Agonist-induced Localization of Gq-coupled Receptors and G Protein-gated Inwardly Rectifying K\(^+\) (GIRK) Channels to Caveolae Determines Receptor Specificity of Phosphatidylinositol 4,5-Bisphosphate Signaling*\(^{[5]}\)

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G protein-gated inwardly rectifying K\(^+\) (GIRK) channels are parasympathetic effectors in cardiac myocytes that act as points of integration of signals from diverse pathways. Neurotransmitters and hormones acting on the Gq protein regulate GIRK channels by phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) depletion. In previous studies, we found that endothelin-1, but not bradykinin, inhibited GIRK channels, even though both of them hydrolyze PIP\(_2\) in cardiac myocytes, showing receptor specificity. The present study assessed whether the spatial organization of the PIP\(_2\) signal into caveolar microdomains underlies the specificity of PIP\(_2\)-mediated signaling. Using biochemical analysis, we examined the localization of GIRK and Gq protein-coupled receptors (GqPCRs) in mouse atrial myocytes. Agonist stimulation induced a transient co-localization of GIRK channels with endothelin receptors in the caveolae, excluding bradykinin receptors. Such redistribution was eliminated by caveolar disruption with methyl-\(\beta\)-cyclodextrin (M\(\beta\)CD). Patch clamp studies showed that the specific response of GIRK channels to GqPCR agonists was abolished by M\(\beta\)CD, indicating the functional significance of the caveolae-dependent spatial organization. To assess whether low PIP\(_2\) mobility is essential for PIP\(_2\)-mediated signaling, we blocked the cytoskeletal restriction of PIP\(_2\) diffusion by latrunculin B. This abolished the GIRK channel regulation by GqPCRs without affecting their targeting to caveolae. These data suggest that without the hindered diffusion of PIP\(_2\) from microdomains, PIP\(_2\) loses its signaling efficacy. Taken together, these data suggest that specific targeting combined with restricted diffusion of PIP\(_2\) allows the PIP\(_2\) signal to be compartmentalized to the targets localized closely to the GqPCRs, enabling cells to discriminate between identical PIP\(_2\) signaling that is triggered by different receptors.

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Several lines of evidence suggest that neurohormonal imbalance plays a role in the generation of atrial fibrillation (1). Simultaneous sympathetic and parasympathetic (sympathovagal) activation facilitates the onset of paroxysmal atrial fibrillation (2). In addition, vasoactive peptide hormones such as endothelin-1 (ET-1)\(^{3}\) (3) and angiotensin II (4) have been implicated in atrial fibrillation. The cardiovascular actions of native peptides and receptors showed a wide range of variety. Unlike ET-1 and angiotensin II, myocardial bradykinin (BK) might be a mechanism to protect the heart during acute myocardial infarction (5, 6). The neurohormonal imbalances have seldom been sufficiently characterized to study their pathophysiology.

One possible candidate for cross-talk for sympathovagal interaction and neurohormonal interaction in cardiac myocytes is the acetylcholine activated K\(^+\) (GIRK) channel. Recently, we and others found that \(\alpha\)\(_1\)-adrenergic receptor agonist inhibits GIRK channels by depleting PIP\(_2\) in the plasma membrane, indicating a novel pathway for sympathetic-parasympathetic interaction (7, 8). ET-1 and angiotensin II can also regulate GIRK channel activity via PIP\(_2\) signaling (8, 9). Thus, GIRK channels might act as points to integrate hormonaland neurotransmitter signals from diverse pathways. However, although GIRK channels are regulated by PIP\(_2\), they do not respond to PIP\(_2\) hydrolysis from stimulation of all Gq-coupled receptors. We found that BK had no effect on GIRK channels, although being capable of hydrolyzing PIP\(_2\) in cardiac myocytes (9). It is possible that these selective responses could discriminate the beneficial effect associated with BK from other Gq protein-coupled receptor (GqPCR) pathways. Until now, the molecular mechanisms underlying receptor specificity have been mostly unknown.

