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# "Protein kinase C activation promotes $\alpha_{1B}$-adrenoceptor internalization and late endosome trafficking through Rab9 interaction. Role in heterologous desensitization"

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Running Title: Protein kinase C promotes $\alpha_{1B}$-adrenoceptor Rab9 interaction

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[1]Abbreviations used: $\alpha_{1B}$-ARs, $\alpha_{1B}$-adrenergic receptors; PKC, protein kinase C; FRET, Förster resonance energy transfer; NA, noradrenaline, PMA, phorbol 12-myristate 13-acetate; S1P, sphingosine 1-phosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DsRed, *Discosoma* spp. red fluorescent protein, EGFP, enhanced green fluorescent protein.

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**Abstract**

Upon agonist stimulation, $\alpha_{1B}$-adrenergic receptors ($\alpha_{1B}$-ARs) couple to Gq, calcium signaling and protein kinase C activation; subsequently, the receptors are phosphorylated, desensitized and internalized. Internalization seems to involve scaffolding proteins, such as β-arrestin and clathrin. However, the fine mechanisms that participate remain unsolved. The roles of protein kinase C and the small GTPase, Rab9, in $\alpha_{1B}$-AR vesicular traffic were investigated by studying $\alpha_{1B}$-adrenergic receptor-Rab protein interactions, using Förster resonance energy transfer (FRET), confocal microscopy, and intracellular calcium quantitation.

In HEK293 cells overexpressing DsRed-tagged $\alpha_{1B}$-ARs and EGFP-tagged Rab proteins, pharmacological protein kinase C activation mimicked $\alpha_{1B}$-AR traffic elicited by non-related agents, such as sphingosine 1-phosphate, i.e., transient $\alpha_{1B}$-AR-Rab5 FRET signal followed by a sustained $\alpha_{1B}$-AR-Rab9 interaction, suggesting brief receptor localization in early endosomes and transfer to late endosomes. This latter interaction was abrogated by blocking protein kinase C, activity resulting in receptor retention at the plasma membrane. Similar effects were observed when a dominant-negative Rab9 mutant (Rab9-GDP), was employed. When $\alpha_{1B}$-adrenergic receptors mutated at protein kinase C phosphorylation sites (S396A, S402A) were used, phorbol ester-induced desensitization of the calcium response was markedly decreased; however, interaction with Rab9 was only partially decreased and internalization was observed in response to phorbol esters and sphingosine 1-phosphate. Finally, Rab9-GDP expression did not affect adrenergic-mediated calcium response but abolished receptor traffic and altered desensitization. Data suggest

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that protein kinase C modulates $\alpha_{1B}$-adrenergic receptor transfer to late endosomes and that Rab9 regulates this process and participates in G protein-mediated signaling turn-off.

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## 1. Introduction

$\alpha_{1B}$-Adrenergic receptors ($\alpha_{1B}$-ARs)[1] are seven transmembrane-domain proteins that mediate many adrenaline and noradrenaline (NA) actions and that participate in health and disease (García-Sáinz et al., 2000; Hieble et al., 1995). $\alpha_{1B}$-ARs couple to Gq, which activates phospholipase C, catalyzing inositol trisphosphate and diacylglycerol generation, calcium signaling and protein kinase C (PKC) activation. $\alpha_{1B}$-ARs function is tightly regulated and their signaling is markedly attenuated upon sustained agonist activation (homologous desensitization) and in response to unrelated agents (heterologous desensitization) acting either through other G protein-coupled receptors, receptor tyrosine kinases, nuclear receptors or by direct stimulation of protein kinases such as PKC (García-Sáinz et al., 2011; García-Sáinz et al., 2000). Agonist-induced $\alpha_{1B}$-AR phosphorylation and desensitization is mainly due to the action of G protein-coupled receptor kinases, whereas second messenger-activated kinases, particularly PKC, are the major mediators of the heterologous process (Castillo-Badillo et al., 2012; Diviani et al., 1997; Diviani et al., 1996; García-Sáinz et al., 2011; García-Sáinz et al., 2000). Receptor phosphorylation appears to be an early step in desensitization, as it triggers recruitment of β-arrestins, clathrin and other proteins, such as Rab GTPases, which participate in receptor internalization (Lefkowitz, 2013). $\alpha_{1B}$-AR internalization seems to involve an initial sequestration away from the plasma membrane followed by endocytosis (reviewed in (Toews et al., 2003).

Rab proteins are monomeric GTPases, considered as key regulators of vesicular transport (Stenmark, 2009; Zerial and McBride, 2001). There are more than 60 different

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types that localize to the cytoplasmic face of specialized membranous organelles (Stenmark, 2009; Zerial and McBride, 2001). These GTPases facilitate docking, fusion and vesicle transport along the cytoskeleton (Stenmark, 2009; Zerial and McBride, 2001). Specifically, Rab5 and Rab9 are key elements in the crossroads defining whether receptors rapidly recycle back to the plasma membrane or whether they are targeted to late endosomes for slow recycling and/ or degradation. Rab5 is an essential regulator of endocytosis; it localizes to endosome and mediates early membrane fusion processes (Zhu et al., 2004). In contrast, Rab9 is present in late endosomes, it is mainly involved in cargo transport between these vesicles and the *trans*-Golgi network (Choudhury et al., 2005; Kloer et al., 2010; Schwartz et al., 2007).

Rab proteins regulate a number of G protein-coupled receptors (GPCRs), including $\beta_1$-ARs (Filipeanu et al., 2006; Gardner et al., 2011), $\beta_2$-ARs (Dong et al., 2010; Filipeanu et al., 2006; Hammad et al., 2012; Lachance et al., 2011; Moore et al., 2004; Parent et al., 2009), $\alpha_{2A}$-ARs (Li et al., 2012), $\alpha_{2B}$ -ARs (Dong et al., 2010; Li et al., 2012), angiotensin II $AT_1$ receptors (Esseltine et al., 2011; Hunyady et al., 2002; Li et al., 2010; Seachrist et al., 2002; Szakadati et al., 2015), Bradykinin $B_2$ receptors (Charest-Morin et al., 2013), thromboxane A2 receptors (Hamelin et al., 2005), the calcium-sensing receptor (Reyes-Ibarra et al., 2007) and metabotropic glutamate receptors of the 1a subtype (mGluR1a) (Esseltine et al., 2012). In some of these cases, there is evidence indicating direct binding of Rab proteins to specific GPCR domains mainly located at their carboxyl termini (Dong et al., 2010; Parent et al., 2009). It has also been observed that G protein βγ subunits interact with Rab11a and that they colocalize at early and recycling endosomes in response to cell activation of lysophosphatidic acid receptors (García-Regalado et al., 2008). However, little

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is known about the mechanism and functional repercussion of the interaction between Rab9 and GPCRs.

The possible role of Rab proteins in the function, desensitization and internalization or $\alpha_1$-ARs has received little attention. It has been reported that Rab1 plays a key role in the transport of $\alpha_1$-ARs from the endoplasmic reticulum to the plasma membrane (anterograde traffic) (Filipeanu et al., 2006). Regarding receptor internalization (retrograde transport), we recently reported that $\alpha_{1B}$-ARs differentially interact with Rab5 and Rab9 during homologous (agonist-induced) and heterologous desensitizations (induced by activation of $S1P_1$ sphingosine 1-phosphate (S1P) receptors), i. e., noradrenaline triggers a strong and progressive association of these receptors with Rab5, and very little with Rab9, whereas activation of $S1P_1$ receptors essentially induced the opposite effect (Castillo-Badillo et al., 2015).

The aims of the present work were to study the roles of Rab9 and PKC in $\alpha_{1B}$-AR trafficking and desensitization. To investigate receptor-Rab interaction, we employed confocal microscopy and Förster resonance energy transfer (FRET), using red fluorescent protein (DsRed)-tagged $\alpha_{1B}$-ARs and enhanced green fluorescent protein (EGFP)-tagged Rab proteins, as described previously (Castillo-Badillo et al., 2015). Our results provide new insights into the roles of PKC and Rab9 in modulating $\alpha_{1B}$-AR vesicular traffic and signaling.

## 2. Materials and Methods.

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*2.1 Materials*

PMA (phorbol 12-myristate 13-acetate; PubChem CID 27924), dl-propranolol (1-naphthalen-1-yloxy-3-(propan-2-ylamino)propan-2-ol; PubChem CID 4946), NA (noradrenaline, norepinephrine; 4-[(1R)-2-amino-1-hydroxyethyl]benzene-1,2-diol; PubChem CID 439260), S1P (sphingosine 1-phosphate; [(E,2S,3R)-2-amino-3-hydroxyoctadec-4-enyl] dihydrogen phosphate; PubChem CID , bisindolylmaleimide I (2-(1-(3-dimethylaminopropyl)indol-3-yl)-3-(indol-3-yl)maleimide; PubChem CID 6419775), hispidin (6-[(E)-2-(3,4-dihydroxyphenyl)ethenyl]-4-hydroxypyran-2-one, PubChem CID 54685921) and DNA purification kits were purchased from Sigma Chemical Co. Polyethyleneimine (PubChem CID 6453551) was obtained from Polyscience, Gö 6976 (12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole; PubChem CID 3501) from Calbiochem (Merck-Millipore) and Lipofectamine from Invitrogen (Thermo Fisher Scientific). Sulfo-NHS-SS-biotin (sodium;1-[3-[2-[5-[(3aS,4S,6aR)-2-oxo-1,3,3a,4,6,6a-hexahydrothieno[3,4-d]imidazol-4-yl]pentanoylamino]ethyldisulfanyl]propanoyloxy]-2,5-dioxopyrrolidine-3-sulfonate; PubChem CID 71571496) was obtained from Pierce (Thermo Fisher Scientific) and the Vectastain® kit from Vector Laboratories. Primary antibodies against the PKC isoforms and β-actin were from Santa Cruz Biotechnology and chemiluminescence kits were from Pierce (Thermo Fished Scientific) (Hernández-Méndez et al., 2014; Morquecho-León et al., 2014). Fura-2/AM, (bis(acetoxymethyl) 2,2'-((2-(5-((acetoxymethoxy)carbonyl)oxazol-2-yl)-5-(2-(2-(bis(2-(acetoxymethoxy)-2-oxoethyl)amino)-5-methylphenoxy)ethoxy)benzofuran-6-yl)azanediyl)diacetate; PubChem 3364574), Dulbecco's modified Eagle's medium, fetal bovine serum albumin, trypsin, antibiotics, and

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other reagents employed for cell culture were from Life Technologies. Other reagents were from previously described sources (Castillo-Badillo et al., 2012; Castillo-Badillo et al., 2015).

*2.2 Plasmids*

The human $\alpha_{1B}$-AR coding sequence was subcloned into pDsRed-Monomer N-1 (Clontech), as described previously (Castillo-Badillo et al., 2015). Proper insertion was confirmed by restriction analysis and sequencing at the Molecular Biology Unit of our Institute. A plasmid coding for $\alpha_{1B}$-AR DsRed containing S396A and S402A substitutions was synthesized commercially for our group by Mutagenex, Inc. EGFP-tagged Rab5, Rab9 wild-type and Rab9 dominant negative GTPases were generated by Dr. Robert Lodge (Institut de Recherches Cliniques de Montréal, Montreal, Canada) (Hunyady et al., 2002) and generously provided to us. All of these constructs are functional, as described previously (Castillo-Badillo et al., 2015).

*2.3 Cells and Transfection*

HEK293 AD cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium containing glutamine and high-glucose supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 U/ml penicillin, and 0.25 μg/ml amphotericin B, at 37°C, under a 95% air/5% $CO_2$ atmosphere. To study Rab-receptor interaction, plasmids for expression of the EGFP-tagged Rab proteins and DsRed-tagged $\alpha_{1B}$-ARs were co-transfected employing polyethyleneimine (Hsu and Uludag, 2012); experiments were carried out 72 h post-transfection. To assess intracellular calcium

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concentration experiments were performed 48 h post-transfection. $DDT_1$-MF2 cells were obtained from the American Type Culture Collection and were cultured in Dulbecco's modified Eagle's medium as described (Molina-Muñoz et al., 2006). Transfection with plasmids for expression of the EGFP-tagged Rab proteins, was performed employing Lipofectamine 2000. Because transfection efficiency was low, cells were transfected four times (Yamamoto et al., 1999) with 1 week interval and subjected to selection in media containing G418 (600 µg/ml). After this time, cells were maintained in buffer containing 300 µg/ml of G418. Using this protocol, the percentage of EGFP-tagged Rab proteins expressing-cells was ~ 60-70%, as evidenced by fluorescence.

