Vol. 197, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1993 Pages 207-213

# DYNAMICS OF EXOCYTOSIS, ENDOCYTOSIS AND RECYCLING IN SINGLE PITUITARY GONADOTROPES

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Received September 6, 1993

***SUMMARY*** The dynamic changes in exocytosis, endocytosis and recycling in single gonadotropes induced by gonadotropin-releasing hormone (GnRH) were visualized and estimated with an impermeable fluorescent membrane probe, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatrien (TMA-DPH), using a digital imaging and confocal laser scanning microscope. $10^{-7}$ M GnRH induced exocytosis and endocytosis within 10 sec and 60 sec, respectively. Recycling of the plasma membrane started at 180-300 sec. Exocytosis and endocytosis in purified gonadotropes changed dose-dependently with $10^{-10}$-$10^{-7}$ M GnRH. These results show that GnRH-induced exocytosis, endocytosis and recycling in gonadotropes maintain dynamic equivalence. The procedure we established will be very useful in studies of the function of secretory cells. © 1993 Academic Press, Inc.

In pituitary cells, regulatory exocytosis and endocytosis are induced by cell-specific hormone-releasing factors such as gonadotropin-releasing hormone (GnRH), thyrotropin-releasing hormone (TRH), corticotropin-releasing hormone (CRH) and growth hormone-releasing hormone (GHRH). However, it has been difficult to directly measure these dynamic changes in single cells. A method for measuring the changes in membrane capacitance electrically was developed for estimation of exocytosis (1-3). In pituitary cells, exocytosis of bovine lactotropes was estimated by this method (4). Exocytosis increases the membrane capacitance, but endocytosis decreases it. Stimulation of cells by releasing factors causes not only exocytosis but also endocytosis. Thus, this method is not adequate for quantitative analysis of exocytosis itself. Recently, a fluorescent probe, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), has allowed estimation of the kinetics of exocytosis (5,6), endocytosis and membrane recycling (7,8) in suspensions of mast cells and macrophages. This probe, which is highly fluorescent in membrane lipids but not at all in solution, binds to and labels only the outer layer of the plasma membrane within a few seconds after addition (9,10). Thus the TMA-DPH fluorescence of a cell is proportional to its membrane surface area. Exocytosis increases the labeled membrane surface area by fusion of secretory granules with the plasma membrane, which results in an

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0006-291X/93 $4.00
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increase in the total cell fluorescence in a TMA-DPH solution. On the other hand, endocytosis moves the membrane fluorescence to intracellular regions but does not affect the total cell fluorescence. Endocytosis is estimated from the fluorescence remaining after washing of the cells, while recycling of endocytotic membranes is estimated from the subsequent decrease in fluorescence. Previous studies were unable to clarify these phenomena directly. A confocal laser scanning microscope has allowed analysis of the fluorescence distribution in optical sections and in three dimensions. In the present study, we visualized the change in fluorescence distribution of TMA-DPH for the first time and characterized exocytosis and endocytosis in single gonadotropes. Furthermore, using a fluorescence microscope with a digital imaging system, we estimated the kinetics of exocytosis, endocytosis and recycling in single gonadotropes.

## *MATERIALS and METHODS*

**Materials** TMA-DPH and 1,6-diphenyl-1,3,5-hexatriene (DPH) were obtained from Molecular Probes. GnRH and [D-pGlu$^{1}$,D-Phe$^{2}$,D-Trp$^{3,6}$]-LH-RH (GnRH antagonist) were obtained from Peninsula Laboratories, Inc.