Several mechanisms were suggested to explain the receptor-specific and target-specific PIP\(_2\) signaling. One of those mechanisms is the PIP\(_2\) microdomain (10). In this scenario, PIP\(_2\) abundance can change independently within a restricted area, so that the activation of a given GqPCR affects only

\(^{3}\) The abbreviations used are: ET-1, endothelin-1; ACh, acetylcholine; BK, bradykinin; B\(_{2}\)R, BK B\(_2\) receptor; Cav, caveolin; ET\(_{2}\)R, ET A receptor; GIRK, G protein-gated inwardly rectifying K\(^+\); GqPCR, Gq protein-coupled receptor; M\(\beta\)CD, methyl-\(\beta\)-cyclodextrin; PIP\(_2\), phosphatidylinositol 4,5-bisphosphate; qss, quasi-steady state; WGA, wheat germ agglutinin; WMN, wortmannin.
those targets that are partitioned in the same microdomain. However, it is unclear whether and how PIP$_2$-sensitive targets are co-localized with specific GqPCRs in atrial myocytes.

Recent data showed that caveolae are important for co-localization of receptors with their signaling partners (11). Caveolae are small invaginations of the plasma membranes, and caveolins are the main structural components of caveolae (12, 13). It has been suggested that by selectively excluding or concentrating signaling proteins, caveolae confer a degree of spatial organization on signal transduction pathways (11, 14). Some receptors and signaling proteins are not fixed in the membrane but are translocated into or out of caveolae upon receptor activation (11, 15–17).

The present study offers the direct evidence that agonist stimulation induces a transient co-localization of GIRK channels with ET A receptor (ET$_A$R) in caveolae, excluding BK B2 receptor (B2R) in mouse atrial myocytes, and that such compartmentalization is essential for receptor specificity in PIP$_2$ signaling. Thus, the PIP$_2$ microdomain created by caveolae plays a key role in the spatiotemporal coding of PIP$_2$ signals, ensuring specificity of GqPCRs in cardiac myocytes.

**EXPERIMENTAL PROCEDURES**

**Cell Isolation**—Mouse atrial myocytes were isolated by perfusing Ca$^{2+}$-free normal Tyrode solution containing collagenase (0.14 mg/ml; Yakult Pharmaceutical, Japan) on a Langendorff column at 37 °C as described (18). Isolated atrial myocytes were kept in high K+, low Cl$^-$ solution at 4 °C until use.

**Electrophysiology**—Membrane currents were recorded from single isolated myocytes in a perforated patch configuration by using nystatin (200 μg/ml; ICN) or ruptured whole cell patch clamp configuration at 35 ± 1 °C. Voltage clamp was performed by using an EPS-8 amplifier (HEKA Instruments) and filtered at 5 kHz. The patch pipettes (World Precision Instruments) were made by a Narishige puller (PP-830; Narishige, Tokyo). The patch pipettes used had a resistance of 2–3 mehmohms when filled with the below pipette solutions. Normal Tyrode solution contained 140 mM NaCl, 5.4 mM KCl, 0.5 mM MgCl$_2$, 1.8 mM CaCl$_2$, 10 mM glucose, and 5 mM HEPES, titrated to pH 7.4 with NaOH. The Ca$^{2+}$-free solution contained 140 mM NaCl, 5.4 mM KCl, 0.5 mM MgCl$_2$, 10 mM glucose, and 5 mM HEPES, titrated to pH 7.4 with NaOH. The high K$^+$ and low Cl$^-$ solution contained 70 mM KOH, 40 mM KCl, 50 mM L-glutamic acid, 20 mM taunine, 20 mM KH$_2$PO$_4$, 3 mM MgCl$_2$, 10 mM glucose, 10 mM HEPES, and 0.5 mM EGTA, adjusted to pH 7.3 with KOH. The pipette solution for perforated patches contained 140 mM KCl, 10 mM HEPES, 1 mM MgCl$_2$, and 5 mM EGTA, titrated to pH 7.2 with KOH. The pipette solution for ruptured whole cell patches contained 20 mM KCl, 110 mM potassium aspartate, 10 mM NaCl, 10 mM HEPES, 1 mM MgCl$_2$, 2 mM MgATP, 5 mM EGTA, and 0.01 mM GTP, titrated to pH 7.2 with KOH. To ensure a rapid solution turnover, the rate of superfusion was kept at >5 ml/min, which corresponded to 50 times bath volume (100 μl).

