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The effect of PKA-mediated phosphorylation of ryanodine receptor on SR Ca^{2+} leak in ventricular myocytes

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ABSTRACT

Functional impact of cardiac ryanodine receptor (type 2 RyR or RyR2) phosphorylation by protein kinase A (PKA) remains highly controversial. In this study, we characterized a functional link between PKA-mediated RyR2 phosphorylation level and sarcoplasmic reticulum (SR) Ca^{2+} release and leak in permeabilized rabbit ventricular myocytes. Changes in cytosolic [Ca^{2+}] and intra-SR [Ca^{2+}]$_{SR}$ were measured with Fluo-4 and Fluo-5N, respectively. Changes in RyR2 phosphorylation at two PKA sites, serine-2031 and -2809, were measured with phospho-specific antibodies. cAMP (10 μM) increased Ca^{2+} spark frequency approximately two-fold. This effect was associated with an increase in SR Ca^{2+} load from 0.84 to 1.24 mM. PKA inhibitory peptide (PKI; 10 μM) abolished the cAMP-dependent increase of SR Ca^{2+} load and spark frequency. When SERCA was completely blocked by thapsigargin, cAMP did not affect RyR2-mediated Ca^{2+} leak. The lack of a cAMP effect on RyR2 function can be explained by almost maximal phosphorylation of RyR2 at serine-2809 after sarcolemma permeabilization. This high RyR2 phosphorylation level is likely the consequence of a balance shift between protein kinase and phosphatase activity after permeabilization. When RyR2 phosphorylation at serine-2809 was reduced to its "basal" level (i.e. RyR2 phosphorylation level in intact myocytes) using kinase inhibitor staurosporine, SR Ca^{2+} leak was significantly reduced. Surprisingly, further dephosphorylation of RyR2 with protein phosphatase 1 (PP1) markedly increased SR Ca^{2+} leak. At the same time, phosphorylation of RyR2 at serine 2031 did not significantly change under identical experimental conditions. These results suggest that RyR2 phosphorylation by PKA has a complex effect on SR Ca^{2+} leak in ventricular myocytes. At an intermediate level of RyR2 phosphorylation SR Ca^{2+} leak is minimal. However, complete dephosphorylation and maximal phosphorylation of RyR2 increases SR Ca^{2+} leak.

1. Introduction

Type-2 isoform of the ryanodine receptor (RyR2) is the primary Ca^{2+} release channel of the sarcoplasmic reticulum (SR) of cardiomyocytes. During systole, synchronized activation of RyR2s by the trans-sarcolemmal inward Ca^{2+} current generates the global Ca^{2+} transient that activates contraction of the heart. During diastole, RyR2s are not completely quiescent, providing a pathway for SR Ca^{2+} leak. Although diastolic SR Ca^{2+} leak is relatively small in comparison with systolic SR Ca^{2+} release [1,2], it might play a protective role against SR Ca^{2+} overload [3]. Thus, accurate RyR2 functioning during different phases of the cardiac cycle is essential for regular heart contraction [4]. RyR2 gating is controlled by several molecular mechanisms and defects in this control can cause SR Ca^{2+} mishandling and contractile dysfunction. For example, increased diastolic SR Ca^{2+} leak can contribute to cardiac arrhythmias in infarcted and failing hearts [5,6].

RyR2 is a target of phosphorylation by different protein kinases, including protein kinase A (PKA). It has been suggested that RyR2 phosphorylation by PKA plays an important role in regulation of SR Ca^{2+} release during β-adrenergic receptor stimulation or in heart failure [5,7–10]. While RyR2 can be phosphorylated at multiple sites [11], phosphorylation of two serines (Ser-2808 and Ser-2030 in small rodents or Ser-2809 and Ser-2031 in rabbit and human) by PKA was suggested to be of functional relevance [5,12,13]. Despite significant efforts, the role of PKA-dependent phosphorylation for the regulation of RyR2 function remains a highly debated topic [14,15]. Originally, it has been suggested that RyR2 phosphorylation by PKA plays a critical role in SR Ca^{2+} mishandling in failing heart. It has been shown that chronic RyR2 phosphorylation at Ser-2808 during heart failure causes dissociation of the small regulatory protein FKBP12.6 from the channel. Such alteration in