**Purification of Gonadotropes** As we reported previously (11), gonadotropes were purified. Female Wistar rats (180-200 g) were decapitated and an anterior pituitary cell suspension was prepared from them. The isolated pituitary cell aggregates were preincubated at 37°C in complete medium (medium 199 supplemented with 10% fetal calf serum) with 1 nM GnRH for 30 min. Then, for labeling experiments, anti-rat LH rabbit antibodies were applied under the same conditions for 20 min. The cells were washed twice with Hanks balanced salt solution (HBSS). Biotinylated anti-rabbit goat IgG was applied for 40 min at room temperature, and then the cells were washed twice with HBSS. Fluorescein-labeled avidin was applied for the next 10min at room temperature, and the cells were then washed twice with HBSS. Gonadotrope-enriched (80-90%) pituitary cells were then obtained with a fluorescence-activated cell sorter (EPICS ELITE, Coulter, USA). Furthermore, after the exocytosis and endocytosis experiments, gonadotropes were identified by an immunofluorescent technic using cells which were fixed with Zamboni fluid (4% paraformaldehyde and 15% saturated picric acid solution in phosphate buffer) at 4°C for 24 hours and treated with 0.3% Triton X-100 for 30 min.

**Measurement of Exocytosis** Purified gonadotropes were plated on glass coverslips which were sealed under the 1.0 cm hole in the bottom of 35-mm culture dishes and coated with poly-D-lysine. The cells were maintained at 37°C in an atmosphere of 95% air-5% $CO_2$ in medium 199 with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml), and used for experiments after 24-48 hours. Gonadotropes were incubated with 1 μM TMA-DPH in HBSS at selected temperatures (4°C or 37°C), and the 430 nm fluorescence of single cells at 350 nm excitation was measured every 10-60 sec. It was preliminarily determined that the TMA-DPH fluorescence of gonadotropes showed a peak intensity of 430 nm emission at 350-360 nm excitation. The changes in fluorescence intensity in individual cells induced by GnRH were analyzed by a digital imaging system (ACAS 470, Meridian, USA). The increase in fluorescence represents the sum total of exocytosis. Various concentrations of GnRH in 1 μM TMA-DPH solution were added to cells. GnRH did not induce any non-specific change in the fluorescence.

**Measurement of Endocytosis** After the cells were washed twice with 4°C HBSS to remove TMA-DPH from the plasma membrane, the fluorescence intensity of the TMA-DPH internalized fraction in the respective cells was measured. There was no difference between two washings and three or more washings. The residual fluorescence after washing the cells was plotted at various times after GnRH stimulation. These fluorescences represent the total amount of endocytosis.

**Measurement of Recycling** After washing twice with 37°C HBSS at various times after the first $10^{-7}$ M GnRH stimulation, the change in the residual fluorescence was analyzed in single gonadotropes. After washing, the fluorescence of cells was measured at 37°C in HBSS in the presence or absence of $10^{-7}$ M GnRH. The decrease in fluorescence is due to

diffusion of the TMA-DPH internalized fraction to the extracellular region as a result of fusion of the plasma membrane and internalized vesicles. Bleaching of fluorescence caused by a hundred times of laser excitation without any stimulations was within 5 % of pre-level of fluorescence intensity.

**Confocal Laser Scanning Microscopy** The distribution of TMA-DPH fluorescence was analyzed with a confocal laser scanning microscope with a 100 x objective lens and a digital imaging system (ACAS570, Meridian, USA). Optical sections were obtained from emission fluorescence limited by a 440nm short-pass filter and a 410nm barrier filter at an excitation wavelength of 350nm from an argon laser. Confocal optics significantly reduced out-of-focus fluorescence thereby permitting optical two-dimensional images with high contrast and high resolution. A confocal image was built by moving the specimen in a pattern with an x-y-z scanning stage past a diffraction-limited laser spot (0.27 μm beam diameter). The fluorescence emissions from the illuminated specimen are projected onto the aperture of a variable pinhole so that only signals from a defined spot are collected. The degree of confocality was controlled by the size of the pinhole (40-100 μm). Points of laser pulse were measured every 0.7 μm, and optical slices were obtained every 1 μm.