**Subcellular Fractionation**—Subcellular fractionation was performed as described previously (19). Briefly, atrial tissue were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 5 mM EGTA, 1 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride) supplemented with protease inhibitor mixture (Roche Applied Science) and fractionated by sequential centrifugation steps. The tissue homogenate was centrifuged at 500 × g for 15 min, and the pellet was discarded. The supernatant was centrifuged at 15,000 × g for 10 min at 4 °C to separate a vesicle-containing soluble fraction and membrane-containing pellet fraction. Samples were separated by 8% SDS-PAGE.

**Western Blotting and Immunoprecipitation**—Western blotting and immunoprecipitation analyses were done as described previously (19). Primary antibodies used were anti-GIRK1 (Alomone Laboratories), anti-M2AChR (Santa Cruz Biotechnology), anti-B2R (BD Transduction Laboratories), anti-ET$_A$R (BD Transduction Laboratories), anti-Cav-3 (BD Transduction Laboratories), anti-EEA1 (BD Transduction Laboratories), and anti-pan-cadherin (AbCam). Protein bands were quantified with ImageJ software (National Institutes of Health).

**Immunocytochemistry**—Immunostaining was performed on isolated mouse atrial myocytes as described previously (20). Briefly, atrial myocytes were plated on laminin (10 μg/ml)-coated coverslips for 3 h at 4 °C, fixed with 4% formaldehyde in phosphate-buffered saline (PBS) on ice, permeabilized in 5% goat serum in PBS with 0.1% Triton X-100 (30 min), incubated with the primary antibody against Cav-3 and WGA-Alexa-633 (overnight), followed by Alexa Fluor 488-conjugated anti-mouse secondary antibody for 1 h. Immunofluorescence was visualized with confocal laser scanning microscopy using a LSM510 apparatus (Zeiss). Cells were randomly selected and used for imaging and analysis, and immunostaining experiments were repeated at least five times. Control experiments performed by using secondary antibody without primary antibody showed no noticeable labeling.

**Confocal Ca$^{2+}$ Imaging**—For confocal Ca$^{2+}$ imaging, freshly isolated mouse atrial myocytes were loaded with Fluo-4 AM (5 μM; Molecular Probes) for 30 min followed by a 10-min washout allowing for deesterification. Fluorescence images were recorded using a Leica TCSSP2 confocal microscope with ×63 water immersion objective (Leica). Fluor-4 was excited with 488-nm laser, and the fluorescence was detected at >505 nm. All experiments were conducted at 35 ± 1 °C.

**Statistics and Presentation of Data**—Results in the text and the figures are presented as mean ± S.E. ($n$ = number of cells tested). Statistical analyses were performed by using Student’s $t$ test. The difference between two groups was considered to be significant when $p < 0.01$.

