JGP Journal of General Physiology

COMMENTARY

How Ca^{2+} influx is attenuated in the heart during a "fight or flight" response

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L-type Ca^{2+} channels are key actors in the various scenes that lead to cardiac contractility (Reuter, 1967; Beeler and Reuter, 1970; Benitah et al., 2010). Their activation during the cardiac action potential allows Ca^{2+} to enter myocytes (Beeler and Reuter, 1970; Ramos-Franco et al., 2016). This Ca^{2+} influx during systole results in an increase in myoplasmic Ca^{2+} concentration that leads to the activation of Ca^{2+} release channels known as ryanodine receptor 2 (RYR2) channels (Pessah et al., 1985; Imagawa et al., 1987). RYR2s are mainly located in the terminal cisternae of the SR (Seifert and Casida, 1986; Inui et al., 1987; Lai et al., 1988). An increase in the open probability (Po) of RYR2 promotes Ca^{2+} release from the SR by a mechanism known as CICR (Ebashi and Endo, 1968; Fabiato and Fabiato, 1975; Fabiato, 1983). Ultimately, this large increase in myoplasmic Ca^{2+} concentration results in cellular contraction. In this issue of *JGP*, Morales et al. investigate the mechanisms involved in regulating Ca^{2+} influx during sympathetic stimulation and, in particular, the role of Ca^{2+}-dependent inactivation.

It has been known for more than 60 years that the autonomic nervous system modulates cardiac contractility (Lee and Shideman, 1959; Katz, 1967; Lindemann and Watanabe, 1985; Cohn, 1989; Henning, 1992). In fact, the sympathetic nervous system increases contractility by releasing the catecholamines epinephrine and norepinephrine, which induce a positive inotropic response (Lee and Shideman, 1959; Evans, 1986; Marks, 2013). When catecholamines bind to β-adrenergic receptors, they promote dissociation of a stimulatory G-protein α_s subunit and subsequent activation of adenyl cyclase (Hildebrandt et al., 1983; Brum et al., 1984). This activation increases the intracellular concentration of cAMP, which promotes dissociation of the catalytic subunit of PKA (Krebs, 1972; Hayes and Mayer, 1981) and phosphorylation of multiple intracellular targets in the myocyte (Collins et al., 1981; Brum et al., 1984; Mundiña de Weilenmann et al., 1987; Suko et al., 1993; Valdivia et al., 1995; Fig. 1 C).

There are two critical proteins that increase cardiac contractility when phosphorylated. One is phospholamban; a protein that, under basal conditions, inhibits the SERCA2-mediated uptake of Ca^{2+} into the SR (Collins et al., 1981; Li et al., 1998; Valverde et al., 2006). Following adrenergic stimulation, phosphorylation of phospholamban at serine 16 by PKA (Chu et al., 2000) and at threonine 17 by CAMKII (Said et al., 2002) relieves its inhibitory effect on SERCA2. The relief of this inhibition increases the rate of Ca^{2+} transport from the cytosol to the SR, thus increasing the Ca^{2+} content of the SR.

A second protein that induces a positive inotropic effect when phosphorylated by PKA is the L-type Ca^{2+} channel ($Ca_V1.2$), which can be phosphorylated at two sites in the C terminus of the α_1 subunit. One site is serine 1928 (De Jongh et al., 1996; Mitterdorfer et al., 1996; Gao et al., 1997; Oliveria et al., 2007), located in the distal C terminus. The other site is serine 1700 (Harvey and Hell, 2013), located in the proximal C terminus (Fig. 1, A and C). However, the sites for PKA phosphorylation are still under discussion.

Interestingly, β subunits can be also phosphorylated. However, because $\alpha_1 1.2$ can interact with different β subunits, all of which are phosphorylated in different ways, it is unclear if PKA phosphorylation of β subunits has a major role in L-type Ca^{2+} channel function (Miriyala et al., 2008; Yang et al., 2019).