## *RESULTS and DISCUSSION*

The changes in TMA-DPH and DPH fluorescences in single gonadotropes were determined under various conditions. DPH is a fluorescent dye that has the same fluorescent characteristics as TMA-DPH, but it is membrane-permeable. Addition of DPH gradually increased the fluorescence intensity of the cells, and this reached a plateau value after 20-25 min. Subsequent GnRH stimulation ($10^{-9}$ - $10^{-6}$ M) did not change the fluorescence intensity (Fig.1). These results explain the facts that (1) DPH, unlike TMA-DPH, is incorporated into all intracellular regions and labels the membranes of the granules and other compartments before GnRH stimulation, (2) the fusion of the plasma membrane and vesicles induced by GnRH did not change the total DPH-fluorescence of a cell, and (3) other changes

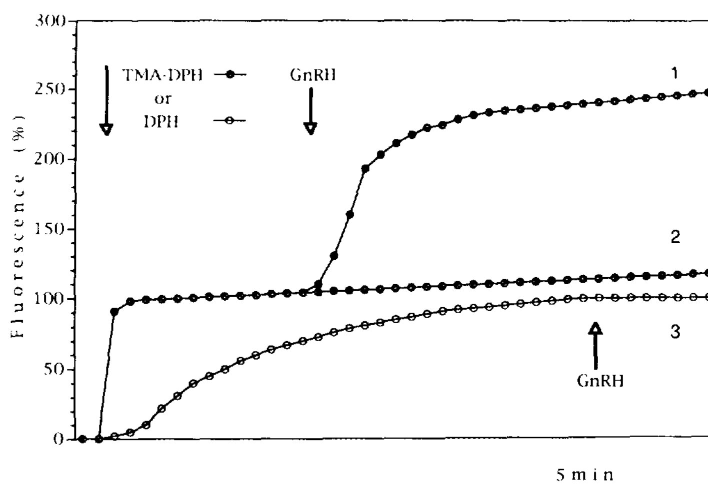


Fig.1. Changes in DPH fluorescence and TMA-DPH fluorescence in single gonadotropes. Lines 1 and 2 indicate the fluorescent changes induced by TMA-DPH (1 μM) and then $10^{-7}$ M GnRH stimulation without and with $10^{-6}$ M GnRH antagonist pretreatment, respectively. Line 3 indicates the change induced by DPH (1 μM) and then $10^{-7}$ M GnRH stimulation. The 430 nm fluorescence of single cells at 350 nm excitation was measured every 60 sec. Y-axis values are shown as the percent of the fluorescence before GnRH stimulation.

induced by GnRH (changes in intracellular ions, enzymes, membrane fluidity, et al.) did not cause nonspecific changes in the fluorescence. Gonadotropes acquired strong fluorescence immediately (within 1 sec) upon addition of TMA-DPH. The fluorescence then remained constant at 4°C and increased only slowly at 37°C in the absence of stimulation (Fig.2). $10^{-7}$ GnRH significantly elevated the fluorescence at 37°C. The increase in fluorescence was almost inhibited by pretreatment with $10^{-6}$ M of a GnRH antagonist (Fig.1). The increase in fluorescence in single gonadotropes at 37°C with and without GnRH stimulation represents regulatory and constitutive exocytosis, respectively. These results suggest that the change in TMA-DPH fluorescence induced by GnRH is due to regulatory exocytosis itself. This is further supported by the fact that the change in total fluorescence is due to an increase in fluorescent regions rather than the fluorescence intensity, as shown later by confocal optical images. On the other hand, exocytosis was not observed at 4 °C, and the process of exocytosis stopped when the temperature of medium was rapidly lowered from 37 °C to 4 °C (not shown).

Next, the characteristics of the changes in fluorescence induced by GnRH were further analyzed in purified gonadotropes. The elevation showed a fast first phase and a slow second phase. $10^{-7}$ M GnRH started the change in fluorescence within 10 sec. The elevated fluorescence reached a plateau value at 600 sec. The slope of the first phase and the time to the plateau values were GnRH dose-dependent ($10^{-10}$ - $10^{-7}$ M) (Fig.2).

