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Long and short distance movements of β_2-adrenoceptor in cell membrane assessed by photoconvertible fluorescent protein dendra2–β_2-adrenoceptor fusion

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ABSTRACT

Local movements of receptors in the plasma membrane have been extensively studied, as it is generally believed that the dynamics of membrane distribution of receptors regulate their functions. However, the properties of large-scale (>5 μm) receptor movements in the membrane are relatively obscure. In the present study, we addressed the question as to whether the large-scale movement of receptor in the plasma membrane at the whole cell level can be explained quantitatively by its local diffusive properties. We used HEK 293 cells transfected with human β2-adrenoceptor fused to photoconvertible fluorescent protein dendra2 as a model system; and found that 1) functional integrity of the dendra2-tagged receptor remains apparently intact; 2) in a mesoscopic scale (~4 μm), ~90% of the receptors are mobile on average, and receptor influx to, and out-flux from a membrane area can be symmetrically explained by a diffusion-like process with an effective diffusion coefficient of ~0.1 $\mu m^2/s$; 3) these mobility parameters are not affected by the activity state of the receptor (assessed by using constitutively active receptor mutants); 4) in the macroscopic scale (4–40 μm), although a slowly diffusing fraction of receptors (with $D<0.01\ \mu m^2/s$) is identifiable in some cases, the movement of the predominant fraction is perfectly explained by the same effective diffusion process observed in the mesoscopic scale, suggesting that the large scale structure of the cell membrane as felt by the receptor is apparently homogeneous in terms of its mesoscopic properties. We also showed that intracellular compartments and plasma membrane are kinetically connected even at steady-state.

1. Introduction

Plasma membrane constitutes a liquid-like array of phospholipids in which integral membrane proteins, including cell surface receptors, can move [1]. Besides this basic property, it is now well understood that the plasma membrane is an organized structure that contains discrete microdomains by which signaling molecules are concentrated and organized together or separated from each other [2]. This compartmentalization eventually contributes to the efficiency or specificity of receptor-mediated signaling [3,4]. In addition to this compartmentalization that is evident in biochemical experiments based on detergent solubilization followed by density gradient centrifugation, physical measurements, such as fluorescence recovery after photobleaching (FRAP), fluorescence correlation spectroscopy (FCS) or single particle (or molecule) tracking (SPT) have revealed that the movements of membrane proteins (or phospholipids themselves) are much slower in cell membrane than in artificial bilayers (see for example [5–7]). The latter observations, which cannot be explained solely by lipid composition and cholesterol content of the cell membrane, or by protein–protein interactions among membrane proteins that are expressed at physiological levels [8], have generally been attributed to the effects of cytoskeleton and scaffold proteins on the apparent diffusion of membrane proteins [9]. This implies an additional level of dynamic compartmentalization of proteins in the plasma membrane, as the membrane proteins may feel the latter effects differentially by specific interactions, or collectively by nonspecific interactions. Hence, as opposed to the Cuatrecasas picture of floating receptors [10], the plasma membrane does not seem to provide a homogeneous two-dimensional liquid continuum, in which membrane receptors diffuse freely to collide and interact with their signaling partners randomly. Rather, the dynamic organization of the membrane and its immediate intracellular vicinity seems to be actively contributing to the regulation of transmembrane signaling by controlling mobility of the receptors in a purposeful manner. Yet, another level of dynamic organization at a larger scale is apparent in highly polarized cells, like neurons; as required by the very nature of their purposes, neurotransmitter receptors are found to be confined to specific (anatomic) areas (i.e. synapses) resisting against diffusion-like processes in the large

Abbreviations: β_2AR, β_2-adrenoceptor; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; FCS, fluorescence correlation spectroscopy; SPT, single particle (or fluorescent molecule) tracking

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continuum of the neuronal cell membrane. Thus, in addition to the mode of compartmentalization mentioned above, the latter implies an ultimately large scale compartmentalization of receptors in the entire plasma membrane of polarized cells, as the mobility of the receptor within an anatomic regions need not to be the same as the one at the boundary. In such cases, receptor concentration in specific areas in the membrane, and consequently the signaling efficiency of the receptor, is expected to be sensitive to, and perhaps to be regulated by, the large scale mobility of the receptors (i.e. the ability of receptor to move from one large region to another). Indeed, it has been shown for AMPA [11], NMDA [12], $GABA_A$ [13] and glycine [14] receptors that dynamic regulation of large scale receptor diffusion, which works against its anatomic confinement, contributes significantly to the fast control of synaptic sensitivity and plasticity in neurons (see [15] for a review). In summary, the cell membrane is dynamically (and hierarchically) organized at many different levels, starting from the very microscopic scale of a few molecules, to the ultimate scale of the entire cell membrane. Obviously, mechanisms that are involved in such a wide range of organization are expected to be variable at different scales.

This new picture of highly organized cell membrane that actively and dynamically contributes to the regulation of transmembrane signaling in many different ways has motivated a considerable amount of work in the last two decades aiming to characterize the modes of movements of G protein-coupled (or other types of) receptors in the cell membrane [16]. A great deal of progress has been made in determining fine structure of cell membrane in terms of receptor mobility by using FRAP, FCS and SPT techniques (reviewed in [9,17]). However, the question as to whether the microscopic models of membrane organization thus constructed apply to the very macroscopic scale of the entire cell membrane has not been addressed so far. Hence, the large scale dynamic structure of cell membrane as seen by the receptor molecules is relatively obscure. In the present study, we therefore investigated the properties of ultimately large-scale (cell-wide) movements of β2AR in the HEK 293 cells in order to see whether the effective diffusion determined in the FRAP-like experiments at a mesoscopic scale (~4 μm) explains the cell-wide movements of β2AR in the plasma membrane.

