Caveolae-Independent Activation of Protein Kinase A in Rat Neonatal Myocytes

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Received February 25, 2005; Accepted April 20, 2005

Abstract. Cardiomyocytes express both β₁- and β₂-adrenergic receptors, and these receptors play a differential role in chronotropic and inotropic effects of the heart. Caveolae are known as an important regulator of G-protein-coupled receptor signaling. In the present report, we examined whether caveolae have a role in β-adrenergic receptor-stimulated cAMP production and protein kinase A activation in neonatal myocytes. Isoproterenol-stimulated cAMP production was mediated by β₁- and β₂-subtypes, which depends on the receptor number of each subtype. However, protein kinase A activation was exclusively mediated by the β₁-subtype. Disruption of caveolae by methyl-β-cyclodextrin treatment did not affect the relative contribution of subtypes to isoproterenol-stimulated cAMP production. β₁-Subtype-mediated protein kinase A activation was also not affected by the disruption of caveolae. These results suggest that β₁-adrenergic receptor-mediated protein kinase A activation is compartmentalized and independent of caveolae.

Keywords: caveolae, β-adrenergic receptor, protein kinase A, cAMP, neonatal myocyte

Introduction

β-Adrenergic receptor (β-AR) is classified into three subtypes: β₁, β₂, and β₃. Cardiomyocytes express β₁- and β₂-syntypes. The number of β₁- and β₂-ARs is 70 – 80% and 20 – 30% of the total β-ARs, respectively. These subtypes couple with heterotrimeric G-protein G, and activate protein kinase A (PKA) through production of cAMP (1). PKA activation predominantly mediated by β₁-AR regulates heart rate and contraction through phosphorylation of key target proteins such as L-type calcium channel, phospholamban, and troponin I (2, 3). However, it has been reported in cardiomyocytes prepared from β₁-AR knockout mice that the effect of isoproterenol on the contraction rate is evident and biphasic with an initial increase followed by a sustained decrease that is inhibited by pertussis toxin (PTX) treatment (4). These data indicate that β₁-AR couples with Gs. It also indicates that β₂-AR couples with Gs as well as Gq. Although β₁- and β₂-ARs increase the contraction rate, there is a difference about the signaling pathway downstream of β₁- and β₂-ARs activation. In cardiomyocytes or β₁-AR transgenic mouse heart, sustained activation of β₁-AR induces apoptosis, but β₂-AR stimulation elicits a cardiac protective effect against apoptosis (5 – 7). These results suggest that the responses regulated by β₁-AR and β₂-AR are mediated by different signal transduction pathways.

There are microdomains on the cell surface that participate in cellular signal transduction. Lipid rafts are small and mobile membrane subdomains, and caveolae are flask-shaped invaginations on the cell surface containing caveolin. Both lipid rafts and caveolae are highly enriched in cholesterol and lipids with saturated acyl side-chains and resist solubilization by non-ionic detergents such as Triton X-100 at low temperatures. Many proteins participating in signaling are localized in the lipid rafts and caveolae (8). The mammalian caveolin gene family consists of three members: caveolin-1, caveolin-2, and caveolin-3. Caveolin-3 is dominantly expressed in muscle tissues including the heart (9). In neonatal mouse cardiomyocytes, caveolin-3 associates...
with β2- but not β1-ARs, and this association depends on cholesterol content (10). It was reported that β1-AR and Goα are localized in the caveolae and other cell surface areas, whereas β2-AR and Goα are localized in the caveolae in cardiomyocytes (11). Another report using adult rat cardiomyocytes showed that caveolin-3 is communoprecipitated with Goα but not Goβ (12). Thus, two β-AR subtypes show differential localization and signaling in cardiomyocytes. In the present study, we examined the role of caveolae in β1- or β2-ARs-mediated signaling in rat neonatal cardiomyocytes.

Materials and Methods

Materials

Isoproterenol, methyl-β-cyclodextrin (MβCD), CGP-20712A, and ICI-118,551 were purchased from Sigma-Aldrich (St. Louis, MO, USA). DMEM and penicillin/streptomycin were from Invitrogen (Carlsbad, CA, USA). Collagenase, liberase, and Fugene 6 were from Roche Diagnostics (Mannheim, Germany).