In similar experiments on pituitary cells of mixed population, a GnRH response was caused in 10 -15 % of all cells, but the response was irregular. Even a high dose ($10^{-7}$ M) of

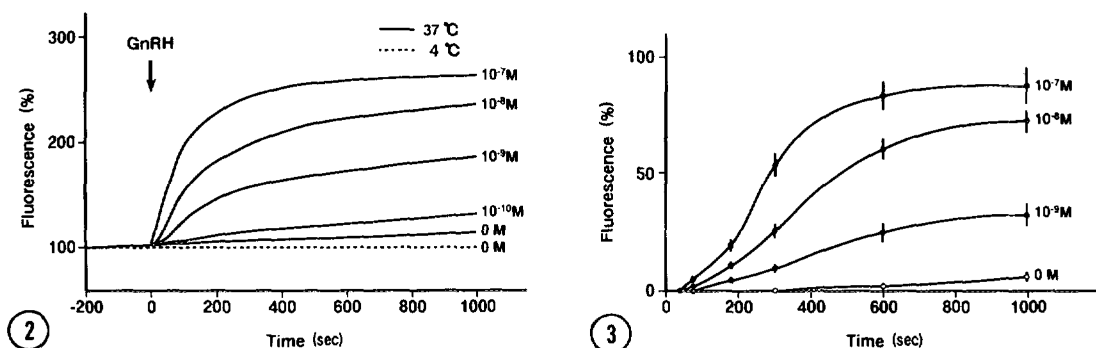


Fig.2. Kinetics of exocytosis induced by GnRH in single gonadotropes. Exocytosis was estimated from the changes in TMA-DPH fluorescence induced by various concentrations ( 0, $10^{-10}$, $10^{-9}$, $10^{-8}$ and $10^{-7}$ M ) of GnRH at 4°C or 37°C. The 430 nm fluorescence of single cells at 350 nm excitation was measured every 10 sec. The respective lines indicate the time-course changes of a cell. The data were obtained by multiple determinations in at least 10 replicate experiments. The data are presented as the percent of the TMA-DPH fluorescence intensity before GnRH stimulation.

Fig.3. Kinetics of endocytosis induced by GnRH in single gonadotropes. Endocytosis was estimated from the residual TMA-DPH fluorescence after washing at various times ( 30, 60, 180, 300, 600 and 1000 sec ) after GnRH stimulation at various concentrations ( 0, $10^{-9}$, $10^{-8}$ and $10^{-7}$ M ). The points and bars in the figure indicate the mean values and ± SEM for 10 cells, respectively. The time of the X-axis is shown with 0 sec being the time of GnRH stimulation. The data are presented as the percent of the TMA-DPH fluorescence intensity before the first GnRH stimulation.

GnRH increased the fluorescence after a time-lag of several minutes in some pituitary cells. (not shown) These results may be due to an indirect response through a paracrine pathway or a poor response by immature cells.

Next, the endocytosis induced by GnRH at 37°C was estimated. This was measured under the condition of stopping exocytosis and endocytosis by washing at 4 °C. Endocytosis was activated by GnRH in a dose-dependent manner from $10^{-9}$ - $10^{-7}$ M. $10^{-8}$ and $10^{-7}$ M GnRH started endocytosis at 30-60 sec after stimulation. $10^{-9}$ M GnRH started endocytosis at 60-180 sec (Fig.3). However the values shown in Fig.3 may be slightly underestimated after the start of recycling because of recycling of the internalized membranes into the plasma membrane.