*2.4 Confocal immunofluorescence microscopy*

This procedure was described in detail previously (Castillo-Badillo et al., 2015). Briefly, cells were cultured in glass-bottomed Petri dishes and treated with the agents and for the times indicated and immediately fixed with 4% paraformaldehyde. When NA was employed, 0.1 µM propranolol was added to block endogenous β-adrenergic receptors. Images of 4-6 independent experiments using different cell cultures were obtained employing a Fluoview Confocal microscope model FV10i (Olympus, LD laser, 405 nm [18 mW], 473 nm [12.5 mW], 635 nm [10 mW], 559nm) with a water-immersion objective (60X). EGFP was excited at 480 nm and the emitted fluorescence was detected at 515-540 nm, the red fluorescent protein, DsRed, was excited at 557 nm and the fluorescence emitted was detected at 592 nm.

*2.5 Förster resonance energy transfer (FRET)*

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Interaction between $\alpha_{1B}$-ARs and Rab proteins was analyzed using FRET by means of the sensitized-emission method, employing a confocal microscope equipped with an automated laser spectral scan FV10i Olympus, as described (Castillo-Badillo et al., 2015). Expression of EGFP- and DsRed-tagged proteins was confirmed for each experiment. EGFP was excited at 480 nm and emitted fluorescence was detected at 515-540 nm; DsRed was excited at 557 nm and emitted fluorescence detected at 592 nm. For the FRET channel analysis, EGFP (but not DsRed) was excited and fluorescence was detected at 592 nm. Images of 4-6 independent experiments using different cell cultures and at least eight photographs per sample were captured to estimate the interaction of Rab proteins with $\alpha_{1B}$-ARs by the FRET index after treatment with either agent. The FRET index (that eliminates "bleed-through" and "false" FRET) was quantified using ImageJ software and the "FRET and Colocalization Analyzer" plug-in (Hachet-Haas et al., 2006). This plug-in functions with 8-bit images (Hachet-Haas et al., 2006) and allows supervised computation of the FRET index by means of a "pixel by pixel" method. ImageJ version 1.47b software was obtained from the National Institutes of Health website (ImageJ, Rasband, W.S., U. S. National Institutes of Health, Bethesda, MD, http://imagej.nih.gov/ij/).

*2.6 Intracellular calcium determinations*

Intracellular calcium concentration was assessed as previously described (Hachet-Haas et al., 2006). Cells were serum-starved for 2 h, then loaded with 2.5 μM of the fluorescent $Ca^{2+}$ indicator, Fura-2/AM, in Krebs-Ringer-HEPES containing 0.05% bovine serum albumin, pH 7.4 for 1 h at 37°C, and subsequently washed three times to eliminate unincorporated dye. Fluorescence measurements were carried out at 340- and 380-nm

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excitation wavelengths and at a 510-nm emission wavelength, with a chopper interval set at 0.5 sec, utilizing an Aminco-Bowman Series 2 Luminescence Spectrometer (Rochester, NY). Intracellular calcium levels were calculated according to Grynkiewicz et al. (Grynkiewicz et al., 1985).

*2.7 Cleavable Biotin Protection Assay*

Experiments were performed as described previously (Reyes-Ibarra et al., 2007). In brief, cells were treated for 30 min at 4 ºC with 30 µg/ml of disulfide-cleavable biotin [sulfo-NHS-SS-biotin], washed to remove free biotin, and pre-warmed at 37 ºC in serum-free culture medium. Incubation was continued for 15 min in the absence or presence of the agents tested. After this, cells were washed and subjected to biotin removal from the plasma membrane by incubating the cells in glutathione reducing buffer (50 mM glutathione, 75 mM NaCl, 75 mM NaOH, and 1% fetal bovine serum) for 20 min at 4 ºC. Unreacted glutathione was quenched using iodoacetamide buffer (50 mM iodoacetamide and 1% BSA in PBS); then cells were washed again and lysed. Immunoprecipitation was performed using an antiserum generated in our laboratory against DsRed (Castillo-Badillo et al., 2015). Samples were denatured in Laemmli's sample buffer (Laemmli, 1970) under nonreducing conditions, subjected to SDS-PAGE, and transferred to nitrocellulose. Biotinylated $\alpha_{1B}$-ARs were visualized using Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and chemiluminescence.

*2.8. Western blot assays*

Western blotting was performed as described previously (Hernández-Méndez et al.,

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2014; Morquecho-León et al., 2014). In brief, cells were washed and lysed; lysates were centrifuged and 12,700 x g, for 15 min and proteins in supernatants were subjected to SDS-PAGE. Proteins were electrotransferred onto nitrocellulose membranes and subjected to immunoblotting. Incubation with primary antibodies was for 12 h at 4 ºC and with secondary antibodies for 1 h at room temperature. Chemiluminescence kits were employed and signals were quantified by densitometric analysis using the Scion software from Scion Corporation.

*2.9 Statistical analysis*

When data were normalized, the reference average of each individual experiment (i. e., the 100% value, usually resulting from 3-4 data) was used to calculate all the data. Statistical comparison between two paired groups was performed by the Student *t* test and when three or more conditions were compared ANOVA with Bonferroni's post-test was used. In both cases the software included in the GraphPad Prism program was employed.

## 3. Results

*3.1 PKC activation triggered $\alpha_{1B}$-AR traffic to early and late endosomes*

In order to assess the effect of the pharmacological activation of PKC in $\alpha_{1B}$-AR traffic, we transiently co-transfected HEK293 cells with: a) a plasmid coding for the DsRed-$\alpha_{1B}$-ARs and b) with those coding for either EGFP-Rab5 or EGFP-Rab9. As depicted in Fig. 1, panel A, EGFP-Rab5 and DsRed-$\alpha_{1B}$-ARs were both present in the cells (see EGFP and DsRed channels). Then we measured FRET index intensities upon stimulation with the PKC activator, PMA. First, we evaluated receptor interaction with EGFP-Rab5, an early endosome marker. As shown in Fig. 1, panel B, 1 µM PMA induced

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$\alpha_{1B}$-AR-Rab5 FRET after 2 min and this decreased toward baseline afterward. A similar kinetics was observed with 1 μM S1P, a positive control for $\alpha_{1B}$-AR heterologous desensitization (Castillo-Badillo et al., 2015). The data indicate that PKC pharmacological activation triggers a receptor traffic route similar to that elicited by receptor-mediated (i. e., through $S1P_1$ receptors) heterologous desensitization. In contrast, 10 μM NA, a positive control for $\alpha_{1B}$-AR homologous desensitization, rapidly and markedly induced FRET, which continued increasing afterward (Fig. 1, panel B), as previously reported (Castillo-Badillo et al., 2015). Representative images obtained from cells incubated for 15 min without any agent (Baseline) or in the presence of 1 μM PMA, 1 μM S1P or 10 μM NA for 15 min are presented in Fig. 1, panel A. Similarly, representative FRET index images corresponding to the time-course of $\alpha_{1B}$-AR -Rab5 interaction are depicted in Supplementary Fig. S1. As shown, receptors appear to be directed mainly toward early endosomes during homologous desensitization and only transiently interact with this Rab5 during the heterologous processes.

Similar experiments were performed to study $\alpha_{1B}$-AR- Rab9 interaction and the pattern observed was completely different, as depicted in Fig. 2, panel A. Representative images presented clear expression (fluorescent signal) of both $\alpha_{1B}$-AR and Rab9 (DsRed and EGFP channels, respectively). FRET was detected after a 15 min stimulation with 1 μM PMA or 1 μM S1P treatments but not in the presence of 10 μM NA (FRET and FRET index channels). The time-course of these effects (Fig. 2, panel B) exhibited significant increase in FRET signal after 2 and 5 min stimulation with 1 μM S1P, reaching a maximum at 15 min (positive control (Castillo-Badillo et al., 2015)). PMA mimicked this effect and NA, employed as a negative control, induced no significant effect (Fig. 2, panel B).

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Representative FRET index images corresponding to the time-course of $\alpha_{1B}$-AR -Rab9 interaction are depicted in Supplementary Fig. S2.

*3.2 Late endosome $\alpha_{1B}$-AR traffic required PKC activity and receptor phosphorylation.*

The data described previously suggested that PKC activation induced $\alpha_{1B}$-AR trafficking to late endosomes. To further define the role of PKC in these processes, we employed two approaches: a) the use of a broad-spectrum PKC inhibitor, bisindolylmaleimide I, and b) PKC down regulation by overnight exposure to PMA (Hernández-Méndez et al., 2014; Krug and Tashjian, 1987; Morquecho-León et al., 2014). In the first approach, HEK293 cells, co-transfected with EGFP-Rab9 and DsRed-$\alpha_{1B}$-AR, were stimulated with 1 μM PMA, 1 μM S1P or 10 μM NA for 15 min in the absence or presence of 1 μM bisindolylmaleimide I. We then assessed $\alpha_{1B}$-AR-Rab9 interaction using FRET. As expected, PMA and S1P induced a clear FRET signal in cells incubated in the absence of bisindolylmaleimide I (Fig. 3, panels A and C). However, the $\alpha_{1B}$-AR-Rab9 FRET signal was abolished in cells incubated with the inhibitor (Fig. 3, panels B and C). FRET levels were similar between non-stimulated cells (baseline) and the negative control (NA), as anticipated (Fig. 3, panel A). In the second approach, cells were incubated overnight with 1 μM PMA to down-regulate PKC isoforms (Hernández-Méndez et al., 2014; Krug and Tashjian, 1987; Lum et al., 2013; Melnikov and Sagi-Eisenberg, 2009; Morquecho-León et al., 2014); this treatment markedly reduced the total amount of PKC α, βI (both conventional isoforms), and ε (novel isoform), but not ζ (atypical isoform); no change was observed either in the amount of the loading control, β-actin (Supplementary Fig. S3). The results of both approaches were essentially identical, i. e., the overnight PMA-treatment abolished PMA- and S1P-induced $\alpha_{1B}$-AR-Rab9 FRET (Fig. 3, panels C

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and D), indicating that PKC activity is necessary to direct $\alpha_{1B}$-AR to late endosomes during heterologous desensitization.

The previous data prompted us to explore whether PKC phosphorylation sites present in $\alpha_{1B}$-ARs participate in this process. Elegant work has already identified these sites at the $\alpha_{1B}$-AR carboxyl tail at S396 and S402 (Diviani et al., 1997). To accomplish this, we transiently co-transfected HEK293 cells with EGFP-Rab9 and wild-type DsRed-$\alpha_{1B}$-ARs or the PKC phosphorylation site-defective double mutant (S396A, S402A), and receptor-Rab interactions were determined using FRET. It was observed that, using the $\alpha_{1B}$-AR mutant, PMA and S1P were able to elicit a clear FRET response, but it was consistently smaller than that detected using the wild-type receptor (Fig. 4, panels A-C). These data suggest that phosphorylation in S396 and/ or S402 participate in $\alpha_{1B}$-AR-Rab9 association and traffic.

*3.3 Rab9 -$\alpha_{1B}$-AR interaction was GTPase activity-dependent*

To define whether Rab9 GDP/GTP exchange was necessary for its association with $\alpha_{1B}$-ARs and their subsequent transport to late endosomes, we evaluated changes in FRET signal in the presence of a GDP-locked dominant-negative form of Rab9 (Hunyady et al., 2002). It was observed that overexpression of this mutant allowed no increase in FRET signal in response to PMA or S1P stimulation, as compared with positive controls expressing wild-type Rab9 (Fig. 5, panels A to C). Data indicate that Rab9 associates with $\alpha_{1B}$-ARs in a process dependent on its GTPase activity. As anticipated, no FRET signal was detected upon NA stimulation (Fig. 5, panels A to C), emphasizing that this process mainly affects vesicular traffic elicited during heterologous $\alpha_{1B}$-AR desensitization.

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*3.4 $\alpha_{1B}$-AR internalization was PKC- and Rab9-modulated*

The possible role of the $\alpha_{1B}$-AR-Rab9 interaction on receptor internalization was studied by employing confocal microscopy. In cells cotransfected with DsRed- $\alpha_{1B}$-AR and wild-type EGFP-Rab9, the receptor was mainly localized at the plasma membrane with some fluorescence also observed in intracellular vesicles (Baseline) (Fig. 6, upper row; representative images evidencing expression of EGFP-Rab proteins are presented in Supplementary Fig. S4). This localization drastically changed (i. e., membrane delineation was markedly decreased and fluorescence accumulation into intracellular vesicles was observed) upon stimulation for 15 min with PMA, S1P or NA (Fig. 6, first column). Fluorescence accumulation in intracellular vesicles was a clearer index of internalization and its quantitation is presented in Fig. 7 (panel A). It can be observed that PMA and NA increased intracellular fluorescence ~ 2-fold and S1P slightly less. The time-course of these actions is shown in Supplementary Fig. S5; PMA and NA rapidly increased receptor internalization whereas the action of S1P took place more slowly. Internalization was confirmed using the disulfide-cleavable biotin protection assay. As shown in Supplementary Fig. S6, the amount of biotin-labeled $\alpha_{1B}$-ARs located intracellularly increased in cells incubated with PMA, S1P or NA for 15 min.

