When One Says Yes and The Other Says No; Does Calcineurin Participate in Physiologic Cardiac Hypertrophy?

Running Title: Calcineurin Controversy

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ABSTRACT

Developing engaging activities that build skills for understanding and appreciating research is important for undergraduate and postgraduate science students. Comparing and contrasting opposing research studies does this, and more: it also appropriately for these cohorts challenges higher-level cognitive processing. Here, we present and discuss one such scenario, that of calcineurin in the heart and its response to exercise training. This scenario is further accentuated by the existence of only 2 studies. The background is that regular aerobic endurance exercise training stimulates the heart to physiologically adapt to chronically increase its ability to produce a greater cardiac output to meet the increased demand for oxygenated blood in working muscles, and this happens by 2 main mechanisms: 1) increased cardiac contractile function and 2) physiologic hypertrophy. The major underlying mechanisms have been delineated over the last decades, but one aspect has not been resolved: the potential role of calcineurin in modulating physiologic hypertrophy. This is partly because the existing research has provided opposing and contrasting findings, one line showing that exercise training does activate cardiac calcineurin in conjunction with myocardial hypertrophy, but another line showing that exercise training does not activate cardiac calcineurin even if myocardial hypertrophy is blatantly occurring. Here, we review and present the current evidence in the field and discuss reasons for this controversy. We present real-life examples from physiology research and discuss how this may enhance student engagement and participation, widen the scope of learning, and thereby also further facilitate higher-level cognitive processing.

SNAPSHOT

Physiologic cardiac hypertrophy is an important exercise adaptation. Several signaling pathways responding to the exercise stress cause this. However, the role of calcineurin has yet to be determined, because the available studies on its role have delivered diametrically opposite results and conclusions. Here, we review the field and present and discuss the controversy, in order to facilitate enhanced teaching and learning in physiology.

KEY WORDS Training, heart, physiologic hypertrophy, athletes' heart, calcineurin.

DEFINITION Controversy: a discussion marked especially by the expression of opposing views; dispute.

INTRODUCTION

For undergraduate and postgraduate science students, it is important to develop skills that enable them to appropriately appreciate, understand, critically evaluate and maybe even conduct research. As educators, we must provide engaging activities that facilitate and support this. Comparing and contrasting opposing research studies is an important aspect in this regard. Apart from highlighting an issue of "known unknowns," it also enhances learning because it requires cognitive processing at a high level in Bloom's Taxonomy (1). This scenario of compare and contrast is simplified and yet accentuated if within a given field, only 2 studies on the matter exist, and they lead to diametrically opposite conclusions, with no further studies able to reconcile the given matters.

Here, we present and discuss one such scenario, that of calcineurin in the heart and its response to exercise training. We review the current evidence and discuss reasons that may have contributed to the lack of clarity, and use this to develop an approach (from a real-life example) that showcases and highlights physiology research as a source to challenge and thereby facilitate higher-level cognitive processing in an innovative manner that extends beyond covering only well-established concepts of physiology. We thereafter propose a framework to follow for the physiology educator that directs the discussion to cover adjacent, but related topics such as research methodologies and experimental research models, clinical utility and drug discovery, critical appraisal of evidence with a view from the theorem of hypothesis testing, falsification and knowledge generation, as well as practical aspects from ethics to publishing; some of which may be specifically tailored to either undergraduate and/or postgraduate students. This may be challenging, but in our experience, it leads to profound intellectual growth.

EXERCISE TRAINING-INDUCED PHYSIOLOGIC CARDIAC HYPERTROPHY

The scientific background is that it is well-established that trained endurance athletes from a variety of sports backgrounds, such as running, cycling, rowing, and cross-country skiing, present with increased cardiac dimensions and myocardial mass, mainly in the form of ventricular hypertrophy (2). This view is supported by longitudinal studies of intense endurance exercise training that show the heart adapts by increasing dimensions and size (3) and therefore may not be inherited but rather at least partly adapted, while experimental animal studies demonstrate that this occurs by a process that involves physiologically beneficial expansion and growth in the myocardium as well as concomitant increases in length and width of cardiomyocytes (4). This results in parallel deposition of sarcomeres, is considered beneficial, and it improves heart contractility and pump performance (5).

The stimulus for physiologic cardiac hypertrophy is that during intense exercise, there is an increased metabolic and oxygen demand in the working skeletal muscles (due to the exercise, with a similar adaptation occurring in pregnancy). This demand can only be met

sufficiently by increasing the supply of oxygenated blood from the heart as arterial blood oxygenation, vascular redistribution, peripheral diffusion, and muscle extraction all have capacity limits well below the demand set by the exercise (6). Hence, and regardless of changes in cardiac stress or workload, the heart must deliver more blood. When this exercise stimulus is frequently repeated, particularly at a high exercise intensity (7), the heart will adapt partly through increased contractile capacity and function, and partly through increased dimensions and mass. From a simplistic perspective, and without further comparison, this is analogous to skeletal muscle hypertrophy in response to increased load demands in the trained muscle (8). Physiologic cardiac hypertrophy is due to its association with highly trained endurance athletes, also sometimes called *athletes' heart* (9).

In contrast, pathologic hypertrophy is typically characterized by either ventricular wall growth or stretching without a concomitant preservation of wall-to-chamber ratio. At a gross organ level, pathologic hypertrophy may appear reminiscent to physiologic hypertrophy, but ensues from pathologic stimuli such as myocardial infarction (MI), valvular diseases, chronic hypertension and metabolic syndromes (10,11) and may in fact precede heart disease with reduced contractility and cardiac pump capacity (12). Pathologic adaptation is however outside the remit of this paper but is mentioned here for clarity.