The fluorescence after washing was subsequently measured at 37 °C in the solution with or without of $10^{-7}$ M GnRH (the same concentration as the first stimulation). The fluorescence decreased slowly in the absence of GnRH but rapidly in the presence of GnRH (Fig.4). This decrease, which is due to diffusion of TMA-DPH into the medium after the fusion of labeled vesicles with the plasma membrane, represents recycling of internalized membranes to the plasma membrane. After washing, recycling occurred only slightly in the absence of GnRH. A significant decrease in fluorescence was caused in the cells washed 300 sec and 600 sec after first GnRH stimulation in the presence of $10^{-7}$ M GnRH, but there was no decrease when the cells were washed at 180 sec. (Fig.4) The results show that recycling started at 180-300 sec after GnRH addition in the presence of $10^{-7}$ M GnRH. On the other hand, the findings shown in Fig.2, that the increase in fluorescence reached a plateau value at 1000 sec after $10^{-7}$ M GnRH stimulation, suggest that almost all of exocytosis replaced recycling of the labeled membrane after 1000 sec.

The changes in the fluorescence distribution of TMA-DPH induced by $10^{-7}$ M GnRH were visualized and characterized with a confocal laser scanning microscope. By addition

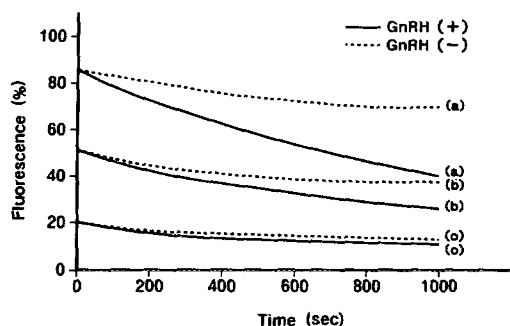


Fig.4. Kinetics of membrane recycling induced by GnRH in single gonadotropes. Recycling was estimated from the subsequent changes in residual fluorescence after washing. The 430 nm fluorescence of single cells at 350 nm excitation was measured every 10 sec. The gonadotropes activated by the first $10^{-7}$ M GnRH addition were analyzed in TMA-DPH-free HBSS in the presence or absence of $10^{-7}$ M GnRH. These cells were washed at 600 sec (a), 300 sec (b) or 180 sec (c) after the first GnRH stimulation. The time of the X-axis is shown with 0 sec as the time of washing. The data are presented as the percent of the TMA-DPH fluorescence intensity before the first GnRH stimulation.

of TMA-DPH to the medium, fluorescence was observed only in the plasma membrane. At 30 sec after GnRH stimulation, the fluorescence inside the membrane became apparent. At 60 sec, the regions of fluorescence were even more expanded inside the cell, but the fluorescence intensity per unit area did not change. At 300 sec, the fluorescence was almost confluent in the cytoplasm, but there was little in the nucleus. After washing out the TMA-DPH, the fluorescence of the membrane disappeared, and only internalized fluorescence remained (Fig.5). These results support the concept that exocytosis increases the regions of TMA-DPH fluorescence by increasing the surface area of the plasma membrane, while endocytosis expands the TMA-DPH fluorescence to intracellular regions. These changes hardly occurred in the cells without GnRH stimulation (not shown). Furthermore, we surmise that, because of the dynamic movement of stained vesicles in the cytoplasm, not only the regions in the intracellular compartments but almost all regions were stained.

Exocytosis in gonadotropes is essential for secretion of LH and FSH and important for the introduction of constituent proteins to the plasma membrane. Endocytosis and recycling of GnRH and other receptors are important for controlling the receptor number and intracellular messenger systems. However, little has been known about these dynamic changes. The

