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Phosphorylation of the Cardiac Ryanodine Receptor by Ca\(^{2+}\)/Calmodulin-Dependent Protein Kinase II

The Dominating Twin of Protein Kinase A?

Jens Kockskämper, Burkert Pieske

Excitation–contraction coupling in the heart relies on Ca\(^{2+}\)-induced Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR). Ca\(^{2+}\) influx via L-type Ca\(^{2+}\) channels during an action potential triggers Ca\(^{2+}\) release from the SR via Ca\(^{2+}\) release channels, or ryanodine receptors (RyR2). Fine tuning of RyR2-mediated SR Ca\(^{2+}\) release is central to cardiac function. When RyR2-mediated Ca\(^{2+}\) release increases, the resulting augmentation of the [Ca\(^{2+}\)], transient causes increased contraction. Uncontrolled openings of RyR2 during diastole, on the other hand, may elicit delayed afterdepolarizations and arrhythmias. Dysfunction of RyR2 may occur under certain pathological conditions, eg, excess sympathetic stimulation, and may contribute to such cardiac diseases as heart failure and atrial fibrillation. Furthermore, mutations in RyR2 can cause stress-induced ventricular tachycardias and sudden death in otherwise healthy individuals. Thus, proper regulation and function of RyR2 is essential for adequate cardiac function.

RyR2: Regulation, Regulation, Regulation

Not surprisingly, because of its crucial role in cardiac excitation–contraction coupling, RyR2 activity is highly regulated. Substances involved in regulation of RyR2 activity include Ca\(^{2+}\), Mg\(^{2+}\), H\(^{+}\), adenine nucleotides, calmodulin, NAD\(^{+}\)/NADH, nitric oxide, and glycolytic intermediates, to name but a few. The list of molecules regulating RyR2 activity is far from complete and will undoubtedly grow longer as research continues. In addition, RyR2 is regulated by phosphorylation. The protein forms a macromolecular complex with regulatory proteins (notably FKBP12.6), cytoskeletal proteins, adapter proteins, kinases, and phosphatases. This allows for tight control and localized regulation of RyR2 activity in the microenvironment of the channel.

Phosphorylation of RyR2 and Cardiac Disease

Regulation of RyR2 activity by phosphorylation is not only important from a physiological point of view to adjust SR Ca\(^{2+}\) release and, ultimately, cardiac output to the varying demands of the body. Phosphorylation-dependent regulation of RyR2 activity is also pathophysiologically important, as it is implicated in potentially life-threatening cardiac diseases such as heart failure and atrial fibrillation. Convincing data indicate that, in the failing heart, RyR2 is hyperphosphorylated. Hyperphosphorylated RyR2 becomes “leaky,” ie, the channel opens more frequently even in diastole, and this has important consequences. First, the SR loses stored Ca\(^{2+}\). As a result, less Ca\(^{2+}\) is available for release, causing reduced [Ca\(^{2+}\)], transients and impaired contractions. This then contributes to cardiac failure. Second, diastolic Ca\(^{2+}\) release can trigger delayed afterdepolarizations (via activation of the electrogenic Na\(^{+}\)/Ca\(^{2+}\) exchanger) and arrhythmias. In fact, approximately half of patients with terminal heart failure die from arrhythmias. It is assumed (although not yet proven) that some of these fatal arrhythmias are caused by sporadic openings of RyR2 in diastole, triggering the aforementioned chain of events. A large body of data support the notion that hyperphosphorylation of RyR2 in heart failure is related to a protein kinase A (PKA)-dependent mechanism, mediated at least in part by reduced local phosphatase activity. However, PKA-dependent hyperphosphorylation of RyR2 and induction of SR Ca\(^{2+}\) leak in cardiac disease are controversial and could not be reproduced by others.

