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All G protein βγ complexes are capable of translocation on receptor activation

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ABSTRACT

Heterotrimeric G proteins transduce signals sensed by transmembrane G protein coupled receptors (GPCRs). A subfamily of G protein βγ subunit types has been shown to selectively translocate from the plasma membrane to internal membranes on receptor activation. Using 4D imaging we show here that Gβγ translocation is not restricted to some subunit types but rather all 12 members of the family of mammalian γ subunits are capable of supporting βγ translocation. Translocation kinetics varies widely depending on the specific γ subunit type, with $t_{1/2}$ ranging from 10 s to many minutes. Using fluorescence complementation, we show that the β and γ subunits translocate as βγ dimers with kinetics determined by the γ subunit type. The expression patterns of endogenous γ subunit types in HeLa cells, hippocampal neurons and cardiomyocytes are distinctly different. Consistent with these differences, the βγ translocation rates vary widely. βγ translocation rates exhibit the same γ subunit dependent trends regardless of the specific receptor type or cell type showing that the translocation rates are intrinsic to the γ subunit types. βγ complexes with widely different rates of translocation had differential effects on muscarinic stimulation of GIRK channel activity. These results show that G protein βγ translocation is a general response to activation of GPCRs and may play a role in regulating signaling activity.

1. Introduction

G protein subunits (α and βγ) are associated with the plasma membrane and are central to the regulation of cell physiology [1]. Early evidence suggested that the subunits are activated by transmembrane GPCRs and regulate the levels of second messengers by regulating effector activity at the plasma membrane. This lead to a long standing model that G proteins are constrained to the plasma membrane where they function as transducers of signals from outside the cell. However recent evidence suggests that G protein subunits are present inside the cell in the Golgi, ER, nucleus, endosomes and mitochondria [2–7]. The mechanism that allows G proteins to reside at both the plasma membrane and intracellular membranes was not clear. Evidence for constitutive shuttling of G protein subunits between the plasma membrane and intracellular membranes [8,9] as well as translocation of some βγ subunit types on receptor activation to the Golgi and ER [10,11] identified mechanisms that allow G proteins to reach various intracellular sites from the plasma membrane. Gβγ translocation was previously observed by using wide field microscopy for relatively short periods of time [10,11]. Here we imaged the properties of all members of the G protein γ subunit family in live cells using more sensitive 4D imaging methods for longer periods of time to ensure that even limited translocation of subunits would be detected. To ascertain that the properties identified were not particular to certain cell types or receptors we examined G protein subunit movement in different cells types on activation of different endogenous receptors. The results showed that the entire family of G protein βγ subunit types demonstrates receptor stimulated translocation, albeit at vastly different rates.

To examine if differential translocation rates of G protein βγ subunit types modulate downstream signaling activity differentially, we examined the effect of two βγ subunit type combinations with widely different translocation rates on muscarinic receptor activation of GIRK (G protein-coupled inwardly-rectifying potassium channel) activity. Gi coupled muscarinic receptors are known to activate GIRK channels through direct interaction of the βγ complex with the channel [12]. We examined the kinetics of channel activation in the presence of a rapidly or slowly translocating γ subunit. These experiments suggested a functional consequence for cells possessing G protein subunit types with differential translocation kinetics.

2. Materials and methods

2.1. Constructs and cell lines

G protein constructs used in this study have been previously described [8,11,13,14]. Receptors and G protein subunits were

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cloned into pcDNA3.1 (Invitrogen), GalT-DsRed was from Clontech, USA. $Venus_{155-239}$-β1 and $Venus_{1-155}$-γ2 have been described before [15] and were from N. Lambert, (Georgia Health Sciences University). $Venus_{1-155}$-γ2 in pcDNA3.1 was cut with BamH1 and XbaI to release the γ2 fragment. γ5 and γ9 PCR fragments respectively were then cut with BamH1 and XbaI and cloned into these sites to make $Venus_{1-155}$-γ5 and $Venus_{1-155}$-γ9. GFP-GPI was from V.R. Caiolfa, (San Raffaele Institute of Research). The HeLa cell line was obtained from ATCC and cultured in the recommended medium. The HL-1 cardiomyocyte cell line was from W. Claycomb, (Louisiana State University Medical Center) and was grown in complete supplemented Claycomb medium (Sigma) [16]. Hippocampal cultures were prepared by coating the glass bottom of 30 mm plastic culture dishes with 0.15% agarose. Cell suspensions were prepared from postnatal day 1–3 rat hippocampus using papain and mechanical dispersion and cultured as described [17]. A-68930, norepinephrine, yohimbine, carbachol, atropine, SDF-1α, AMD3100, isoproterenol, N6-cyclopentyladenosine were from Sigma. Quantitative PCR was performed as previously described [13].