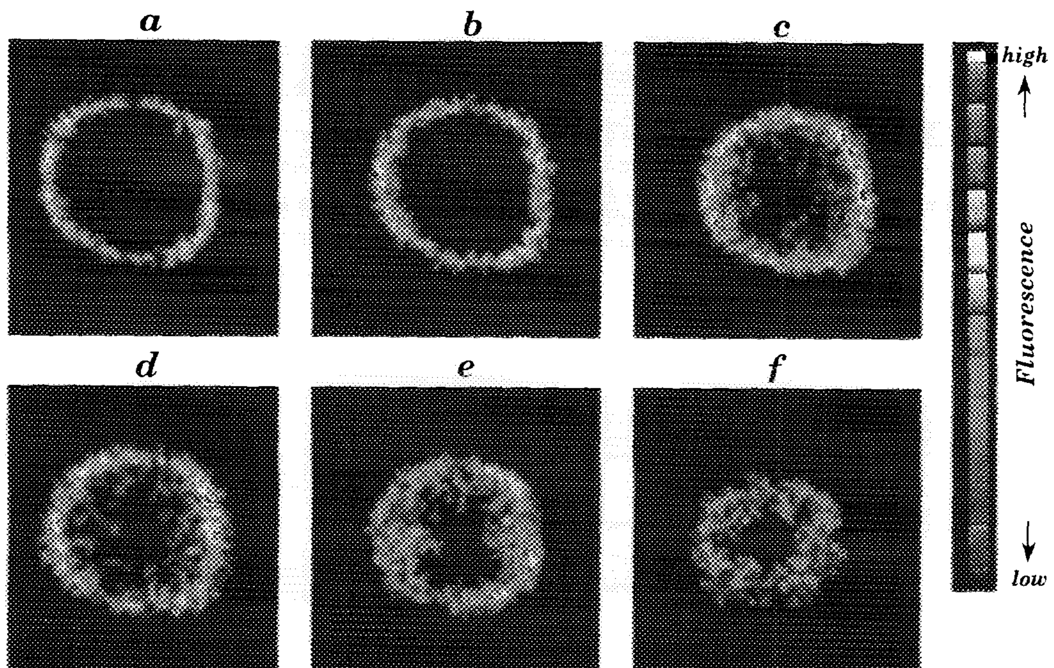


Fig.5. Optical imaging of the distribution of TMA-DPH fluorescence by a confocal laser scanning microscope. The fluorescence is coded using the spectrum from red to blue, as calibrated by the right-hand scale. *a*. Immediately after addition of TMA-DPH. *b*. 200 sec after addition of TMA-DPH and before $10^{-7}$ M GnRH stimulation. *c*. 30 sec after addition of GnRH. *d*. 60 sec after addition of GnRH. *e*. 300 sec after addition of GnRH. *f*. Immediately after washing at 600 sec after addition of GnRH. Optical images of the maximum diameter at respective times in an identified cell were selected. The Z-axis levels of these images were the same.

kinetics of exocytosis in gonadotropes have been estimated on the basis of LH and FSH secretion. However, little is known regarding LH and FSH secretion by single gonadotropes or exocytosis itself. There are several reports on regulatory endocytosis induced in gonadotropes by GnRH (12-14). They demonstrated binding and internalization of ferritin-coupled or radioiodinated GnRH to gonadotropes. Previous studies have suggested that receptor-bound GnRH is aggregated, internalized and then routed to lysosomes, secretory granules or Golgi complex. On the other hand, Schvartz and Hazum detected endocytosis and recycling of photoaffinity-labeled GnRH receptors by gel electrophoresis (15). However, the dynamics of endocytosis and recycling in living gonadotropes is not well understood.

We have developed a method by which exocytosis, endocytosis and recycling in single secretory cells can be measured using a fluorescence dye, TMA-DPH, and a fluorescence microscope with a digital imaging analyzer. TMA-DPH has been used for estimation of exocytosis, endocytosis and recycling (5-8), but they were theoretical estimations. For the first time, we observed the movement of TMA-DPH fluorescence using a confocal laser scanning microscope, showing that the changes were caused by exocytosis and endocytosis. We surmise that internalization of receptor-bound GnRH is a part of all endocytosis, and that exocytosis, endocytosis and recycling maintain a balance by the exchange of membrane parts between the plasma membrane and the intracellular membrane structures. Our present studies have, for the first time, clarified the time-related changes in exocytosis, endocytosis and recycling in single gonadotropes. Our techniques for evaluating exocytosis, endocytosis and recycling are very useful and will have a wide range of applications in studies of endocrine cell functions.

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