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COMMENTARY

Why make a strong muscle weaker?

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Importance of studying drug effects at myofibrillar level

Hypertrophic cardiomyopathy (HCM) affects at least 1 out of 500 individuals of the general population and is the most common cause of sudden cardiac death in people under 30 yrs old (Maron and Maron, 2013). HCM is mostly caused by mutations in sarcomeric proteins with a large phenotypic variability among patients. While the primary cause of HCM is still poorly understood, it is often considered that HCM associates with a hypercontractile state of the ventricle (Spudich, 2019). Therefore, partly inhibiting contractile force in an HCM heart at the sarcomeric level appears as a promising clinical and pharmaceutical aim. This implies the necessity of understanding the mechanism of action of HCM-specific modulators like the myosin-inhibitor mavacamten (MAVA), known as MYK-461, and their effect on the sarcomeric contractile function of striated muscles (Sparrow et al., 2019; Toepfer et al., 2019; Awinda et al., 2020; Awinda et al., 2021; Sparrow et al., 2020; Scellini et al., 2021). Fast kinetic chemo-mechanical studies on sarcomeric function are possible with subcellular myofibrils (MFs) because they rapidly reach diffusional equilibrium with their surrounding environment and therefore allow activation, as well as relaxation, kinetics to be studied in detail. In addition, functional studies with MFs can provide insights in contractile function of cardiomyocytes (CMs) in the absence of Ca^{2+}-handling systems and upstream signaling (Stehle et al., 2009; Stehle and Iorga, 2010; Scellini et al., 2021).

Scellini et al. (2021) investigated the inhibitory effect of the drug MAVA on ventricular MFs from human hearts and compared it to fast skeletal MFs from rabbit psoas muscle. They observed a quick and reversible decrease of steady-state isometric force of ventricular (IC_{50} ~0.5 µM) and skeletal (IC_{50} ~10 µM) MFs at maximal Ca^{2+} activation (pCa 4.5) below physiological temperature (15°C). In the slowly contracting human ventricular MFs, MAVA had no inhibitory effect on the kinetics of force development but accelerated kinetics of relaxation upon Ca^{2+} removal. In fast rabbit skeletal MFs, MAVA's effects on sarcomeric force kinetics were different—it decreased the rate constant of force development and had no significant influence on relaxation (Scellini et al., 2021).

Why make a strong muscle weaker?

Hypercontractility of HCM heart muscle, commonly reflected by an increased force response to calcium (i.e., higher Ca^{2+}-sensitivity of force), is often accompanied by diastolic dysfunction (Maron and Maron, 2013). In such cases, partial inhibition of force and acceleration of sarcomeric relaxation by MAVA will most likely be beneficial for the HCM ventricle. MAVA also decreased force generated by fast rabbit skeletal MFs (Scellini et al., 2021). Thus, it is important to expand investigations to human fast and slow skeletal muscles as well as to human atrial muscles because differences in the isoform profile of sarcomeric proteins may distinctly modulate the effects of MAVA on force kinetics.

Studies such as the one by Scellini et al. (2021) are of particular value for addressing disease mechanisms and potential treatments because MFs can be isolated from myocardial- and skeletal-muscle biopsies of HCM patients (e.g., Kirschner et al., 2005; Kraft et al., 2013), CMs derived from human induced-pluripotent stem cells (e.g., Pioner et al., 2016; Iorga et al., 2018), or HCM-animal models carrying missense mutations in sarcomeric proteins (e.g., Iorga et al., 2008; Green et al., 2016; Awinda et al., 2021). Potentially beneficial effects of HCM drugs like myosin-inhibitor MAVA can therefore be assessed for different types of HCM-related mutations (Green et al., 2016). This strategy is particularly important because there is evidence that different HCM mutations can lead to hypocontractility (i.e., reduced Ca^{2+}-sensitivity of force or ATPase activity) or other alterations of force generation and relaxation (e.g., Kirschner et al., 2005; Moore et al., 2012; van Dijk et al., 2012; Kraft et al., 2013). For example, an HCM-linked myosin mutation (R712L) decreased the working stroke, which was rescued by a myosin activator (omecamtiv mecarbil) instead of an inhibitor (Snoberger et al., 2021). Some HCM-related mutations in cardiac troponin T (cTnT) caused an increase in Ca^{2+}-sensitivity of force, which was, however, dependent on the mutation location and dose level of the mutant protein (Schuldt et al., 2021). For example, R278C-cTnT at low and intermediate levels induced hypercontractility, while at high levels it

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resulted in hypocontractility of ventricular CMs (Schuldt et al., 2021).

