β2-Adrenergic cAMP Signaling Is Uncoupled From Phosphorylation of Cytoplasmic Proteins in Canine Heart

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Background—Recent studies of β-adrenergic receptor (β-AR) subtype signaling in vitro preparations have raised doubts as to whether the cAMP/protein kinase A (PKA) signaling is activated in the same manner in response to β2-AR versus β1-AR stimulation.

Methods and Results—The present study compared, in the intact dog, the magnitude and characteristics of chronotropic, inotropic, and lusitropic effects of cAMP accumulation, PKA activation, and PKA-dependent phosphorylation of key effector proteins in response to β-AR subtype stimulation. In addition, many of these parameters and L-type Ca2+ current (ICa) were also measured in single canine ventricular myocytes. The results indicate that although the cAMP/PKA-dependent phosphorylation cascade activated by β1-AR stimulation could explain the resultant modulation of cardiac function, substantial β2-AR–mediated chronotropic, inotropic, and lusitropic responses occurred in the absence of PKA activation and phosphorylation of nonsarcolemmal proteins, including phospholamban, troponin I, C protein, and glycogen phosphorylase kinase. However, in single canine myocytes, we found that β2-AR–stimulated increases in both ICa and contraction were abolished by PKA inhibition. Thus, the β2-AR–directed cAMP/PKA signaling modulates sarcolemmal L-type Ca2+ channels but does not regulate PKA-dependent phosphorylation of cytoplasmic proteins.

Conclusions—These results indicate that the dissociation of β2-AR signaling from cAMP regulatory systems is only apparent and that β2-AR–stimulated cAMP/PKA signaling is uncoupled from phosphorylation of nonsarcolemmal regulatory proteins involved in excitation-contraction coupling. (Circulation. 1999;99:2458-2465.)

Key Words: receptors, adrenergic, beta ■ contractility ■ relaxation ■ phospholamban ■ troponin I

β-Adrenergic receptor (β-AR) stimulation by catecholamines enhances cardiac performance in response to an increased peripheral demand, eg, during stress or exercise. In various mammalian species, at least 2 β-AR subtypes, β1-AR and β2-AR, coexist in cardiomyocytes, and their stimulation modulates cardiac function.1-4 The classic view of β-adrenergic signaling mechanism is that β-ARs selectively couple to G protein, activating adenyl cyclase and enhancing cAMP production, leading to protein kinase A (PKA) activation, which phosphorylates a multitude of regulatory proteins, including the L-type Ca2+ channels, phospholamban (PLB) in the sarcoplasmic reticulum (SR) membrane, the myofilament proteins (troponin I [TnI] and C protein), and metabolic enzymes, eg, glycogen phosphorylase kinase. Phosphorylation of these proteins not only increases cardiac contractility but also accelerates relaxation. In addition, a more rapid, cAMP-independent, direct coupling of β-AR–stimulated G, and L-type Ca2+ channels has been proposed.5,6 Recent studies on cardiac β-AR subtype stimulation have raised doubts as to whether the above signaling scheme pertains equally to both β1- and β2-ARs. Although it is known that β2-AR is coupled to adenyl cyclase more efficiently than β1-AR,7,8 the functional relevance of β2-AR signaling to modulate contractility or heart rate (HR) has been questioned because of a relatively smaller number of β2-AR than β1-ARs.7,4 In addition, in vitro studies have revealed a substantial diversity among species with respect to the magnitude of the β2-AR effects and more importantly, with respect to the role of cAMP/PKA signaling.9-13 More recent studies in rat myocytes have shown that the β2-AR–mediated inotropic effect is due to augmentation of ICa, by a local cAMP/PKA-dependent mechanism, because it is blocked by PKA inhibition.14,15 However, it is not clear whether the β2-AR–induced local cAMP signaling in rat myocytes can be generalized to other mammals. In particular, it has been argued that the β2-AR–induced positive inotropic
effect in canine heart may be cAMP-independent, because cAMP production is not increased during β2-AR stimulation.