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RyR2 structure leads to increased SR Ca^{2+} leak [16] as a result of prolonged subconductance openings of RyR2 [5]. Moreover, transgenic mice with Ser-2808 replaced with alanine (RyR2-S2808A mice) were more resistant to development of heart failure [17], whereas mice with a mutation that mimics RyR2 phosphorylation at Ser-2808 developed cardiomyopathy associated with SR Ca^{2+} mishandling [18]. However, other studies have shown little or no effect of PKA-mediated RyR2 phosphorylation on SR Ca^{2+} release and cardiac pump function. It has been shown that RyR2 phosphorylation had no effect on local SR Ca^{2+} release events (Ca^{2+} sparks) in transgenic animals lacking PKA effect on SERCA-mediated SR Ca^{2+} uptake [19]. Moreover, other studies in RyR2-S2808A mice did not find any significant difference in cardiac function during adrenergic receptor activation or heart failure progression [20,21]. Several recent studies of SR Ca^{2+} release in cardiomyocytes and RyR2 gating in lipid bilayers suggest that the effect of PKA-mediated RyR2 phosphorylation on RyR2 function is more complex than previously assumed. It has been shown that RyR2 is significantly phosphorylated at Ser-2808/9 under "basal" condition (in the absence of adrenergic stimulation or PKA activation) [13,20,22,23] and reduction in RyR2 phosphorylation increases SR Ca^{2+} release, causing SR Ca^{2+} depletion [24]. Therefore, it seems that RyR2 dephosphorylation, rather than phosphorylation, increases the channel activity. Studies of RyR2 activity in lipid bilayers revealed an even more complex pattern of RyR2 regulation by phosphorylation. It has been found that minimum and maximum RyR2 phosphorylation at Ser-2808 both increase RyR2 activity, suggesting a V-shaped dependence of RyR2 activity from its PKA-dependent phosphorylation level [22]. However, a study of a transgenic mouse model susceptible to cardiac arrhythmias suggested that 50% phosphorylation of RyR2 at Ser-2808 had a more detrimental effect on heart function and SR Ca^{2+} handling than complete dephosphorylation or maximum phosphorylation (i.e. suggesting a bell-shaped relationship between RyR2 activity and its phosphorylation level) [25]. Thus, it is unclear whether these opposite findings are due to significant differences between the acute effect of RyR2 phosphorylation observed in bilayer experiments or the chronic effect of RyR2 phosphorylation in transgenic animals.

We have recently developed an experimental approach for direct measurement of RyR2 function in the cellular environment under well-controlled cytosolic conditions [2,3,26]. Here we applied this approach to investigate a possible functional link between RyR2 phosphorylation by PKA at two sites (Ser-2809 and Ser-2031) and RyR2-mediated Ca^{2+} release in rabbit ventricular myocytes. The obtained results revealed that RyR2 phosphorylation by PKA has a complex effect on SR Ca^{2+} release. Between the two PKA sites on RyR2, Ser-2809 was more sensitive to PKA activation. We found that phosphorylation of Ser-2809 at ~75% results in the lowest SR Ca^{2+} leak rate. Maximum phosphorylation and maximum dephosphorylation of Ser-2809 both increase SR Ca^{2+} leak. Unless RyR2 is regulated by PKA via some other unidentified phosphorylation sites, these findings suggest that RyR2 has the lowest SR Ca^{2+} leak rates at a specific intermediate phosphorylation level at Ser-2809.

2. Materials and methods

2.1. Myocyte isolation

All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committees of Loyola University and Rush University Medical Center, and comply with USA regulations on animal experimentation. Ventricular myocytes were isolated from hearts of New Zealand White rabbits (31 animals were used in this studies) according to the procedure described previously [27]. Chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. All experiments were performed at room temperature (20–24 °C).

2.2. Intracellular Ca^{2+} imaging in permeabilized myocytes

To record cytosolic [Ca^{2+}] ($[Ca^{2+}]_i$) and intra-SR [Ca^{2+}] ($[Ca^{2+}]_{SR}$) we used the high affinity Ca^{2+} indicator Fluo-4 and the low affinity Ca^{2+} indicator Fluo-5N, respectively (both indicators were obtained from Thermo Fisher Scientific). Changes in $[Ca^{2+}]_i$ and $[Ca^{2+}]_{SR}$ were measured with laser scanning confocal microscopy (Radiance 2000 MP, Bio-Rad, UK or LSM 410, Carl Zeiss, Germany) equipped with a ×40 oil-immersion objective lens (N.A. = 1.3). Fluo-4 and Fluo-5N were excited with the 488 nm line of an argon ion laser and fluorescence was measured at >515 nm.

