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Forum Minireview

New Molecular Mechanisms for Cardiovascular Disease: Cardiac Hypertrophy and Cell-Volume Regulation

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Abstract. Cardiac hypertrophy is an increase in the muscle volume of the ventricle due to the enlargement of cardiac cells. Physiological cardiac hypertrophy is the normal response to healthy exercise, and pathological hypertrophy is the response to increased stress such as hypertension. Intracellular and extracellular aniosmotic conditions also change cell volume. Since persistent cell swelling or cell shrinkage during aniosmotic conditions results in cell death, the ability to regulate cell volume is important for the maintenance of cellular homeostasis. Cell swelling activates a regulatory volume decrease (RVD) response in which solute leakage pathways are stimulated and solute with water exits cells, reducing the cell volume towards the original value. In cardiac cells, one of the essential factors for cell-volume regulation is the volume-regulated anion channel (VRAC). However, the relationship between cardiac hypertrophy and cell-volume regulation is not clear. In this review, we introduce our recent findings showing that the impairment of VRAC current is exhibited in ventricular cells from mice with cardiac hypertrophy induced by transverse aortic constriction. Similar results were shown in caveolin-3–deficient mice, which develop cardiac hypertrophy without pressure overload. These results suggest that VRAC will be a new target for protection from the development of cardiac hypertrophy.

Keywords: hypertrophy, volume regulation, cardiac cell, anion channel, Cl^- current, cardiovascular disease

1. Introduction

Cardiac hypertrophy is defined as an increase in cardiac mass. The primary cellular basis of cardiac hypertrophy is an enlargement of the cardiac myocyte, which is clearly distinguished from hyperplasia, in which the cells remain the same size but increase in number (1). The hypertrophy is classified as "physiological" hypertrophy when it occurs in healthy individuals following exercise and is not associated with cardiac damage, or "pathological" hypertrophy (2). Physiological hypertrophy is largely mediated by signaling through peptide growth factors, IGF-1 and growth hormone; the former acts in a phosphoinositide 3-kinase (PI3K)-dependent manner and the latter acts predominantly via increased production of IGF-1 (3). In the case of pathological hypertrophy, although the increased heart size is initially a compensatory process to stress such as hypertension, sustained hypertrophy can induce attenuation of left ventricular function and ultimately lead to heart failure via many molecules being activated during biomechanical stress, such as Gq protein, mitogen-activated protein (MAP) kinases and protein kinase C (PKC), Ca^{2+}/calmodulin (CaM), and the nuclear factor of activated T cells (NFAT) (3). Thus, it is critical to define and distinguish the pathways for adaptive regulation to hypertrophy.

At the level of a single cell, image analysis showed that the enlargement of cardiac myocytes occurs in ischemia, septic shock, dilated cardiomyopathy, and failure (4). For the maintenance of cell volume, the balance between intracellular and extracellular osmolarity in such pathological states is extremely important. The osmolarity of body fluid is tightly controlled under normal conditions, and osmotic changes can occur in several pathological conditions. Mammalian cells, including

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cardiac myocytes, can adjust their volumes when challenged by a number of different stimuli (4). This cell-volume regulation is a basic property of all mammalian cells and is of particular importance in cardiac cells in heart, where it is directly linked to cell death (5). The volume-regulated outwardly rectifying anion channel (VRAC) is found in almost all mammalian cells and is implicated in cell-volume regulation (5). Recent work has provided fascinating evidence that cell-volume regulation and VRAC may play an important role in cardiac hypertrophy. Thus, this review focuses on cell-volume regulation and VRAC in normal and hypertrophied cardiac myocytes.