Employing fluorescence detection, it was observed that when cells were incubated with bisindolylmaleimide I, receptor fluorescence remained at the plasma membrane after treatments with PMA or S1P. In contrast, internalization was observed in NA-treated cells (Fig. 6, second column; Fig. 7, panel B). Essentially the same results were obtained both in cells in which PKC was down-regulated by overnight PMA treatment (Fig. 6, third column; Fig. 7, panel C) as well as when the GDP-locked dominant-negative form of Rab9 was

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expressed (Fig. 6, fourth column; Fig. 7, panel D), i. e., PMA- and S1P-induced internalizations were markedly attenuated, whereas that triggered by NA was not affected. These data unraveled PKC and Rab9 GTPase activities as relevant for $\alpha_{1B}$-AR internalization during heterologous desensitization. The role of the PKC phosphorylation sites identified was studied employing the DsRed-$\alpha_{1B}$-AR (S396A, S402A) mutant co-transfected with wild-type EGFP-Rab9. Interestingly, this mutant receptor internalized in response to PMA,S1P and NA.

*3.5 Rab9 regulates $\alpha_{1B}$-AR desensitization*

In order to determine the repercussion of $\alpha_{1B}$-AR-Rab9 interaction in calcium signaling, cells were transfected with plasmids coding for DsRed-$\alpha_{1B}$-AR alone or together with plasmids coding for EGFP-Rab proteins. . . NA-triggered intracellular calcium increases were observed in all the conditions studied. As anticipated, in cells expressing $\alpha_{1B}$-ARs and only the endogenous Rab proteins, PMA induced a marked desensitization of the response to NA (~ 70-80%) (Fig. 8, panel A), whereas transfection of the different Rab proteins decreased the magnitude of such desensitization (Fig. 8, panels B-D). In particular, expression of the dominant negative mutant, Rab9-GDP, resulted in marked resistance to the action of PMA (i. e., the action of the active phorbol ester was not statistically significant) (Fig. 8, panel C). In contrast, expression of the Rab5-GDP dominant-negative mutant did not alter the effect of PMA to such an extent (Fig. 8, panel D). NA-induced calcium response was only marginally affected by PMA in cells expressing the $\alpha_{1B}$-AR (S396A, S402A) mutant (Fig. 8, panel D).

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*3.6 Studies using $DDT_1$-MF2 cells*

We explored the possibility that some of the actions observed using transfected HEK293 cells could also be detected in a cell line that endogenously expressed $\alpha_{1B}$-ARs. $DDT_1$-MF2 cells abundantly express these receptors and have been used as a cellular model to study their actions and regulation (García-Sáinz et al., 2004; Leeb-Lundberg et al., 1985). In addition, the ability of active phorbol esters to desensitize $\alpha_{1B}$-ARs was first shown in isolated rat hepatocytes (Corvera and García-Sáinz, 1984; Corvera et al., 1986), but their actual receptor phosphorylation was initially shown using $DDT_1$-MF2 cells (Leeb-Lundberg et al., 1985). We confirmed the ability of PMA to block $\alpha_{1B}$-AR action and studied the ability of a series of PKC inhibitors. PMA-induced $\alpha_{1B}$-AR desensitization was essentially blocked by bisindolylmaleimide I (general PKC inhibitor) and Gö 6976 (PKC α- and β-selective inhibitor (Martiny-Baron et al., 1993)) and was reduced by hispidin (PKC β-selective inhibitor (Gonindard et al., 1997)) (Supplementary Fig. S7, panel A); the inhibitors themselves did not alter NA action (data not shown). As expected, overnight treatment with PMA essentially abolished the ability of acute addition of the phorbol ester to desensitize NA action in $DDT_1$-MF2 cells (Supplementary Fig. S7, panel B). We also tested the effect of expression of the Rab-EGFP constructs on the ability of PMA to desensitize $\alpha_{1B}$-ARs in $DDT_1$-MF2 cells. Transfection of these constructs was not very efficient in these cells; therefore, we had to use multiple transfections and the selection protocol described under Material and Methods, to obtain cells with 60-70% Rab-EGFP expression, as evidenced by fluorescence microscopy. Using these cells, we observed that expression of Rab9 (Fig. 9, panel B) and in particular the Rab9-GDP mutant (Fig. 9, panel C) decreased PMA-induced $\alpha_{1B}$-AR desensitization; expression of Rab5-GDP did not affect

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this PMA action (Fig. 9, panel D); which is remarkably similar to what was observed using HEK293 cells (Fig. 8).

## 4. Discussion

The possible role(s) of Rab GTPases in $\alpha_1$-AR action, vesicular traffic and its regulation has received little attention. To the best of our knowledge, our previous publication (Castillo-Badillo et al., 2015) is the only one dealing with retrograde traffic, thus far. Data in our present work indicate that direct activation of PKC with phorbol esters induces Rab9- $\alpha_{1B}$-AR interaction with great similarity to that observed with S1P (Castillo-Badillo et al., 2015). In other words, similar patterns of $\alpha_{1B}$-AR-Rab interaction take place when PKC is activated either physiologically or by pharmacological means. In contrast, this was not observed when cells were stimulated with the natural agonist, NA, emphasizing differences between homologous and heterologous desensitizations. Rab9- $\alpha_{1B}$-AR interaction and internalization are blocked by bisindolylmaleimide, a PKC inhibitor and also by the down regulation of this enzyme (induced by overnight incubation with PMA). In both cases, Rab9- $\alpha_{1B}$-AR interaction required Rab9 GTPase activity. PMA and S1P induced $\alpha_{1B}$-AR internalization that was markedly diminished by the same treatments, i. e., PKC inhibition, PKC downregulation, and the expression of a GDP-locked dominant-negative Rab9 mutant. These data are consistent with the current idea that PKC is a major mediator of $\alpha_{1B}$-AR heterologous desensitization, including receptor phosphorylation and internalization; this can be induced by S1P (Castillo-Badillo et al., 2012; Castillo-Badillo et

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al., 2015) and by a large variety of hormones and neurotransmitters acting through different families of receptors (see (Castillo-Badillo et al., 2012; Castillo-Badillo et al., 2015; García-Sáinz et al., 2011; García-Sáinz et al., 2000) and references therein). A working model is presented in Fig. 10. The PKC-down regulation studies and the pharmacological profile of the PKC inhibitors used suggest that conventional PKC isoforms are participants in the actions described, which is consistent with what we have observed for $LPA_1$ and $S1P_1$ receptors (Hernández-Méndez et al., 2014; Morquecho-León et al., 2014).

Major issues include how the cellular machinery "senses" when a receptor is internalized in response to agonist activation or to other processes and how this event is linked to the different internalization pathways. Although this is currently unknown, accumulating evidence suggests that the actual sites phosphorylated in a given receptor can vary (Butcher et al., 2014; Tobin et al., 2008; Torrecilla et al., 2007). Such variation has been denominated the "phosphorylation bar code" (Butcher et al., 2014; Tobin et al., 2008; Torrecilla et al., 2007) and it has been proposed that such variations can favor some cellular processes over others, including apoptosis, proliferation, differentiation or metabolic changes, to mention a few. As indicated, different phosphorylation sites have been detected for PKC and G protein-coupled receptor kinases in $\alpha_{1B}$-ARs (Diviani et al., 1997); these authors performed their work with the hamster ortholog (sites S394 and S400) which are conserved in human $\alpha_{1B}$-ARs (corresponding to S396 and S402). When the phosphorylation sites-defective $\alpha_{1B}$-AR mutant (S396A, S402A) was used, the interaction with Rab9 was clearly diminished, but not abolished. These data suggest that such sites participate, but might not be the only ones, in the receptor-Rab interaction. Internalization of the $\alpha_{1B}$-AR mutant (S396A, S402A) during heterologous desensitization (i. e., under the

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action of of PMA or S1P) was observed. Interestingly, the ability of PMA to block $\alpha_{1B}$-AR-mediated increase in intracellular calcium was markedly attenuated. This raises the possibility that other sites or elements could participate in receptor-Rab protein interaction and internalization. It should be kept in mind that differences might exist among receptor orthologs and in cellular models in which the experiments are performed (Butcher et al., 2011; Tobin et al., 2008). Current evidence indicates that Rab5 is involved in the initial endocytosis of vesicles formed from the plasma membrane and their subsequent interaction with early endosomes (Bhattacharya et al., 2004; Nielsen et al., 1999; Novick and Zerial, 1997; Zerial and McBride, 2001); this suggests that agonist-activation targets $\alpha_{1B}$-ARs to early endosomes and fast recycling back to the plasma membrane. In contrast, Rab9 is a protein characteristic of late endosomes (Bhattacharya et al., 2004; Nielsen et al., 1999; Novick and Zerial, 1997; Zerial and McBride, 2001). This suggests that PKC activation leads initially to a brief interaction of $\alpha_{1B}$-ARs with early endosomes subsequently migrating to late endosomes. Such differential traffic might have functional consequences.

As already mentioned, Rab proteins appear to regulate the signaling and traffic of a variety of GPCRs and in some cases a direct Rab association with these receptors has been documented, particularly to the GPCR carboxyl termini (Dong et al., 2010; Parent et al., 2009). Esseltine et al. (Esseltine et al., 2011) previously reported that several Rab proteins bind to a common site in the Angiotensin II $AT_1$ receptor carboxyl tail and that Rab4, in particular, regulates receptor phosphorylation, desensitization and resensitization. Regarding the present work, the evidence provided by the FRET analysis indicates that the $\alpha_{1B}$-AR and Rab9 constructs are in close proximity (i. e., 1-10 nm, the limit for FRET to take place). FRET is a collision-free, but distance-dependent photophysical process and one

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of the few non-invasive approaches currently used to analyze interaction among molecular species and its use in the biological sciences is expanding exponentially (Shrestha et al., 2015). The FRET-based approach employed in this work was developed for single cell analysis using confocal fluorescent microscopy and it was also validated using cell suspensions using fluorescence emission scanning (Castillo-Badillo et al., 2015). Other approaches have been successfully used to study GPCR-Rab interactions including co-immunoprecipitation and colocalization. Using those procedures we observed that, under baseline conditions, there was a very strong signal, which did not allow us to detect any changes with the different treatments (Castillo-Badillo et al., 2015). This was confirmed in the present study also and even using cells that endogenously express $\alpha_{1B}$-ARs (data not shown). Our interpretation is that signaling complexes (signalosomes) are already preformed (detected by co-immunoprecipitation) and that FRET allowed us to detect organization changes within those complexes. More structural work will be necessary to define whether there is a physical interaction between $\alpha_{1B}$-ARs and Rabs, and the domain(s) and specific residues involved.

Although it is clear that Rab9 modulates $\alpha_{1B}$-AR traffic to late endosomes, as evidenced by the receptor internalization data, we possess no clear explanation of the fact that Rab9 expression diminished the ability of PMA to desensitize NA-induced $\alpha_{1B}$-AR-mediated calcium signaling, and why such a diminution was even more pronounced in cells expressing the Rab9 dominant-negative mutant. One possibility is that Rab activity could be involved in PKC-mediated $\alpha_{1B}$-AR desensitization. However, this seems unlikely because PMA action is usually rather fast, occurring in the range of a few seconds to 2-3 min, and preceding any significant receptor internalization. A more plausible possibility is

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that the interaction of Rab9 with the receptor might represent a physical obstacle for PKC access to the receptor. Were this the case, the difference observed with the dominant-negative mutant could be due to higher level of expression, changes in cell localization, higher affinity for the receptor, or a combination of these or to other factors. This aspect remains a puzzling but interesting observation meriting further exploration.

The interaction between GPCRs and Rabs appears to be rather complex, due to the following: a) various Rab proteins may compete for the binding sites present in GPCRs (Esseltine et al., 2011), b) GPCRs are not only "cargo" proteins but also may function as GEFs (Guanine nucleotide Exchange Factors, that facilitate the exchange of GDP for GTP) modifying the activity of Rab proteins (Seachrist et al., 2002) and c) ligands (agonist / antagonists) might modulate these actions by altering receptor conformation, thus changing GPCR-Rab interactions qualitatively and/or quantitatively (Seachrist et al., 2002). Additionally, receptor activation triggers signaling cascades leading to Rab phosphorylation by protein kinases (Chiariello et al., 1999; Fitzgerald and Reed, 1999; Karniguian et al., 1993; Pavarotti et al., 2012) or prenylation by geranylgeranyltransferases (Lachance et al., 2011); such covalent modifications might alter Rab localization and function. There is already evidence for the PKC-catalyzed phosphorylation of some Rab proteins (Fitzgerald and Reed, 1999; Pavarotti et al., 2012). Preliminary evidence indicates that Rab9 is phosphorylated under baseline conditions and that such phosphorylation was not increased in cells treated with PMA (unpublished observation). Further work will be required to properly define whether Rab9 phosphorylation plays a role and the protein kinases involved.

