Regulation of cardiac alternans by β-adrenergic signaling pathways

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Florea SM, Blatter LA. Regulation of cardiac alternans by β-adrenergic signaling pathways. Am J Physiol Heart Circ Physiol 303:H1047–H1056, 2012. First published August 17, 2012; doi:10.1152/ajpheart.00384.2012.—In cat atrial myocytes, β-adrenergic receptor (β-AR) stimulation exerts profound effects on excitation-contraction coupling and cellular Ca2+ cycling that are mediated by β1- and β2-AR subtypes coupled to G proteins (Gα and Gβγ). In this study, we determined the effects of β-AR stimulation on pacing-induced Ca2+ alternans. Ca2+ alternans was recorded from single cat atrial myocytes with the fluorescent Ca2+ indicator indo-1. Stable Ca2+ alternans occurred at an average pacing frequency of 1.7 Hz at room temperature with a mean alternans ratio of 0.43. Nonselective β-AR stimulation as well as selective stimulation of β1/Gα, β2/Gα, + Gβγ, and β2/Gα coupled pathways all abolished pacing-induced Ca2+ alternans. β1-AR stimulation abolished alternans through stimulation of PKA and Ca2+/calmodulin-dependent protein kinase II, whereas β2-AR stimulation exclusively involved PKA and was mediated via Gβγ, whereas a known second pathway in cat atrial myocytes acting through Gβγ and nitric oxide production was not involved in alternans regulation. Inhibition of various mitochondrial functions (dissipation of the mitochondrial membrane potential or inhibition of mitochondrial F1/F0-ATP synthase, mitochondrial Ca2+ uptake via the mitochondrial Ca2+ uniporter, and Ca2+ extrusion via mitochondrial Na+/Ca2+ exchange) enhanced Ca2+ alternans; however, β-AR stimulation still abrogated alternans, provided that sufficient cellular ATP was available. Selective inhibition of mitochondrial or glycolytic ATP production did not prevent β-AR stimulation from abolishing Ca2+ alternans. However, when both ATP sources were depleted, β-AR stimulation failed to decrease Ca2+ alternans. These results indicate that in atrial myocytes, β-AR stimulation protects against pacing-induced alternans by acting through parallel and complementary signaling pathways.

Cardiac alternans is defined as a beat-to-beat alternation in contraction amplitude (mechanical alternans), action potential duration (electrical or action potential duration alternans), and Ca2+ transient amplitude (Ca2+ alternans) at a constant stimulated frequency (e.g., Ref. 57). Alternans has been recognized as a risk factor for cardiac arrhythmia and sudden cardiac death (47–49), including atrial fibrillation (20, 37), and although it is considered a multifactorial process, it has become increasingly clear that alternans is ultimately linked to disturbances in myocardial Ca2+ homeostasis and impaired intracellular Ca2+ concentration ([Ca2+]i) regulation (for reviews, see Refs. 5, 6, 12–14, 16, 31, 36, and 43). Based on the theory of cardiac Ca2+ signaling, computational studies, and experimental data (for reviews and references, see Refs. 54 and 56), two parameters have emerged as critically relevant to the generation of alternans at the cellular level: fractional Ca2+ release from the sarcoplasmic reticulum (SR), dependent on SR load, and the efficiency of beat-to-beat cytosolic Ca2+ sequestration. Ca2+ sequestration depends on the availability of adequate ATP supplies to fuel Ca2+-pumps [particularly sarc(endo)plasmic reticulum Ca2+-ATPase (SERCA)] and to serve as a substrate for phosphorylation processes as well as on organelles capable of storing Ca2+. We have previously shown that conditions that are accompanied by reduced ATP generation promote the generation of alternans. Our previous studies showed that inhibition of glycolysis (5, 23, 28, 29) as well as mitochondrial homeostasis and impaired intracellular calcium; mitochondria

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signaling (52), and 2) NO can inhibit the β₂/Gₛ/cAMP/PKA pathway via protein nitrosylation (10). Thus, the goal of the present study was to elucidate the interplay between these specific β-AR signaling pathways and their downstream targets, mitochondrial function, and cellular energy (ATP) sources for the regulation of pacing-induced Ca²⁺ alternans in atrial myocytes.

METHODS

Chemicals and solutions. Chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. ICI-118,551 was from AstraZeneeca (Wilmington, DE), CGP-37157 was obtained from Tocris (Ellisville, MO), and KN-92, KN-93, spermine NONO-ate (SNO), and W-7 were from Calbiochem (San Diego, CA). During experiments, cells were continuously superfused with standard Tyrode solution containing (in mM) 140 NaCl, 5 KCl, 1 MgCl₂, 10 glucose, 10 HEPES, and 2 CaCl₂ (pH 7.3 adjusted with NaOH). All inhibitors, agonists, and antagonists were added to normal Tyrode solution from stock solutions.