Cell culture and adenovirus infection

Cardiomyocytes were isolated from 1 – 2-day-old Sprague-Dawley rats as described previously (13). Cells were plated on 2% gelatin-coated plates and cultured in DMEM with 5% FBS at the density of 3 × 10^5 cells/mL. After 24 h, the cells were infected with recombinant adenoviruses for 2 h at 37°C. After 24 h starvation, cells were incubated overnight in culture medium containing 1 μCi/mL [³H]-adenine (Perkin-Elmer Life Sciences, Turku, Finland). The cells were stimulated in DMEM containing 20 mM HEPES (pH 7.4) and 1 mM 3-isobutyyl-1-methylxanthine (IBMX) at 37°C. The reaction was stopped, and [³H]-cAMP was isolated with Dowex 50 and alumina columns (14). Cyclic AMP production was calculated as the ratio of [³H]-cAMP to total [³H]-nucleotides per well and normalized as fold stimulation.

Determination of VASP (vasodilator-stimulated phosphoprotein) phosphorylation

Cardiomyocytes in 6 wells dishes were infected with adenovirus expressing Flag-tagged VASP at 50 MOI (multiplicity of infection). Forty-eight hours after infection, cells were stimulated with isoproterenol and harvested with RIPA buffer, and VASP was detected by immunoblotting analysis using anti-Flag antibody (mouse monoclonal IgG 1:10,000 dilution) from Sigma-Aldrich.

Plasmid construction and production of adenoviruses

The plasmids encoding human β1- and β2-ARs and a mutant of regulatory subunit of PKA (PKAδR1α) were provided by Drs. Robert J. Lefkowitz and Stanley McKnight, respectively. As PKAδR1α binds the catalytic subunit of PKA, but cannot bind cAMP, it behaves as a dominant negative mutant of the regulatory subunit of PKA. The tag of hemagglutinin (HA) was introduced at the amino-terminus of β1- and β2-ARs (15). Adenoviruses expressing VASP, GFP, and PKAδR1α were produced as described (16).

Statistical analyses

The experiments were repeated at least three times, and the results are shown as the mean ± S.E.M. P values are calculated with Student’s t-test.

Results

Localization of β1-, β2-ARs and caveolin-3

At first, we examined the localization of β1-, β2-ARs and caveolin-3 in cardiomyocytes. Figure1 shows immunostaining of caveolin-3, which is a marker of caveolae (Fig. 1: C and D). Caveolae were stained as punctate. However, this punctate staining was changed to a diffused pattern by the treatment with 10 mM MβCD that extracts cholesterol from the caveolae and disrupts them (data not shown). Figure 1 also shows the localization of HA-tagged β1- and β2-ARs expressed in cardiomyocytes (Fig. 1: A and B). We could not detect
significant amount of HA-tagged β1- and β2-ARs because the transfection efficiency by Fugene 6 was less than 2%. Localization of β1-AR does not match that of caveolin-3, indicating that β1-AR is largely localized outside of the caveolae (Fig. 1E). In contrast with β1-AR, a portion of β2-AR was localized in the caveolae (Fig. 1F). Therefore, β1-AR is almost exclusively localized outside of the caveolae and β2-AR is localized in the caveolae and non-caveolae areas.