Therefore, we compared cardiac chronotropic, inotropic, and lusitropic responses to β1-AR versus β2-AR stimulation in intact dogs and in ventricular myocytes. We systematically characterized the phosphorylation status of major cytoplasmic regulatory proteins (PLB, TnI, C protein, and glycogen phosphorylase) as well as cAMP levels or PKA activation after β-AR subtype stimulation to determine whether β1-AR signaling is global or is restrained to certain domains. Furthermore, in isolated canine ventricular myocytes we determined whether cAMP/PKA signaling is essential for cardiac β2-AR–mediated Ica, and contractile responses using PKA inhibitors.

**Methods**

**Materials**
Norepinephrine (NE), reserpine, and H-89 were purchased from Sigma Chemical Co and Rp-cAMPS from Biolog Life Science Institute. Zinpterol (Zin) was kindly supplied by Bristol-Myers Inc, ICI 118,551 (ICI) by Imperial Chemical Inc, CGP 20712A (CGP) by Ciba-Geigy, and bisoprolol (Bis) by Merck.

**Measurements of β-AR Subtype Effects on HR, Left Ventricular Contractility, and Relaxation in the Intact Dog**
β-AR subtype stimulation was studied in adult anesthetized (pentothal), open-chest beagle dogs. Forty-eight hours before the experiments, the dogs were reserpinized (1.5 mg/kg body wt) to reduce the influence of endogenous catecholamines. NE (5 μg/kg body wt) and Zin (50 μg/kg body wt) were injected as a bolus into the left ventricle. The β1-AR antagonist Bis (0.6 mg/kg body wt) was administered intravenously 10 minutes before Zin to block reflex β1-AR stimulation and possible background catecholamine influence. This dose of Bis completely blocked the response to NE. The β1-AR antagonist ICI (0.2 mg/kg body wt) was used 5 minutes before Zin in the presence of Bis. NE and Zin doses were chosen because preliminary experiments showed that they elicited a maximum inotropic response. HR, left ventricular pressure (LVP), and dP/dt were continuously recorded before and after drug administration. The half-time of relaxation (t1/2) was derived from fast-speed recordings of LVP. At 30 seconds after the drug injection, free-wall left ventricular tissue was freeze-clamped.

**Electrophysiological and Contractile Measurements in Isolated Cardiomyocytes**
Left ventricular myocytes were isolated from hearts of beagle dogs with a standard enzymatic technique.1 The cells were suspended in HEPES buffer (pH 7.4) containing (mmol/L) HEPES 20, CaCl2 1, KCl 5, MgSO4 1.3, and NaH2PO4 1.2. 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.16 Ica was measured via the whole-cell patch-clamp technique using an Axopatch 1D amplifier (Axon Instruments Inc). Cells were voltage-clamped at −40 mV to inactivate Na+ and T-type Ca2+ channels. K+ currents were inhibited by 4 mmol/L 4-aminopyridine and 5.4 mmol/L CsCl instead of KCl in the buffer and the pipette solution containing (mmol/L) CsCl 100, NaCl 10, TEA-Cl 20, HEPES 10, Mg-ATP 5, and EGTA 5; pH 7.2 adjusted with CsOH. Ica was elicited by 300-ms pulses from a holding potential of −40 to 0 mV at 0.1 Hz at 23°C.

**Protein Phosphorylation Assays**
Heart tissue was homogenized in 10 volumes buffer containing (mmol/L) histidine HCl 5 (pH 7.4), EDTA 10, Na4P2O7 50, NaF 25, DTT 0.2, and PMSF 0.1. For backphosphorylation assays,17 0.75 mol/L KCl was included, and homogenates were centrifuged at 150 000g for 30 minutes. The resulting supernatants, containing contractile proteins, and the homogenate were stored at −80°C.

