Chapter 5

Voltage-Independent Calcium Channels, Molecular Sources of Supraventricular Arrhythmia

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1. Introduction

Since its identification over one hundred years ago, atrial fibrillation has been shown to occur frequently in the general population and is now recognized as an important medical problem in developed societies. Three major hypotheses to explain cardiac rhythm disorders like atrial fibrillation have been proposed during this time and one of these three, impulse reentry has become predominate. The two other explanations, designated as focal source hypotheses, have been relegated to a secondary role in understanding arrhythmia. Despite widespread acceptance of the reentry hypothesis, however, current non-invasive anti-arrhythmic drugs based on this mechanism poorly prevent or reverse atrial fibrillation or other types of arrhythmia. One interpretation of this paradoxical clinical result is that mechanisms other than reentry initiate arrhythmias like atrial fibrillation in real-life settings. As a consequence, current non-invasive therapeutics may neither target nor effectively suppress important but unrecognized non-reentrant mechanisms that provoke clinical arrhythmia. We have found that challenging isolated non-automatic left atrial muscle, left ventricular papillary muscle, and perfused heart in sinus rhythm with an activator of the voltage-independent Orai calcium channels provokes high frequent tachycardia and fibrillation. Thus the Orais and related voltage-independent calcium channels may be unexpected sources of arrhythmia. This manuscript provides (a) a synopsis of the identification of atrial fibrillation as a clinical entity, (b) an overview of the development of the three current hypotheses for arrhythmia, and (c) our hypothesis that dysregulated voltage-independent calcium channels may be a fourth means to provoke electrical instability in heart muscle.

2. Historical perspective

Atrial fibrillation was first observed as compromised heart mechanical output. By the late 1800s clinicians including Nothnagel, and later MacKenzie and Hering, noted and analyzed abnormal or absent 'a waves' in venous pressure tracings but had not specifically correlated these abnormalities with atrial dysfunction [1,2,3]. Kymographic analyses of the pulse waves of their patients allowed these investigators to report examples of irregularly irregular pulse intervals and pulse heights, a surrogate for ventricular force generation. Towards the end of the nineteenth century physicians also came to realize that these mechanical disturbances often occurred persistently in some patients. Thus by the outset of the twentieth century these types of abnormal pressure wave recordings were grouped into the clinical conditions of *delirium cordis* or the more definitive *pulsus irregularis et inaequalis perpetuus* [4]. Vulpian, Krehl, and Hering were initial proponents of the notion that such irregularities resulted from the defective mechanical output of the atria [5,6,7]. The translational research of Cushny and Edmunds provided the first direct validation of this hypothesis when in 1907 they correlated chance observations of atrial *delirium* made in the dog laboratory with clinical recordings of *pulsus irregularis et inaequalis perpetuus* [8].

In the early 1900s clinical and experimental string galvanometer data formed the basis for the idea that disorganized atrial electrical activity caused both the loss of venous 'a waves' and the appearance of the fine pulsatile activity which define *pulsus irregularis et inaequalis perptuus*. Specifically, string galvanometer tracings published in 1906 by Einthoven [9] and in 1908 by Hering [10] demonstrated that mechanical *pulsus irregularis et inaequalis perpetuus* occurred in humans who lacked p-waves, had F-waves, and had irregularly timed but otherwise normal QRS complexes. These initial reports coupled with the extensive electrocardiographic analyses of Rothberger and Winterberg published in 1909 [11] provided the electrical equivalent of the venous wave data of Cushny and Edwards. That is, the electrocardiographic measurements Rothberger and Winterberg acquired from animals undergoing experimental atrial fibrillation were identical to recordings obtained from patients with *pulsus irregularis et inaequalis perpetuus*. This work together with the earlier report of MacWilliam [12] that faradic stimulation produced atrial fibrillation led to the acceptance of the view that the complete disruption of atrial electrical activity caused the irregular pulse waves that characterize *pulsus irregularis*. Thomas Lewis built on and expanded this work in his elegant electrocardiographic characterization of atrial flutter and fibrillation in humans and in animals [*e.g.*,13]. By 1920 it had been accepted that the organized electrical activity observed using string galvanometers or electrocardiographs sparked rhythmic heart contraction and conversely that disordered electrical activity caused abnormalities like *pulsus irregularis et inaequalis perpetuus*. Importantly, it was accepted that *pulsus irregularis* arose from disturbances that occurred specifically in the atria since hearts in atrial fibrillation often produced normal but irregularly timed QRS complexes.

During the evolution of this explanation for clinical *pulsus irregularis et inaequalis perpetuus* experimentalists developed the initial, non-vitalist explanation for atrial fibrillation and other cardiac rhythm disorders. Engelmann in 1896 [14] and Winterberg in 1906 [15] proposed

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an original hypothesis centered round the seemingly logical view that a solitary ectopic depolarization occurring spontaneously in a small number of heart cells confined to a specific region of the atria (*or ventricle*) could produce a 'premature' atrial (*or ventricular*) contraction. They reasoned that if such spontaneous electrical activity also could occur repeatedly and at a sufficiently rapid rate then such a 'focus' could likewise produce tachycardia or fibrillation. Variations of this 'focal' hypothesis included multiple 'heterotopic centers' depolarizing at rates sufficient to produce flutter or fibrillation or, as Rothberger proposed [16], a single heterotopic center which depolarized at extremely rapid rates. Several early experimentalists noted that the refractory period of heart muscle must shorten to accommodate rapid ectopic activity and that such abnormal electrical activity might occur at rates fast enough to preclude regular heart muscle contraction [17], foreshadowing the idea of fibrillatory conduction. While these focal source hypotheses were logical, a molecular mechanism through which non-automatic atrial (*or ventricular*) muscle might spontaneously or automatically depolarize was not known at that time. Thus non-focal, that is reentrant hypotheses to explain arrhythmia came to the fore and now dominate this field of inquiry. Nonetheless, the challenge to identify all molecular mechanisms that cause quiescent heart muscle to excite independently of normal sinus rhythm still remains at the center of arrhythmia research today just as it did over one hundred years ago. Thus we seek to *define mechanisms which provoke quiescent heart cells to depolarize (a) independently of normal sinus rhythm, (b) at sporadic or rapid rates, (c) in an organized manner or (d) in an apparently chaotic way, and (e) over inconstant periods of time including apparent perpetuity.* There are three such mechanisms currently known and our data suggest the existence of a fourth one.

3. Mechanism 1: Impulse reentry

Reentry occurs when electrical impulses conduct abnormally through the heart and re-excite quiescent heart muscle (Figure 1, center & right). Multiple experimentalists in the early twentieth century began creating this hypothesis for arrhythmia with the assumption that the mechanisms for normal electrical impulse generation and propagation they were discovering at that time fully explain the production of heart abnormal electrical activity. This hypothesis was formed from experiments Mayer published in 1906 [18] which demonstrated the fundamental event of impulse reentry. He showed that rings of excitable jellyfish tissue exposed to an external electrical impulse would produce recirculating electrical waves when unidirectional impulse block was imposed on these preparations. Subsequently in 1913 and 1914 Mines and Garrey [19,20] published similar results they acquired in heart muscle. Their data provided the initial evidence supporting the view that recirculating electrical impulses produce arrhythmia. Based on this work and their own original observations [17], Lewis, Drury and Ilescu proposed in 1921 [21] that the 'circus movement' of normal electrical activity might explain the five characteristics of atrial fibrillation (*and other arrhythmia*) noted at the end of the preceding paragraph. This simple and elegant circus creation contributed to establishing the impulse reentry hypothesis which has since metamorphosed into the accepted explanation for atrial fibrillation and for other clinically relevant rhythm disorders.

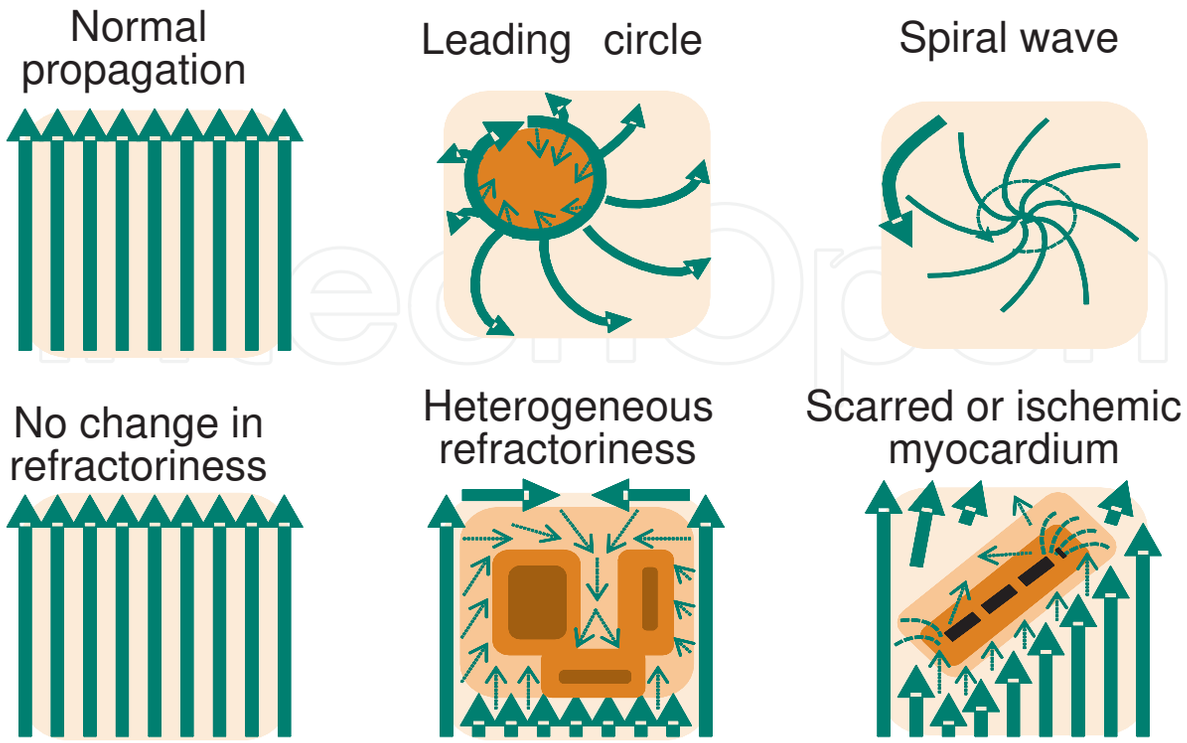

Figure 1. Impulse Reentry.*Left:* Normal heart muscle conduts impulses homogeneously. Uniform fields of excitable and refractory muscle limit impulse recirculation. *Center: Leading circle model:* Inhomogeneous rates of impulse conduction (Small v large arrows) create contiguous regions of excited and quiescent myocardium with altered refractoriness (Light v dark boxes). Electrical activity may recirculate in these regions if unidirectional conduction block were present. *Right: Spiral wave model:* Impulses circulating around scar could encounter local conduction inhomogeneities which cause impulse wavenreak &reentrant rotors.

Lewis realized that variants of the circus hypothesis might arise in pathological settings. In particular he mentioned that the primary arrhythmogenic circuit could fragment to produce secondary or offspring ectopic sources and that local variation in the rate of conduction of ectopic impulses could provoke disorganized fibrillation [17, *see page 591 Figure III and page 592 Figures V & VI*]. Several decades later Moe, Abildskov [22], and others embellished this general circus view of impulse recirculation to explain the persistent nature of fibrillation or other high frequency arrhythmias. Based on his own data and on his careful examination of the earlier high-speed cinematographic work of Wiggers [23], Moe proposed that local variations in impulse conduction induced faradically or arising in diseased heart could cause impulses to fragment and purposelessly but persistently meander through excitable regions of atrial (*or ventricular*) muscle. Moe proposed that such 'wandering wavelets' of impulse reentry were the fundamental cause of atrial fibrillation.

This 'wandering wavelet' hypothesis has been modified or superseded during the 50 years since it was first proposed. Work published by Allessie in 1977 [24] demonstrated that local variations in the refractory period of heart muscle can produce 'leading circle' reentry. In his model, impulses circulate like a pinwheel around a small region of refractory, non-excitable myocardium, producing vortices of electrical activity that emanate from a central inexcitable

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core. In this view recirculating abnormal electrical emanations disrupt normal electrical activity and cause arrhythmia. Allessie indeed observed 'leading circle' or 'functional' reentry under experimental conditions in which acetylcholine markedly shortened atrial action potential duration and either bursts of high frequency stimulation or ectopic impulses administered during so-called 'vulnerable periods' induced a type of atrial fibrillation. This hypothesis of 'leading circle reentry' has evolved since 1977 and variants of it are now the dominant means to explain arrhythmia [25]. In particular, one current 'state-of-the-art' view proposes that the interaction of normal or ectopic impulses with refractory objects of an appropriate size leads to impulse fractionation and impulse reentry (Figure 1, right). This type of 'wavebreak' allows for impulse recirculation to occur around fixed anatomical sites like papillary muscles, around scar tissue or around myocardium that poorly conducts electricity. These rotors of fractionated impulse can remain fixed in or meander through heart muscle. Elegant imaging methodologies produced from decades of engineering coupled with mathematical-biophysical modeling have theorized, searched for and characterized wavebreak. The high frequency pin-wheel rotors of electrical activity that this model posits have sometimes been directly observed in the aftermath of high frequency burst stimulation. Their direct observation has been reported less frequently in myocardial pathologies like ischemia where arrhythmia arises 'naturally' in the absence of either burst pacing or exquisitely timed ectopic stimulations. Regardless, the intellectual flexibility and elegance of the wavebreak construct allowed for the development of multiple concepts espoused as fundamental mechanisms for arrhythmia including atrial fibrillation [25].

The current iteration of the impulse reentry hypothesis thus proposes that arrhythmia is the response of contiguous regions of discontinuously excitable and non- or poorly excitable heart muscle to an external depolarizing influence (Figure 1). That is, reentry requires (a) extremely localized inhomogeneity in the conduction properties of heart muscle such as might occur in the border between scar tissue and viable myocardium, (b) pathological conditions that affect the biophysical properties of the voltage-dependent sodium or potassium channels or (c) decreasing the activity of proteins like connexins which would impose conduction heterogeneities on the heart. This dominant hypothesis to explain atrial fibrillation or other arrhythmia views these disorders from a vantage point developed in the early twentieth century. Arrhythmia in this view begins primarily as a disturbance in heart electrical activity. The fact that faradic methods like burst-pacing or stimulation during an 'electrically vulnerable period' remain mainstays in inducing arrhythmia and that arrhythmia is assessed by the electrocardiograph or by other devices that measure myocyte electrical activity sustains the opinion that rhythm disorders are mainly or solely electrical problems. An alternate view might ask whether the voltage-dependent ion channels that produce heart electrical activity are themselves regulated by voltage-independent cell signaling events. Might such cell signaling events drive arrhythmia? That is, *can cardiac non-electrical sources cause heart electrical problems*?

The creative hypothesis proposed by Engelmann to explain arrhythmia emphasized that changes or defects in small regions of heart muscle might generate sporadic or high frequency focal ectopic impulses. The inability to identify a candidate mechanism for focal ectopy at

the turn of the twentieth century, the identification of impulse reentry in jellyfish, and its ascendance as a facile, malleable explanation for arrhythmia caused the focal view to fall into disfavor. By the middle of the twentieth century only few proponents supported it, in particular investigators like Rothberger, Scherf, and Kisch [16,26,27]. They continued to present data which showed that focal (*or cellular*) sites of spontaneous depolarization could provoke arrhythmia just as well as impulse reentry. From the 1920s through the 1950s Scherf repeatedly reported that focal administration of toxins like aconitine or alkaloids like veratradine incite cardiac rhythm disturbances that mimic atrial (*or ventricular*) fibrillation and atrial flutter. It is important to note that these pharmacological agents initiate arrhythmia by modifying sodium channel gating properties to disrupt this gatekeeper of the action potential. Jervell and Lange-Nielsen published a groundbreaking report in 1957 [28] which first documented the long QT syndrome and laid the foundation for research on the genetic basis for arrhythmia. Dessertenne [29] and others greatly developed the appreciation that genetic mutation can alter the biophysical properties of voltage-dependent sodium and potassium channels in a manner analogous to the pharmacological approach of Scherf. Consequently, in addition to changes in the gross electrical properties of heart muscle proposed to underlie wavebreak and impulse reentry, pharmacological or genetic modification of ion channels came to be accepted as potential sources of clinical arrhythmia. But this toxin and genetic view have at least three critical limitations when used as evidence to support a cell-based focal hypothesis of arrhythmia

Toxins and alkaloids modify the biophysical properties of the sodium channel to provoke arrhythmic activity. These changes in channel properties at the site of toxin administration may provoke conditions that favor impulse reentry. Thus these pharmacological approaches might incite arrhythmia in a reentrant manner analogous to faradic sources.

