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EDITORIAL

β-adrenergic receptors in cardiac muscle: it takes two to tango

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This editorial refers to 'Compartmentalized β_1-adrenergic signalling synchronizes excitation–contraction coupling without modulating individual Ca^{2+} sparks in healthy and hypertrophied cardiomyocytes', by H.-Q. Yang *et al.*, doi:10.1093/cvr/cvaa013.

Cardiac output is appropriately adjusted by changes of heart rate and muscle force to cover the varying metabolic needs of the body. Adjustments of inotropy are accomplished by fine-tuning the activity of the autonomic nervous system (ANS), most prominently the sympathetic system. Extracellular messengers communicating the sympathetic ANS signals comprise the catecholamines epinephrine (acting as a circulating hormone) and the synaptic neurotransmitter norepinephrine. In cardiac muscle, both catecholamines impinge on β-adrenergic receptors (β-ARs), which are mainly of the β_1-type (75–80%) and β_2-type (20–25%) in cardiomyocytes. After activation of these extracellular G-protein coupled receptors, the intracellular signals branch out via second messengers and several signalling pathways to reach a range of specific but distinct subcellular targets. Many of these signalling pathways contain intertwined positive and negative feedback loops and often exhibit substantial cross-talk. A complex task for this kind of regulation is the challenge to translate a single signal carried by one (or very few) molecular entities into a variety of specific and appropriate cellular and subcellular responses. On the level upstream of the receptors, this is accomplished by using the two subtypes of receptors but also by modulating the amplitude (i.e. agonist concentration and timing), the frequency and the time-course of the signals [e.g. acute (min) or chronic (days) stimulation]. The receptors themselves also undergo adjustments, for example via post-translational modifications, or by means of fine-tuning their membrane abundance and location via changes of their trafficking and degradation.

After translation of these extracellular signals into an intracellular second messenger an analogous problem is usually present. There is a single second messenger which should specifically activate targets and orchestrate an array of cellular responses. Besides varying the amplitude and frequency of such second messenger signals, a possible way out of this dilemma is to establish subcellular spatially limited signalling domains (or microdomains), by constraining the signal locally. A prominent example and archetype for this are the highly localized elementary Ca^{2+} signals termed Ca^{2+} sparks. Similarly, it has been recognized for quite a long time that also the second messenger cAMP can initiate responses confined to a subcellular region or microdomain (see *Figure 1*, upper panel).[1]

In the present publication[2] and in a complementary paper presented recently,[3] the authors move this concept one step further to examine the subcellular regulation of cardiomyocyte excitation–contraction coupling (ECC) and Ca^{2+} signalling inside the micro- and even a nanodomain of the dyadic cleft between the sarcolemma and the membrane of the sarcoplasmic reticulum (SR), and how activations of β_2- and β_1-ARs mutually interact within the dyadic cleft and this nanodomain.

An early and striking observation in these single-cell voltage-clamp and confocal Ca^{2+} imaging studies was that initial β_2-AR stimulation suppressed most of the well-characterized changes of cardiac ECC commonly seen when adding a β_1-AR agonist. During β_2-AR stimulation, β_1-AR activation was merely able to increase the Ca^{2+} current via L-type Ca^{2+} channels (LTCCs) located in the sarcolemma. In other words, the widely known functional enhancements of Ca^{2+} signalling resulting from β_1-AR stimulation and mimicking the 'fight- or flight' reaction were suppressed and not observed during concomitant β_2-AR stimulation. Thus, targets located in the SR membrane just a few nanometers away were excluded. With a range of sophisticated experiments combining a wide spectrum of techniques, such as classical pharmacology, specific inhibitory peptides, western blotting, and transgenic animal approaches, the decisive event for this functional segregation was identified. It became evident that despite a complex sequence of events happening after β_2-AR stimulation, there is a common denominator and final converging mechanism for this suppression. As it turned out, this was the result of preventing cAMP access to the protein kinase A (PKA) located on the RyR macromolecular complex, only a few nanometers away from the LTCCs. Consequently, these PKA molecules were not activated and did not phosphorylate the regulatory sites on the RyRs (and on phospholamban, which is further away from the cAMP source). How could the cAMP signal be limited to such a tiny subsarcolemmal domain? It is well-established that cAMP can be subcellularly 'fenced off' and its diffusion limited by phosphodiesterases (PDEs), which efficiently metabolize and inactivate this second messenger.[1] Indeed, inhibiting PDEs pharmacologically in the present studies relieved the limitation of β_1-AR generated signals and allowed RyR phosphorylation to occur. Further experiments

