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WNK4 kinase negatively regulates the surface expression of Muscarinic M3 receptor

Woo Young Chung, Hyun Woo Park, Dae Keon Heo, Kyung Hwan Kim, Min Goo Lee, Joo Young Kim *

Department of Pharmacology and Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul 120-752, Republic of Korea

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ABSTRACT

With-No-Lysine [K] 4 (WNK4) kinase regulates the surface expression of various ion transporters. Not only ion transporters but G-protein coupled receptors (GPCR) can function properly when their expression level is appropriate at the plasma membrane. In this study, we examined the role of WNK4 kinase in the regulation of muscarinic receptor 3 (M_3R) using physiological and biochemical experiments. Measurement of the pilocarpine-responsive $[Ca^{2+}]_i$ change demonstrated that WNK4 kinase decreased the activity of M_3R through its reduced surface expression. Kinase domain of WNK4 bound with the third intracellular region of M_3R whereas its negative regulation was independent on the kinase activity. Comparable to wild-type WNK4, kinase-inactive $WNK4^{D318A}$ mutant also reduced the surface expression of M_3R, whereas the kinase domain of $WNK4_{1-441}$ failed to reduce the surface expression of M_3R. In accordance with surface biotinylation experiments, non-permeable immunostaining of M_3R also showed that M_3R surface expression is independent on the kinase activity of WNK4. Interestingly, comparison of the half life of total and surface M_3R revealed that only the half life of total M_3R, but not surface M_3R was decreased by WNK4 kinase. Nevertheless, the rate of decrease in surface M_3R always exceeded that of total M_3R. Taken together, these results suggest that WNK4 kinase negatively regulates the anterograde trafficking of M_3R through kinase-independent mechanism.

1. Introduction

WNK kinases are unique serine/threonine kinases that lack the lysine [K] in their catalytic region [1]. WNK4 is one of the four members of the WNK family constituted by 1221 amino acids in human. The kinase domain is located at the N-terminus and other parts are composed of the regulatory region which is predicted to mediate protein–protein interaction [2]. WNK4 kinase became well-known as the causative gene of PHAII [2] that exhibits hypertension, hyperkalemia, hypercalciuria and metabolic acidosis due to the abnormal regulation of diverse ion transporters [3]. Heterologus expression studies in Xenopus oocyte and mammalian cells showed that WNK4 reduced the surface expression of ion transporters, such as NCC, ROMK, and TRPV5 [3–5].

Not only ion transporters but the G-protein coupled receptor (GPCR) can function properly once they are expressed appropriately at the plasma membrane. Compared with previous studies focused on the endocytotic internalization pathways of GPCR, the regulatory mechanisms underlying its anterograde trafficking are not well understood [6]. M_3R is a GPCR with seven hydrophobic transmembrane helices which is known to internalize via arrestin independent-, clathrin mediated-mechanisms [7,8]. Thus, the present study aimed to investigate the anterograde trafficking of M_3R.

The mechanisms of WNK4 kinase regulation have been most frequently studied in regard to Na^+ Cl^- co-transporter (NCC) from two points of view [9,10]. The first one is the dependency of its kinase activity. WNK4 kinase has been known to mediate the phosphorylation of NCC through down-stream kinases including SPAK and OSR1 [5,9]. The second one is an endocytosis [11,12] or lysosomal degradation [13] related mechanism. Most early reports of WNK4 kinase effects on NCC showed that WNK4 decreased the surface expression of NCC without altering its total protein amount and glycosylation status [3,10,14,15]. However, it is necessary to define whether WNK4 kinase facilitates the endocytosis of ion transporters from the surface or instead hinders their movement toward the cell surface, because both cases result in decreased surface expression of ion transporters. In the present study, we showed that WNK4 kinase decreased the total abundance of M_3R without affecting the stability of surface M_3R. Despite the fact that the stability of surface M_3R was not altered by WNK4, its surface abundance was always reduced by WNK4 co-expression. These data indicate that the action of WNK4 kinase on M_3R trafficking takes place during its maneuver toward the cell surface. Our findings provide a novel negative regulator in the anterograde trafficking of M_3R.

Abbreviations: WNK4, with-no-lysine[K] kinase 4; M_3R, muscarinic M3 receptor; GPCR, G-protein coupled receptor; PHAII, pseudohypoaldosteronism type II; HEK, human embryonic kidney; SAPK, STE20/SPS1-related proline/alanine-rich kinase; ORS, oxidative stress responsive kinase; NCC, Na^+ Cl^- co-transporter.

* Corresponding author. Tel.: +82 2 2228 1734; fax: +82 2 313 1894.
E-mail address: jooyoungkim@yuhs.ac (J.Y. Kim).

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2. Material and methods

2.1. Materials

HEK 293 cells stably expressing M3R were produced by G418 500 μg/ml treatment for positive selection of neomycin resistant gene in pcDNA3.1(+) 3xHA M3R. 3xHA tagged M3R clone was provided by the UMR (University of Missouri-Rolla) cDNA resource center, and WNK4 clone was kindly provided by Chou-Long Huang at UTSW. The M3R deletion mutants and WNK4 D318A mutant were generated by site-directed mutagenesis. Lifopectamine Plus reagents are used for transient expression. All chemical were purchased from Sigma or Invitrogen unless specified otherwise.

2.2. Immunocytochemistry

Immunostaining of transfected cells was performed as previously [16]. Briefly, cells were fixed with methanol. Nonspecific bindings were blocked by incubation for 1 h at room temperature then, anti-HA (Santacruz), anti-FLAG (Sigma), or anti-Myc (Santacruz) antibody and fluorophore-tagged secondary antibodies in the blocking medium were treated sequentially. To create a non-permeable condition, cells were treated with a blocking solution for 1 h. The primary antibody was treated on ice for 1 h, followed by three rounds of PBS washing. The cells were then fixed with 3.7% formaldehyde in PBS for 30 min, then fluorophore-tagged secondary antibodies were treated to detect only surface existing primary antibodies. Images were obtained with a confocal microscopy (Zeiss LSM 700).