**Backphosphorylation**
Supernatants (40 μg protein) were phosphorylated in a medium containing (mmol/L) histidine HCl 40 (pH 6.8), MgCl2 10, NaF 15, and EGTA 1 and 0.75 μmol/L catalytic subunit of PKA in the presence of 50 μmol/L [γ-32P]ATP. The reaction was initiated by the addition of [γ-32P]ATP and was stopped after 5 minutes with 50 mmol/L H3PO4, 0.5 mmol/L ATP, and 15% trichloroacetic acid. After centrifugation (2000g, 20 minutes), the precipitate was directly processed for electrophoresis. The gels were exposed to x-ray films, and the respective bands for TnI and C protein were cut out to quantify the 32P incorporation. The backphosphorylation data are expressed as pmol phosphate incorporation/μg protein.

**Site-Specific PLB Phosphorylation**
After homogenization, proteins were solubilized in sample buffer (50 mmol/L H3PO4, 5 mmol/L EDTA, 2% SDS, 1% mercaptoethanol, and 10% glycerol, pH 6.8 adjusted with Tris). Under these conditions, PLB is fully dissociated into its monomeric form. Proteins were resolved by 7.5% urea/SDS-PAGE and transferred to polyvinylidene difluoride membranes (Serva). Membranes were incubated with 5% dried milk in TBST (50 mmol/L Tris, 150 mmol/L NaCl, 0.1% Tween-20) and then with the phosphorylation site–specific PLB antibodies (PS-16 or PT-17, 1:10 000, PhosphoProtein Research). The immunoreaction was detected by chemiluminescence (Amersham).

PLB phosphorylation was also examined in canine ventricular myocytes incubated for 10 minutes with NE (10 μmol/L to 1 μmol/L) or Zin (0.1 to 1 μmol/L); 15% SDS was added, and samples were processed as described above. In some experiments, cells were pretreated for 5 minutes with ICI (100 nmol/L) or CGP (300 mmol/L).

**Other Assays**
cAMP levels were analyzed in trichloroacetic acid extracts, purified by column chromatography.17 PKA activity of the soluble and particulate fractions was analyzed by a modified method of Murray et al.18 Homogenates were centrifuged at 6000g for 5 minutes; the resulting supernatant was taken to represent the soluble PKA activity, and the resuspended pellet, the particulate fraction. PKA activity is expressed as the activity ratio of malantide phosphorylation in the absence and presence of cAMP.2.8 PKA activity is expressed as the activity ratio of malantide phosphorylation in the absence and presence of cAMP.2.8 Protein concentration was determined by the method of Lowry et al.,20 using ovalbumin as standard.

**Statistics**
Results are presented as mean±SEM. Statistical significance was determined by unpaired or paired t test or ANOVA when appropriate. Values of P<0.05 were considered to be statistically significant.

**Results**

**β-AR Signaling in the Intact Dog**

Both β1-AR and β2-AR Stimulation Enhance Cardiac Function
Representative tracings of the effects of NE and Zin are illustrated in Figure 1. The cardiac responses (HR, LVP, and ±dP/dt) reached a maximal level at 20 to 30 seconds after the drug bolus. The average values of HR, LVP, +dP/dt, +dP/dt/P, and t1/2 under control conditions and after varying drug regimens are listed in the Table. Figure 2A shows the responses to β1-AR stimulation in intact dog. After injection of the β1-AR agonist, NE, HR, and +dP/dt/P were markedly
increased, whereas t_{1/2} was significantly reduced, consistent with previous studies. The effects of NE were completely prevented by the β₁-AR antagonist Bis (0.6 mg/kg body wt) (Figure 2A and Table), indicating that in intact dog, similar to rat ventricular myocytes, the NE-induced cardiac response is mediated by β₁-AR stimulation. It is noteworthy that HR and +dP/dt/P were reduced 32% and t_{1/2} increased 23% by pretreatment with Bis (Table), whereas ICI had no additional effect, suggesting that under basal conditions, only β₁-AR stimulation is tonically involved in the cardiac regulation. The increase in t_{1/2} over the control level after Bis (Table) represents, at least in part, the background β₁-AR activation under these experimental conditions.