**Effect of MβCD on cAMP production by β1- and β2-ARs stimulation**

We next examined cAMP contents by receptor stimulation. Stimulation of β1- and β2-ARs increases cAMP through activation of Gs and adenylyl cyclase. As a β1-selective agonist was not commercially available, cardiomyocytes were stimulated with non-selective β1-AR agonist isoproterenol in the presence of a subtype-selective antagonist such as CGP-20712A (β1-selective antagonist) or ICI-118,551 (β2-selective antagonist). CGP-20712A inhibited cAMP accumulation in a dose-dependent manner (Fig. 2A). ICI-118,551 also inhibited cAMP production. However, the inhibition curve by ICI-118,551 was biphasic. The two phases of the inhibition curve reflect the differential affinities of ICI-118,551 for β1- and β2-ARs. Using human hearts and [125I]-iodocyanopindolol, Kᵢ values of ICI-118,551 for β1- and β2-ARs are reported to be 8.1 × 10⁻⁸ M and 8.0 × 10⁻¹⁰ M, respectively (17). The affinities of CGP-20712A for β1- and β2-ARs are 7.0 × 10⁻⁹ M and
proterenol-stimulated VASP phosphorylation is mediated in the presence of 10^{-6} M. Based on the inhibition curves, approximately 100 times higher affinity for CGP-20712A or 10^{-3} M for CGP-20712A has approximately 430 times higher affinity for CGP-20712A. PKA activation by such as mitogen-activated protein kinase. We examined PKA and PKA activates many downstream signaling proteins in response to isoproterenol stimulation was almost completely inhibited by PKA activation was assessed by the phosphorylation state of VASP. VASP was originally discovered as a substrate for PKA and protein kinase G in human platelets and for PKA and protein kinase G in human platelets and is a useful tool to detect the activation of these kinases, as the mobility of VASP on SDS-polyacrylamide gel is retarded by phosphorylation. In cardiomycocytes expressing Flag-tagged VASP, isoproterenol stimulation resulted in VASP phosphorylation. This phosphorylation was almost completely inhibited by PKAαRIα (a dominant negative mutant of the regulatory subunit of PKA) (Fig. 3C). This result indicates that isoproterenol-stimulated VASP phosphorylation is mediated only by PKA activation. To determine the relative contribution of β_{1}- and β_{2}-ARs to PKA activation, cardiomycocytes were stimulated with isoproterenol in the presence of CGP-20712A or ICI-118,551. CGP-20712A inhibited PKA activation in a dose-dependent manner, similar to the inhibition curve of cAMP production (Fig. 3: A and B). However, ICI-118,551 did not inhibit PKA activation up to 10^{-6} M. These data show that PKA is mainly activated through β_{1}-AR. It also indicates that β_{2}-AR-stimulated cAMP production does not participate in PKA activation, although β_{2}-AR stimulation increases cAMP contents.

We next examined whether disruption of caveolae influences PKA activation. To evaluate β_{1}- and β_{2}-AR-mediated PKA activation separately, we included CGP-20712A or ICI-118,551 in the concentration of 10^{-6} M. β_{1}-Adrenergic receptor-stimulated VASP phosphorylation was strongly inhibited by CGP-20712A and partially inhibited by ICI-118,551 under the present conditions (Fig. 3: A and D). When the cells were treated with MβCD, isoproterenol-stimulated PKA activation was slightly but not significantly decreased (Fig. 3D). However, PKA activation in the presence of CGP-20712A or ICI-118,551 in MβCD-treated cells was essentially the same as that of the untreated cells. This result suggests that PKA activation by β_{1}-AR stimulation is independent of caveolae.

Discussion

The present study demonstrated the localization of β_{1}- and β_{2}-ARs and the role of caveolae on β_{2}-AR-mediated cAMP production and PKA activation in rat neonatal myocytes. β_{1}-AR was localized exclusively in the non-caveolae areas, and β_{2}-AR was localized in the caveolae and non-caveolae areas. Stimulation of β_{2}-ARs with isoproterenol increased cAMP production, and the ratio of β_{1}- and β_{2}-subtype-mediated cAMP production was almost equal to the proportion of expression level of each subtype. Cyclic AMP production through β_{1}- and β_{2}-ARs was enhanced by the disruption of caveolae. However, β_{2}-AR-stimulated PKA activation was almost exclusively mediated by the β_{1}-subtype, and independent of caveolae.