β-AR stimulation protocols. Four different β-AR stimulation protocols were used based on our previous studies (10, 51) on β-AR signaling in cat atrial myocytes. Nonselective β₁/β₂-AR stimulation (termed here β-AR stimulation) was achieved with isoprotrenol (ISO; 0.1 μM). For specific stimulation of β₁-AR-dependent signaling (β₁-AR stimulation), cells were treated with ISO (0.1 μM) in combination with the β₂-AR subtype blocker ICI-118,551 (0.01 μM). Simultaneous activation of β₂/Gₛ and β₂/Gₐ signaling (β₂-AR stimulation) was achieved with the combination of ISO (0.1 μM) and the specific β₁-AR inhibitor atenolol (0.01 μM), whereas for selective β₂/Gₛ stimulation, fenoterol (41, 42) was used (0.1 μM). Concentrations were chosen based on our previous studies (10, 51) on β-AR signaling in cat atrial myocytes.

Myocyte isolation. Cat atrial myocytes were isolated according to previously described methods (58). All protocols and procedures were approved by the Institutional Animal Care and Use Committee. Adult cats of either sex were anesthetized with pentobarbital sodium (50 mg/kg), and hearts were excised, mounted on a Langendorff apparatus, and retrogradely perfused with oxygenated collagenase-containing solution (37°C).

[Ca²⁺]ᵢ measurements. For [Ca²⁺]ᵢ measurements, atrial myocytes were loaded with the membrane-permeable form of the fluorescent Ca²⁺ indicator indo-1 AM (5 μM, Invitrogen/Molecular Probes, Carlsbad, CA) in standard Tyrode solution for 15–20 min at room temperature followed by a >20-min deesterification interval in dye-free media. Indo-1-loaded cells were excited at 360 nm, and emission signals were collected simultaneously at 410-nm fluorescence (F₄₁₀) and 485-nm fluorescence (F₄₈₅) using photomultiplier tubes. Fluorescence signals were background subtracted, and [Ca²⁺]ᵢ changes were expressed as changes in the ratio (R) of F₄₁₀ to F₄₈₅. Ca²⁺ transients and Ca²⁺ alternans were elicited by electrical field stimulation (voltage set at ~50% above the threshold for contraction to ensure that every electrical stimulus was captured by the myocyte) using a pair of platinum electrodes. Pacing-induced stable Ca²⁺ alternans was elicited by increasing the stimulation frequency from 0.5 to 1 Hz followed by further increases in stimulation frequency in 0.1-Hz increments until stable alternans was observed. Experimental protocols were repeated when individual cells revealed stable alternans for ~1 min at a given stimulation frequency. The degree of Ca²⁺ alternans was quantified as the alternans ratio. The alternans ratio was defined as 1 - S/L, where S/L is the ratio of the small-amplitude (S) to large-amplitude (L) Ca²⁺ transient during a pair of alternating Ca²⁺ transients (28, 59). Thus, the alternans ratio had values between 1 and 0, with 0 indicating no alternans and 1 indicating the highest possible degree of alternans, with only every other stimulation resulting in a measurable Ca²⁺ transient.

RESULTS

Effect of β-adrenergic stimulation on pacing-induced Ca²⁺ alternans. Stable Ca²⁺ alternans was induced by incrementally increasing the pacing frequency. Ca²⁺ alternans was observed at stimulation frequencies of >1 Hz. The average pacing frequency where stable maintained alternans was observed in cat atrial myocytes at room temperature was 1.7 Hz. The average alternans ratio was 0.43 ± 0.02 under control conditions.

In the heart, β-AR stimulation exerts positive inotropic effects and enhances the efficacy of E-C coupling through the regulation of intracellular Ca²⁺-handling proteins and Ca²⁺ signaling pathways. Stimulation of atrial myocytes revealing stable pacing-induce Ca²⁺ alternans with the nonselective β-AR agonist ISO (0.1 μM) abolished alternans (alternans ratio decreased from 0.43 ± 0.02 to 0.03 ± 0.01), as shown in Fig. 1A. Furthermore, ISO increased the Ca²⁺ transient amplitude of both the small and large alternans Ca²⁺ transient by 232% and 85%, respectively, indicative of the positive inotropic effect of β-AR stimulation.