In summary, our data indicate that PKC pharmacological activation triggered a

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weak and transient $\alpha_{1B}$-AR-Rab5 interaction and a progressive and sustained receptor association with Rab9 (suggesting GPCR targeting to late endosomes). The following elements were necessary to elicit such $\alpha_{1B}$-AR-Rab9 interaction: 1) PKC activity, 2) $\alpha_{1B}$-AR PKC-mediated phosphorylation at S396 and/ or S402 (evidenced by the use of an $\alpha_{1B}$-AR mutant), and 3) Rab9 GTPase activity. The lack of any of these elements impaired receptor internalization. Finally, we found that the expression of wild-type Rab9 or the dominant-negative mutant of this GTPase altered PMA-induced $\alpha_{1B}$-AR desensitization.

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**Authorship contributions**

*Participated in research design:* Alfonzo-Méndez, Reyes-Cruz and García-Sáinz.

*Conducted experiments:* Alfonzo-Méndez, Hernández-Espinosa, Carmona-Rosas and Romero-Ávila.

*Contributed new reagents or analytic tools:* Reyes-Cruz.

*Performed data analysis:* Alfonzo-Méndez, Hernández-Espinosa, Carmona-Rosas and Romero-Ávila.

*Wrote or contributed to the writing of the manuscript:* Alfonzo-Méndez, Reyes-Cruz and García-Sáinz.

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References

Bhattacharya M, Babwah AV and Ferguson SS (2004) Small GTP-binding protein-coupled receptors. *Biochem Soc Trans* **32**(Pt 6): 1040-1044.

Butcher AJ, Hudson BD, Shimpukade B, Alvarez-Curto E, Prihandoko R, Ulven T, Milligan G and Tobin AB (2014) Concomitant action of structural elements and receptor phosphorylation determine arrestin-3 interaction with the free fatty acid receptor FFA4. *J Biol Chem* **289**(26): 18451-18465.

Butcher AJ, Prihandoko R, Kong KC, McWilliams P, Edwards JM, Bottrill A, Mistry S and Tobin AB (2011) Differential G-protein-coupled Receptor Phosphorylation Provides Evidence for a Signaling Bar Code. *J Biol Chem* **286**(13): 11506-11518.

Castillo-Badillo JA, Molina-Muñoz T, Romero-Ávila MT, Vázquez-Macías A, Rivera R, Chun J and García-Sáinz JA (2012) Sphingosine 1-phosphate-mediated alpha1B-adrenoceptor desensitization and phosphorylation. Direct and paracrine/autocrine actions. *Biochim Biophys Acta* **1823**(2): 245-254.

Castillo-Badillo JA, Sánchez-Reyes OB, Alfonzo-Méndez MA, Romero-Ávila MT, Reyes-Cruz G and García-Sáinz JA (2015) alpha1B-Adrenergic Receptors Differentially Associate with Rab Proteins during Homologous and Heterologous Desensitization. *PLoS One* **10**(3): e0121165.

Charest-Morin X, Fortin S, Lodge R, Roy C, Gera L, Gaudreault RC and Marceau F (2013) Inhibitory effects of cytoskeleton disrupting drugs and GDP-locked Rab mutants on bradykinin B(2) receptor cycling. *Pharmacol Res* **71**: 44-52.

Chiariello M, Bruni CB and Bucci C (1999) The small GTPases Rab5a, Rab5b and Rab5c are differentially phosphorylated in vitro. *FEBS Lett* **453**(1-2): 20-24.

Choudhury A, Marks DL and Pagano RE (2005) Use of Rab GTPases to study lipid trafficking in normal and sphingolipid storage disease fibroblasts. *Methods Enzymol* **403**: 166-182.

Corvera S and García-Sáinz JA (1984) Phorbol esters inhibit alpha 1 adrenergic stimulation of glycogenolysis in isolated rat hepatocytes. *Biochem Biophys Res Commun* **119**(3): 1128-1133.

Corvera S, Schwarz KR, Graham RM and Garcia-Sainz JA (1986) Phorbol esters inhibit alpha 1-adrenergic effects and decrease the affinity of liver cell alpha 1-adrenergic receptors for (-)-epinephrine. *J Biol Chem* **261**(2): 520-526.

Diviani D, Lattion AL and Cotecchia S (1997) Characterization of the phosphorylation sites involved in G protein-coupled receptor kinase- and protein kinase C-mediated desensitization of the alpha1B-adrenergic receptor. *J Biol Chem* **272**(45): 28712-28719.

Diviani D, Lattion AL, Larbi N, Kunapuli P, Pronin A, Benovic JL and Cotecchia S (1996) Effect of different G protein-coupled receptor kinases on phosphorylation and desensitization of the alpha1B-adrenergic receptor. *J Biol Chem* **271**(9): 5049-5058.

Dong C, Yang L, Zhang X, Gu H, Lam ML, Claycomb WC, Xia H and Wu G (2010) Rab8 interacts with distinct motifs in alpha2B- and beta2-adrenergic receptors and differentially modulates their transport. *J Biol Chem* **285**(26): 20369-20380.

Esseltine JL, Dale LB and Ferguson SS (2011) Rab GTPases bind at a common site within the angiotensin II type I receptor carboxyl-terminal tail: evidence that Rab4

regulates receptor phosphorylation, desensitization, and resensitization. *Mol Pharmacol* **79**(1): 175-184.

Esseltine JL, Ribeiro FM and Ferguson SS (2012) Rab8 modulates metabotropic glutamate receptor subtype 1 intracellular trafficking and signaling in a protein kinase C-dependent manner. *J Neurosci* **32**(47): 16933-16942a.

Filipeanu CM, Zhou F, Fugetta EK and Wu G (2006) Differential regulation of the cell-surface targeting and function of beta- and alpha1-adrenergic receptors by Rab1 GTPase in cardiac myocytes. *Mol Pharmacol* **69**(5): 1571-1578.

Fitzgerald ML and Reed GL (1999) Rab6 is phosphorylated in thrombin-activated platelets by a protein kinase C-dependent mechanism: effects on GTP/GDP binding and cellular distribution. *Biochem J* **342 ( Pt 2)**: 353-360.

García-Regalado A, Guzmán-Hernández ML, Ramírez-Rangel I, Robles-Molina E, Balla T, Vázquez-Prado J and Reyes-Cruz G (2008) G protein-coupled receptor-promoted trafficking of Gbeta1gamma2 leads to AKT activation at endosomes via a mechanism mediated by Gbeta1gamma2-Rab11a interaction. *Mol Biol Cell* **19**(10): 4188-4200.

García-Sáinz JA, Romero-Ávila MT and Alcántara-Hernández R (2011) Mechanisms involved in alpha(1B) -adrenoceptor desensitization. *IUBMB Life* **63**: 811-815.

García-Sáinz JA, Romero-Ávila MT, Molina-Muñoz T and Medina LC (2004) Insulin induces alpha1B-adrenergic receptor phosphorylation and desensitization. *Life Sci* **75**(16): 1937-1947.

García-Sáinz JA, Vázquez-Prado J and Medina LC (2000) Alpha 1-adrenoceptors: function and phosphorylation. *Eur J Pharmacol* **389**(1): 1-12.

Gardner LA, Hajjhussein H, Frederick-Dyer KC and Bahouth SW (2011) Rab11a and its binding partners regulate the recycling of the ss1-adrenergic receptor. *Cell Signal* **23**(1): 46-57.

Gonindard C, Bergonzi C, Denier C, Sergheraert C, Klaebe A, Chavant L and Hollande E (1997) Synthetic hispidin, a PKC inhibitor, is more cytotoxic toward cancer cells than normal cells in vitro. *Cell Biol Toxicol* **13**(3): 141-153.

Grynkiewicz G, Poenie M and Tsien RY (1985) A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J Biol Chem* **260**(6): 3440-3450.

Hachet-Haas M, Converset N, Marchal O, Matthes H, Gioria S, Galzi JL and Lecat S (2006) FRET and colocalization analyzer--a method to validate measurements of sensitized emission FRET acquired by confocal microscopy and available as an ImageJ Plug-in. *Microsc Res Tech* **69**(12): 941-956.

Hamelin E, Theriault C, Laroche G and Parent JL (2005) The intracellular trafficking of the G protein-coupled receptor TPbeta depends on a direct interaction with Rab11. *J Biol Chem* **280**(43): 36195-36205.

Hammad MM, Kuang YQ, Morse A and Dupre DJ (2012) Rab1 interacts directly with the beta2-adrenergic receptor to regulate receptor anterograde trafficking. *Biol Chem* **393**(6): 541-546.

Hernández-Méndez A, Alcántara-Hernández R, Acosta-Cervantes GC, Martínez-Ortiz J, Avendaño-Vázquez SE and García-Sáinz JA (2014) Conventional protein kinase C isoforms mediate phorbol ester-induced lysophosphatidic acid LPA1 receptor phosphorylation. *Eur J Pharmacol* **723**: 124-130.

Hieble JP, Bylund DB, Clarke DE, Eikenburg DC, Langer SZ, Lefkowitz RJ, Minneman KP and Ruffolo RR, Jr. (1995) International Union of Pharmacology. X. Recommendation for nomenclature of alpha 1-adrenoceptors: consensus update. *Pharmacol Rev* **47**(2): 267-270.

Hsu CY and Uludag H (2012) A simple and rapid nonviral approach to efficiently transfect primary tissue-derived cells using polyethylenimine. *Nat Protoc* **7**(5): 935-945.

Hunyady L, Baukal AJ, Gaborik Z, Olivares-Reyes JA, Bor M, Szaszak M, Lodge R, Catt KJ and Balla T (2002) Differential PI 3-kinase dependence of early and late phases of recycling of the internalized AT1 angiotensin receptor. *J Cell Biol* **157**(7): 1211-1222.

Karniguian A, Zahraoui A and Tavitian A (1993) Identification of small GTP-binding rab proteins in human platelets: thrombin-induced phosphorylation of rab3B, rab6, and rab8 proteins. *Proc Natl Acad Sci U S A* **90**(16): 7647-7651.

Kloer DP, Rojas R, Ivan V, Moriyama K, van Vlijmen T, Murthy N, Ghirlando R, van der Sluijs P, Hurley JH and Bonifacino JS (2010) Assembly of the biogenesis of lysosome-related organelles complex-3 (BLOC-3) and its interaction with Rab9. *J Biol Chem* **285**(10): 7794-7804.

Krug E and Tashjian AH, Jr. (1987) Time-dependent changes in protein kinase C distribution and disappearance in phorbol ester-treated human osteosarcoma cells. *Cancer Res* **47**(9): 2243-2246.

Lachance V, Cartier A, Genier S, Munger S, Germain P, Labrecque P and Parent JL (2011) Regulation of beta2-adrenergic receptor maturation and anterograde trafficking by an interaction with Rab geranylgeranyltransferase: modulation of Rab geranylgeranylation by the receptor. *J Biol Chem* **286**(47): 40802-40813.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**(5259): 680-685.

Leeb-Lundberg LM, Cotecchia S, Lomasney JW, DeBernardis JF, Lefkowitz RJ and Caron MG (1985) Phorbol esters promote alpha 1-adrenergic receptor phosphorylation and receptor uncoupling from inositol phospholipid metabolism. *Proc Natl Acad Sci U S A* **82**(17): 5651-5655.

Lefkowitz RJ (2013) A brief history of G-protein coupled receptors (Nobel Lecture). *Angew Chem Int Ed Engl* **52**(25): 6366-6378.

Li C, Fan Y, Lan TH, Lambert NA and Wu G (2012) Rab26 modulates the cell surface transport of alpha2-adrenergic receptors from the Golgi. *J Biol Chem* **287**(51): 42784-42794.

Li H, Yu P, Sun Y, Felder RA, Periasamy A and Jose PA (2010) Actin cytoskeleton-dependent Rab GTPase-regulated angiotensin type I receptor lysosomal degradation studied by fluorescence lifetime imaging microscopy. *J Biomed Opt* **15**(5): 056003.

Lum MA, Pundt KE, Paluch BE, Black AR and Black JD (2013) Agonist-induced down-regulation of endogenous protein kinase c alpha through an endolysosomal mechanism. *J Biol Chem* **288**(18): 13093-13109.

Martiny-Baron G, Kazanietz MG, Mischak H, Blumberg PM, Kochs G, Hug H, Marme D and Schachtele C (1993) Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. *J Biol Chem* **268**(13): 9194-9197.

Melnikov S and Sagi-Eisenberg R (2009) Down-regulating protein kinase C alpha: functional cooperation between the proteasome and the endocytic system. *Cell Signal* **21**(11): 1607-1619.

Molina-Muñoz T, Romero-Ávila MT and García-Sáinz JA (2006) Insulin-like Growth Factor-I induces {alpha}1B-Adrenergic Receptor Phosphorylation Through G{beta}{gamma} and Epidermal Growth Factor Receptor Transactivation. *Mol Endocrinol* **20**(11): 2773-2783.