In any event, PKA phosphorylation of L-type Ca^{2+} channels increases their Po (Langer, 1983; Bean et al., 1984; Brum et al., 1984; Sperelakis, 1984). This increase in Po results from a change in the modal gating of $Ca_V1.2$ (Yue et al., 1990; Delcour and Tsien, 1993; Shirokov et al., 1998). Under voltage-clamp conditions, the increase in Po can be as large as three times (Yue et al., 1990). Therefore, the myocyte needs to have a mechanism that limits an excessive influx of Ca^{2+} upon phosphorylation.

There are two negative feedback mechanisms that can limit the positive inotropic actions of catecholamines. Specifically, $Ca_V1.2$ can reduce its own Po by two different inactivation

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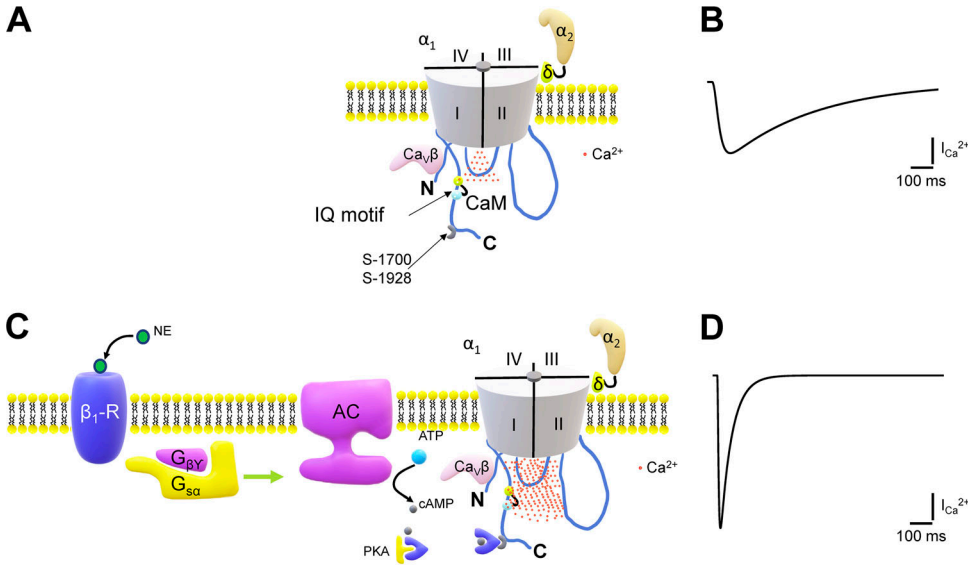

Figure 1. **Overview of the molecular regulation of L-type Ca^{2+} currents. (A)** The central molecular components of a $Ca_V1.2$ channel. The pore-forming subunit α_1 and the regulatory subunits α_2, δ, and Ca_V β. The interaction of Ca_V β and the N terminus of α_1 are essential in defining the VDI. On the other hand, CaM binding site at the IQ motif located at the C terminaus is the protein locus involved in CDI. Interestingly, the PKA phosphorylation sites (S-1700 and S-1928) are also located at the C terminus. **(B)** The activation and inactivation of a numerically simulated L-type Ca^{2+} current. **(C)** The norepinephrine (NE) activation of the G-protein coupled receptor complex that finally leads to PKA phosphorylation of α_1. The scheme illustrates that when α_1 is phosphorylated, there will be an increase in the Ca^{2+} current that will promote a local increase in the free Ca^{2+} concentration on the cytosolic face of the channel. This elevation in Ca^{2+} concentration will increase the probability of binding between Ca^{2+} and CaM, a critical event that promotes CDI. **(D)** Upon PKA phosphorylation of the α_1 subunit, there will be an increase both in the amplitude of the current and in the rate of CDI.

