Endocytosis-Dominated Membrane Area Decrease Requires Rab5 Protein in Rat Melanotrophs

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ABSTRACT: Eukaryotic cells internalize extracellular macromolecules by endocytosis and it was shown that Rab5 protein is required for this process. While it is clear that endocytosis consists of vesicle fission from the plasma membrane, the role of Rab5 protein in the plasma membrane surface area changes is still unclear. Here we studied whether Rab5 is required for membrane surface area changes in rat melanotrophs—cells deriving from the pituitary pars intermedia. The presence of this protein in melanotrophs was probed by immunocytochemistry and its putative role in membrane area dynamics was monitored electrophysiologically with membrane capacitance measurements as this parameter directly reflects changes in membrane surface area. We found that Rab5 protein exists in melanotrophs. At \([\text{Ca}^{2+}]_i < 3\ \mu\text{M}\), endocytosis-dominated membrane capacitance decrease was found to be blocked by microinjection of specific Rab5 antibody. At high \([\text{Ca}^{2+}]_i\), Rab5 antibody did not affect the steady-state increase in membrane capacitance, while it elevated the rate of membrane capacitance increase, which is consistent with an inhibition of endocytosis.

KEYWORDS: melanotrophs; endocytosis; membrane capacitance; Rab 5; small GTPase

INTRODUCTION

Rab5 protein is a key component of the machinery that regulates both transport from the plasma membrane to early endosomes and lateral fusion between early endosomes. 1 Rab5 protein is required in the homotypic fusion of early endosomes with each other in vitro 2 and in vesicle assembly with clathrin. 1 Rab5 protein intracellularly locates to both the plasmalemma and the membrane of early endosomes. 3 Many cell processes involve Rab5 protein, such as trafficking of endothelin receptors in Chinese hamster ovary (CHO) cells, 4 actin remodeling by receptor tyrosine kinas-
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es, adenovirus endocytosis, and indirect trafficking of EGF receptors in HeLa cells, transferrin receptors in hepatocytes, “kiss and run” fusion between phagosomes and endosomes and between lysosomes, internalization of β-2 adrenergic receptors and angiotensin II type 1A receptor in human embryonic kidney cells (HEK 293), and dopamine D2 receptors in COS-7 cells. Endocytotic pathway abnormalities of Rab5 protein cause Alzheimer’s disease neurons by deposition of β-amyloid protein in brain or have possible implications in development of growing astrocytes.

In recent experiments it was shown that Rab5-specific antiserum can specifically inhibit early endosome fusion in vitro. While Rab5 regulates endocytosis at the level of early endosome fusion, the mechanism by which Rab5 exerts its function on endocytosis and consequently on the plasma membrane area remains to be established. Furthermore, the function(s) of Rab5 protein in rat melanotrophs and specifically the role of Rab5 protein in electrophysiologically determined plasma membrane area changes have not been reported yet.

In our experiments, we examined the effect of Rab5 protein on endocytotic activity of cultured rat melanotrophs. These cells secrete different important peptide hormones from posttranslational processing of pro-opiomelanocortin, such as β-endorphin and α-melanocyte stimulating hormone. Specific Rab5 antibodies, previously shown to inhibit endosome-to-endosome fusion were microinjected into single melanotrophs to inhibit endocytosis. We used the patch-clamp technique and measured changes in membrane capacitance (Cm) to monitor the secretory activity of single cells. The cytosol was dialyzed with various Ca2+ concentrations.

We found the presence of Rab5 protein in rat melanotrophs. Inactivation of Rab5 protein inhibited endocytosis. When exocytosis was stimulated and endocytosis inhibited, exocytosis reached the saturation level faster.