MOLECULAR SIGNALING FOR PHYSIOLOGIC CARDIAC HYPERTROPHY

The molecular pathways that activate, maintain and modulate physiologic cardiac hypertrophy have also been well described, albeit not all aspects are equally well understood. There is, however, good evidence indicating that the entire cell machinery from modulation of gene transcription to protein translation and subsequent post-translation are all processes that contribute to the physiologic hypertrophy phenotype. This evidence comes from a number of different approaches, including studies of experimental animals including genetically modified animals undergoing different types and modes of exercise training (4,7,13–24), cell-in-a-dish-based studies of growth factor-stimulated C2C12 muscle mimics (25,26), as well as studies of exercise training in humans (27,28). It is beyond the scope of this review to exhaustively present and discuss those studies; however, because we ultimately point out an area of controversy, we here also highlight that at least several molecular pathways have been convincingly shown to associate with physiologic hypertrophy (Figure 1):

1: Mitogen-activated protein kinase (MAPK): In the early phases of an exercise training program, activated gene transcription via MAPK have been shown to induce myocardial hypertrophy, whereas intriguingly, during the continued chronic phase of the exercise training, MAPK responses to exercise slowly deteriorated despite continued hypertrophy generation (14), suggesting that MAPK-activation may transiently be important in the early, but not maintenance, phase of the exercise training-induced physiologic

hypertrophy. The transient, but not chronic activation may be beneficial, given chronic MAPK activation typically has been linked to pathologic hypertrophy and maladaptive remodeling in the heart (29).

2: Calcium-calmodulin-dependent protein kinase-II (CaMKII): Further activation of muscle and myofilament-specific gene transcription has also been observed after exercise training. The increased contractile work that the heart has to execute during exercise initiates calcium-linked regulation due to excitation-transcription coupling. Intracellular calcium-bound calmodulin activating CaMKII phosphorylates histone deacetylase (HDAC) to relieve suppression of nuclear myocyte enhancer factor-2 (Mef2) during exercise training (30-34). In line with this, exercise has also been shown to associate with deoxyribonucleic acid (DNA)-demethylation (16,35), which taken together are thought to contribute positively to myocardial gene transcription during exercise training in a manner that induces physiologic hypertrophy. Other putative processes that also enhance gene transcription include intracellular metabolic and hypoxic challenges linked to exercise, as well as endocrine/paracrine or autocrine hormonal events (14).

3: Micro-RNA: The activation of nuclear gene transcription produces increased amounts of messenger-ribonucleic acid (mRNA) transcripts (or what we commonly understand as expressed genes), which for a phenotype effect must be exported to the ribosome for mRNA-directed amino acid assembly and protein synthesis in the ribosome. After the relatively recent discovery of short non-coding microRNAs (and also longer non-coding RNAs with similar function) and their ability to post-transcriptionally negatively regulate gene transcription, it became clear that the nucleus-ribosome transfer of mRNA is also subject to modulation, including in the heart (27,36,37). This modulation occurs by microRNA complementary binding to said mRNA, and since it physically carries RNA-induced silencing complex (RISC), it denatures and silences mRNA and thereby post-transcriptionally decreases gene transcription. Exercise training has been shown to reduce the expression of specific myocardial microRNAs (27,38), which therefore also may become permissive for physiologic hypertrophy.

4: Insulin-like growth factor-1 (IGF1)-phosphoinositide-3 kinase (PI3K)-protein kinase-B (Akt)-mammalian target of rapamycin (mTOR): Perhaps the most compelling evidence of molecular activation that modulates physiologic hypertrophy is related to IGF1-PI3K-Akt-mTOR-signaling; a major modulator of protein synthesis, but also indicated to regulate nuclear gene transcription, including in muscle (17,39). Its role in controlling physiologic hypertrophy has been established by studies using a variety of exercise training modes, such as running and swimming, in experimental animals (17,24,39-41), after transgenic knock-in, over-expression, knock-out or pharmacological inhibition of IGF1-PI3K-Akt-mTOR axis targets. These studies demonstrate a central role in IGF1-PI3K-Akt-mTOR for permitting exercise training-induced physiologic hypertrophy (24,39), whereas in vitro C2C12 cells hypertrophy after IGF1-stimulation,

but in the presence of mTOR-inhibitor Rapamycin fail to respond to IGF-1 stimulation (42).

5: Heat shock protein (HSP): Post-translational modification with repercussions to possibly enhanced protein stability have also been indicated to increase in the heart after exercise training, at least in the aspects that are governed by HSPs, which in a series of proteomics experiments have been observed to over-express in trained hearts (43,44). More directly however, in vitro experiments have also suggested that HSP70 may in addition co-regulate hypertrophy development in itself and not just chaperone cellular maintenance, by maintaining HDAC phosphorylation (45), which taken together with specifically CAMKII-induced transcription as well as general induction of gene transcription and translation provides a cellular environment that is conducive to physiologic hypertrophy.

6: Peroxisome proliferator-activated receptor (PPAR)-coactivator-1α (PGC1α): Adjacent and complementary to activation of cardiomyocyte hypertrophy is the notion that this requires energy (46,47). Hence, alongside the processes that lead to architectural changes and cell and tissue growth, adaptation of cellular bioenergetics to facilitate the former may also be required. Some evidence has suggested involvement of the transcription factor PPAR and its coactivator PGC1α, downstream of MAPK, calcium and a host of other signaling events (46). This pathway is activated by exercise training in several organ systems including the heart (46,48,49) and is thought to profoundly increase cell metabolic energy production (47). Hence, PGC1α-activation may not directly control physiologic hypertrophy, but indirectly it at least partly facilitates this by permitting conditions to supply the additional cellular energy required by the hypertrophy process. On the other hand, direct pharmacologically-induced activation of PPAR-β leading to cardiac growth without any sign of pathology (hence, physiologic hypertrophy) however indicates a possible role of this molecular cascade for also inducing physiologic hypertrophy (50). However, it should be pointed out that not all studies have been able to show increased cardiac PGC1α after exercise training, despite presence of physiologic hypertrophy, but in this case, other intracellular energy systems that enhance generation of adenosine triphosphate (ATP) increase, such that a phenotype of higher metabolic state still occurs (20).