2. The mechanism of cell-volume regulation

Since persistent cell swelling or cell shrinkage during aniosmotic conditions results in cell death, the ability to regulate cell volume is important for the maintenance of cellular homeostasis. The high water permeability of plasma membranes of several cell types is due to the presence of aquaporin water channels (6). Water flux through the aquaporins is driven by a shift in extracellular or intracellular osmolarity, and most cell types are able to maintain their volume following the osmotic gradient (Fig. 1A). Swollen cells release KCl and water, thereby reducing the cell volume towards the original value, via the process of regulatory volume decrease (RVD) (Fig. 1B). In contrast, shrunken cells generally gain NaCl and cell water, thereby increasing cell volume towards the original value, via the process of regulatory volume increase (RVI). Accumulated Na^+ is then exchanged for extracellular K^+ by the Na^+-K^+ pump. Several transporter pathways have been reported to be responsible for the RVI-associated NaCl gain and the RVD-associated KCl loss. Na^+-K^+-$2Cl^-$ cotransporter, Na^+/H^+, Cl^-/HCO_3^-, and Cl^-/OH^- exchangers have a supporting role in RVI. RVD occurs through parallel activation of K^+ and Cl^- channels and activation of electroneutral cotransporters such as K^+-Cl^- cotransporter and K^+/H^+ and Cl^-/HCO_3^- exchanger. In cardiac cells, the action of K^+ channels and VRAC is the principal mechanism for RVD (5, 7).

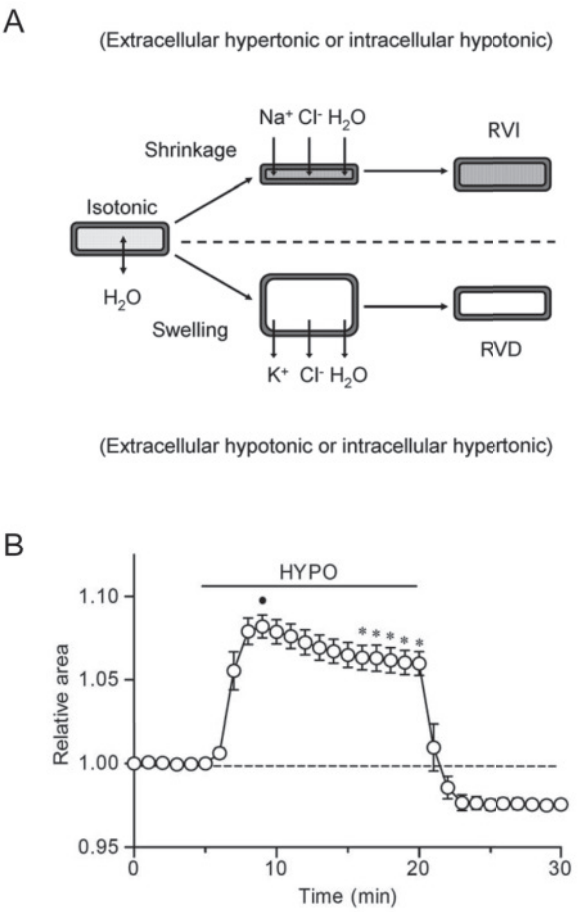

Fig. 1. Cell-volume regulation in the heart. A: Cell shrinkage is triggered by extracellular hypertonicity or intracellular hypotonicity. Subsequently, regulatory volume increase (RVI) occurs through uptake of NaCl and osmotically obliged water. Cell swelling induced by extracellular hypotonicity or intracellular hypertonicity is countered by regulatory volume decrease (RVD), which involves the cellular loss of Cl^- via the activation of VRAC. Modified from Ref. 5. B: A typical time course of RVD in guinea-pig ventricular cells. The cells were initially bathed in isotonic modified Tyrodes solution, and then hypotonic (70% of control) solution (HYPO) was applied during the period indicated by the bar. The values are relative to those obtained before application of HYPO. Each point represents a mean ± S.E.M. (n = 7). *Significantly smaller than the cell area at the maximum cell swelling (denoted by the dot) with $P < 0.05$. Data from a previous report (Ref. 7).