In addition to PKA, Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) is also associated with the RyR2 macromolecular complex. Furthermore, CaMKII can phosphorylate RyR2 in vitro and in vivo, and expression of CaMKII is increased in human heart failure. What then is the role of CaMKII phosphorylation in regulation of RyR2? Is it just a bystander, or does it have significant functional relevance, such that CaMKII-dependent RyR2 (hyper)phosphorylation may induce a “leaky” channel?

Surprising CaMKII

In this issue of Circulation Research, Guo et al pursue this question using state-of-the-art techniques to measure RyR2 activity and phosphorylation in ventricular myocytes from wild-type and genetically altered mice. For their experiments, the authors use permeabilized myocytes and confocal Ca\(^{2+}\) imaging to record elementary Ca\(^{2+}\) release events, so-called Ca\(^{2+}\) sparks, as a readout for RyR2 activity. Ca\(^{2+}\) sparks arise from the concerted opening of a functional cluster of several RyR2. As such, Ca\(^{2+}\) sparks are well suited to characterize elementary RyR2 function in a cellular context. This approach offers the advantage to study RyR2 activity and regulation in its native membrane environment. At the same time, there is reasonably good control over the composition of the cytosolic solution. Under these conditions, Guo et al...
observe that CaMKII activation increases the frequency of Ca\(^{2+}\) sparks, similar to PKA activation, and this is associated with phosphorylation of RyR2. At first glance, therefore, it appears as if both kinases act as functional twins, exerting almost identical (potentially adverse) effects on RyR2-mediated Ca\(^{2+}\)-release. Closer examination, however, reveals that CaMKII and PKA act distinctly differently. An important determinant of Ca\(^{2+}\) spark frequency is the loading state of the SR—the higher the Ca\(^{2+}\) load, the higher the open probability of RyR2. However, RyR2 is not the only target of CaMKII and PKA. Both have pleiotropic effects, one of these the phosphorylation of phospholamban, which, in turn, stimulates the activity of the SR Ca\(^{2+}\) pump and thereby increases SR Ca\(^{2+}\) load. Thus, direct effects on RyR2 are difficult to discern from indirect effects mediated by elevated SR Ca\(^{2+}\) load. To circumvent this problem, Guo et al use myocytes from phospholamban knockout mice. In these myocytes, the confounding effect of increased SR Ca\(^{2+}\) load after treatment with either CaMKII or PKA is absent and direct effects on RyR2 can be studied in isolation. This experimental approach reveals a surprise: whereas CaMKII is still able to increase Ca\(^{2+}\) spark frequency in the absence of elevated SR Ca\(^{2+}\) load in phospholamban-deficient cells, PKA is not. (The latter finding confirms previous results from the same laboratory.) Further examination of Ca\(^{2+}\) spark characteristics shows that CaMKII also increases duration and spatial spread, which the authors suggest to be attributable to longer openings of RyR2. Thus, according to the study by Guo et al, CaMKII and PKA may exert distinct effects on RyR2 function. CaMKII appears to be able to increase Ca\(^{2+}\) spark frequency directly via phosphorylation of RyR2, i.e., in the absence of increased SR Ca\(^{2+}\) load. PKA-mediated effects on spark frequency appear to be indirect, on the other hand, and rely predominantly on elevation of SR Ca\(^{2+}\) load.

Potential Mechanisms Underlying Different Effects of CaMKII and PKA on RyR2 Function

Obviously, both CaMKII and PKA are able to phosphorylate RyR2 with possibly different functional consequences. What is the molecular mechanism underlying these differences? This question is not addressed by Guo et al.\(^9\) and awaits resolution. One potential mechanism is phosphorylation of different serine or threonine residues on RyR2, resulting in different alterations in gating behavior of the channel. RyR2 is a huge molecule containing a number of residues as potential targets for protein kinases. Current focus is on serine 2809,\(^11\) serine 2815,\(^12\) and serine 2030.\(^13\) Whereas some studies show that CaMKII and PKA might act on different serine residues,\(^12\) others demonstrate that both can phosphorylate the same residues.\(^14\) Clearly, further studies on this issue are warranted. Another mechanism (not mutually exclusive) might be the degree of phosphorylation of a particular serine residue. A recent study indicates that the phosphorylation degree of serine 2809 may influence the gating properties of RyR2, with maximal and minimal phosphorylation resulting in unique alterations of RyR2 gating.\(^11\) Taken together, although some promising insights have been provided al-ready, it is still not well understood how a particular kinase may elicit specific alterations in RyR2 function.