mechanisms; voltage-dependent inactivation (VDI; Cota et al., 1984; Kass and Sanguinetti, 1984; Lee et al., 1985; Zhang et al., 1994; Ferreira et al., 1997, 2003) and Ca^{2+}-dependent inactivation (CDI; Tillotson, 1979; Lipp et al., 1987; Lacampagne et al., 1996; Peterson et al., 2000). These inactivation mechanisms not only have physiological importance, but are also critical in preventing pathological events during catecholaminergic stimulation (Zhang et al., 2014). For example, in the absence of these mechanisms, excessive Ca^{2+} influx leads to SR Ca^{2+} overload in myocytes. This overload increases the probability of spontaneous SR Ca^{2+} release events during diastole. Thus, β-adrenergic stimulation can induce delayed diastolic depolarizations, which can trigger extrasystolic action potentials and eventually ventricular tachycardias and arrhythmias (Katra and Laurita, 2005; Curran et al., 2010; Ko et al., 2017).

VDI is mediated by the interaction between the pore-forming Ca_V α_1 subunit and Ca_V β subunits (Restituito et al., 2000; Wei et al., 2000; Kobrinsky et al., 2004; Jangsangthong et al., 2010; Fig. 1, A and C). On the other hand, CDI is primarily mediated by the Ca^{2+} sensor calmodulin (CaM; Zühlke et al., 1999; Peterson et al., 2000; Pitt et al., 2001). CaM has four helix-loop-helix domains (EF-hands) grouped within two lobes with low and high affinity for Ca^{2+} (Chin and Means, 2000). There is a Ca^{2+}-dependent CaM-binding sequence, the IQ motif, in the cytoplasmic C-terminal tail of the channel's α_1 subunit, which is critical for CDI (Peterson et al., 1999; Qin et al., 1999; Zühlke et al., 1999; Fig. 1, A and C). Although both inactivation mechanisms are physiologically relevant, there has been controversy about which of the two mechanisms have the larger impact on $Ca_V1.2$ inactivation during the cardiac action potential (Findlay, 2004; Grandi et al., 2010). Moreover, a phenomenon that is even less understood is what happens with the L-type channel inactivation during adrenergic stimulation (Morotti et al., 2012; Kumari et al., 2018).

In the current issue of *JGP*, Morales et al. (2019) use a novel conjunction of molecular biology, electrophysiological approaches, and mathematical modeling to investigate the mechanisms involved in controlling Ca^{2+} influx during catecholaminergic stimulation. Specifically, the authors test the hypothesis that CDI is the central mechanism limiting adrenergic stimulation of L-type Ca^{2+} current. This hypothesis, presented in Fig. 1, postulates that Ca^{2+} ions permeating through L-type Ca^{2+} channels will locally increase the cytosolic Ca^{2+} concentration and induce a certain degree of CDI in the absence of a sympathetic stimulus (Fig. 1, A and B). However, in the presence of a catecholaminergic stimulus, there will be an increase in current permeating through the Ca^{2+} channels due to an increase in $Ca_V1.2$ Po. This increase in Ca^{2+} current will not only augment Ca^{2+} influx into the myocyte, but also will increase the local cytosolic Ca^{2+} concentration. This local increase in Ca^{2+} will promote more CaM binding to Ca^{2+}, leading to an increase in CDI. Thus, catecholaminergic stimulation will lead to an increase the amplitude of the Ca^{2+} current and also accelerate the rate of inactivation of the channel (Fig. 1, B and D).

In their paper, Morales et al. (2019) explore the role of VDI by overexpressing the Ca_V β_2a subunit, known to dramatically slow down VDI (Restituito et al., 2000; Wei et al., 2000). In a different set of experiments, the authors explore the relevance of CDI by inducing the expression of a mutated calmodulin (CaM_{34}), known to abolish CDI (Lee et al., 2003). The role of Ca_V β_2a

and/or the action of CaM34 are evaluated in experiments performed in neonatal cardiomyocytes. Specifically, myocytes were voltage clamped using the waveform of a self-action potential (sAP-Clamp; Banyasz et al., 2011, 2012), recorded from the same cell in control and isoproterenol-treated conditions.