2.3. Immunoprecipitation and immunoblotting

For immunoprecipitation, transfected HEK lysates (~500 μg of protein) were mixed with the appropriate antibodies and incubated overnight at 4 °C in lysis buffer. Immune complexes were precipitated by protein G beads (Pierce) and subsequently washed three times with lysis buffer. The eluted immunoprecipitates or lysates (30 μg of protein) were separated by 4–12% pre-made gradient SDS-PAGE, then the transferred protein into the PVDF membrane was immunoblot with the appropriate antibody.

2.4. Surface biotinylation assay and chasing the surface M3R stability

Cell surface biotinylation of M3R was performed as previously described [11,17]. Briefly, transfected HEK293T cells were washed with ice-cold PBS-CM (PBS containing 0.1 mM CaCl2 and 1 mM MgCl2) and the cell surface proteins were then biotinylated by 1 mg/ml sulfo-NHS-SS-biotin (Pierce) in PBS-CM for 30 min at 4 °C. Then, the free biotin was quenched with 1% bovine serum albumin in PBS and excessive washing with PBS were following. The cells were lysed, and Streptavidin-bead (Pierce) was added to capture the biotinylated proteins. For the stability chasing experiments, 36 h post-transfected cells were biotinylated in sterile conditions and incubated in DMEM media for 0, 14, 24, or 48 h. The

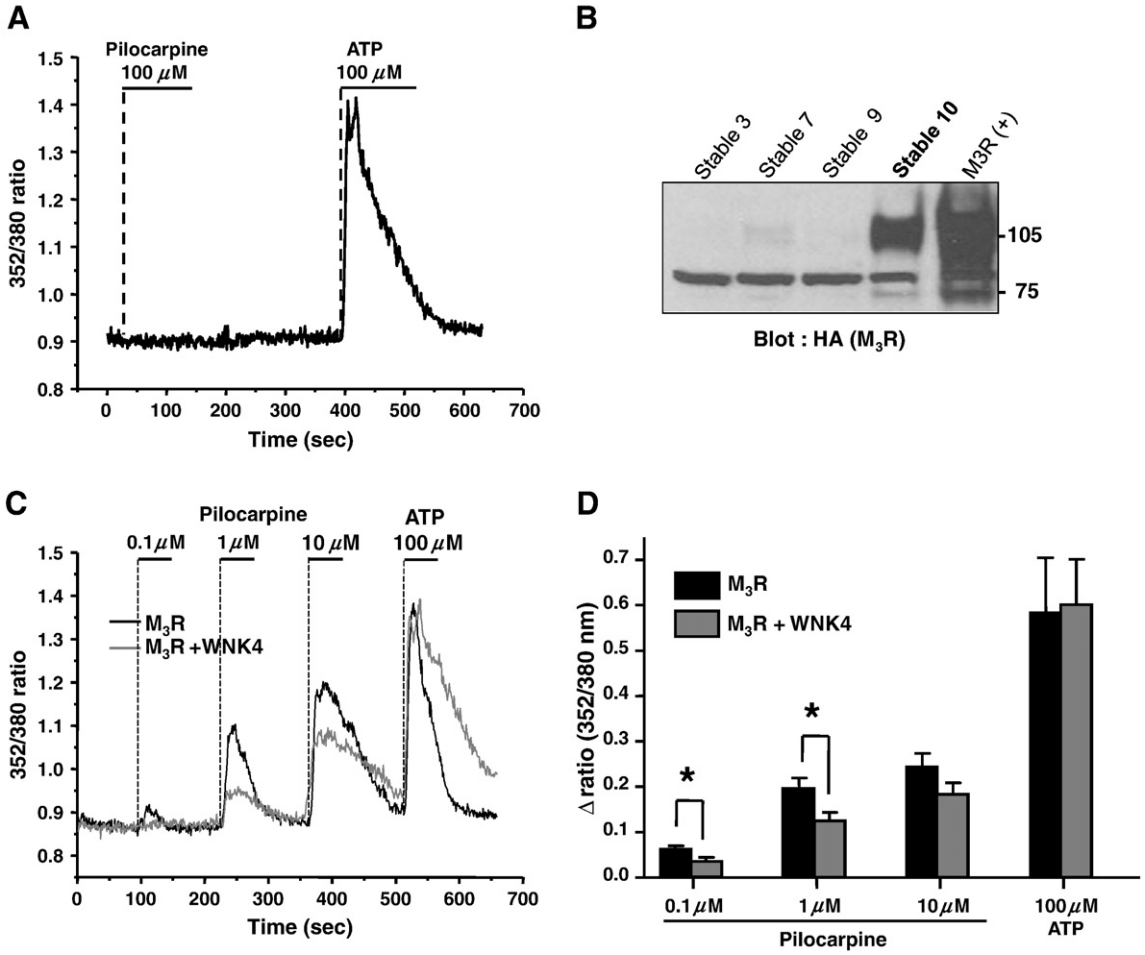

Fig. 1. Decreased activity of M_3R by WNK4 kinase. Pilocarpine induced intracellular $[Ca^{2+}]_i$ evocation via M_3R activity. A. Absence of endogenous M_3R activity in HEK cells. B. Generation of monoclonal M_3R/HEK stable cells. C. Decreased M_3R activity by WNK4 co-expression. D. Summary of the fifteen independent experiments from (B). *$P<0.05$.