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Review Article

CARDIAC CALSEQUESTRIN AS A POSSIBLE TARGET FOR ARRHYTHMIA

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ABSTRACT

The term arrhythmia encapsulates all the conditions which result in the change in rhythm of the heart. It is classified broadly into two classes; tachycardia, with increased and rhythm of heart and bradycardia, with decreased rhythm of the heart. In most cases, the arrhythmias are not serious and asymptomatic, however, in some cases it leads to heart attack which becomes fatal. The present review focuses on stepwise understanding of functioning of the heart and its excitation-contraction (EC) coupling followed by the mechanism of arrhythmia and the present line of medications. The systematic flow of blood in and out of the cardiac chambers is due to the rhythmic contraction and relaxation of cardiac muscle, i.e. the EC coupling of cardiac myocyte. Any disruption in the ion flow during the EC coupling causes arrhythmia. Ca^{2+} ions act as the trigger for the excitation-contraction coupling,; therefore, understanding its movement is an essential to understand the rhythm disorders of the heart. Calsequestrin (CASQ) is the most abundant calcium buffering protein present in the sarcoplasmic reticulum of skeletal and cardiac muscles. This review further focuses on CASQ; its structure and functions. Moreover, it describes the association of CASQ with arrhythmia. With the calcium binding the CASQ attains its linear polymeric structure on the neutralization of its highly electronegative surface. The protein binds calcium with high capacity and intermediate affinity which releases and uptakes calcium reversibly during the EC coupling. Mutation in CASQ genes has been associated with catecholaminergic polymorphic ventricular tachycardia, and moreover, there are quite a few molecules which are known to bind to CASQ and bring about changes in ionic buffering properties. Therefore under suitable optimized conditions CASQ could be chosen as a novel target for cardiac arrhythmia. Scrutinizing the scope of CASQ this review presents the first in depth study suggesting cardiac CASQ as a possible target for arrhythmia.

Keywords: Calsequestrin, Arrhythmia, Tachycardia, Bradycardia, Catecholaminergic polymorphic ventricular tachycardia, Excitation-contraction coupling.

INTRODUCTION

Cardiac arrhythmias are the reason for the most sudden cardiac deaths across the globe [1-3]. The term arrhythmia encapsulates all the conditions which result in the change in rhythm of the heart. It is classified broadly into two classes; tachycardia, with increased and rhythm of heart and bradycardia, with decreased rhythm of the heart. In most cases, the arrhythmias are not serious and asymptomatic, however, in some cases it leads to heart attack which becomes fatal. Up to 50% of patients have sudden death as the very first manifestation of cardiac disease [4]. The rhythm of the heart is controlled by excitation-contraction (EC) coupling [5,6]. EC coupling is the result of the differential electric signal across the membrane [6]. The electrical signal is generated at the sinoatrial (SA) node, which travels via specific pathways to the right and the left atrium causing the atria to contract and pump blood to the lower ventricles and thereby to the body. This electrical signal is generated with the differential movement of ions. With the generation of action potential in the cardiac myocyte, calcium ions are released which in turn triggers a larger release of Ca^{2+} from the sarcoplasmic reticulum (SR) via the ryanodine receptors (RyR). This process is called as calcium-induced calcium release (CICR) [7]. With the increase in myoplasmic Ca^{2+}, myofilament gets activated to initiate contraction and thereafter the reuptake of Ca^{2+} occurs causing it to relax. Within the SR, there are located various calcium binding proteins, which releases its calcium via the RyR. Calsequestrin (CASQ) is the major calcium ion buffering protein present in the SR of skeletal and cardiac muscles. CASQ is a highly acidic protein which binds calcium upon polymerization, and releases calcium upon depolymerization, with the neutralization of its surface [8]. In the absence of calcium ions it is present as a random coil with the addition of the calcium ions it forms the thioredoxin folds of the monomers which further dimerizes and finally polymerizes [9]. The buffering ability of CASQ is directly related to its structure and, therefore, the understanding of the structure is important. The primary sequence of both CASQs is highly homologous [10]. The protein attains from three thioredoxin folds of the monomeric unit with the disordered C-terminal and N-terminal. The protein dimerizes with two contacts; the front to front and the back to back [10]. The front to front contact is established when the N-terminal of one unit inserts the other and vice versa. This contact is stabilized by salt bridging interactions [11]. C-terminals are supposed to play an important role in the back to back interaction. However, since the structure and orientation of the C-terminal are not reported, the back to back interaction is not clearly understood. Understanding of this could be the key to understand the structural details and give an insight that how changes in CASQ2 bring about arrhythmia and how it could be modulated to cure the same. Ablations in CASQ2 are known to cause a form of tachycardia, namely, catecholaminergic polymorphic ventricular tachycardia (CPVT). 15 different mutations in CASQ2 are seen to be associated with CPVT. The CPVT mutations L167H, D307H, P308L, and R33Q occurs in all the three domains of CASQ2, hence inhibits polymerization pattern, impairs Ca^{2+} buffering and causes arrhythmia [12-18]. CPVT is associated with the overexpression of the protein [11,19], inducing Ca^{2+} leak. Therefore, modulation of the calcium release properties is an important mode for treating arrhythmia. Some classes of molecules with different medicinal values are reported to bind CASQ and modify the calcium release properties. However, the binding of these molecules are known to confer cardio-toxicity [20]. Based on the structure of the polymeric CASQ, various classes of molecules could be designed as plausible antiarrhythmic agents.