In control cells, the authors show very nicely that application of 100 nM isoproterenol shortens the action potential by increasing the rate of inactivation of the L-type Ca^{2+} current recorded with sAP-Clamp (Figs. 1 and 2 in Morales et al. [2019]). This suggests that modifying the rate of inactivation of the L-type current has a critical effect on the duration of the action potential. Additionally, in experiments performed in the absence of isoproterenol, the authors demonstrate that molecular interventions that alter the rate of inactivation of L-type Ca^{2+} channels dramatically prolong the duration of the action potential and Ca^{2+} currents. The expression of CaM_{34} increases action potential duration by more than five times (Fig. 5 in Morales et al. [2019]), and overexpression of CaV β_2a increases action potential duration by more than three times (Fig. 6 in Morales et al. [2019]). These results confirm that the rate of inactivation of L-type Ca^{2+} currents defines the duration of the action potential in neonatal rat myocytes.

Figs. 8 and 9 in Morales et al. (2019) show the conclusive experiment designed to evaluate which of the L-type Ca^{2+} current inactivation mechanisms is dominant. The results presented in Fig. 8 illustrate that, in the absence of CDI induced by overexpression of CaM_{34}, isoproterenol has a significantly smaller effect than when VDI is impaired by the expression of Ca_V β_2a. This, along with isoproterenol's significantly larger effect in myocytes when CDI is not altered (Fig. 9), clearly indicates that CDI is the main mechanism for L-type Ca^{2+} channel inactivation during adrenergic stimulation.

As previously stated, these experiments were conducted in neonatal rat cardiomyocytes, a model that significantly differs from adult ventricular myocytes (Escobar et al., 2004; Pérez et al., 2005; Snopko et al., 2007). For example, neonatal myocytes have a reduced expression of Kv 4.X channels (Kilborn and Fedida, 1990; Wang and Duff, 1997; Kobayashi et al., 2003) and the regulatory subunit KChIP (Kobayashi et al., 2003; Jia and Takimoto, 2006). These K^+ channels define a transient K^+ outward current (Ito; Guo et al., 1999; Teutsch et al., 2007; Rossow et al., 2009) that can reshape action potential repolarization. Thus, because of the presence of Ito, we can expect that both VDI and CDI will have a smaller effect on action potential duration in adult myocytes.

Previous studies have also shown that CICR is not critical for defining intracellular Ca^{2+} dynamics during excitation–contraction coupling in neonatal cardiac myocytes (Escobar et al., 2004), primarily because the tubular system is not fully developed (Di Maio et al., 2007). This reduced SR Ca^{2+} release in neonatal myocytes further supports the hypothesis presented by Morales et al. (2019): Ca^{2+} release from the SR would likely augment the effect of CDI (Lacampagne et al., 1996). Indeed, SR Ca^{2+} release is dramatically increased during adrenergic stimulation, not only due to a larger triggering signal, but also because the intra SR Ca^{2+} content is higher. The increase in luminal SR Ca^{2+} content is mediated by an increase in Ca^{2+} influx into the myocyte during each action potential. In addition, SR Ca^{2+} content is further elevated due to phosphorylation of phospholamban, which in turn increases the SERCA2 transport rate. These findings suggest that the mechanism proposed by Morales et al. (2019) will be even more relevant in controlling Ca^{2+} influx during β–adrenergic stimulation in an adult heart.

In summary, Morales et al. (2019) use a novel and powerful approach to shed light upon a fundamental physiological and pathophysiological puzzle: how to prevent Ca^{2+} overload, and the pathological consequences of this overload, during a "fight or flight" response.

Acknowledgments

We thank Drs. Alicia Mattiazzi, Rafael Mejia-Alvarez, and Maria Elena Zogbhi for their valuable discussions.

This study was supported by University of California, Merced graduate funding. A.L. Escobar is funded by University of California, Merced grant no. Castle-20095-442167 and National Institutes of Health grant no. 1R01GM132753-0.

The authors declare no competing financial interests.

Author contributions: A.L. Escobar conceived the comments and wrote the manuscript. M. Bazmi and A.L. Escobar contributed to writing the manuscript and making figures. The manuscript was approved by both authors. Both authors agree to be accountable for all aspects of the work, all persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Eduardo Ríos served as editor.

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