While cat atrial myocytes have both β₁- and β₂-subtypes of the AR (53), our previous study (51) revealed that the β₁-subtype is not involved in E-C coupling regulation in cat atrial tissue. Therefore, in the following experiments, we focused on β₁- and β₂-AR-mediated signaling pathways. As shown in Fig. 1B, selective stimulation of the β₁-AR pathway by the combined application of ISO (0.1 μM) and the β₂-AR blocker ICI-118,551 (0.01 μM) abolished Ca²⁺ alternans to the same degree as ISO alone. Similarly, selective stimulation of the β₂-AR pathway [with ISO in the presence of the β₁-AR blocker atenolol (0.01 μM)] or exclusively of the β₂/Gₛ pathway [with the selective β₂/Gₛ agonist fenoterol (0.1 μM)] abolished Ca²⁺ alternans, as shown in Fig. 1, C and D, respectively. Similar to nonselective β-AR stimulation, the selective activation of β₁- and β₂-ARs had a positive inotropic effect on the Ca²⁺ transient amplitude. Thus, the combined or selective activation of β-AR subtypes leads to an abolishment of pacing-induced Ca²⁺ alternans. With all four stimulation protocols, the ARs dropped to similarly low values between 0.03 and 0.04 (Fig. 1E), indicative of the complete absence of Ca²⁺ alternans.

β-AR effects on Ca²⁺ alternans involves PKA. It is well established that β₁- and β₂-AR-mediated effects on E-C coupling are mediated by Gₛ proteins coupled to AC, which generates cAMP, which, in turn, activates PKA. PKA is involved in the phosphorylation processes of Ca²⁺-handling proteins [L-type voltage-gated Ca²⁺ channels (LTCC), SERCA, and the ryanodine receptor (RyanR) Ca²⁺-release channel], which profoundly affects Ca²⁺ signaling during E-C coupling. We there-
fore tested the effect of PKA inhibition alone and in combination with β-AR stimulation on Ca\(^{2+}\) alternans. As shown in Fig. 2A, the PKA inhibitor H-89 (1 μM) enhanced Ca\(^{2+}\) alternans and increased the alternans ratio by 76%, to 0.72 ± 0.06. These data suggest that basal PKA activity exerts partial protection against alternans. Subsequent β-AR stimulation in the presence of the PKA blocker decreased Ca\(^{2+}\) alternans, but with different efficiency depending on which β-AR subtype was activated. β\(_1\)-AR stimulation completely abolished Ca\(^{2+}\) alternans (Fig. 2A, top), whereas selective activation of the β\(_2\)/G\(_s\) pathway, while showing a partial decrease of alternans, failed to lower the alternans ratio below control levels (Fig. 2A, middle). The summary data of changes of the alternans ratio are shown in Fig. 2A, bottom.

The effect of PKA inhibition was tested with a different protocol, as shown in Fig. 2B. Here, β-AR stimulation preceded the application of the PKA inhibitor. As expected, both β\(_1\)-AR, β\(_2\)-AR, and selective β\(_2\)/G\(_s\) stimulation completely abolished Ca\(^{2+}\) alternans. Subsequent application of H-89 (10 μM) caused the maximal degree of alternans during β\(_2\)/G\(_s\) stimulation, with an alternans ratio approaching 1. In contrast, during β\(_1\)-AR stimulation, PKA inhibition also enhanced Ca\(^{2+}\) alternans beyond control levels (alternans ratio: 0.72 ± 0.15); however, the effect was less pronounced than during β\(_2\)/G\(_s\) stimulation. Taken together, these data indicate that β\(_2\)/G\(_s\) stimulation abolished alternans through a PKA-dependent pathway, whereas the decrease of alternans mediated by β\(_1\)-AR signaling appeared only partially to be dependent on PKA. CaMKII is a second crucial intracellular kinase that regulates Ca\(^{2+}\)-handling proteins through phosphorylation, and it has been shown that in the heart, β\(_1\)-AR stimulation activates a dual signaling pathway mediated by cAMP/PKA and CaMKII (50). Therefore, in the following set of experiments, we tested the involvement of CaMKII in β-AR-mediated regulation of Ca\(^{2+}\) alternans.