We used the irreversibly photoconvertible florescent protein dendra2 [18] to tag β2AR, which enabled us to label β2AR instantaneously and irreversibly in desired regions of a living cell under a confocal microscope. We compared the results obtained in different spatial scales and geometries quantitatively by means of numerical simulations. Besides the main question we asked in the present study, we also discussed the utility of photoconvertible tags in investigating receptor dynamics not only in the cell membrane, but also in the entire cell including intracellular compartments.

2. Material and methods

2.1. Materials

Cell culture media, fetal bovine serum and antibiotics were purchased from Biochrom (Germany). Complete Mini protease inhibitor cocktail and Pwo DNA polymerase were purchased from Roche Diagnostics GmbH (Manheim, Germany). Isobutylmethylxanthine, MTT (methylthiazolyldiphenyl-tetrazolium bromide) and (−) isoproterenol were purchased from Sigma (Taufkirchen, Germany). [^{125}I]-iodocyanopindolol was purchased from Amersham-Pharmacia (Sweden). Lipofectamine 2000 and Geneticin were purchased from Invitrogen (Karlsruhe, Germany). All standard reagents (buffers, salts, detergents, etc.) were from Sigma-Aldrich (Taufkirchen, Germany) or Fisher Scientific (NJ, USA) at appropriate purity.

2.2. DNA constructs, cells and transfection

Full length cDNA encoding β2AR–dendra2 fusion protein was constructed by amplifying the cDNA of wild type or mutated human β2AR from the original vector hβ2AR-pCDNA3.1(+), and by inserting the resulting fragment (amplified without the stop codon) into the multiple cloning region of the dendra2-N vector (kindly provided by Konstantin Lukyanov, Russia) between the unique XhoI-HindIII sites. The final construct that encodes the β2AR fused to dendra2 protein from the C-terminus of the former, starts with β2AR and ends with the dendra2 protein, with a 19 -mer linker (β2AR–KLRILQSTVPRARDPPVAT–dendra2) between the two proteins. Site specific mutations were performed by standard PCR-based strategies using mismatched primers and Pwo DNA polymerase. We used a two-step procedure to insert the constitutively active β2AR mutant (CAMβ2AR) into the dendra2-N vector, as the CAM mutations (L272A, H269K, K267R, L266S in human β2AR) introduced an additional XhoI site in the β2AR sequence. Mutations and integrity of the final fusion constructs were verified by DNA sequencing.

All experiments were carried out in HEK 293 cells. Cells were grown in DMEM (Dulbecco's modified Eagle's media) supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml) and 10% (v/v) fetal bovine serum, at 37 °C in a humidified atmosphere with 5% CO_2. All transfections were carried out by using Lipofectamine 2000 reagent as described by the manufacturer. Stable transfectants were selected by geneticin (750 μg/ml). Expression level of transfected proteins was assessed by ^{125}I-iodocyanopindolol binding. Relative expression levels of different cells, or different populations of cells, were assessed by evaluating the average fluorescence of the dendra2 protein in the cells. Functional integrity of the β_2AR–dendra2 fusion protein was confirmed by agonist binding and cAMP accumulation assays. Cells that were used in fluorescence measurements were grown on glass cover slips in standard conditions as described above, except that the cultures were incubated in serum-free conditions at least 2 h before the experiments.

2.3. Confocal microscopy, photoconversion and fluorescence measurement procedures

We performed all fluorescence measurements in living cells that were grown on glass cover slips in serum-free DMEM medium by means of a confocal microscope system (Leica TCS SP5) equipped with argon-ion and He–Ne lasers, and an additional mercury lamp in a diverse optical path. We used a 63× water immersion objective with a numerical aperture of 1.2 (Leica, HCX PL APO) throughout the experiments. Photoconversion of the dendra2 protein from green to red form was achieved either by using the 488 nm line of the argon-ion laser or the UV band of the mercury arc lamp. In the case of laser-induced photoconversion either a region of interest was scanned repeatedly for 1–2 s with high power laser (20% of the total power of the laser), or a target point was constantly irradiated for 100 ms with an enhanced-power static laser beam. The latter strategy was used in FRAP-like experiments (see Section 2.4.1 below). In the case of broad-band photoconversion, low-pass-filtered light of the mercury arc lamp was focused and confined to a small region in the visual field by using the field diaphragm of the microscope, and a region of interest was illuminated for 1–2 s right before switching to the data collection mode. The latter strategy was used when photoconversion was desired in relatively large and thick regions of the sample. Green and red forms of the dendra2 protein were detected, respectively, by using 488 nm line of argon ion laser (Ex.) and 509–540 nm band of the monochromator (Em.), and 543 nm line of He–Ne laser (Ex.) and 560 nm high-pass region of the monochromator (Em.). Simultaneous detection of green and red spectrums were achieved by fast switching between the two configurations at each scanning line. Under these conditions, green channel is blind to the red, and red channel is blind to the green form of the dendra2 protein. Unless indicated otherwise, data were collected with a spatial resolution of 512 or 1024 pixel per scanning line (with 8 bit of intensity depth) and a time resolution of 2 s/frame with a pinhole aperture of 1 Airy unit. Under the detection conditions of low-intensity 488 nm laser (0.3% of the total laser power) no spontaneous photoconversion or significant photobleaching was observed during the experiments.