NORMAL FUNCTIONING OF HEART: EC COUPLING IN CARDIAC MYOCYTE

The heart is a specialized organ which pumps blood to the body and to the lungs, to do so it has to contract and relax for more than 1,00,000 times a day without tiring or stopping [21]. The heart consists of 4 chambers; the upper two chambers are called the atria, and the lower two chambers are called the ventricle. The right atrium receives deoxygenated blood from the body through the superior and inferior vena cava. Electrical impulse is generated at the SA node which causes it to contract. Source–sink relationships are significant for the accurate functioning of the SA node. However exactly how the generated depolarizing "source" current impels depolarization and activates the neighboring atrial tissue (current

"sink") remains unclear [22]. With the contraction of SA node the blood flows from the SA node to the right ventricle through the tricuspid valve and the electrical impulse passes to the rest of the heart through the atrioventricular (AV) node. Blood now flows from the right ventricle to the lungs where it gets oxygenated and returns back to the left atrium and flows to the left ventricle and finally distributed to the body. This pumping action is a result of electrical impulses that arises due to the ion flow across the cardiac myocyte. The systematic flow of blood in and out of the cardiac chambers is due to the rhythmic contraction and relaxation of cardiac muscle, i.e. the EC coupling of cardiac myocyte.

Therefore, EC coupling can be defined as the process that links the action potential (AP) to contraction in skeletal and cardiac muscles [24]. The AP (Fig. 1) in skeletal muscles is generated in brain while the action potential in cardiac muscles is initiated in the SA node. Despite this, the EC coupling in skeletal and cardiac muscles have a similar series of events with some minute structural and functional differences. The focus of the present review is on cardiac functioning so EC coupling in cardiac myocyte is highlighted here. The series of events (Figs. 1 and 2) starts with the opening of voltage-gated ion channels. The AP generated at the SA node is propagated from a neighboring myocyte through the gap junctions and activates voltage-gated Ca^{2+} ion channels which causes substantial influx of Ca^{2+} triggering a larger release of Ca^{2+} from SR via RyR. Then, the myofilament gets activated to initiate contraction and thereafter reuptake of Ca^{2+} occurs causing it to relax. There are five phases of action potential (Fig. 1).

- The resting membrane potential or the Phase 4 is when the membrane potential is not stimulated, therefore, is seen as a horizontal line. Herein the potential in a cardiomyocyte is −90 mV because of constant outward leak of K^+ via the inward rectifier channels.
- During Phase 0 the cardiac myocyte is electrically simulated from the neighboring cardiomyocytes, which in turn leads to the opening of fast Na^+ channels. The Na^+ ions depolarize the membrane rapidly to 0 mv and little more transiently, wherein the L-type Ca^{2+} channels open up.
- Phase 1 is marked by the inactivation of Na^+ ion channels. The minor downward deflection is due to the movement of K^+ and Cl^- across the membrane.

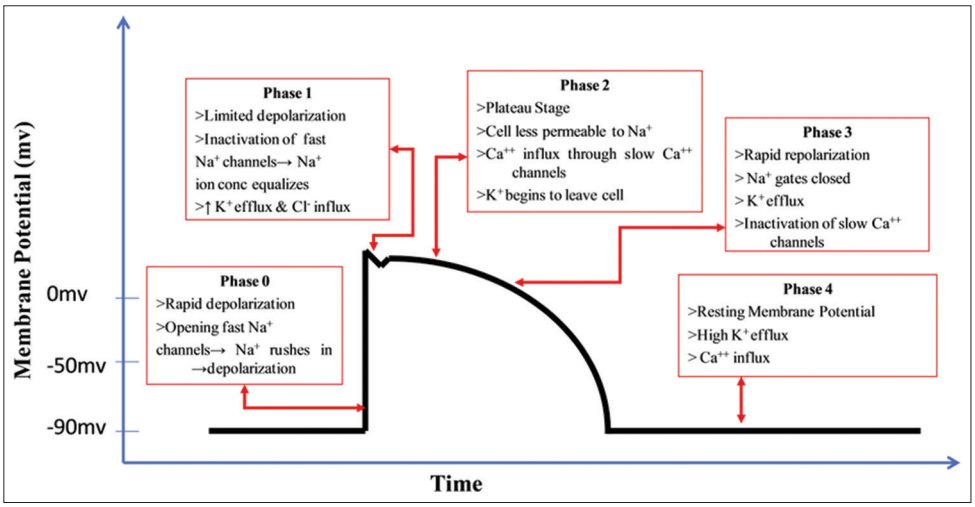

Fig. 1: Phases of action potential [23]