Role of calmodulin and CaMKII in the regulation of Ca\(^{2+}\) alternans. Inhibition of the CaMKII signaling pathway on pacing-induced Ca\(^{2+}\) alternans is shown in Fig. 3. Exposure to the calmodulin antagonist W-7 (25 μM) led to a profound enhancement of Ca\(^{2+}\) alternans, again suggesting that basal kinase activity protects against the development of a high degree of alternans. In the example shown in Fig. 3A, top, W-7 almost completely abolished the small-amplitude alternans Ca\(^{2+}\) transient, and, on average, the alternans ratio increased to 0.90 ± 0.05. Similarly, the CaMKII antagonist KN-93 (1 μM) enhanced Ca\(^{2+}\) alternans and increased the alternans ratio to 0.77 ± 0.06 (Fig. 3A, bottom), whereas KN-92, the inactive analog of KN-93, serving as a negative control, had only a marginal effect on the alternans ratio. Subsequent β\(_1\)-AR stimulation decreased Ca\(^{2+}\) alternans, but in the presence of W-7 and KN-93, the alternans ratio remained above control levels (0.69 ± 0.18 and 0.67 ± 0.10, respectively). In contrast, in the presence of the inactive analog KN-92, β\(_1\)-AR stimulation was capable of completely abolishing Ca\(^{2+}\) alternans.

Fig. 1. Effects of β-adrenergic receptor (β-AR) stimulation on pacing-induced Ca\(^{2+}\) alternans. Pacing-induced Ca\(^{2+}\) alternans recordings are from individual atrial myocytes before and during β-AR stimulation. A: nonselective β-AR stimulation with isoproterenol (ISO; 0.1 μM); B: β\(_1\)-AR-specific stimulation using ISO (0.1 μM) combined with the β\(_2\)-AR antagonist ICI-118,551 (0.01 μM); C: β\(_2\)-AR-specific stimulation with ISO (0.1 μM) combined with the β\(_1\)-AR antagonist atenolol (0.01 μM). D: specific stimulation of the β\(_2\)/G\(_s\) pathway with fenoterol (0.1 μM). Bottom recordings show Ca\(^{2+}\) transients at expanded times (a, b, and c) from the top recordings. E: summary of selective and nonselective β-AR stimulation on alternans ratio. Numbers in parentheses indicate numbers of individual atrial myocytes tested. *P < 0.001 vs. control (CTL).
Fig. 2. β-AR effects on Ca$^{2+}$ alternans mediated by PKA. A, top and middle: recordings of Ca$^{2+}$ alternans before and during inhibition of PKA with H-89 (1 μM) followed by β₁-AR (top) or β₂/Gr stimulation (middle). Bottom, average alternans ratios in the presence of H-89 and during subsequent β₁-AR and β₂/Gr stimulation. B, top: recordings of Ca$^{2+}$ alternans where cells were first exposed to β-AR stimulation followed by treatment with H-89 (10 μM). Bottom, average alternans ratios. During β₂-AR stimulation, H-89 caused the maximal degree of alternans, with the alternans ratio approaching 1. Black circles mark the timing of electrical stimulation when the small-amplitude Ca$^{2+}$ transient was strongly reduced in amplitude. Numbers in parentheses indicate numbers of individual atrial myocytes tested. [Ca$^{2+}$], intracellular Ca$^{2+}$ concentration; R, ratio of fluorescence at 410 nm to fluorescence at 485 nm.

Figure 3B shows the reversed protocol. β₁-AR stimulation abolished pacing-induced Ca$^{2+}$ alternans, as expected; however, the subsequent application of W-7 and KN-93 increased the alternans ratio above control levels, to 0.78 ± 0.11 and 0.76 ± 0.13, respectively. The inactive analog KN-92 had no influence on the abolishing effect of β₁-AR stimulation (Fig. 3B, bottom). These data indicate that the effect of β₁-AR stimulation to abolish alternans largely depends on CaMKII activity and, to a lesser extent, on the cAMP/PKA pathway, whereas the β₂-AR-dependent effects on Ca$^{2+}$ alternans primarily involve PKA. This was further confirmed by the observation that in the presence of KN-93, β₂-AR stimulation still lowered the alternans ratio below control levels (data not shown).

Combined inhibition of PKA and CaMKII is required to abolish β₁-AR effects on Ca$^{2+}$ alternans. After pacing-induced Ca$^{2+}$ alternans was enhanced by the inhibition of PKA (alternans ratio in the presence of H-89: 0.72 ± 0.06; Fig. 4), subsequent β₁-AR stimulation reversed the effect of H-89 and decreased the alternans ratio below control levels (alternans ratio: 0.13 ± 0.09). Subsequent exposure to W-7 reversed the effect of β₁-AR stimulation. In the example shown in Fig. 4, top, combined inhibition of PKA and CaMKII almost completely abolished the small-amplitude Ca$^{2+}$ transient despite β₁-AR stimulation. On average, the alternans ratio rose to 0.72 ± 0.12. In conclusion, the results indicate that β₁-AR stimulation protects against alternans by invoking two complementary signaling pathways, one involving cAMP/PKA and the other depending on CaMKII activity.

