Biophysical Chemistry 161 (2012) 50–53

Contents lists available at SciVerse ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

Ligand-induced clustering of EGF receptors: A quantitative study by fluorescence image moment analysis

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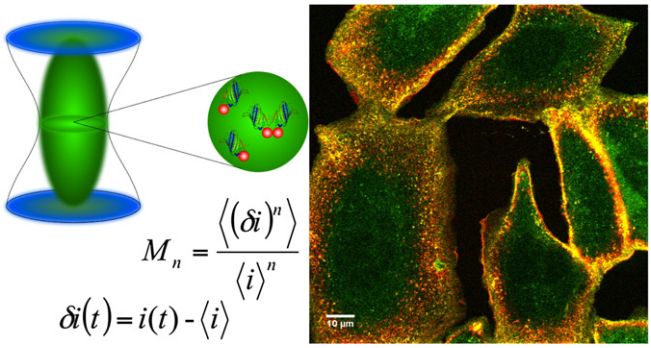
GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 27 October 2011
Received in revised form 15 November 2011
Accepted 16 November 2011
Available online 25 November 2011

Keywords:
Moment analysis
SpIDA
Epidermal growth factor receptor
Receptor tyrosine kinase
Protein oligomerization
Fluorescence fluctuation analysis

ABSTRACT

Fluorescence microscopy is widely used in the life sciences, but largely for qualitative imaging. Here we apply a bioanalytical technique, fluorescence image moment analysis, to demonstrate how the distribution of the fluorescent molecules can be measured directly from confocal microscopy images. We measured the oligomerization state of EGF-eGPF receptors expressed in CHO-K1 cells in situ.

1. Introduction

In order to maintain homeostasis, membrane receptors in cells are in a constant state of flux, rarely remaining in a single state for any extended period of time. Many receptors are present in the cell in a mixture of different oligomerization states, which poses quantification challenges to most standard fluorescence imaging methods due to the diffraction limit of light and typical receptor expression levels (membrane densities). Spatial domain techniques like image correlation spectroscopy (ICS) [1] are often biased by the dominant receptor population and are not able to accurately resolve complex mixtures of receptor populations. In contrast, higher order moment analysis of fluorescence intensity fluctuations allows one to determine the number densities

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doi:10.1016/j.bpc.2011.11.003

and fluorescence intensity brightness ratios of mixed populations from singe images and thus determine the oligomerization state of fluorescently labeled proteins in situ. Validation and characterization of this method has been previously published demonstrating its suitability for quantitative receptor cluster analysis [2].

To further demonstrate the capabilities of fluorescence image moment analysis, here we apply this method to the study of the epidermal growth factor receptor (EGFR), a member of the receptor tyrosine kinase (RTK) super family [3]. These receptors are responsible for growth, proliferation, differentiation, apoptosis and angiogenesis and are key regulators in normal cell processes [4]. Aberrant signaling in this receptor family has been linked to the development of solid state tumors and other types of cancer. Thus RTK receptors are an important target for therapeutic intervention. In order to evaluate the effectiveness of drug agents, a full understanding of normal receptor signaling dynamics is needed including processes such as receptor clustering, internalization and down regulation. Currently there remain important gaps in the understanding of RTK activation and function in relation to endosomal signal transduction and receptor trafficking [4].

It has previously been shown that fine control of EGFR and other RTK trafficking is exercised through a combination of both the type of ligand, the concentration of the ligand, and the extent of homo and heterodimerization of activated receptors [4,5]. It is currently understood that ligands bind to monomeric EGFR forming a ligand activated dimer, or to inactive preformed dimers [6]. In both cases ligand binding initiates the phosphorylation of tyrosine residues in the cytoplasmic kinase domain ultimately leading to the activation of downstream signaling partners responsible for gene regulation and mitogenic signaling [7]. Low doses of the epidermal growth factor (EGF) ligand (0.25 nM) lead to activated receptors being sequestered and internalized through clathrin dependent pathways, while high doses of EGF (3 nM) lead to activated receptor internalization occurring through both clathrin dependent and independent (caveolea) pathways [4]. Additionally, it has been shown that in receptor overexpression systems (>50,000/cell), activated EGFR can significantly accelerate the endocytosis of receptor complexes in a clathrin independent manner [8]. Growth factor function is carefully controlled by negative feedback, which terminates signaling pathways. One of the key mechanisms for termination of receptor signaling is the process of down-regulation (or loss of protein from the cell surface) [9,10]. EGF ligand induced down-regulation in human fibroblasts was found to decrease the total amount of EGFR present on the surface of plasma membrane [9]. Failure to attenuate signaling by receptor down-regulation could be one of the major mechanisms by which EGFR becomes oncogenic [7]. In the current study, we present the application of fluorescence image moment analysis combined with spatial fluorescence intensity distribution analysis [11] to fully characterize the chain of internalization and down-regulation events starting from ligand mediated EGFR dimerization and subsequent formation of large receptor clusters.