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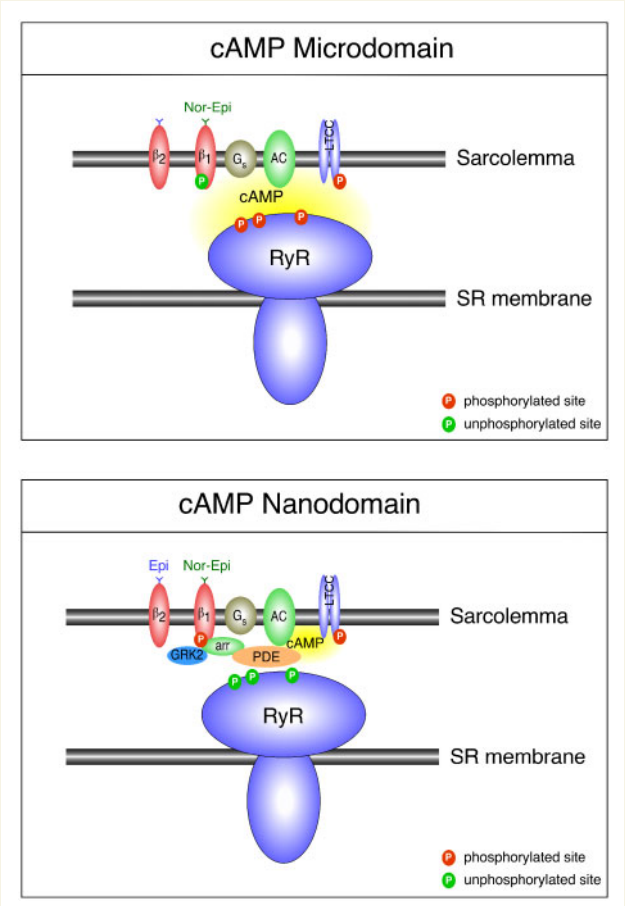

Figure 1 Microdomains and nanodomains of cAMP. Arrangement of key elements for offside compartmentalization in the dyadic cleft (not to scale). (*Upper panel*) When only the β_1-ARs are activated (e.g. by nor-epinephrin), a microdomain of cAMP is established. This allows for phosphorylation to occur on the LTCC and on the RyR (red dots). This will result in changes of spark properties. (*Lower panel*) After β_2-AR activation (e.g. by epinephrin) G-protein coupled receptor kinase 2 (GRK-2) will phosphorylate β_2-AR at the C-terminal, allowing β-arrestin-1 to bind and to scaffold PDE4 between adenylate cyclase (AC) and the PKA phosphorylating sites on the RyR, which therefore remains unphosphorylated (green dots). Spark properties will remain unchanged. For details see text.

were designed to define the molecular signals and proteins involved in this precisely targeted PDE activity. Taken together, the results suggest the following sequence of events to establish the cAMP nanodomain (see also *Figure 1*, lower panel). The initial β_2-AR activation recruits G-protein receptor coupled kinase 2 (GRK-2) to the dyadic cleft, where it phosphorylates β_1-ARs at the C-terminal end. This post-translational receptor modification in turn allows β-arrestin-1 to bind to the β_1-AR. Arrestin-1 will subsequently scaffold PDE molecules in a location where they can form a hedge and interfere with cAMP movement from the adenylate cyclase to the RyRs (and further). Thus, activating β_1-ARs alone creates a cAMP microdomain reaching many elements of cardiac ECC, while simultaneous (or preceding) β_2-AR activation will modify the β_1-AR response to establish a highly restricted nanodomain, which the authors termed 'offside compartmentalization'.