**RESULTS**

**Caveolar Disruption Abolishes Receptor Specificity of GIRK Channel Regulation**—From acutely isolated mouse atrial myocytes, membrane currents were recorded using a nystatin-perforated whole cell patch clamp technique, at a holding potential of −40 mV. When acetylcholine (ACh, 100 μM) was applied to the bath solution, $I_{GIRK}$ was promptly activated and
underwent variable degrees of desensitization (Fig. 1A, top). Consistent with previous data (7, 9), desensitization recovered after washout of ACh, so I_{GIRK} during a second ACh stimulation (I_2) was similar to that during the first stimulation (I_1) (Fig. 1B, right). The amplitudes of I_1 measured at its peak (I_{2,peak}) were 84.4 ± 2.4% of those of I_1 (I_{1,peak}) (n = 9; supplemental Fig. 1) and quasi-steady-state amplitudes of I_2 (I_{2,qss}) were 87.6 ± 2.3% of those of I_1 (I_{1,qss}) (n = 9, Fig. 1B). To investigate the effect of GqPCR-mediated signaling on I_{GIRK}, GqPCR agonists were applied together with ACh in the second stimulation, and I_2 was compared with I_1. Fig. 1A (middle) illustrates typical examples of these experiments showing the effects of ET-1 (30 nM). Addition of ET-1 during the second stimulation (I_2) obtained from the experiment shown in A, was superimposed, showing the effect of the drugs on I_{GIRK}. C, top, the second application of ACh was after 10-min treatment of MβCD. MβCD pretreatment did not affect I_{GIRK}. Middle, MβCD pretreatment attenuated the effects of ET-1 on I_{GIRK}. D, data are summarized for I_{2,qss}/I_{1,qss} in cells pretreated with MβCD, * p < 0.05, compared with ACh alone. Right, current traces obtained from the experiment shown in C are superimposed. Two representative traces for ET-1 or BK stimulated cells are shown.

that, as previously reported for these cells (9), GqPCR-induced regulation of I_{GIRK} was receptor-specific.

If caveolae compartmentalize GIRK channels to ET_ARs, excluding BRs, the response of GIRK channels should be forced to be nonspecific by disruption of caveolae. To test this scenario, mouse atrial myocytes were pretreated with MβCD, which disrupts caveolae by depleting cholesterol (21, 22). We confirmed that treatment with 3 mM MβCD for 10 min did not affect I_{GIRK}. The response to ACh following MβCD treatment (I_2) was similar to that to ACh before MβCD application (I_1) (Fig. 1C, top). I_{2,peak} and I_{2,qss} were 87.0 ± 5.5% (n = 8; supplemental Fig. 1) and 85.7 ± 2.3% (Fig. 1D) of those of I_1 (I_{1,peak} and I_{1,qss}), respectively, which were not significantly different from the relative I_{2,peak} and I_{2,qss} under control. Then, the effects of ET-1 and BK on I_{GIRK} were examined in MβCD-treated cells. As in the control, GqPCR agonists accelerated the decay phase of I_{GIRK}, without significant changes in peak amplitude. However, the inhibitory effect of ET-1 (30 nM) on I_{GIRK} was significantly attenuated and its effect varied in a wide range among cells (Fig. 1, C, middle, and D, right). On average, the I_{2,qss} was 53.8 ± 9.3% (n = 9) of the I_{1,qss} in MβCD-treated cells, which was significantly different from the result in the control (24.6 ± 5.9%, n = 6, p < 0.05). A dose-response curve performed in the presence after MβCD
pretreatment was shifted to the right, with decrease of its maximum response (supplemental Fig. 2). BK stimulation also showed variable effects on \( I_{\text{GIRK}} \) following M\( \beta \)CD treatment. In some cases, BK, which showed a minimal effect on \( I_{\text{GIRK}} \) under control, induced a robust inhibition of \( I_{\text{GIRK}} \) (Fig. 1C, bottom). On average, BK reduced \( I_{\text{GIRK}} \) to 54.4 ± 12.1% (\( n = 7 \)) of \( I_{\text{GIRK}} \) (Fig. 1D). BK suppression of \( I_{\text{GIRK}} \) was greater than the meager BK effect in control conditions and indistinguishable from the extent of ET-1-induced inhibition after M\( \beta \)CD pretreatment (\( p > 0.05 \), Fig. 1D). Thus, these data showed that the receptor-specific inhibition of \( I_{\text{GIRK}} \) by GqPCR agonists was not observed when caveolae was disrupted, suggesting that caveolae are signaling platforms where receptor-specific regulation of GIRK channel is taking place in cardiac myocytes.