Mutations of voltage-dependent ion channels also might create conditions for functional or anatomic impulse reentry. Indeed reentry is invoked to explain genetically-linked arrhythmia including the long QT syndromes [30].

Even if toxin-induced arrhythmia were purely a focal event, this approach to induce arrhythmia does not identify the cellular process which might alter the biophysical properties of the sodium or other voltage-dependent ion channels to recapitulate the arrhythmogenic effects of aconitine or veratradine.

The development of a robust focal explanation for arrhythmia requires the identification of cellular mechanisms that destabilize quiescent atrium (*or ventricle*) to produce sporadic, tachycardic or fibrillatory ectopic electrical activity. There are two mechanisms now accepted to generate such abnormal electrical impulses.

4. Mechanism 2: Triggered afterdepolarization

The first is afterdepolarization or triggered activity. This ectopic event (a) arises within stressed or failing atrial (*or ventricular*) myocytes, (b) appears to require specific changes in

intracellular signaling and post-translational protein modification including phosphorylation, (c) is hypothesized to depend on changes in intracellular calcium homeostasis, and (d) needs a preceding action potential as a triggering event.

The groundbreaking work of Arvanataki in 1939 [31] provided the initial evidence for afterdepolarization. This series of papers demonstrated that spontaneous electrical activity occurred in a wide range of excitable cells including snail muscle when these preparations were stimulated at extremely rapid rates and the pacing stimulus then was abruptly stopped. Studies reported by Bozler in 1943 [32] expanded on this breakthrough work, demonstrating that cardiac muscle also can afterdepolarize. The two types of afterdepolarization are designated as early or delayed events.

Early afterdepolarization occurs either during the Phase II plateau or during Phase III repolarization of a prolonged action potential. Increased late sodium current [33] or decreased potassium channel activity, lowered 'repolarization reserve' [34], may prolong the duration of the action potential. Numerous studies show that early afterdepolarization occurs more readily with increased late sodium current compared to decreased repolarization reserve even though action potential durations are similarly prolonged. Interesting to a focal view of arrhythmia described later on, stimulating Gαq receptors greatly increases the frequency at which early afterdepolarization occurs in muscles with decreased repolarization reserve. The molecular basis for this curious effect has not been conclusively established. Early afterdepolarization occurs most often at low rates of muscle stimulation and materializes much less frequently as the stimulation rate increases toward normal. Thus arrhythmia that arises in settings of bradycardia or in conditions where heart rate is highly variable is often ascribed to early afterdepolarization. In addition, early afterdepolarization is a likely source for premature atrial (*or ventricular*) contraction and more complex arrhythmia when genetic mutation or pharmacological intervention prolongs the myocardial QT interval.

Delayed afterdepolarization is the second type of triggered activity. By contrast to early afterdepolarization, muscle or myocytes with normal action potentials that have returned to their Phase IV resting potential generate this type of abnormal impulse. Delayed afterdepolarization usually arises following high frequency burst stimulation of heart or myocytes or when heart calcium stores are greatly increased. Depending on the precise experimental condition, afterdepolarization can occur as a solitary event, as a few afterdepolarizations or as ectopy that lasts for seconds or longer. This latter type of event has been termed 'sustained triggered activity' [33]. Hypotheses for afterdepolarization must explain isolated events, sustained activity, and the transition between the two. That is, how can a single isolated ectopic event lead to sustained tachycardic or fibrillary activity?

Schmitt and Erlanger initially explained premature contraction of intact muscle using the impulse reentry hypothesis [35]. In their view, electrical impulses might recirculate through junctions in the Purkinje system or around a region of the heart if both somehow came to possess unidirectional impulse block and altered conduction properties. They envisioned a scenario wherein recirculation could occur once or in a sustained manner depending on the electrical characteristics of the recirculating loop. The observation of afterdepolarization in isolated myocytes indicated that mechanisms besides the gross physiological ones of reentry

might also initiate triggered activity. January and others [36] proposed voltage-dependent sodium or calcium channel window currents as potential mediators of early afterdepolarization. In their view, the biophysical properties of these voltage-dependent ion channels favor channel reopening during their prolonged exposure to the membrane potentials of the action potential plateau phase. For a wide range of reasons reviewed by Salama and others [37,38], neither of these purely electrical explanations adequately explain the production or the properties of early afterdepolarizations. Window currents also appear to be a less likely explanation for delayed afterdepolarizations which occur from resting potentials. Pogwizd among others [39] hypothesized that decreased activity of the inwardly rectifying potassium channel could sensitize heart muscle to depolarizing influences during diastole. This enhanced sensitivity would favor myocyte delayed afterdepolarization during Phase IV. All of these explanations, however, view afterdepolarization as essentially an electrical phenomenon. That is, they hold that the voltage-dependent ion channels which produce normal electrical activity are the sole cause for the ectopic electrical instability of afterdepolarization. An alternate view of afterdepolarization began to evolve from data first reported in 2000 [40,41] which proposed that abnormalities in the calcium homeostasis responsible for muscle contraction might cause afterdepolarization.

The mechanism which couples myocyte excitation and contraction remained unresolved into the 1970s [42]. The experiments of Fabiato established that the passage of small amounts of calcium across the myocyte plasma membrane initiated the rapid release of a much larger myocyte calcium store sequestered within the lumen of the sarcoplasmic reticulum (SR) [43]. This calcium release causes the rapid elevation of cytosolic free calcium which induces myofilaments to shorten. The subsequent accumulation of this free cytosolic calcium back into the SR lumen promotes muscle relaxation. This process of calcium-induced calcium release is the mechanism through which myocyte electrical depolarization promotes contraction. Particularly important details of this process were provided by the molecular and electrophysiological studies of the voltage-dependent slow calcium channel by Fleckenstein and others [44], the SR ryanodine receptor calcium release channel by Fleischer and others [45], and the SR calcium ATPase by MacLennan, Katz, Tada, and others [46-49].

Beta-adrenergic receptor stimulation provokes the phosphorylation of several myocyte proteins critical for excitation-contraction coupling including SR phospholamban. Phosphorylation of phospholamban dissociates it from the SR calcium ATPase which activates this transporter and enhances the sequestration of cytosolic calcium into the SR lumen [48]. As a result, SR calcium stores increase which contributes both to the positive inotropic effect of beta-adrenergic stimulation and to the production of delayed afterdepolarizations. Myocyte calcium stores likewise increase in response to increased cytosolic sodium, for example following exposure to the Na/K-ATPase inhibitor ouabain. Excess sodium exits myocytes via the plasma membrane sodium-calcium exchange transporter leading to myocyte calcium loading. As first quantitated by Pitts and by Reeves [50,51], this transporter facilitates the electrogenic exchange of three sodium ions for one calcium ion.

Fleischer [45] and others defined the mechanism through which calcium egresses from the SR. They demonstrated that the alkaloid ryanodine binds with high affinity to an SR calci-

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um release channel, locks it into an open state, and permits the leakage of SR calcium. Using ryanodine binding as a molecular probe, they identified and purified the ryanodine receptor calcium release channel and demonstrated its central role in calcium-induced calcium release. The development of reporter molecules that measure intracellular free calcium, molecules such as aequorin by Blinks [52] and fura-2 by Grynkiewicz and Tsien [53], allowed the interrogation of the intracellular calcium dynamics of cardiac calcium-induced calcium release.

This myocyte calcium-handling system also offers a cell-based mechanism for triggered activity. Marks [40] and then others [41,54,55], proposed that slow leakage of SR calcium through dysfunctional ryanodine receptors might incite afterdepolarization especially delayed afterdepolarization. This 'calcium leak' hypothesis for triggered arrhythmia (Figure 2) takes advantage of the localization of the ventricular SR ryanodine receptor calcium release channel in SR terminal cisternae near the myocyte T-tubule. It posits that SR calcium leak stimulates calcium efflux on the electrogenic sodium-calcium exchanger which would depolarize myocytes during diastole. Thus conditions that (a) increase the content of myocyte calcium stores, (b) create a steady-state leak of SR calcium or (c) create a preferential leak of calcium during diastole would raise myocyte resting membrane potential to more positive values and reach threshold. Delayed aftedepolarization would result. To some degree this general model may also hold in atrial myocytes that lack well developed T-tubules. Here junctional ryanodine receptors appose the atrial myocyte plasma membranes [54]. Increases in ryanodine receptor calcium leak have been reported in experimentally and pathologically challenged atrial myocytes, indicating that calcium leak might be a generally applicable cause for delayed afterdepolarization. How the disruption of calcium homeostasis generates early afterdepolarization remains under active investigation.

'Hyperphosphorylation' of the ryanodine receptor is proposed to incite its leakiness. Using experimental systems as diverse as lipid bilayers and failing hearts the inventive work of Marks [40] and others supported protein kinase A as the agent that hyperphosphorylates the ryanodine receptor and causes leakiness. Work from the laboratory of Bers [41] and others [54,55] highlighted isoforms of calmodulin-dependent protein kinase II (CaMKII) as a second potential initiator of ryanodine receptor hyperphosphorylation/leakiness. The ryanodine receptor is a large protein critical to the normal function of heart muscle. Thus it is not unexpected that many cell factors regulate its properties including its leakiness; redox stress and the interactions between the FKBP12.6 protein and the ryanodine receptor are two such factors [40,56].

5. Germane questions about 'calcium leak' & afterdepolarization

Several questions arise about the logical & widely accepted calcium-leak hypothesis for triggered arrhythmia.

Does the accepted axis of [SR calcium leakage→electrogenic calcium efflux] describe the entire mechanism for afterdepolarization or does afterdepolarization result from more compli-

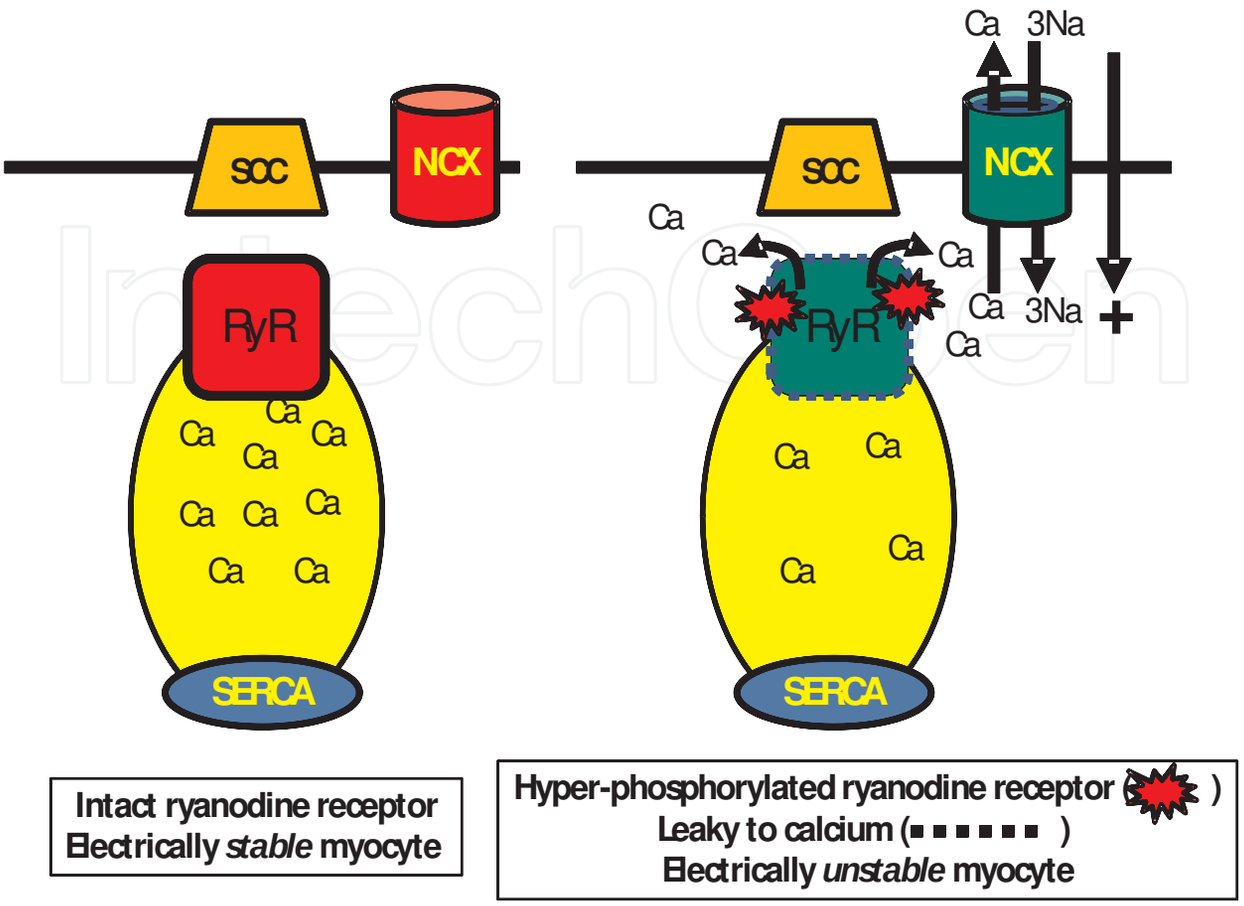

Figure 2. Model for 'Calcium Leak' Afterdepolarization. *Left:* Ryanodine receptors (RyR) are impermeant to calcium except during the action potential when 'trigger calcium' enters myocytes via the voltage-dependent calcium channel (*SCC*). *Right:* Hyper-phosphorylated ryanodine receptors are leaky to calcium. This depletes SR stores. Leaked calcium leaves myocytes on the sodium calcium exchanger (*NCX*). Electrogenic calcium efflux drives positive charges into the myocyte which acts as a depolarizing influence. At impulse threshold, afterdepolarization would occur.

cated molecular pathways? Numerous observations in the literature support the latter view. For example, Ben-David and Zipes [57] showed that reduced repolarization reserve effectively prolongs the action potential duration of intact heart but does not produce a high incidence of arrhythmia. By contrast, alpha-adrenergic agonists provoke fulminant early afterdepolarization and complex arrhythmia in intact hearts with low repolarization reserve. Beta-adrenergic stimulation of these hearts does not provoke arrhythmia. Both Kimura and co -authors and Molina-Viamonte and colleagues [58,59] reported that alpha-adrenergic stimulation provoked delayed afterdepolarization in calcium loaded or ischemic Purkinje fibers. These authors concluded that a specific alpha 1-adrenergic pathway is involved in inducing triggered activity in the setting of ischemia and reperfusion. Finally Lo and co-authors [60] among others report that alpha- and beta-adrenergic receptor stimulation provokes afterdepolarization in intact pulmonary veins and that CaMKII inhibitors block this triggered activity. While SR calcium leak might account for these results, one or more

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events specific to alpha-adrenergic receptor stimulation/Gαq signaling might also exacerbate afterdepolarization in hearts with reduced repolarization reserve.

- Can the stimulation of Gαq-coupled signaling by means other than the alpha-1 receptor enhance afterdepolarization in isolated pulmonary veins or in hearts with reduced repolarization reserve? There is evidence indicating this is the case [61]. Pharmacological and molecular dissection of the interaction between voltage-independent Gαq-coupled signaling and early- or delayed-afterdepolarization might reveal new mechanisms for arrhythmia.
- Does alpha-adrenergic stimulation of Purkinje fibers, isolated pulmonary veins, and normal heart muscle with reduced repolarization reserve 'hyperphosphorylate' ryanodine receptors compared to normal preparations. If 'hyperphosphorylation' were not to occur, then additional molecular mechanisms contribute to afterdepolarization.
- In the particular case of the pulmonary veins, does calcium loading by approaches other than beta-adrenergic stimulation, approaches like slow calcium channel activation, provoke spontaneous ectopic activity?
- Does 'arrhythmogenic' calcium activate afterdepolarization solely as a charge carrier or as a signaling intermediate that accelerates ryanodine receptor calcium leak? Anderson and co-authors reported in 1998 [62] that CaMKII inhibitors prevent afterdepolarization in intact and isolated cardiac preparations, a result since widely validated [63]. Whether CaMKII acts by hyperphosphorylating the ryanodine receptor or whether it has multiple arrhythmogenic targets remains open to investigation.
- What source of calcium activates CaMKII to provoke triggered activity? Is this source calcium leaked from the SR or might alternate mean exist to activate arrhythmogenic calmodulin and CaMKII?

