α1A-Adrenergic Receptor Induces Activation of Extracellular Signal-Regulated Kinase 1/2 through Endocytic Pathway

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Abstract

G protein-coupled receptors (GPCRs) activate mitogen-activated protein kinases through a number of distinct pathways in cells. Increasing evidence has suggested that endosomal signaling has an important role in receptor signal transduction. Here we investigated the involvement of endocytosis in α1A-adrenergic receptor (α1A-AR)-induced activation of extracellular signal-regulated kinase 1/2 (ERK1/2). Agonist-mediated endocytic traffic of α1A-AR was assessed by real-time imaging of living, stably transfected human embryonic kidney 293A cells (HEK-293A). α1A-AR was internalized dynamically in cells with agonist stimulation, and actin filaments regulated the initial trafficking of α1A-AR. α1A-AR-induced activation of ERK1/2 but not p38 MAPK was sensitive to disruption of endocytosis, as demonstrated by 4°C chilling, dynamin mutation and treatment with cytochalasin D (actin depolymerizing agent). Activation of protein kinase C (PKC) and C-Raf by α1A-AR was not affected by 4°C chilling or cytochalasin D treatment. U73122 (a phospholipase C [PLC] inhibitor) and Ro 31–8220 (a PKC inhibitor) inhibited α1B-AR- but not α1A-AR-induced ERK1/2 activation. These data suggest that the endocytic pathway is involved in α1A-AR-induced ERK1/2 activation, which is independent of Gq/PLC/PKC signaling.

Introduction

α1A-Adrenergic receptor (α1A-AR) is one of 3 members of the α1-AR subfamily (α1A, α1B, and α1D) of G protein-coupled receptors (GPCRs) [1–3]. α1A-AR plays a key role in physiological effects such as contraction of vascular and cardiac muscle, contraction of the spleen, liver glycogenesis, or melatonin secretion in the pineal gland [2–5]. Mice with cardiac-restricted overexpression of the wild-type α1B-AR that were treated with α1-AR agonist (phenylephrine [PE]) exhibited poor survival, markedly exaggerated cardiac hypertrophy, myocardial fibrosis, and suppressed left ventricular function [6]. In contrast, animals with α1A-AR overexpression showed improved survival and even abrogated cardiac remodeling in response to thoracic aorta constriction-induced pressure overload or myocardial infarction [7,8]. The activation of extracellular signal-regulated kinase (ERK), a regulator of myocyte survival, is critical in mediating α1-AR survival signaling in cardiac myocytes [9–11]. Recent studies of selective inactivation of α1-ARs indicate that the activation of ERK1/2 induced by α1A-AR is critical for cardiomyocyte survival. Reconstitution of α1A-AR but not α1B-AR induced ERK1/2 activation and rescued α1ABKO myocytes from cell death induced by norepinephrine, doxorubicin, and H_2O_2 [12]. The

observation that α1A-AR specifically restored ERK1/2 activation in α1ABKO myocytes suggests that α1A-AR and α1B-AR activate ERK1/2 through differential mechanisms. However, studies to date have not consistently identified major differences in immediate signaling responses initiated by α1A-AR and α1B-AR.

α1A-AR is regulated by many mechanisms, including phosphorylation, protein-protein interaction, protein traffic, and transcription [13]. After stimulation by their ligands, α1-ARs activate intracellular effectors, including phospholipase C β (PLCβ), inositol trisphosphate, protein kinase C (PKC), mitogen-activated protein kinase (MAPK) and calcium signals, often through a heterotrimeric G protein-dependent manner [14,15]. An α1A-AR variant, which was unable to couple to Gq, could also induce calcium influx when coactivated by β2-AR [16]. Thus, α1A-AR, even though uncoupled from Gq, may remain competent for induction of signaling events through yet unknown pathways.

Increasing evidence has shown the existence of receptor signaling from the endocytic process. For instance, activation of ERK1/2 via epidermal growth factor receptor (EGFR) and β2-AR were suppressed in cells transfected with dynamin-mutant K44A (Dyn-K44A), which is defective in GTPase activity [17,18]. Signaling from GPCR inside the cell is persistent and appears to trigger specific downstream effects [19]. Visualizing and tracking

receptors stimulated by agonists in living cells contributes to understanding the molecular mechanisms of receptor signaling [20]. However, the association of α_{1A}-AR endocytic trafficking and activation of MAPKs is still unknown.

We aimed to investigate whether an endocytic process is involved in ERK1/2 activation induced by α_{1A}-AR. By real-time tracking, we found a time-dependent dynamic pattern of α_{1A}-AR endocytosis with stimulation and the involvement of the cytoskeleton, especially actin-filaments, in this process. This relationship was further examined by colocalization of α_{1A}-AR with reorganized cytoskeletons. We provide evidence for an involvement of endocytosis in α_{1A}-AR-induced activation of ERK1/2, which differs from that of α_{1B}-AR via a G_q/PLC/PKC pathway.

Materials and Methods

Materials

Cytochalasin D, nocodazole, PE and U73122 were from Sigma (St. Louis, MO). Ro 31–8220, prazosin and phorbol 12-myristate, 13-acetate (PMA) were from Calbiochem (La Jolla, CA). Phospho-p38 MAPK (Thr180/Tyr182), -p42/44 MAPK (Thr202/Tyr204), -PKC (pan) (Ser660), and -C-Raf (Ser338) antibodies were from Cell Signaling Technology (Beverly, Mass). Antibodies against ERK1/2, PKC (pan), C-Raf, p38, FLAG-tag and HA-tag were from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse and anti-rabbit antibodies were from Beijing Zhongshan Golden Bridge Biotechnology. Alexa 488-conjugated WGA, Alexa 555 and 633 IgG and Alexa 488-conjugated phalloidin were from Invitrogen. All other chemicals were of analytical grade.