To measure specific β₂-AR–mediated effects, Bis (0.6 mg/kg body wt) was infused before the β₂-AR agonist Zin (50 μg/kg body wt). In the presence of Bis, Zin still significantly increased both HR and contractility and decreased t_{1/2} (Table, Figures 1B and 2B). Although the relative augmentations of HR and +dP/dt/P induced by β₂-AR stimulation were comparable to those of β₁-AR stimulation, the β₂-AR–induced decrease in t_{1/2} was relatively smaller (Figure 2). The β₂-AR–mediated responses were specifically abolished by the β₂-AR antagonist ICI (Figure 2B and Table), further confirming that the effects of Zin in the presence of Bis are mediated by β₂-AR. It is noteworthy that although β₂-AR stimulation significantly increased +dP/dt, it had no significant effect on LVP. The difference between the effects of Zin and NE on LVP is probably attributable to an additional vasodilatory effect of β₂-AR stimulation.

cAMP Accumulation Induced by β₂-AR Stimulation

In the freeze-clamped heart tissue of the same dogs as used for the functional studies, both β₁-AR stimulation by NE and β₂-AR stimulation by Zin in the presence of the β₁-AR

| Effects of β-AR Subtype Stimulation on Cardiac Function |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | HR, bpm          | LVP, mm Hg      | +dP/dt, mm Hg/s | +dP/dt/P, s⁻¹   | t_{1/2}, ms     |
| Control (n=11)  | 156.2±8.7        | 154.3±14.1      | 3901±400        | 23.5±1.1        | 231.1±7.1       |
| Bis (n=7)       | 105.9±8.9*       | 127.2±18.2      | 1559±116*       | 15.9±2.3        | 283.5±18.1*     |
| NE (n=11)       | 182.0±9.4†       | 237.0±13.7†     | 13754±492†      | 58.0±2.6†       | 136.1±2.2†      |
| NE + Bis (n=6)  | 93.5±5.4*        | 154.9±14.8      | 1789±172*       | 13.1±0.7        | 266.7±2.5       |
| Zin + Bis (n=8) | 148.3±8.5†       | 129.9±19.8      | 3551±525†       | 33.4±4.1†       | 197.2±9.4†      |
| Zin + Bis + ICI (n=3) | 106.2±8.6 | 96.6±38.9       | 1554±122        | 21.9±6.3        | 264.7±8.9       |

Doses used were Bis, 0.6 mg/kg; NE, 5 μg/kg; Zin, 50 μg/kg; and ICI, 0.2 mg/kg. Data are mean±SEM from 3 to 11 dogs.

*P<0.05 vs control; †P<0.05 vs Zin+Bis; ‡P<0.05 vs Bis, by ANOVA.
antagonist Bis markedly increased total cellular cAMP to a similar extent (Figure 3). The NE- and Zin-induced augmentations in cAMP were completely blocked by Bis or ICI, respectively (Figure 3).

**β₂-AR Stimulation Fails to Increase PKA Activity**

Next, we characterized the effects of β-AR subtype stimulation on PKA activation. Both the soluble and the particulate fractions were examined, because previous studies have suggested that the particulate fraction contains the functional component mediating protein phosphorylation.²¹ NE significantly increased PKA activity by 60% to 70% in both fractions (Figure 4), and this was inhibited in the particulate fraction by Bis. This biochemical result is in good agreement with the functional results described above (Figures 1 and 2 and Table). In sharp contrast, PKA activity was not elevated by Zin plus Bis in either the soluble or the particulate fraction, despite the significant increases in HR, contractility, and accelerated relaxation as well as the enhanced cAMP levels. To further investigate this difference in β-AR subtype signaling, we measured the cAMP-dependent phosphorylation of several PKA target proteins.