2.2.1. Measurements of Ca^{2+} sparks

Myocytes were permeabilized with 0.005% saponin [28,29]. The experimental solution containing Fluo-4 pentapotassium salt (30 μM) was composed of (in mM): K-aspartate 100; KCl 15; KH_2PO_4 5; MgATP 5; EGTA 0.35; $CaCl_2$ 0.067; $MgCl_2$ 0.75; phosphocreatine 10; HEPES 10; creatine phosphokinase 5 U/ml; dextran (MW: 40,000) 4%, and pH 7.2 (KOH). Free [Ca^{2+}] and [Mg^{2+}] of this solution were 150 nM and 1 mM, respectively. Ca^{2+} sparks were recorded in line scan mode (3 ms per scan; pixel size 0.12 μm). Ca^{2+} sparks were detected and analyzed using SparkMaster [30]. The analysis included Ca^{2+} spark frequency (sparks × s^{-1} × $(100\ \mu m)^{-1}$), amplitude ($\Delta F/F_0$), full duration at half-maximal amplitude (FDHM; ms), and full width at half-maximal amplitude (FWHM; μm). F_0 is the initial fluorescence recorded under steady-state conditions and $\Delta F = F - F_0$.

2.2.2. Measurements of SR Ca^{2+} leak

SR Ca^{2+} leak as a function of $[Ca^{2+}]_{SR}$ was measured in permeabilized myocytes as described previously [2]. The SR was loaded with the low-affinity Ca^{2+} dye Fluo-5N after incubating myocytes with 5 μM of Fluo-5N/AM for 2.5 h at 37 °C followed by 1 h of dye wash out [27]. Fluo-5N was excited with minimum laser energy of an argon ion laser (to minimize dye photobleaching). To improve signal-to-noise ratio of the low intensity Fluo-5N signal, fluorescence was collected with an open pinhole and averaged over the entire cellular width of an individual 2-D image (pixel size 0.2 μm). At the end of each experiment minimum Fluo-5N fluorescence (F_{min}) was measured after depletion of the SR with 10 mM caffeine in the presence of 5 mM EGTA. Maximum Fluo-5N fluorescence (F_{max}) was measured following an increase of [Ca^{2+}] to 10 mM in the presence of caffeine [2]. Caffeine keeps RyRs open allowing [Ca^{2+}] equilibration across the SR membrane [31]. The Fluo-5N signal was converted to [Ca^{2+}] using the formula: $[Ca^{2+}]_{SR} = K_d \times (F - F_{min}) / (F_{max} - F)$, where K_d was 390 μM [2]. SR Ca^{2+} leak was measured as the changes of total $[Ca^{2+}]_{SR}$ ($[Ca^{2+}]_{SRT}$) over time ($d[Ca^{2+}]_{SRT} / dt$) after complete SERCA inhibition with thapsigargin (TG; 10 μM). $[Ca^{2+}]_{SRT}$ was calculated as: $[Ca^{2+}]_{SRT} = B_{max} / (1 + K_d / [Ca^{2+}]_{SR}) + [Ca^{2+}]_{SR}$; where B_{max} and K_d were 2700 μM and 630 μM, respectively [32]. The rate of SR Ca^{2+} leak ($d[Ca^{2+}]_{SRT} / dt$) was plotted as a function of $[Ca^{2+}]_{SR}$ for each time point during $[Ca^{2+}]_{SR}$ decline.

2.3. Western Blot analysis

Equal amounts of freshly isolated myocyte suspension were treated with different compounds (e.g. cAMP, staurosporine) under the same experimental conditions as for [Ca^{2+}] measurements. After treatment, cells were lysed in Laemmli buffer (Sigma-Aldrich). An equal amount of total lysate from each sample was subjected to 4–15% SDS-PAGE and transferred to nitrocellulose membranes for Western Blot analysis. Changes in RyR2 phosphorylation level at two PKA sites (Ser 2809 and 2031) and the CaMKII site (Ser 2815) were quantified using phospho-specific antibodies RyR-PS2809, RyR-PS2031 and RyR-PS2815 (Badrilla, Leeds, UK). The signal was normalized to total RyR2 level measured with the antibody C34 (DSHB, University of Iowa, USA). The secondary antibody was HRP-conjugated, therefore the RyR2 band was visualized