Triggered afterdepolarization often begins as an isolated event but evolves into more robust and continuous ectopy, so-called sustained triggered activity. This transition depends on the duration of high-frequency burst pacing, the dose of pharmacological activators of the late sodium current, or the apparent timing of R-on-T phenomena. How does the transition from afterdepolarization to complex arrhythmia like tachycardia or fibrillation actually occur? It is now generally accepted that these transitions arise from abnormalities in impulse conduction. In this view, ectopic afterdepolarization triggers reentry in arrhythmogenic 'substrate,' heart muscle that conducts impulses heterogeneously. This facile explanation, however, may not address all potential causes for the transition from isolated to complex ectopy. Might afterdepolarization and sustained activity be manifestations of a common cell arrhythmogenic signaling pathway?

Do both 'isolated' and 'sustained' triggered activities require CaMKII signaling? That is, could myocytes or Purkinje cells express an arrhythmogenic pathway in which CaMKII and afterdepolarization lie upstream of a second calcium-linked mechanism whose stimulation elicits CaMKII-independent 'sustained' ectopic activity?

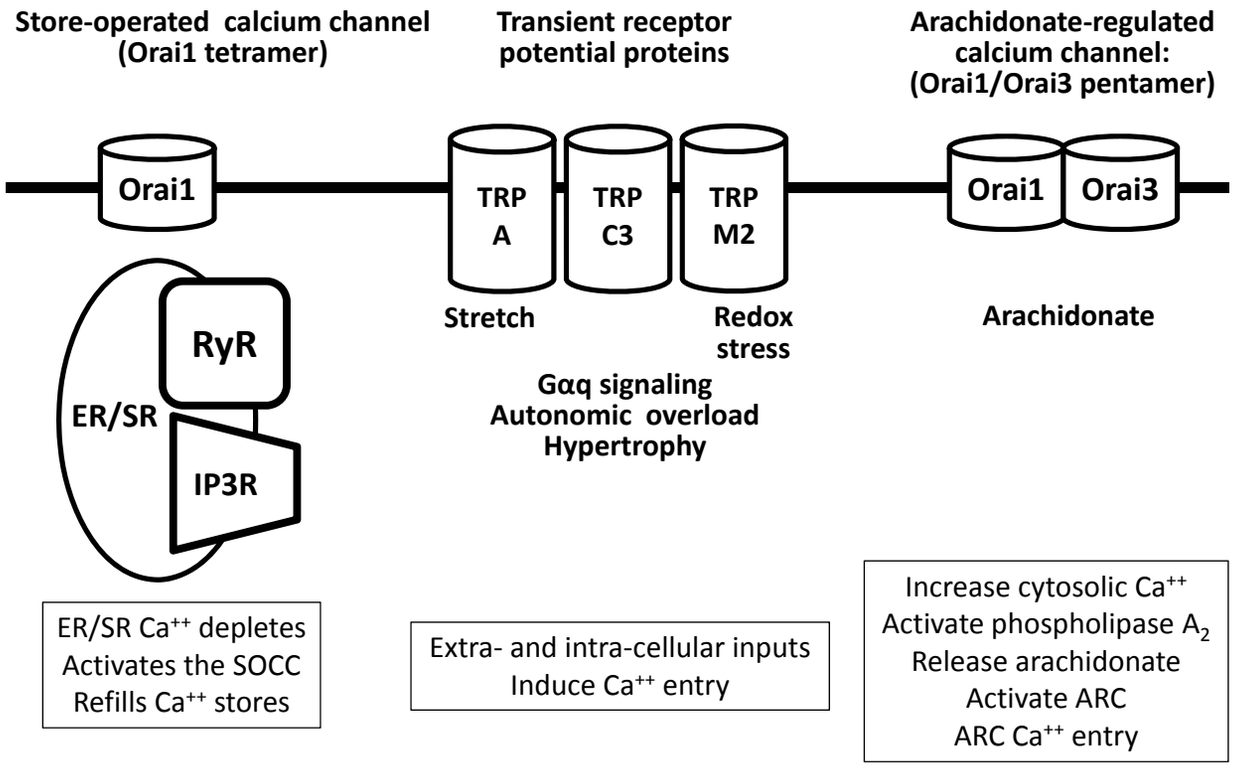

Figure 3. Four Families of Voltage-Independent Calcium Channels.*Left:* IP3Rs allow calcium release from intracellular ER/SR stores. This generates intracellular signals. ER store depletion activates calcium entry via the Orail a/o Orai1/TRPC1 store-operated calcium channel (*Left box*). *Center:* The transient receptor potential channels permit calcium entry into cells in response to a wide range of influences pertinent to atrial fibrillation (*Middle box*). These calcium signals mediate the phenotypic response of atria to stretch or to autonomic signaling. *Right:* Orail and Orai3 create an arachidonate-sensitive calcium channel. This channel permits calcium entry in response to stress signals that activate eicosanoid metabolism (*Right box*).

The well-documented role of calcium in arrhythmogenesis and the central role of SR calcium in heart muscle contraction focused the 'calcium leak' hypothesis on the SR ryanodine receptor as the source of arrhythmogenic calcium. At the time of its formulation only the ryanodine receptor, the voltage-dependent slow calcium channel, and the sodium-calcium exchanger were accepted to greatly affect cytosolic calcium in atrial or ventricular myocytes and Purkinje cells. Now extensive work in non-excitable cells has established the voltage-independent inositol-tris-phosphate receptors (IP3R), the transient receptor potential protein (TRP) channels and the Orai channels are the predominant means to generate cell calcium signals (Figure 3).

Stating our proposition succinctly, do after depolarization and complex arrhythmia arise from cell processes other than those which produce excitation and the ECG (voltage-dependent ion channels) or myocyte contraction (calcium-induced SR calcium release)? Might myocardial non-electrical, voltage-independent processes provoke myocardial electrical instability including after depolarization?

Reports in the literature and our data suggest they do. Myocytes and Purkinje cells express the cellular calcium transporters, kinases, lipases and other proteins that initiate and regulate voltage-independent calcium entry and calcium signaling. These include the IP3Rs, the

TRP channels, the Orai channels, and Stim1. This signaling system normally regulates the Gαq-coupled growth response, stress responses, and other events in all cells including myocytes. Our data and that of others lead to an initial hypothesis that voltage-independent calcium signaling assumes an additional, apparently untoward task in cells like myocytes or Purkinje cells that highly express voltage-dependent ion channels. This task is the activation of a calcium-dependent arrhythmogenic signaling pathway. This putative pathway is normally silent until appropriate arrhythmogenic stimuli or pharmacological activators rouse it into action. Depending on the intensity of the activation challenge, we believe this complex pathway can co-opt the activity of voltage-dependent ion channels to produce isolated afterdepolarization, afterdepolarization that leads to sustained activity, and high frequency sustained ectopic activity. In this view, solitary afterdepolarizations are focal events that result from the activation of one part of a broader calcium-dependent arrhythmogenic pathway. The activation of an interrelated downstream part of this pathway provokes high-frequency focal tachycardia or fibrillation. The two parts of this putative pathway functionally interact which allows the transition between afterdepolarization and complex arrhythmia. This interaction might transpire in a manner analogous to that described by Shuttleworth [64] for the sequential activation of voltage-independent calcium signaling and calcium entry pathways in non-excitable cells. In heart the putative calcium signaling events that cause afterdepolarization would gradually deplete cell voltage-independent calcium stores specific for calcium signaling. This depletion stimulates voltage-independent calcium entry via the Orai channels. We suggest that this type of calcium entry activates sustained ectopic activity.

6. Mechanism 3: Typical abnormal automaticity

Abnormal automaticity occurs when ectopic sites in the atria (*or ventricle*) spontaneously depolarize independently of normal sinus rhythm or without a preceding triggering event. Investigators like Vassalle [65] have made important contributions to our current understanding of this type of ectopy. Typical abnormal automaticity occurs during hypoxia and ischemia when myocytes partially depolarize from their resting potential of ~-85 to about -65mV. An additional mechanism for abnormal automaticity takes advantage of the fact that the hyperpolarization-activated 'funny currents', which contribute to normal automaticity, are expressed throughout the heart [66]. The activation of atrial or ventricular funny currents might induce spontaneous depolarization akin to the sinoatrial pacemaker but the properties of these ectopic channels indicate that they are inactive in normal myocytes. How ectopically expressed funny channels might spring to life to provoke focal abnormal automaticity is unresolved.

Several reports suggest the existence of alternate, atypical forms of abnormal automaticity and that atypical automaticity may be an unrecognized contributor to arrhythmogenesis. For example, in 1999 Nuss and co-workers [67] reported that myocytes isolated from failing hearts produced sporadic, spontaneous depolarizations while normal myocytes did not. These ectopic depolarizations occurred from normal resting potentials, did not require a preceding external stimulation, and occurred independently of any significant change in in-

tracellular calcium homeostasis. Furthermore, the spontaneous action potentials these 'failing' cells produced showed no Phase 4 depolarization which might occur if cell funny currents had somehow become active. One interpretation of this provocative report is that pathological conditions like failure change the fundamental properties of ventricular myocytes, transforming normal, non-automatic myocytes into cells that are capable of an atypical automatic activity. This change survives cell isolation indicating it is reasonably permanent and possibly acutely reversible. Nuss did not define a mechanism to transform non-automatic (*normal*) myocytes to sporadically or rapidly automatic (*failing*) ones. The voltage-independent arrhythmogenic pathway we describe in some detail below is one candidate mechanism.

Robichaux and others [68] assessed the arrhythmogenic mechanisms that underlie experimental fibrillation and reported that reentry does not predominant either soon after the induction of faradic fibrillation or several minutes after the start of fibrillation. Rather they showed that organized sources of relatively regular high frequency ectopic activity drives long-duration ventricular fibrillation. Others also have reported that focal sources of non-reentrant activity predominate during experimental ventricular fibrillation [69]. Automatic activity was among the proposed explanations for both sets of data. It is possible that an atypical form of automaticity underlies these results and affords an unrecognized means to produce sporadic or high frequency myocardial electrical instability.

7. Summary of the mechanisms for arrhythmia

The three current mechanisms for arrhythmia assume that abnormalities in (a) the well-defined process of normal cardiac excitation-contraction coupling, (b) the propagation of electrical waves through the heart or (c) the electrical response of heart muscle to enormous faradic insults circumscribe all the properties of the myocardium needed to fully explain clinical arrhythmia including atrial fibrillation. In other words, all other non-electrical cell processes are by-standers in arrhythmogenesis and they little influence heart muscle electrical stability. None of these three theories is a true focal hypothesis for arrhythmia as envisioned by Engelman and championed by Scherf and others.

Our fourth view of arrhythmia proposes that non-electrical cell signaling events can destabilize the electrical activity of myocytes and conduction system cells. Such destabilization produces isolated focal ectopic events or high frequency focal tachycardia or fibrillation. Thus our unconventional hypothesis for arrhythmia proposes that heart muscle can produce electromechanical activity in two ways. First is by the well-defined pathway of sinus rhythm and impulse conduction. This pathway for normal heart electromechanical activity integrates heart function with systemic physiology. Second, the activation of a cellular 'arrhythmogenic' signaling pathway can transform non-automatic myocytes into cells that spontaneously produce sporadic or high frequency electrical activity independent of external regulators like sinus rhythm or systemic physiology. Aberrant or exuberant myocyte or Purkinje cell voltage-independent calcium homeostasis is one means we have identified to

activate this cryptic arrhythmogenic signaling pathway. The novel fourth mechanism outlined below satisfies the requirements for a purely focal hypothesis for arrhythmia.

8. Mechanism 4: Relevant overview of voltage-independent calcium homeostasis

Cell calcium entry and cell calcium homeostasis are divided operationally into voltage-independent and voltage-dependent domains. Voltage-independent calcium homeostasis regulates non-excitable and excitable cell signaling events that are critical to cell growth, survival, and death. Four families of proteins control the generation and propagation of these calcium signals thereby allowing cells to respond appropriately to challenges or changes in their environment. Two families of plasma membrane calcium transporters permit voltage-independent calcium entry in response to extra- or intra-cellular signals. While cell membrane potential influences these carriers, they are not voltage-gated proteins. A third family of intracellular calcium release channels interacts functionally with these transporters. A fourth family maintains the cell calcium stores used to continually generate calcium signals, a task critical for cell viability. None of these families of voltage-independent proteins is now widely believed to greatly influence heart excitability. Our data and that of others directly challenge this view. They propose that deranged voltage-independent calcium homeostasis and we suggest the dysregulated activity of one family of voltage-independent calcium channels can provoke heart muscle electrical instability.

The first family is the well-characterized Gαq-coupled receptor proteins (Figure 4). A broad range of agonists including bioactive peptides like angiotensin II, bioactive lipids like prostaglandins, and hormones like norepinephrine stimulate this family of receptors. Agonist binding to a specific Gαq-coupled receptor activates a plasma membrane phosphatidylinositoyl-specific phospholipase C. This lipase generates two active intermediates for voltage-independent calcium signaling. Water-soluble inositol-1,4,5-trisphosphate is the first intermediate as defined in the elegant work of Berridge in the 1970s [70]. The second is the membrane-bound lipid diacylglycerol. A highly complex interaction among G-protein regulators, inositol phosphate kinases and phosphatases, and diacylglycerol kinases and lipases set the rate of production and the steady-state levels of these signaling intermediates.

Inositol-1,4,5-trisphosphate diffuses from the environ of the cytosolic face of the plasma membrane and binds with high affinity to the IP3Rs, the second family of proteins central to voltage-independent calcium homeostasis. The ~300kDa IP3Rs are membrane proteins inserted into the endoplasmic reticulum of non-excitable cells and the SR of excitable cells, and are active as tetramers. IP3Rs are calcium release channels that regulate the egress of pools of calcium stored within the lumen of the endoplasmic reticulum or the SR. The IP3R calcium release process is highly regulated and depends on factors including lumen calcium content, cytosolic free calcium, the post-translational modification of the receptor, and the binding of regulator proteins like bcl-2 [71]. IP3R calcium release contributes to cytosolic calcium signaling events through the information encoded in the amplitude of released calci-

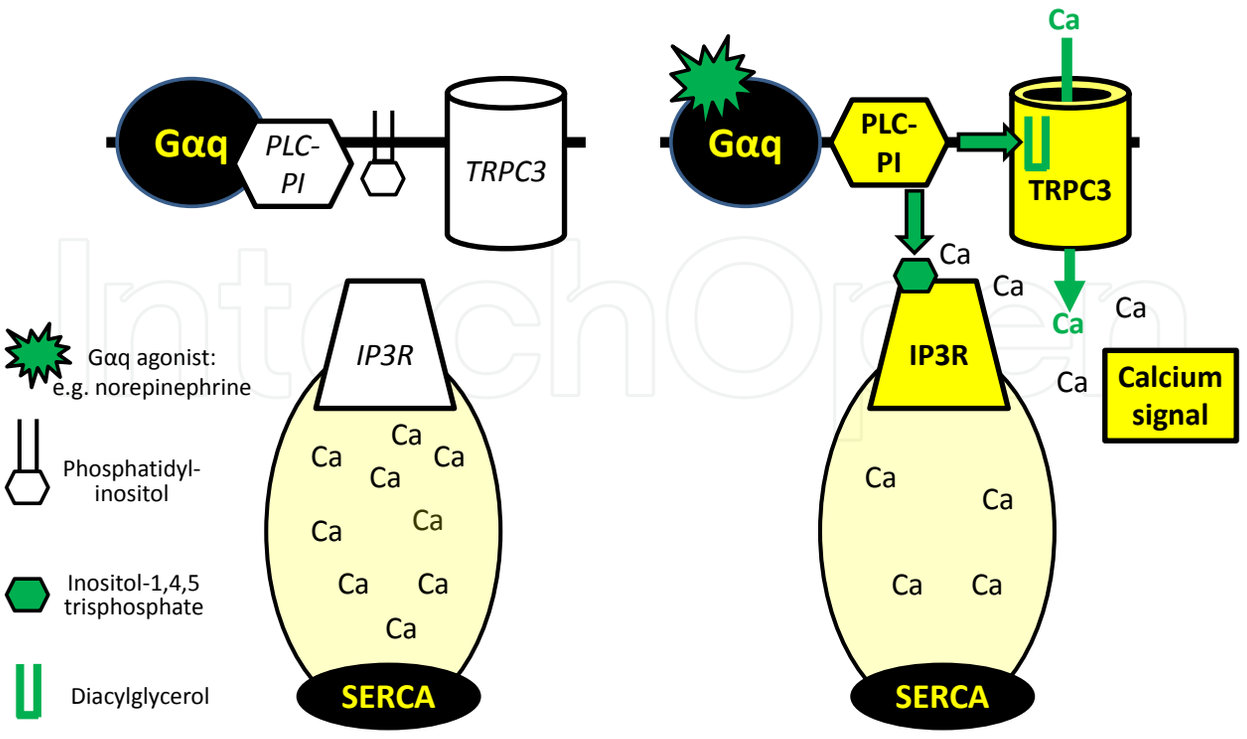

Figure 4. Model of Gag Signaling. Agonist occupation of specific Gαq receptor proteins activate a phosphatidylinositol specific phospholipase C (PLC-PI). Active lipase hydrolyzes plasma membrane phosphatidylinositol. This produces diacylglycerol and inositol-1,4,5-trisphosphate. Both intermediates activate calcium signaling: the former via plasma membrane TRPC3, the latter by binding to the IP3R which initiates SR/ER calcium release.

um and the frequency at which release occurs. Whether the IP3Rs and the ryanodine receptor access identical calcium stores in excitable cells remains an actively investigated question.