Role of downstream targets of β₂-AR signaling in the protection against alternans. β₂-ARs couple to both G$_{i}$ and G$_{s}$ proteins (25, 60, 61). As detailed in the Introduction, in cat atrial myocytes, the β₂/G$_{s}$ signaling pathway couples via PI3K/Akt to eNOS and leads to NO production. The downstream effects of NO are twofold and result in enhanced cAMP/PKA...
Nitric oxide (NO) and phospho-(L-NAME) or supplied NO in excess with an exogenous NO donor (SNO) resulted in a complete inhibition of the Ca^{2+}/H^{+} pathway (and thus NO) does not play a significant role in the regulation of alternans. This was further confirmed by the observation that in the presence of pertussis toxin to disrupt $G_i$ regulation (61), $\beta_2/G_{iG}$ stimulation fully abolished alternans (data not shown) and that even in the presence of excessive amounts of NO, $\beta_2$-AR stimulation led to an abrogation of alternans (Fig. 5A). In addition, inhibition of PDE III by milrinone (Fig. 5B) completely abolished Ca^{2+} alternans and had a strong positive inotropic effect on the Ca^{2+} transient amplitude, presumably by increasing cytosolic Ca^{2+} levels. $\beta_2$-AR stimulation in the presence of milrinone had no additional effect on the alternans ratio since the alternans ratio was already nearly 0 (data not shown). In conclusion, these data indicate that $\beta_2$-AR-mediated abolishment of Ca^{2+} alternans is mediated by the $\beta_2/G_{iG}/AC/cAMP/PKA$ pathway.

Under $\beta$-AR stimulation, normal contraction of myocytes and intracellular Ca^{2+} cycling are critically dependent on the balance between myocardial energy demand and energy supply from mitochondrial and glycolytic sources. Thus, in the next experiments, we explored the role of $\beta$-AR signaling in Ca^{2+} alternans during metabolic inhibition.

Alternans depends on the interaction between mitochondrial function and $\beta$-AR signaling. We have previously shown that inhibition of various mitochondrial functions (ranging from inhibition of the electron transport chain, $F_1/F_0$-ATP synthase, and mitochondrial dehydrogenases to mitochondrial Ca^{2+} transport) enhances the propensity and degree of Ca^{2+} alternans, suggesting that mitochondrial function, specifically ATP production, plays a critical role in the generation of alternans (16).

Consistent with our previous study, as shown in Fig. 6, inhibition of mitochondrial ATP production by block of mitochondrial $F_1/F_0$-ATP synthase with oligomycin (A), dissipation of the mitochondrial membrane potential with FCCP (B), inhibition of mitochondrial Ca^{2+} uptake via the mitochondrial Ca^{2+} uniporter with ruthenium red (C), or Ca^{2+} extrusion via the mitochondrial Na^{+}/Ca^{2+} exchanger with CGP-37157 (D) all enhanced Ca^{2+} alternans and increased the alternans ratio to values between 0.53 and 0.80 (E). $\beta$-AR stimulation in the presence of oligomycin, FCCP, or ruthenium red lowered the alternans ratio and decreased and even abolished alternans. There were no significant differences between $\beta_1$- and $\beta_2$-AR-mediated signaling. In contrast, $\beta$-AR stimulation after the inhibition of Ca^{2+} extrusion with CGP-37157 failed to significantly decrease alternans. We (16) have previously shown that treatment with CGP-37157 led to cyclosporine-sensitive opening of the mitochondrial permeability transition pore (MPTP), which is linked to irreversible impairment of mitochondrial function via inhibition of cGMP-dependent PDE III but also inhibition of the $\beta_2/G_{iG}/AC/cAMP/PKA$ pathway (1, 10). Since NO plays a key role in the $\beta_2/G_{iG}$ signaling pathways, we applied a blocker of eNOS [N\textsuperscript{\text{-}}-nitro-L-arginine methyl ester (L-NAME)] or supplied NO in excess with an exogenous NO donor (SNO). As shown in Fig. 5A, neither L-NAME nor SNO had a profound effect on the alternans ratio of pacing-induced Ca^{2+} alternans. Selective stimulation of the $\beta_2/G_{iG}$ pathway in the presence of L-NAME or SNO resulted in a complete abolishment of Ca^{2+} alternans, suggesting that the $\beta_2/G_{iG}$ pathway is sufficient for complete protection against alternans during $\beta_2$-AR stimulation. Concomitant stimulation of the $\beta_2/G_{iG}$ pathway had no additional effect, indicating that the $\beta_2/G_{iG}$ pathway (and thus NO) does not play a significant role in the regulation of alternans. This was further confirmed by the numbers in parentheses indicate numbers of individual atrial myocytes tested.
function. Presumably due to the irreversible nature of MPTP opening, β-AR stimulation failed to protect against Ca\(^{2+}\) alternans. In contrast, these results indicate that potentially reversible inhibition of mitochondrial functions can be counteracted through β-AR signaling.