It is also relevant to note that during physiologic hypertrophy following exercise training, no evidence of activation of embryonic or fetal gene programs have surfaced, in contrast to its common activation during pathologic hypertrophies (51).

The mechanisms of control of physiologic cardiac hypertrophy described so far (Figure 1) have been reasonably well established. However, one well-described signaling pathway that may potentially also play a regulatory role to physiologic hypertrophy is calcineurin, but a link to exercise training has not yet received the same attention afforded other signal pathways, albeit its role in pathologic hypertrophy is better understood (12).

The attempts to study it in the setting of exercise training have in contrast provided contradictory and diametrically opposite results, which thereby may have inadvertently to some degree thwarted the field.

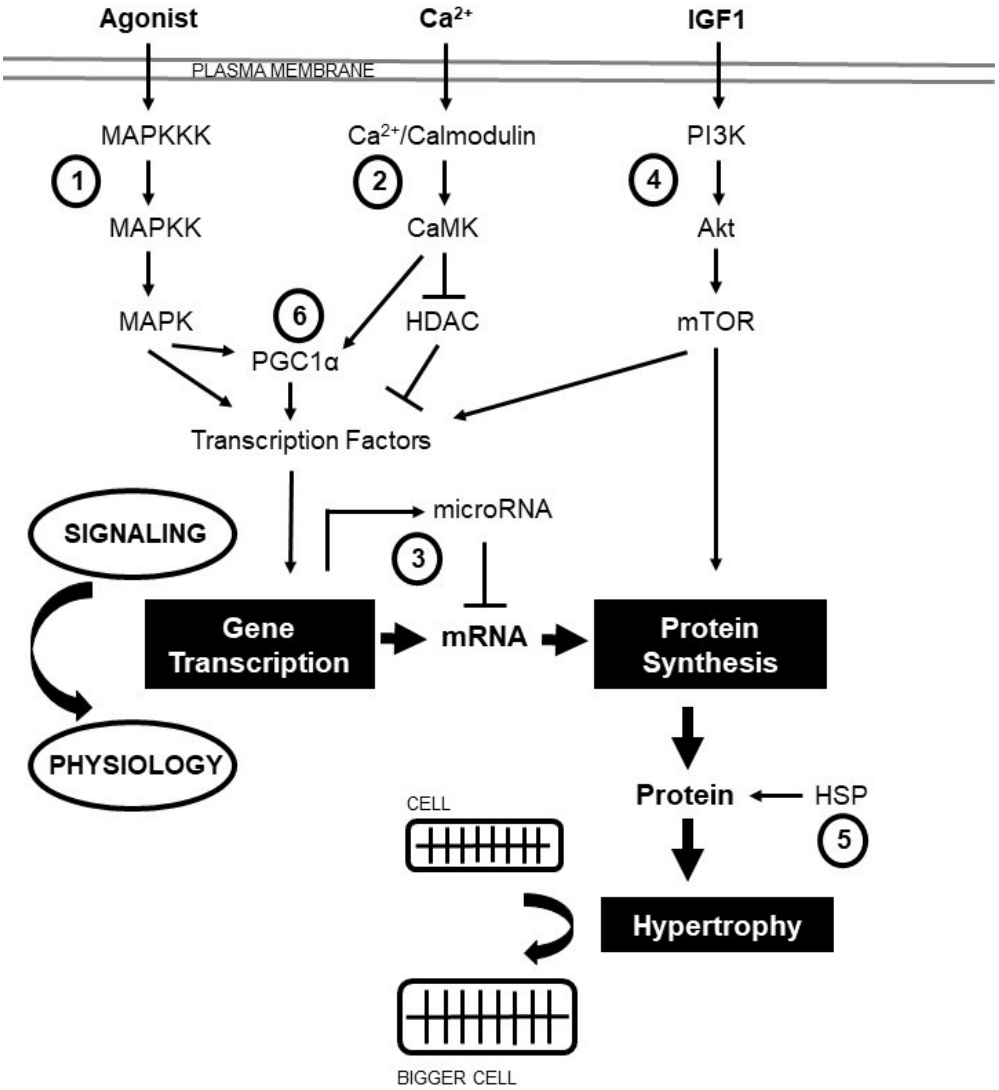

Figure 1: A model of established molecular pathways relevant in physiologic cardiac hypertrophy, activated in response to several distinct extra- and intracellular signals, leading to physiologic cellular hypertrophy after modulation via gene transcription and/or protein synthesis and maintenance. MAPK(K(K)): mitogen activated protein kinase (kinase (kinase)); CaMK: Ca^{2+}-calmodulin dependent protein kinase; HDAC: histone deacetylace; IGF1: insulin like growth factor-1; PI3K: phosphoinositide-3 kinase; Akt: protein kinase B; mTOR: mammalian target of rapamycin; mRNA: messenger ribonucleic acid; HSP: heat shock protein. Numbers link to text description.

CALCINEURIN

Calcineurin is a heterodimeric, calcium-calmodulin dependent, serine-threonine protein phosphatase, also known as protein phosphatase-3 or calcium-dependent serine-threonine phosphatase. It is made up of both catalytic (calcineurin A) and regulatory (calcineurin B) subunits (52). Functionally it is vital in the development of both cardiac and skeletal muscle. Calcineurin dephosphorylates the transcription factor nuclear factor of activated T cells (NFAT), which thereby influences gene transcription. Calcineurin can be stimulated by a variety of endocrine, autocrine and paracrine hypertrophic stimuli