Cell culture, plasmids and transfection

HEK-293A cell lines were obtained from Invitrogen. Receptor constructs and HEK-293A cells stably transfected with α_{1A}-AR or α_{1B}-AR were described previously [21]. Dyn-K44A was a gift from Ming Zhao (La Jolla Institute for Molecular Medicine, San Diego, CA). Amphiphysin I construct was a gift from Pietro De Camilli (Yale University School of Medicine, New Haven, CT). Transfection involved use of Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Membrane receptor labeling and tracking

FLAG-tagged receptors were labeled with anti-FLAG monoclonal antibody (12.5 µg/ml) for 10 min and then Alexa FLour® 555 goat anti-mouse IgG (Invitrogen) (3.75 µg/ml) for 10 min as described previously [21,22]. Before fluorescence experiments, cells were washed 3 times in phosphate buffered saline (PBS) buffer (pH 7.4; 37°C). Live imaging involved use of a wide-field fluorescence microscope equipped with a 100×/1.40NA Plan Apochromat objective (Olympus, Japan) and a 14-bit, back-illuminated, electron-multiplying charge-coupled device camera (Andor iXon DU-897 BV). The microscope was also equipped with a cell incubation system (INU-ZIL-F1, TOKAI HIT), which ensured live-cell imaging at 37°C in 5% CO_2. Fluorescence was excited at 532-nm by an argon laser (Melles Griot, Carlsbad, CA). Movies were acquired at a frame rate of 20 Hz by use of MetaMorph software (Molecular Devices). Trajectories from cells observed under the given labeling procedure were plotted and resolved as described previously [21].

Fluorescence microscopy

After drug treatment, cells were fixed for 15 min in 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100. After washes with PBS, cells were incubated for 25 min with TRITC-labeled phalloidin (Sigma). The samples were viewed under a laser scanning confocal microscope (TCS SP2, Leica Microsystems) with a Plan-Apo 63×/1.32 oil immersion objective (Leica Microsystems); images were collected by use of Leica TCS SP2 v2.611537. The 488- and 532-nm laser beam was focused by a Leica Apochromat with <200 lW power irradiation. The pinhole size was 1 airy unit.

Western blot analysis

Protein expression was examined by western blot analysis as previously described [23]. Briefly, samples were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. After being blocked, blots were probed with the appropriate primary antibodies overnight at 4°C or for 2 h at room temperature, then washed and incubated with HRP-conjugated secondary antibody. Bands were visualized by use of a super-western sensitivity chemiluminescence detection system (Pierce). Autoradiographs were quantitated by densitometry (Science Imaging System, Bio-Rad).

Results

Tracking α_{1A}-AR stimulated by agonist in single living cells in real time

We studied the dynamic properties and mechanisms of receptor transport in HEK-293A cells stably transfected with a FLAG-tagged α_{1A}-AR construct. α_{1A}-AR was detected on the surface of living HEK-293A–α_{1A}-AR cells by use of a monoclonal primary antibody and Alexa-555 IgG (Fig. 1A). After incubation with PE, an α_1-AR agonist, some of the α_{1A}-AR particles trafficked inward in the cells. From recorded movies, we tracked the trajectories of trafficking α_{1A}-AR particles. Figure 1B shows 2 sample trajectories of α_{1A}-AR particles (as marked in Fig. 1A) with directed movement within 8 sec on PE stimulation. To quantify the velocities of α_{1A}-AR movements, we plotted the mean square displacement (MSD) versus time (Fig. 1C), which also showed the directional movement of these particles.

We then resolved the velocities at different time after PE stimulation [21]. Figure 1D shows the time-dependent velocity distribution of endocytic α_{1A}-AR with PE stimulation during 1 hour (10-min intervals). At the early stage of the activation (first 30 min), the receptor mainly moved at a low velocity at a peak of about 0.3 µm/s. After stimulation for 40 to 60 min, movements became much faster, with high velocity trajectories increased gradually. The main peak of the highest velocity was about 0.8 µm/s. Thus, in general, the active movement of α_{1A}-AR vesicles was slower at the early phase of endocytosis and faster at the later phase.