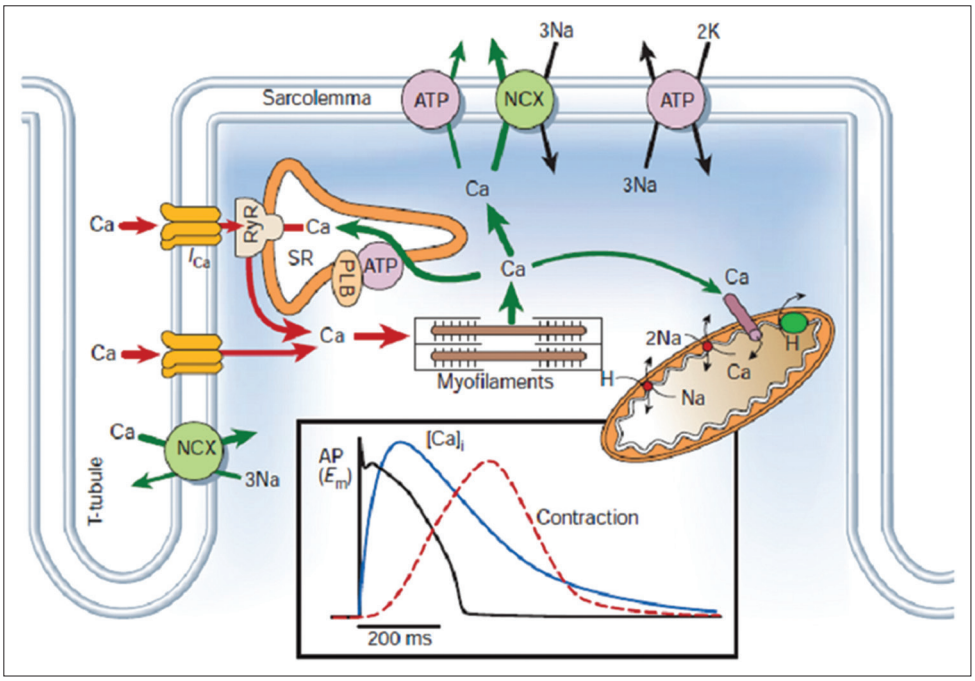

Fig. 2: Ca^{2+} transport in ventricular myocytes during excitation-contraction coupling [6]

- The plateau region is designated as Phase 2 where there is a balanced inward movement of Ca^{2+} and outward movement of K^{+}. With the increase in Ca^{2+} concentrations inside cell, the contraction of the cardiac muscles takes place. The Ca^{2+} ions binds to the troponin complex, activating the contractile apparatus and hence the heart contracts. Contraction stops when the release of Ca^{2+} ends and $[Ca]^{2+}$ returns to its diastolic value. The Ca^{2+} is removed from the cytosol by two different mechanisms; (a) reuptake through SERCA pump and (b) through NCX. Moreover to a small extent through sarcolemmal Ca^{2+} ATPase pump and mitochondrial Ca^{2+} uptake [25].
- During the Phase 3 the Ca^{2+} influx is stopped, and there is efflux of K^{+} causing polarization of the surface.

CARDIAC ARRHYTHMIA

The rhythm of the heart is generated due to the difference in electric potential across the membranes of cardiac myocyte. Any change in this rhythm is referred as arrhythmia. In general if the rate of the heart is slowed down it is called badycardia, and if there is an increased rhythm, it is called tachycardia. Most cases of arrhythmia do not require any treatment; however, of all the deaths caused by cardiovascular diseases, arrhythmia accounts to 50%. It could be either asymptomatic or have a fluttering feeling in chest, feel like missing heartbeat, feel tired, have a headache and even shortness of breath in acute conditions.

Mechanisms of arrhythmogenesis

Arrhythmias arise due to diverse reasons but at the cellular level, it is caused due to the disorders of impulse generation, disorders of impulse conduction or the permutation of both. There are three described reasons for arrhythmia which includes; abnormal automacity, triggered activity and reentry (Fig. 3) [26,27].

Only a few specific cardiac cells, like SA nodal cells, AV nodal cells, and His-Purkinje system has the ability to have pacemaker activity or automaticity [28]. Any enhancement or suppression of this activity may lead to clinical arrhythmias. In general, SA node has the fastest firing rate, while the others are called as "subsidiary pacemaker cells" which fires at slower rates. Interaction of three factors determines the rate:

- The threshold potential at which AP is initiated
- The maximum diastolic potential
- Moreover, the rate or slope of Phase 4 depolarization [27].

Any alteration in whichever of these may change the rate of impulse initiation [27,29], by bringing changes in the ionic movement differentially. Unlike normal automaticity, abnormal automaticity arises from non-pacemaker myocardial cells, which begins to spontaneously and abnormally initiate an impulse. Abnormal automaticity is believed to be the result of reduced resting membrane potential bringing it closer to the threshold potential. The two main causes that lead to abnormal automaticity are ischemia and electrolyte imbalances across the membranes. Triggered activity is defined as "the impulse initiation caused by after depolarizations which could be either delayed after depolarization (DAD) or early after depolarization (EAD) [30]." Herein the functional expression of SERCA is reduced while the Na^{+}/Ca^{2+} (NCX) activity is increased [31]. DAD arises during the Phase 4, in membrane voltage that occurs subsequent to completion of repolarization of the AP. The group of conditions that lifts the diastolic intracellular $[Ca]^{2+}$ are the reasons for these oscillations. This in turn cause Ca^{2+} mediated oscillations to trigger a new AP, if they reach the stimulation threshold [32]. This threshold is affected by RyR open probability; if there is an increase in the probability the threshold lowers [31]. The amplitude and rate of the DADs increases, as the cycle length decreases, and therefore, is expected to initiate arrhythmias triggered when DADs increase the heart rate. The EADs are generated in the ventricle during the Phase 2 or during the repolarization, i.e. Phase 3. During the normal electrical activity, the signal propagates to the entire heart. But if in certain conditions, a cluster of isolated fibers is not activated by the initial wave, they can recover excitability in time to be depolarized before the impulse dies out. They can, therefore, act as a link to re-excite areas that were previously depolarized but have already recovered from the initial depolarization [27,29]. This process is commonly denoted as "reentrant excitation, circus movement, reciprocal, echo beats or reentry reciprocating tachycardia (RT)." This refers to a repetitive propagation of the wave of activation, returning to its site of origin to reactivate that site [27,30].

