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Visualizing the endocytosis of phenylephrine in living cells by quantum dot-based tracking

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ABSTRACT

To study the intracellular receptor-drug transportation, a fluorescent probe consisting of phenylephrine −polyethylene glycol-quantum dots conjugate was employed to track endocytosis process of phenylephrine in living cells. This type of movement was studied by continuously filming fluorescent images in the same cell. We also calculated the movement parameters, and divided the endocytosis process into 6 stages. Furthermore, the movement parameters of this probe in different organelles were determined by co-localization of the probe fluorescent images and different cellular organelles. After comparing the parameters in cellular organelles with these in 6 stages, the whole endocytosis pathway was demonstrated. These results verified that this probe successfully tracked the whole intracellular dynamic endocytosis process of phenylephrine. Our method realized the visual tracking the whole receptor-mediated endocytosis, which is a new approach on investigating the molecular mechanisms and kinetic properties of intracellular receptor-drug transportation.

1. Introduction

G-protein coupled receptors (GPCRs) form the largest class of cell-surface receptors and they are important drug targets with over 40% of current marketed drugs acting on [1–5]. Since the therapeutic effect of most drugs is achieved by targeting GPCRs, the study of receptor-drug interaction is of great importance, especially the transportation of intracellular receptor-drug. α_{1B}-adrenoceptor (α_{1B}-AR) is one subtype of α_1-AR, which can be used as a model to study the intracellular receptor-drug transportation in living cells, since the target receptor is distributed on the cell membrane surface [6].

Phenylephrine (PE) is a selective agonist toward α_1-AR, which has no selectivity toward subtypes. PE could specifically bind to α_{1B}-AR, activate receptor, produce adrenaline effects, and induce receptor-mediated receptor-drug internalization [7,8]. The receptor-mediated internalization of PE is a complex, multistep process: PE binding to membrane α_1-AR, entering the cells by a clathrin-mediated endocytosis and then being transported into the living cell via actin filaments, microtubules and endosomes [9]. However, despite intensive efforts in investigating the transport process of PE, many crucial features of PE traffic remain ambiguous. Among these are important and general questions for the transport mechanism: what is the whole transport pathway in live cell, how does PE move in different stages in the whole process, and where is the final destiny of PE in live cells?

Conventional approaches to investigate the intracellular transportation rely on fluorescently tagging the organelles or biomolecules of interest using various organic fluorophores [10]. While plenty of dynamic and structural information has been obtained by organic fluorophores, their rapid photobleaching and broad, overlapping emission spectra sometimes limit this application in living-cell studies [11]. Recent advances in the development of engineered fluorescent proteins provide an alternative approach to monitor the dynamic behavior of living cells at the molecular level. However, fluorescent proteins also suffer in photobleaching when used in long-term observation [12]. To observe dynamic processes in living cells for long time, more robust living cell imaging agents are highly demanded [13–15]. Owing to the unique photophysical properties including high quantum yields, superior photostability, broad absorption spectra, and narrow emission

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spectra, semiconductor quantum dots (QDs) have the potential for sensitively long-term tracking individual cellular events [16,17]. It has been demonstrated that the uptake of bioconjugated QDs can be tracked in living cells [18–20]. Kuo et al. used QDs conjugated with nuclear localization signaling peptides to track the transportation across the plasma membrane into the nucleus, and further measured the transportation trajectories and movement parameters to study the uptake mechanism of targeted delivery [11].

In this assay, we used QDs as fluorescent labels to track long-term endoctyosis processes of PE in living cells. Single-particle tracking technique was used to study *in-situ* and real-time dynamic process in living cells, which can reveal interesting biological interactions and elucidate the mechanisms of particle motions in living cells [21–25]. Our method realized the visual tracking of whole receptor-mediated endocytosis, which is of great importance for the study of receptor–ligand interactions, especially the molecular mechanisms and dynamic properties.

2. Materials and methods

2.1. Materials and apparatus

Streptavidin-QDs_{605} (SA-QDs), Dulbecco's modified Eagle's medium (DMEM), Fetal Bovine Serum (FBS), CellLight Actin-GFP BacMam 2.0 (C10506), CellLight Early Endosomes-GFP BacMam 2.0 (C10586), CellLight Late Endosomes-GFP BacMam 2.0 (C10588), CellLight Lysosomes-GFP BacMam 2.0 (C10507) and CellLight Tubulin-GFP BacMam 2.0 (C10509) were obtained from Invitrogen Co. (Eugene, OK, USA). Trypsin (>250 NF U mg^{-1}) was obtained from Amresco Inc. (Solon, OH, USA). PE-PEG_{2200}-bio was synthesized previously. HEK293α_{1B} cells were a gift from Professor Youyi Zhang (Institute of Vascular Medicine, Peking University Third Hospital, Beijing 100191, China). Penicillin and streptomycin were purchased from Shandong Lukang Pharmaceutical Co., Ltd. The phosphate buffered saline solution (PBS, pH = 7.2) consisted of 2.4×10^{-3} M Na_2HPO_4, 4.9×10^{-4} M NaH_2PO_4 and 0.15 M NaCl. Coverslips were purchased from Cole–Parmer (Vernonhills, Illinois, USA). Cytochalasin D and nocodazole were purchased from Sigma (St. Louis, MO, USA). 6-well plates were purchased from Nest Biotech Co., Ltd. All solutions were prepared by doubly distilled water. All other reagents were of analytical grade.