Possible Consequences of RyR2 Phosphorylation by CaMKII

CaMKII phosphorylation enhances RyR2 activity, as shown by Guo et al\(^9\) and by several other investigators. This could lead to augmented SR Ca\(^{2+}\) release during systole as well as diastole. In resting myocytes, Guo et al not only observe a CaMKII-induced increase in Ca\(^{2+}\) spark frequency, they also note the occurrence of spontaneously propagating SR Ca\(^{2+}\) release events (miniwaves), demonstrating the arrhythmogenic potential of CaMKII phosphorylation. Thus, stimulation of CaMKII may be a double-edged sword. Moderate, short-term stimulation could augment SR Ca\(^{2+}\) release during excitation–contraction coupling, whereas excessive or long-term stimulation might favor SR Ca\(^{2+}\) loss and the development of arrhythmias. Furthermore, the net effect of CaMKII stimulation on SR Ca\(^{2+}\) release may also depend on other factors such as additional stimulation or inhibition of other kinases or phosphatases.

Therapeutic Implications: Seal the Leak!

According to the present study by Guo et al.,\(^9\) CaMKII appears to be the dominating kinase, which phosphorylates RyR2 to increase SR Ca\(^{2+}\) leak in mice. Similar results have been obtained in a rabbit heart failure model.\(^15\) Whether these effects of CaMKII on RyR2 are present and relevant in human heart failure remains to be shown. Because PKA-dependent hyperphosphorylation of RyR2 has been implicated in human heart failure,\(^1\) the relative importance of CaMKII versus PKA for induction of SR Ca\(^{2+}\) leak in the failing human heart is still unclear. This is particularly true in light of recent findings raising doubts about the functional importance of PKA-dependent phosphorylation of RyR2 in heart failure.\(^7,15–17\) Future studies will have to clarify this issue. Irrespective, however, of whether CaMKII or PKA or both are involved, one point becomes increasingly clear: Ca\(^{2+}\) leak via (hyperphosphorylated) RyR2 would be a serious problem in the failing heart.\(^18\) In conjunction with the well-documented reduction of Ca\(^{2+}\) uptake (via reduced sarcoplasmic-endoplasmic reticulum Ca\(^{2+}\)-ATPase [SERCA] activity and expression), it can account for both reduced contractility and an increased propensity toward arrhythmias during heart failure (Figure, top). Current therapeutic heart failure strategies aim at increasing SR Ca\(^{2+}\) uptake (Figure, A), via overexpression of SERCA or knockout of phospholamban, for example. This approach, however, could prove insufficient as it might not only increase SR Ca\(^{2+}\) load but also, as a direct consequence, increase SR Ca\(^{2+}\) leak (Figure, A). Increased leak, in turn, would limit the effect on load and, even worse, might increase the likelihood of arrhythmias. Therefore, a more promising therapeutic approach could be to reduce the leak (Figure, B). This could likewise increase load but, more importantly, it would significantly reduce the likelihood of arrhythmias. First studies using stabilizers of RyR2 have yielded promising results in animal models of heart failure,\(^19,20\) although the plot continues to thicken, with new results casting doubt on the specificity, mechanisms, and
utility of the compounds in question.\textsuperscript{21} A thorough understanding of how CaMKII and PKA regulate RyR2 phosphorylation and function is essential for developing such therapies, and the study of Guo et al is an important step in this direction.

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**Disclosures**
None.

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