Diacylglycerol the second signaling intermediate is hydrophobic so it remains intercalated in membranes following its release from plasma membrane phosphatidylinositol. Diacylglycerol first was believed to signal by activating a protein kinase C. Subsequent work has shown that it also activates members of the third family of voltage-independent calcium signaling proteins which may be germane to arrhythmia, the TRPC family of calcium channels [72,73].

The TRP channels were first identified in drosophila where they play a central role in vision transduction [74]. Subsequent work from the laboratories of Birnbaumer [75], Montell [76], and others identified multiple families of mammalian TRP channels including the classical (TRPC), the melatonin, the vallinoid, and the ankyrin repeat forms. TRP channels contain six transmembrane domains and an ion pore domain which selects calcium over sodium under most conditions. Diacylglycerol released following Gαq receptor stimulation binds to TRPC3 and TRPC6, activates these channels, and permits cell calcium entry. The TRPC1 channel may participate in cell signaling as a subunit of the store-operated calcium channel (SOCC) which maintains cell calcium stores [75]. The TRPM3 &TRPA channels appear to activate when cells are stretched while TRPM2 responds to increased oxidant stress [75]. Calci-

um entering cells through the TRP channels spark downstream signaling responses to receptor stimulation or to environmental challenges. As muscle stretch and oxidant stress contribute to the pathophysiology of atrial fibrillation [77], calcium entry linked to TRP channels may contribute to the hypertrophy and fibrosis that accompany atrial fibrillation.

In 1986 Putney raised a critically important question about voltage-independent calcium homeostasis [78]. He noted that calcium release events initiated by inositol-1,4,5-trisphosphate could deplete intracellular calcium stores. This depletion would disrupt continued calcium signaling. Putney proposed that cells must contain a mechanism to sense the calcium content of their stores and promote calcium entry in response to store depletion. This logical proposition was widely accepted. Electrophysiological and calcium imaging protocols clearly demonstrate that depleting cell calcium stores in calcium-free media provokes a dramatic calcium entry when external calcium is restored to these cells. That is, calcium store depletion activates a cell mechanism to replenish these stores. The initial hypotheses to explain SOCC calcium entry included a calcium-inducible factor, a direct-coupling mechanism between the store and the channel involving the actin cytoskeleton, and an indirect coupling mechanism [79]. In 2005, however, the elegant molecular mechanism for SOCC calcium entry came into focus. Dziadek and colleagues [80] identified stromal interaction molecule 1 (Stim1) which subsequently was shown to be a sensor for the lumenal calcium of the endoplasmic reticulum. Stim1 resides mainly in the endoplasmic reticulum, contains a single transmembrane domain, and has a calcium-binding EF-hand domain positioned within the lumen of the endoplasmic reticulum. In unstimulated cells, Stim1 distributes throughout the endoplasmic reticulum membrane. Depletion of calcium from the endoplasmic reticulum lumen by any mechanism including calcium release through the IP3R causes Stim1 to translocate to plasma membrane-endoplasmic reticulum junctions. Here Stim1 docks with plasma membrane SOCCs and activates SOCC calcium entry which repletes cell calcium stores [81].

Controversy exists about the exact molecular constituents of the SOCC. In one current paradigm plasma membrane Orai1 proteins constitute the SOCC. This model proposes that Orai1 distributes throughout the plasma membrane of cells with full calcium stores, calcium replete cells, and is inactive. Calcium store depletion causes Stim1 to translocate to endoplasmic reticulum-plasma membrane junctions. Stim1 there binds and activates Orai1 to allow calcium entry. In this model the active channel is an Orai1 tetramer [82]. An alternate model posits a complex of Orai1 and TRPC1 or other isoforms of TRPC as the active calcium channel which responds to cell store-depletion but not to extracellular depolarizing influences [75]. Regardless of this debate, the Orai proteins are the fourth family of proteins critical to voltage-independent calcium homeostasis whose tightly regulated function allows continued physiological calcium signaling.

How might this general SOCC pathway relate to current views of arrhythmia? As one example, SR calcium store depletion through 'leaky' ryanodine receptor might initiate [Stim1-Orai1/TRPC1] voltage-independent calcium entry in an effort to maintain SR or other myocyte calcium stores. This type of calcium entry may exacerbate the driving force for afterdepolarization posited by Marks and others(Figure 5). It also might provoke more serious unexpected forms of electrical instability which we outline below.

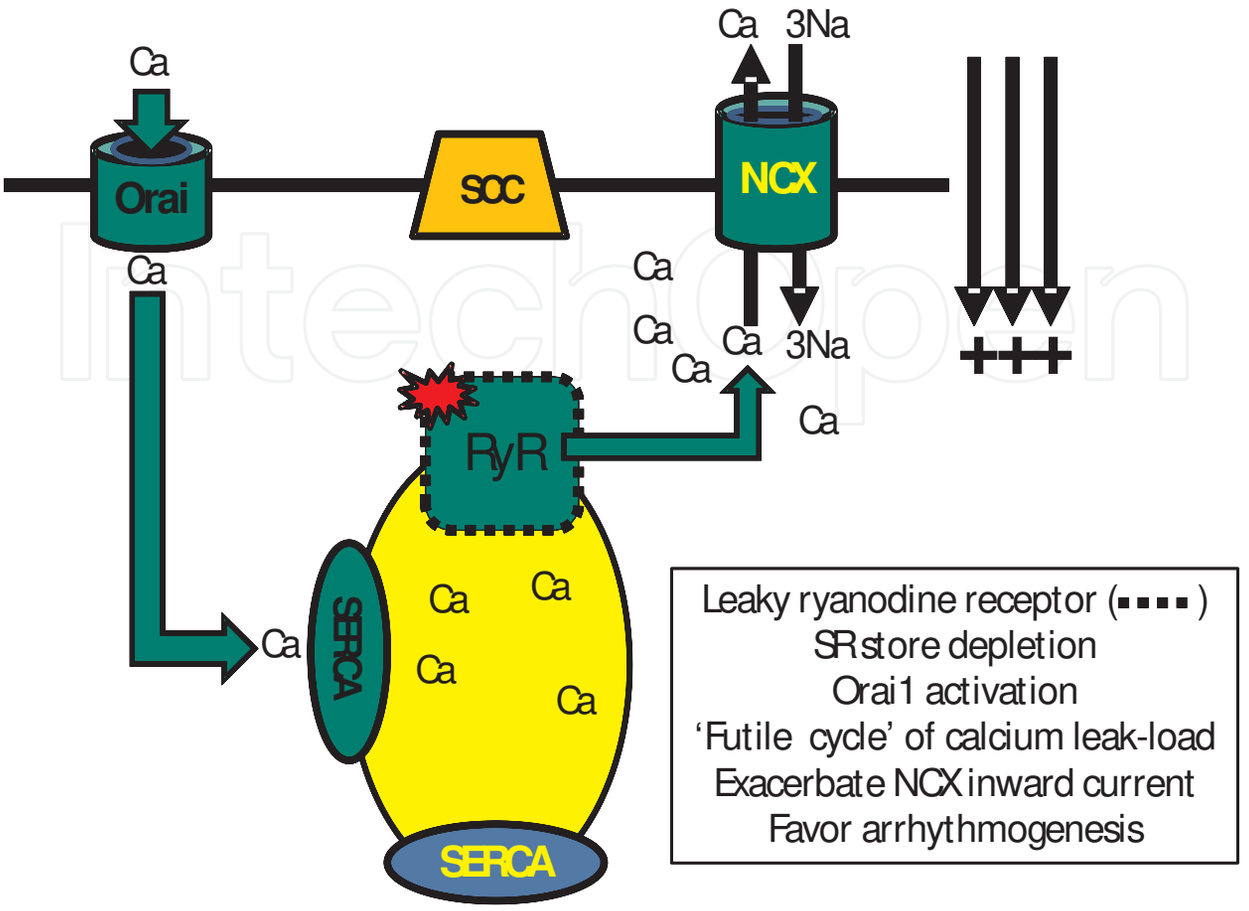

Figure 5. Potential Interaction between Orai Calcium Entry and Arrhythmogenic SR Calcium Leak. Calcium leak through the ryanodine receptor. (*RyR & right green arrow*) depletes SR calcium. This depletion may activate Orail-linked calcium entry (*Left green arrow*) to maintain E-C patency & muscle function. A futile cycle of calcium entry-leak may exacerbate NCX-linked calcium efflux and cell depolarization, driving it more frequently or more quickly toward threshold (*Rightmost black arrows*).

Orai2 and Orai3 are the remaining members of this fourth family. Orai2 is a pseudogene and has garnered some interest. By contrast, Shuttleworth first demonstrated that Orai3 is an important participant in voltage-independent calcium signaling [64]. Elegant work from his lab group shows that pentamers of Orai3 and Orai1 form an arachidonate regulated calcium channel (ARC). Arachidonate binding to ARC causes channel activation and permits voltage-independent calcium entry. Like the SOCC, ARC also requires Stim1 but it uses the small pool of Stim1 present in the plasma membrane. The arachidonate which activates ARC can arise from several sources. In cell culture experiments it is usually added exogenously. Calcium-dependent cytosolic phospholipase A_2 is a key source of cellular free arachidonate in physiological settings. Importantly, the arachidonate arising from the action of calcium-dependent cytosolic phospholipase A_2 on cell phospholipids is a key source for inflammatory prostaglandins and leukotrienes. CaMKII phosphorylates and activates this phospholipase [83]. Thus two possibilities emerge. First, myocyte calcium loading may activate CaMKII which then phosphorylates cytosolic calcium-dependent phospholipase A_2;

second, the arachidonate this lipase produces may activate ARC, voltage-independent calcium entry, the production of inflammatory molecules, and possibly ectopic activity.

The past 50 years of research in heart calcium and arrhythmogenesis have focused principally on voltage-dependent calcium homeostasis. Indeed there are only few reports which identify atrial, ventricular, sinoatrial or Purkinje cell expression of the molecular constituents of voltage-independent calcium homeostasis. Even fewer of these reports detail the unique intracellular distribution of these proteins in the different types of heart cells or study how this pattern of distribution might contribute to arrhythmogenesis.

Bootman and co-authors [84] provide convincing evidence that atrial myocytes contain predominately the type 2 IP3R. They show that atrial myocytes express about 10-fold more IP3R than do ventricular myocytes. An impressive observation they and others report is that this calcium release channel distributes mainly in the junctional SR near to the sarcolemmal membrane and that these IP3Rs associate with the junctional ryanodine receptors that encircle each atrial myocyte. Bootman and others suggest that these IP3Rs sensitize ryanodine receptor calcium release and may participate in the response of atria to inotropic Gαq receptor agonists.

Only little is known about the expression of the TRP channels, the Orai channels, and the Stim proteins in normal atrial muscle and pulmonary veins. To our knowledge how pathological situations like paroxysmal or sustained atrial fibrillation affect the expression of these calcium channels and channel regulators has not been investigated. This is important information as these families of proteins control the induction of hypertrophy, the response to stretch, fibrosis, and the intrinsic pathway for apoptosis. A complete evaluation of these signaling proteins in normal and diseased atria would dissect the molecular mechanisms through which atria responds to clinically relevant stressors and how these responses may favor dysfunction including electrical instability like atrial fibrillation.

Ventricular myocytes contain much lower levels of the IP3Rs relative to atria. Of interest Mohler [85] and others report that ventricular IP3Rs preferentially associate with the parajunctional SR of the T-tubule. The purpose or consequence of the specific localization of these calcium release channels is actively investigated. The responsiveness of heart muscle to Gαq stimulation increases during hypertrophy and heart failure. These results are in keeping with reports that the expression of ventricular IP3Rs increases in these diseases. Using probes specific for the type 1 IP3R, Marks [86] showed that the ventricular content of these channels nearly triples in failing heart while characteristically the content of the ryanodine receptor decreases by a factor of at least two. Little is known about the expression or functional properties of ventricular TRP channels, Orai channels, and Stim proteins either in normal or diseased heart.

By comparison with the paucity of work in ventricular myocytes, in 1994 Volpe [87] provided the first evidence that IP3Rs are highly expressed in the conduction system. Subsequent elegant and thorough analyses by Boyden, ter Keurs and colleagues demonstrated an intricate distribution of the IP3Rs and the ryanodine receptors in the Purkinje cells of the conduction system [88]. Much like atrial myocytes, the IP3Rs distribute at the periphery of

Purkinje cells. Here they associate with ryanodine receptors within specific regions of the cytoplasm just below the Purkinje plasma membrane. Boyden, ter Keurs and co-authors speculate that this striking arrangement plays a role in the arrhythmogenic potential of the conduction system. Establishing this critically important conclusion is a clear priority in arrhythmia research. Little is known about Purkinje cell expression of the TRP channels, the Orai channels or Stims. One could speculate that Stim1, Orai1 and TRPC1 might be highly expressed in the conduction system as they are functionally related to the IP3Rs. One question of potential importance is whether the marked increase in IP3R expression reported in failing heart occurs in the Purkinje system, in myocytes or in both. Furthermore, it would be useful to determine whether the expression of Orai1, Stim1, and TRPC1 respond similarly to 'failure' as do the IP3Rs. If the expression of these three IP3R partners were to increase, then the activity or hyperactivity of voltage-independent calcium signaling may contribute to the increased arrhythmogenicity seen in heart failure, as Boyden and ter Keurs speculate [88].

Ju and co-authors [89] and Demion and co-authors [90] reported that the sinoatrial node expresses the TRP channels which play a role in normal automaticity. A more detailed analysis of the expression of other voltage-independent calcium signaling proteins and how they contribute to normal automaticity is clearly required. To our knowledge nothing is known of the expression or activity of voltage-independent calcium signaling proteins in the muscular sleeves of the pulmonary or other supraventricular vessels. Since alpha-adrenergic agonists induce afterdepolarization and automatic activity in these anatomical structures, characterizing 'muscular sleeve' TRP channel, Orai channel, Stim, and IP3R expression should aid in establishing whether these channels contribute to paroxysmal atrial fibrillation.

9. Is voltage-independent calcium signaling a focal source of arrhythmia?

Experimental evidence acquired in intact animals, in intact heart muscle, and intact pulmonary veins coupled with clinical studies of human arrhythmia strongly suggest that Gαq-coupled receptor stimulation and by inference voltage-independent calcium signaling can initiate afterdepolarization and more complex arrhythmia. However, no attempt was made in these intact preparations to positively connect the calcium signaling linked to IP3Rs, the TRP channels or the Orai channels to atrial electrical instability.

Bootman and Blatter [84,91] acquired such evidence in isolated atrial myocytes. They demonstrated that Gαq agonists like endothelin-1 and pharmacological activators of the IP3Rs provoke ectopic calcium sparks, calcium waves, spontaneous calcium transients, and calcium alternans in atrial myocytes. Both groups concluded that exuberant calcium release from IP3Rs sensitizes the junctional ryanodine receptors of atrial myocytes, increasing their susceptibility to spontaneous calcium release events. Importantly, low concentrations of 2APB that block both the IP3Rs and the TRP channels suppress abnormal atrial myocyte calcium release. Blatter then showed [92] that the genetic ablation of the atrial myocyte type 2 IP3R suppresses 'arrhythmogenic' calcium release in atrial myocytes treated with endothelin-1.

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Together these data support and extend earlier intact animal studies and provide striking evidence that voltage-independent calcium homeostasis contributes to atrial arrhythmogenic calcium signaling.

The depletion of inositol-1,4,5-trisphosphate sensitive calcium stores which likely occurs with high levels of Gαq stimulation provokes SOCC calcium entry [64,78,81,82]. Thus while disturbed inositol-1,4,5-trisphosphate-linked calcium signaling is arrhythmogenic, it remains open to question whether (a) calcium release through IP3Rs, (b) the attendant increase in SOCC calcium entry or (c) both provoke ectopy. Furthermore whether these ectopic calcium release events produce myocyte depolarization in a 1:1 manner remains to be established as well as the mechanism through which ectopic depolarization might occur. It is also important to define whether the cause for abnormal depolarization in these myocytes is solely or mainly calcium efflux on the sodium-calcium exchanger or if other calcium signaling events are involved.