The objective-type total internal reflection fluorescence microscope (TIRFM) consisted of an inverted microscope (Model IX81, Olympus, Japan) with a high-numerical-aperture (60×, NA = 1.45) TIRFM oil-immersion objective (PlanApo TIRFM, Olympus, Japan), a multiline Ar ion laser with an output power of 10 mW for 488 nm (Model IMA 101040ALS; Melles Griot, Carlsbad, CA, USA), a TIRF illuminator and a 16-bit thermoelectrically cooled Electron-Multiplying CCD (EMCCD, Cascade 512B, Tucson, AZ, USA) was employed. QDs and green fluorescent protein (GFP) were excited with a 488 nm laser. A 573–603 nm and a 510–550 nm band-pass filter were used for QDs and GFP emission, respectively.

3. Methods

3.1. Preparation of PE-PEG-QDs

PE-polyethylene glycol-QDs (PE-PEG-QDs) were prepared by reacting SA-QDs with PE-PEG_{2200}-bio at the molar ratio of 1:32 in double distilled water and then diluted in DMEM.

3.2. Cell culture

HEK293α_{1B} cells and HEK293 cells were cultured in DMEM supplemented with 10% of FBS, 100 U mL^{-1} penicillin, and 100 U mL^{-1} streptomycin at 37 °C/5% CO_2. Cells were plated on coverslip 10–12 h before adding the complex.

3.3. Specificity of the PE-PEG-QDs

To ensure that the endocytosis of PE-PEG-QDs was dominated by PE, HEK293α_{1B} cells were treated with 1.4 nM of SA-QDs (the same amount in 45 nM PE-PEG-QDs) at 37 °C for 30 min. After repeated washing with PBS to remove non-specific bound QDs, cells were taken for visualization. The method was a combination of TIRFM at the apical cell surface and the intracellular fluorescence microscopy (FM) coupled with objective focusing [26]. The fluorescent images of single PE-PEG-QDs not only at both apical and basal plasma membrane, but also in the cell interior could be obtained. Imaging acquisition was controlled by MetaMorph software (Universal Imaging, Downingtown, PA, USA).

3.4. The internalization process of PE-PEG-QDs

HEK293α_{1B} cells were treated with 45 nM of PE-PEG-QDs at 37 °C for 30 min. After repeated washing with PBS to remove non-specific bound QDs, cells were taken for visualization.

3.5. Inhibition studies

To clarify the transport pathway of PE-PEG-QDs, a series of uptake inhibition studies were carried out. To block actin filaments, the cells were exposed in DMEM with 40 μM cytochalasin D (cyto-D) for 30 min before experiment. To block microtubules, the cells were exposed in DMEM with 60 μM nocodazole for 30 min before experiment. After repeated washing with DMEM to remove non-specific bound QDs, cells were taken for visualization.

3.6. The movement of PE-PEG-QDs in cellular organelles

HEK293α_{1B} cells were incubated with 2 μL Actin-GFP, Early Endosomes-GFP, Late Endosomes-GFP, Lysosomes-GFP, Tubulin-GFP respectively at 37 °C overnight, then incubated with 45 nM P-PEG-QDs at 37 °C for 15 min. After repeated washing with PBS, cells were taken for visualization using a 573–603 nm and a 510–550 nm band-pass filter for QDs and GFP, respectively. Co-localization of the overlapping images from the two channels was used for analyzing the movement of PE-PEG-QDs in different organelles.

3.7. Data analysis

To investigate the dynamic behavior of a PE-PEG-QDs particle, Mean Square Displacement (MSD) was calculated from x–y coordinates of individual tracking position data [27]. MSD values of individual tracking are defined by the following equation.

$$\mathrm{MSD}(n\Delta t) = \frac{1}{N-n}\sum_{i=1}^{N-n}\left[(x_{i+n}-x_i)^2+(y_{i+n}-y_i)^2\right]$$

where x_i and y_i are positions on frame i, N is the total number of frames. Δt is the time between frames. $n\Delta t$ is the time interval over which the MSD is calculated. Thus MSD is a function of time. To get the information for the diffusion coefficients and the velocities, MSD were fitted by the following equation.

$$\mathrm{MSD} = 4Dt,$$

$$v = \frac{s}{t}$$

where D is diffusion coefficient and v is velocity.

When MSD was fitted versus time, the linear plot of MSD produced represents free diffusion. When the change in MSD was nonlinear with time, the movement is directed diffusion or confined diffusion. A decreasing slope indicates confined movement. On the other hand, directed diffusion produces an MSD plot with an increasing slope.