Moore RH, Millman EE, Alpizar-Foster E, Dai W and Knoll BJ (2004) Rab11 regulates the recycling and lysosome targeting of beta2-adrenergic receptors. *J Cell Sci* **117**(Pt 15): 3107-3117.

Morquecho-León MA, Bazúa-Valenti S, Romero-Ávila MT and García-Sáinz JA (2014) Isoforms of protein kinase C involved in phorbol ester-induced sphingosine 1-phosphate receptor 1 phosphorylation and desensitization. *Biochim Biophys Acta* **1843**(2): 327-334.

Nielsen E, Severin F, Backer JM, Hyman AA and Zerial M (1999) Rab5 regulates motility of early endosomes on microtubules. *Nat Cell Biol* **1**(6): 376-382.

Novick P and Zerial M (1997) The diversity of Rab proteins in vesicle transport. *Curr Opin Cell Biol* **9**(4): 496-504.

Parent A, Hamelin E, Germain P and Parent JL (2009) Rab11 regulates the recycling of the beta2-adrenergic receptor through a direct interaction. *Biochem J* **418**(1): 163-172.

Pavarotti M, Capmany A, Vitale N, Colombo MI and Damiani MT (2012) Rab11 is phosphorylated by classical and novel protein kinase C isoenzymes upon sustained phorbol ester activation. *Biol Cell* **104**(2): 102-115.

Reyes-Ibarra AP, García-Regalado A, Ramírez-Rangel I, Esparza-Silva AL, Valadez-Sánchez M, Vázquez-Prado J and Reyes-Cruz G (2007) Calcium-sensing receptor endocytosis links extracellular calcium signaling to parathyroid hormone-related peptide secretion via a Rab11a-dependent and AMSH-sensitive mechanism. *Mol Endocrinol* **21**(6): 1394-1407.

Schwartz SL, Cao C, Pylypenko O, Rak A and Wandinger-Ness A (2007) Rab GTPases at a glance. *J Cell Sci* **120**(Pt 22): 3905-3910.

Seachrist JL, Laporte SA, Dale LB, Babwah AV, Caron MG, Anborgh PH and Ferguson SS (2002) Rab5 association with the angiotensin II type 1A receptor promotes Rab5 GTP binding and vesicular fusion. *J Biol Chem* **277**(1): 679-685.

Shrestha D, Jenei A, Nagy P, Vereb G and Szollosi J (2015) Understanding FRET as a research tool for cellular studies. *Int J Mol Sci* **16**(4): 6718-6756.

Stenmark H (2009) Rab GTPases as coordinators of vesicle traffic. *Nat Rev Mol Cell Biol* **10**(8): 513-525.

Szakadati G, Toth AD, Olah I, Erdelyi LS, Balla T, Varnai P, Hunyady L and Balla A (2015) Investigation of the fate of type I angiotensin receptor after biased activation. *Mol Pharmacol* **87**(6): 972-981.

Tobin AB, Butcher AJ and Kong KC (2008) Location, location, location...site-specific GPCR phosphorylation offers a mechanism for cell-type-specific signalling. *Trends Pharmacol Sci* **29**(8): 413-420.

Toews ML, Prinster SC and Schulte NA (2003) Regulation of alpha-1B adrenergic receptor localization, trafficking, function, and stability. *Life Sci* **74**(2-3): 379-389.

Torrecilla I, Spragg EJ, Poulin B, McWilliams PJ, Mistry SC, Blaukat A and Tobin AB (2007) Phosphorylation and regulation of a G protein-coupled receptor by protein kinase CK2. *J Cell Biol* **177**(1): 127-137.

Yamamoto M, Okumura S, Schwencke C, Sadoshima J and Ishikawa Y (1999) High efficiency gene transfer by multiple transfection protocol. *Histochem J* **31**(4): 241-243.

Zerial M and McBride H (2001) Rab proteins as membrane organizers. *Nat Rev Mol Cell Biol* **2**(2): 107-117.

Zhu G, Zhai P, Liu J, Terzyan S, Li G and Zhang XC (2004) Structural basis of Rab5-Rabaptin5 interaction in endocytosis. *Nat Struct Mol Biol* **11**(10): 975-983.

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FOOTNOTE

**Financial support.**

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Figure Legends

**Fig 1.** $\alpha_{1B}$-AR-Rab5 interaction. Panel A, images of cells coexpressing DsRed-$\alpha_{1B}$-ARs and EGFP-Rab5. Cells were incubated for 15 min in the absence of any agent (Baseline, upper row) or presence of 1 μM PMA (PMA, second row), 1 μM sphingosine 1-phosphate (S1P, third row) or 10 μM noradrenaline (plus 0.1 μM propranolol) (NA, fourth row). Cells were fixed and observed in a fluorescence confocal microscope. The following images are presented: EGFP fluorescence (EGFP was excited and its fluorescence recorded; first column), DsRed fluorescence (DsRed was excited and its fluorescence recorded; second column), "FRET channel" (EGFP was excited, the laser to excite DsRed remained off, and DsRed fluorescence was recorded; third column) and "FRET index" (images processed with the "FRET and Colocalization Analyzer", fourth column). Scale bars: 15 μm. Panel B, quantitative analysis of the $\alpha_{1B}$-adrenergic receptor-Rab5 interaction time-course. Plotted are the means and vertical lines representing the S.E.M of 6 images for each condition; 3 experiments were performed utilizing different cell preparations. PMA (1 μM red line and symbols); S1P 1 (1 μM, brown line and symbols), and NA (10 μM plus 0.1 μM propranolol, blue line and symbols). * $P < 0.001$ vs. their respective baseline (Time 0'); ** $P < 0.001$ vs. their respective baseline (Time 0') and also $P < 0.001$ vs. treatments with S1P or PMA for the same time.

**Fig 2.** $\alpha_{1B}$-AR-Rab9 interaction. Panel A, Images of cells coexpressing DsRed-$\alpha_{1B}$-ARs and EGFP-Rab9. Other indications as in Fig. 1. Panel B, quantitative analysis of the $\alpha_{1B}$-adrenergic receptor-Rab9 interaction time-course. Plotted are the means and vertical lines

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representing the S.E.M of 5 images for each condition for each of the 5 experiments performed using different cell preparations. * $P < 0.001$ vs. their respective baseline (Time 0') and also $P < 0.001$ vs. treatments with S1P or PMA for the same time. Other indications as in Fig. 1.

**Fig. 3.** Role of PKC on $\alpha_{1B}$-AR-Rab9 interaction. Panel A, quantitative analysis of the $\alpha_{1B}$-adrenergic receptor-Rab9 interaction. Cells were preincubated in the absence of any agent (None, open bars), in the presence of 1 μM bisindolylmalimide I (BIM, dashed bars) for 15 min, or the night before the experiment with 1 μM PMA (PMA-ON [overnight], black bars); after this pretreatment cells were challenged for 15 min with vehicle (Baseline), 1 μM PMA, 1 μM S1P or 10 μM NA (plus propranolol) and the samples were fixed for image acquisition. Plotted are the means and vertical lines representing the S.E.M of 5 images for each condition for each of the 4 experiments performed using different cell preparations. * $P < 0.001$ vs. their respective baseline. Panels B and D, representative images of cells pretreated with bisindolylmaleimide for 15 min (BIM, panel B ) or with PMA overnight (PMA-ON, panel D). Other indications as in Fig. 1. Panel C, representative FRET index images of cells preincubated in the absence of any agent (None), in the presence of 1 μM bisindolylmaleimide (BIM) for 15 min or overnight with 1 μM PMA (PMA-ON) black bars); after this pretreatment cells were challenged for 15 min with vehicle (Baseline), 1 μM PMA, 1 μM S1P or 10 μM NA (plus propranolol) and the samples were fixed for image adquisition. Other indications as in Fig. 1.

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**Fig. 4.** Role of $\alpha_{1B}$-AR PKC phosphorylation sites on $\alpha_{1B}$-AR-Rab9 interaction. Panel A, quantitative analysis of the $\alpha_{1B}$-adrenergic receptor-Rab9 interaction. Cells expressing wild-type DsRed-$\alpha_{1B}$-ARs (open bars, WT) or the PKC phosphorylation site-defective DsRed-$\alpha_{1B}$-AR mutants (S396A, S402A) (dashed bar, Mut) were challenged for 15 min with vehicle (Baseline), 1 µM PMA, 1 µM S1P or 10 µM NA (plus propranolol). Plotted are the means and vertical lines representing the S.E.M of 6 images for each condition for each of the 4 experiments performed using different cell preparations. * $P < 0.001$ vs. their respective baseline; ** $P < 0.05$ vs. its respective baseline and also $P < 0.05$ vs. PMA wild-type; *** $P < 0.001$ vs. its respective baseline and also $P < 0.05$ vs. S1P wild-type. Panels B, representative images of cells expressing the PKC phosphorylation sites-defective DsRed-$\alpha_{1B}$-AR mutant (S396A, S402A) challenged for 15 min with vehicle (Baseline), 1 µM PMA, 1 µM S1P or 10 µM NA (plus propranolol). Other indications as in Fig. 1. Panel C, representative FRET index images of cells expressing DsRed-$\alpha_{1B}$-ARs wild-type (WT) or the PKC phosphorylation sites-defective mutant (S396A, S402A) (Mut) challenged for 15 min with vehicle (Baseline), 1 µM PMA, 1 µM S1P or 10 µM NA (plus propranolol). Other indications as in Fig. 1.

**Fig. 5.** Role of Rab9 GTPase activity on $\alpha_{1B}$-AR-Rab9 interaction. Panel A, quantitative analysis of the $\alpha_{1B}$-AR-Rab9 interaction. Cells expressing wild-type DsRed-$\alpha_{1B}$-ARs and either EGFP-Rab9 wild-type (open bars, Rab9-WT) or the GDP-locked dominant-negative mutant (dashed bar, Rab9-GDP) were challenged for 15 min with vehicle (Baseline), 1 µM PMA, 1 µM S1P or 10 µM NA (plus propranolol). Plotted are the means and vertical lines

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representing the S.E.M of 6 images for each condition for each of the 4 experiments performed using different cell preparations. * $P < 0.001$ vs. their respective baseline. Panels B, representative images of cells expressing the GDP-locked Rab9 mutant (Rab9-GDP) were challenged for 15 min with vehicle (Baseline), 1 μM PMA, 1 μM S1P or 10 μM NA (plus propranolol). Other indications as in Fig. 1. Panel C, representative FRET index images of cells expressing Rab9-WT and the GDP-locked mutant (Rab9-GDP) challenged for 15 min with vehicle (Baseline), 1 μM PMA, 1 μM S1P or 10 μM NA (plus propranolol).

**Fig. 6.** Internalization of $\alpha_{1B}$-ARs. Representative images (Red Channel) of cells expressing DsRed $\alpha_{1B}$-ARs (first four columns) and the PKC phosphrorylation site-defective DsRed-$\alpha_{1B}$-AR mutant (S396A, S402A) (fifth colum); cells also expressed wild-type EGFP-Rab9 (columns 1-3 and 5) or the GDP-locked mutant (Rab9-GDP, fourth column). Preincubation was with 1 μM bisindolylmaleimide I for 15 min (BIM, second column) or overnight with 1 μM PMA (PMA-ON, third column). Cells were challenged for 15 min with vehicle (Baseline), 1 μM PMA, 1 μM S1P or 10 μM NA (plus propranolol). Images showing Rab9 expression are presented in Supplementary Fig. S4).

