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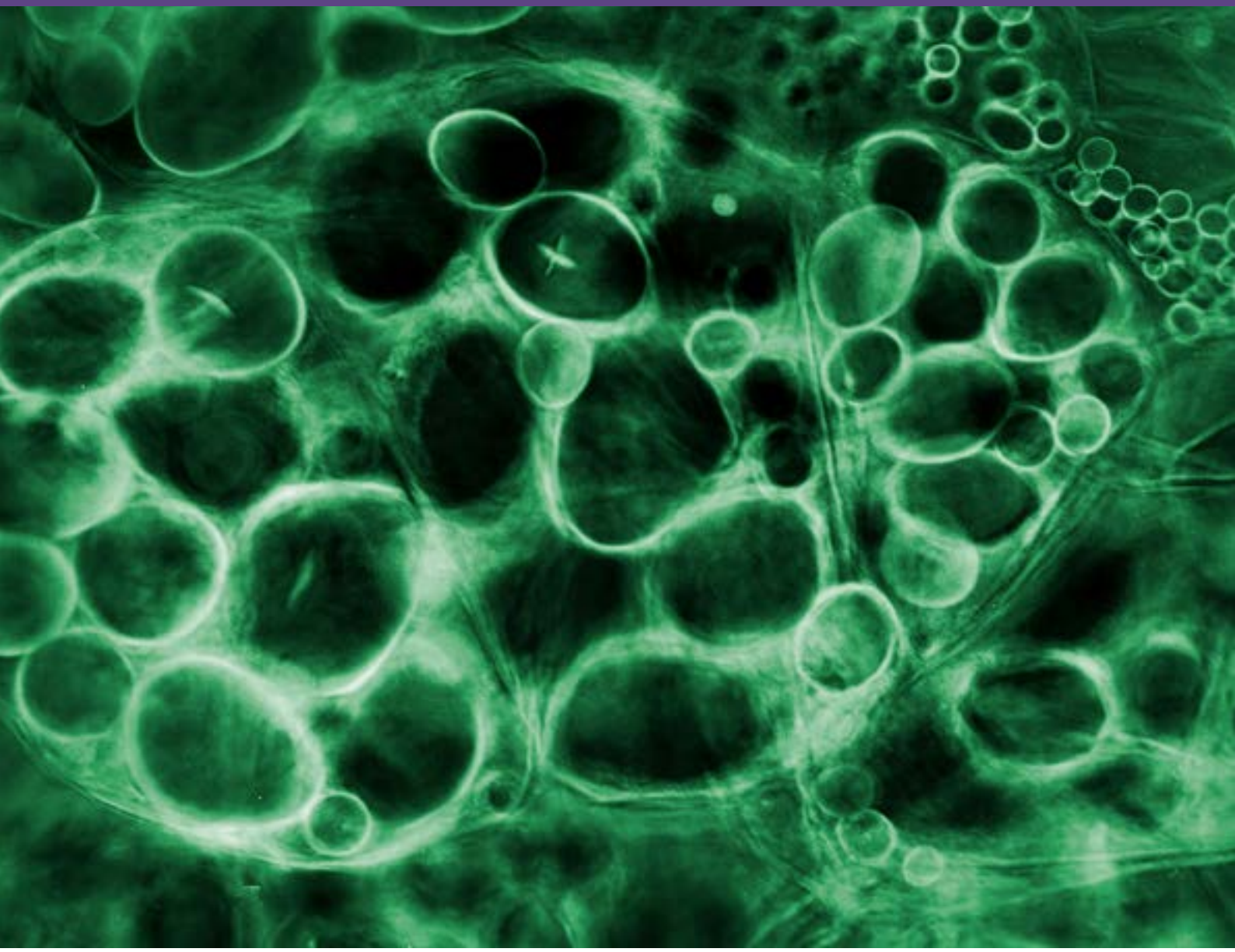

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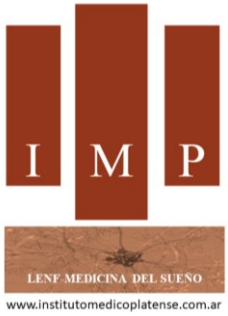

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CAMKII: CARDIAC PHYSIOLOGICAL FUNCTIONS

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Introduction

Calcium-calmodulin-dependent protein kinase (CaMKII) is a Ser/Thr kinase whose activity is triggered by calcium (Ca^{2+}), in response to various distinct signaling pathways in different organs, including the pancreas, liver, brain, and heart. CaMKII was discovered in the late sixties. Interestingly, the initial mentions of this kinase were nearly simultaneous in both the brain and the heart, despite it is more abundant in the brain, where it plays a crucial role in memory and learning processes. [1, 2]

CaMKII is unique in that it is very widely distributed and can phosphorylate a broad range of proteins. These properties promptly suggested that CaMKII regulates many physiological responses to agonists that elevate intracellular Ca^{2+}, leading to its designation as the "multifunctional Ca/CaM-dependent protein kinase" or "CaM-dependent multiprotein kinase".