Present medications

The current line of treatment of arrhythmia encompasses surgical procedures, electrical shock, and medications. Anti-arrhythmic agents are the drugs that are capable in reverting any irregularity in cardiac rhythm back to normal [33]. Progress in the understanding of the voltage-gated ion channels led to the determination of molecular effects of drugs and improved the prediction of drug effects on function [34]. The medications intend to change the cardiac action potential back to normal by preventing the ion channels opening and decrease the flow of ions across the cardiac membrane [35]. These drugs generally have an affinity for the ion channel protein and change its conformational state. There have been many attempts to classify anti-arrhythmic agents [34,36]; however, complexity arises since most of the drugs have multiple modes of action. The most widely accepted classification is Singh Vaughan Williams according to their general effect (Fig. 4). According to which the drugs are classified into four classes Class I, II, III, and IV. In this review, we have highlighted on the calcium channel blockers with brief in insight to other classes of drugs.

Class I also known as sodium channel blocker, these drugs can assist preventing arrhythmias by transforming a unidirectional block to bidirectional block. This could, however, be the cause of promotion of reentrant arrhythmias [34]. These drugs are further divided into three subclasses.

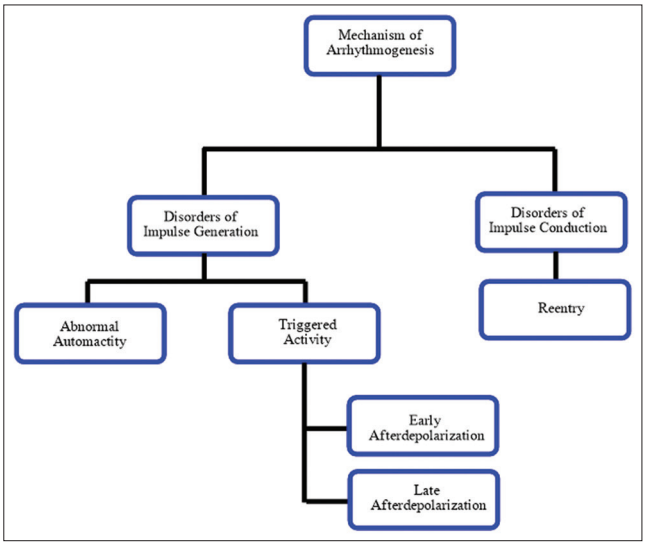

Fig. 3: Mechanisms of arrhythmias [26]

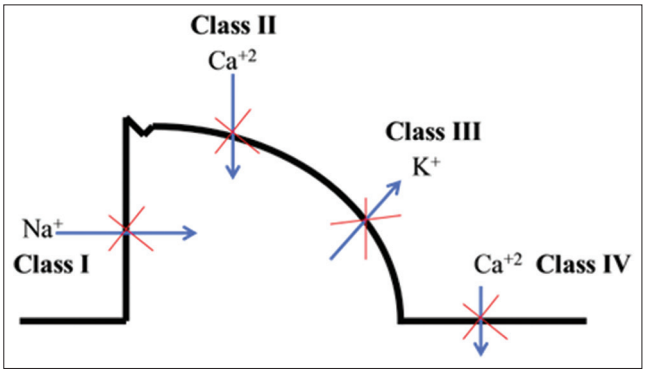

Fig. 4: Singh Vaughan Williams classification of anti-arrhythmic agents [36]

- Class Ia: It inhibits depolarizing effect by blocking Na^+ ion channels by lengthening the action potential (increases the refractory period). It is used to treat ventricular arrhythmias and preventing paroxysmal recurrent atrial fibrillation (Fig. 5).
- Class Ib: It inhibits depolarizing effect by blocking Na^+ ion channels by shortening the action potential (decreases the refractory period). It is used for treating and preventing myocardial infarction and also for the treatment of certain ventricular arrhythmias (Fig. 6).
- Class Ic: It works by inhibiting Na^+ ion channels however without changing the action potential duration. It interacts slowly with the Na ion channels. It is used for the treatment of recurrent tachyarrhythmias and preventing paroxysmal atrial fibrillation (Fig. 7).

