G<sub>i</sub> Protein-mediated Functional Compartmentalization of Cardiac β<sub>2</sub>-Adrenergic Signaling*  

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In contrast to β<sub>1</sub>-adrenoceptor (β<sub>1</sub>-AR) signaling, β<sub>2</sub>-AR stimulation in cardiomyocytes augments L-type Ca<sup>2+</sup> current in a cAMP-dependent protein kinase (PKA)-dependent manner but fails to phosphorylate phospholamban, indicating that the β<sub>2</sub>-AR-induced cAMP/PKA signaling is highly localized. Here we show that inhibition of G<sub>i</sub> proteins with pertussis toxin (PTX) permits a full phospholamban phosphorylation and a de novo relaxant effect following β<sub>2</sub>-AR stimulation, converting the localized β<sub>2</sub>-AR signaling to a global signaling mode similar to that of β<sub>1</sub>-AR. Thus, β<sub>2</sub>-AR-mediated G<sub>i</sub> activation constricts the cAMP signaling to the sarcolemma. PTX treatment did not significantly affect the β<sub>2</sub>-AR-stimulated PKA activation. Similar to G<sub>i</sub> inhibition, a protein phosphatase inhibitor, calyculin A (3 × 10<sup>-8</sup> M), selectively enhanced the β<sub>2</sub>-AR but not β<sub>1</sub>-AR-mediated contractile response. Furthermore, PTX and calyculin A treatment had a non-additive potentiating effect on the β<sub>2</sub>-AR-mediated positive inotropic response. These results suggest that the interaction of the β<sub>2</sub>-AR-coupled G<sub>i</sub> and G<sub>α</sub> signaling affects the local balance of protein kinase and phosphatase activities. Thus, the additional coupling of β<sub>2</sub>-AR to G<sub>i</sub> proteins is a key factor causing the compartmentalization of β<sub>2</sub>-AR-induced cAMP signaling.

The classical view of β<sub>AR</sub> signal transduction is that agonist-bound β-AR selectively interact with stimulatory G proteins (G<sub>S</sub>), which, in turn, activate adenyl cyclase to enhance cAMP formation. Subsequently, PKA phosphorylates a multitude of regulatory proteins involved in cardiac excitation-contraction coupling, including L-type Ca<sup>2+</sup> channels (1, 2), the sarcoplasmic reticulum Ca<sup>2+</sup> pump regulator PLB (3, 4), and myofilament proteins (5, 6), producing positive inotropic and lusitropic effects. In addition, PKA also phosphorylates and activates the endogenous protein phosphatase inhibitor 1 (7), which further ensures the action of protein kinases by protein phosphatase inhibition.

Although cardiac β<sub>1</sub>-AR signaling follows the scheme described above, recent studies have revealed a dissociation of β<sub>2</sub>-AR-mediated positive inotropic as well as lusitropic effects from global cAMP accumulation in several mammalian species (8–11). In addition, it has been demonstrated that in contrast to β<sub>1</sub>-AR, β<sub>2</sub>-AR stimulation fails to induce a cAMP-dependent phosphorylation of non-sarcomembral proteins involved in excitation-contraction coupling and energy metabolism (e.g. phospholamban, the myofilament proteins, troponin I, C protein, and the cytosolic protein glycogen phosphorylase kinase), but it does activate sarcolemmal L-type Ca<sup>2+</sup> channels (10–12), suggesting that β<sub>2</sub>-AR signaling is localized. More direct evidence supporting the localized β<sub>2</sub>-AR signaling has emerged from single L-type Ca<sup>2+</sup> channel recordings. Employing cell-attached patch clamp technique, the activity of single L-type Ca<sup>2+</sup> channels has been measured in response to specific β-AR subtype agonist outside (remote) or inside (local) the patch pipette. In contrast to the diffusive effect of β<sub>1</sub>-AR stimulation, β<sub>2</sub>-AR stimulation by zinterol only locally activates the L-type Ca<sup>2+</sup> channel but has no remote effect (13). Taken together, these previous studies have led to the hypothesis that the β<sub>2</sub>-AR-induced cAMP signaling is compartmentalized to a subsarcolemmal space and cannot be transmitted to cytoplasmic and SR PKA target proteins. Alternatively, the signal is transmitted to cytosolic proteins but local inactivation occurs at these sites.