**PLB Phosphorylation**

PLB plays an important role in modulating SR Ca²⁺ pump activity,²²–²⁴ and its phosphorylation at Ser16 and Thr17 is mediated by PKA and Ca²⁺/calmodulin-dependent kinase II, respectively.²⁵,²⁶ Phosphorylation site–specific PLB antibodies²⁷ were used in the present study to determine whether β-AR subtype stimulation differentially regulates PLB phosphorylation. In the absence of β-AR stimulation, only a minor PLB phosphorylation is detectable. β₁-AR but not β₂-AR stimulation increased the PKA-catalyzed PLB phosphorylation at Ser16 (Figure 5). The NE-induced PLB phosphorylation was blocked by the β₁-AR antagonist (Figure 5). The lack of PLB phosphorylation after β₂-AR stimulation is consistent with the lack of PKA activation by Zin (Figure 4) and in agreement with previous studies that detected different PLB mobility forms.¹³ However, previous studies did not distinguish the phosphorylation sites. To test whether a
abolished by Bis. Thus, though phosphorylation of PLB at Thr17 either (Figure 5), even indicating a significant increase in the in vivo phosphorylation. A 42.6 \pm 4.0\% reduction in vivo, as manifested by a decrease in t_{1/2}, without increasing the effects of phosphorylation technique (Figure 6). In this assay, \(^{32}\)P incorporation was detected in animals exposed to NE, and C protein in response to NE (Figure 6). A 62.9 \pm 1.3\% decrease in \(^{32}\)P incorporation, detected only in NE-treated animals, indicates an increased phosphorylation state of this protein in vivo in response to \(\beta_2\)-AR stimulation. This phosphorylation was also entirely abolished by Bis. Thus, \(\beta_1\)-AR but not \(\beta_2\)-AR stimulation induced PKA-catalyzed phosphorylation of the myofilament proteins TnI and C protein.

**Conversion of Phosphorylase b to Phosphorylase a**

In addition to modulation of HR and contractility, \(\beta_2\)-AR stimulation is also critically involved in the regulation of cardiac energy metabolism. For example, glycogen phosphorylase, an important enzyme involved in glycogen synthesis and breakdown, is activated by phosphorylase kinase–dependent phosphorylation, which can be activated by PKA-dependent phosphorylation. We demonstrated, for the first time, that glycogen phosphorylase did not respond to \(\beta_2\)-AR stimulation, whereas NE induced a marked augmentation in the conversion of phosphorylase b to phosphorylase a (Figure 7). This result suggests that \(\beta_2\)-AR but not \(\beta_2\)-AR stimulation is linked to energy metabolism in association with the increased cardiac performance. Thus, the results from Figures 5 through 7 indicate that none of the nonsarcolemmal PKA target proteins examined are accessible to the \(\beta_2\)-AR signaling pathway. However, it is noteworthy that the sarcolemmal L-type \(\mathrm{Ca}^{2+}\) channel phosphorylation cannot be determined by any presently available techniques.

**\(\beta_2\)-Adrenergic Signaling in Single Canine Cardiomyocytes**

To verify the results obtained in the intact dog and to measure \(I_{\text{Ca}}\), we performed additional studies in isolated canine myocytes. Consistent with the results from the intact dog, both \(\beta_2\)-AR stimulation by NE 0.1 \(\mu\)mol/L and \(\beta_2\)-AR stimulation by Zin 1.0 \(\mu\)mol/L produced positive inotropic effects, which were totally abolished by the \(\beta_2\)-AR antagonists CGP 300 nmol/L and ICI 100 nmol/L, respectively (Figure 8A). The average dose-response curves of contraction and t_{1/2} to \(\beta_2\)-AR subtype stimulation are shown in Figure 9. Both NE and Zin significantly increased contraction amplitude and abbreviated t_{1/2} of contraction in a dose-dependent manner with a higher sensitivity to \(\beta_2\)-AR stimulation (Figure 9). The small decrease in contractility at the highest NE dose is probably due to the occurrence of spontaneous oscillations. In contrast to the observation in the intact dog, \(\beta_2\)-AR stimulation had no significant effect on cAMP accumulation (Figure 10A), which is consistent with previous studies in isolated canine myocytes. In the intact dog, the \(\beta_2\)-AR agonist Zin at any concentration tested failed to induce PLB phosphorylation at

**Phosphorylation of Myofilament Proteins**

The \(\beta_2\)-induced, cAMP-dependent phosphorylation of the myofilament protein TnI was assessed with the backphosphorylation technique (Figure 6). In this assay, \(^{32}\)P incorporation is used to indicate the in vivo protein phosphorylation status, i.e., a lesser \(^{32}\)P incorporation indicates a greater agonist-induced phosphorylation. A 42.6 \pm 4.0\% reduction in \(^{32}\)P incorporation was detected in animals exposed to NE, indicating a significant increase in the in vivo phosphorylation state. However, \(\beta_2\)-AR stimulation did not alter the amount of \(^{32}\)P incorporation and therefore had no effect on TnI phosphorylation.