Association of M2AChR/GIRK Channel Complexes with Cav-3 during ACh Stimulation—The next experiment examined whether GIRK channels and GqPCRs are localized to caveolae. Initially, the presence of caveolae in mouse atrial myocytes was confirmed. Consistent with a previous study (21), immunostaining using the antibody against caveolin-3 (Cav-3, green), a marker for caveole of cardiac myocytes, clearly revealed Cav-3 along the peripheral sarcolemma and T tubules, coinciding with the location of the membrane dye WGA (red) (Fig. 2A).

The spatial resolution of confocal microscopy is inadequate to localize proteins to caveolae definitively given the small size of caveole (50–100 nm). Therefore, co-immunoprecipitation experiments were used to determine whether GIRK channels were localized to caveolae. Co-immunoprecipitation experiments showed that GIRK1 protein, the GIRK channel subunit, interacts with Cav-3 (Fig. 2B), suggesting the localization of GIRK1 at caveolae. Stimulation of M2AChR with ACh significantly increased the association of GIRK1 with Cav-3. This effect was prevented by the caveolar disruption with M\( \beta \)CD, suggesting that this process requires intact caveolae. In contrast, the amount of M2AChR that was co-immunoprecipitated with the GIRK channels was not affected by either ACh alone or combined treatment of M\( \beta \)CD and ACh. Consistent with a previous report (23), these data suggest that GIRK channels form stable complexes with M2AChR. The marginal effect of M\( \beta \)CD treatment on the responses of GIRK channels for ACh may be due to the relatively strong association of M2AChR and GIRK proteins (Fig. 1D). The expression levels of Cav-3 and M2AChR were not changed after agonist stimulation. ACh stimulation did not affect the localization of GIRK1 proteins (Fig. 2C). Taken together, these data support the suggestion that ACh treatment may induce the movement of GIRK1 proteins on the membrane to Cav-3 inside the caveole structure.

Agonist Stimulation Increases Caveolar Association of ET\(_A\)R and Decreases B\(_R\)R Association—The next experiment examined the association of GqPCRs with Cav-3. ET\(_A\)R and B\(_R\)R mediate ET-1- or BK-induced PIP\(_2\) hydrolysis in cardiac myocytes, respectively (24, 25). Although treatment of ET-1 (30 nm, 4 min) increased the association of ET\(_A\)Rs with Cav-3 (\( p < 0.01 \)), BK application (10 \( \mu \)M, 4 min) decreased the association of B\(_R\)Rs with Cav-3 (\( p < 0.05 \)) (Fig. 3A). M\( \beta \)CD pretreatment abrogated both of the ET-1 and BK effects (\( p < 0.05 \)), suggesting that both processes require an intact caveole structure.

Next, it was determined whether ET\(_A\)R and GIRK channels interact with each other in caveolae. ET\(_A\)Rs proteins were co-immunoprecipitated with GIRK1, and the association of ET\(_A\)R with GIRK1 was increased by concurrent stimulation of ET\(_A\)Rs and M2AChR/GIRK channel signaling pathway by ET-1 and ACh treatment (\( p < 0.05 \)) (Fig. 3B). M\( \beta \)CD pretreatment blocked this process (\( p < 0.05 \)), suggesting that the association of ET\(_A\)R and GIRK proteins requires the caveolae.

Cytoskeletal Restriction of PIP\(_2\) Diffusion Is Necessary for PIP\(_2\) Signaling—Our previous simulation study (26) showed that the low mobility of PIP\(_2\) promotes locally high [PIP\(_2\)] changes and limits PIP\(_2\) signal into neighboring regions, contributing to the formation of PIP\(_2\) microdomains. However, there is no direct evidence that low PIP\(_2\) mobility is necessary for PIP\(_2\)-mediated signaling in cardiac myocytes. To examine whether the change in PIP\(_2\) mobility affects GqPCR-induced GIRK channel regulation in mouse atrial myocytes, latrunculin B, an inhibitor of actin filament polymerization, was utilized to manipulate PIP\(_2\) mobility. PIP\(_2\) diffusion increases...
markedly upon cytoskeletal disruption (26). When atrial myocytes were pretreated with latrunculin B (10 μM) for 30 min, the regulation of GIRK channels by ET-1 was completely abolished, so that $I_{1}$ and $I_{2}$ are almost completely overlapped (Figs. 4A).