3. Characteristics of VRAC current

Cardiac VRAC is one of the most functionally important membrane anion channels for cell-volume regulation (8 – 11). This is because VRAC is a channel protein of volume-regulated outwardly rectifying Cl^- current, which is sometimes referred to as VRAC current. The VRAC current is now known to be ubiquitously expressed in mammalian cells (9). An increase in cell volume activates VRAC, which contributes to the regulation of cell volume. This regulation is an essential cellular function coupled to a variety of physiological processes, such as cell proliferation, differentiation, migration, and apoptosis, in most animal cell types (12).

In general electrophysiological experiments, VRAC current is induced by increased cell volume. The macroscopic current is outwardly rectifying with both a physiologic and a symmetric Cl^- gradient and shows time-independent activation over the physiologic voltage range, but is partially inactivated at strongly positive potentials

(5). The Cl^- equilibrium potential in cardiac cells is between −65 and −35 mV under normal physiological conditions (9). Thus, membrane Cl^- channels have the unique ability, compared with cation channels, to contribute both inward as well as outward current during cardiac action potential (9, 13, 14). Consequently, activation of VRAC current should decrease the action potential duration (APD) and thereby the effective refractory period (ERP), and should depolarize membrane potential (15); it may also contribute to the development of stretch-induced membrane depolarizations and arrhythmias (9, 14).

4. Regulation of VRAC current

It is still unknown whether VRAC is regulated by cell swelling or membrane stretch (8). Activation of VRAC current is delayed 30 – 90 s after swelling, and it is inferred that signaling cascades participate in its activation (16 – 18). In isotonic conditions, VRAC can be activated by reducing intracellular ionic strength, by intracellular GTPγS, or by application of shear stress in endothelial cells (10), but studies have yet to be undertaken in cardiac cells. In most cells, activation of VRAC current does not appear to require phosphorylation, since channels can be activated in the absence of cytoplasmic Ca^{2+} and Mg^{2+} and in the presence of nonhydrolyzable analogs of ATP (11). However, the role of PKA phosphorylation of VRAC in heart is still controversial (9). Although phosphorylation by protein kinases does not appear to play a direct role in channel activation, they may modulate channel activity by direct phosphorylation of the channel or some accessory protein that regulates channel activity (9). Another potentially important regulatory mechanism of VRAC current is phosphorylation by tyrosine protein kinase. It has been reported that VRAC current in canine atrial cells may be regulated by tyrosine protein kinase (19). However, it is still unknown whether or not the substrate for tyrosine phosphorylation is the channel itself or another regulatory protein. Other reported signals are involved in PKC, Src, focal adhesion kinase, and the stretch activated angiotensin II type 1 receptor signaling cascade, which involves trans-activation of epidermal growth factor receptor kinase, PI3K, NADPH oxidase, superoxide anion, and ultimately H_2O_2 (20).

Recently, we found that intracellular dialysis of anti-phosphatidylinositol 3,4,5-trisphosphate (PIP3) antibody (21) strongly inhibited the activation of VRAC current. Phosphoinositides (PIs), including PIP3 and phosphatidylinositol 4,5-bisphosphate (PIP2), serve as important second messengers in many intracellular signaling cascades. It has been shown that the phosphoinositides directly and/or indirectly bind several types of membrane transporters and channels (22). Interestingly, cell swelling is accompanied by activation of PI3K, which is an enzyme that converts PIP2 to PIP3 (23). The attenuation of VRAC current by the inhibition of PI3K has also been shown in various types of cells (5). Inhibition of VRAC was shown in cells with intracellular application of LY294002 (a PI3K inhibitor) or anti-PIP2 antibody. PIP3, but not PIP2, restored the VRAC current suppressed by LY294002 or anti-PIP2 antibody, whereas intracellular PIP3 or PIP2 influenced neither the basal background current in isotonic solution nor the VRAC current in hypotonic solution (24). According to these results, we hypothesize that two distinct steps are required for VRAC activation. In the first step, PIP3 (or related compounds) can change from membrane stretch–insensitive to –sensitive VRAC. In the next step, the VRAC in the latter can be opened in hypotonic solutions. Alternatively, the mechanical stretch might cause a change from the closed to pre-opened VRAC, and then PIP3 (or related compounds) might open the channel. Furthermore, the question arises as to how PIP3 can be involved in such downstream reactions. In this regard, it is noteworthy that most of these signaling proteins such as GDP–GTP exchange factors for Rac or serum- and glucocorticoid-regulated protein kinase (SGK) structurally include pleckstrin-homology or phox-homology domains and that PIP3 binds to these domains to transduce intracellular signals (25). It is possible that similar PIP3-dependent processes are involved in the activation of VRAC current in mouse cardiac cells.