2.2. Live cell imaging

All the imaging was performed using an Andor-Leica spinning disk laser confocal imaging system. It consisted of a Leica DMI 6000B microscope with adaptive corrective focus (AFC) that prevents drift in long term experiments, a Yokogawa CSU X1 spinning disk unit and an Andor iXon EM CCD camera. Excitation was controlled with 4 solid state lasers: 445, 488, 515 and 595 nm. Excitation and emission wavelength filters (Semrock) were as follows: CFP fluorescence – 445 excitation and 478/30 emission; GFP fluorescence – 488 excitation and 515/20 emission; YFP fluorescence – 515 excitation and 528/20 emission, Red fluorescence – 595 excitation and 628/20 emission. Cells were cultured and transiently transfected either in 29 mm glass bottom culture dishes (In vitro Scientific) or on 40 mm glass coverslips. Images were acquired with a 63× objective (1.4 NA). Confocal planes of cells for Z stacks were imaged at 0.4 μm intervals and the topographic Z projection images of maximum intensity were created using Andor iQ 2.5 software. Mean pixel fluorescence intensity changes in the entire cell or in selected areas of the cell were determined using Andor iQ 2.5 software. Z stacked images were acquired at 15–40 s intervals before and after addition of agonist to activate an endogenous receptor. When measuring fluorescence in internal membranes, the top few planes were removed to avoid interference from the plasma membrane (Fig. S1).

2.3. Electrophysiology

Whole-cell patch-clamp current recordings were performed with an EPC 9 amplifier driven by the *Pulse* program (Heka-Electronik) using pipettes with a resistance of 2–3 MΩ, pulled from filamented borosilicated glass capillaries (WPI, 1B150F-4) using a micropipette laser puller (P-2000 Sutter Instruments, Novato, CA). Cells were clamped at −50 mV and bathed in an extracellular solution containing (millimolar): NaCl 120, KCl 20, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, pH 7.4, while the intracellular solution was (millimolar): K gluconate 110, KCl 20, NaCl 10, $MgCl_2$ 1, Mg ATP 2, EGTA 2 GTP 0.3, pH 7.4. Approximately 2 min after the whole cell configuration was established, the cell membrane capacitance was measured in voltage-clamp by using the automatic compensation circuitry of the EPC-9 amplifier at a holding potential of −50 mV. Series resistance was electronically compensated (80%). Drugs were applied by a gravity driven perfusion system allowing switching between different test solutions. Solution exchange time with this system is typically <0.5 s. All experiments were performed at room temperature (25 °C).

Transiently transfected HL-1 cells with GFP tagged γ subunits or GPI membrane marker were visualized with a Nikon Eclipse TE2000-U epifluorescence microscope using a 40× oil immersion objective (0.6 NA). GFP fluorescence was detected with D492/18 excitation and D535/30 emission filters (Chroma) Images were recorded at 1 s exposures with 4 binning using a Hamamatsu CCD Orca-ER camera (12 bit). Images were acquired using MetaMorph software and cells with approximately equal intensities of GFP were selected for patch clamping. During agonist application, cells were clamped at −80 mV (nominal $E_K = -49$ mV). Agonist application elicited an inward current reaching a steady-state plateau. After removal of the agonist, the current decays to the initial baseline. Current activation and deactivation kinetics were fitted to a single exponential function $A\exp(-t/\tau) + C$, where A is the current amplitude at the start of the fit, t is time, τ is the activation or the deactivation time constant, and C is the steady-state asymptote. Currents were analyzed using IgorPro (WaveMetrics). The time course of the current was fitted to the indicated equations by using Origin v. 7.5. Results are expressed as mean ± SEM. Statistical differences between means were analyzed using the Kolmogorov–Smirnov test ($^{*}p < 0.05$ and $^{**}p < 0.01$).

3. Results and discussion

3.1. Translocation of all members of the G protein γ subunit family can be detected

Earlier results showed that six members of the γ subunit family support receptor mediated βγ translocation from the plasma membrane [11]. Based on wide field imaging, it was concluded that γ2, γ3, γ4, γ7, γ8 and γ12 were not capable of translocation. Here we examined Gβγ translocation using confocal microscopy and 4D imaging. Translocation of all members of the γ subunit family was examined in HeLa cells by activating two endogenous receptors, CXCR4 and α2 adrenergic (α2AR). Cells were imaged using a spinning disk confocal microscope for >10 min after receptor activation. To obtain a 3D view of the translocation, confocal images along the Z axis were captured and stacked as described in Section 2.

Fig. 1A shows the translocation of FP (Fluorescence Protein)-γ2 to intracellular membranes when CXCR4 receptors in HeLa cells were activated with SDF-1α. Translocation occurred over a relatively long period of time (Fig. 1A, B) compared to subunit types that we previously categorized as those capable of translocation. The $t_{1/2}$ for translocation as determined by time lapse 3D imaging was about 180 s. The closely related γ3 subunit also translocated on a similar time scale ($t_{1/2} \sim 170$ s) to the Golgi when HeLa endogenous α2 adrenergic receptors in HeLa cells were activated with norepinephrine (Fig. 1C, D). This result suggested that the receptor stimulated translocation of γ2 is not a peculiarity specific to that subunit type. 3D images showed that on translocation γ3 colocalized with a Golgi marker – galactosyltransferase (GalT-dsRed) (Fig. 1E). The slow translocating γ subunit types thus translocate predominantly to the Golgi similar to the rapidly translocating γ subunit types examined earlier [10,11].

We then examined γ4, γ7, γ8 and γ12 subunit types which had previously been thought to be incapable of translocation. The result of activating G proteins in HeLa cells with SDF-1α and norepinephrine showed that these subunits were also capable of translocation (Table 1, Fig. S2). When the rate of translocation of each of the twelve members of the γ subunit family was examined (Table 1) the $t_{1/2}$ varied widely from 10 s in the case of γ9 to 290 s in the case of γ3. For each γ subunit we examined the reverse translocation as well by deactivating the receptor with an antagonist. The ability of