It has been reported that the localization of FLAG-tagged β_{2}-AR is well-matched to caveolin-3 in mouse neonatal cardiomyocytes (10). However, the present result showed that only a portion of β_{2}-AR is localized in the caveolae (Fig. 1). One of possibilities to explain this difference is the level of expression. When the amount of β_{2}-AR exceeds the cell’s capacity to accommodate it, β_{2}-AR may not traffic to the proper domain in cells. The myocytes prepared from different species may also explain this difference. We used rat neonatal myocytes in the present study. However, they used mouse neonatal myocytes prepared from β_{1}- and β_{2}-ARs knockout mice (10). Mouse neonatal myocytes may have the ability...
to accommodate larger amounts of β-ARs. Species difference of β-ARs may be another factor for this difference. We expressed human β1- and β2-ARs, and they expressed mouse β1- and β2-ARs. As mouse β1-AR but not human β1-AR internalizes upon agonist stimulation (19, 20), mouse β-ARs translocate to a specific domain more efficiently than human β-ARs. In the literature, there is controversy about the localization of β-AR subtypes. Ostrom et al. reported that HA-tagged β1-AR heterogeneously expressed in cardiomyocytes is detected as a single 72-kDa band in the caveolae fraction (21). However, Rybin et al. reported that endogenous β1-AR in cardiomyocytes is identified as multiple species that distribute over the caveolae and non-caveolae fractions (11). The precise mechanism to explain these differences is unknown.

Stimulation of β1- and β2-ARs increases cAMP production through Gs and adenylyl cyclase. The present results showed that cAMP production mediated by β2-AR accounted for about 20% of the isoproterenol-stimulated cAMP production (Fig. 2A), and this percentage is approximately equal to the abundance of β2-AR (β2-AR expression is 20 – 30% of total β-ARs). The present result also showed that the contribution of β2-AR-
mediated cAMP production is not affected by disruption of caveolae. β2-AR activates adenylyl cyclase more efficiently than β1-AR (22), and Rybin et al. suggest the spatial proximity of β2-ARs with the adenylyl cyclase in caveolae. Thus, β2-AR may selectively couple with adenylyl cyclase (11). However, the present results suggest that the localization of β2-AR in the caveolae does not contribute to cAMP production linked to PKA activation in rat neonatal cardiomyocytes.

Although β1-AR was not localized in the caveolae, β1-AR-induced cAMP production was enhanced by the disruption of caveolae with MβCD. Why was β1-AR-mediated cAMP production enhanced by MβCD treatment? Neonatal cardiomyocytes express two types of adenylyl cyclase: adenylyl cyclase type 5 (AC-V) and type 6 (AC-VI) (23). These subtypes of AC distribute mediated cAMP production is not affected by disruption of caveolae. β2-AR activates adenylyl cyclase more efficiently than β1-AR (22), and Rybin et al. suggest the spatial proximity of β2-ARs with the adenylyl cyclase in caveolae. Thus, β2-AR may selectively couple with adenylyl cyclase (11). However, the present results suggest that the localization of β2-AR in the caveolae does not contribute to cAMP production linked to PKA activation in rat neonatal cardiomyocytes.

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Regulatory subunit type II of PKA is abundant in caveolae, while regulatory subunit type I is excluded from caveolae in rat neonatal cardiomyocytes and canine cardiac tissues. The present result showed that β1- and β2-ARs stimulation increased cAMP production, but PKA was activated only by β1-AR stimulation, which was independent of caveolae. Therefore, β1-AR may activate a specific subtype of PKA that is excluded from caveolae. Catecholamine action in the heart is predominantly mediated by β1-AR, and consequent PKA activation alters contractile function by phosphorylating L-type calcium channel, phospholamban, and troponin I. These phosphorylation events may also depend on a specific PKA subtype.

In conclusion, we examined the effect of MβCD on cAMP production and PKA activation. We showed that caveolae do not play a major role in PKA activation induced by β1-AR stimulation. These results support the recent concept that β1- and β2-ARs transduce different signaling pathways in myocytes.

Acknowledgments

We thank Drs. Lefkowitz and McKnight for plasmids encoding human β1- and β2-ARs, and PkaδR1α. This study was supported in part by a grant (to M.N. and H.Ku.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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