2. Experimental

The experimental section is fully described in the Supplementary data.

3. Results and discussion

Two CHO-K1 cell lines stably expressing approximately 100,000 and 600,000 eGFP tagged EGFR per cell were used for the experiments (no endogenous expression). Room temperature conditions were specifically chosen to slow down cellular kinetics for imaging without perturbing signaling pathways [12]. We applied spatial intensity distribution analysis (SpIDA), an image analysis method that can measure monomer/dimer distributions from fluorescence images of cells [11], to measure an increase in EGFR dimer density as a function of time post ligand stimulation (see Fig. 1A–B). No significant difference in quantal brightness (ε) was measured between images of mGFP (monomeric control) and those of EGFR-eGFP in serum starved

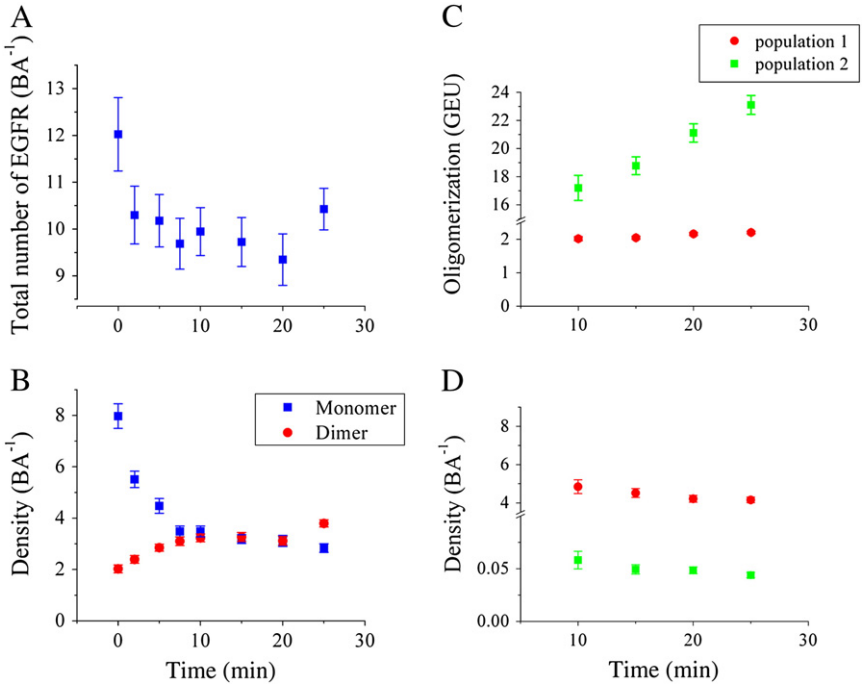

Fig. 1. Time series plots of two-population spatial fluorescence intensity fluctuation analysis of EGFR-eGFP number density of monomeric and oligomeric populations following stimulation with 100 nM EGF in CHO-K1 cells stably expressing 600,000 EGFR-eGFP receptors per cell. (A) Total number density time series recovered with SpIDA; (B) number density time series of monomeric and dimeric populations recovered with SpIDA; (C) oligomerization state and (D) the number density time series of dimeric and oligomeric populations recovered with fluorescence image moment analysis from ROIs exhibiting cluster formation. Two different oligomer populations were resolved (population 1 and 2). Cells were stimulated with EGF ligand and then chemically fixed with 4% PFA. Each data point represents the mean of measurements on 38 cells while the error bars represent the standard error of the mean. BA = beam focus area; GEU = GFP equivalent unit of quantal brightness.