such as angiotensin-II, endothelin-1 and catecholamine activation of G protein-coupled receptors that stimulate a calcium influx through activation of stretch-activated calcium channels, or via excitation-transcription coupling calcium release via plasma membrane-bound L-type calcium channels or sarcoplasmic reticulum-bound ryanodine receptors (12,53; Figure 2). Hypertrophic stimuli also trigger release of calcium through inositol triphosphate-mediated endoplasmic reticulum calcium channels. After entry, calcium ions bind to and activate calmodulin, which consequently binds to calcineurin, thereby dephosphorylating NFAT (Figure 2). There are 4 NFATS activated by calcineurin and these are located in the cytoplasm. Dephosphorylated NFAT is translocated to the nucleus and activates genetic programs, including induction of cardiac hypertrophy (54-56). Other actions involving NFAT are linked to immune function, whereby NFAT also activates and differentiates T cells (57). However, dephosphorylated NFAT can be phosphorylated by glycogen synthase kinase-3 (GSK3), thereby inhibiting cardiac hypertrophy. GSK3 is inhibited by phosphorylation of serine-9 by Akt, downstream of PI3K, which has been found to be activated in several scenarios including pressure overload hypertrophy (58,59) as well as exercise training (17,60), as previously described. Calcineurin can be also inhibited by several mechanisms. Most notably, cyclosporin A (CsA) blocks NFAT activation indirectly by conformationally changing cyclophilin A, which prevents activation of calcineurin. FK506 inhibition also works similarly through FK506 binding protein-12 (61). The link between calcineurin and cardiac hypertrophy was first elucidated using transgenic mice expressing activated NFAT and calcineurin in 1998. These mice developed cardiac hypertrophy and eventually heart failure, suggesting calcineurin played an important role in myocardial hypertrophic signalling (62). The next, and perhaps the more controversial, step in calcineurin discovery would however revolve around its potential involvement in physiologic cardiac hypertrophy.

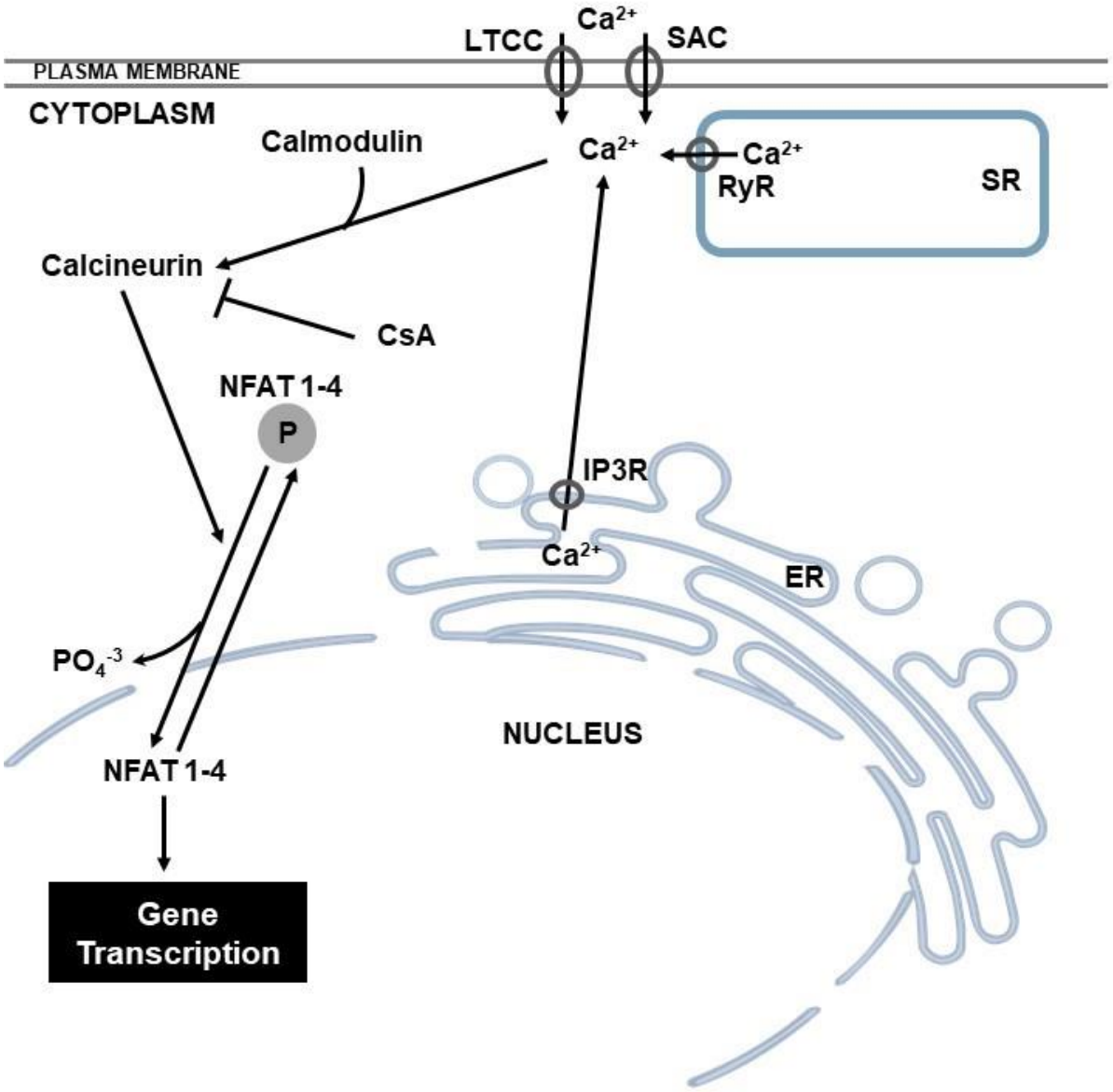

Figure 2: A model of the established calcineurin pathway for regulating gene transcription, and as it may be potentially relevant in physiologic cardiac hypertrophy, activated by calcium-bound calmodulin. Downstream, calcineurin dephosphorylates the nuclear factor of activated T cells (NFAT), which allows for NFAT entry to nucleus where it functions as a transcription factor. Calcineurin may also be inhibited, most notably by cyclosporin A (CsA). ER: endoplasmic reticulum; IP3R: inositol triphosphate receptor; LTCC: L-type calcium channel; RyR: ryanodine receptor; SAC: stretch-activated (calcium) channel; SR: sarcoplasmic reticulum.