Class II: This class of anti-arrhythmic drugs is also known as Beta Blockers. These drugs bind to the beta receptors and increase the AV conduction. It is used to reduce myocardial infarction mortality and prevent the recurrence of tachyarrhythmias (Fig. 8). These drugs are presently the most widely used drugs.

Class III: This class of anti-arrhythmic drugs is also known as Potassium Channel Blockers. They prolong the refractory period by prolonging the action potential duration. They are used for the treatment of reentrant arrhythmias (Fig. 9). However, no drug in this class is solely a potassium channel blocker. The most extensively used drug in this class "sotalol" which is also a beta blocker. "Amiodarone" yet another important drug, inhibits various other channels as well.

Class IV: This class of anti-arrhythmic drugs is also known as calcium channel blockers (CCBs). They inhibit the inward flow of extracellular calcium by interacting with ion-specific channels present in the cell wall. When calcium ion concentration inside the cell decreases, the contractility reduces and thereby the SA and AV conduction decreases [37]. There are three chemical classes of CCBs (Fig. 10): (i) Phenylalkylamines (e.g., verapamil), (ii) benzothiazepines (e.g., diltiazem), and (iii) dihydropyridines (e.g., amlodipine, felodipine, lercanidipine, nicardipine, nifedipine, nimodipine) [38]. These molecules targets the three types of calcium channels, the voltage sensitive, receptor-operated and stretch operated. The calcium ions regulation depends on both the entry and exit of calcium across the plasma membrane and also on the sequestration and release of calcium inside the cell. Calcium channels are generally funnel-shaped, membrane spanning that functions like ion selective valves. In addition to the voltage-gated calcium channels there are also receptor operated calcium channels. Every channel has an outer and inner gate. With the change in channel macromolecule conformation, the activation, and the inactivation gates moves either in or out of an occluding position, which determines the opening and closing of the channel pore. Most of the CCBs target the voltage-gated calcium channels receptor operating

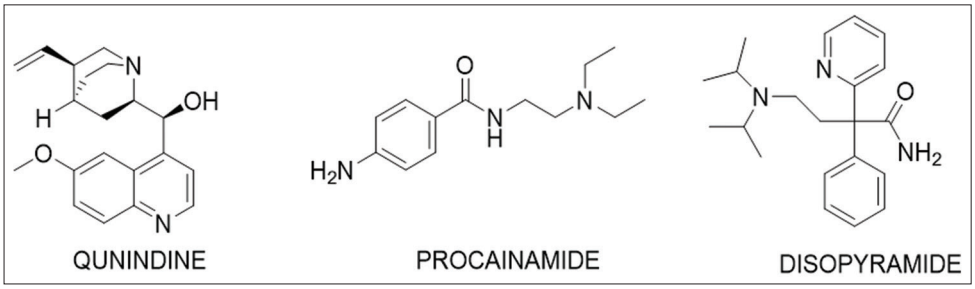

Fig. 5: Examples of Class Ia sodium channel blocking drugs

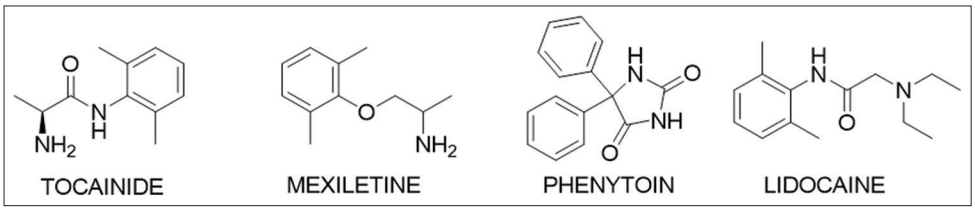

Fig. 6: Examples of Class Ib sodium channel blocking drugs

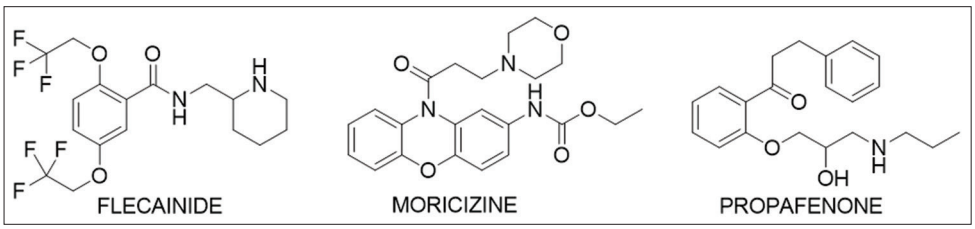

Fig. 7: Examples of Class Ic sodium channel blocking drugs

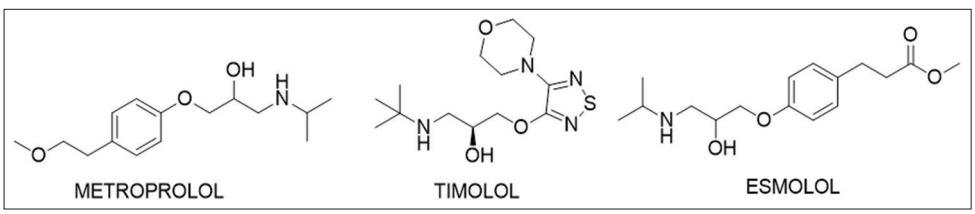

Fig. 8: Examples of Class II drugs (beta blockers)