Depletion of cellular ATP sources diminishes the ability of β-AR stimulation to protect against alternans. A common result of inhibition of diverse mitochondrial functions is a decrease in mitochondrial ATP production and exhaustion of cellular energy reserves. The diminished ATP supply potentially affects ATP-dependent Ca\(^{2+}\) transport processes, such as cytosolic Ca\(^{2+}\) sequestration. Decreased cytosolic Ca\(^{2+}\) sequestration has been identified as a key factor causing cardiac alternans. We have previously shown that selective inhibition of glycolytic ATP production (5, 23, 28, 29) or ATP synthesis by mitochondrial Ca\(^{2+}\) extrusion via the mitochondrial Na\(^+/\)Ca\(^{2+}\) exchanger with CGP-37157 (CGP; 2.5 μM) followed by β\(_2\)/Gs stimulation. In contrast, these results indicate that potentially cytosolic Ca\(^{2+}\) removal. Decreased cytosolic Ca\(^{2+}\) sequestration has been identified as a key factor causing cardiac alternans. We have previously shown that selective inhibition of glycolytic ATP production (5, 23, 28, 29) or ATP synthesis by mitochondrial Ca\(^{2+}\) extrusion via the mitochondrial Na\(^+/\)Ca\(^{2+}\) exchanger with CGP-37157 (CGP; 2.5 μM) followed by β\(_2\)/Gs stimulation. β\(_1\)-AR stimulation failed to protect against alternans. In contrast, when both sources of ATP were abolished, β\(_1\)-AR stimulation failed to decrease alternans (Fig. 7B). β\(_2\)/Gs stimulation-dependent decrease of Ca\(^{2+}\) alternans is capable of decreasing Ca\(^{2+}\) alternans during inhibition of mitochondrial ATP production with oligomycin. Similarly, β\(_2\)/Gs stimulation was effective in abolishing Ca\(^{2+}\) alternans that were previously enhanced by inhibition of glycolytic ATP formation. In contrast, when both sources of ATP were abolished, β\(_2\)/Gs stimulation failed to decrease alternans (Fig. 7B). Furthermore, diastolic [Ca\(^{2+}\)\(_i\)] substantially increased and Ca\(^{2+}\) transients were prolonged, consistent with compromised cytosolic Ca\(^{2+}\) removal. As shown in Fig. 7C, separate or combined inhibition of glycolytic and/or mitochondrial ATP production consistently enhanced Ca\(^{2+}\) alternans. When only one of the two cellular ATP sources was inhibited, β\(_2\)/Gs stimulation could overcome the inhibitory effect and was able to decrease alternans. However, when both ATP sources were abolished, β\(_2\)/Gs stimulation failed to protect against alternans.

In conclusion, our results indicate that β-AR stimulation protects against proarrhythmic Ca\(^{2+}\) alternans and involves parallel and complementary pathways regulated by both β\(_1\)- and β\(_2\)-AR subtypes. β-AR stimulation-dependent decrease of ATP.
alternans involves both PKA and CaMKII and depends on a sufficient ATP supply.

DISCUSSION

In this study, we investigated the interplay between β-AR signaling, mitochondrial function, and the occurrence of Ca2+ alternans in atrial tissue. The key findings of our investigation were as follows.

First, pacing-induced Ca2+ alternans could be decreased by several distinct pathways linked via G proteins to β1- and β2-AR subtypes. The effect of β-AR stimulation involved activation of the kinases PKA and CaMKII. β1-AR stimulation was linked via Gs to PKA and CaMKII activation. β2-AR stimulation only activated PKA via Gs, similarly to β1-AR stimulation; however, the previously identified β2/Gi/eNOS/NO signaling pathway in atrial myocytes had no effect on alternans. Furthermore, basal PKA and CaMKII activity exerted partial regulation of Ca2+ signaling pathway in atrial myocytes had no effect on alternans.

Second, pacing-induced Ca2+ alternans was enhanced by disruption of mitochondrial function; however, β-AR stimulation could still decrease Ca2+ alternans in the presence of compromised mitochondria, except when mitochondrial impairment included MPTP opening.