5. Molecular identity of VRAC

Despite its well-characterized properties, the molecular identity of VRAC has yet to be determined. Several candidate proteins have been identified including P-glycoprotein, volume-sensitive chloride conductance regulatory protein, phospholemman, ClC-2, and ClC-3 (8). However, for each of these, several negative findings have been reported (9, 10). Cardiac ClC-2 may be responsible for the cell swelling– or extracellular acidosis–activated inwardly rectifying Cl^- current (26 – 28). A more recent study showed that activation of VRAC is reduced in the colonic epithelium and in salivary acinar cells from mice lacking expression of TMEM16A, a molecular candidate for the Ca^{2+}-activated Cl^- current (29), whereas another group showed preliminary data that TMEM16 is not VRAC (30).

6. VRAC in cardiac hypertrophy

The progression to cardiac hypertrophy causes remodeling of the cytoskeleton and extracellular matrix and

activates multiple neurohormonal and intracellular signaling cascades (e.g., tyrosine kinases, PKA, PKC, protein phosphatases, MAP kinases, and endothelin), almost all of which modulate VRAC (15), as described above. The first recording of chloride current in hypertrophic ventricular cells was carried out in rat left ventricular cells with surgical constriction of the abdominal aorta (31). Time-independent Cl^- current was found in the hypertrophied cells. Baumgarten and co-workers identified that this is Cl^- current passing through VRAC. They found that the sarcolemmal surface area (an index of cell volume) is larger, as evidenced by an approximately 20% increase in membrane capacitance (another index of cell volume) in ventricular myocytes from a pacing-induced congestive heart failure (CHF) canine model and showed that the basally activated Cl^- current in the CHF cells exhibits similar properties to VRAC (32). Moreover, they showed preliminary data that a tamoxifen-sensitive VRAC was also persistently activated in human atrial myocytes obtained from patients with right atrial enlargement and/or elevated left ventricular end-diastolic pressure (33). Thus, this group concluded that hypertrophied cells behave like swollen cells and that VRAC may become a target for pharmacologic therapy. A similar result was shown in cultured neonatal rat ventricular hypertrophic myocytes induced by cyclic mechanical stretch (34). In contrast, in rabbit hypertrophied ventricular myocytes produced by combined volume and pressure overload, the current density of VRAC was reduced by approximately 50% (35). The difference among these groups might be due to the type of stress, period of continued stress to the heart, development of pathological hypertrophy, or degree of heart failure. Thus, we examined the current amplitude of VRAC in single hypertrophic myocytes freshly isolated from left ventricle of mice with transverse aortic constriction (TAC) for 4 and 8 weeks (36).

TAC-treated mice developed cardiac hypertrophy within 4 weeks. As shown in Fig. 2A, 4 weeks of TAC treatment induced an increase in cell capacitance, an index of cell volume. In parallel, the heart-to-body weight ratio of 4-week TAC mice was significantly larger than that of sham-operated mice (7.67 ± 0.862 mg/g ($n = 16$) and 5.50 ± 0.262 mg/g ($n = 9$), respectively; $P < 0.05$). Next, we compared the change in the cell volume during hypotonic challenge between the cells from 4-week TAC mice and those from sham-operated mice. The relative cell area on captured video images was used as an index of cell volume (37). In cells from sham-operated mice, RVD is shown upon switching the bathing solution from isotonic (310 mOsm) to hypotonic solution (200 mOsm), for which the cell area increased as expected. However, the cells from 4-week TAC mice also inflated in response to the hypotonic solution, but there was little RVD in these cells (Fig. 2B). Re-application of isotonic solution decreased the cell area in both types of swollen cells. In