Hirose and co-authors [93] used transgenesis to obtain molecular and pharmacological evidence that dysregulated Gαq-coupled calcium signaling profoundly disrupts atrial and ventricular electrical stability. They employed a mouse model developed by Mende [94] which transiently overexpresses constitutively active Gαq in a heart-specific manner. The atria of these genetically modified mice are grossly enlarged and exhibit paroxysmal or persistent fibrillation. To establish that deranged diacylglycerol metabolism caused these atrial abnormalities, Hirose created a second mouse which overexpresses both Gαq and diacylglycerol kinase ζ. Such a double transgenic would accelerate diacylglycerol phosphorylation to phosphatidic acid, reduce heart content of diacylglycerol, and thus TRPC3 signaling. Mice harboring both transgenes had essentially normal atrial anatomy and electrical activity. The current reentry hypothesis for atrial fibrillation would propose that the electrical instability observed in the atria of Gαq overexpressors results from atrial enlargement and from the high levels of fibrosis observed in these muscles. In this electrocentric view, transgenically increasing diacylglycerol kinase activity would suppress atrial fibrillation by restoring normal atrial size and by reducing arrhythmogenic atrial scarring/abnormal conduction. Curiously, reentry also proposes electrical abnormalities like fibrillation should not occur in muscles as small as mouse atria (*or ventricle*) [20,22,24]. Vaidya and authors [95] first reported a similar egregious violation of Garrey's 'critical mass' tenet for reentry when they reported the occurrence of faradic fibrillation in mouse heart. They postulated unusual forms of wavebreak to account for this unexpected result.

Hirose and co-authors addressed this possible interpretation of their data in a follow-on paper [96]. Here they investigated how Gαq overexpression affected ventricular electrical stability and heart failure. They observed that mice which overexpress constitutively active Gαq exhibit heart failure and sustained or paroxysmal ventricular tachycardia and fibrillation. Some of the ventricular arrhythmia recorded in these transgenic mice may result from the irregularly irregular electrical activity produced by fibrillating atria but much of this ectopy appeared to originate in the ventricles themselves. Importantly, they reported that the acute administration of SKF-96365, a TRP and Orai channel inhibitor [97], reverses ventricular fibrillation and restores sinus rhythm in Gαq transgenic mice. This result could only oc-

cur if SKF-96365 also effectively suppressed atrial fibrillation in these animals. It is vital to remember that the atria of these transgenic mice treated acutely with SKF-96365 remained grossly enlarged and fibrotic. This single result, obtained in a model which mimics the high autonomic drive associated with atrial fibrillation, dissociates fibrillation from atrial enlargement and fibrosis.

Hirose's data argue that a focal, non-reentrant mechanism can produce atrial and ventricular fibrillation. Specifically, the genetic activation of Gαq-coupled signaling promotes cardiac hypertrophy which would enlarge the atria in Gαq transgenic mice. Atrial fibrosis may result from enhanced Gαq signaling or from the activation of specific gene programs. This transgenic intervention enhances heart diacylglycerol content and consequently the activity of TRPC3/6. Exuberant Gαq stimulation, voltage-independent calcium entry and signaling might deplete or disrupt voltage-independent calcium stores initiating compensatory SOCC calcium entry. The acute administration of SKF-96365 would block calcium entry via TRPC3/6 and/or the Orai1/3 channels. Thus calcium entry via voltage-independent calcium channels or arrhythmogenic signaling events downstream of these channels may cause atrial and ventricular fibrillation in this model.

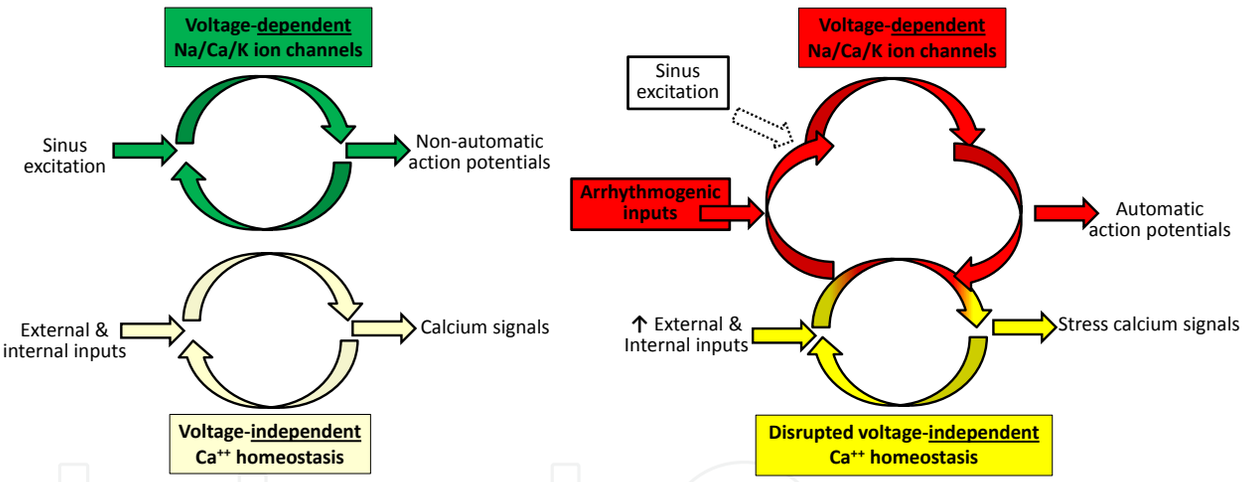

Figure 6. General Model for a Voltage-Independent Mechanism for Arrhythmia. *Left:* **Normal:** - Voltage-dependent ion channels regulate excitation-contraction coupling in normal cells while voltage-independent calcium signaling controls growth and apoptosis. *Right:* **Arrhythmogenic**: In stressed cells, voltage-independent calcium signaling subserves a novel, untoward function. It co-opts voltage-dependent ion channels to act independently of external electrical impulses and produce high frequency ectopic depolarizations. These arrhythmogenic foci of myocytes electrically capture the heart, subvert organized sinus rhythm & cause arrhythmia.

The reentry hypothesis would propose that rhythm disturbances in Gαq overexpressing mice occur because hypertrophy and fibrosis provide an 'arrhythmogenic substrate' that inhomogeneously conducts electrical impulses. In a reentrant view fibrosis, hypertrophy, and arrhythmia cannot be completely dissociated. By contrast, a focal view proposes that arrhythmia arises from cell signaling events that may be functionally distinct from those that produce fibrosis or hypertrophy; these three events may be dissociable. Hirose's SKF-96365 data support a focal view. If the atrial and ventricular fibrillation in these mice were self-

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sustaining and provoked by atrial enlargement and fibrotic substrate, they should not have reversed abruptly or at all. That they did suggests that cell events may indeed drive this arrhythmic activity.

These data in humans, intact animals, preparations of pulmonary vascular tissue, and in isolated myocytes pinpoint voltage-independent calcium homeostasis as an underappreciated source of arrhythmia (Figure 6). That is, these types of signaling events when regulated and occurring at normal levels allow hearts to increase mass in response to hypertrophic stimuli. By contrast, the dysregulation or hyperactivity of one or more aspects of voltage-independent calcium entry or downstream signaling appears to elicit spontaneous sporadic or high frequency ectopic depolarizations in intact atria and ventricle. Consequently some arrhythmia might be purely a cell's response to extra- or intra-cellular conditions that disrupt voltage-independent calcium homeostasis. Note that in contrast to 'calcium leak' models which often require burst pacing to induce atrial (*or ventricular*) arrhythmia [40,54,55], the disruption of voltage-independent calcium homeostasis results in intact heart muscle spontaneously producing profound complex arrhythmia.

While provocative these evidences for a focal mechanism for arrhythmia leave unanswered at least four questions.

- Which part or parts of voltage-independent calcium homeostasis underlie this arrhythmic activity, (a) calcium release through the IP3R, (b) calcium entry via one of more of the TRP channels, (c) calcium entry via the Orai channels and/or (d) calcium signaling downstream of these channels?
- Can this novel mechanism for arrhythmia account for the gamut of ectopic activities from sporadic depolarization to paroxysmal or sustained tachycardia to fibrillation?
- How might pathological stimuli or high autonomic activity favor the activation of this arrhythmogenic mechanism?
- What is the final molecular initiator of this putative focal mechanism for arrhythmia?

Work from our laboratory has begun to address these questions using the following rationale.

Lewis [98,99], Putney [78], Shuttleworth [64] and others identify voltage-independent calcium homeostasis as a dynamic process that depends on the inter-relationship between multiple families of calcium channels and the filling state of intracellular calcium stores. In this model Gαq agonists provoke calcium entry via TRPC3 as well as the release of calcium from internal stores regulated by IP3Rs. These calcium entry and release events sum to generate intracellular signals which are then terminated by re-accumulation of calcium into the endoplasmic reticulum lumen. The net flux of calcium out of the reticular lumen is a sum of all inputs experienced by a cell under any particular physiological or pathophysiological conditions. As one or more of these agonist signals increases in intensity, the local or the net calcium content of the reticular calcium stores begins to decrease. As stores deplete, the [Stim1-Orai1/TRPC1] channel complex activates to refill them, permitting continued calcium signaling. Excessive or continual calcium store depletion initiates a strong SOCC calcium entry response. Earlier studies suggest a potent arrhythmic effect associates with excessive or

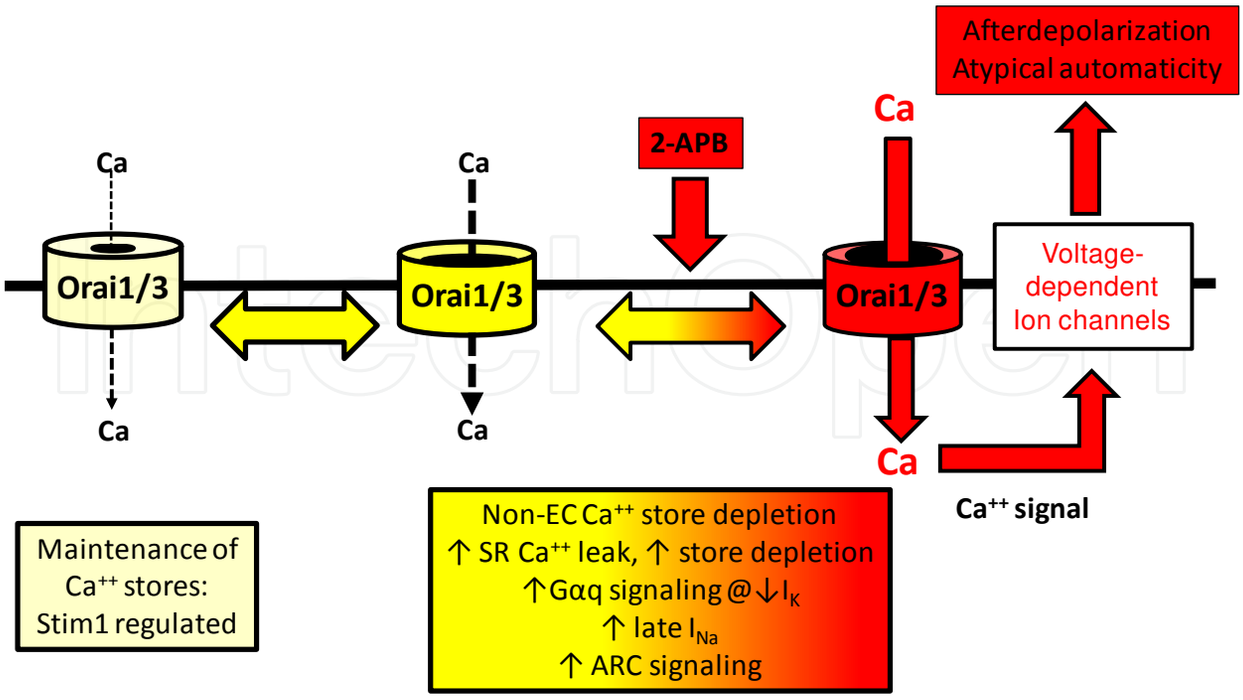

Figure 7. Model for Orai Arrhythmogenesis.*Left:* Under normal conditions Orais are tightly regulated and not arrhythmogenic. *Center*: Low level dysregulation (or activation) of Orais by numerous factors (*Lower right box*) will produce a progressive arrhythmic effect. At intermediate levels the calcium signal will provoke afterdepolarization. *Right*: At high levels of Orai opening the calcium signal co-opts myocyte voltage-dependent ion channels to provoke ~20Hz atypical automaticity and fibrillation. 2APB causes automatic tachycardia and fibrillation in this manner.

dysregulated activation of this overall pathway [57-61]. The interpretation of this data focused on calcium release through the IP3R and showed that pharmacological blockade or genetic ablation of this protein suppresses ectopic electromechanical activity. However, (a) Gαq stimulation, (b) voltage-independent calcium release from the IP3Rs, and (c) Orai-linked SOCC calcium entry are interrelated events [64,98]. Thus exuberant arrhythmogenic Gαq stimulation [96] or IP3R calcium release [91,92] will activate Orai-linked calcium entry (Figure 7). Previous experiments did not fully address whether the first two of these voltage-independent events or the third one, Orai-linked calcium entry, might drive arrhythmic activity. Thus we wished to test whether increased Orai channel opening might be an unrecognized arrhythmic principle (Figure 7). This requires a means to activate the Orais.

Putney reported [100] that 2-aminoethoxydiphenyl borate (2APB) activates calcium entry in non-excitable cells apparently by a store-operated mechanism. Subsequent work [101-102] conclusively demonstrated that 2APB pharmacologically opens Orai1 with an EC_{50} of 20μM and Orai3 with an EC_{50} of 13μM. 2APB also alters the ion conduction properties of these channels to enhance sodium transport. We took advantage of this Orai channel opener to interrogate in a crude manner whether activating voltage-independent calcium channels might underlie a focal mechanism for arrhythmia.

We found that 2APB provokes a novel type of arrhythmic activity [103-106] which appears to satisfy the demands of the focal source hypothesis of Engelmann and Scherf. In particular,

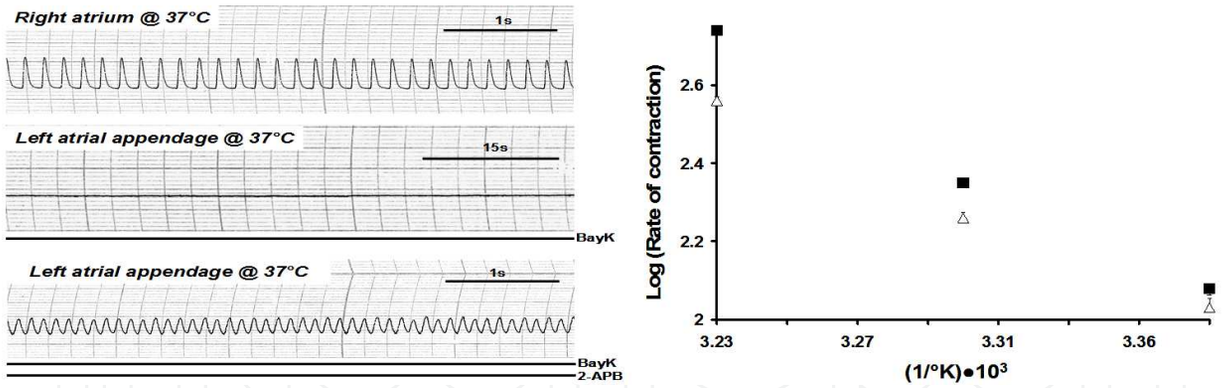

Figure 8. 2APB Activation of 10Hz Atypical Automaticity in Superfused Rat Left Atria. *Left. Upper:* The mechanical function of an unpaced rat right atrium superfused at 37°C. Spontaneous sinoatrial node-driven normal automaticity occurs at —6Hz in this muscle. *Middle:* An unpaced rat left atrial appendage superfused with 300nM Bayk at 37°C. This left atrium and all others do not contract under this condition. *Bottom.* An unpaced rat left atrium superfused with 300nM BayK and 20μM 2APB. This muscle produces spontaneous mechanical activity at 10Hz. *Right.* Summary of groups of unpaced right atria (Δ; n=7) superfused at 23, 30, and 37°C and unpaced left atria superfused with BayK and 20μM 2APB (■; n=9) at 23, 30, and 37°C. Left atria treated with BayK and 2APB perfused at 37°C spontaneously contract at rates of 543±13 contractions/minute.

intact, superfused normal rat left atria and rat left ventricular papillary muscles begin to spontaneously contact when they are challenged with 2APB at concentrations greater than 10μM. This ectopic activity takes several minutes to arise following muscle exposure to 2APB but once initiated it occurs persistently until this borinate is removed from the superfusate. Increasing muscle calcium by several disparate means including slow channel activation and ouabain markedly increases the rate of this ectopic activity [104 *see Table I*]. Under well-defined conditions isolated left atria and left ventricular papillaries can produce persistent ectopic activity at rates of at least 10 to 12 Hz at 37°C (Figure 8). These rates are similar to those reported for arrhythmic drivers of clinical and experimental arrhythmia [107]. Reentrant mechanisms are usually invoked to explain such drivers but cell-based focal means may also exist to provoke persistent high frequency ectopy. The disruption of heart muscle electromechanical stability by 2APB is not self-sustaining as this high frequency ectopy stops immediately after the removal of this molecule from the superfusate. One implication from this reversible destabilization is that a cell-based voltage-independent mechanism for arrhythmia would produce electrical instability only as long as it remains stimulated. Paroxysmal or persistent arrhythmia thus might result if the pathological disturbance of voltage-independent calcium homeostasis were ephemeral or unrelenting in nature regardless of the presence or absence of arrhythmogenic 'substrate.' Hirose's work and our data in fibrillating hearts discussed in a following section substantiate this speculation.