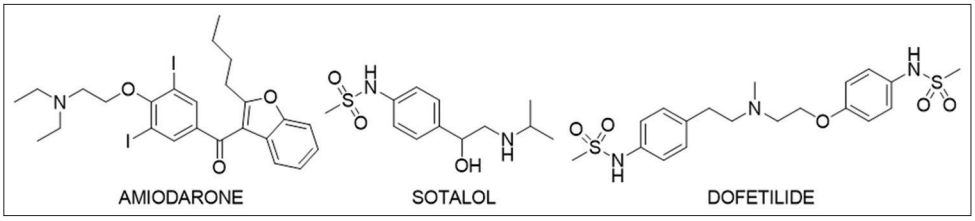

Fig. 9: Examples of Class III drugs (potassium channel blockers)

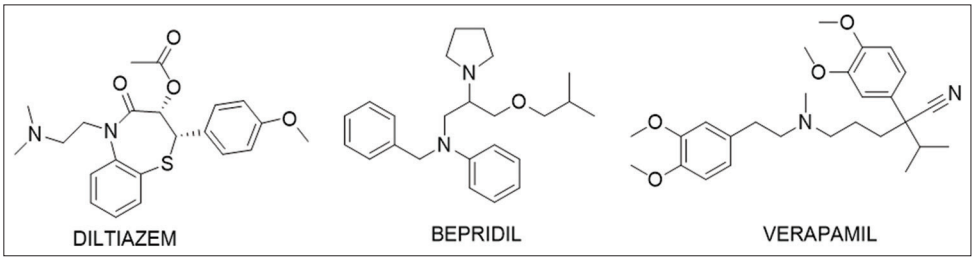

Fig. 10: Examples of calcium channel blockers (Class IV drugs)

calcium channels does not appear to be a preferred target. Tsien *et al*. identified three types of voltage-gated calcium channels; the L-type or the long lasting, T-type or the transient, tiny channels and the N-type or the neuronal which is neither L-type nor T-type. The classification is based on their conductances, activation and inactivation kinetics and sensitivity to toxins and drugs. The most common target is the L-type Ca^{2+} ion channels and dihydropyridines are the most common class of molecules which targets this channel. The L-type Channels are present in the T-tubule of cardiac myocyte, through which there is an intial influx of Ca^{2+} ions into the cell. However, major challenge in the presently used CCBs is that they cause self-poisoning by reducing the contractions, which leads to death [39,40]. Therefore, many antidotes have also been developed to treat the CCBs poisoning [38].

The various drug resistances and toxicities give an insight to explore a novel target and thereby a novel chemical class as a possible new CCB and a new target receptor. There is various calcium binding proteins present inside the SR, which is responsible for buffering of calcium. CASQ is the most abundant and comprises more than 25% of calcium binding protein present in the SR. This review indents to explore CASQ as a novel target and therefore its structure and function are discussed in details in the following sections.

Class V: This class of drugs includes all those which operate with unknown mechanisms. These drugs are not a part of conventional VW classification.

The attempt to design anti-arrhythmic drugs generally focuses on reducing the mortality rate by diminishing the associated symptoms. However, the major challenge is that the complexity in the altering electrical signals poses a threat of malignancy.

Emerging concepts in novel calcium ion modulating anti-arrhythmic drugs.

The present line of medications has serious limitations including proarrhythmic potential and drug toxicity. Implantable Cardioverter Defibrillator (ICDs) is the only line of treatment which is known to reduce mortality in patients with ventricular arrhythmias [34].

The new targets for anti-arrhythmia treatment interfere with the Ca^{2+}/calmodulin-dependent protein kinase II, the Na/Ca exchanger (NCX), RyR and the late component of Na current ($I_{Na\text{-}Late}$), all of them are related to intracellular calcium handling in the cardiac myocyte [31].

The main contributors for the removal of Ca^{2+} removal from the cytoplasm of the cardiomyocytes are the NCX and SERCA. The known inhibitors of NCX include KB-R7943 and SEA-0400 [31]. These drugs seem to be neutral on ionotropy due to the counteracting effects of the negative hemodynamics and positive ionotropy [41,42].

The RyR is an important Ca^{2+} release channel through which the calcium binding protein inside the SR releases the Ca^{2+} ions during the Phase 2 of the action potential. Spontaneous release of the calcium ions causes CPVT and hence is a new target for arrhythmias. Drugs like carvedilol [43] and dantrolene [44] which targets RyR have shown high potentials. propafenone and flecainide successfully prevent arrhythmia asymptomatic patients by acting as a RyR blocker [45].

Sodium current is composed of an early component and late component. The Na ion channels of this component are rapidly activated and deactivated. Anolazine is a potential $I_{Na\text{-}late}$ inhibitor and has shown its potential in EAD. Other potential drugs in this class include GS-967 and sophocarpine [31].