**Fig. 7.** Quantitation of intracellular fluorescence as an index of $\alpha_{1B}$-AR internalization. Data obtained from cells expressing DsRed $\alpha_{1B}$-ARs (panels A-D) or the PKC phosphrorylation site-defective DsRed-$\alpha_{1B}$-AR mutant (S396A, S402A) (panel E); cells also expressed wild-type EGFP-Rab9 (panel A-C and E) or the GDP-locked mutant (Rab9-

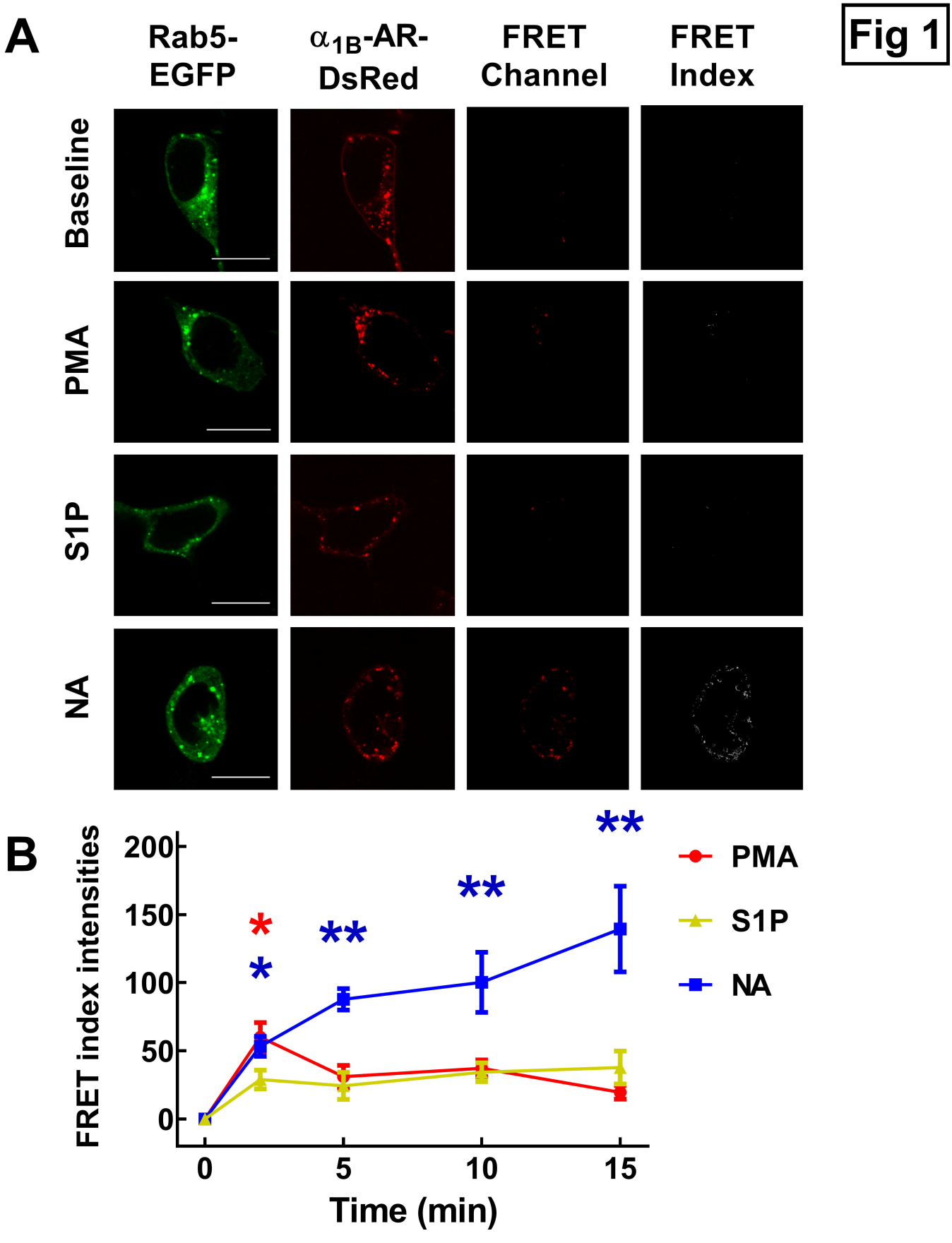
GDP, panel D). Preincubation was with 1 μM bisindolylmaleimide I for 15 min (BIM, panel B) or overnight with 1 μM PMA (PMA-ON, panel C). Cells were challenged for 15 min with vehicle (B, Baseline), 1 μM PMA, 1 μM S1P or 10 μM NA. In all panels plotted are the means with vertical lines representing the S.E.M. of 5-6 experiments using different cell preparations; in each experiment 5 different images were analyzed (i.e, 25-30 samples in each condition). * $P < 0.001$ vs. B (baseline); ** $P < 0.01$ vs. B (baseline).

**Fig. 8.** Effect of Rab9 expression on NA-induced $\alpha_{1B}$-AR-mediated intracellular calcium increases and PMA action. Cells expressing either DsRed-$\alpha_{1B}$-ARs and a) no exogenous Rab protein (No Rab, vector, panel A); b) wild-type EGFP-Rab9 (Rab9-WT, panel B); c) the GDP-locked EGFP-Rab9 mutant (Rab9-GDP, panel C); d) the GDP-locked EGFP-Rab5 mutant (Rab5-GDP, panel D); or the DsRed-$\alpha_{1B}$-AR mutant (S396A, S402A) with no exogenous Rab protein (panel E) were used. Cells were preincubated for 5 min in the absence of any any agent (open bars) or the presence of 1 μM PMA (dashed bars); cells were then challenged with 10 μM NA (plus propranol). Plotted are the means and vertical lines representing the S.E.M of 8-10 determinations using different cell preparations. * $P < 0.001$ vs. absence of PMA , ** $P < 0.01$ vs. absence of PMA .

**Fig. 9.** Effect of Rab9 expression on NA-induced $\alpha_{1B}$-AR-mediated intracellular calcium increases and PMA action. $DDT_1$-MF2 cells transfected to express a) no exogenous Rab protein (No Rab, panel A); b) wild-type EGFP-Rab9 (Rab9-WT, panel B); c) the GDP-locked EGFP-Rab9 mutant (Rab9-GDP, panel C); and d) the GDP-locked EGFP-Rab5

mutant (Rab5-GDP, panel D) were used. Cells were preincubated for 5 min in the absence of any any agent (open bars) or the presence of 1 μM PMA (dashed bars); cells were then challenged with 10 μM NA. Plotted are the means and vertical lines representing the S.E.M of 6-9 determinations using different cell preparations. * $P < 0.001$ vs. absence of PMA.

**Fig. 10.** Working model for the possible roles of PKC and Rab proteins in receptor internalization.


A
Rab5-EGFP
α1B-AR-DsRed
FRET Channel
FRET Index
Baseline
PMA
S1P
NA
B
FRET index intensities
200
150
100
50
0
0
5
10
15
Time (min)
*
*
**
**
**
PMA
S1P
NA

A
Rab9-EGFP
α1B-AR-DsRed
FRET Channel
FRET Index
Baseline
PMA
S1P
NA
B
FRET index intensities
200
150
100
50
0
0
5
10
15
Time (min)
PMA
S1P
NA

Fig 2

Fig 3

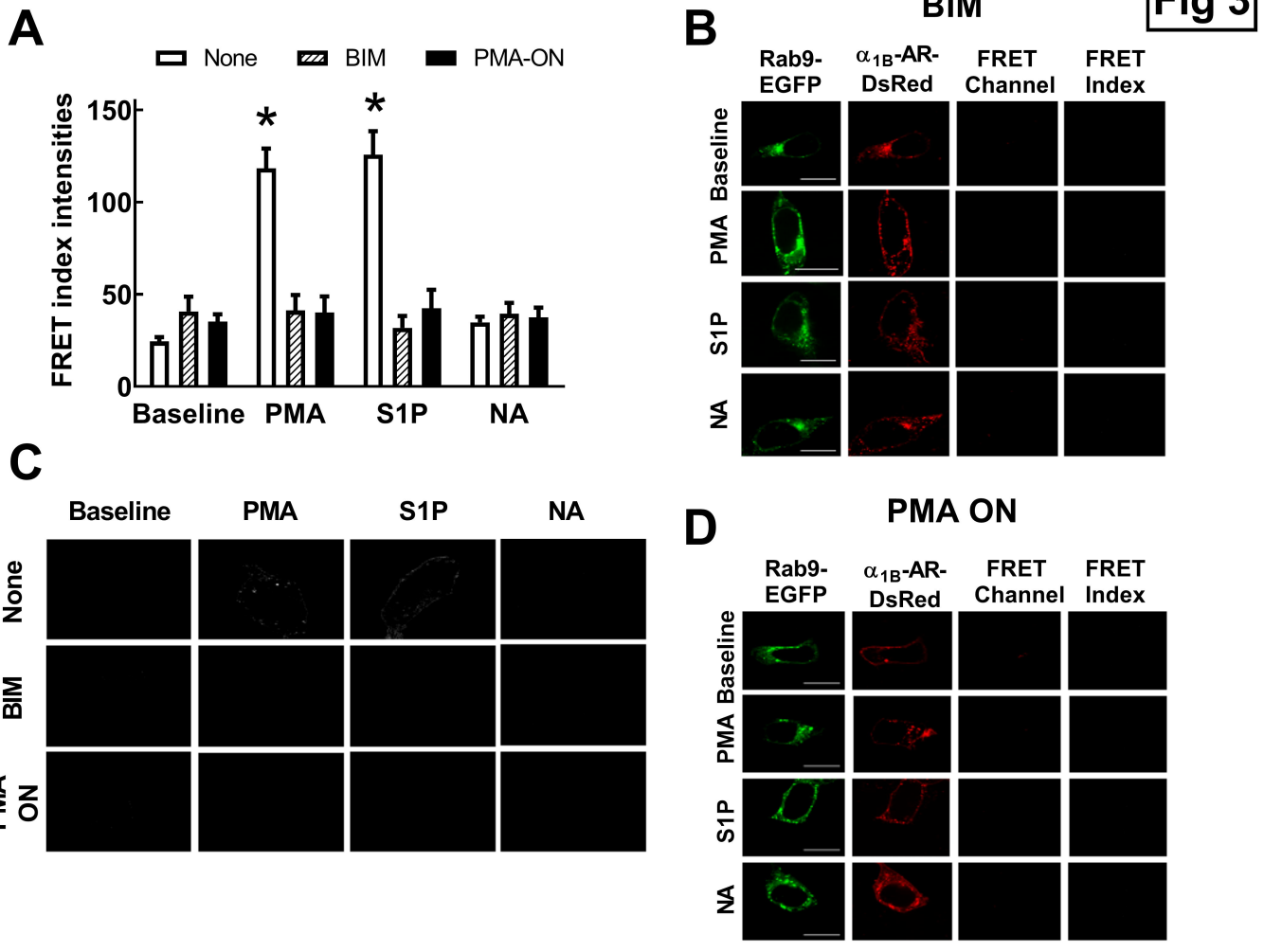
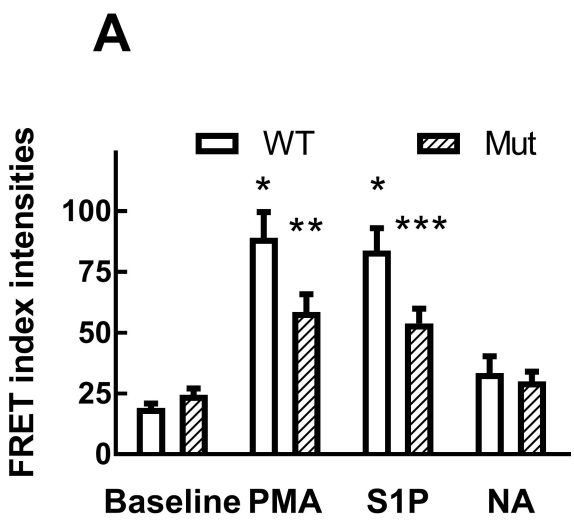
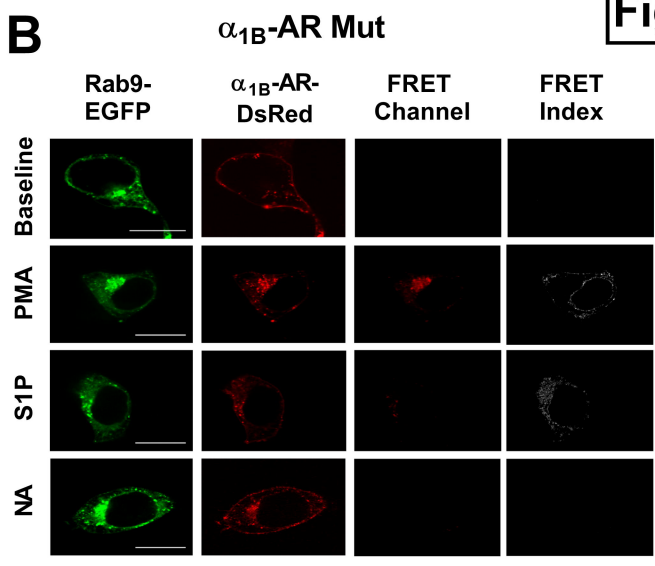

A
None
BIM
PMA-ON
FRET index intensities
150
100
50
0
*
*
Baseline
PMA
S1P
NA
B
BIM
Rab9-EGFP
α1B-AR-DsRed
FRET Channel
FRET Index
Baseline
PMA
S1P
NA
C
Baseline
PMA
S1P
NA
None
BIM
PMA ON
D
PMA ON
Rab9-EGFP
α1B-AR-DsRed
FRET Channel
FRET Index
Baseline
PMA
S1P
NA