Actin filament-mediated endocytosis of α_{1A}-AR

We used confocal microscopy to determine the association of endocytic receptors with cytoskeleton, actin and microtubules, respectively. α_{1A}-AR vesicles largely colocalized with F-actin after 20-min PE stimulation (Fig. 2A). With 50-min PE stimulation, some of the α_{1A}-AR vesicles colocalized with microtubules. With higher resolution imaging, we observed a more relevant relation between reorganized actin and α_{1A}-AR; at 20 min after PE stimulation, small actin patches and tails appeared in the cells (Fig. 2B). Most of the actin patches showed colocalization of a α_{1A}-AR vesicle. Actin may use α_{1A}-AR-associated actin patches as polymerization sites, as was reported for virus internalization [24]. The changes were transient, and after 50-min stimulation, most of actin patches and tails disappeared. And α_{1A}-AR vesicles became
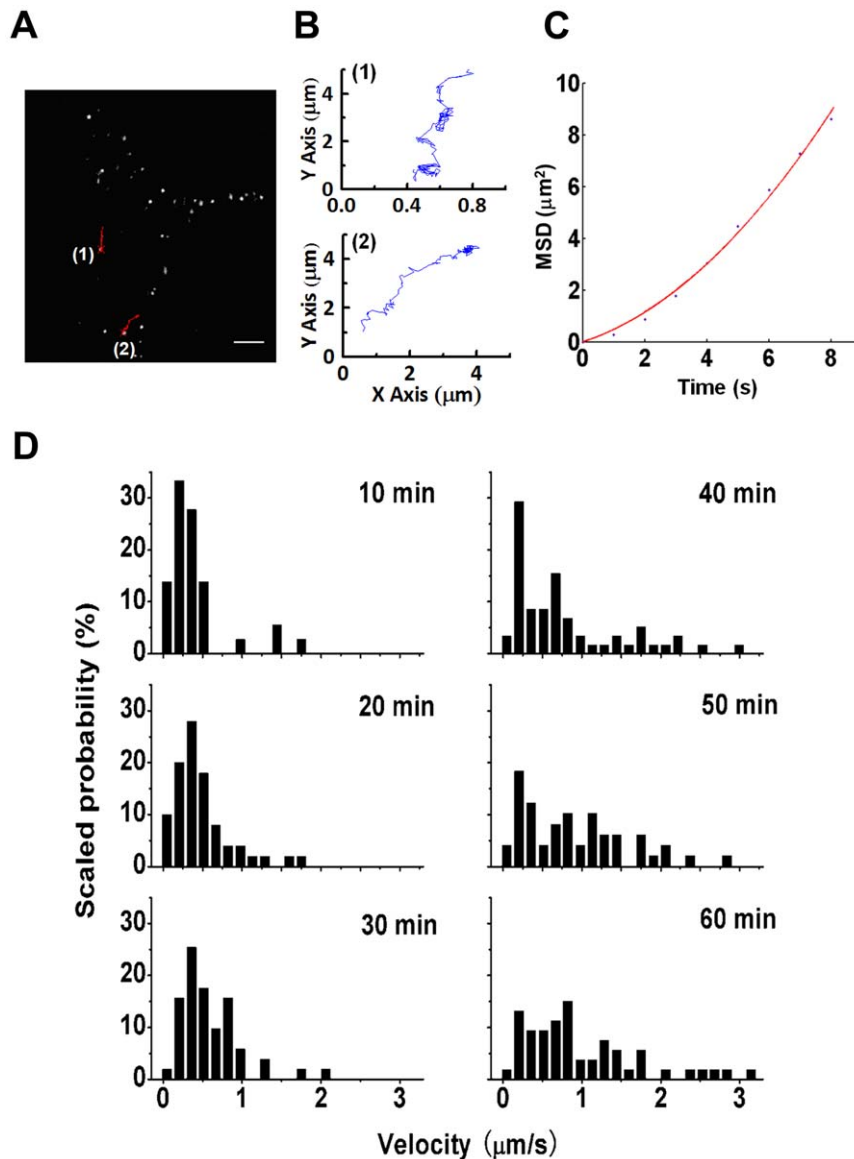
Figure 1. Tracking α₁A-AR in response to agonist stimulation. (A) α₁A-ARs were detected with anti-FLAG antibody and Alexa-555 IgG in live HEK-293A–α₁A-AR cells at 37°C. Images were captured after 30-min stimulation with 10 μM phenylephrine (PE). Two sample trajectories of α₁A-AR particles are shown with red lines (1 and 2). Bar: 10 μm. (B) The trajectories in (A) were plotted (1 and 2, respectively). (C) The plot of the mean square displacement ($<r^2>$) against time (t) to the trajectory in Fig. B(2). The red line is a fit by $<r^2> = 4Dt+(vt)^2$. Directed movement was confirmed by the superlinear MSD-Δt plots. (D) Velocities of directional movements of α₁A-AR resolved from tracked trajectories at various times after 10 μM PE stimulation plotted in probability histograms. (n = 36, 61, 51, 58, 49 and 53 trajectories in separated cells, respectively).
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located on the filamentous actin. Thus, PE-induced α₁A-AR endocytic trafficking in the early phase depends on F-actin.

To justify the role of F-actin in regulation of α₁A-AR endocytosis, cytochalasin D was used before PE stimulation to inhibit the actin polymerization. Incubated for 5 min with 5 μM cytochalasin D, α₁A-AR congregated on membrane even after PE stimulation (Fig. 2C). It provides further evidence that α₁A-AR endocytosis is regulated by actin filaments.

Receptor endocytosis is required for ERK1/2 activation induced by α₁A-AR