$I_{2}$,qss in the presence of ET-1 was 89.7 ± 5.0% ($n = 6$) of the $I_{1}$,qss, which is significantly different from that under control condition (24.6 ± 5.9% ($n = 6$), $p < 0.01$, Fig. 4C). BK also had no effect on $I_{GIRK}$ (Fig. 4B). The relative amplitude of $I_{2}$,qss ($I_{2}$,qss/$I_{1}$,qss) in the presence of BK was 91.3 ± 3.9% ($n = 3$; Fig. 4C).

To rule out the possibility that latrunculin B affects PIP$_2$-mediated signal by blocking the dynamic targeting, the association of GIRK channels with Cav-3 in latrunculin B-pretreated cardiac myocytes was examined. ACh stimulation increased the association of Cav-3 with the GIRK channel regardless of latrunculin pretreatment (Fig. 4D), and latrunculin B also did not affect the increase in the association of ET$_{AR}$ to Cav-3 during ET$_{AR}$ stimulation (Fig. 4E). These data indicate that latrunculin treatments did not inhibit the caveolar targeting of GIRK channels or GqPCRs. Taken together, these data suggest that latrunculin B might affect GqPCR-induced GIRK regulation by disrupting the barrier to PIP$_2$ diffusion, suggesting the critical importance of low PIP$_2$ mobility in efficient PIP$_2$-mediated signaling.
PIP<sub>2</sub> Regeneration Is Not Involved in Receptor Specificity of GIRK Channel Regulation—An alternative hypothesis of receptor specificity for GIRK channel regulation is that as exemplified by the N-type Ca<sup>2+</sup> channel modulation in SCG neuron (27), B<sub>2</sub>Rs promote activity of PI4-kinase, which compensates for the PIP<sub>2</sub> depletion induced by the phospholipase C pathway. If this is true in atrial myocytes, then it should be possible to for B<sub>2</sub>R to modulate GIRK channels in atrial myocytes by acute PI4-kinase blockade. To test this possibility, 10 μM BK was applied in the presence of 50 μM wortmannin (WMN) (Fig. 5A). This brief treatment (~10 min) by WMN should be sufficient to block PI4-kinase but would not be long enough to cause substantial PIP<sub>2</sub> depletion by itself, as evidenced by the only minor rundown of I<sub>GIRK</sub> during WMN application (7). Although this maneuver indeed conferred to B<sub>2</sub>Rs the ability to suppress I<sub>GIRK</sub> with the same potency as for muscarinic M1 receptors in SCG neurons (28), it did not cause BK to induce an inhibition of I<sub>GIRK</sub> (Fig. 5A). In the WMN-treated cells the qss amplitude of I<sub>L</sub> in the presence of BK was 92.2 ± 2.1% (n = 10) of I<sub>L,qss</sub>. This was not significantly different from I<sub>L,qss</sub>/I<sub>L,qss</sub> in the control experiments (91.8 ± 1.6%, n = 10, p > 0.05).

The potent Gq regulation of PIP<sub>2</sub> synthesis was mediated via increases of [Ca<sup>2+</sup>]i, as acting as the stimulus (28). Therefore, an experiment was done to assess whether blocking the increase of intracellular Ca<sup>2+</sup> would allow B<sub>2</sub>R modulation of GIRK channels. Fig. 5B shows a perforated patch experiment on atrial myocytes pretreated with BAPTA-AM (10 μM, 30 min), a membrane-permeant Ca<sup>2+</sup>-specific chelator. The cells did not respond to BK stimulation; I<sub>L,qss</sub> was 91.3 ± 2.0% (n = 10) of I<sub>L</sub>, similar to control cells (p > 0.05). BAPTA under ruptured whole cell conditions produced similar results (90.9 ± 2.5%, n = 6). The data are summarized in Fig. 5C.