non-stimulated cells indicating that the vast majority of the EGFRs were in a monomeric state prior to stimulation. An increase in EGFR-eGFP dimer density was observed as a function of time following stimulation with EGF ligand (see Fig. 1B). Beyond the 10 min time point, the formation of large EGFR clusters proximal to the membrane was observed (Fig. S4). Next, fluorescence image moment analysis was applied to CLSM images of CHO-K1 cells expressing EGFR-eGFP (600,000/cell). Only ROIs which contained large visible clusters were analyzed (see Fig. 1C–D). Two distinct populations of oligomers were resolved in the measurements: a diffuse dimer population (2.1 ± 0.1 GEU) (GEU = GFP equivalent unit of quantal brightness, see Supplementary data for details) and a population of larger oligomers (20 ± 1 GEU) (see Fig. 1C). The detection of a dominant dimer population is consistent with the SpIDA data shown in Fig. 1B. The decrease in the measured oligomer density at the membrane can be explained by the loss of receptors due to internalization.

Previous ligand accessibility studies suggest that there is limited propagation of small molecules (ligands, dyes, etc.) under the central regions of well adherent CHO-K1 cells compared to the edges of the same cells [13]. Our experiments involving stimulation of EGF receptors with Atto 655 tagged EGF ligand (see Fig. S1) supported the findings of the ligand accessibility studies, and suggested no change in receptor distribution has occurred in the central regions of basal membranes of CHO-K1 cells expressing EGFR-eGFP 12 min post stimulation. However, clustering of EGFR in the central regions of basal membranes of CHO-K1 cells stably expressing EGFR-eGFP was observed at an early time (1 min) post stimulation with high doses of EGF ligand (Fig. 2). Stimulation of cells expressing 100,000 EGF receptors per cell with 20 nM EGF (or higher) resulted in formation of EGFR clusters homogeneously distributed at or near the surface of the basal membranes. Multiple experiments were conducted where the CHO-K1 cells were stimulated with increasingly high doses of EGF. These studies revealed that a higher dose of EGF ligand (200 nM and higher) is required to achieve a similar distribution of EGFR oligomers in the central basal regions at early activation time in cells expressing 600,000 EGFRs/cell. These observations suggest that EGF receptors are down-regulated by the cells according to a mechanism that depends on the total percent ligand occupancy of the receptors. Since the central regions of basal membranes are largely inaccessible to EGF ligands, formation of the large EGFR oligomers almost certainly occurs because of ligand stimulation of the receptors on the apical membrane. However, there is the possibility that there are alternative cell signaling pathways involved in the activation and clustering of EGF receptor ultimately leading to down-regulation.

4. Summary

We applied the two-population fluorescence intensity moment analysis to central regions of basal membranes which were exhibiting homogeneous distributions of EGFR clusters at early stimulation times (Fig. 3A). For comparison purposes, we applied the two-population fluorescence intensity moment analysis to ROIs of basal membranes which showed the presence of EGFR clusters after stimulating the cells with 1 nM EGF ligand for 20 min (Fig. 3B). In both cases, the measured values of the quantal brightness of the dominant (less bright) population, ε_1 suggests that the majority of basal membrane EGFR was in the dimeric state upon stimulation with EGF ligand. This is in good agreement with the dose response curves presented in Ref. [14]. We repeated the same measurements in cells that express 100,000 EGFRs/cell which were activated with 100 nM EGF ligand. The results of the two-population fluorescence intensity moment analysis applied to central regions of basal membranes is shown in Fig. S6. For comparison purposes, we applied the two-population fluorescence intensity moment analysis to ROIs of basal membranes which showed the presence of EGFR clusters after stimulating the cells with 1 nM EGF ligand for 20 min (Fig. S6B). Non-parametric t-tests were carried out on the data sets obtained with the moment analysis. For cells expressing 600,000 EGFRs/cell, the average size of EGFR clusters formed due to down-regulation was not significantly different from that due to the receptor internalization. For cells expressing 100,000 EGFRs/cell, the average size of EGFR clusters formed due to down-regulation was found to be significantly smaller than the average size of those formed during internalization (8.7 ± 0.6 GEU vs. 15.5 ± 1.7 GEU). The saturating % occupancy of the EGF receptors on the apical membrane of cells with low expression level of EGFR can explain this observed difference.