2APB induces a unique atypical automaticity in non-automatic heart muscle. Specifically, 2APB provokes high frequency ectopic action potentials and muscle contraction even when added to the superfusate of quiescent left atria or left ventricular papillary muscles [105 *see Figure 3*]. That is, non-automatic heart muscle which normally requires external stimulation to produce action potentials and contract will do both spontaneously, at high-frequency, and in the absence of a triggering depolarizing stimulus if these quiescent muscles are ex-

posed to 2APB. The action potentials produced by these spontaneously contracting muscles are identical to those produced by electrically paced untreated muscles [105 *see Figure 2 & Table I*]. That is, exposing non-automatic normal left atria and papillary muscles to >10μM 2APB causes them to produce spontaneous normal action potentials and muscle contractions at extremely high frequency in the absence of an external electrical stimulus. Under all other conditions, these muscles require an external electrical stimulus to generate an action potential and contract (Figure 8 Left; *middle panel*). These ectopic action potentials also occur from normal resting potentials and have no visible Phase 4-type depolarization. These criteria rule that 2APB induces neither a triggered activity nor typical abnormal automaticity as defined earlier. Heart muscle thus may contain a cryptic pathway whose activation transforms non-automatic tissue to a fully automatic state.

Several pharmacological studies assessed whether 2APB provokes atypical automaticity through the activation of Orai channels. SKF-96365, an inhibitor of calcium entry via the TRP and the Orai channels, completely prevents or reverses 2APB-linked atypical automaticity [105 *see Figure 3 & 5*]. Importantly, if paced left atria or papillary muscles are exposed to 2APB, they produce electromechanical activity independently of the pacing stimulus. That is, they produce both paced and 'spontaneous' electromechanical events. SKF-96365 added to the superfusate stops only the spontaneous ectopy which occurs independently of pacing. These isolated muscles then follow the pacing stimulus faithfully, requiring external pacing to contract or produce action potentials [105 *see e.g. Figure 5*]. ML-7, a congener of a second inhibitor of Orai-linked calcium entry [108], also suppresses this high frequency atypical automaticity as do two calmodulin inhibitors [105 *see Figure 6*]. These data suggest that the Orai channels and cell calcium signaling participate in converting non-automatic muscles to an automatic state. These atrial and papillary muscles weighed 3 to 5mg wet-weight. Thus they were unlikely to support reentry based on the criteria of Garrey and Moe. The high frequency automaticity they produced following the presumed activation of the Orai channels could, however, form focal sites of paroxysmal or permanent electrical instability in intact heart muscle.

Some current hypotheses for focal arrhythmia require high levels of SR calcium (SR calcium load) to drive afterdepolarization or more complex arrhythmia [40, 41,55]. In support of this view, increased muscle calcium has long been known to favor arrhythmogenesis. However, SR calcium load decreases in heart failure, a condition that also exhibits high rates of arrhythmia. Thus muscle calcium load appears to not always associate with ectopic activity. Despite this paradoxical complication, a great deal of exquisite experimental expertise has defined SR calcium leak rates in relation to SR calcium load with an eye toward explaining arrhythmogenic activity in failing heart [109]. Rates of 2APB atypical automaticity increase with increases in muscle calcium, so we wished to test whether SR calcium stores were, in fact, critical to the persistence of atypical automaticity. Left atria were challenged with BayK 8644 to increase their calcium content and then with 2APB to provoke high frequency atypical automaticity. These unpaced muscles produced persistent action potentials and contractions at a very fast rate (Figures 9A & 9B). Treating these muscles with 800nM ryanodine greatly reduces their force of contraction, evidence for ryanodine receptor opening and near

complete SR calcium store depletion (Figure 9C). Interestingly, these muscles continue to produce spontaneous action potentials at a high rate (Figure 9D) with no discernible difference in their characteristics compared to action potentials produced in 'calcium loaded' muscles (cp. Figures 9B & 9D). Treating these spontaneously contracting muscles with SKF-96365 abolished any residual automatic contractions (Figure 9E), and these muscle now required pacing to produce action potentials (Figure 9F, *cp. Rest with 3Hz pacing*).

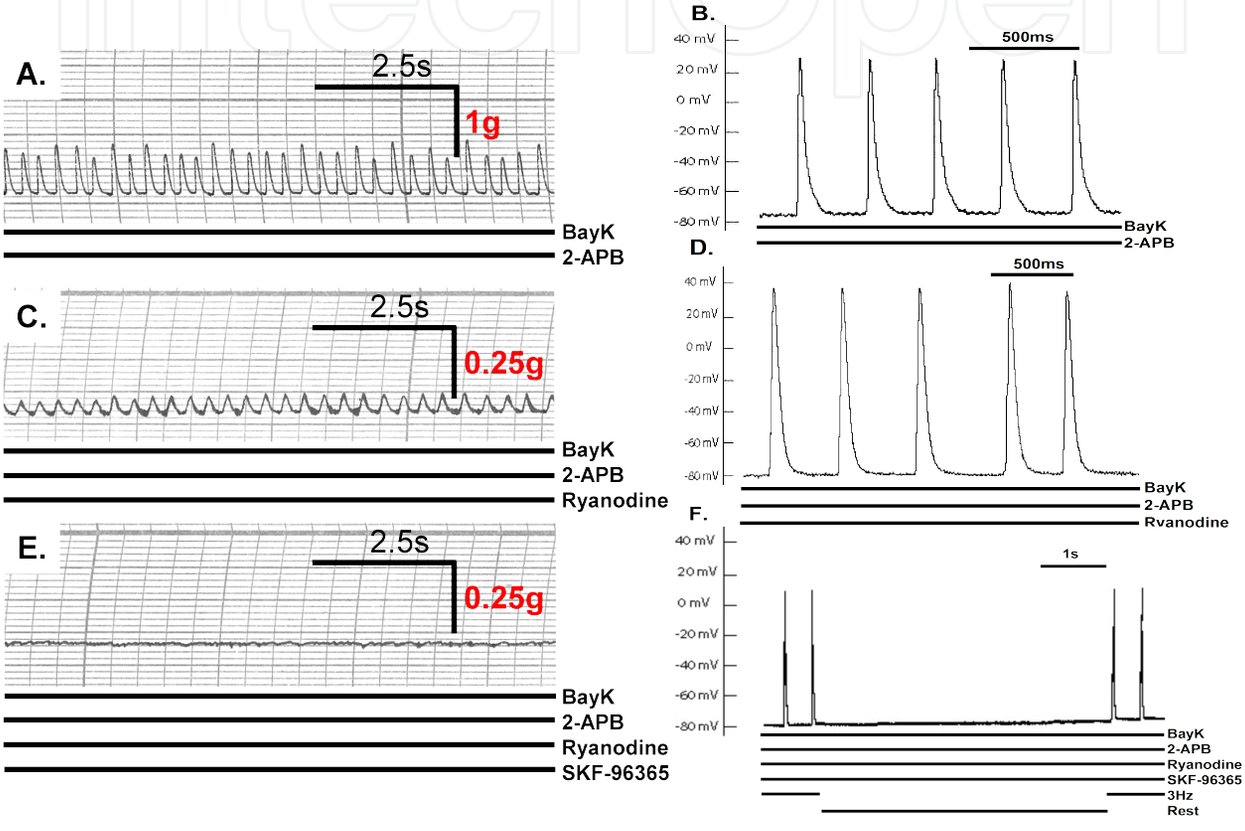

Figure 9. Ryanodine Depletion of Left Atrial SR Calcium Does Not Affect 2APB Atypical Automaticity. (A) Mechanical function of an *unpaced* left atrium treated with 300nM BayK 8644 (BayK) and 22μM 2APB (2APB). Atypical mechanical automaticity is observed. **(B)** Action potentials from an *unpaced* left atrium treated as in (A). Spontaneous, automatic action potentials are recorded. **(C)** Mechanical function of a left atrium treated as in (A), ~8min after exposure to 800nM ryanodine (*Ryanodine*). Ryanodine depresses mechanical function by opening the ryanodine channel which leaks SR calcium (*N.B.* force scales on right of (A) & (C)). Automatic mechanical activity persists but at low levels. **(D)** Electrical activity of a left atrium treated as in (C). Spontaneous electrical activity continues unabated. **(E)** Mechanical function of an atrium treated as in (C) followed by SKF-96365 (*SKF-96365: 50μM*). SKF-96365 blocks spontaneous mechanical activity. **(F)** Electrical activity of a left atrium treated as in (E) in the absence (*Rest*) or presence (3Hz) of 3Hz pacing. No action potentials are recorded in the presence of SKF-96365; it blocks spontaneous ectopy. Pacing is required to generate action potentials (3Hz).

We then compared how an alternate calcium efflux enhancer, caffeine affected the rate of atypical automaticity. In these experiments left atria were not 'loaded' with calcium before they were exposed to 2APB; loading was avoided to assess how the disruption of normal calcium stores affected automaticity.

Impressively, 10mM caffeine markedly increased the rate of left atrial spontaneous contraction from 106±25 to 362±38 contractions per minute (Figure 10). This significant increase in the rate of atypical automaticity was transient as after 3 to 5 minutes of exposure to these conditions the rates of automaticity decrease to 10±7 per minute. Normal paced mechanical function remained intact albeit at lower forces of contraction because of caffeine treatment (Data not shown).

This notable result leads to four interesting speculations. First, the rates of atypical automaticity measured in caffeine-treated muscles at 30°C are about 50% faster than those measured for 'calcium loaded' atria at the same temperature [104-105]. Thus calcium loading does not produce the most rapid rates of automaticity, caffeine a calcium efflux agent does. Second, the rate of atypical automaticity does not greatly slow with the depletion of ryanodine-sensitive SR calcium. Thus a distinction must exist between ryanodine and caffeine in their interaction with the source of atypical automaticity. Third, if the rate of automatic activity observed with caffeine treatment exhibits identical Q_{10}s as our earlier data (Figure 8), then this type of atypical automaticity might reach rates of ~15Hz at 37°C. Fourth, ryanodine calcium stores do not appear to greatly influence atypical automaticity indicating that this form of ectopic activity may occur readily at low and at high muscle calcium loads.

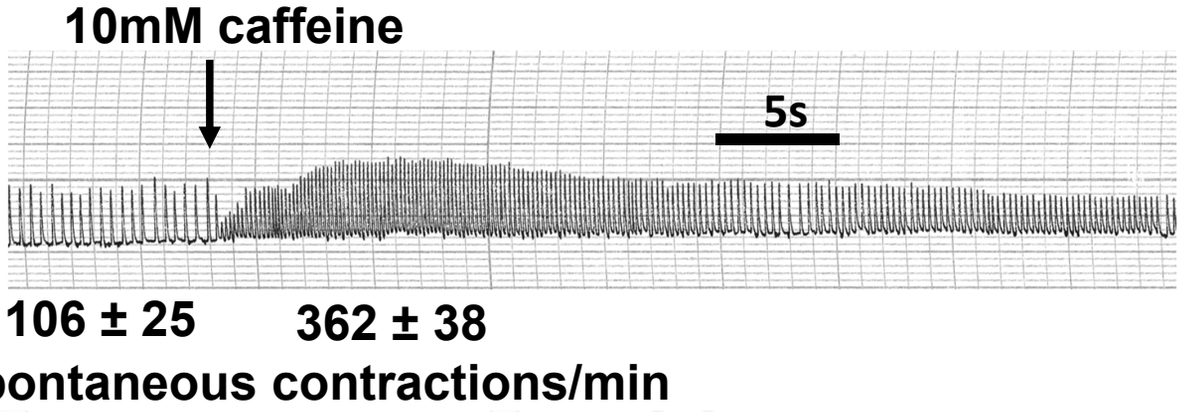

Figure 10. Caffeine Transiently Accelerates the Rate of Atypical Automaticity. Rat left atria (n=6) were exposed to 30μM 2APB in the absence of calcium loading agent. These muscles spontaneously contracted sporadically at a rate shown at the left of the figure. Muscles then were rapidly exposed to superfusate containing 30μM 2APB and 10mM caffeine. This treatment greatly increased the rate of atypical automatic activity to over 350 contractions per minute. After 3-5 minutes under these conditions, the rate of automaticity decreased to 10 contractions per minute.

The literature contains one possible explanation for the difference between the ryanodine and the caffeine responses observed in automatically contracting left atria. Corda noted that while ryanodine affects calcium leak from internal stores it does not activate a voltage-independent store-operated response [110]. By contrast, caffeine does. That is, in their experimental system, exposing cultured cells to caffeine provoked a prominent entry of calcium presumably through the Orai-linked store-operated channel. This result supports a contention that voltage-independent calcium entry and downstream signaling are the source for atypical automaticity (Figure 11). Many more experiments are needed to establish this possibility.

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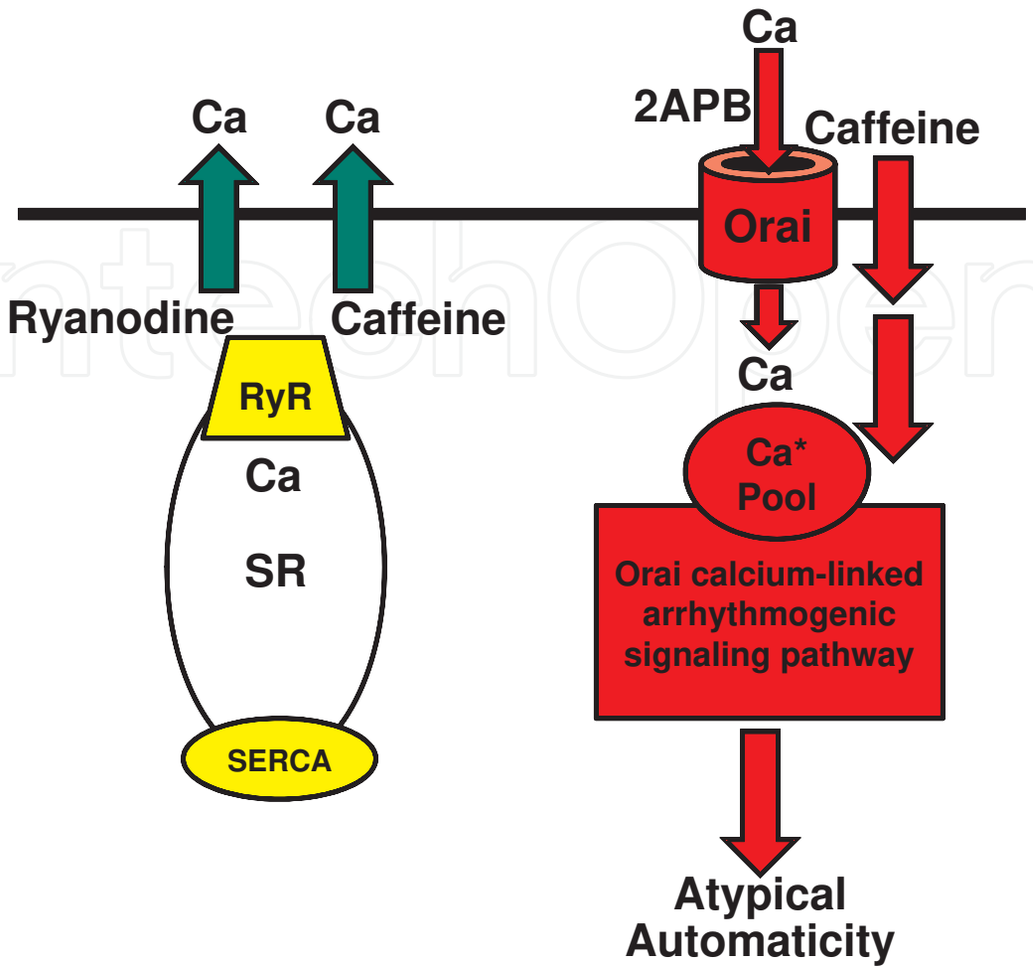

Figure 11. Interpretation of Ryanodine-Caffeine Effects on Atypical Automaticity.*Left.* Both ryanodine and caffeine deplete SR calcium stores. *Right.* Caffeine accelerates store-operated calcium entry in some experimental settings [110]. We suggest this latter effect of caffeine enhances the rates of automaticity (Figure 10).