CALCINEURIN CONTROVERSY

In 2000, Eto et al (63) published findings that convincingly demonstrated that in rats, calcineurin was activated in physiologic left ventricular hypertrophy (LVH) following 10 weeks of chronic exercise (Table 1). This was a considerable effect, in the order of 150%

or a 2.5-fold increase vs sedentary controls, and of course of statistical and most likely also biological significance. Furthermore, the study also found calcineurin was activated in developing pathologic LVH (44.9±6.7 pmol/min/mg after 1 week of aortic constriction vs 22.1±3.7 pmol/min/mg after surgical placebo (sham)); i.e. a 100% or 2-fold increase. In contrast, calcineurin activity was not elevated in decompensated cardiac hypertrophy (29.0±3.4 pmol/min/mg after 4 weeks of aortic constriction vs 18.4±0.5 pmol/min/mg in sedentary or 22.1±3.7 pmol/min/mg in sham). The exercise protocol involved exercising rats by running 2.4±0.7 km/day on a bespoke voluntary running wheel for 10 consecutive weeks, and importantly, the exercise protocol also increased cardiac contractile capacity.

However, in 2004, a conflicting study by Wilkins et al (64) found that in mice, calcineurin activity was not significantly elevated in physiologic LVH (~1800 μg^{-1} after 20 days of swim training and ~1700 μg^{-1} after 14 days of running vs ~2000 μg^{-1} in sedentary mice; Table 1). In fact, the experiments even showed downregulation of calcineurin in the early phase of exercise training (~1000 μg^{-1} after 3-14 days of swimming). In contrast, calcineurin activity was upregulated in pathologic LVH (~8000 μg^{-1} after 8 weeks of aortic constriction vs ~2800 μg^{-1} in sham). For the exercise effect, physiologic LVH was stimulated by daily exercise training either in the form of swimming in increments that increased by 10 minutes/day until day 9, after which mice then swam 2x90-minute sessions for 12 more days, making 20 days in total, or running on voluntary wheels for 5-7 km/day (or more specifically, /night), whereas pathologic LVH was initiated by aortic constriction.

No other studies have before or after assessed calcineurin responses in the heart simultaneous to development of physiologic hypertrophy, such that we are left with these 2 studies to compare and contrast. Likewise, no subsequent study has been published that would swing confidence one way or the other or otherwise enable us to make a decisive informed decision to trust one over the other. Furthermore, both come from renowned research groups with a long established history in the techniques and models; both studies are all things considered expertly executed and fairly represented in the publications, and the publications themselves appear in *Circulation* and *Circulation Research*, both globally leading journals in the field with the highest bars and strictest criteria for accepting and publishing manuscripts, and incidentally, from the same organization (*American Heart Association*, also a globally leading authority in all matters of the heart). Hence, asserting credence to one over the other is near-impossible, and so we as readers are caught in a limbo in the middle. What shall we believe?

Table 1. Summary table comparing Eto et al (63) and Wilkins et al (64). LVH: left ventricular hypertrophy, LAD: left anterior descending, NFAT: Nuclear Factor of Activated T Cells, RLU: relative light units, CsA: cyclosporin A.

AUTHOR	ETO ET AL (63)	WILKINS ET AL (64)
YEAR	2000	2004
RESEARCH QUESTION	Is calcineurin activated in physiologic or pathologic cardiac hypertrophy?	Is calcineurin involved in physiologic or pathologic cardiac hypertrophy?
ANIMAL MODEL	Wistar Rats	Transgenic Mice
STUDY TYPE	Experimental Animal Study	Experimental Animal Study
INTERVENTIONS	Exercise training (running) Sedentary regime Pressure overload (1- and 4-week aortic constriction) Surgical placebo (sham)	Exercise training (running and swimming) Pressure overload (8-weeks aortic constriction) Myocardial infarction (left anterior descending artery ligation) Surgical placebo (sham)
OUTCOME MEASURES	Relative phosphatase enzyme assay Echocardiogram Hemodynamic measurements Heart/body weight ratio Hematoxylin and eosin-stained myocardial sections	Relative NFAT-luciferase reporter activity (NFAT-luciferase transgenic mice and luciferase assays) Heart/body weight ratio Masson's trichome stained myocardium Protein and mRNA expression
KEY RESULTS	Calcineurin upregulated in physiologic LVH (46.4±8.3 pmol/min/mg in exercise vs 18.4±0.5 pmol/min/mg in control)	No significant calcineurin increase in physiologic cardiac hypertrophy (1700-1800 RLU/μg in exercise vs ~2000 RLU/μg in control; ~1000 RLU/μg in early phase of exercise)
STUDY STRENGTHS	Large sample size Low variability Confirmed findings with CsA as negative control Utilized imaging and hemodynamic measurement to confirm physiologic hypertrophic and functional changes in myocardium Published in a world-leading journal	Large sample size Low variability Assessment of calcineurin activation at different stages of physiologic cardiac hypertrophy In-vivo and in-vitro reporter activity may offer improved accuracy of calcineurin quantification Multiple exercise protocols Published in a world-leading journal
STUDY WEAKNESSES	Exercise protocol was less standardized Reduced quality echocardiogram images Relied on single enzyme assay	Lack of hemodynamic monitoring In vivo reporting may be confounded by other activators of NFAT

SO DOES CALCINEURIN PARTICIPATE IN PHYSIOLOGIC CARDIAC HYPERTROPHY?

Eto et al's (63) "yes" to the central question here regarding calcineurin involvement in physiologic LVH contradicts the majority of current literature (60,65-69), as well as Wilkins et al (64), although it is only Eto et al (63) and Wilkins et al (64) of those studies that have designed and dedicated their studies to this question. The central conclusion

from these studies is that calcineurin has not been found to participate in exercise adaptation in a regulatory manner that would stimulate physiologic cardiac hypertrophy. In fact, it was even reported from a microarray analysis with subsequent mRNA and protein expression confirmation in left ventricular myocardial samples after exercise training-induced cardiac hypertrophy that the gene calcineurin-inhibitor (Cain) increased significantly with exercise training (15). If the latter is true, calcineurin activity would most likely have been suppressed after exercise training, which was also demonstrated biochemically after loaded and unloaded free-wheel running in mice (70).