Finally, the abolishing effect of β-AR stimulation on Ca2+ alternans depended on sufficient cellular ATP reserves and energy supplies. Selective inhibition of glycolysis or mitochondrial ATP production via oxidative phosphorylation enhanced Ca2+ alternans; however, alternans could still be decreased by β-AR stimulation, suggesting that one ATP source could compensate for the other. Severe depletion of cellular ATP reserves by combined inhibition of glycolytic and mitochondrial ATP production prevented β-AR stimulation from abrogating Ca2+ alternans, suggesting that the protective effect of β-AR signaling ultimately depends on a sufficient ATP supply.

The three β-AR signaling cascades investigated here have been previously established in atrial myocytes (10, 51). In these studies, the importance of these signaling pathways for E-C coupling was demonstrated for the regulation of ion currents (L-type Ca2+ current and ATP-dependent K+ current). The present study extended these earlier observations by investigating how these pathways regulate or modulate Ca2+ alternans in atrial myocytes.

An important finding of our study was that the abolishment of Ca2+ alternans by β-AR stimulation involved the activation of cellular kinases. Whereas β1-AR effects depended on both PKA and CaMK activity, β2-AR-mediated effects appeared to involve solely PKA. Both kinases have well-established phosphorylation targets, including Ca2+-handling proteins that play a key role in E-C coupling and mediate the inotropic effects of β-AR stimulation. Among these proteins, the activity of LTCCs, RyRs, and SERCA are all enhanced by β-AR stimulation-dependent phosphorylation. While β-AR stimulation enhances LTCC currents, Ca2+ entry, and SR Ca2+ load and release, it does not seem to have a direct effect on LTCC as far as alternans are concerned, because the L-type Ca2+ current typically does not alternate during alternans in atrial myocytes (23, 45; but see Refs. 17 and 32). Thus, the abolishing effect appears to be downstream of LTCC augmentation. A prime candidate for a downstream target is beat-to-beat Ca2+ sequestration. It has been convincingly shown that cellular processes, transport pathways, and organelles that clear cytosolic Ca2+ protect against alternans and that conditions that impair cytosolic Ca2+ sequestration promote alternans (for reviews and references, see Refs. 54 and 56). β-AR stimulation strongly enhances SERCA activity, which is accomplished via phosphorylation of the SERCA inhibitory protein phospholamban (30, 33). Phospholamban phosphorylation, principally by PKA (although there is evidence for a role of CaMKII), reduces its inhibitory interactions with SERCA and leads to a dramatic increase in SERCA activity.

Along the same line of arguments, intact mitochondrial function plays an important role in the protection against proarhythmic alternans. Mitochondria participate in Ca2+ signaling during E-C coupling in two main ways: as a potential Ca2+ sink that can take up and store significant amounts of Ca2+ and as the quantitatively most important source of ATP production that fuels phosphorylation processes or serves as a regulatory cofactor of ion channels and transporters. While it is still a matter of debate (for a discussion of this controversy, see Refs. 9 and 40) as to whether mitochondrial Ca2+ uptake and extrusion make a significant contribution to the beat-to-beat regulation of [Ca2+], (and therefore may also make a direct