MATERIALS AND METHODS

Cell Culture

Cell cultures of melanotrophs from the rat pituitary pars intermedia were isolated (male Wistar rats, 200–300 g) and prepared by methods described. Cells were plated on poly-L-lysine-covered glass cover slips and kept in an incubator at 37°C, in 95% humidity and 5% CO2 in a cell culture medium (a mixture of α-minimal essential medium (αMEM), Dulbecco’s modified Eagle’s medium (DMEM), and F-12 medium (GIBCO, UK) for 1–5 days before experimentation. Then the coverslips were moved into the recording chamber mounted onto an inverted microscope (Opton IM35, Zeiss). The recorded medium in the chamber consisted of recording solution (in mM): NaCl, 131.8; CaCl2, 1.8; KCl, 5; MgCl2, 2; HEPES/NaOH, 10; D-glucose, 10; NaH2PO4·2H2O, 0.5; NaHCO3, 5; at pH 7.2. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

Electrophysiological Recording

Using the whole-cell patch-clamp technique, the cells were voltage clamped at a holding potential of –70 mV. Membrane capacitance (Cm) was recorded using a two-
phase lock-in amplifier (1,600 Hz, 1 mV peak-to-peak) incorporated into a patch-clamp amplifier (SWAM Cell, Henigman, Slovenia). A DC current (low pass, 1 Hz, –3 dB), holding potential and real and imaginary admittance signals (low pass, 1 Hz, –3 dB) were used in this calculations. The reversal potential for cell membrane current was –50 mV and did not change during a recording. The plots of the passive cell parameters, access conductance (G_a), parallel combination of leak (G_m) and membrane conductance (C_m) were derived by computer-aided reconstruction following an analogue-to-digital conversion (CED 1401, Cambridge, UK) using a PC computer. The software was written by Dr. J. Dempster (University of Strathclyde, Glasgow, UK). Recordings were made at room temperature with pipette resistances between 1 and 4 Ω. The basic solution in the recording pipette contained (in mM): KCl, 150; MgCl_2·6H_2O, 2; HEPES, 10; Na_2ATP, 2; at pH 7.2. To this, various amounts of 13.5 mM stocks of DBB (1,2-bis (o-amino-5-bromophenoxy)ethane-N,N,N′,N′-tetraacetic acid) and Ca^{2+}-DBB were added to obtain various Ca^{2+} activities. Intracellular Ca^{2+} concentration (Ca^{2+}_i) was calculated assuming an apparent dissociation constant (K_d) for the Ca^{2+}-DBB complex of 1.62 μM. If only DBB (4 mM) was added to the pipette solution, intracellular Ca^{2+} was taken as 0 μM. Total DBB concentration was 4 mM. DBB buffer is pH independent (7.0–7.5) and binds Ca^{2+} quickly.

**Microinjection of Rab5 Antibodies and Proteins**

Antibodies were microinjected by using a Transjector 4657 with a micromanipulator (Eppendorf, Germany). Pulses (20–30 hPa, 0.3–0.5 sec) were applied with a compensation pressure set to 12 hPa. Microinjection solution consisted of intracellular solution, purified monoclonal Rab5 antibody (from Dr. Marino Zerial, Heidelberg, Germany), 2% streptomycin and fluorescence marker RITC (rhodamine isothiocyanate)-dextran. Fluorescence marker was used to make the treated cells visible under the fluorescence microscope. Antibodies were injected into cells together with RITC, and then cells were placed in an incubator for 1 to 2 hours at 37°C before patch-clamp experiment. Pipettes were prepared with a horizontal puller (P-97, Sutter Instruments, California, USA).

**Immunocytochemistry**

Cells were washed with phosphate buffered saline (PBS) at pH 7.4, then cells were fixed in 4% paraformaldehyde solution in PBS for 15–20 min at room temperature. Cells were added 0.1% Triton X-100 solution in 4% paraformaldehyde for 10 minutes. Cells were again washed with PBS. We prepared 10% goat serum in 3% BSA and placed it on coverslips for 60 min in 37°C. After washing with PBS, monoclonal primary antibodies for Rab5 protein were added and incubated during the night at 4°C. We washed again with PBS before secondary antibodies were added ( antimouse IgG) marked with fluorescein-isothiocyanate (FITC). Cells were incubated 45 min at 37°C and washed with PBS. Mounted coverslips were viewed with laser confocal microscope LSM 510 (Zeiss, Germany).
Rab5 Protein Is Present in Rat Melanotrophs