Fig 4


A
WT
Mut
FRET index intensities
100
75
50
25
0
*
**
*
***
Baseline
PMA
S1P
NA


B
α1B-AR Mut
Rab9-EGFP
α1B-AR-DsRed
FRET Channel
FRET Index
Baseline
PMA
S1P
NA

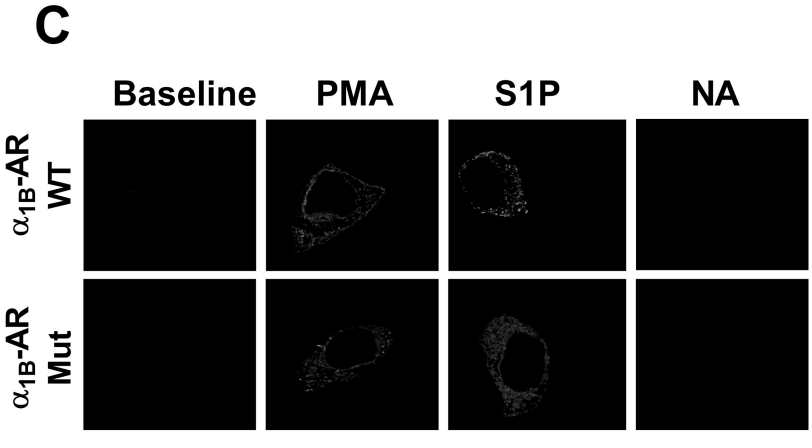
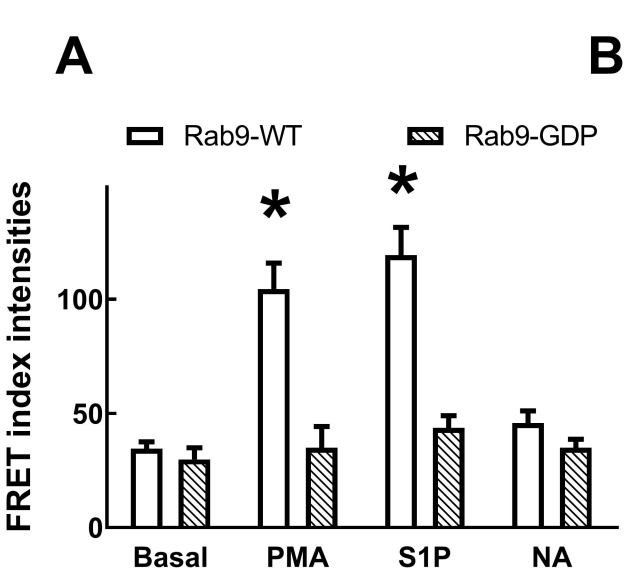
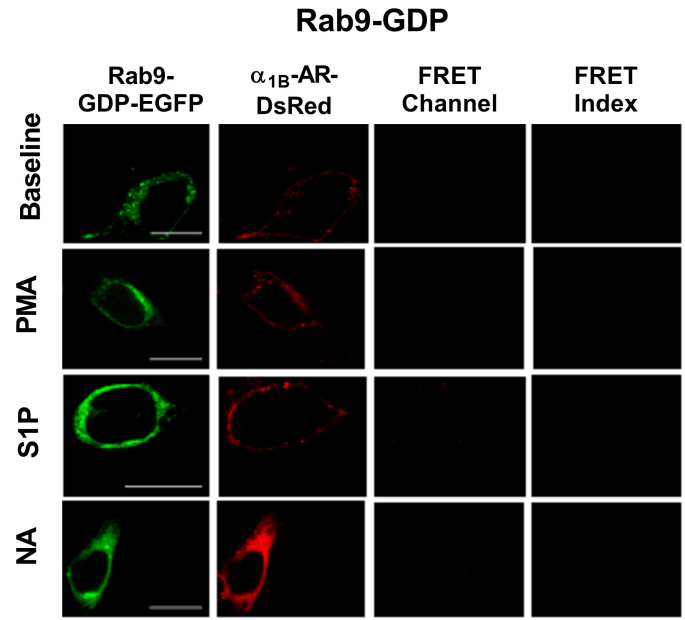

C
Baseline
PMA
S1P
NA
α1B-AR WT
α1B-AR Mut

Fig 5


A
Rab9-WT
Rab9-GDP
FRET index intensities
100
50
0
*
*
Basal
PMA
S1P
NA


B
Rab9-GDP
Rab9-GDP-EGFP
α1B-AR-DsRed
FRET Channel
FRET Index
Baseline
PMA
S1P
NA

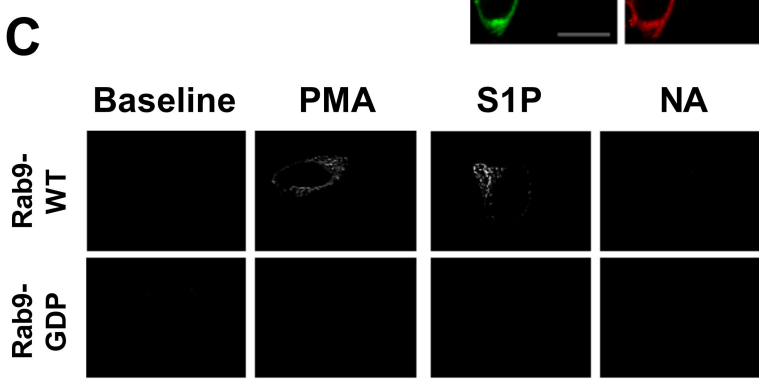

C
Baseline
PMA
S1P
NA
Rab9-WT
Rab9-GDP

Fig 6

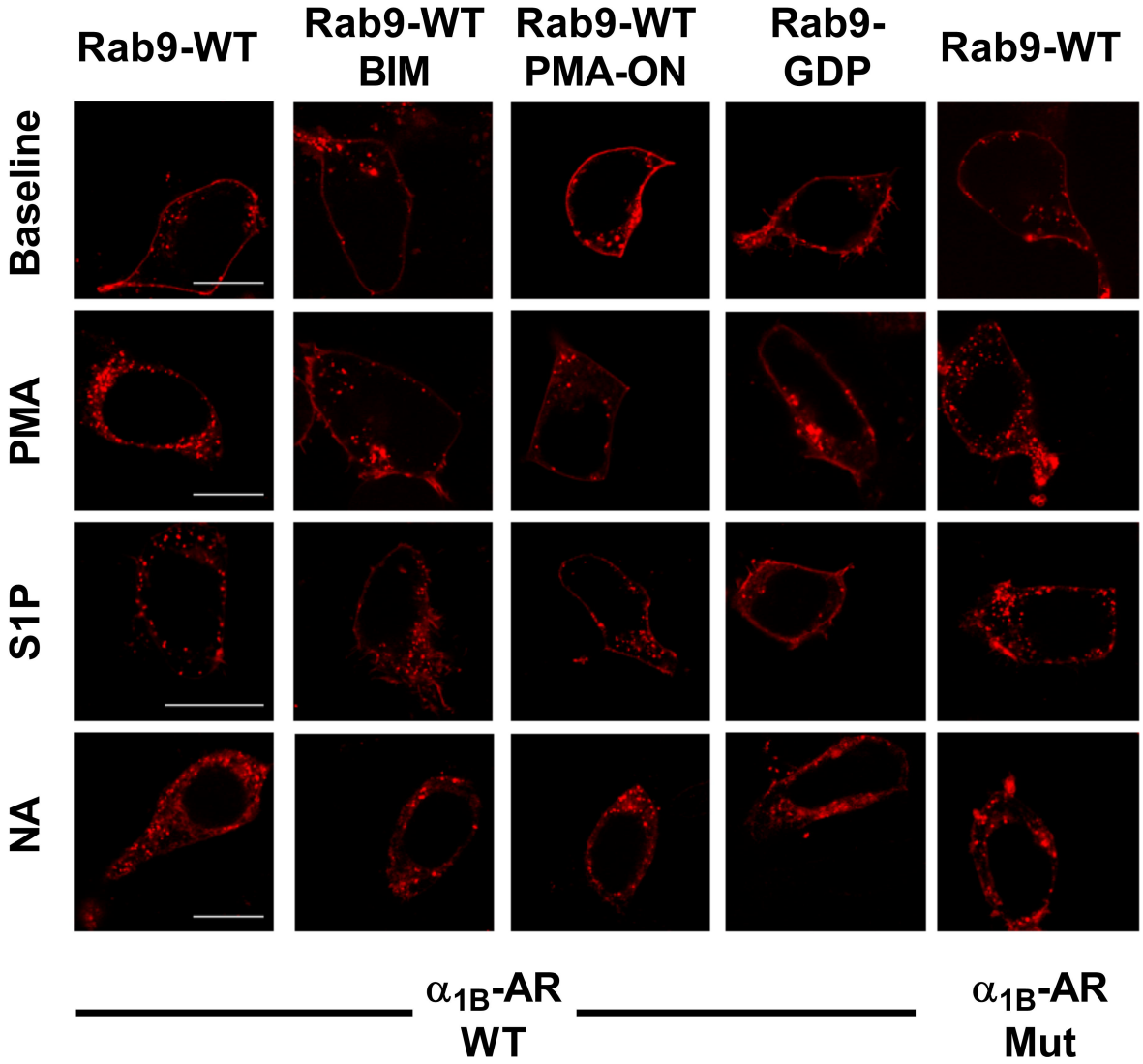

Rab9-WT
Rab9-WT BIM
Rab9-WT PMA-ON
Rab9-GDP
Rab9-WT
Baseline
PMA
S1P
NA
α1B-AR WT
α1B-AR Mut