Next, the ability of BK to mobilize Ca<sup>2+</sup> from intracellular IP<sub>3</sub> stores in atrial myocytes was assessed. [Ca<sup>2+</sup>]i, was measured in undialyzed cells with the membrane-permeant dye Fluo-4 AM (Fig. 5D). In contrast to BK stimulation in neurons, Ca<sup>2+</sup> mobilization was not detectable in atrial myocytes. Taken together, unlike stimulation of B<sub>2</sub>Rs in SCG neurons, B<sub>2</sub>Rs in atrial myocytes may not induce a compensatory PIP<sub>2</sub> synthesis during concurrent PIP<sub>2</sub> depletion.

DISCUSSION

The results of the present study demonstrate that the agonist-induced association of GIRK channel and selective receptors to caveole is necessary for receptor-specific regulation of GIRK channels. In contrast to B<sub>2</sub>Rs, ET<sub>1</sub>Rs are clustered with GIRK channels to form discrete signaling complexes during receptor stimulation. The low PIP<sub>2</sub> mobility limits the propagation of the PIP<sub>2</sub> signal and confines PIP<sub>2</sub> depletion to ET<sub>1</sub>Rs and co-localized GIRK channels, ensuring specificity of the signaling pathway.

Signaling Microdomains for PIP<sub>2</sub>—The results show that agonist stimulation induced signaling complexes linking ET<sub>1</sub>Rs and GIRK channels, excluding B<sub>2</sub>Rs. The signaling pathway involved in GIRK channel regulation is illustrated schematically in Fig. 6. Immunoprecipitation analysis suggests that M2AChR stimulation can induce the targeting of the M2AChR-GIRK channel complex to Cav-3, most probably via their lateral redistribution along the cell surface (Fig. 2). Immunoprecipitation data for GqPCR localization suggest that agonist stimulation might also induce the targeting of ET<sub>1</sub>Rs to Cav-3 but diminish B<sub>2</sub>R binding to Cav-3 (Fig. 3). By binding GIRK channel and ET<sub>1</sub>R receptors in caveole, Cav-3 might bring both GIRK channel and ET<sub>1</sub>R in close proximity to each other, enabling these proteins to interact. This notion is supported by the fact that co-precipitation of ET<sub>1</sub>R and GIRK channels was increased after simultaneous activation of both pathways. In contrast, B<sub>2</sub>R dissociated from Cav-3 during B<sub>2</sub>R stimulation. By losing the scaffolding network, they could not interact with GIRK channel complexes.

Caveolar disruption eliminated the receptor specificity of PIP<sub>2</sub>-mediated GIRK channel regulation (Fig. 1), suggesting that compartmentalization of GIRK channels and GqPCRs by caveole may be important for specificity of PIP<sub>2</sub> signals. However, co-localization of GIRK channels with their signaling partners can be attributed to, but is not solely sufficient, to cause receptor-specific regulation of GIRK channels. The present results show that when PIP<sub>2</sub> mobility was high, GqPCR-induced regulation of GIRK channel was abolished (Fig. 4), suggesting that low mobility of PIP<sub>2</sub> may be necessary for GqPCR-mediated regulation of GIRK. Thus, it is conceivable that only when combined with low PIP<sub>2</sub> mobility, can complexes of ET<sub>1</sub>Rs with GIRK channels induce changes in [PIP<sub>2</sub>]i in close proximity to GIRK channels high enough to induce a strong inhibition of GIRK channels. In line with this, B<sub>2</sub>Rs, which are physically excluded and remote from GIRK channel domains, might fail to deplete PIP<sub>2</sub> near GIRK channels. This could prevent B<sub>2</sub>R from inhibiting GIRK channels. Furthermore, the possibility that receptor specificity of PIP<sub>2</sub> signaling could be induced by differential abilities of GqPCRs.
Microdomains Define Specificity of PIP₂ Signaling