Fig. 7. Effects of β-AR stimulation on Ca2+ alternans during inhibition of glycolytic and mitochondrial ATP production. A: effects of inhibition of glycolysis with pyruvate (Pyr; 10 mM) followed by nonselective β-AR stimulation with ISO (0.1 μM) on pacing-induced Ca2+ alternans. B: effects of combined inhibition of glycolytic (Pyr) and mitochondrial (Oligo; 1 μg/ml) ATP production followed by nonselective β-AR stimulation with ISO. C: summary data of average alternans ratios during glycolytic and/or mitochondrial inhibition followed by β-AR stimulation. β-AR stimulation could overcome the effect of separate inhibition of glycolytic or mitochondrial ATP generation; however, during inhibition of both ATP sources, β-AR stimulation failed to decrease Ca2+ alternans. Numbers in parentheses indicate numbers of individual atrial myocytes tested. 2-DG, 2-deoxyglucose.
contribution to Ca\(^{2+}\) alternans), there is no doubt that impairment of mitochondrial function at various levels generates conditions that favor alternans. Dissipation of mitochondrial membrane potential and inhibition of mitochondrial function at the level of the electron transport chain, F\(_{1}/F_{0}\)-ATP synthase, or Ca\(^{2+}\) uptake and extrusion all enhance Ca\(^{2+}\) alternans (for a review, see Ref. 16; see also Figs. 6 and 7 in the present study). In addition, altering mitochondrial function changes the cellular redox environment (8), and redox modification of the RyR can cause alternans (3). These results, together with the observation that inhibition of glycolysis also induced electromechanical and Ca\(^{2+}\) alternans (see Refs. 23, 28, and 29; see also Figs. 6 and 7 in the present study), suggest that a sufficient ATP supply is crucial for the prevention of alternans and emphasizes a key role of kinases and phosphorylation processes in this context. Our results indicate that selective elimination of one of the two ATP sources per se enhanced Ca\(^{2+}\) alternans but was not sufficient to prevent the protective effect of \(\beta\)-AR signaling against Ca\(^{2+}\) alternans. This observation suggests that if at least one ATP source is functional, the amounts of ATP produced are sufficient to sustain the \(\beta\)-AR effects on Ca\(^{2+}\) signaling. Even though the bulk cellular ATP pool is depleted dramatically during inhibition of mitochondrial ATP production (since mitochondria generate up to 90% of cellular ATP in cardiac myocytes, which is primarily consumed to sustain contractile activity), glycolysis-derived ATP appears to suffice to support \(\beta\)-AR effects on Ca\(^{2+}\) alternans. This may in part be explained by the observation that ATP derived from glycolysis is used preferentially by membrane ion transport mechanisms. The close physical association of glycolytic enzymes with ATP-dependent ion transporters and channels form functional microcompartments where source and utilization sites for ATP are intimately linked. Such arrangements have been demonstrated for Na\(^{+}\)/K\(^{+}\)-ATPase (18), ATP-dependent K\(^{+}\) channels (55), and the SERCA pump (62) and have been proposed for the RyR Ca\(^{2+}\)-release channel as a potential contributing factor to alternans (5, 23, 28, 29).

Modulation of RyR function through \(\beta\)-AR stimulation represents an additional mechanism through which \(\beta\)-AR stimulation protects against alternans. RyRs are subject to phosphorylation by PKA and CaMKII (2, 19, 22, 34, 35). The signaling events that occur downstream of \(\beta\)-AR stimulation and their effects on RyR activity remain highly contentious (for reviews, see Refs. 21 and 63), and the ramifications of RyR phosphorylation for cardiac function in health and disease have remained a highly debated and controversial issue (for a recent update on the debate, see Ref. 4). Nonetheless, we (45) have previously shown that in atrial myocytes, refactororiness of SR Ca\(^{2+}\) release via RyRs and beat-to-beat alternations in the kinetics of RyR and SR Ca\(^{2+}\)-release restitution constitute a key defining factor of cardiac alternans. Factors that sensitize the RyR to Ca\(^{2+}\)-induced Ca\(^{2+}\) release can accelerate restitution and therefore can overcome Ca\(^{2+}\) alternans. This can be demonstrated with the RyR-sensitizing agent caffeine at low concentrations, and the effects were strikingly similar to the effects of ISO stimulation (45). Thus, enhanced Ca\(^{2+}\) sequestration together with \(\beta\)-AR-mediated effects on the RyR appear to act in tandem to protect against pacing-induced Ca\(^{2+}\) alternans. The abolishment of pacing-induced Ca\(^{2+}\) alternans by \(\beta\)-AR stimulation is controversial and not unique to atrial tissue. As discussed in the Introduction, \(\beta\)-AR stimulation has also been shown to facilitate alternans. At the cellular level, it is believed that this can occur when a strong effect of \(\beta\)-AR stimulation on SR Ca\(^{2+}\) load and fractional SR Ca\(^{2+}\) release outweighs the protective effect of cytosolic Ca\(^{2+}\) sequestration (54). Furthermore, we (23) have previously shown that in feline ventricular myocytes, ISO stimulation reversibly abolished electrical (action potential duration) and mechanical (cell shortening) alternans, where the alternations in action potential duration subsided in concert with the changes in contraction, indicating that electrical and mechanical (or Ca\(^{2+}\)) activity are tightly coupled during alternans.

In summary, we demonstrated here that \(\beta\)-AR stimulation abolishes pacing-induced Ca\(^{2+}\) alternans even under conditions where inhibition of mitochondrial function facilitates Ca\(^{2+}\) alternans. The protective effect of \(\beta\)-AR stimulation is mediated by parallel and complementary signaling cascades. The redundancy in the \(\beta\)-AR signaling-mediated protection against proarrhythmic alternans represents an inherent safety mechanism to prevent arrhythmogenic Ca\(^{2+}\) release in the heart during enhanced sympathetic tone.


**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


63. Yamaguchi N, Meissner G. Does Ca2+/calmodulin-dependent protein kinase deltac activate or inhibit the cardiac ryanodine receptor ion channel? *Circ Res* 100: 293–295, 2007.