In addition, the phosphorylation of another myofilament component, C protein, was studied with the same method (Figure 6). A 62.9 \pm 1.3\% decrease in \(^{32}\)P incorporation, detected only in NE-treated animals, indicates an increased phosphorylation state of this protein in vivo in response to \(\beta_2\)-AR stimulation. This phosphorylation was also entirely abolished by Bis. Thus, \(\beta_1\)-AR but not \(\beta_2\)-AR stimulation

![Figure 6. Phosphorylation of myofilament proteins induced by \(\beta_2\)-AR subtype stimulation. PKA-catalyzed phosphorylation of TnI and C protein in response to NE (\(\beta_1\)-AR) or Zin (\(\beta_2\)-AR) was measured by backphosphorylation. Values are mean \pm SEM for n=3 to 6 experiments. \(*P<0.05\) vs control, \(\dagger P<0.05\) vs Zin+Bis.](image1)

![Figure 7. Conversion of phosphorylase b to phosphorylase a induced \(\beta_2\)-AR subtype stimulation. \(\beta_2\)-AR subtype–induced conversion of phosphorylase b to phosphorylase a by NE (\(\beta_2\)-AR) or Zin (\(\beta_2\)-AR) was measured in heart tissue. Values are mean \pm SEM for n=3 to 6 experiments. \(*P<0.05\) vs control, \(\dagger P<0.05\) vs Zin+Bis.](image2)
either Ser16 (Figure 10B) or Thr17 (data not shown). The above in vivo and in vitro results indicate that in contrast to β1-AR activation, β2-AR–stimulated contractile and relaxant effects are not accompanied by an increase in cAMP or cAMP-dependent phosphorylation of myofilament or SR proteins. The critical question, then, is how β2-AR stimulation increases contractility and accelerates cardiac relaxation without phosphorylation of PLB and myofilament proteins. The β2-AR–mediated contractile effects are mediated by an augmentation of I_{Ca}, as shown by a typical example in Figure 11A and the average data in Figure 11B.

Figure 8. Contractile responses induced by β-AR subtype stimulation and PKA inhibition in canine ventricular myocytes. A, Representative continuous chart recordings of contraction amplitude in response to specific β-AR subtype agonists (NE, 50 nmol/L and Zin, 1 μmol/L) and blockers (CGP, 300 nmol/L and ICI, 100 nmol/L). An upward deflection indicates cell shortening. B, Average contractile response to NE and Zin in presence and absence of H-89 (2 μmol/L). Data are presented as mean±SEM for n=3 to 12 cells. *P<0.05 vs control.

Figure 9. Dose-response curves of inotropic (A) and relaxant (B) effect in response to β-AR subtype stimulation in single canine ventricular myocytes. Data are shown as percent of control (mean±SEM for n=8 to 10, except n=4 for highest NE dose). A single dose of β-AR subtype agonists was applied for each cell to avoid desensitization.

Figure 10. β-AR subtype-induced cAMP accumulation and cAMP-dependent Ser16 PLB phosphorylation in isolated cardiomyocytes. A, Dose-response curve for β-AR subtype–mediated cAMP accumulation; B, Ser16 PLB phosphorylation. Values are mean±SEM of n=3 to 10 experiments.