Encoded by four different genes (α, β, γ, and δ), CaMKIIδ is the predominant isoform in the heart, with two splice variants in the adult myocardium: δ_B, mainly localized in the nucleus, and δ_C, primarily found in the cytosol.

Given its role in phosphorylating proteins related to Ca^{2+} handling and excitationcontraction coupling (ECC), CaMKII is believed to play an important role in physiological processes. However, unlike its well-established role in memory and learning, the physiological significance of CaMKII on cardiac function is less acknowledged. Indeed, its better-known associations lie in non-physiological actions within the heart, particularly in various cardiac pathologies such as heart failure (HF), myocardial infarction, and cardiomyopathies, leading to contractile dysfunction and malignant arrhythmias. [3-9]

This mini review aims to provide a concise summary of CaMKII's role on cardiac physiology.

Structure of CaMKII and activation pathways

CaMKII has a dodecameric structure formed by two overlapping hexameric rings with a central association domain from which emerge, from the upper and lower rings, the regulatory and kinase domains of each monomer. In the absence of calcium, the kinase or catalytic domain is inactive as it binds to the inhibitory domain, largely impeding the kinase's access to substrates and ATP. The binding of the complex formed by calmodulin (CaM) with 4 calcium ions (Ca/CaM) peels off the regulatory segment from the kinase domain, allowing the activity of the kinase and its access to substrates. The displacement of the inhibitory domain also exposes the phosphorylation site (Thr^{286}) of the neighbor subunits. This autophosphorylation of the kinase prevents the reassociation of the regulatory domain, and therefore, the kinase remains active without the need for Ca^{2+} and CaM binding **(Figure 1)**, *i.e.* in a Ca^{2+}-independent manner. In addition, CaMKII may remain active by oxidation, glycosylation, and nitrosylation at different sites (See for review [11]).