To test whether endocytosis is involved in the α₁A-AR induced signaling, we first examined the activation of ERK1/2 and p38

MAPK with PE stimulation. ERK1/2 and p38 MAPK phosphorylation significantly increased at 10 and 20 min after PE treatment and then decreased to the basal level (Fig. 3A,B). PE also caused a secondary increase of p38 MAPK phosphorylation after 50-min treatment. We then used 4°C incubation to inhibit α₁A-AR endocytosis [25,26]. α₁A-AR remained on the membrane after PE stimulation at 4°C (Fig. 3C). α₁A-AR endocytosis was markedly inhibited at 4°C as compared with at 37°C. 4°C chilling almost completely abrogated the α₁A-AR-induced ERK1/2 activation, whereas activation of p38 was not modified (Fig. 3D). To ensure that the ERK1/2 was not defective in phosphorylation at 4°C incubation, we measured PMA-induced activation of ERK1/2 in 4°C. PMA activated both PKC and ERK1/2 at 4°C and at 37°C
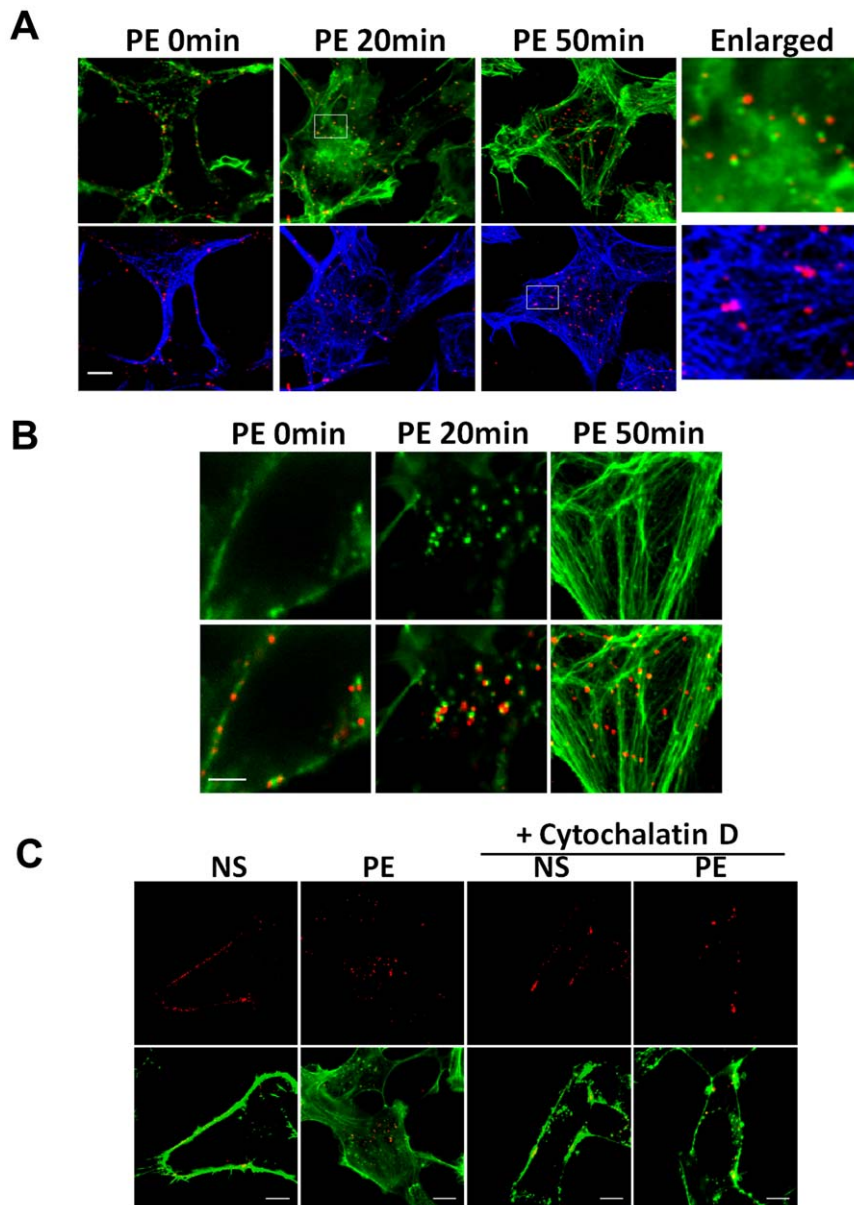
Figure 2. α$_{1A}$-AR endocytosis is regulated by cytoskeleton. (A) Colocalization of α$_{1A}$-AR with F-actin and microtubules after agonist stimulation. Cells were stimulated with 10 μM PE for 20 or 50 min. Untreated cells were used as control. α$_{1A}$-AR was labeled with anti-FLAG antibodies and Alexa 555 IgG (*red*). F-actin was labeled with Alexa 488-conjugated phalloidin (*green*). Microtubules were labeled with antibodies and Alexa 633 IgG (*blue*). Last column: 5× magnification of selected boxed regions. Bar: 10 μm. (B) High-resolution imaging of colocalization of α$_{1A}$-AR with reorganized actin after stimulation. Cells were treated with agonist for 20 or 50 min, and then labeled with antibodies against α$_{1A}$-AR and Alexa 488-conjugated phalloidin against F-actin (*red*) (bottom row). Bar: 5 μm. (C) Inhibition of α$_{1A}$-AR endocytosis by Cytochalasin D. HEK-293A–α$_{1A}$-AR cells were pre-incubated with cytochalasin-D (Cyto-D; 5 μM, 5 min), then stimulated with 10 μM PE for 20 min. F-actin was stained by Alexa 488-conjugated Phalloidin (*green*), α$_{1A}$-ARs were detected with anti-FLAG antibody and Alexa-555 IgG (*red*). NS: no stimulation. Bar: 10 μm.
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(Fig. 3E), which suggests that the failure to activate ERK1/2 by α$_{1A}$-AR at 4°C was not due to a defect in ERK1/2 signaling but rather to a defect in receptor endocytosis.