Thus heart may possess two ways to produce action potentials that provoke contraction. First is the well-known and long-studied pathway whereby an external input derived either from the sinoatrial node or from a pacing stimulus causes myocyte depolarization. Second, an activator of the voltage-independent Orai channels appears to uncover a pathway whose activation allows non-automatic muscles to produce normal action potentials and muscle contraction independent of an external stimulus. This atypical automaticity can occur sporadically or at high frequency depending on the calcium loading of isolated muscles.

All of these experiments were performed in intact superfused muscles. Consequently, we harbored concern that conditions like hypoxia might influence our results. To address this point rat hearts were perfused in the Langendorff mode to test how 2APB affects well-oxygenated muscle. These perfusions also assessed (a) whether high frequency 2APB-induced automaticity requires muscle calcium loading and (b) how 2APB affects hearts with an intact conduction system in sinus rhythm. The first point arises because the isolation of atria or

papillary muscles might unload unique cell calcium pools that are critical for instigating atypical automaticity. Hence the effect of the calcium loading of isolated muscles to increase the rate of 2APB automaticity [104] might be mistaken to reflect loading of the SR pool involved in contraction rather than the concurrent loading of a pool central to atypical automaticity. The second point addresses questions by Boyden and ter Keurs [88].

Perfused hearts yield several important and impressive results. First, the perfusion of hearts with 5μM 2APB generates spontaneous, sporadic electrical activity from multiple sites, a conclusion based on the morphology of these ectopic depolarizations [106 see Figure 2]. Increasing the perfusate 2APB concentration produces striking changes in the electromechanical activity of these hearts [http://www.dom.uab.edu/pwolkowicz/IPH_Fibrillation2-APB.mov]. After a few minutes of perfusion with 22μM 2APB hearts begin to produce spontaneous but broad QRS complexes and contract at rates that increase from sinus rhythm (~5Hz) to upwards of 12Hz [106 *see Figures 3 & 4*]. Heart mechanical function briefly follows this ectopic electrical activity but electro-mechanical dissociation occurs as ectopic electrical activity continues to steadily increase in rate to apparent values of ~20Hz. These hearts lose the ability to generate organized mechanical activity which may reflect a form of fibrillatory conduction originating from high frequency focal sources [25,111]. A brief but impressive increase in diastolic pressure from 5mm to ~60mmHg accompanies electromechanical dissociation. A minute or so later fibrillating hearts reach and then maintain a resting tension of 30mmHg [106 *see Figures 3 & 4*]. Coronary flow remains normal during fibrillation and persistent contracture lessening the possibility that ischemia occurs in these preparations.

The Orai and TRP channel inhibitor SKF-96365 reverses this ventricular fibrillation in an intriguing way [106 *see Figure 5*] [http://www.dom.uab.edu/pwolkowicz/IPH_SKF-96365-Reversal.mov]. After a few minutes of perfusion with 2APB and 20μM SKF-96365, the electrical disorganization recorded by our bipolar electrodes begins to resolve. The resolution of electrical fibrillation occurs quickly over about one second but at first it is only a transient event as electrical instability reappears immediately after this initial flash of stability. As the time of perfusion with SKF-96365 increases, periods of stable electrical activity get longer until sinus rhythm is restored. In all cases, the temporal interface between fibrillation and electrical quiescence produce a rapid decrease in resting tension from 30mmm to about 8mmHg, mechanical quiescence, and then normal, potentiated mechanical contractions [106 *see Figure 5*]. This very rapid restoration of normal diastolic pressure and electro-mechanical quiescence most likely are not caused by a decrease in bulk cytosolic calcium throughout the heart. We suspect this change likely reflects the interdiction by SKF-96365 of signaling events initiated by 2APB. Note that 2APB remains in the perfusate throughout these experiments. These electrical results remarkably mimic those reported by Hirose for Gαq overexpressing mice [96]. Together these data indicate that SKF-96365 suppresses fibrillation caused by genetic enhancement of the initiation site for voltage-independent calcium signaling [96], the Gαq receptor, and by perfusion with a pharmacological activator of Orai channel calcium entry, which might occur in Gαq transgenic mice for reasons stated earlier. The concentrations of 2APB used in our work inhibit both the IP3Rs and many of the relevant TRPC channels. Thus the voltage-independent Orai channels, either Orai1 or Orai3, appear

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as a likely source of arrhythmia. If the model for the functional inter-relationship of voltage-independent calcium channels and calcium pools proposed by Shuttleworth [64], Lewis [98] and others is correct, then (a) inputs which excessively stimulate the Orai channels or (b) a yet-to-be-identified cell signaling system which mirrors the action of 2APB on the Orai channels might create focal sources of high frequency atypical automaticity in muscle as small or smaller than 3mg wet-weight. The duration of this automaticity would depend on the presence of input signals that stimulate voltage-independent calcium entry.

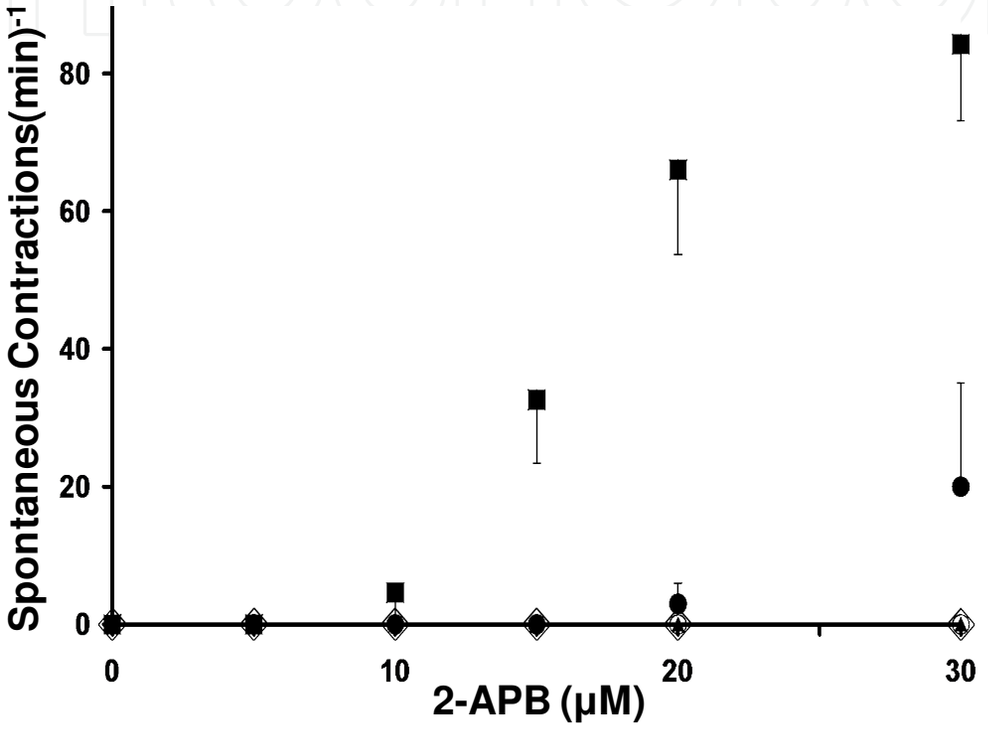

Figure 12. BcI2 Antagonists Block 2APB Atypical Automaticity. Rat left atria (n=5 per group) were superfused and paced [105]. Atria were left untreated (▪) or were pre-incubated with 80μM methoxy-antimycin A3 (•) 80 μM HA-14-1 (◊), 30 μM EGCG (o), or 30 μM gossypol (x). Increasing concentrations of 2APB were added to the superfusate for 3min at each concentration. The pacing stimulus then was stopped and the rate of spontaneous atrial contraction was recorded. All four bcl2 antagonists suppressed 2APB automaticity.

If voltage-independent calcium entry via the Orai channels initiates atrial and ventricular electrical instability *in vivo*, then this putative fourth mechanism would offer (a) a dynamic means to explain arrhythmia and (b) a mechanism which extends the three current hypotheses to explain these disorders. Dynamism arises because a multitude of physiological and pathophysiological inputs directly stimulate or indirectly activate the Orai channels [64,75,98]. The apparent arrhythmogenicity of calcium entry via the Orai channels may extend the 'calcium leak' hypothesis for arrhythmia (Figure 5). Specifically, certain types of SR calcium leak may favor the compensatory activation of voltage-independent calcium entry [112]. Thus coupling SR calcium leak and voltage-independent calcium entry may increase the depolarizing influence which calcium leak exerts on compromised myocytes. This could contribute to the increased propensity for arrhythmia observed in 'leaky' failing hearts.

With respect to 'abnormal' automaticity, exuberant Orai channel activation might produce automatic foci under conditions that do not require partial myocyte depolarization. Regarding reentry, if dysregulated Orai channels were *in vivo* sources of >10Hz atypical automaticity, then they could lead to ectopy at rates that promote atrial (*and ventricular*) fibrillatory conduction which begins at 6Hz [111]. Finally, if this fourth mechanism of voltage-independent arrhythmogenesis were to hold in Purkinje cells, it might explain some types of idiopathic ventricular fibrillation [113].

We are cognizant that the available data allow for only the barest of frameworks for the hypothesis that voltage-independent calcium entry and signaling are important sources of focal arrhythmia. Undoubtedly multiple unexpected cellular signaling intermediates participate in this pathway and they may provide new targets for anti-arrhythmics. For example, bcl-2 is a small protein that regulates the intrinsic pathway for apoptosis through its interaction with mitochondria. By contrast, Distelhorst [114] has championed the concept that bcl-2 binding to the IP3R suppresses calcium signals related to apoptosis while enhancing signals related to cell survival. Sub-sets of bcl-2 bind to the endoplasmic reticulum and the plasma membrane [115]. Thus bcl-2 might play a role in the atypical automaticity which 2APB induces. To test this possibility rat left atria were superfused and paced at 0.1Hz as previously described [105]. Left atria (n=5 per group) were then left untreated or were pretreated with 80μM HA14-1 and methoxyantimycin A, two cell permeable inhibitors of bcl-2, or with 30μM of two naturally occurring bcl-2 inhibitors EGCG and gossypol [116-119]. Increasing concentrations of 2APB from 0 to 30μM were added to the superfusate and the rate of spontaneous activity was recorded after three minute incubation at any concentration. All four bcl-2 inhibitors significantly or completely prevented atypical automatic activity (Figure 12). EGCG and gossypol were tested for their ability to reverse high frequency atypical automaticity in left atria treated with BayK 8644 and 20μM 2APB. Both naturally occurring bcl-2 inhibitors reversed this automatic activity but did not affect normal paced muscle contraction (Figure 13). Thus bcl-2 may play a role in this type of ectopy but these provocative data require significant follow-on experiments to more firmly establish this conclusion.

The evidence summarized in this review suggests the existence of a cell mechanism to generate focal arrhythmia. Some limitations must be addressed to afford a more sound footing for the concept that focal, cellular sources of arrhythmia arise from the activation of voltage-independent calcium channels.

- How does the activation of voltage-independent calcium channels produce electromechanical instability in intact heart muscles (Figure 7, Calcium signal)? Does voltage-independent calcium entry *per se*, that is calcium acting as a charge carrier, activate this high frequency ectopy? Or, do calcium signaling events specific to this type of channel lead non-automatic heart muscle to become automatic? We favor the latter view. Supporting this possibility calmodulin inhibitors suppress atypical automaticity in heart muscles treated with 2APB [105]. By contrast CaMKII inhibitors do not [105]. Thus calmodulin targets other than CaMKII may be involved in this automatic activity.
- What molecular entities lie between voltage-independent calcium signaling and automatic depolarization at rates of 10-12Hz? While a current view would favor calcium ions themselves as the arrhythmogenic principle, we have preliminary data that will be pub-

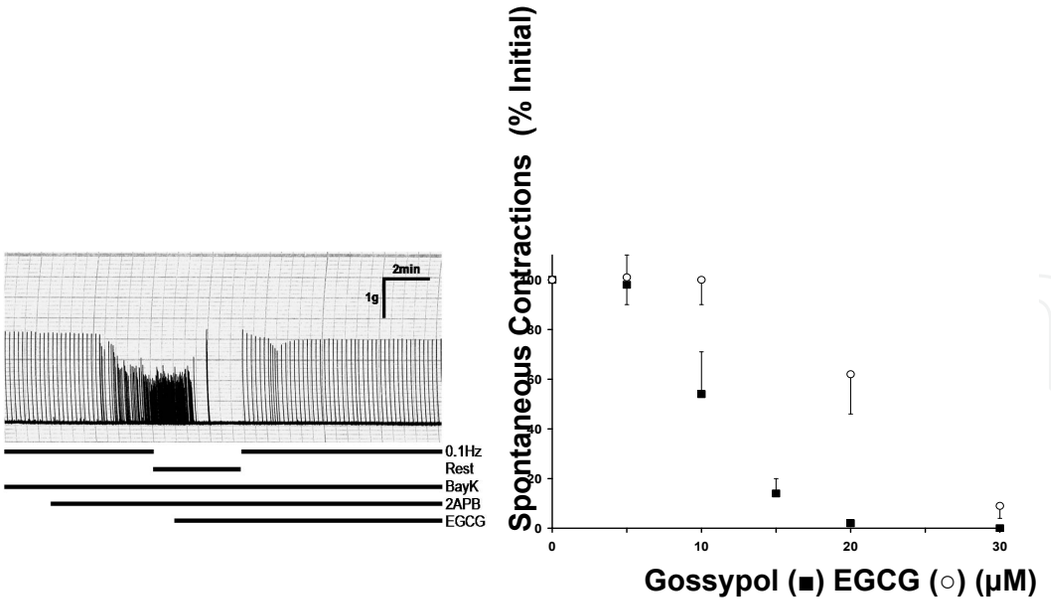

Figure 13. Naturally Occurring bcl2 Antagonists Suppress 2APB Atypical Automaticity.*Left.* Rat let atrium was superfused, paced at 0.1Hz, and treated with 300nM BayK 8644 [105]. 2APB (22μM) was added where indicated (2APB). After two to three minutes this atrium began to spontaneously contract at a high rate (*dark area in center of trace*). The pacing stimulus then was stopped. (*Rest*). Atypical automatic activity persists unabated. EGCG (30μM) was added where indicated (EGCG). Shortly thereafter, atypical automaticity ceased and this *now* **unpaced** muscle became quiescent. The 0.1Hz pacing stimulus then was reinstated and the left atrium faithfully followed it (*Right 0.1Hz*). *Right.* Average of exeriments like the one shown to the left where increasing concentrations of gossypol and EGCG were used to staunch atypical automaticity. Gossypol induced a 50% reduction at 10μM while EGCG required 25μM.

lished under separate auspices which demonstrate that these signaling events profoundly change the fundamental characteristics of heart voltage-dependent ion channels. We believe these fundamental changes underlie the fourth mechanism for arrhythmia we propose here.

- Can this voltage-independent mechanism provide a connection between afterdepolarization and sustained triggered activity/high frequency atypical automaticity? To test this possibility we investigated whether voltage-independent calcium channels also participate in the triggered activity that occurs with increased late sodium current. We find that atria treated with appropriate concentrations of sea anemone toxin type II [33] produce triggered early afterdepolarization in a steady-state manner. Our preliminary data to be published elsewhere show that (a) the ARC channel inhibitor LOE-908, (b) an antibody that binds plasma membrane Stim1 which is required for ARC channel activity, (c) the Orai inhibitor SKF-96365, (d) the CaMKII inhibitor KN-93, and (e) several inhibitors of the calcium-dependent cytosolic phospholipase A_2 all suppress late sodium current-induced triggered activity. Importantly, we assessed whether SKF-96365 shortens action potential duration in atria treated with sea anemone toxin. It does not. Also neither LOE-908 nor KN-93 suppresses the high frequency automaticity which 2APB induces.