Immunocytochemistry showed the expression of Rab5 protein in cytosol and plasma membrane of rat melanotrophs. Cells treated with specific Rab5 antibodies were stained fluorescently green (FIG. 1, right down), while control cells were barely visible (FIG. 1, right up). Notice the different green contrast between nucleus and cytosol with the plasmalemma, displaying the presence of Rab5 protein only in the plasmalemma and cytoplasm, but not in the nucleus. The location of Rab5 protein is consistent with previous reports.¹

Endocytosis and Exocytosis Were Balanced at ~3 µM [Ca²⁺]ᵢ

To investigate the effect of different [Ca²⁺]ᵢ on endocytotic and exocytotic activity, we measured changes in membrane capacitance (Cₘ) with the standard patchclamp technique.²¹,²⁵ This technique allows the dialysis of cytosol with pipette solution. To determine [Ca²⁺]ᵢ value, at which the steady-state rates of endocytosis and exocytosis are balanced, we changed [Ca²⁺]ᵢ using the DBB buffer. The analysis was performed by measuring the change in Cₘ 300 sec after the establishment of the whole-cell recording relative to the resting Cₘ determined at the time of the whole-cell recording establishment and by measuring the rate of change in Cₘ 10 sec after the establishment of the whole-cell recording. Results are shown on FIGURE 2. Cy-
tosol dialysis with high calcium ([Ca$^{2+}$]$_i$ = 20 µM) induced an increase in C$_m$ by 19.2 ± 4.6% ($N = 6$; mean ± S.E.M.; Fig. 2A), whereas at low calcium ([Ca$^{2+}$]$_i$ = 0.02 µM) a decrease in C$_m$ of –29.4 ± 5.0% ($N = 5$; Fig. 2B) relative to the resting C$_m$ was recorded. As noted from Figure 3B, the steady-state rates of exocytosis and endocytosis appear to be balanced in these experiments at around [Ca$^{2+}$]$_i$ = 3 µM, since C$_m$ was –7.1 ± 5.9% ($N = 5$, not significantly different from zero (Fig. 2B, left panel). At [Ca$^{2+}$]$_i$ = 3 µM the rate of increase was –1.9 ± 0.3 fF sec$^{-1}$ ($N = 5$; see Fig. 2B right panel).

**FIGURE 2.** Calcium-dependence of the membrane capacitance change in rat melanotrophs. (A) Time courses of membrane capacitance (C$_m$) in control cells at 20 µM and 0 µM [Ca$^{2+}$]$_i$. Numbers adjacent to both traces indicate resting membrane capacitance (dotted line). (B) Relative changes in membrane capacitance ($\Delta$% C$_m$) after 300 sec of cytosol dialysis in control cells at different [Ca$^{2+}$]$_i$: 0, 1, 3, 5, 10, 20 µM. Number in brackets near the symbols show the number of cells tested, error bars on diagrams indicate S.E.M. (C) Maximal rate of C$_m$ changes (dC$_m$/dt) in control cells at different [Ca$^{2+}$]$_i$: 0, 1, 3, 5, 10, 20 µM.
Anti Rab5 Antibody Inhibited Endocytosis And/or Stimulated Exocytosis

It was reported previously that Rab5-specific antiserum blocks endocytosis. Therefore, we studied whether Rab5 antibody introduction into single cells affects the time-course of $C_m$ changes in melanotrophs dialyzed with low and high Ca$^{2+}$-containing pipette solutions, respectively (FIG. 3). The results show that at low calcium the changes in $C_m$ were significantly different in Rab5 antibody-treated cells in comparison with controls ($-29.4 \pm 5.0\%$, $N = 5$ in controls and $-2.5$...

**FIGURE 3.** Inhibition of endocytosis with a specific Rab5 antibody. Membrane capacitance recording of a control cell and a cell microinjected with a specific Rab5 antibody in low (A) and high cytosolic [Ca$^{2+}$]$_i$ (B). Numbers adjacent to traces indicate the resting membrane capacitance (dotted line). Relative membrane capacitance change (C) and the rate of membrane capacitance change (D) in control cells and cells microinjected with specific Rab5 antibody or non-specific antibody. Empty bars show the membrane capacitance changes at low calcium and gray bars at high calcium. Relative changes were measured as indicated in Figure 2. Numbers in brackets near the bars show numbers of cells tested, error bars are S.E.M. Statistically significant differences between the control and Rab5 antibody treated cells are indicated with asterisks (Student’s $t$ test, $P < 0.01$).
± 2.9%, N = 6; in Rab5 antibody–treated cells, P < 0.01, Fig. 3C). The responses in Rab5 antibody–treated cells appears to be specific since pre-injection of a non-specific antiserum resulted in C_m changes similar to controls (−17.5 ± 9.8%, N = 4). Interestingly, there was no statistically significant difference between control cells and Rab5 antibody–treated cells at high calcium (19.1 ± 4.6%, N = 6 and 21.1 ± 8.3%, N = 5) measured 300 sec after the start of cytosol dialysis (Fig. 3C).