However, several other lines of inquiry provide at least circumstantial support to Eto et al (63), suggesting calcineurin may have a role to play in forming the exercise training-induced physiologically hypertrophied heart:

1) Mice overexpressing myocyte-enriched calcineurin-interacting protein-1 (MCIP1), which rendered calcineurin ineffective, had a decreased hypertrophic response to exercise (71). Further studies supported this notion by showing calcineurin in the absence of MCIP1 induced LVH, but prevented pressure-overload hypertrophy, indicating possible dual roles in physiologic and pathologic hypertrophies (72,73). Yet further support comes from the finding that calcineurin-deficient mice present with 12% reduced heart sizes (74). These studies in and of themselves lend support to Eto et al (63), especially given there may be a required calcineurin threshold to induce hypertrophy (10). Conversely, it could be argued calcineurin could have a supplementary role in physiologic LVH, including the possibility that calcineurin also could influence or promote LVH through a transcriptional regulatory partner such as AP-1 or GATA4 (62); hence the contradictory results may also depend on how calcineurin is quantified.

2) In skeletal muscle, calcineurin induces slow twitch muscle hypertrophy, which is the corollary to physiologic cardiac hypertrophy (75).

3) In humans, polymorphisms in the calcineurin genes may be among the (admittedly numerous) potential genetic variant candidates that could explain inherited phenotype variations as well as differences in trainability between individuals (76). Whether the described cardiac phenotype effect transcends to include hypertrophy remains unknown, but it keeps the hypothesis of calcineurin participation alive.

4) In a different scheme, cardiac hypertrophy activated by PPAR-β stimulation, a corollary to physiologic hypertrophy, was mediated via calcineurin activation and included downstream modulation of NFAT (31). In this study, calcineurin activation and downstream NFAT translocation *per se* was not measured after exercise training, but the hypertrophied heart after PPAR-β stimulation and calcineurin-NFAT activation was at a gross, histologic and cellular level indistinguishable from the hypertrophied heart resulting from 5 weeks of exercise training.

METHODOLOGIES OF THE CALCINEURIN CONTROVERSY

The studies employed different primary methods. Eto et al (63) measured calcineurin activation using a phosphatase enzyme assay, whereby free inorganic phosphate is isolated by cation-exchange chromatography and quantified by liquid scintillation counting. This technique quantifies phosphate release, which in this case occurs from a calcineurin-specific substrate (^{32}P-labelled cAMP-dependent protein kinase regulatory subunit type-II phosphopeptide) controlled by measuring before and after addition of a calcineurin autoinhibitory peptide. This is therefore *per se* not a specific measure of calcineurin activity, but the comparison of phosphate release in a state with calcineurin inhibition versus a state without calcineurin inhibition generates a proxy index of phosphatase activity specific to calcineurin (77). Although experimental and biologic conditions can lead to some confounding, though these are controlled as much as possible in the experiments as a matter of routine standardization, the technique has become accepted as a reliable marker of calcineurin activity after validation against western gel electrophoresis, enzyme-linked immunosorbent assay (ELISA) immunoassays of protein or peptide expressions, or polymerase chain reaction (PCR)-based measures of transcript expressions (77), with very low variability between these methods. However, calcineurin is sensitive to oxidation during lysis by sonication, which has not been extensively validated, and moreover, calcineurin activation also depends on the levels of calcium and calmodulin. Therefore, there are reasons that could render the enzymatic phosphatase assay inaccurate.

In comparison, Wilkins et al (64) used NFAT-luciferase transgenic mice to assess calcineurin. These were generated by inserting a transgene containing high-affinity NFAT binding sites upstream of a luciferase reporter, which was subsequently validated by showing a high level of expression in the heart. A commercial luciferase assay with luminometry (bioluminescence) then measured NFAT-dependent luciferase reporter activity, which thereby indicates the amount of NFAT binding and therefore generates a proxy index of activated calcineurin. However, other undiscovered signalling mechanisms could potentially also regulate NFAT and therefore confound the signal, while peptide molecules that inhibit NFAT activation without affecting calcineurin phosphatase levels could distort the results (52,78). Therefore, using a surrogate measure like NFAT-luciferase establishes a correlation but cannot be considered a direct cause-effect measure of calcineurin regulation.

Both Eto et al (63) and Wilkins et al (64) subsequently attempted to verify their primary results. Wilkins et al (64) verified the efficacy of the NFAT-luciferase reporter by crossing NFAT-luciferase transgenic mice with calcineurin-activated transgenic mice, which reported a 6-10-fold increase in NFAT activity. In contrast, Eto et al (63) did not verify the accuracy of the phosphatase enzyme assay - a technique Wilkins et al (64) is somewhat critical of – but Eto et al (63) did validate their findings by additional experiments in the presence of the calcineurin antagonist CsA (79) that showed reduced physiologic

hypertrophy in exercising rats but only partially reduced hypertrophy in pressure-overload hypertrophy, which implies calcineurin may have played a role in modulating the physiologic cardiac hypertrophy. While factors remain unclear regarding CsA and cardiac hypertrophy, others (80,81) as well as Wilkins et al (64) have also found CsA fails to prevent hypertrophy, suggesting it might only influence calcineurin and not hypertrophy.

Based on the issues discussed in the preceding paragraphs, it is therefore probably fair to state that both sets of methodologies have inherent sources of potential error and confounding including different levels of variability, but both the phosphatase and luciferase detection assays represent accepted gold-standard technologies. They have been developed, validated and are supplied with appropriate protocols by the respective manufacturers, and they are characterized by high sensitivity and accuracy. It should also be noted that the reported variability of the specific measurements in the results of both studies was remarkably low, adding credence to their conclusions. Although neither method was ideal, another study (82), using a third method of immunoprecipitation with specific protein-binding antibodies, recorded similar findings to Wilkins et al (64) where they converged. However, both phosphatase and luciferase assays could be more suitable than immunoprecipitation assays, which may be adversely affected by potential isoform phenotype shifts that may occur during the development of physiologic hypertrophy. Additionally, a number of other studies have used and therefore validated NFAT-Luciferase reporter activity to measure calcineurin levels (29,83-85). These therefore in hindsight indicate Wilkins et al (64)'s method may be more accurate, but nonetheless, it is still on the basis of this difficult to discard the results from Eto et al (63), especially given the robust magnitude of change, low variability of effect and validation with CsA.