CASQ

CASQ was first isolated by MacLennan and Wong, 1971, and the protein was believed to sequester Ca^{2+} and thus was named, CASQ [46]. It is the major Ca^{2+} reservoir [47], present in the internal SR of skeletal and cardiac muscles. The two isoforms, i.e., the skeletal and cardiac; are encoded from two different genes [48,49]. The cardiac isoform is additionally present in slowtwitch muscle and accounts for 25% of the total share of CASQ [50]. CASQ binds calcium with high capacity and low affinity [9] which allows repetitive contractions required for any muscle movement [51] and keep the free [Ca^{2+}] below the inhibitory concentration [52]. CASQ is present as a linear polymer and is anchored to the surface membrane by binding to calcium release channel, ryanodine receptor (RyR) either directly or *via* transmembrane protein triadin and junctin. Although it was speculated that the only function of CASQ2 is to act as Ca^{2+} buffer, nevertheless it has proven to be participating many far more complex roles. It plays a major task of coordinating the rate of Ca^{2+} release, participate in phosphorylation and oxidative folding. However, the major role is to buffer Ca^{2+} and all the other functions derived from its tendency to bind a more number of Ca^{2+}. The disruptions of the calcium binding properties induced by mutations are known to cause lethal CPVT. There are 15 known mutations in CASQ2 which are related to cause CPVT [53]. CASQ2 mutations, influences the properties of Ca^{2+} dependent regulation of RyR2 and contributes to cardiac arrhythmogenesis [54]. To understand the calcium handling in the protein, it is necessary to understand its structural details.

CASQ sequence

By the term primary structure of proteins, it implies the linear sequence of its amino acids. This term was coined by Linderstrom-Lang in 1951. Conventionally, the sequence is reported starting from N-terminal end to the C-terminal end. The two isoforms of CASQ: "Skeletal" and "cardiac" [46,55,56] are present in the smooth, skeletal and cardiac muscles. Both CASQ1 and CASQ2 have been completely sequenced in many species[57-63], and shows significant resemblance although they are encoded from different genes [60]. There is a substantial sequence homology between the skeletal and cardiac isoforms (Fig. 11).

Sequence similarity in between the various skeletal and cardiac isoforms are over 80% (Fig. 11) Therefore if the crystal structure of one species is known it could be extrapolated to others which have high homology with the template.

Secondary and tertiary structure

CASQ consists of three thioredoxin folds similar to the topology of *Escherichia coli* [10]. Each individual domain consists of five beta sheets sandwiched between four alpha helices, which take the shape of a disk with 32-35Å radium (Fig. 12). However, this folding is highly dependent