![Diagram of signaling microdomain for specific PIP₂ signal.](Image)

FIGURE 6. Schematic diagram of signaling microdomain for specific PIP₂ signal. Stimulation of M2AChR/GIRK pathway and ET₄R induce the recruitment of each component to Cav-3, which allow ET₄R to interact with GIRK channels (upper). In contrast, B₂R are dissociated from Cav-3 during B₂R stimulation. By losing scaffolding network, they cannot interact with GIRK channel complexes any more (lower).

...to induce a compensatory PIP₂ synthesis during concurrent PIP₂ depletion could be ruled out (Fig. 5). In summary, given a diffusion-limited PIP₂ signal, the geometric relationship between GIRK channels and GqPCRs determines the precise nature of the physiological response to this signal.

Caveolae as Organizer of GIRK Channel Signaling Network—
Caveolae are abundantly present in cardiac myocytes, including ventricular, atrial, and nodal cells (12). Although the caveolae of ventricle modulate excitation-contraction coupling, β₂-adrenergic signaling, and ATP-sensitive K⁺ channel regulation (21, 22, 29), the role of caveolae in the atria remains unclear. The current data showed that caveolae can act as signaling platforms linking GIRK channels and GqPCRs in mouse atrial myocytes. To our knowledge, this is the first study to show the role of caveolae in the receptor specificity of PIP₂ signaling in native cardiac myocytes.

The ability of caveolae to coordinate signaling pathways is related to the ability of selectively recruiting a certain set of signaling molecules while excluding others. Still, the mechanism responsible for reversible caveolin-protein interaction is unclear. It is conceivable that GIRK channels might be passively recruited to Cav-3 together with M2AChR, based on the fact that GIRK channels were occupied in stable association with M2AChR (Fig. 2) (23), which is known to translocate into the caveolae upon stimulation (30). In the case of ET₄R, it is possible that its palmitoylation after agonist stimulation increases the affinity of ET₄R for the caveolar membrane (31, 32). In contrast to these receptors, B₂R seems to translocate out of, rather than into, caveolae with receptor activation. These data conflict with reports showing the translocation of B₂Rs into caveolae upon agonist stimulation in A431 cells (33) and HEK293 cells (34). This discrepancy implies that the redistribution of GPCR to stimulation differs depending on cell type (35). Experimental manipulations such as exposure time to agonist also need to be considered; presently, the effects of acute stimulation (4 min) on the localization of receptor were observed, whereas others focused on the effects of prolonged simulation (10–30 min) (33, 34). Indeed, receptor trafficking out of caveolae is not uncommon. A₁-adenosine receptors of cardiac myocytes (15) and adrenergic β₂-receptors were reported to exit caveolae upon receptor stimulation. Thus, dynamic targeting of GPCRs and stimulus-dependent protein-protein associations could be the logical mechanisms to ensure specificity of signaling, at the same time permitting similar pathways to exist in different parts of the same cell (Fig. 6).

Because caveolae contribute to the fine spatial control of PIP₂ signaling, changes in the caveolae density/function will perturb the compartmentalization of PIP₂ signals, thereby affecting the ability of cardiac cells to produce the receptor-specific GIRK channel responses. Given that changes in caveolin expression, location, and caveolae density are seen with age, in cardiovascular diseases including cardiac hypertrophy, cardiac failure, and diabetes and following the use of drugs such as statins for treatment of cardiovascular disease (36), this has wide implications. It is possible that dysregulation of the caveolar organization could contribute to arrhythmia or heart failure by affecting GIRK signaling complexes and consequently resulting in sympathovagal imbalance (37, 38). This hypothesis is strongly supported by the previous study reporting the development of heart failure in Cav-3⁻/⁻ mice (11).

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