Thus related panels of voltage-independent calcium signaling inhibitors suppress early afterdepolarization and/or 2APB automaticity. We propose that the calcium loading which oc-

curs with increased late sodium current stimulates cytosolic phospholipase A_2 to produce the arachidonate ligand for ARC (Figure 14). Calcium entry through the voltage-independent ARC calcium channel may activate CaMKII which participates in provoking early afterdepolarization. Conditions in which calcium stores begin to deplete would stimulate voltage-independent calcium entry through Orai1 [64]. Greater store depletion resulting from exuberant ARC channel activity would lead to fulminant voltage-independent calcium entry through the Orai1 calcium channel [64] and possibly a 'sustained triggered activity' which resembles 2APB-linked automaticity.

Are there intracellular, naturally occurring activators of Orai1 or Orai3 that mimic 2APB? At present none are known but such Orai-activators would link cell signaling with focal arrhythmogenesis. The interrelationship between ARC and store-operated voltage-independent calcium entry [64] may be one such link.

The following four figures outline a putative mechanism through which voltage-independent calcium signaling might induce afterdepolarization, sporadic and high-frequency atypical automaticity.

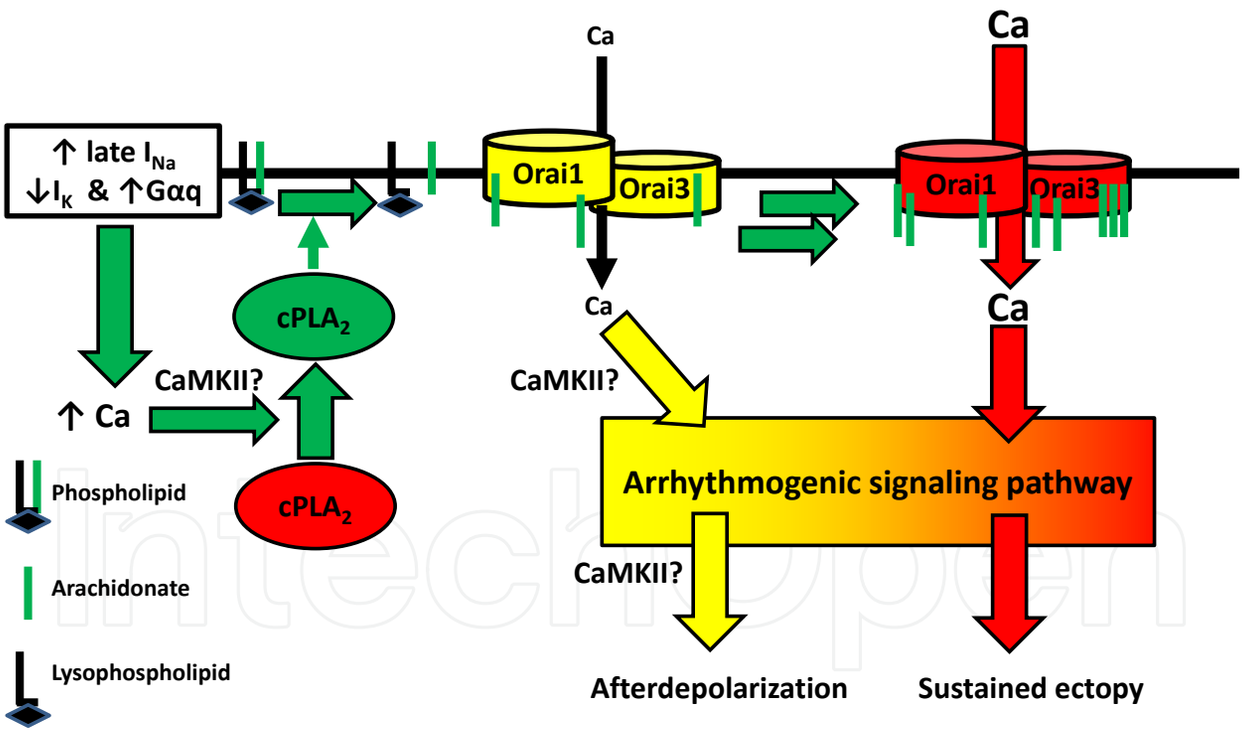

Figure 14. Model linking voltage-independent ARC channel and late sodium current early afterdepolarization. *Left.* Late sodium current loads myocytes first with sodium and then with calcium [33]. This loading activates cytosolic phospholipase A_2 (cPLA$_2$) to hydrolyze membrane phospholipids and release free arachidonate. *Right*. This eicosonoid activates the Orail/Orai3 pentamer ARC channel which permits calcium entry into myocytes. This also disturbs intracellular calcium stores [64]. CaMKII may participate in this pathway by phosphorylating cPLA$_2$ or elsewhere. Low level ARC activity leads to afterdepolarization. More intense ARC activity depletes myocyte calcium stores, opens Orais and provokes high frequency sustained triggered activity. Both events require the co-opting of cardiac voltage-dependent ion channels to produce spontaneous depolarization.

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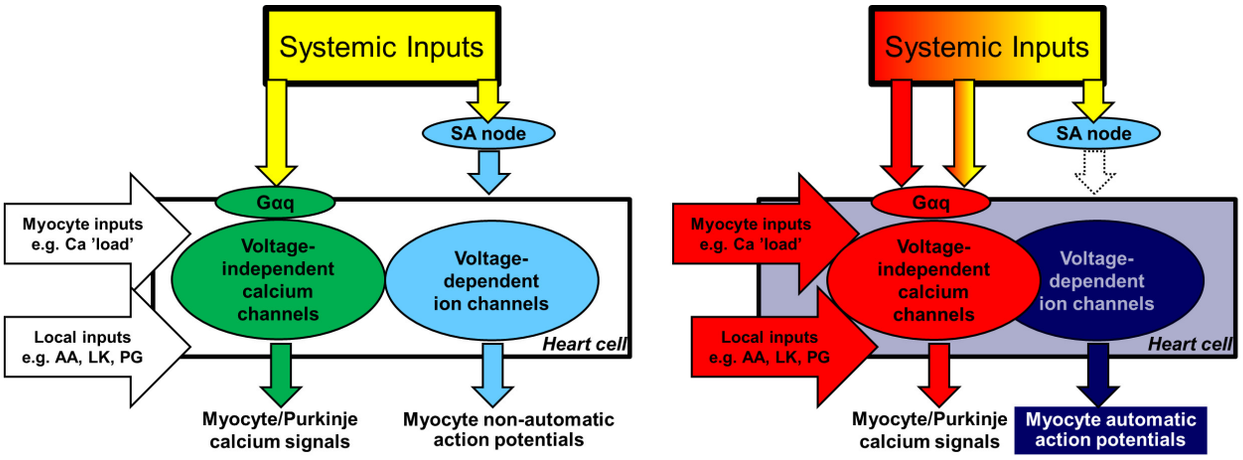

Figure 15. A Putative Mechanism for Focal Ectopy. *Left.* In excitable cells, voltage-independent & voltage-dependent ion channels co-exist. Both sets of channels respond to common or distinct inputs to produce (a) calcium signals for cell homeostasis and (b) action potentials. These two events are generally considered as separate and non-interacting entities [22,25,55,64,75,76]. *Right.* A wide range of systemic, myocyte, and local inputs may provoke the 'inappropriate' interaction of these two systems. This results in voltage-independent signaling events co-opting myocyte voltage-dependent ion channels. Myocytes transform from a non-automatic to an automatic state. This change can occur for an inconstant time period and it is reversible. AA=arachidonate; LK=leukotrienes; PG=prostaglandins

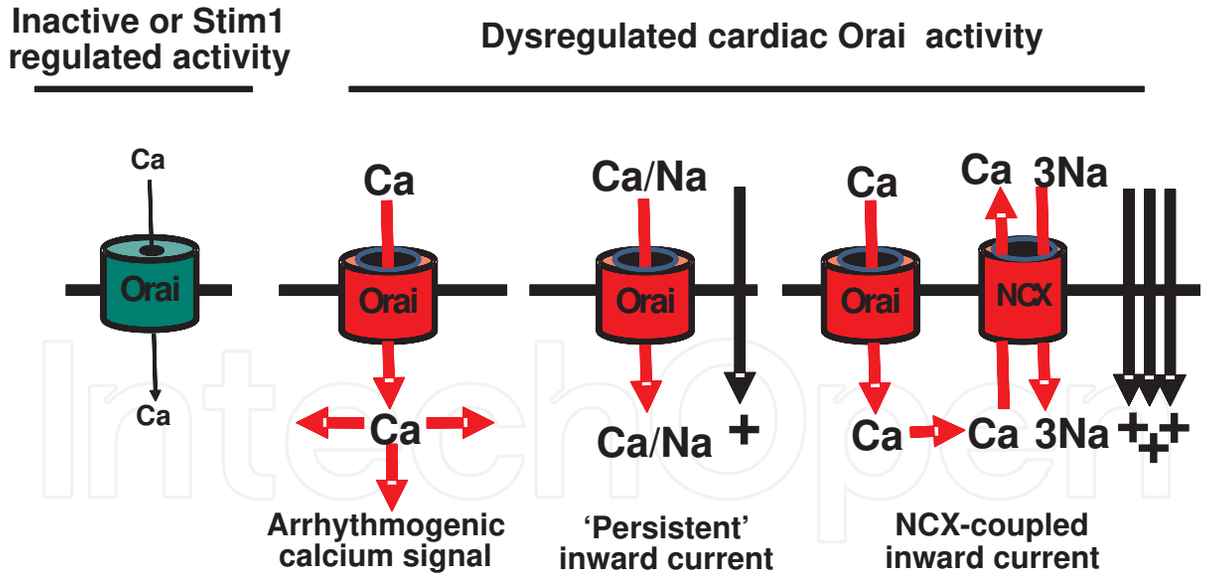

Figure 16. Potential Arrhythmogenic Mechanisms for Dysfunctional Orai Channels. Our data suggest dysfunctional Orais may be a source of focal arrhythmia. *Left.* These proteins normally are well-regulated by the Stim1 calcium sensing system & faithfully fulfill their function of refilling cell calcium stores. *Second from left.* Dysregulated Orals will enhance calcium transport which might activate a cryptic arrhychmogenic calcium signaling pathway. **We favor this view.** *Second from Right & Right*. Dysregulated Oral calcium transport may generate a persistent inward current which acts independent of changes in membrane potential associated with the action potential or Orai calcium entry may couple with the sodium-calcium exchanger (NCX) to create a futile calcium cycle that drives myocyte depolarization. Such a general type of mechanism is similar to that proposed by Huo to explain 2APB ectopy [120].

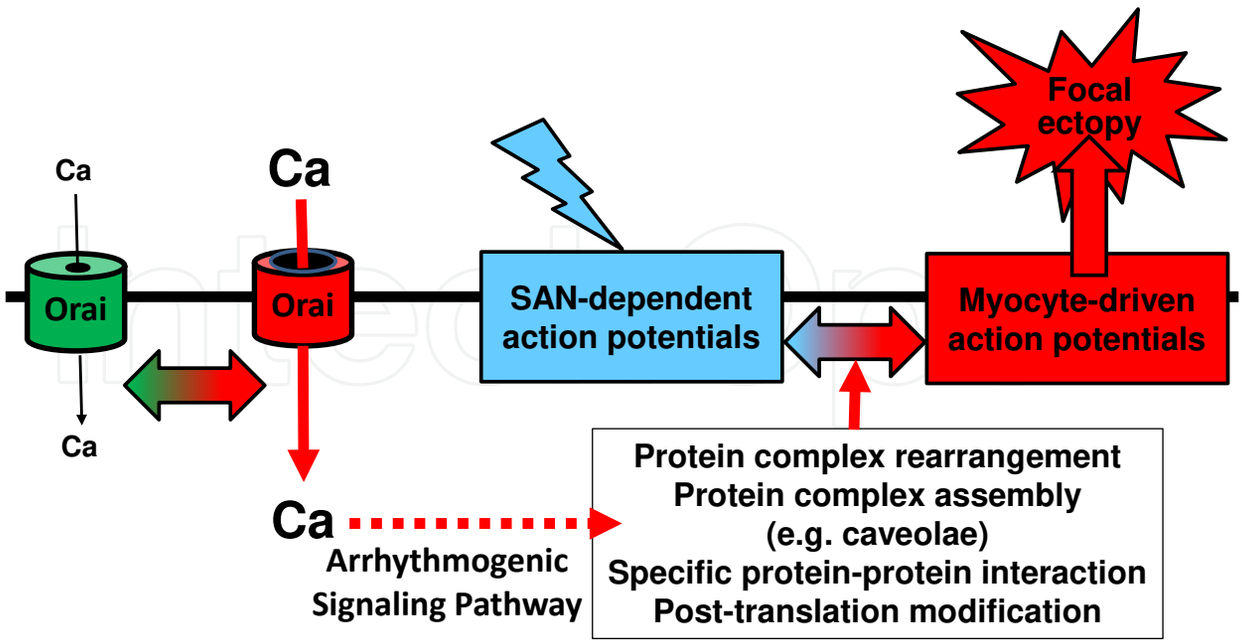

Figure 17. Potential Cellular Mechanism for Orai Atypical Automaticity. Our preliminary data favor a mechanism for Orai atypical automaticity in which calcium entry activates a signaling pathway that modifies the properties of the voltage-dependent ion channels critical to myocyte excitation. We do not have a preferred view as to how this modification occurs but the lower open box notes four possibilities we are now investigating

10. Conclusions

Atrial (*and ventricular*) arrhythmias are disturbances in electrical activity that disrupt the regular, rhythmic contraction of the upper (*or lower*) heart chambers. These electrical instabilities arise from normal or dysfunctional heart muscle, from the heart specialized conduction system or from the 'muscular sleeves' of major supraventricular vessels. Because arrhythmia is recorded as abnormal electrical activity and since experimental faradic stimulation can initiate arrhythmia, it is viewed as having a purely electrical origin. Indeed the dominant explanations for arrhythmia hypothesize it originates from (a) changes in the electrical properties of heart muscle that alter electrical impulse conduction, (b) changes in the properties of the voltage-dependent ion channels responsible for normal electrical activity which alter impulse conduction, (c) changes in the myocytic milieu that permit normal voltage-dependent ion channels to spontaneously generate electrical impulses or (d) changes in myocyte calcium homeostasis that alter heart resting membrane potential or otherwise indirectly enhance muscle excitability.

During the past fifty years, however, it has become clear that all cells contain a staggering array of interrelated signaling pathways and processes which regulate normal and 'abnormal' cell functions. Like all other cells, heart cells, too, express voltage-independent calcium signaling pathways which underlie cardiac processes like inotropy and diseases like hypertrophy or atherosclerosis. The three earlier hypotheses for arrhythmia noted above ignore

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the importance of voltage-independent cell signaling in heart ectopy. That is, they assume that voltage-independent cell signaling does not influence cardiac voltage-dependent ion channels or affect arrhythmia. One corollary of this dominant electrocentric view of arrhythmia is that the fundamental biophysical properties of voltage-dependent ion channels measured in normal muscle are the only properties these proteins can evince, that these ion channels are essentially immutable activities. However, clinical and experimental data suggest that one or more signaling events greatly influence the electrical properties of heart muscle and somehow increase its ability to generate ectopic electrical impulses.

Our data demonstrate that a recognized activator of the voltage-independent Orai calcium channels provokes persistent or paroxysmal tachycardia at rates of up to 12Hz in non-automatic rat left atrial or left ventricular papillary muscles. This activator also induces a reversible type of fibrillation in intact perfused rat hearts. These data lead to the hypothesis outlined here that calcium entry through voltage-independent ion channels, specifically through the Orai channels, and/or calcium signaling events downstream of these channels elicit ectopic electrical activity in atrial (*and ventricular*) muscle. This hypothesis implies that a wide range of extracellular and intracellular signals may disrupt heart muscle electrical stability through their actions on voltage-independent calcium homeostasis that enhance voltage-independent calcium channel activity. This hypothesis provides a framework for future experimental tests of whether voltage-independent calcium signaling related to autonomic activity, to stress or to calcium store filling state are key molecular sources for arrhythmia. Importantly, our data to be published elsewhere indicate that dysregulated voltage-independent calcium signaling alter the fundamental characteristics of voltage-dependent ion channels, transforming them from non-automatic activities that require an external depolarizing influence to automatic activities that spontaneously depolarize heart muscle. If rigorously validated, this fourth putative arrhythmogenic mechanism would satisfy the 'focal source' hypothesis for arrhythmia.

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