Figure 11. Effect of PKA inhibition on β2-AR–stimulated augmentation of I_{Ca}. A, Typical superimposed I_{Ca} traces before and 5 minutes after exposure to Zin (1 μmol/L) in absence and presence of Rp-cAMPS (100 μmol/L). B, Average effects of Rp-cAMPS on I_{Ca}. Values are mean±SEM for n=4 to 6 experiments (baseline I_{Ca}, 0.72±0.07 [n=8] and 0.77±0.09 [n=11] nA in absence and presence of Rp-cAMPS, respectively).
To determine whether the $\beta_2$-AR–induced augmentation of $I_{Ca}$, in the absence of phosphorylation of PLB and other proteins remote from the sarcolemma, is mediated by a localized cAMP signaling, we examined effects of PKA inhibitors on $\beta_2$-AR–mediated contractile and $I_{Ca}$ responses. The PKA inhibitor H-89 2 $\mu$mol/L$^{28}$ completely reversed the positive inotropic (Figure 8B) and relaxant (not shown) effects induced by $\beta_2$-AR and $\beta_2$-AR stimulation, whereas H-89 alone had no significant effect on basal contraction and did not block the positive inotropic effect of high extracellular Ca$^{2+}$ (4.0 $\mu$mol/L) (data not shown). Furthermore, the involvement of cAMP/PKA in the $\beta_2$-AR–mediated $I_{Ca}$ response was separately investigated by dialysis of an inhibitory cAMP analogue, Rp-cAMPS (100 $\mu$mol/L). As shown in Figure 11, the $\beta_2$-AR agonist Zin–induced increase in $I_{Ca}$ was totally abolished by Rp-cAMPS, whereas it had no significant effect on the basal $I_{Ca}$. These results indicate that even though there are no detectable increases in PKA activation and PKA-dependent phosphorylation of cytoplasmic proteins, $\beta_2$-AR–stimulated augmentation of $I_{Ca}$ and the contractile and relaxant effects in the canine heart do require cAMP-dependent PKA activation, which is apparently localized within or near the sarcolemma, and cannot be measured by presently available techniques.

### Discussion

To determine the functional importance of $\beta_2$-AR stimulation in vivo, we compared $\beta_2$-AR– and $\beta_2$-AR–mediated HR contractile and relaxant responses in anesthetized, reserpinized intact dogs. $\beta_2$-AR was selectively stimulated by Zin in combination with a $\beta_2$-AR antagonist to inhibit potential tonic background or any reflex $\beta_2$-AR stimulation. The results demonstrate, for the first time, that in an intact animal model, $\beta_2$-AR stimulation produces a significant increase in HR and inotropic and lusitropic responses.

A similar increase in cellular cAMP after $\beta_2$-AR or $\beta_2$-AR subtype stimulation was observed in the intact dog, consistent with the $\beta_2$-AR–mediated increase in adenylate cyclase activity in dog LV biopsies.$^{29}$ In contrast, the present and previous studies in single canine ventricular myocytes$^{13}$ have demonstrated that Zin had no effect on total cAMP accumulation over a wide dose range, even though mixed $\beta_2$-AR stimulation or specific $\beta_1$-AR stimulation (Figure 10) still markedly increases cAMP levels in these myocytes.$^{13}$ This difference in cAMP accumulation between isolated cardiomyocytes and heart tissue might be due to a $\beta_2$-AR–stimulated cAMP production in other cell types, such as vascular smooth muscle cells or endothelial cells, in which higher levels of $\beta_2$-AR are present.$^{30}$

Previous studies have shown that in rat and canine ventricular myocytes, $\beta_2$-AR stimulation induces a contractile effect and an increase in $I_{Ca}$ without enhancing PLB phosphorylation.$^{12,13}$ Here, we systematically examined the phosphorylation of major regulatory proteins involved in cardiac excitation-contraction coupling as well as in energy metabolism. Specifically, we examined PKA target proteins located within different subcellular compartments to determine whether there is a uniform pattern or alternatively, a substrate-dependent phosphorylation after $\beta_2$-AR subtype stimulation. The present results illustrate that $\beta_2$-AR stimulation failed to elicit a detectable PKA activation, conversion of phosphorylase $b$ to $a$ in the cytoplasm, or PKA-dependent phosphorylation of PLB in the SR and myofilament proteins. In contrast, the $\beta_2$-AR–stimulated cardiac effects are well correlated with an increase in PKA activity and phosphorylation of these key regulatory proteins. These observations, together with the results of recent studies,$^{13}$ clearly show that the $\beta_2$-AR and the $\beta_2$-AR signaling pathways are not identical in nature. Indeed, it has already been proposed that the $\beta_2$-AR–mediated cardiac effects might be cAMP-independent, mediated through a direct interaction between G$\alpha$ and voltage-sensitive Ca$^{2+}$ channels$^{5,6}$ or an increase in myofilament sensitivity to cytosolic Ca$^{2+}$ via increasing intracellular pH.$^{31}$