In consequence, the widespread hypothesis that HCM arises from hypercontractility of the sarcomere may not be universal (Snoberger et al., 2021), and other primary functional alterations of the contractile process could be associated with HCM-mutations in myosin and other sarcomeric proteins. What might be common to heterozygous HCM mutations in proteins with burst-like expression of their two alleles (like myosin, cardiac isoform of the myosin binding protein C [cMyBP-C], and cTnI), and which alter the biomechanical function of the sarcomere, is the variable ratio of mutant and wild-type mRNA and protein from cell to cell, most likely inducing contractile imbalance among CMs (Kraft and Montag, 2019; Montag and Kraft, 2020). Therefore, important questions remain: (1) could MAVA or other drugs that target HCM be beneficial for HCM ventricles with a mutation in myosin or another sarcomeric protein that does not induce hyper-contractile function? And (2), how will MAVA or other drugs affect CM and myocardial function if variable fractions in the HCM-related mutant sarcomeric protein are expressed from cell to cell? The work of Scellini et al. (2021) provides a reliable approach for drug assessment at sarcomeric level to further understand the primary effects of drugs on specific HCM-related mutations.

Consistent with previous x-ray diffraction studies (compare Kraft and Montag, 2019 and references therein), it was suggested that, in relaxed skeletal muscle at high temperature (35°C), most myosin heads are in an ordered configuration, while at low temperature (10°C) about half of myosin heads are less ordered (Caremani et al., 2019). Upon Ca^{2+}-activation of actin thin filaments, the fraction of myosin heads that can be kinetically modulated to enter "force-generating states" (Fig. 1) seems more reduced at low temperature than at high temperature, and therefore the resulting isometric force is lowered by cooling (Caremani et al., 2019). Considering a two-state cross-bridge model (Fig. 1; Brenner, 1988), this would indicate that more cross-bridges are in "non–force-generating states" at low temperature than at high temperature, as there might be a smaller probability for disordered myosin heads to bind strongly to actin when thin filaments are turned on in the presence of Ca^{2+}.

It was proposed that some myosin inhibitors, like MAVA (Anderson et al., 2018) or blebbistatin (Wilson et al., 2014), shift the "disordered" ⇔ "ordered" equilibrium more toward the ordered state and stabilize it. However, there was no direct evidence for this shift induced by MAVA using fast skeletal and slow cardiac MFs at 15°C (Scellini et al., 2021). Nevertheless, such equilibrium shift might be less relevant at physiological temperature as most of the heads will be already in the ordered state in relaxed muscle at very low Ca^{2+}. As an HCM-related mutation may destabilize the ordered state at physiological temperature to some extent, MAVA could eventually compensate by repopulating the ordered state, in addition to its intrinsic inhibitory effect on myosin (Kawas et al., 2017).

After Ca^{2+} removal, the fast, second phase of MF relaxation, having a larger amplitude than the first, slower phase, is characterized by the *fast* k_{rel} rate constant. This describes kinetics of