The mechanism for the local control of β<sub>2</sub>-AR mediated signaling remains unclear. In many biological systems, G<sub>i</sub> and G<sub>S</sub> proteins cross-talk and operate as a complementary system. This balance system is usually regulated through different receptor families. For instance, activation of muscarinic receptors or adenosine receptors, prototypic G<sub>i</sub>-coupled receptors, markedly antagonizes the positive inotropic effect of β-AR stimulation (14, 15). Interestingly, promiscuous G protein coupling of β<sub>2</sub>-AR to both G<sub>i</sub> and PTX-sensitive G proteins (G<sub>i2</sub> and G<sub>α3</sub>) has been demonstrated in intact cardiomyocytes (16, 17). This coupling of β<sub>2</sub>-AR to G<sub>i</sub> proteins and its downstream pathway might interplay with the β<sub>2</sub>-AR/G<sub>i</sub>, signaling and contribute to the localization of β<sub>2</sub>-AR signaling near the sarcolemmal membrane. Thus, in the present study, we intended to determine whether the localized β<sub>2</sub>-AR signaling is mediated by the additional coupling of β<sub>2</sub>-AR to G<sub>i</sub> proteins and, if so, to elucidate potential underlying mechanisms.

EXPERIMENTAL PROCEDURES

Measurements of Cell Length—Single ventricular myocytes were isolated from rat hearts by a standard enzymatic technique (18). The cells were suspended in HEPES pH 7.4 buffer containing (in mmol/liter) 20 HEPES, 1 CaCl<sub>2</sub>, 137 NaCl, 5 KCl, 15 dextrose, 1.3 MgSO<sub>4</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>. In some experiments, cells were separately or simultaneously treated with 1.5 μg/ml PTX (Sigma) for 0 h at 37 °C to block G<sub>i</sub> protein activation (15) or 3 × 10<sup>-8</sup> M calyculin A (Calbiochem) for 20 min at 23 °C to inhibit protein phosphatases. Cells were stimulated at 0.5 Hz at 37 °C, and cell length was monitored from the bright-field image by an optical edge-tracking method using a photodiode array (model 1024 SAQ, Reticon) with a 3-ms time resolution (18).
S.E. Statistical significance was determined by Student's t test or analysis of variance when appropriate. Values with \( p < 0.05 \) were considered to be statistically significant.

RESULTS

The \( \beta_2 \)-AR agonist, zinterol (\(10^{-5} \) m), increased the whole cell \( L \)-type \( Ca^{2+} \) current (\( I_{Ca} \)) to 161 \( \pm \) 8.8% (\( n = 7 \), \( p < 0.05 \)) of control in single rat cardiomyocytes (Fig. 1a), which was completely abolished by the \( \beta_2 \)-AR antagonist ICI 118,551 (10). To delineate a role of cAMP-dependent PKA activation in the modulation of \( L \)-type \( Ca^{2+} \) channels, an inhibitory cAMP analog, \( R_{P} \)-cAMPS (10\(^{-4} \) m), was used to specifically block PKA activation. In the presence of \( R_{P} \)-cAMPS (\( 10^{-4} \) m) zinterol failed to augment \( I_{Ca} \) (Fig. 1b), indicating that the cAMP-dependent PKA activation is obligatory for \( \beta_2 \)-AR-mediated modulation of \( L \)-type \( Ca^{2+} \) channels.

PLB, the main modulator of cardiac relaxation, is phosphorylated following cardiac \( \beta_2 \)-AR stimulation at two adjacent phosphorylation sites, Ser\(^{16} \) (Fig. 2a) and Thr\(^{17} \), catalyzed by PKA and \( Ca^{2+} \)/calmodulin-dependent kinase, respectively (3, 4, 20). In contrast, the \( \beta_2 \)-AR agonist, zinterol, even at a maximal concentration (\( 10^{-5} \) m for 10 min), had only a very minor effect on the PKA-mediated Ser\(^{16} \) phosphorylation of PLB, as detected with phosphorylation site-specific PLB antibodies (21) in the Western blot (Fig. 2, a and b). The dose-response relation and the time course of Ser\(^{16} \) PLB phosphorylation are shown in Fig. 2a and Fig. 3c, respectively. A maximal concentration (\( 10^{-5} \) m) of the \( \beta_2 \)-AR agonist, zinterol, only induced a minor increase in Ser\(^{16} \) PLB phosphorylation even if the incubation time was extended to 20 min (Fig. 3c). Concomitantly, the \( \beta_2 \)-AR-mediated increase in contractility occurred in the absence of a significant relaxant effect (Fig. 3a). Thus, the failure of \( \beta_2 \)-AR stimulation to induce PLB phosphorylation is the apparent mechanism for the absence of a lusitropic effect (Fig. 3c). These data illustrate that whereas both \( \beta_1 \) and \( \beta_2 \)-AR share the common second messenger cAMP, they exhibit differences with respect to PKA-mediated protein phosphorylation and relaxant effects (8–12).