However, PKA inhibition completely abolished the contractile and $I_{Ca}$ responses to $\beta_2$-AR as well as $\beta_2$-AR stimulation in single canine myocytes, indicating that $\beta_2$-AR stimulation still requires cAMP/PKA signaling to augment $I_{Ca}$ and to produce its positive inotropic and relaxant effects. Because $\beta_2$-AR–directed cAMP signaling modulates $I_{Ca}$, without influencing the phosphorylation of the regulatory proteins examined, including its nearest neighbor, PLB, and because there is no detectable elevation of PKA activation in the intact dog after $\beta_2$-AR stimulation, we propose that the $\beta_2$-AR–activated cAMP/PKA signaling may be highly localized to a subsarcolemmal microdomain, in the vicinity of L-type Ca$^{2+}$ channels, but is excluded from the bulk cytoplasm. The observation of localized $\beta_2$-AR cAMP/PKA signaling in rat$^{14}$ and canine myocytes is in general agreement with the previous notion in frog cardiomyocytes that cAMP generated by local stimulation of $\beta_2$-ARs or direct activation of adenylate cyclase has different abilities to modulate remote L-type Ca$^{2+}$ channels.$^{32}$ The local regulation of L-type Ca$^{2+}$ channels is supported by the close spatial association of the channels with adenylate cyclase and PKA.$^{33,34}$

The specific mechanisms mediating the local control of $\beta_2$-AR–stimulated cAMP/PKA signaling in canine hearts cannot be determined from the present results. Our previous studies in rat and mouse ventricular myocytes have shown that $\beta_2$-AR dually couples to G$_{i}$ and G$_{s}$, and that inhibition of $\beta_2$-AR coupling to G$_{i}$ enhances the $\beta_2$-AR–induced contractile and relaxation effects in these cells.$^{10,35}$ More importantly, in rat myocytes, an inhibition of G$_{i}$ function by pertussis toxin rescues the $\beta_2$-AR–mediated Ser16 PLB phosphorylation,$^{36}$ suggesting that the coupling of $\beta_2$-AR to G$_{i}$ might explain the localized nature of the $\beta_2$-AR–stimulated cAMP signaling.

It is widely accepted that $\beta$-adrenergic relaxant effects are closely associated with an increase in PLB phosphorylation, causing an increase in SR Ca$^{2+}$ pump activity through a removal of its inhibitory effect on the pump.$^{22,23}$ In addition, an augmentation in Tnl phosphorylation after $\beta$-AR subtype stimulation might contribute to the relaxant effect.$^{37}$ Another novel finding of the present study is that the Zin-induced acceleration of relaxation occurs both in vivo and in vitro without any increase in Tnl or PLB phosphorylation at either Ser16 or Thr17. Thus, our data indicate that neither PLB nor Tnl phosphorylation is involved in the $\beta_2$-AR–induced relax-
ant effect in canine hearts. Further studies are required to identify mechanisms underlying the β2-AR–stimulated cardiac relaxation in dog.

In conclusion, we have shown that stimulation of both β2-AR subtypes enhances canine cardiac function in vivo and in vitro via cAMP/PKA-dependent signaling. However, the β2-AR–coupled cAMP/PKA signaling pathway differs from that of β1-AR because it apparently affects only sarcolemmal L-type Ca2+ channels and not SR, myofilament, and cytosolic proteins. Taken together, these results suggest that the β2-AR–activated cAMP/PKA signaling might be localized to a subsarcolemmal microdomain.

References


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