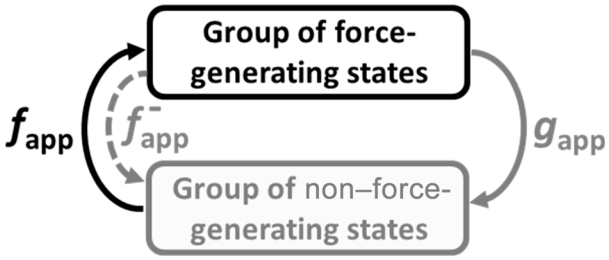

Figure 1. **Simplified scheme of the two-state cross-bridge model proposed by Brenner.** Force-generating and non–force-generating states enclose all possible sub-states that are eligible to be populated by strong actin-interacting cross-bridges and by weak or non–actin-interacting cross-bridges, respectively. An equilibrium is established between the sub-states corresponding to each of the two groups of states. f_{app} represents the apparent rate constant of cross-bridges to enter force-generating states, which is modulated by the fast, dynamic, Ca^{2+}-dependent equilibrium between turned-on and turned-off forms of the regulated actin units (therefore, f_{app} depends on Ca^{2+} concentration; Brenner and Chalovich, 1999; Stehle and Iorga, 2010). g_{app} represents the apparent rate constant of cross-bridges to leave force-generating states, which is independent of $[Ca^{2+}]$ (Brenner, 1988; Stehle and Iorga, 2010). If P_i concentration is negligible and f^-_{app} can therefore be ignored, the rate constant of force redevelopment is $k_{tr} = f_{app} + g_{app}$, and the rate constant of the first, isometric MF relaxation phase is *slow* $k_{rel} = g_{app}$. Active isometric force (or tension) is $P = n \cdot F_0 \cdot f_{app} / (f_{app} + g_{app})$, where n is the maximum number of cross-bridges that are able to participate in turnover in a half-sarcomere at a given temperature, F_0 is the mean force produced by a single actin–myosin cross-bridge in the force-generating states, and $f_{app} / (f_{app} + g_{app})$ is the steady-state fraction of turning over cross-bridges in the force-generating states (Brenner, 1988).

cross-bridges leaving force-generating states under reduced mechanical load (compared with the first relaxation phase during which sarcomeres remain isometric), because sarcomere lengthening partially releases the serial stress in MFs (Stehle et al., 2003; Stehle et al., 2009). Therefore, the rapid and large drop of myofibrillar force is likely to contribute to the ventricular pressure decay at the onset of diastole, while *slow* k_{rel} (=g_{app}, the probability of cross-bridges to leave force-generating states), describing kinetics of the first relaxation phase, is related to tension cost (i.e., ATPase/force).

In HCM myocardium with ischemia due to microvascular dysfunction, there is the possibility of elevated intracellular ADP concentration compared with healthy myocardium (Maron et al., 2009). This would favor existence of additional cross-bridges in force-generating states due to strong actin–myosin interactions inducing an increase of myofilament Ca^{2+} sensitivity. It has been shown that, in the presence of ADP, partial activation of the thin filaments by a small fraction of strong-binding cross-bridges can also delay and slow down myofibrillar relaxation (Stehle et al., 2003). Thus, elevated ADP will contribute to impaired diastolic relaxation in HCM and decrease ventricular compliance at diastolic Ca^{2+} levels (Stehle et al., 2003; Sequeira et al., 2015). Scellini et al. (2021) showed that MAVA induces essentially full relaxation at elevated ADP-levels and thus could be beneficial, particularly for diastolic function in HCM hearts under ischemic conditions.

This may be similar in other conditions, inducing residual, calcium-independent active force generation, and thus diastolic dysfunction in HCM. Some HCM-related missense or truncating

mutations in troponin I showed elevated Ca^{2+}-independent residual force (pCa ≥ 7.5) that impaired sarcomeric relaxation (compare Stehle et al., 2009 and references therein). This residual force was diminished by BDM (Iorga et al., 2008), which shifts cross-bridges to prepower stroke non–force-generating states, and the relaxation process was restored. Similar effects are expected from MAVA-treatment, as also proposed in Scellini et al. (2021).

Furthermore, cardiac MFs that were partially relaxed to a low level of force (e.g., ≤6% of F_{max}) showed delayed and slower relaxation (i.e., reduced *fast* k_{REL}) than MFs which were fully relaxed (Stehle et al., 2009). Thus, if there is incomplete inactivation of cross-bridges due to elevated diastolic Ca^{2+} levels, it feeds back on relaxation kinetics (Stehle et al., 2009; Scellini et al., 2017). This is highly relevant in HCM since in some studies with CMs from HCM patients, diastolic Ca^{2+} concentrations were slightly higher than in healthy control CMs, impacting diastolic function (Coppini et al., 2013). Like other myosin inhibitors, such as BDM (Scellini et al., 2017), MAVA had no effect on passive force in the virtual absence of Ca^{2+} at 15°C with both fast skeletal and slow ventricular MFs stretched to different sarcomere lengths (Scellini et al., 2021). Yet, in another study at physiological temperature, MAVA decreased the low force generated by skinned human ventricular strips at pCa 8.0, suggesting that this force arose from some residual force generating cross-bridges (Awinda et al., 2020). In consequence, MAVA most likely will not compensate for alterations in pure passive properties of the HCM myocardium, probably due to changes in titin phosphorylation or fibrosis, which also contribute to diastolic dysfunction, aside from the active component (i.e., few force generating cross-bridges) that seems to be the main "target" of MAVA.