What about the experimental designs? They are broadly similar - exercise and aortic or coronary constriction was used to induce physiologic and pathologic LVH (Table 1). These are all protocols widely used for evoking profound cardiac stress (17,21,27,81). However, running training has been shown to provide a greater stimulus for modulating the cardiac proteome than swimming (69). Although this difference may not explain the discrepancy in their results, especially since both exercise protocols evoked substantial hypertrophy responses, it may contribute to diverging molecular signals.

Furthermore, rats and mice are good, well-established animal models of cardiac function, and both have over a long time been used for experimental studies that include surgical and other interventional procedures, whereas mice remain the model of choice for genetic manipulation. Importantly, almost all human genes are linked to murine orthologs. Both species, but perhaps especially mice, breed quickly, further making them a popular choice. However, their cardiac contractility differs more drastically from humans than other small rodents. In contrast, rats make very successful models of pressure-overload cardiac hypertrophy (86).

CONCLUDING DISCUSSION ON CALCINEURIN CONTROVERSY

The role of calcineurin-dependent transcription remains unclear in physiologic cardiac hypertrophy following exercise training. Eto et al (63)'s findings are contradictory to Wilkins et al (64), whereas other available studies in this and related fields do not provide further clarity. Several confounding factors have been identified that may explain the controversy. Though it is feasible that calcineurin could play a role in the development of physiologic cardiac hypertrophy, there are however also a number of other probable mechanisms that altogether have received more scientific attention and now present with a broader evidence base. Any potential calcineurin activity is therefore not sole and most likely supplementary to other regulators, and in any case appears to have a greater role in pathologic remodeling and hypertrophy.

These controversies within the literature highlight the importance of further research within this area. The senior author, J. D. Molkentin, of the Wilkins et al 2004 paper (64) wrote an insightful follow-up 9 years after the initial study, which reviewed more recent advances within the area and assessed how the results of the initial study compared to new development (51). The follow-up suggested that the current body of literature and more recent studies agreed that calcineurin remained inactive in physiologic cardiac hypertrophy, although it acknowledged that calcineurin had some physiologic importance as it was vital in promoting myocyte proliferation and survival. We however point out the obvious, this statement comes from one of the proponents of the initial controversy and though it may well be correct, does not offer an independent perspective.

In our review, we have sought to provide a balanced viewpoint to the question of whether or not calcineurin activation in the heart may contribute to development of physiologic cardiac hypertrophy following exercise training; the central question motivating the Eto and Wilkins studies (63,64). The strongest evidence perhaps suggests that no definitive regulatory role has yet been convincingly identified, but the positive findings reported by Eto et al (63) have also at least circumstantially been supported by several lines of studies, so a lack of a participating role cannot be ruled out. Admittedly, in our review, we may have inadvertently sought to find supporting evidence for a role rather than the opposite, but this is perhaps natural given this would challenge rather than confirm the current dogma, and for this we apologize to the reader.

COMPARISON TO OTHER CONTROVERSIES

The calcineurin controversy is not unique; in fact, several controversies showcase areas with "live" scientific theoretical battlegrounds that also keep moving science forward by way of debate. However, we suggest that the calcineurin controversy suits teaching purposes given its simplicity with two diametrically opposing conclusions on a basic research question and hypothesis. For a wider context and discussion though, we suggest

further examples of scientific controversies that may be explored to strengthen the desired educational aim:

The cholesterol hypothesis and statins for primary prevention of cardiovascular mortality: statin therapy is prescribed for protection against cardiovascular disease because they are thought to lower low-density lipoprotein-cholesterol (87). However, the rationale for this has come under scrutiny. For instance, clinical trials have been criticized for methodological flaws that potentially render the reported results invalid (88), clinical data are not being released for independent verification (87), guidance from governing and advisory health authorities may be questioned due to ties to the pharmaceutical industry (89,90), and finally, the basis of the cholesterol hypothesis in itself has been questioned (91). Thus, while there is more agreement on statins for secondary prevention, the debate surrounding primary prevention remains divisive, and would be worthy of debate. As a controversy, it differs from the calcineurin story in that it is more complex, has a rather more clinical than physiologic foundation, and thus requires a wider background for full appreciation, but may serve wider purposes in exploring scientific themes.

Asymmetrical dimethylarginine (ADMA): ADMA endogenously inhibits nitric oxide (NO) synthase such that increased circulating ADMA impairs endothelial function and thereby may increase risk of cardiovascular disease (92). However, a paradox was reported in 2017 when free ADMA was shown to have a weak potency for inhibiting endothelial NO synthase (93). This represents a potential controversy that is physiologic in nature, but so far may have less applicability in this context since the weight of evidence is far in favor of one side, i.e. ADMA as an inhibitor of NO synthase (e.g. 94), whereas the calcineurin controversy remains more balanced.

HOW CAN THIS SUPPORT PHYSIOLOGY TEACHING?

On calcineurin, the research so far is inconclusive and we cannot yet with confidence know its role in physiologic cardiac hypertrophy, but this provides an opportunity for physiology education, especially for skills associated with advanced higher-level cognitive processing. Following teaching the relevant physiology (broadly cardiovascular and exercise physiology) in advanced (in our experience this would be senior/final year) undergraduate or postgraduate cohorts, we also suggest follow-up topics:

- History of science and research, including generation and development of the scientific method and scientific techniques, e.g. in this case bioluminescence, originally found in fireflies and now used in numerous research applications, as well as other physiologic, biochemical, and life science/biologic methods.