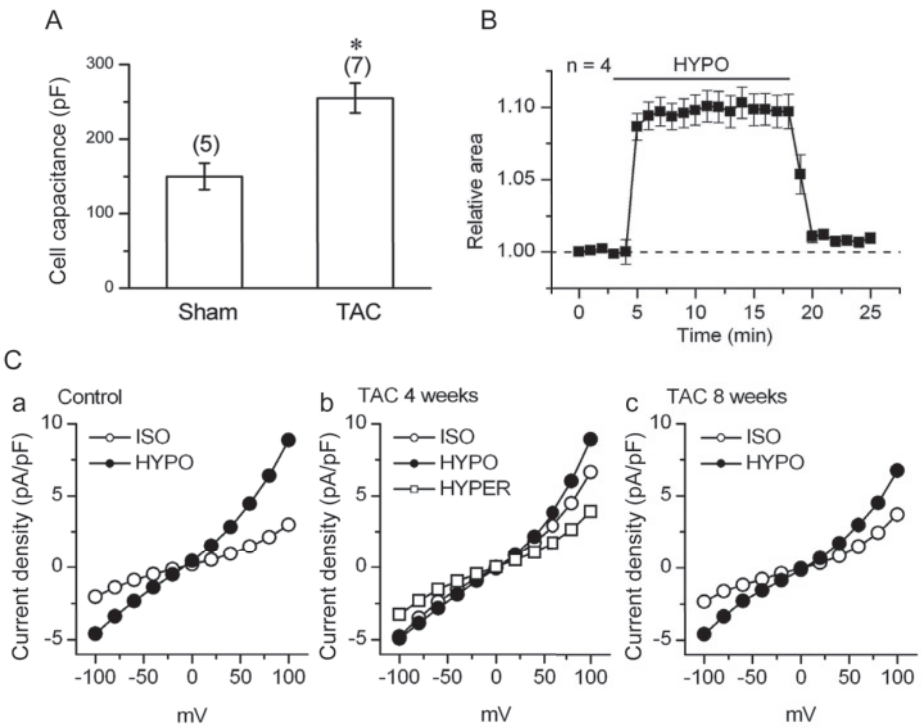

Fig. 2. Volume regulation and VRAC in cells from TAC-induced hypertrophic mice. A: Mean data showing the cell capacitance (pF) of ventricular myocyte from sham-operated mice and mice 4 weeks after TAC treatment. *Significantly different with $P < 0.05$ according to an unpaired *t*-test. Numbers in parentheses indicate numbers of cells examined. B: Time course of change in the relative cell area observed in the myocytes of 4-week TAC mice exposed to HYPO. The cells were initially bathed in isotonic solution, and then hypotonic (HYPO) solution was applied during the period indicated by the bar. C: Typical I-V recording of whole cell currents in myocytes from mice after 4-week sham-operation (a), 4-week TAC operation (b), and 8-week TAC operation (c). Currents were recorded in isotonic (ISO), hypotonic (HYPO), or hypertonic (HYPER) solutions. The current density is measured in pA/pF, that is, membrane current (pA) divided by cell capacitance (pF). All experiments were performed at 36.5 ± 0.5°C.