To further define the role of endocytosis in α$_{1A}$-AR induced signaling, we tested the effect of the mutant Dyn-K44A, used to induce trafficking defects of receptors in many cells [27,28], on ERK1/2 and p38 activation. Overexpression of HA-tagged Dyn-K44A in HEK-293A–α$_{1A}$-AR cells suppressed the activation of ERK1/2 but not p38 (Fig. 3F, 3G). Therefore, endocytosis is required for α$_{1A}$-AR-induced ERK1/2 but not p38 MAPK activation.

Actin organization is involved in ERK1/2 activation induced by α$_{1A}$-AR

Actin filaments play a pivotal role in α$_{1A}$-AR trafficking, especially in the early phase of endocytosis (Fig. 1, 2). Therefore, we tested the contribution of actin polymerization to α$_{1A}$-AR-induced activation of ERK1/2. We pretreated cells with cytochalasin D or nocodazole before PE stimulation to disrupt to disrupt organizing actin or microtubules, respectively. In cells treated for 5 min with 5 μM cytochalasin D, filamentous form of actin was depolymerized and was replaced by aggregated actin, with microtubules appearing normal (Fig. 4A). Treatment with
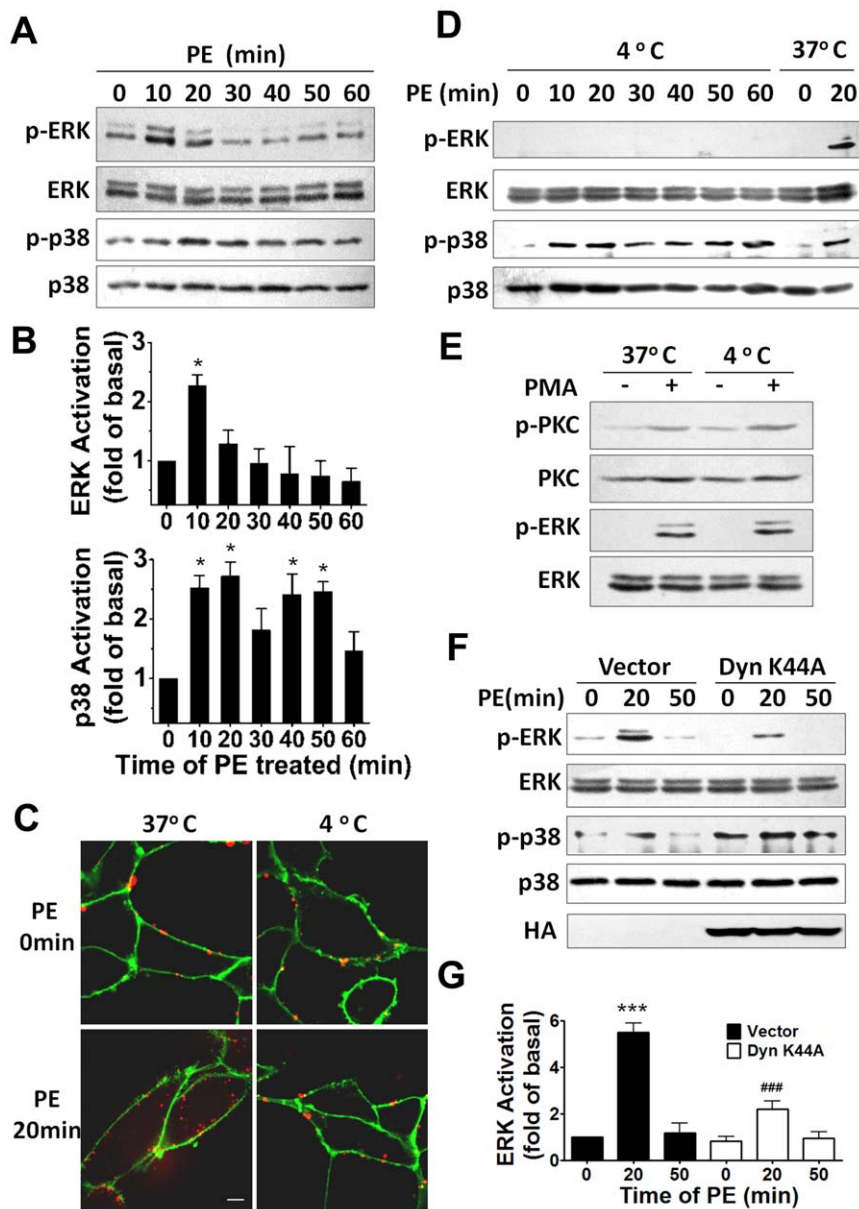
Figure 3. Receptor endocytosis is involved in ERK1/2 activation by α1A-AR stimulation. (A) Representative western blot showing activation of ERK1/2 and p38 after 10-μM PE treatment for the indicated times. (B) Relative ERK1/2 and p38 activeity after PE stimulation are shown. Data are means±SEM of results obtained in three independent experiments. Statistical significance of the difference was assessed using one-way ANOVA analysis. *, $p < 0.05$ versus 0 min. (C) Effect of 4°C chilling on agonist-induced α1A-AR endocytosis. Cells were incubated in 4°C or 37°C for 30 min then stimulated with 10 μM PE for 20 min. α1A-ARs were detected with anti-FLAG antibody and Alexa-555 IgG (*red*), and plasma membrane was labeled with Alexa 488-conjugated WGA (*green*). (D) Western blot analysis of ERK1/2 and p38 phosphorylation with PE stimulation for the indicated times at 4°C. Stimulation at 37°C is a control. (E) Phorbol 12-myristate, 13-acetate (PMA)-induced activation of protein kinase C (PKC) and ERK1/2 at 4°C. Cells were incubated in 4°C or 37°C for 30 min then stimulated with 0.1 μM PMA for 20 min. (F) Dynamin mutation inhibits the activation of ERK1/2 by α1A-AR. HEK-293A–α1A-AR cells were cultured and infected with Dyn-K44A or vectors. After 40 h, cells were treated for 20 min with 10 μM PE. Protein expression of phospho-ERK1/2 and total ERK1/2 and p38 were measured. Expression of Dyn-K44A (HA-tagged) was identified with blotting of HA-tag. (G) Quantification of relative ERK1/2 activation corresponding to (F) was performed by densitometric analysis. Data are means±SEM of results obtained in three independent experiments. Statistical significance of the difference was assessed using one-way ANOVA analysis. ***, $p < 0.001$ versus Vector 0 min. ###, $p < 0.001$ versus Vector 20 min.
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20 μM nocodazole for 30 min resulted in a loss of organizing microtubule but left F-actin intact. These data confirm the specific effects of cytochalasin D and nocodazole on depolymerizing F-actin and microtubules, respectively. Treatment with cytochalasin D impaired the ability of α1A-AR to activate ERK1/2 but that with nocodazole did not affect the ERK1/2 activation at 20 min

of PE stimulation and even increased the phosphorylation levels of ERK1/2 at a later stage of stimulation (Fig. 4B). However, activation of p38 seemed insensitive to the disruption of F-actin or microbules.

To further justify the role of endocytic trafficking in the ERK1/2 activation, we overexpressed amphiphysin, found to be critical
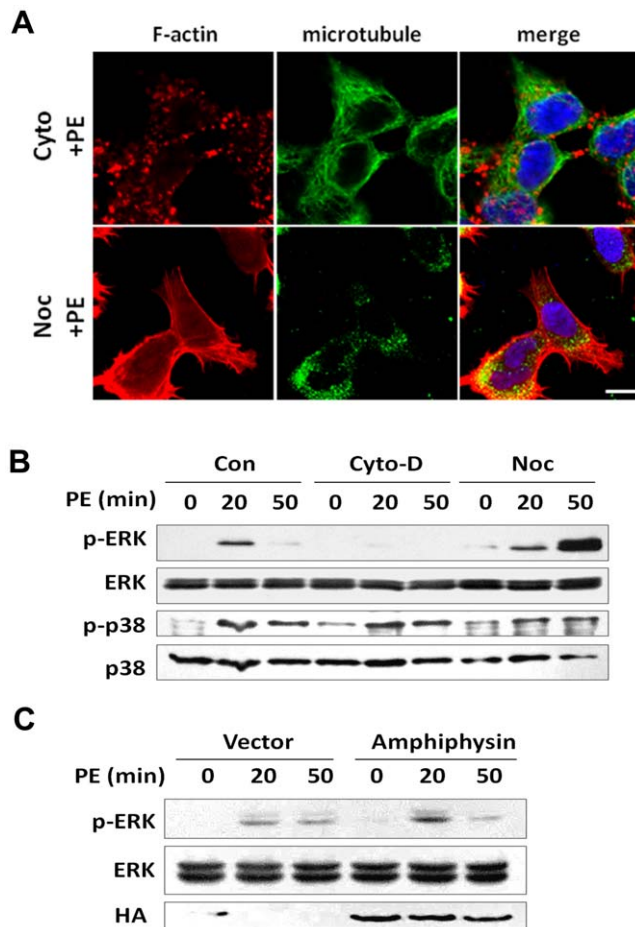
Figure 4. Actin organization is involved in ERK1/2 activation induced by α$_{1A}$-AR. (A) Effect of actin and microtubule-disrupting drugs on cytoskeleton organization after agonist stimulation. HEK-293A–α$_{1A}$-AR cells were pre-incubated with cytochalasin-D (Cyto-D; 5 μM, 5 min) or nocodazole (noc; 20 μM, 20 min) at 37°C, then stimulated with 10 μM PE for 20 min. F-actin was stained by TRITC-conjugated Phalloidin (red), and microtubules were stained with anti-α-tubulin antibody and Alexa-488 IgG (green). Nuclei were stained with Hochest-33342 (blue). Bar: 10 μm. (B) Effect of cytoskeleton disruption on the activation of ERK1/2 and p38 after α$_{1A}$-AR stimulation. HEK-293A–α$_{1A}$-AR cells were pre-incubated with Cyto-D (5 μM, 5 min) or nocodazole (20 μM, 20 min) in 37°C, then treated with 10 μM PE for 20 and 50 min. Cell lysates were immunoblotted for the phosphrylation of ERK1/2 and p38. (C) Overexpression of amphiphysin enhanced the activation of ERK1/2 after agonist stimulation. HEK-293A–α$_{1A}$-AR cells were transfected with amphiphysin plasmid, then treated with 10 μM PE for 20 and 50 min. Cell lysates were immunoblotted for analysis of phospho and total ERK1/2.
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for actin polymerization [29], in HEK-293A–α$_{1A}$-AR cells. Overexpressed amphiphysin increased the phosphrylation level of ERK1/2 at 20- but not 50-min stimulation (Fig. 4C), which agrees with results showing that F-actin preferentially mediates the early-stage but later-stage trafficking of α$_{1A}$-AR (Fig. 1). These data identified the association of F-actin in the ERK1/2 activation with α$_{1A}$-AR agonist stimulation.