Fig 7

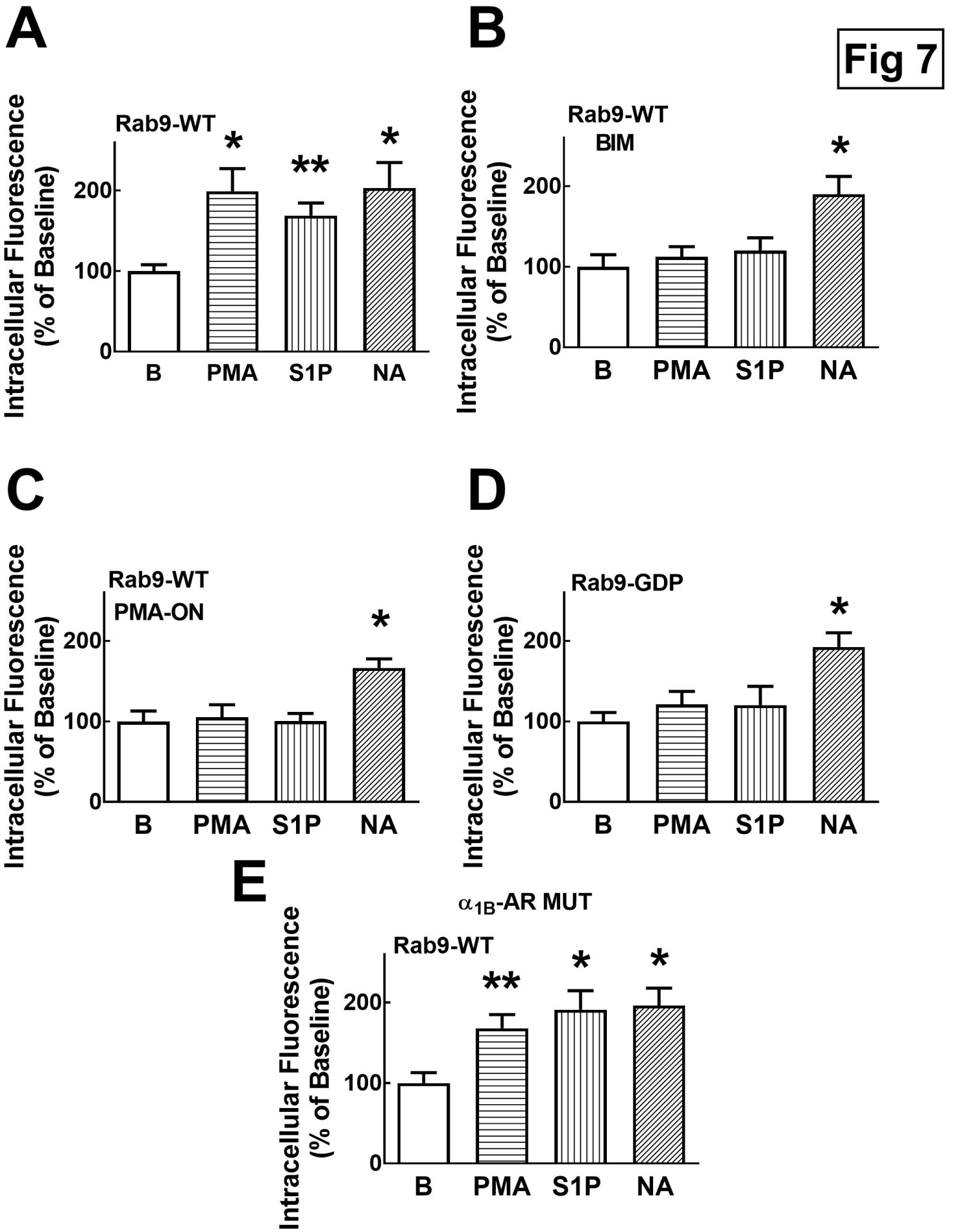
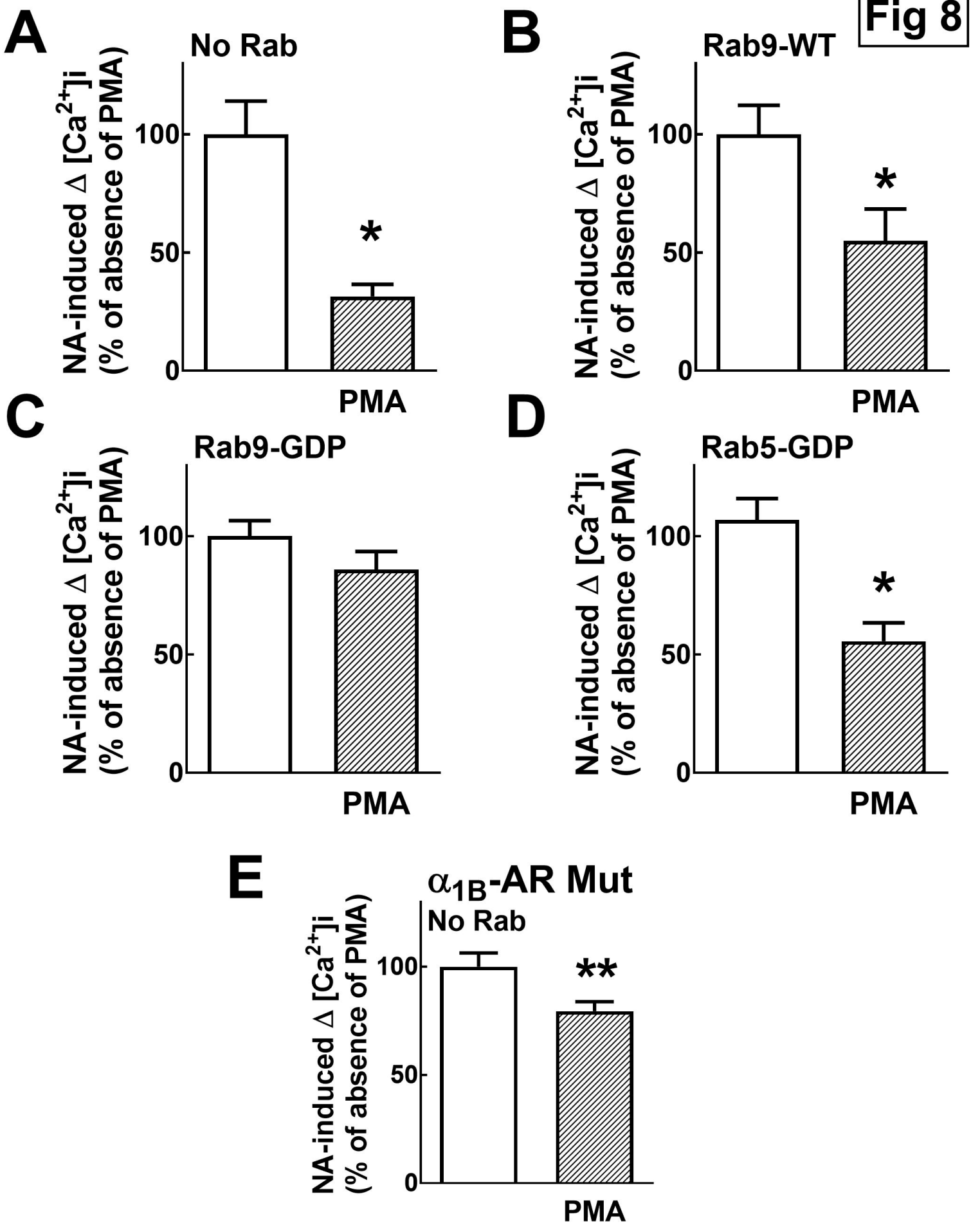
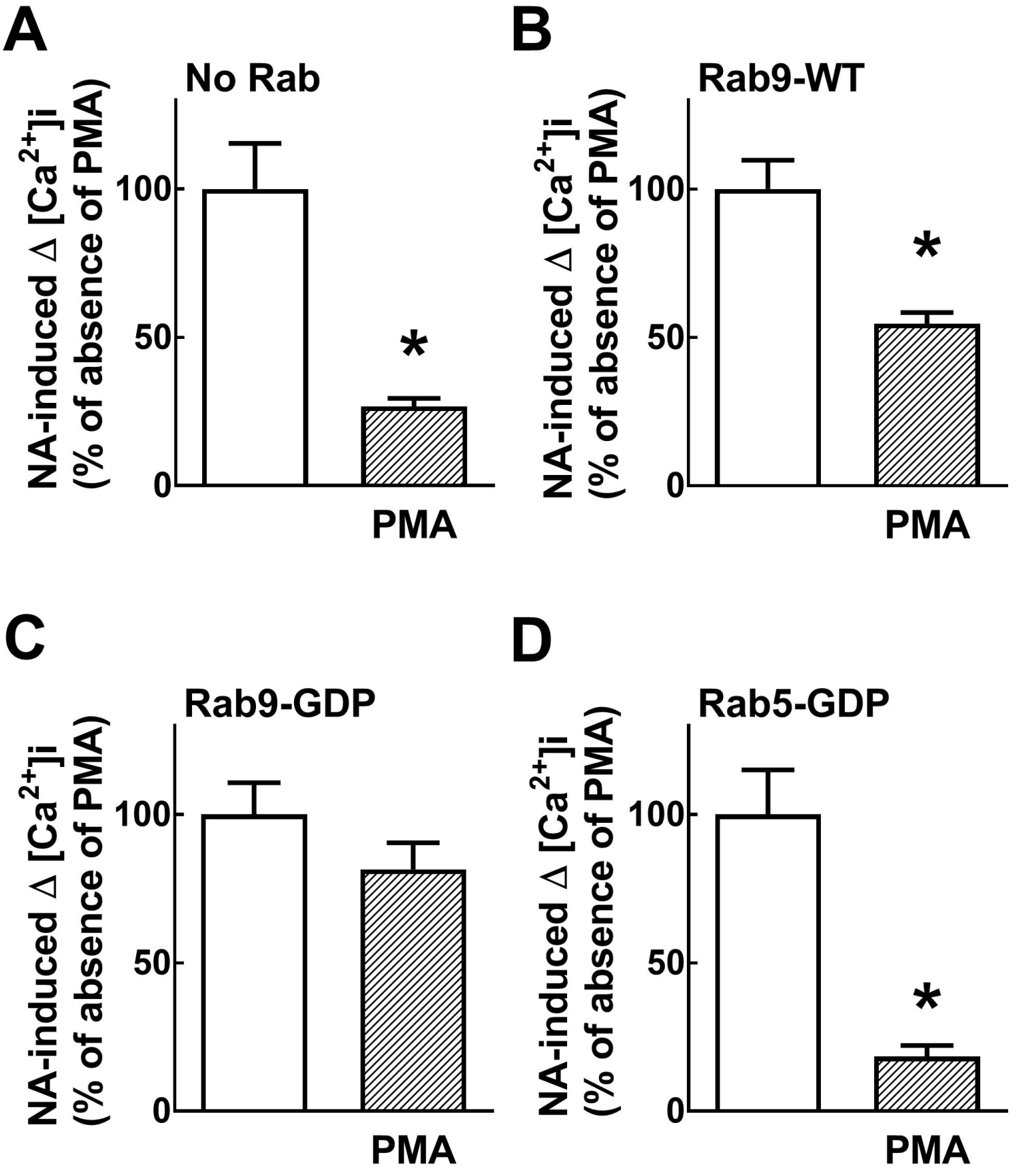

A
Rab9-WT
Intracellular Fluorescence (% of Baseline)
200
100
0
B
PMA
S1P
NA
*
**
*
B
Rab9-WT
BIM
Intracellular Fluorescence (% of Baseline)
200
100
0
B
PMA
S1P
NA
*
C
Rab9-WT
PMA-ON
Intracellular Fluorescence (% of Baseline)
200
100
0
B
PMA
S1P
NA
*
D
Rab9-GDP
Intracellular Fluorescence (% of Baseline)
200
100
0
B
PMA
S1P
NA
*
E
α1B-AR MUT
Rab9-WT
Intracellular Fluorescence (% of Baseline)
200
100
0
B
PMA
S1P
NA
**
*
*

Fig 8


A
No Rab
NA-induced Δ [Ca2+]i
(% of absence of PMA)
100
50
0
*
PMA
B
Rab9-WT
NA-induced Δ [Ca2+]i
(% of absence of PMA)
100
50
0
*
PMA
C
Rab9-GDP
NA-induced Δ [Ca2+]i
(% of absence of PMA)
100
50
0
PMA
D
Rab5-GDP
NA-induced Δ [Ca2+]i
(% of absence of PMA)
100
50
0
*
PMA
E
α1B-AR Mut
No Rab
NA-induced Δ [Ca2+]i
(% of absence of PMA)
100
50
0
**
PMA

# $DDT_1$-MF2

Fig 9



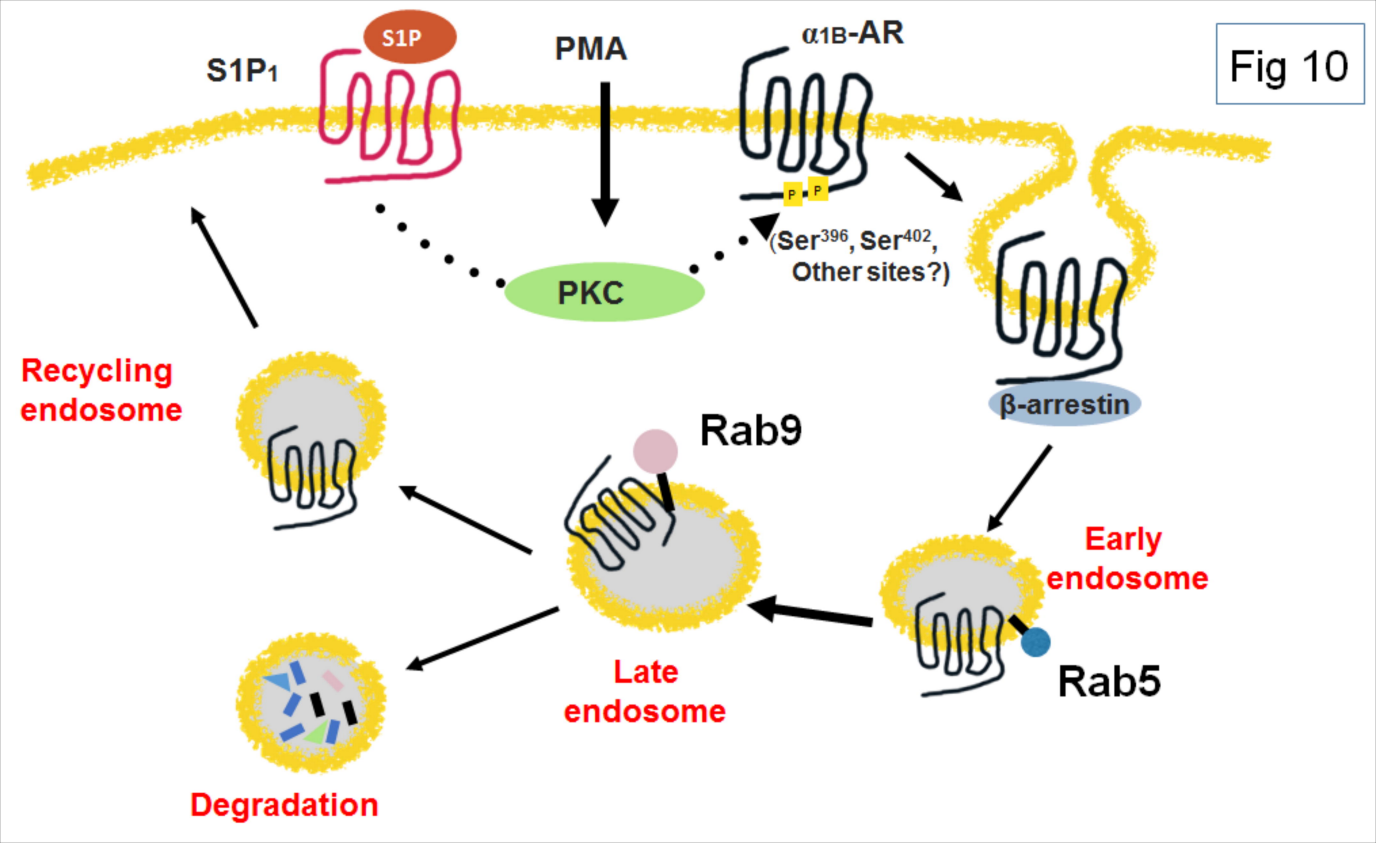


Fig 10
S1P
S1P1
PMA
α1B-AR
P
P
(Ser396, Ser402,
Other sites?)
PKC
β-arrestin
Recycling
endosome
Rab9
Early
endosome
Rab5
Late
endosome
Degradation