cells from sham-operated mice, the level of cell area attained with the reapplied isotonic solution was smaller than the initial level, showing excessive cell shrinkage. This undershoot of the relative cell area below the initial level is attributed to a loss of solutes from the intracellular medium during RVD. In contrast, there was no clear undershoot of cell area in the TAC-induced hypertrophic cells that showed little RVD (Fig. 2B). It has been reported that Cl^- efflux through VRAC was implicated in RVD in ventricular myocytes (7). Therefore, we examined VRAC currents in ventricular myocytes from sham-operated and TAC-operated mice. VRAC currents were recorded in isotonic, hypotonic, or hypertonic solutions with 400-ms voltage-clamp steps to membrane potentials between −100 and +100 mV in +20-mV steps from a holding potential of −40 mV every 6 s. The hypotonic solution (210 mOsm) and the pipette solution were prepared as previously described (38). Appropriate amounts of mannitol were added to this solution to make isotonic (310 mOsm) or hypertonic (360 mOsm) solutions. Figure 2C shows the typical current–voltage (I-V) relationships of VRAC in sham-operated, 4-week TAC, and 8-week TAC mice. In 4-week TAC mice (Fig. 2Cb), we found basal current activation. The I-V relationships of the current showed the moderate outward rectification and the reversal potentials (E_{rev}) was almost 0 mV, the value of which was close to the predicted Cl^- equilibrium potential under symmetrical Cl^- condition (105 mM). DIDS, a popular anion-channel inhibitor, suppressed the basal current (data not shown). Accordingly, the basal activated current in ventricular cells of 4-week TAC mice may reflect persistent activation of VRAC current in the isotonic condition. The VRAC currents also developed in the cells derived from 4-week TAC mice, but their amplitude in these cells was much smaller than that of VRAC currents in cells from sham-operated mice (Fig. 2Ca). This property is similar to basal activation of VRAC current in cells from the canine CHF model (32). Interestingly, the basal activation of VRAC current in cells from 8-week TAC mice was smaller than that from 4-week TAC mice, and the hypotonic solution induced a VRAC current (difference current) that was also smaller than that from sham-operated mice (Fig. 2Cc). This decrease in hypotonic VRAC current without basal activation is observed in rabbit hypertrophied ventricular cells after treatment of volume and pressure overload (35) and spontaneous hypertrophic ventricular cells from caveolin-3–deficient mice (39). Several signaling molecules, including phosphatidylinositol lipids, are localized to caveolae in cardiac cells, and caveolin-3 is a muscle-specific protein integrated in the caveolae (40). Recently, we found that an impairment of PIP3 production is responsible for the attenuation of cardiac VRAC currents in caveolin-3–deficient mice (39). These results support our hypothesis that VRAC in an early adaptive stage of cardiac hypertrophy is basally activated by persistent mechanical stretch of the cell membrane and that attenuation of mechanical sensitivity of VRAC by long-tern mechanical stretch of the plasma membrane is a key for transformation to a non-adaptive stage.

7. Closing remarks

Recent studies using physiological and pharmacological techniques indicate that Cl^- channels contribute to several cardiac diseases including cardiac hypertrophy. It is well known that the coupling of mechanical stretch and electrical activity in the heart has been termed mechanoelectrical feedback and is believed to play a role in arrhythmias observed with pathological conditions including congestive heart failure and left ventricular hypertrophy. Furthermore, it has been suggested that VRAC modulates arrhythmogenesis, myocardial injury, ischemic preconditioning, and apoptosis. Given these findings, cardiac VRAC represents an important novel target for therapeutic approaches to cardiac diseases. Despite these developments, a lot of questions remain unanswered for the assessment of cell-volume regulation in healthy and diseased hearts. Transgenic mice are clearly invaluable experimental models for understanding the relationships between cardiac VRAC and hypertrophy. Hume and co-workers showed that at 3 weeks after knock-down of heart-specific ClC-3 using the tetO-Cre inducible approach, mice exhibited significant cardiac hypertrophy (4). Although this evidence strongly supports our hypothesis about the interaction of VRAC and hypertrophy, the truth does not seem to be so simple. ClC-3 may be an intracellular Cl^-/H^+ antiporter member of the ClC family (8). In addition, Nox1 (an integral membrane subunits of NADPH oxidase) and ClC-3 colocalize to early endosomes in vascular smooth muscle cells, and both are required for tumor necrosis factor (TNF-α)- and interleukin (IL-1β)-dependent ROS generation and for the subsequent activation of the transcription factor nuclear factor kappa B (NF-κB) (41). Further studies are necessary to clarify the molecular basis for VRAC as well as to completely elucidate the pathophysiological role of cardiac VRAC current during the progression of cardiac hypertrophy.

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