ERK1/2 activation by α$_{1A}$-AR is independent of the G$_q$/PLC/PKC pathway

One of the earliest events in the signaling cascade initiated by α$_{1A}$-AR is G$_q$-mediated activation of PLC, with a resulting

increase in PKC phosphorylation [14]. To test whether the G$_q$/PLC/PKC pathway is involved in the α$_{1A}$-AR-induced activation of ERK1/2, we examined the phosphorylation level of PKC and its downstream C-Raf molecule by α$_{1A}$-AR with 4°C incubation or cytochalasin-D treatment. As compared with 37°C, 4°C incubation had no effect on the α$_{1A}$-AR-activation of PKC or C-Raf, but it inhibited the activation of ERK1/2 (Fig. 5A). Similarly, pretreatment with cytochalasin-D before PE did not impair α$_{1A}$-AR-induced PKC and C-Raf activation (Fig. 5B). Thus, α$_{1A}$-AR endocytosis was not involved in the G$_q$/PLC/PKC pathway.

To detemine the role of G$_q$/PLC/PKC signaling pathway in α$_{1A}$-AR-induced ERK1/2 activation, HEK-293A–α$_{1A}$-AR cells were treated with U73122 before PE exposure (Fig. 6A). U73122 is a pharmacological agent commonly used to demonstrate a role of G$_q$ activation of PLC, which inhibits the PLC-dependent process [30]. U73122 suppressed PKC phosphorylation but did not alter the activation of ERK1/2 with PE stimulation. To compare the role of G$_q$/PLC/PKC signaling in the activation of ERK1/2 by α$_{1A}$-AR and α$_{1B}$-AR, we also examined the effect of U73122 in HEK-293A cells stably transfected with α$_{1B}$-AR. U73122 inhibited both PKC phosphorylation and ERK1/2 activation in HEK-293A–α$_{1B}$-AR cells with PE stimulation (Fig. 6B). The PKC inhibitor Ro 31–8220 used before PE stimulation showed inhibition of PKC but not ERK1/2 activation with α$_{1A}$-AR induction (Fig. 6C), and inhibition of both PKC and ERK1/2 with α$_{1B}$-AR induction (Fig. 6D). The effects of U73122 and Ro 31–8220 on HEK-293A–α$_{1A}$-AR or –α$_{1B}$-AR cells suggest that the G$_q$/PLC/PKC pathway is required for ERK1/2 activation induced by α$_{1B}$-AR but not α$_{1A}$-AR.

Discussion

GPCRs activate MAPKS through distinct pathways in cells, and endosomal signaling has an important role in receptor signal transduction. We investigated the involvement of endocytosis in α$_{1A}$-AR-induced activation of ERK1/2 in HEK-293A cells. Agonist-mediated endocytic trafficking of α$_{1A}$-AR was assessed by real-time imaging of living, stably transfected cells. α$_{1A}$-AR was internalized dynamically in cells with agonist stimulation, and actin filaments regulated the initial trafficking of α$_{1A}$-AR. α$_{1A}$-AR-induced activation of ERK1/2 but not p38 MAPK was sensitive to disruption of endocytosis. α$_{1A}$-AR-induced activation of PKC and C-Raf by was not affected by endocytosis disruption. Thus, the endocytic pathway is involved in α$_{1A}$-AR-induced ERK1/2 activation and is independent of G$_q$/PLC/PKC signaling.