On the other hand, measurements of the rate of increase in C_m appeared statistically different, both at low and high [Ca^{2+}]_i pipette solutions when Rab5 antibody was microinjected into melanotrophs (Fig. 3). The rate of C_m changes at low [Ca^{2+}]_i were in control cells −20.7 ± 1.6 fF sec\(^{-1}\) (N = 5 and in Rab5 antibody–treated cells 1.0 ± 1.0 fF sec\(^{-1}\) (N = 7), significantly different (P < 0.001). Furthermore, at high [Ca^{2+}]_i the rate of C_m changes in control cells were 4.1 ± 0.5 fF sec\(^{-1}\) (N = 6), significantly different from those recorded in Rab5 antibody–treated cells (23.0 ± 3.2 fF sec\(^{-1}\), N = 5; P < 0.001; Fig. 3D).

DISCUSSION

The aim of our work was to study the role of Rab5 protein on surface membrane area regulation in rat melanotrophs. Using immunocytochemistry and confocal microscopy, we showed that Rab5 protein is present in rat melanotrophs, located on the plasmalemma and in the cytoplasm (Fig. 1). This observation correlates with the distribution previously observed in BHK\(^{1,2,28}\) and HeLa cells.\(^{28}\)

To test whether Rab5 protein plays a role in determining area of the plasma membrane of rat melanotrophs, we used the patch-clamp technique to monitor changes in membrane capacitance, a parameter linearly related to changes in membrane surface area. An increase in surface area reflects an increase in exocytosis and reverse, a decrease in surface area reflects an increase in endocytosis.\(^{21}\) As reported,\(^{29}\) we found that high [Ca^{2+}]_i in the pipette solution resulted in a C_m increase, whereas at low [Ca^{2+}]_i a C_m decrease was recorded (Fig. 2). At [Ca^{2+}]_i ~3 µM no increase in C_m was observed, indicating that the rates of both exocytosis and endocytosis were balanced under these experimental conditions (Fig. 2B). If cells were pre-injected with specific Rab5 antibodies, a decline in C_m was no longer recorded, if cells were dialyzed with a solution containing a low concentration of Ca^{2+} (Fig. 3), which is consistent with a block of endocytosis. Furthermore, if cells were dialyzed with a solution containing a high concentration of Ca^{2+}, pre-injection of the Rab5 antibody resulted in an enhanced rate of C_m increase in comparison to controls (Fig. 3), which is likely due to a block of endocytosis. Thus the Ca^{2+}-induced increase in C_m is under these conditions mainly determined by exocytosis. The effects of Rab5 antibody appears to be specific since a non-specific antiserum did not affect the time-course of C_m significantly.

Thomas and colleagues\(^{22}\) proposed a tight coupling between exocytosis and endocytosis in melanotrophs. Releasing caged Ca^{2+} by UV flash photolysis triggers Ca^{2+}-dependent C_m increases with two kinetic components.\(^{23}\) Each one is followed by a decline in C_m determined by endocytosis, enabling the cell to maintain the membrane area constant. Which of the two kinetic components of endocytosis includes Rab5 protein remains to be elucidated in further investigations.
In summary, we have studied the role of Rab5 protein on the dynamics of the plasma membrane area in cultured rat melanotrophs by the patch-clamp technique. We found that Rab5 protein is present in rat melanotrophs and that the pre-injection of Rab5 protein antibodies resulted in the modulation of membrane area changes consistent with a block of endocytosis by this antibody, revealing directly at the cellular level a role of this protein in membrane area dynamics.

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