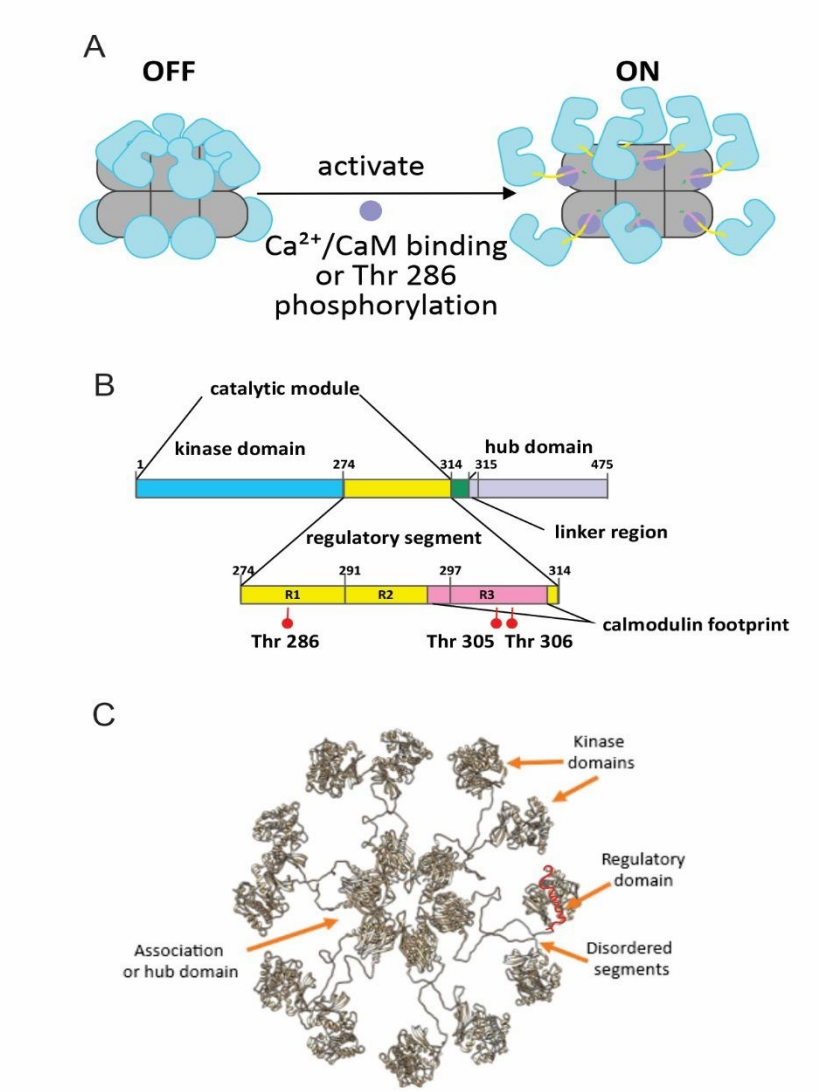

Figure 1: CaMKII Structure. A. Cartoon showing the architecture of CaMKII holoenzyme. The hub domain oligomerizes CaMKII into two stacked hexameric rings. The inactive holoenzyme has a compact configuration. Upon activation by Ca/CaM, or phosphorylation of Thr286 in the regulatory segment (purple circles), the kinase domains are extended from the hub assembly. B. The domains of CaMKII. Each subunit of CaMKII consists of a kinase domain, a regulatory segment, a variable linker region, and a hub domain. The regulatory segment houses the CaMbinding region as well as a regulatory autophosphorylation site, Thr^{286} and $Thr^{305/306}$ sites (Modified from Stratton et al., 2014). [10] **C.** Model of the extended conformation of CaMKIIδ with the kinase domains variably distributed around the hub. In red: the regulatory domain (Courtesy of Dr. Gustavo Parisi).

Another important point regarding its activation, is how CaMKII integrates Ca^{2+} stimuli to become active. When Ca^{2+} levels increase, it interacts with calmodulin (CaM) to form a Ca/CaM complex which then binds and activates CaMKII. As Ca^{2+} levels return to baseline, it breaks apart from the complex, Ca^{2+} and CaM quickly dissociates, leading to the kinase inactivation. A train of high-frequency Ca^{2+} pulses allows CaMKII to integrate Ca^{2+} signals and become phosphorylated. [12] In this scenario, the dissociation of CaM is greatly slowed due to a substantial increase in CaM affinity, a phenomenon known as CaM trapping.

The main physiological cardiac function of CaMKII: The fight-or-flight response

The main physiological role of CaMKII in the heart is its involvement in the fightor-flight response. During physical and emotional exercise, there is an increase in heart rate (HR) and cardiac output, which are essential for maintaining normal body homeostasis and ensuring long-term survival. The

fight-or-flight response is typically associated with the release of catecholamines, stimulation of βadrenoceptors (βAR), increase in cyclic adenosine monophosphate (cAMP), and activation of protein kinase A (PKA), **(Figure 2A)**. However, the rise in PKA activity leads to an increase in intracellular Ca^{2+}, subsequently activating CaMKII, and, as will be explained below, both PKA and CaMKII participate in the fight-orflight response **(Figure 2B)**.

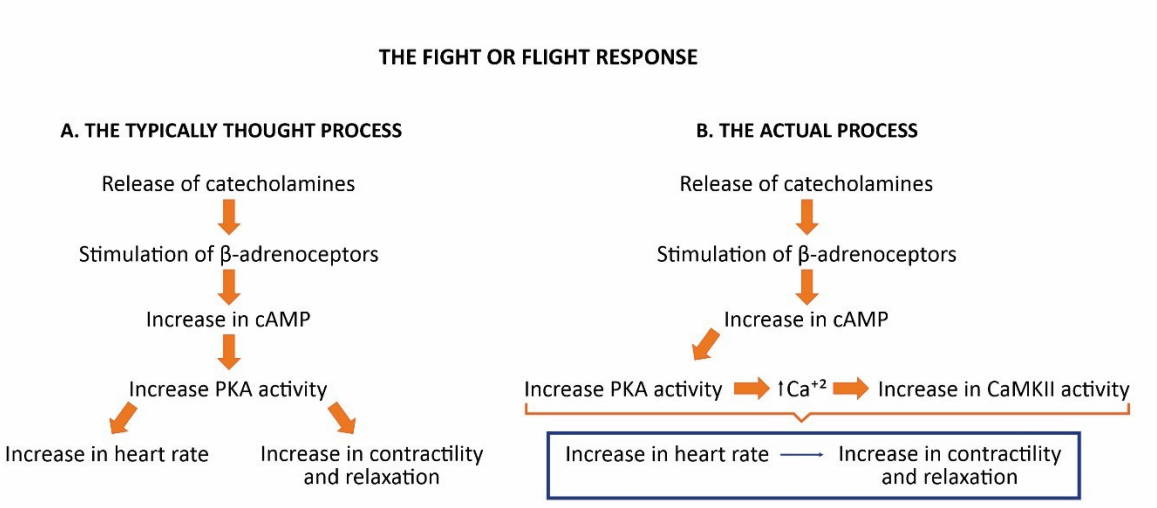

Figure 2. Mechanisms of the fight-or-flight response at the heart level. **A.** The typically thought pathway. **B.** The actual pathway, in which CaMKII is involved.