- Measurement issues, including exploration of suitability, differences, benefits and limitations of each, and specific aspects of accuracy, precision, sensitivity, reproducibility, and validity.
- Research models, including the use of experimental animals, associated ethics and the 3R (replacement, reduction, refinement) framework for performing more humane animal research, specific concerns about applying exercise and stress stimuli to animals, and concepts of transgenic and gene-modified animals.
- Research design, including appropriate and fit-for-purpose research protocols, the theorem of hypothesis testing and falsification, statistics, inference, and extrapolation to humans.
- Clinical utility and drug discovery, including exploration of potential targets for therapy originating from basic research.
- Practical aspects of performing, disseminating, and publishing research, including confirmation bias, positive versus negative results, and ethics and practice of publishing in scientific journals.
- Research existing in a state of flux, where discussions like the current are necessary to move science forward. This could include a broad range of aspects including exploration of other controversies, but also personification such as asking "what must the researchers have felt when the results from the second study (64) contradicted the first study (63)?"

From the calcineurin controversy itself and its associated topics, we have experience with introducing several activities to the classroom, which may be delivered in either teacher- or student-led modes; active use of Table 1 for tasking and researching these activities as well as break-out group-work is encouraged:

- Pro/con-style debates: Students either individually or in groups are tasked with presenting and defending their side on the argument, in this case either of Eto et al (63) or Wilkins et al (64) articles. Students should be given time to appropriately research the article and associated topics before the debate to prepare their arguments. Within a group, students could take responsibility for specific focused aspects, such as the respective methodologies, validity and reliability of specific measurements, results, interpretations, and robustness of conclusions. This is the activity we have the most experience and enjoyed the most success with, as it is easy to organize, immediately intuitive to the students, and often creates lively discussions, with the engaged students showing deep thinking and in-depth research on various aspects of the 2 articles in question.
- Discussion: Critical appraisal of strengths and weaknesses of the presented research, including discussions of how to interpret results and evidence, and planning for future research to resolve or generate reconciliation on the controversy. In our experience, this is often best achieved by open-ended journal-club-style discussion without pro/con debates, but may also be incorporated into other modes of teaching delivery by short, focused discussion segments.

- Voting: Following debate or discussion, students vote on which side of argument they believe in. This may be extended to Oxford-style voting, where the audience votes before and after the debate, and the side that gains the most votes wins. We have tried voting with mixed success; for this to be successful, we have found it relies on passionate advocates on both sides of the argument, which are not always present. However, an interesting discussion ensued after voting, when the audience was asked which parts of the arguments swayed their vote, with a variety of expected and unexpected replies.
- Grant-writing: A grant-writing exercise or assessment may be introduced, where the task is to design a research proposal to reconcile the controversy. Teachers, students outwith this class, or the class itself, especially if taking turns between different topics, may serve as a mock grant-reviewing panel. In our experience, this requires students be adept at research design and is more suitable for postgraduate students.

IMPLEMENTATION TO TEACHING

We suggest the described approach is implemented as a class activity following delivery of the background exercise and cardiovascular physiology. A workplan includes:

- Formulate aims and intended learning objectives (ILOs): critical appraisal and compare and contrast of published research studies; develop graduate attributes.
- Introduce concepts of physiologic cardiac hypertrophy, molecular signaling mechanisms, and calcineurin, all provided in this article.
- Briefly present the contradictory Eto et al (63) and Wilkins et al (64) articles.
- Students research the articles as group work, with support on critical appraisal for research articles available including checklists for points to cover (95,96):
    - Identify hypothesis/aim.
    - Evaluate methodologies (examples include design, ethics, sample size, models, protocols, outcome measures, analysis and statistics).
    - Evaluate results and appropriateness of conclusions, crosscheck to hypothesis/aim.
    - Consider strengths and weaknesses.
- Students present findings.
- Class discussion/debate, possibly extended to voting and post-discussion activities such as mock grant-writing.
- Formal or informal evaluation of activity: did students achieve ILOs, did activity engage/attract interest/lead to discussion?

CONCLUSION

In our experience, the approach described in the current paper leads to learning of important skills and development of critical and reflective attributes in both senior undergraduate and postgraduate student cohorts, such as ability to compare and

contrast, analyze and critically appraise, and evaluate opposing views. This was achieved by breaking up classes and seminars with interactive, community-centered activities that resulted in a high degree of engagement with lively and entertaining discussions that we believe both students and educators enjoyed and benefitted from. If nothing else, it provided variety to the classroom, which may also suit both learners and teachers (97). Thus, in the end something good comes out of the stalemate.

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FIGURE LEGENDS

Figure 1: A model of established molecular pathways relevant in physiologic cardiac hypertrophy, activated in response to several distinct extra- and intracellular signals, leading to physiologic cellular hypertrophy after modulation via gene transcription and/or protein synthesis and maintenance. MAPK(K(K)): mitogen activated protein kinase (kinase (kinase)); CaMK: Ca2+-calmodulin dependent protein kinase; HDAC: histone deacetylace; IGF1: insulin like growth factor-1; PI3K: phosphoinositide-3 kinase; Akt: protein kinase B; mTOR: mammalian target of rapamycin; mRNA: messenger ribonucleic acid; HSP: heat shock protein. Numbers link to text description.

Figure 2: A model of the established calcineurin pathway for regulating gene transcription, and as it may be potentially relevant in physiologic cardiac hypertrophy, activated by calcium-bound calmodulin. Downstream, calcineurin dephosphorylates the nuclear factor of activated T cells (NFAT), which allows for NFAT entry to nucleus where it functions as a transcription factor. Calcineurin may also be inhibited, most notably by cyclosporin A (CsA). ER: endoplasmic reticulum; IP3R: inositol triphosphate receptor; LTCC: L-type calcium channel; RyR: ryanodine receptor; SAC: stretch-activated (calcium) channel; SR: sarcoplasmic reticulum.