescence recovery after photobleaching technique as described in the Materials and Methods section. Only one FRAP curve was obtained from each cell. The number of runs indicated in the figures was averaged and the data were fit to Eqn 1 using Inplot. (A) TMR-α_0, (B) TMR-$\beta\gamma$, (C) normalized % recovery of TMR-α_0 and TMR-$\beta\gamma$ and (D)DiI.

complete dequenching of R_{18} after fusion shows that virtually all of the vesicles in the cell pellet have actually fused to the cell. Also, the lipid from the vesicles must have access to a cellular pool of lipid at least ten-times as large as the amount of lipid in the incorporated vesicles. Second, the full mobility in FRAP measurements of the lipid probe, DiI, is inconsistent with a significant fraction of vesicles adhering to the surface of the cells or being endocytosed as the vesicle size is much smaller than the bleaching spot so that DiI in vesicles would not appear to be mobile.

G proteins incorporated into the outer leaflet of the plasma membrane might have a significantly different lateral mobility than G protein in the inner leaflet of the plasma membrane. As mentioned above, microinjection, which would result in all of the labelled protein in the intracellular space was not practical. While the fusion method resulted in both intracellularly and extracellularly oriented label, it had the advantage that all of the fluorescent protein starts out in the plasma membrane. Indeed, the 50% of label accessible to anti-fluorescein antibody after fusion with cells is consistent with the expected random distribution of the G protein. Antibody-quenching of fluorescein-labelled βγ subunits was used to show that the low mobile fraction of labelled βγ subunits was not due to protein on the extracellular surface. In addition, the exogenous G proteins readily interacted with endogenous receptors. Full recovery of the high affinity binding of the α_2 agonist [^{125}I]PIC was observed. These reconstitutions used amounts of G protein which increased total cellular G protein content to 5–10 times the normal amount. Since about half of the incorporated G protein is probably in the wrong orientation to interact with the receptor, this is a relatively efficient reconstitution. Many reconstitution studies that utilize unpurified receptors have used 1000-fold excesses of G protein to achieve full effects [19, 44, 45].

For the small amount of α and βγ subunits which are mobile, the translational diffusion coefficients are rather high, 2–4 × 10^9 cm^2/s. The greater mobile fraction of TMR-α_o appears to be due to dissociation of the heterotrimers, perhaps induced by intracellular guanine nucleotides. This is consistent with the confocal results in which there was much more fluorescence in the intracellular region when the heterotrimer was introduced with label in the α subunit (i.e. TMR-α_o/βγ). In contrast, labelling with TMR-βγ/α_o was entirely consistent with the expected plasma membrane location showing intense fluorescence at the edge of cells. This implies that the heterotrimer is initially inserted in the plasma membrane then the βγ subunits either stay in the membrane or are rapidly reinserted into the membrane if they leave. In contrast, the α subunits appear to be able to move away from the plasma membrane. The ability of α subunit to be released more easily from the membrane than βγ has been shown previously in lipid vesicles [46] and isolated cell membranes [47–49]. Also, α subunits have been found in many subcellular compartments in cells. It may be that the high D_T observed for the mobile fraction of TMR-α is due to free α monomers in the cytosol or a very high mobility in intracellular membranes. The high D_T value could also result if the α subunit associates with the membrane through its N-terminal myristoyl tail. Exogenous proteins attached to lipids and attached to the cytosolic face of plasma membranes have been shown to have diffusion coefficients which approach that of the lipids to which they are attached [50].

What is the location of the intracellular TMR-α subunit? Bokoch *et al.* [12] reported the existence of a small amount of cytosolic G protein in neutrophils. They found that the cytosolic protein is an uncomplexed G protein alpha subunit, based on the ability of exogenous G protein βγ subunits to increase its ADP-ribosylation and upon its hydrodynamic behaviour. Volpp *et al.* [10] and Khachatrian *et al.* [11] also reported the presence of free α subunit in the cytosol of human neutrophils. Our data show that little of the TMR-α_o is localized in the cytosol, rather it is in intracellular organelle membranes or associated with structural proteins which pellet on centrifugation. By immunocytochemistry and subcellular fractionation methods, G protein α subunits have been found in mitochondria [51], endoplasmic reticulum [52], Golgi [53–57], secretory granules [58–60], and lysosomes [51]. The lack of any specific structural patterns (i.e. perinuclear location for Golgi or stringy shape for association with mitochondria or cytoskeletal proteins), however, makes it difficult to predict exactly where TMR-α_o is localized in the intracellular region. The NG-108–15 cells used in this study are undifferentiated making the identification of structures somewhat more difficult.

The movement of TMR-α away from the plasma membrane occurred in the absence of receptor stimulants. This suggests that there may be basal activation of G proteins in unstimulated NG-108–15 cells. A basal level of activation of G proteins by unstimulated receptors is commonly found in reconstitution studies [61]. Even in NG-108–15 membranes, evidence has been presented for opioid receptor stimulated GTPase activity in the absence of exogenous agonist [62]. However, more recently Costa *et al.* have proposed that such basal activation of G proteins by opioid receptors doesn't occur in intact NG-108–15 cells [63]. Our data suggest that there is significant basal activation of G_o in these cells since the α and βγ subunits do not remain associated in the cell. We can not state which receptor, if any, is responsible for this dissociation or if it is simply due to the high level of GTP in cytoplasm.

The collision-coupling model [64] and derivatives thereof [14] described by Tolkovsky and Levitzki implied that the β-adrenergic receptor and adenylyl cyclase (and implicitly the G protein) were mobile in cells. The very low mobility of the G protein βγ subunit raises questions about this commonly used model of G protein function. However, Levitzki and colleagues clearly recognized that different receptors have different modes of coupling. Specifically, the adenosine receptor seemed more tightly coupled [65] to adenylyl cyclase than the β adrenergic receptor.

The data reported in this paper include the first measurements of the mobility of G protein subunits in cell membranes. The surprisingly immobile nature of the βγ subunits requires that we reassess some of the currently accepted models of signal transduction by guanine nucleotide binding proteins. Some questions raised by the immobility of the βγ subunits include: do βγ subunits act as anchors on which a high molecular weight signal transduction complex including receptors and effectors is assembled [1, 6]? Is the ability of βγ subunits to form such complexes important in their specificity for receptors in intact cells [66, 67]? Is the high diffusion coefficient of the mobile fraction of α subunit responsible for the great efficiency of signal transduction? The answers to these questions will require additional studies in intact cells to determine which proteins are in proximity to G protein subunits.

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