Role of CaMKII in the increase in heart rate

The sinoatrial node (SAN) demonstrates intrinsic HR, also known as intrinsic firing activity, which persists in the presence of dual blockade of the autonomic nervous system. The primary mechanism governing HR regulation at rest relies on the superimposed effects of the autonomic system on the intrinsic HR. Parasympathetic vagus nerves slow the heart through impulses, while sympathetic nerves increase HR. The heightened HR is contingent upon both, direct cardiac sympathetic innervation and circulating catecholamines released from the adrenal medulla.

The essence of pacemaking is an autonomous diastolic depolarization (DD), causing a slow increase in membrane potential toward an excitation "threshold," building phase 4 or diastolic depolarization phase of the pacemaker action potential **(Figure 3)**. Initially, it was believed that the main current involved in DD was the inward current (*If*) carried by the ion channel HCN4 [13], proposed as the unique "pacemaker current". [14] Later, it was shown that several voltagedependent channels, including L-type (CaV1.2/1.3) and T-type (CaV3.1/3.2), Ca^{2+} channels, and the K^+ channels, like ERG and KvLQT1, substantially contribute to DD (See for review [15]). Further experiments from Lakatta's laboratory described a novel mechanism producing spontaneous action potentials in SAN cells, the "Ca^{2+} clock." It was shown that in SAN cells, Ca^{2+} release from the sarcoplasmic reticulum (SR), occurs in diastole spontaneously, sporadically, rhythmically, and locally. These local Ca^{2+} releases emerge under basal conditions in the form of Ca^{2+} sparks from RyR2, without requirement of SR Ca^{2+} overload or sympathetic stimulation, as is the case of non-automatic ventricular or atrial myocytes. During the ensuing diastole, Ca^{2+} increases at a variable rate, reaching a crescendo immediately before the oncoming action potential. Diastolic local Ca^{2+} releases interact with the electrogenic sodiumcalcium exchanger (NCX), which mainly exchanges a single outward Ca^{2+} ion with three inward Na^+ ions, resulting in a net inward current that depolarizes the membrane potential. This current, progressively and exponentially increases the rate of DD toward threshold for the firing of an action potential via activation of Ltype Ca^{2+} channels (Bogdanov et al., 2001) [16], and also T-type Ca^{2+} channels in species expressing this channel. These experiments gave rise to a new paradigm to explain automaticity in pacemaker cells: the Ca^{2+} clock works synergistically with ionic membrane

currents ("membrane clock"), constituting a coupled clock system *(Figure 3A)* (See for reviews [17, 18]). Thus, the "membrane clock' refers to molecules located on the cell surface membrane, the "Ca^{2+} clock" refers to molecules associated with the SR. Low-voltage activated Ca^{2+} channels (Cav1.3), T-type Ca^{2+} channels (Cav3.1) and L-type Ca^{2+} channels (Cav1.2), as well as INCX are members of both clocks.

At this point, two different questions are pertinent:

1 Which of the two systems, if any, is crucial for DD to occur?
2 Why does spontaneous release occur in the pacemaker cells in the absence of SR Ca^{2+} overload or sympathetic stimulation, as is the requirement for ventricular myocytes? [3,8]

Referent to the first question, different type of evidence indicates that the main determinant of spontaneous diastolic membrane depolarization is the SR Ca^{2+} load, which determines the periodicity of local Ca^{2+} releases from RyR2. *If*, would be only required to prevent membrane potential from hyperpolarizing to a degree that compromises clock signaling during the DD phase.[19]

Concerning the second question, it is known that spontaneous beating ceases when either PKA or CaMKII are inhibited [20], indicating that both proteins are involved in spontaneous SR Ca^{2+} release. Younes et al. (2008) [21] showed that in pacemaker cells, there is an increased basal adenylyl cyclase activity, which increases cAMP and activates PKA and CaMKII, both of which ultimately modulate intracellular Ca^{2+} dynamics and transmembrane potential in SAN cells. Indeed, Li et al. (2016) [22] showed that the basal level of autophosphorylated CaMKII in rabbit SAN cells was significantly higher than that in ventricular myocytes, whereas CaMKII-dependent PLN and RyR2 phosphorylation were 3 and 10 times greater, respectively, than in ventricular myocytes.

Increased HR is a fundamental physiological component of the "fight-or-flight" response to βAR stimulation. For many years, the consensus was that the "fightor-flight" mechanism induces an increase in HR by the activation of the If current, enhancing DD in the SAN cells. However, this perspective shifted after two main findings:

1 The discovery of the coupled-clock system.
2 The demonstration that HCN4 knockout mice still exhibit physiological HR increases with the β-agonist isoproterenol (Iso), indicating the involvement of *If*-independent pathways crucial to SAN fight-or-flight responses.