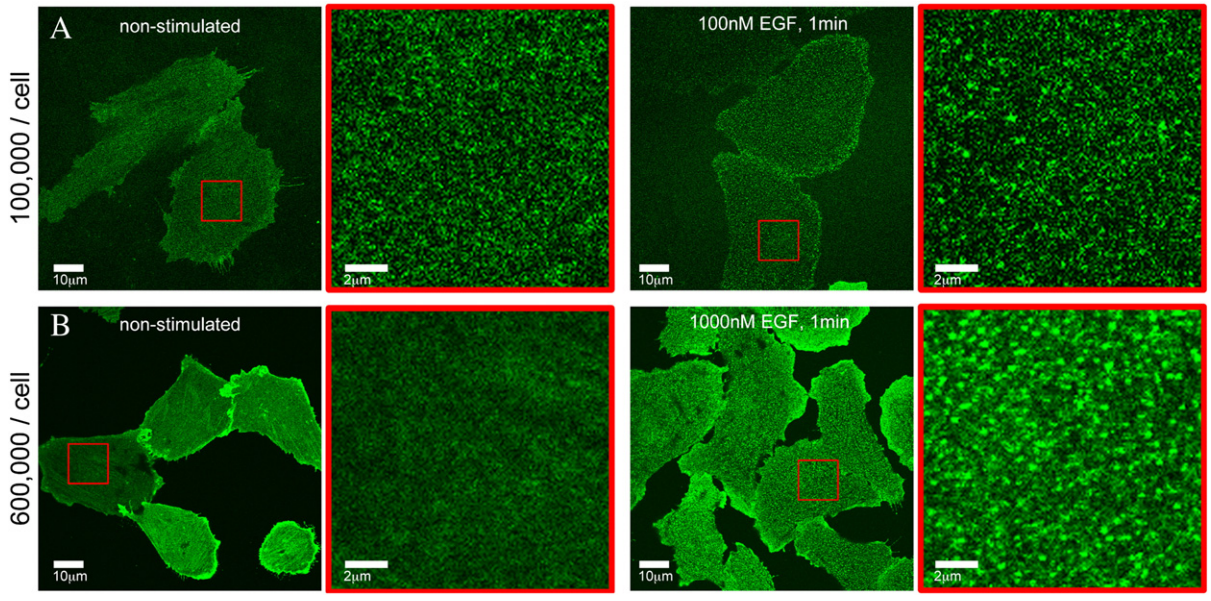

Fig. 2. Visualization of down-regulation of EGF receptors mediated by high doses of EGF ligand. CLSM images of basal membranes of CHO-K1 cells stably expressing EGFR-eGFP (A — 100,000; B — 600,000 receptors per cell). EGF receptors undergo down-regulation following treatment with doses of EGF ligand added to the growth medium. The cells were chemically fixed with 4% PFA after 1 min stimulation with EGF ligand. The experiments showed that down-regulation was both ligand concentration and receptor surface density dependent. A homogeneous distribution of EGFR clusters is observed across the whole area of the basal plasma membrane including the central regions which are not directly accessible by ligands as shown in Ref. [13]. The image contrast was enhanced in both panels A and B for visualization purposes. Red squares indicate ROIs selected for the higher zoom images.