Based on our recent finding that \( \beta_2 \)-AR dually couples to \( G_{i} \) and \( G_{s} \) proteins (16, 17), we hypothesized that the additional coupling of \( \beta_2 \)-AR to \( G_{s} \) might interfere with the \( \beta_2 \)-AR/G\(_{i}\) signaling, contributing to the restriction of \( \beta_2 \)-AR-mediated cAMP signaling to a subsarcolemmal domain. To test this hypothesis, we examined the effect of \( G_{i} \) protein inhibition by PTX...
on the PLB phosphorylation following β2-AR stimulation and its functional consequences. Whereas PTX itself had only a negligible effect on the basal Ser16 PLB phosphorylation (Fig. 2b), β2-AR stimulation with zinterol in PTX-treated cardiomyocytes markedly increased PLB phosphorylation in a dose-dependent manner (EC50 = 48.6 ± 1.8 nM) (Fig. 2b), with a maximal increase of 6.5-fold, comparable with that induced by the β1-AR agonist norepinephrine (NE at 10−7 M) (37.9 ± 4.7 and 36.7 ± 7.7 in arbitrary units, respectively). Fig. 3a shows the time courses of β2-AR effects on contraction amplitude and duration (t50) in both PTX-treated and non-treated cells. In addition to the 1.5-fold potentiation of the β2-AR inotropic response, inhibition of Gα function allowed zinterol to induce a de novo relaxant effect in rat cardiomyocytes. The β2-AR-induced lusitropic effect in PTX-treated cells is highly comparable with that of β1-AR stimulation in control cells (Fig. 3, a and b). Furthermore, the time course of the β2-AR-induced Ser16 PLB phosphorylation was tightly correlated to the time course of the relaxant effect in PTX-treated cardiomyocytes, both reaching a steady state within 5 min (Fig. 3c). In contrast, neither the β2-AR-mediated contractile nor its relaxant response was affected by PTX (Fig. 3b). These results strongly suggest that the β2-AR/Gi, coupling functionally compartmentalizes the β2-AR/Gi-mediated cAMP signaling, altering the quality as well as the magnitude of its cellular response.

To elucidate further the mechanism underlying the Gα-mediated spatial control of β2-AR signaling, we measured the PKA activity following β2-AR subtype stimulation. Similar to β2-AR stimulation, β2-AR activation also significantly increased the PKA activity ratio in both soluble and particulate fractions (Fig. 4). This suggests that, unlike β1-AR, β2-AR-mediated increases in cAMP accumulation (8, 10) and PKA activation (Fig. 2a) are dissociated from Ser16 PLB phosphorylation (Fig. 2). Surprisingly, PTX did not significantly affect the response of PKA in either fraction (Fig. 4), suggesting that the cross-talk of Gα and Gβ signaling following β2-AR stimulation may occur downstream of PKA (see “Discussion”). We therefore examined the potential involvement of protein phosphatases in β2-AR-mediated Gα signaling. Rat cardiomyocytes were treated with calcullin A (3 × 10−8 m) for 20 min to inhibit protein phosphatases. Control experiments showed that calcullin A at this concentration had only a marginal effect on contraction amplitudes, whereas it had no effect on the submaximal β2-AR (NE, 10−6 M)-stimulated contractile response (Fig. 5). Thus, the effects of protein phosphatase inhibition are similar to that of Gα inhibition by PTX, enhancing the s contractile response in a β2-AR-specific manner (Fig. 3). This result strongly suggests that protein phosphatases are likely to be involved in the β2-AR/Gi-directed signaling. This conclusion was further substantiated by the observation that calcullin A failed to potentiate further the β2-AR-mediated contractile response if the Gα pathway is disrupted by PTX treatment (Fig. 5). Therefore, protein phosphatases may serve as a novel subcellular compartment of cAMP/PKA signaling.