Currently, it is understood that βAR agonist stimulation modulates the "coupledclock system" intrinsic to pacemaker cells within the SAN as well as HCN4 channels. This regulation involves cAMP, PKA, and CaMKII (refer to **Figure 3B**). While the primary mechanism for HCN4 activation is typically through direct cAMP action, Liao et al. [23] have also indicated modulation of these channels by PKA in mouse SNA cells. Wu et al. (2009) [24] demonstrated that CaMKII is necessary for βAR-mediated increases in HR and suggested that *If* alone was insufficient to sustain physiological chronotropic responses to βAR stimulation in the absence of other depolarizing currents that require normal SR Ca^{2+} release. Further studies showed that ablation of individual RyR2 or PLN PKA- or CaMKIItarget sites failed to affect heart rate in vivo or in SAN cells. These results indicate that fight-or-flight HR increases are facilitated by redundant PKA and CaMKII catalyzed phosphorylation of target proteins. These studies also demonstrated that superinhibitory PLN mutants significantly lowered SR Ca^{2+} content and limited HR acceleration, whereas PLN deficiency prevented reduction in SR Ca^{2+} content and normalized HR acceleration during CaMKII inhibition. These findings provide additional insight into our previous understanding of HR regulation, suggesting that a minimum SR Ca^{2+} content is required in SAN cells for physiological fight-or-flight heart rate increases. [25] **Figure 3B** provides a summary of SAN regulation by both kinases.