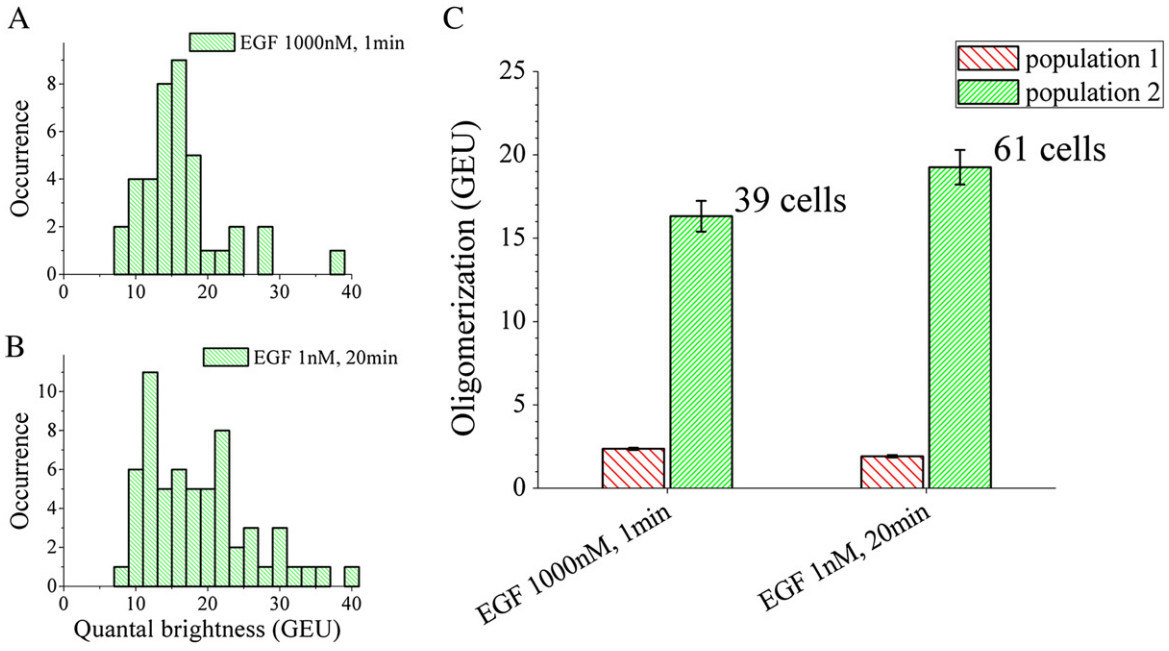

Fig. 3. Fluorescence intensity moment analysis of EGFR cluster size formed due to ligand mediated internalization and down-regulation of the receptors (600,000 EGFR/cell). (A) CLSM images of central regions of basal membranes were analyzed with two-population fluorescence intensity moment analysis. EGF receptors undergo down-regulation due to a saturating dose of EGF ligand (1000 nM) added to the growth medium. The cells were chemically fixed with 4% PFA after 1 min stimulation with EGF ligand. (B) Two-population fluorescence image moment analysis of subregions of plasma membranes exhibiting formation of large clusters resulting from EGFR endocytosis. The cells were stimulated with 1 nM EGF ligand for 20 min and then chemically fixed. The histograms show the recovered values of quantal brightness normalized to GEU. All of the measurements summarized in each histogram were carried out under identical collection conditions. (C) Side-by-side comparison of the brightness values of EGFR-eGFP clusters forming as a result of down-regulation and endocytosis respectively. The error bars represent the standard error of the mean obtained from multiple cells as indicated. GEU = GFP equivalent unit of quantal brightness.

In this study, we carried out pharmacodynamic experiments of ligand-induced activation of EGF receptors and the distribution of oligomers was measured using the spatial fluorescence moment and intensity distribution analysis. Measurements on a large number of cells stimulated under identical conditions provided sufficient population information for reliable statistics so conclusions could be drawn about the distributions of receptor oligomerization size. The two CHO-K1 cell lines examined in our studies, were stably transfected with EGFR-eGFPwith two distinct expression levels — 100,000 and 600,000 receptors per cell respectively. The large difference in total EGFR between the two stable lines allowed us to correlate the impact of the receptor expression level and the ligand concentration on the EGF induced dimerization and higher order oligomerization. We found EGFR to be predominantly monomeric prior to ligand stimulation in serum starved cells. The results of monomer–dimer SpIDA measurements applied to CLSM images of ligand stimulated CHO-K1 cells stably expressing EGFR-eGFP validated the functionality of the EGFR-eGFP construct. SpIDA revealed an increase of EGFR dimer density at early activation times (1 min post stimulation with the EGF ligand) demonstrating the ligand mediated activation of the receptor. We were interested in determining whether the expression levels of the EGFR-eGFP in the cell could result in indirect receptor clustering in cells, from either auto-oligomerization or distal activation of receptors. By using fluorescence image moment analysis in combination with ligand accessibility studies, we differentiated between clustering occurring as a result of the direct receptor activation, and clustering which is likely a result of down-regulation of surface EGFR on the membrane due to high doses of EGF ligand. Taken together the measurements suggest that an equilibrium exists between down-regulation and internalization of the ligand activated EGFRs which depends on the total ligand percent occupancy of the receptors. This has important implications for biologically relevant pharmodynamic studies especially for studies of abnormal signaling by EGFR in cancer cell lines that typically overexpress this RTK (e.g. A431 epidermoid carcinoma cells).

Acknowledgments

We acknowledge Dr. T. M. Jovin and Dr. D. Arndt-Jovin (Max Planck Institute for Biophysical Chemistry) for providing the transfected CHO cell line used in these studies. PWW acknowledges the Natural Sciences and Engineering Research Council of Canada (NSERC), the Canadian Institutes for Health Research (CIHR) and the Canadian Foundation for Innovation (CFI) for funding support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bpc.2011.11.003.

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