**DISCUSSION**

Recent advances in β2-AR signaling have provided evidence for a novel subcellular compartmentalization of cAMP signaling. Specifically, although both β1- and β2-AR stimulation enhance cAMP accumulation (8, 10) and PKA activity and modulate τC50 via cAMP/PKA-dependent signaling, the β2-AR stimulation is uncoupled from the phosphorylation of more remote proteins (8, 9, 11). This indicates that the signaling may be highly localized to sarcolemmal microdomains or that it can be transmitted to cytoplasmic sites but locally inactivated there. The key question then is what mechanism enforces the tight spatial control of β2-AR-mediated cAMP signaling. In principle, a localized cAMP signaling could arise from localization of signaling components, e.g., localization of cAMP by phosphodiesterases (22, 23) or PKA by specific anchoring proteins of PKA (24, 25). A close spatial association of Ca2+ channels with adenyl cyclase and PKA (26, 27) could provide a structural...
Thus, inhibition of Gi proteins converts the magnitude but also the quality of its cardiac response. It is well established that activation of protein phosphatases (7). In other words, the Gi protein activation could functionally counterbalances cellular effects of protein kinases. Interestingly, calyculin A, a non-selective protein phosphatase inhibitor, mimics the PTX effect. Both interventions, G_i inhibition by PTX or protein phosphatase inhibition by calyculin A, had a non-additive potentiating effect on β_2-AR-mediated contractile response when applied together, suggesting that PTX and calyculin A act on a common signaling pathway. Therefore, the negating and spatial restricting effects of β_2-AR-activated G_i proteins on the G_i-directed signaling might be mediated by a modulation of the protein phosphatase/kinase balance. For instance, a high dephosphorylation rate of non-sarcolemmal proteins following β_2-AR stimulation might negate the PKA-mediated phosphorylation of PLB (and other cytoplasmic proteins).

G_i-mediated COMPARTMENTALIZATION OF β_2-AR SIGNALING

In the present study, we demonstrated that, apart from localization of signaling molecules of the cAMP/PKA cascade (22–25), an interaction between functionally opposing signal transduction pathways can also create compartmentalization of receptor-mediated signaling. In particular, following inhibition of G_i function by PTX treatment, β_2-AR stimulation markedly increased Ser^16 PLB phosphorylation and elicited a modulation of the protein phosphatase/kinase balance. For instance, a high dephosphorylation rate of non-sarcolemmal proteins following β_2-AR stimulation might negate the PKA-mediated phosphorylation of PLB (and other cytoplasmic proteins).

investigated the potential involvement of protein phosphatases in the cross-talk of the β_2-AR-stimulated G_i/G_s signaling. Interestingly, calyculin A, a non-selective protein phosphatase inhibitor, mimics the PTX effect. Both interventions, G_i inhibition by PTX or protein phosphatase inhibition by calyculin A, had a non-additive potentiating effect on β_2-AR-mediated contractile response when applied together, suggesting that PTX and calyculin A act on a common signaling pathway. Therefore, the negating and spatial restricting effects of β_2-AR-activated G_i proteins on the G_i-directed signaling might be mediated by a modulation of the protein phosphatase/kinase balance. For instance, a high dephosphorylation rate of non-sarcolemmal proteins following β_2-AR stimulation might negate the PKA-mediated phosphorylation of PLB (and other cytoplasmic proteins).
(30, 31). Between these extremes, β2-AR stimulation in rat cardiomyocytes induces significant increases in ICa and contractility in the absence of phosphorylation of cytoplasmic regulatory proteins. PTX treatment further enhances the β2-AR contractile response (17) and restores its ability to phosphorylate cytoplasmic regulatory proteins in this species. The situation in canine myocytes is similar to that of rat myocytes, except that β2-AR does induce lusitropic as well as inotropic effects in the absence of cytoplasmic protein phosphorylation (9, 11).

The aforementioned data also illustrate that the same signaling molecule, cAMP, mediates remarkably different cardiac functional responses following β1- and β2-AR stimulation (8–12). Analogously, it has been shown that intracellular Ca2+ located in different subcellular compartments may mediate distinctly different and sometimes even opposing cellular functions. For instance, a global elevation in cytosolic Ca2+ in arterial smooth muscle cells causes vasoconstriction, but Ca2+ sparks near the sarcolemma induce relaxation (32). In this case, local Ca2+ gradients are possible, because various endogenous binding sites buffer Ca2+ of discrete origins. Thus, physical or functional compartmentalization of ubiquitous intracellular messengers, such as cAMP and Ca2+, creates specificity and diversity of a given receptor-mediated signaling.

In summary, we have demonstrated that, in addition to the potentiation of the inotropic response, inhibition of G function by PTX induces a de novo lusitropic effect and PLB phosphorylation following β2-AR stimulation in rat ventricular myocytes. These results suggest a contribution of β2-AR/Gi-coupled signaling to the compartmentalization of β2-AR/Gi-stimulated cAMP/PKA signaling, possibly through a protein phosphatase-dependent mechanism. In addition, the present study demonstrates that compartmentalization of a common second messenger-directed signaling allows for selective modulation of a variety of target proteins and cellular processes, creating signaling specificity and versatility among closely related G protein-coupled receptors.

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