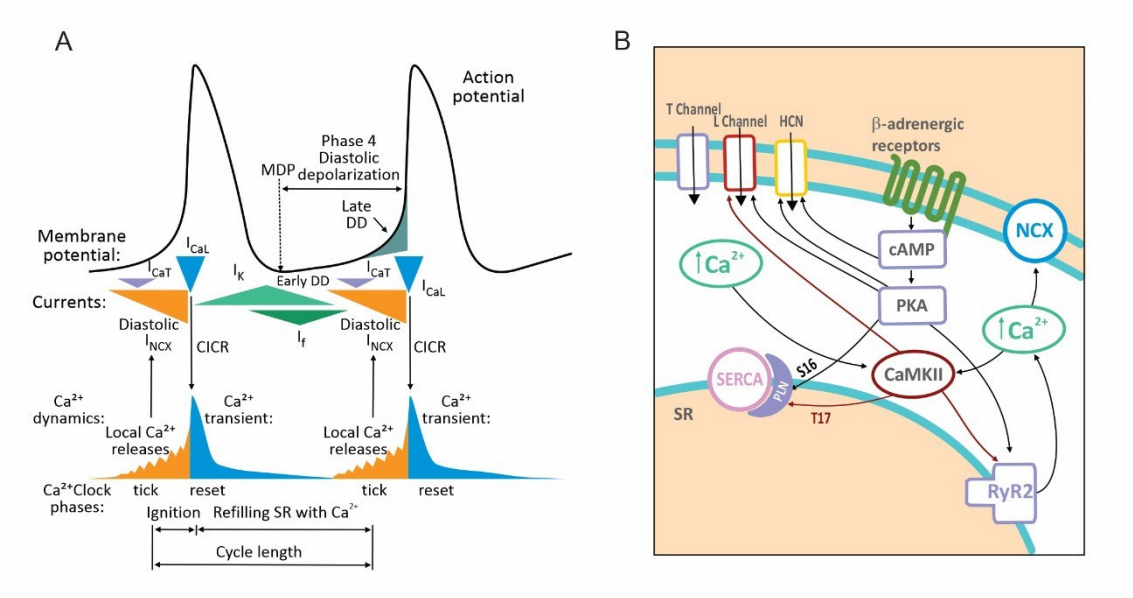

Figure 3. A. CaMKII in Sino auricular node (SAN) physiology. A. A schematic interplay of the membrane clock and Ca^{2+} clock mechanisms. **Top**: Typical action potential of SAN cells (black trace). Middle: Schematic representation of the timing and magnitude of the different components of the "membrane clock". **Bottom:** Different components of the "Ca^{2+} clock". It can be seen that, during phase 4, total cytosolic Ca^{2+} gradually builds due to accumulation of Ca^{2+} in the form of spontaneous local Ca^{2+} releases emanating from the SR (tick orange zone). Ca^{2+} is removed from the cell through the Na^{+}-Ca^{2+} exchanger (NCX) promoting an increase in positive charges that produces the late phase of diastolic depolarization (DD). Toward the end of diastole, activation of L-type Ca^{2+} channels causes Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum via RyR2, resulting in the whole cell Ca^{2+} transient (Reset light blue zone). MDP, maximum diastolic potential; DD, diastolic depolarization; ICa,T, T-type voltage-dependent Ca^{2+} current; ICa,L, Ltype voltage-dependent Ca^{2+} current; INCX, NCX current; IK, delayed rectifier potassium current; I_f funny current; SERCA, sarco-endoplasmic reticulum ATPase; LCRs, local Ca^{2+} releases. (Modified from Manfredi et al., 2013) [17] **B.** Fight-or-flight stimulation (e.g., isoproterenol) increases cyclic adenine monophosphate (cAMP). cAMP directly activates HCN4 channels (HCN) [26] and protein kinase A (PKA), which phosphorylate L-type Ca^{2+} channels (L Channels), phospholamban (PLN) and ryanodine receptors (RyR2) to increase sarcoplasmic reticulum (SR) Ca^{2+} content and release from SR. Increased Ca^{2+} release activates Ca^{2+}-calmodulin kinase II (CaMKII) -which in turn phosphorylates the same targets than PKA- and accelerates NCX activity, which increases the DD rate of SAN action potentials. SERCA, sarco-endoplasmic reticulum ATPase. Ser^{16}: PKA-Phosphorylation site of PLN. Thr^{17}: CaMKII phosphorylation site of PLN (Modified from Wu and Anderson 2014 [27]).

Role of CaMKII in the Increase in cardiac contractility and relaxation

During the fight-or-flight response, the increase in HR increases cardiac output and relaxation rate. But, in addition, βAR activity directly enhances cardiac force and relaxation, by phosphorylating key proteins of the ECC, *i.e.* the L-type Ca^{2+} channels, PLN and the RyR2. Both, PKA and CaMKII, phosphorylate these proteins (See for review [28]). PKA also phosphorylates troponin I, which contributes to the relaxant effect of β-agonists. As stated above, the positive and relaxant effects of βAR stimulation was (and is) usually credited to PKAdependent phosphorylation (See for instance [27]). However, experiments from our laboratory clearly showed that CaMKII-dependent phosphorylation of at least two of these proteins, PLN and RyR2, play a significant role in both effects.

PLN is a small protein in the SR that negatively regulates SERCA2a because, in the dephosphorylated state, under rest conditions, it tonically inhibits SERCA2a. Phosphorylation of PLN relieves this inhibition. PLN is phosphorylated by several kinases, among which are CaMKII and PKA. *In vitro* experiments also showed that these phosphorylations were independent, and their effects are additive. Similar results were described in the intact heart in our laboratory [29]. Decreasing Ca^{2+} influx to the cell by lowering extracellular Ca^{2+} concentration and perfusion with the Ca^{2+} channel blocker nifedipine, showed that CaMKIIdependent PLN phosphorylation decreases in association with a decrease in the relaxant action of Iso. These findings indicate that CaMKII contributes to the relaxant effect of βAR stimulation (and therefore to the increase in SR Ca^{2+} load and inotropic action) **(Figure 4A)**.

One therefore might ask: If CaMKII contributes to the inotropic and relaxant effect of β-adrenoceptor stimulation, why is this effect not usually recognized? Several reasons might account for this under-acknowledged action of CaMKII:

1 As stated above, CaMKII is downstream of PKA: It requires the PKAdependent increase in Ca^{2+} to become active.

2 The PKA-induced phosphatase inhibition favors CaMKII and substrate phosphorylation.

3 PKA is capable of performing all necessary functions at low levels of βAR stimulation **(Figure 4B)**.

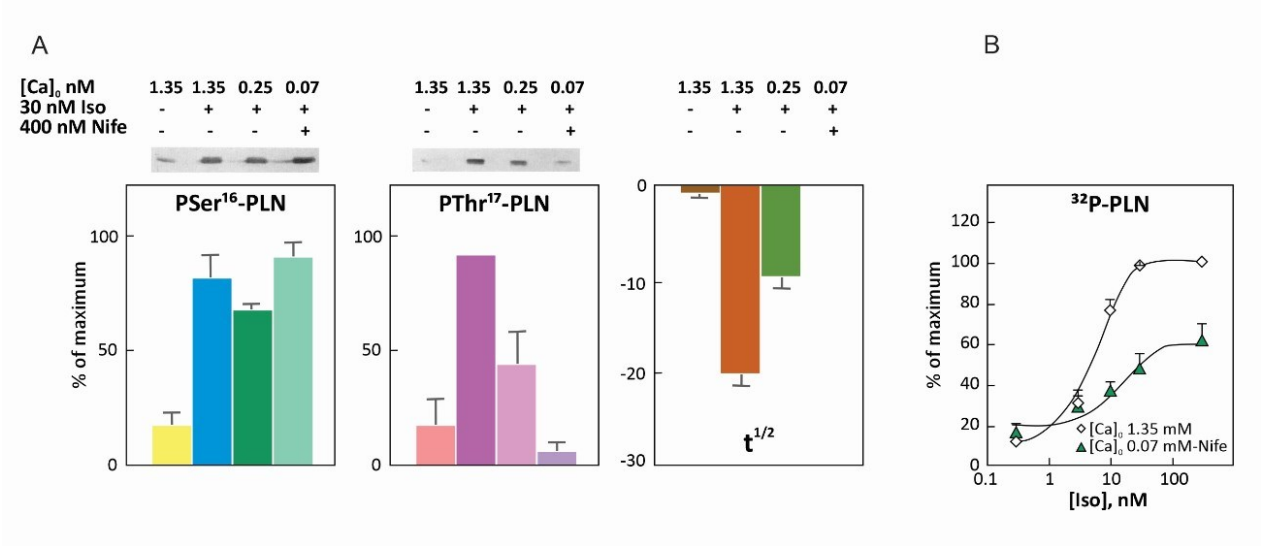

Figure 4. CaMKII dependent phosphorylation of phospholamban (PLN) participates in the relaxant effect of β-adrenergic stimulation. A. Two first panels: Blots and overall results of the immunodetection of the site-specific phosphorylated phospholamban ($PSer^{16}$-PLN and $PThr^{17}$-PLN) in the absence (lane a) or the presence (lanes b to d) of 30 nM isoproterenol (Iso) simultaneously given with interventions that gradually decrease the Ca^{2+} supply to the myocardium, low extracellular Ca^{2+} (Low $[Ca]_o$) with or without nifedipine (Nife). The decrease in Ca^{2+} supply to the cell only affects the phosphorylation of Thr^{17} site of PLN, the CaMKII site. The third panel shows the decrease in half relaxation time ($\mathbf{t}_{1/2}$) in association with the decrease in CaMKII dependent phosphorylation of PLN (Modified from Mundiña-Weilenmann et al., 1996). [29] **B.** CaMKII-dependent phosphorylation of PLN contributes to total PLN phosphorylation at the higher isoproterenol concentrations. Overall results of experiments showing the Isoproterenol concentration-dependent increase of phospholamban (PLN) phosphorylation, assessed by ^{32}P incorporation in isolated SR vesicles from rat hearts at two different extracellular Ca^{2+} ([Ca]o). ^{32}P incorporation into phospholamban was significantly decreased at the higher isoproterenol concentrations in hearts perfused at low extracellular Ca^{2+} (Modified from Said et al., 2002). [30]

A second target that we explored was the SR Ca^{2+} release channels (RyR2 receptors). RyR2s are phosphorylated by different kinases, among which is CaMKII that phosphorylates RyR2 mainly at the Ser2814 site (Ser2815 for some species), and to a lesser extent, at the Ser2808 residue (Ser2809 for some species). Working in the perfused rat heart, we have shown that the binding of ^{3}[H]ryanodine to RyR2 increased with Iso, (of note, ^{3}[H]ryanodine binds to RyR2 only in the open state). This binding decreased when Iso was perfused in the presence of KN-93 or low extracellular Ca^{2+}. Iso also increases the velocity of SR Ca^{2+} release measured in SR vesicles isolated from hearts treated with Iso, and KN 93 decreases this velocity **(Figure 5A)**. [31]

Whether the increased activity of RyR2 phosphorylation contributes to βadrenergic-induced positive inotropic effect is a matter of controversy, and we believe it is a semantic problem that depends on the definition of inotropism (See for further discussion [32]). In any case, our experiments showed that CaMKII-dependent phosphorylation of RyR2 enhances SR-Ca^{2+} release. In summary, the fight-orflight response is the main known physiological function of CaMKII, due to its crucial role in pacemaker cells increasing HR and its contribution to the relaxant and contractile effect of βAR stimulation. CaMKIIdependent phosphorylation of PLN and RyR2 receptors may contribute to this effect.