Real-time microscopy with high-tempo resolution has novel implications in the investigation of the behavior of receptors. For single particle tracking in this study, α$_{1A}$-AR was detected on the surface of living HEK-293A–α$_{1A}$-AR cells by use of a monoclonal primary antibody and Alexa-555 IgG. It should be aware of the potential effects of this labeling method on receptor trafficking and signaling. We found that the labeling with antibodies did not impair the calcium response of α$_{1A}$-AR among the labeled cells, as compared with non-labeled ones [31]. And it did not affect the ERK1/2 signaling (See Supporting Information Figure S1). Previously, we showed α$_{1A}$-AR transport with an average step size of 33 nm [21]. In the present work, α$_{1A}$-AR showed a spatial and time-dependent trafficking pattern with PE stimulation. Near the plasma membrane, the α$_{1A}$-AR particles were transported a relatively short distance (as shown in Fig. 1A and 1B, trajectory 1) within 8 sec. Inside the cells, the α$_{1A}$-AR particles moved farther (as shown in Fig.1A and 1B, trajectory 2) in the same time interval. Long-distance transportation allows α$_{1A}$-AR-containing vesicles to reach late endosomes or lysosomes, which were mostly located
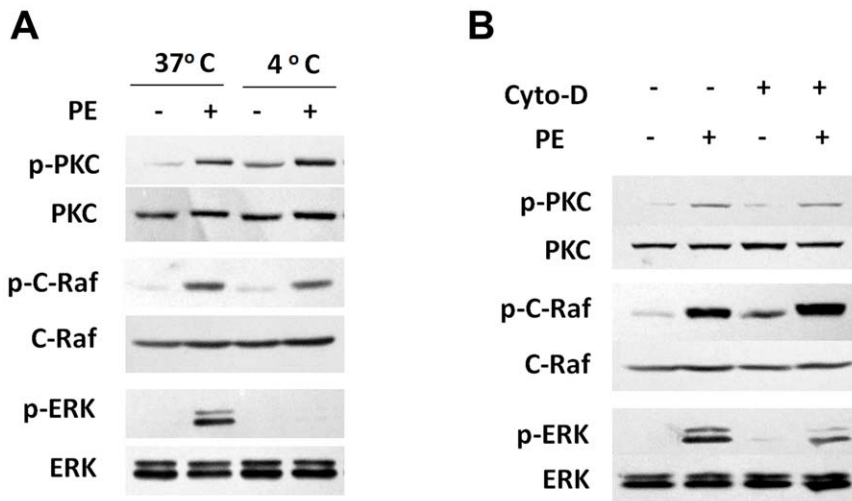
Figure 5. PKC and C-Raf activation with α_{1A}-AR stimulation is not impaired by 4°C chilling or F-actin disruption. (A) HEK-293A–α_{1A}-AR cells were pre-incubated at 4°C or 37°C for 30 min, then stimulated with 10 μM PE for 20 min. PKC, C-Raf and ERK1/2 activity was measured by western blot analysis. (B) HEK-293A–α_{1A}-AR cells were pre-incubated with or without 5 μM cyto-D for 5 min, then stimulated with 10 μM PE for 20 min. PKC, C-Raf and ERK1/2 activity was measured by western blot analysis.
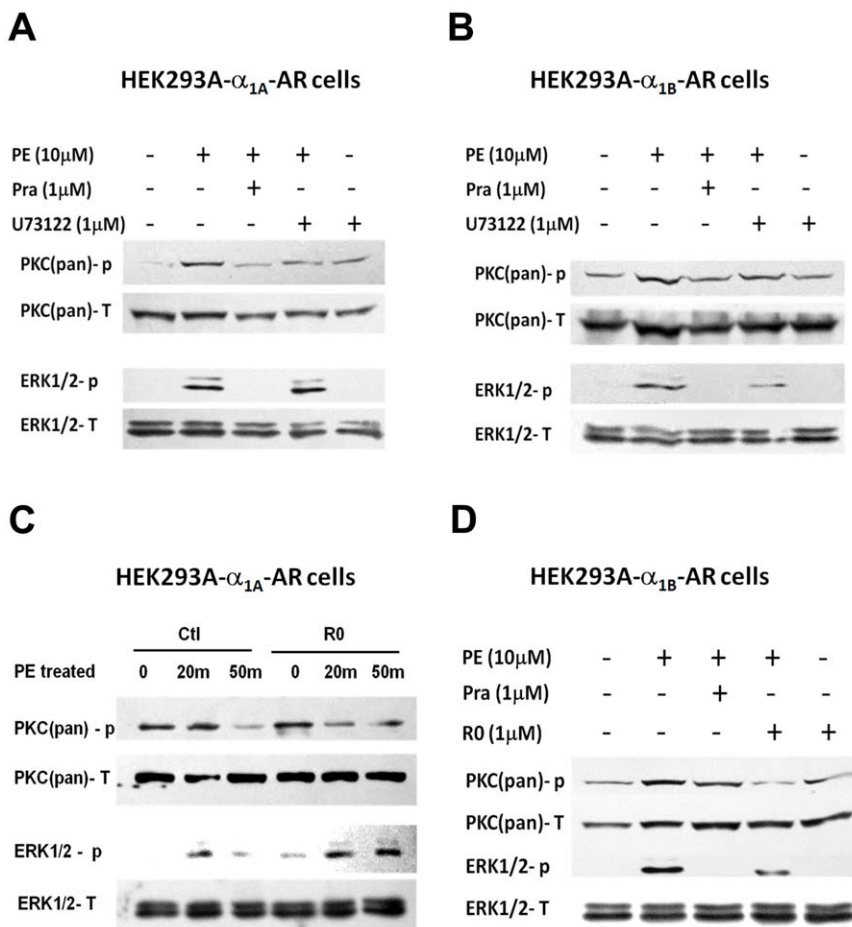doi:10.1371/journal.pone.0021520.g005

Figure 6. ERK1/2 activation by α_{1A}-AR is independent of G$_q$/PLC/PKC pathway. (A, B) Inhibition of phospholipase C (PLC) by U73122 affected ERK1/2 activation by α_{1B}-AR but not α_{1A}-AR. HEK-293A–α_{1A}-AR or –α_{1B}-AR cells were pre-incubated with 1 μM U73122 or prazosin or not for 30 min. Then cells were treated with 10 μM PE for 20 min. PKC and ERK1/2 activity was measured by western blot analysis. (C, D) Inhibition of PKC by Ro 31–8220 affected ERK1/2 activation by α_{1B}-AR but not α_{1A}-AR. HEK-293A–α_{1A}-AR or –α_{1B}-AR cells were pre-incubated with 1 μM Ro 31–8220 or prazosin or not for 30 min, then were treated with 10 μM PE for 20 min. PKC and ERK1/2 activity were measured by western blot analysis.
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near the nucleus. At the early stage of the activation, the receptor mainly moved at a slow velocity, at a peak of about 0.3 µm/sec, which is consistent with the velocity of a single myosin motor walking along actin *in vitro* [32,33]. After 40- to 60-min stimulation, the main peak of the higher velocity was about 0.8 µm/sec, which is similar to the value reported for movement along microtubules under *in vitro* conditions [34,35]. Thus, the movement of α₁A-AR vesicles mainly depends on actin at the early phase of endocytosis and on microtubules at the later phase. Confocal microscopy further verified that endocytic traffic of α₁A-AR was mediated by reorganized actin (Fig. 2A, 2B).