Why does MAVA inhibit force differently in fast and slow MFs?

According to the previously described two-state cross-bridge model (Fig. 1; Brenner, 1988), ≥25–30% of maximum Ca^{2+} activation, experimental evidence indicated that Ca^{2+} regulation of isometric steady-state parameters (e.g., force and ATPase activity) is mediated through changes in cross-bridge turnover kinetics (Fig. 1) while the maximum number of cross-bridges participating in active cycling remains unchanged at a given temperature (rate modulation principle). At lower Ca^{2+} activation levels, cross-bridges could also be switched in and out of the turnover process by a recruitment mechanism (Brenner, 1988). At higher Ca^{2+} levels, regulatory units on actin are in a dynamic equilibrium for turning actin on and off with very fast rate constants. These rates are much faster compared with cross-bridge turnover (Brenner and Chalovich, 1999), and the equilibrium is Ca^{2+}-dependent, meaning that for the apparent rate constant f_{app}, the probability of cross-bridges to enter force-generating states is modulated by $[Ca^{2+}]$ (Brenner, 1988; Stehle and Iorga, 2010).

Changes in the f_{app}/g_{app} ratio modulate Ca^{2+}-sensitivity of force and the apparent cooperativity (Brenner, 1988). As a consequence, any (patho)physiological or pharmacological intervention (e.g., an HCM-related mutation in sarcomeric proteins, change of phosphorylation level or of isoform profile of sarcomeric proteins, or presence of MAVA) affecting f_{app}/g_{app} ratio can impact the force–pCa relation without a substantial alteration of cross-bridges available for turnover (n) at a given temperature when $[Ca^{2+}]$ is not too low.

In fast skeletal MFs, f_{app} seems substantially larger than in slow ventricular MFs when compared with g_{app} (Scellini et al., 2021) and the difference could be assigned, at least in part, to distinct myosin and other sarcomeric protein isoforms in the two muscle types. Since the rate constant of force redevelopment k_{tr} (and also k_{act}) in MFs equals the sum $f_{app} + g_{app}$ at negligible P_i concentrations (Brenner, 1988), an intervention like MAVA that seems to affect both f_{app} (reduction) and g_{app} (increase; Scellini et al., 2021) could be distinctly reflected on k_{tr} (or k_{act}) in different MFs types. In MFs from fast skeletal muscle, MAVA affected mainly k_{tr} by reducing f_{app} (e.g., by decreasing the P_i release rate) while a possible increase in g_{app} (e.g., by accelerating ADP release rate) was not detectable (Scellini et al., 2021). Only in ventricular MFs did g_{app} seem to be increased as indicated by a faster *slow* k_{rel} at negligible P_i concentrations (Scellini et al., 2021). Possible slowing of f_{app} was below detection limit (as indicated by unchanged k_{tr} in cardiac MFs), presumably due to blunting by an increase of g_{app}.

In conclusion, the work by Scellini et al. (2021) shows the potential of further myofibril studies with MAVA or other drugs targeting HCM-mechanisms at the sarcomeric level. Such studies allow analysis of the effects of the drugs (1) on different striated muscle types (human fast/slow skeletal MFs, atrial/ventricular MFs), which will also reveal adaptations in sarcomeric function among protein isoforms, and (2) on cardiac MFs or CMs carrying HCM-related mutations in sarcomeric proteins that induce either hyper- or hypocontractility (higher or lower calcium sensitivity, respectively) or other changes of the force-generating mechanism. In combination with clinical data, these insights could lead to administration of drugs (MAVA) in a personalized manner.

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