Even though there were no substantial changes observed in the cellular Ca^{2+} transients after establishing the cAMP nanodomain, in this article presented in *Cardiovascular Research*,[2] the authors examined whether and how the detected modifications of LTCC and the establishment of a nanodomain could affect ECC on the cellular and near molecular level. Particular attention was given to conditions where the Ca^{2+}-induced Ca^{2+} release mechanisms could be compromised, such as, for example, during heart failure. In initial experiments on the cellular level, they noted a shorter delay from the depolarization to the appearance of Ca^{2+} spikes, and the spikes were much better synchronized throughout the cell after β_2-AR stimulation. Ca^{2+} spikes can be recorded in the presence of intracellular EGTA. Unlike Ca^{2+} sparks, which are rapidly blurred by Ca^{2+} diffusion, Ca^{2+} spikes remain better confined spatially and directly reflect Ca^{2+} release flux from the SR. This shorter delay and the better synchronization could of course have several reasons, including an enhanced Ca^{2+} sensitivity of the RyRs resulting from their phosphorylation. However, this seemed unlikely based on the results described above. To obtain a more detailed picture and assess the Ca^{2+} release events on a near molecular level the authors applied the loose-seal patch-clamp technique. By simultaneously recording Ca^{2+} sparklets, fluorescence signals resulting from Ca^{2+} influx via a single LTCC, and the Ca^{2+} sparks subsequently triggered by these sparklets, they could demonstrate that the changes in Ca^{2+} spark timing were not the result of shorter delays between the sparklets and the triggered sparks during β_2-AR activation. Other Ca^{2+} spark parameters, such as amplitude or duration, were not changed. All things considered, there was no change of the RyR Ca^{2+} sensitivity, as one would have noticed after RyR phosphorylation. Thus, the changes of spike delays and synchronization were entirely due to the larger current via LTCCs. This observation also provides an important confirmation that the increase of the Ca^{2+} current after β_2-AR is indeed mediated by channels that are situated in the dyadic cleft abutting the RyRs, and not somewhere further away in the sarcolemma.

While these subtle improvements of spark synchronization only resulted in minimal alterations of the cellular Ca^{2+} transient, the larger Ca^{2+} influx and enhanced coupling could become important under conditions were ECC is compromised, such as in pathological cardiac conditions. Indeed, a test for the functional ECC reserve by curtailing the triggering Ca^{2+} current led to a deterioration of the Ca^{2+} signals in untreated cells, while after establishment of the cAMP nanodomain ECC was much more robust. Similarly, in cardiomyocytes isolated from hypertrophied rat hearts developing heart failure (after TAC), Ca^{2+} signalling also turned out to become sensitive to changes of Ca^{2+} current during β_2-AR activation, unlike the cells from sham operated animals. And this potentially beneficial effect of β_2-AR was entirely dependent on the enhanced Ca^{2+} current. Taken together, compartmentalized cAMP signalling helped to stabilize ECC under conditions in which Ca^{2+} signalling across the dyadic cleft might be compromised, such as during heart failure. This feature may be one of the physiologically relevant aspects of the offside compartmentalization of cAMP nanodomains in the heart. In summary, the findings of these publications provide detailed insight into subcellular features of second messenger signalling by cAMP.

However, intuitively it is puzzling and difficult to conceptualize such a nanodomain with a range covering only a small fraction of the dyadic cleft. Thus, cAMP could merely diffuse (or move) a few nanometers. Indeed, mathematical modelling of cAMP diffusion and degradation by PDEs has indicated that such nanodomains cannot exist when cAMP is

able to diffuse freely.[4] Interestingly, in the restricted space of the dyadic cleft, such a limited diffusion has already been proposed for Na^+ many years ago, whereby Na^+ entering via Na^+ channels would accumulate in a subsarcolemmal 'fuzzy space', thereby activating the Na^+-Ca^{2+} exchange in the Ca^{2+} influx mode.[5] When considering the spatial boundary conditions in the dyadic cleft, it appears that this tiny volume is very much packed with protein masses, predominantly by the huge RyR macromolecular complex, but also by other proteins involved in Ca^{2+} signalling and maintenance of the structural integrity of the cleft. Indeed, there seems to be very little water volume available for cAMP to move and diffuse freely. This space crammed with proteins would therefore introduce a substantial extent of hindrance towards diffusion. Such a tortuosity can impede diffusion and thereby extend the distances beyond the width of the dyadic cleft. In addition, the special arrangement of the cAMP signalling system with PKA attached to specific sites on the RyR my means of anchoring proteins will, together with the PDEs fence, result in a cAMP 'nano-signalosome', in which cAMP could be efficiently trapped.

To obtain further details regarding this cAMP nano-signalosome, more information will be required. Additional insight might be gained using recently developed genetically engineered fluorescent cAMP indicators. These probes can be linked to a protein of interest and will report the cAMP concentration in the immediate vicinity of a specific site on this protein. Thus, the spatial precision of such a probe will be submolecular and will not be limited by optical resolution (or super-resolution).[6,7]

Hopefully, the detailed understanding of these nanoscopic signalling events and their relevance for cardiac diseases might help to develop new treatment modalities, particularly for diseases that are accompanied by an elevated level of circulating stress-related catecholamines.

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