Disruption of endocytosis by 4°C chilling, dynamin mutation and F-actin depolymerizing all indicated that the endocytic process was associated in α₁A-AR-induced activation of ERK1/2. Many studies have implied a critical role for the ERK1/2 signaling pathway in cardiac myocytes [9–11]. Recently, α₁A-AR was shown to signal through ERK1/2 to promote cardiac hypertrophy [36] or survival signals [12]. α₁A-AR but not α₁B-AR rescued α1ABKO cardiac myocytes from cell death, and only α₁A-AR could mediate ERK1/2 activation in the myocytes [12]. Activation of phosphoinositide-3-kinase (PI3K) and Ras protein induces activation of a series of growth or proliferation-related protein kinase cascades [37]. Investigation of the role of PI3K and Ras in the activation of ERK1/2 by 2 α₁-ARs in NIH3T3 cells revealed that overexpression of a dominant-negative Ras mutant attenuated the α₁B-AR– but not α₁A-AR-mediated activation of ERK1/2. And overexpression of a dominant-negative PI3K mutant (p85 subunit) attenuated α₁A-AR- but not α₁B-AR-induced ERK1/2 activation [38]. Therefore, α₁A-AR and α₁B-AR differentially activate downstream effectors, which further underscores the complexity of α₁-AR signaling pathways. We found endocytic trafficking involved in α₁A-AR induced ERK1/2 activation and that it was independent of Gq/PLC/PKC signaling. By contrast, α₁B-AR-induced ERK1/2 activation required Gq/PLC/PKC signaling, as was previously reported [39]. Thus, α₁A-AR uses pathways different from those of α₁B-AR to activate ERK1/2.

A number of studies have found a non-Gq-protein signaling pathway in α₁A-AR function. An α₁A-AR mutant defective in Gq coupling could also activate calcium influx when coactivated with β₂-AR [16]. Thus, the functional interaction of these 2 receptors involves heterodimer formation and/or the presence of unidentified non-Gq signaling events in response to α₁A-AR stimulation. Activation of calcium influx and PKC may be key events coupling Gq- and Gi-coupled receptor activation to MAPK activation [40]. Berts et al. showed that the Ca²⁺ chelator BAPTA dose-dependently abolished norepinephrine (NE) -stimulated Ca²⁺ responses but not ERK1/2 activation. The potent PKC inhibitor bisindolylmaleimide I dose dependently inhibited ERK1/2 activation by phorbol ester tumor-promoting agent but not NE. Thus, Ca²⁺ release and PKC activation are neither necessary nor sufficient for α₁A-AR-mediated activation of ERK1/2 in PC12 cells stably transfected with α₁A-AR [41]. Therefore, ERK1/2 activation induced by α₁A-AR may be independent of traditional Gq-coupled second-messenger pathways. We found that α₁A-AR-induced ERK1/2 activation was unaffected by the PLC inhibitor U73122 and PKC inhibitor R0 31–822, which further identifies a non-Gq signaling pathway involved in α₁A-AR-mediated ERK1/2 activation.

Arrestins are critically important for desensitization, endocytosis, and G protein-independent signaling of GPCRs [42]. One of the early evidences that β-arrestins are active participants in signaling was the observation that dominant-negative mutants of β-arrestins inhibited β₂-AR-induced activation of ERK1/2 [18].

β-arrestins similarly participate in ERK1/2 signaling by other GPCRs, including neurokinin-1 receptor, protease activated receptor 2, angiotensin II type 1A receptor, and vasopressin V2 receptor [43–46]. The stability of receptor–β-arrestin complex controlled the mechanism and extent of ERK1/2 activation [44]. However, results from both coimmunoprecipitation experiments and β-arrestin translocation assays indicated that the agonist-induced interaction of α₁A-AR with β-arrestins was much weaker than that of α₁B-AR. In addition, α₁A-AR did not bind AP50, a subunit of the clathrin adaptor complex AP2. Moreover epinephrine-induced increase of the association of the α₁A-AR and β-arrestin 1 or 2 was not statistically significant [47]. Thus, defining the role of β-arrestins in the endocytosis of α₁A-AR and α₁A-AR-induced ERK1/2 activation is important. PI3K has a potential role in the α₁A-AR induced ERK1/2 activation [38] and has been found to regulate intracellular vesicular transport at multiple steps [48,49]. PI3K may be involved in α₁A-AR mediated ERK1/2 activation through an endocytosis pathway but remains to be elucidated.

In summary, we identified a putative role of endocytosis in the regulation of the MAPK pathway under α₁A-AR stimulation in cells. Through analysis of receptor tracking in live cells and confocal microscopy, we conclude that the α₁A-AR endocytic process is actin-related. Endocytosis and actin reorganization are involved in α₁A-AR induced activation of ERK1/2 but not p38, and Gq/PLC/PKC signaling is not required in this process. Thus, we reveal a novel pathway of ERK1/2 activation in α₁-AR subtypes. We provide the first biological evidence for a role of endocytosis in the signaling of the α₁-AR family, which challenges the classical view that α₁A-AR act through Gq/PLC/PKC signaling. Moreover, the mechanism by which α₁A-AR induces ERK1/2 activation differs from that of α₁B-AR. We provide a possible molecular explanation for the difference between α₁A-AR and α₁B-AR in activation of ERK1/2 in cardiac myocytes. Because receptor vesicles could link to various intracellular membrane compartments in the endocytic process, the distinct spatiotemporal profile of ERK1/2 activation induced by α₁A-AR has profound implications in α₁A-AR-mediated survival signaling in cardiac myocytes. In-depth studies of this signaling pathway should add great potential for developing more efficacious and/or safer treatment of heart failure and other clinical conditions.

Supporting Information

Figure S1 Labeling with antibodies did not impair the ERK1/2 signaling induced by α₁A-AR stimulation. HEK-293A–α₁A-AR cells were pre-incubated with or without anti-FLAG antibody and Alexa-555 IgG. After phenylephrine (PE) treatment, the phosphorylation level of ERK1/2 did not show significant difference among labeled and unlabeled cells. Prazosin (Pra, antagonist of α₁A-AR) inhibited the PE-induced ERK1/2 activation in both. (TIF)

Acknowledgments

Author Contributions

Conceived and designed the experiments: FL XZ QH YZ. Performed the experiments: FL KH XY NX ZL. Analyzed the data: FL KH YZ. Contributed reagents/materials/analysis tools: XY NX ZL MX. Wrote the paper: FL YZ.

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