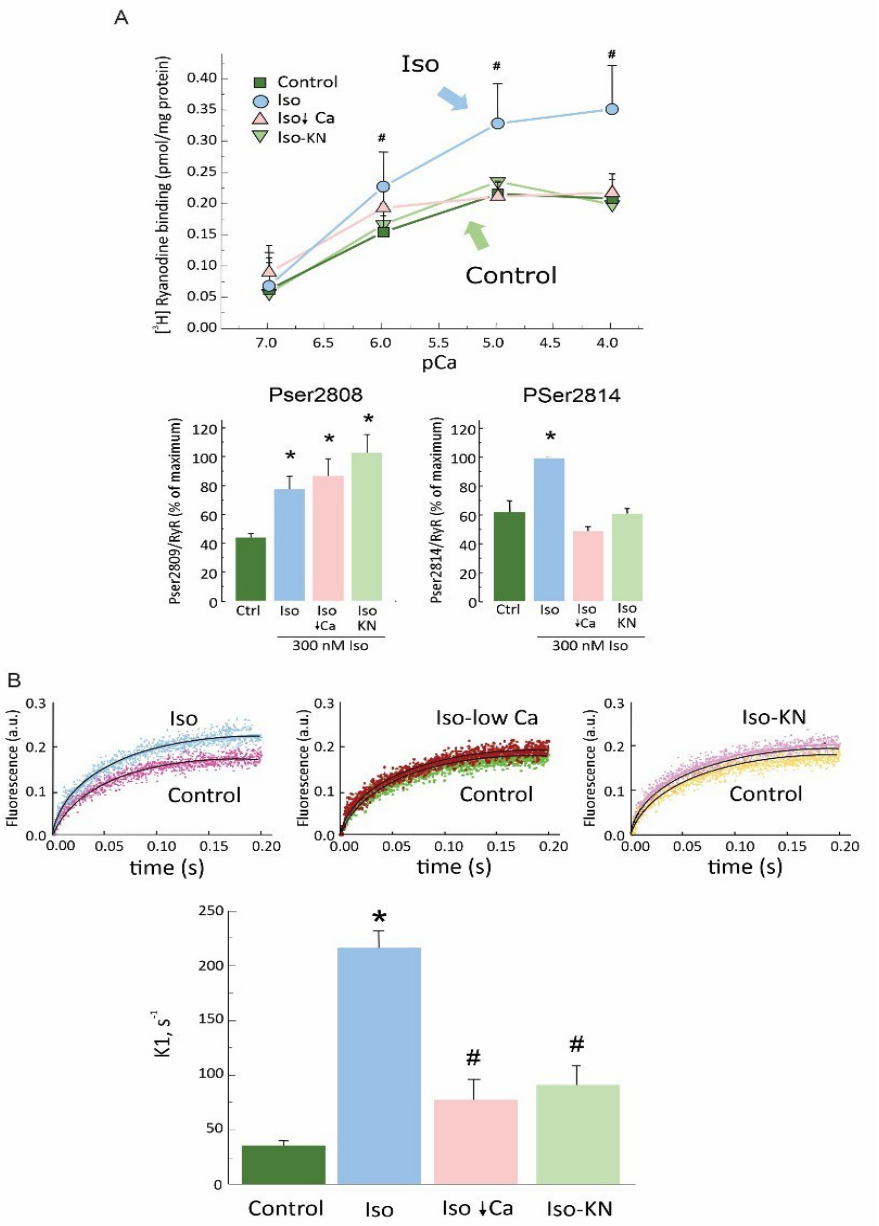

Figure 5. Ca^{2+}/calmodulin kinase II increases ryanodine binding and Ca^{2+}-induced sarcoplasmic reticulum Ca^{2+} release kinetics during β-adrenergic stimulation A. Above: Overall results of experiments performed in the perfused rat heart, showing that Isoproterenol (Iso) increased [^{3}H]ryanodine binding to SR vesicles isolated from these hearts, and that this increase did not occur in the presence of KN-93 (Iso-KN) or low extracellular Ca^{2+} (Iso ↓ Ca). Below: Phosphorylation of RyR2 in rat hearts after perfusion with isoproterenol in the absence (Iso) and the presence of low extracellular Ca^{2+} (Iso ↓ Ca) or CaMKII-inhibition (Iso-KN). (Modified from Ferrero et al., 2007). [31] **B**. Representative Ca^{2+} release records (above) and overall results (below) obtained in SR vesicles isolated from rat hearts. The increase in Ca^{2+} release kinetics produced by isoproterenol (Iso) was significantly decreased by low extracellular Ca^{2+} ($[Ca]_o$) plus nifedipine (Iso-low Ca) or by CaMKII-inhibition(Iso-KN). (Modified from Ferrero et al., 2007). [31]

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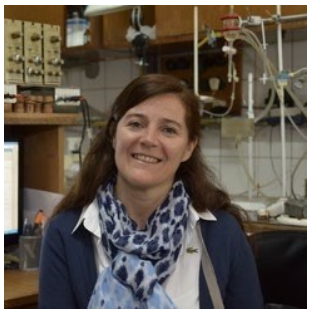

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