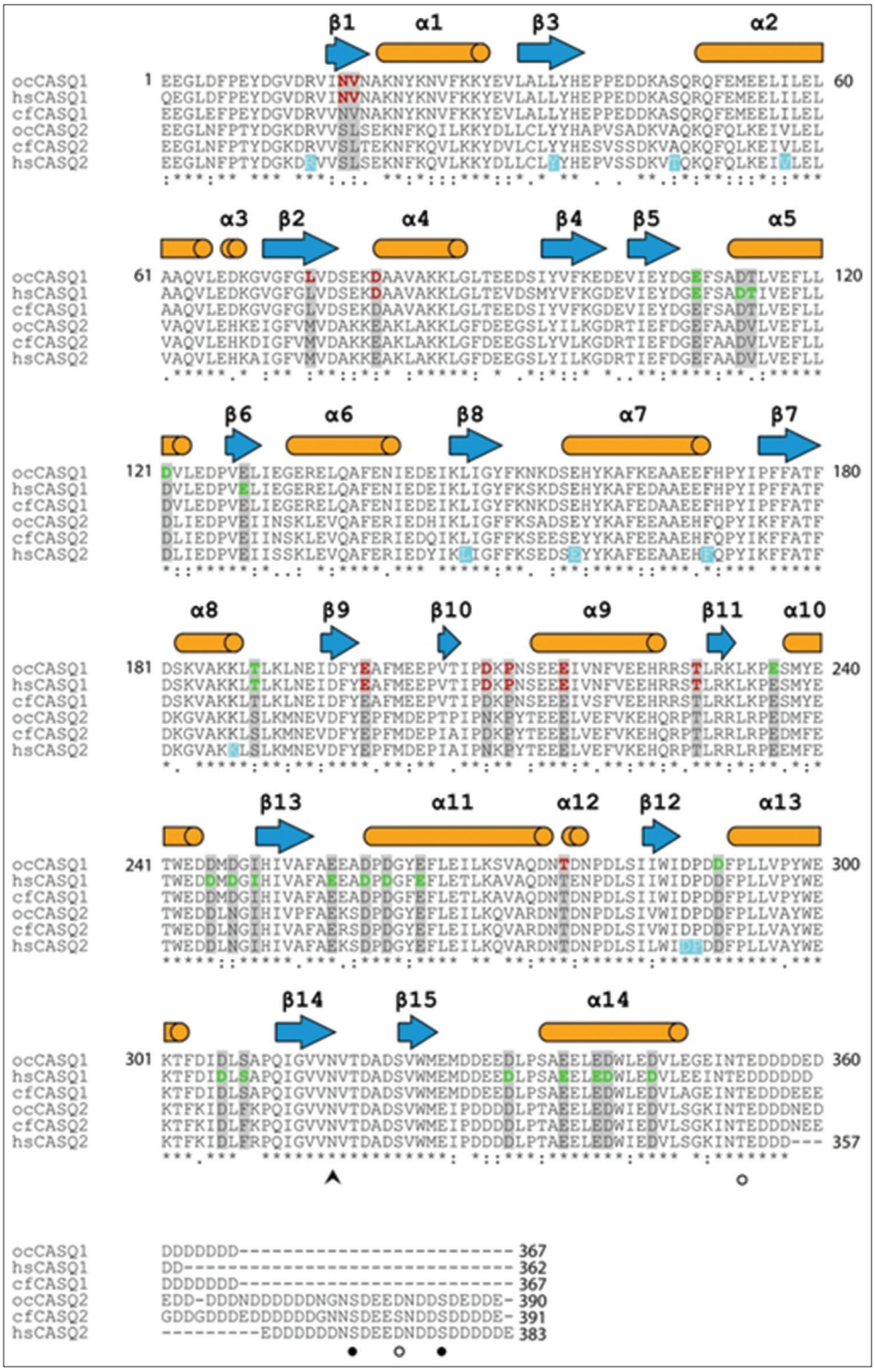

Fig. 11: Comparison of calsequestrin (CASQ) amino acid sequence from rabbit human and canine isoforms representing a very homology between species and also in between CASQ1 and CASQ2 [64]

on calcium ion concentration. The thioredoxin folds are formed at the calcium concentration of ~10 µM [10]. This rigidity of the structure is required for its stability while in binds Ca^{2+} ions with high capacity [65].

Each domain has a hydrophobic core, and acidic residues are present in the exterior to form an electronegative potential surface (Fig. 13). The interior of each domain is composed of the high aromatic amino acid composition. Interactions between aromatic groups provide the exceptional stability within the interiors of the protein, and the greatest stability arises from edge to face interactions between aromatic amino acids [66]. The stable cores shaped by the hydrophobic interactions between aromatic residues could be needed to offset the instability from large net negative charges [51].

These domains are connected to each other by loops, which is mostly composed of acidic residues. This makes the overall core hydrophilic as well. Cations are certainly required to stabilize the acidic center of CASQ. This may provide an explanation for the fact that CASQ is more susceptible to protease at low salt concentration [67,68]. The N-terminal is partly

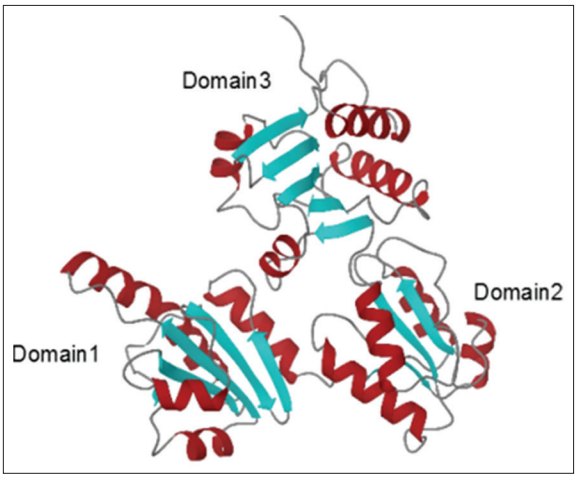

Fig. 12: Schematic representation of the structural elements present in calsequestrin (CASQ) indicating the thioredoxin folds of CASQ

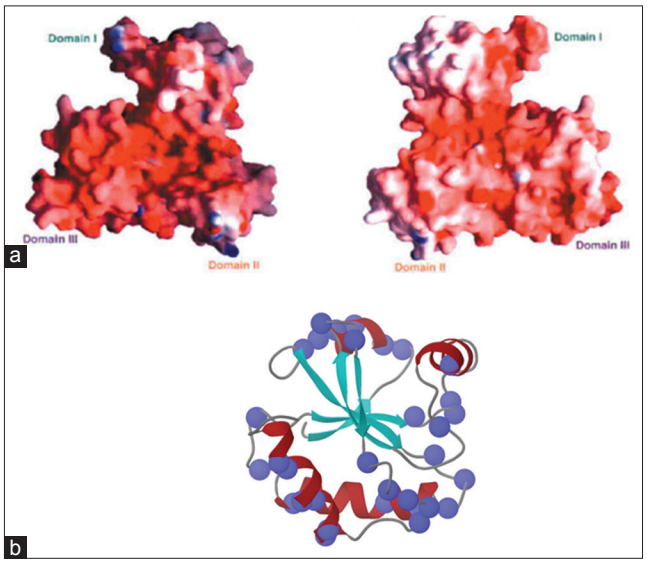

Fig. 13: (a) Front and back view of the electrostatic surface the red color indicating the highly electronegative surface of calsequestrin (CASQ) [10], (b) blue balls indicate the acidic residues. These acidic residues are solely confined to the surface giving the hydrophilic character to CASQ

disordered while C-terminal is completely disordered. The structure of the body and N-terminal for both the isoforms are similar while the structure of C-terminal is very different. The C-terminal of CASQ1 consists of 10-14 acidic residues and C-terminal of CASQ2 is composed of 35-45 acidic residues. Functionally, although both isoforms functions to buffer Ca^{2+} ions, however, CASQ2 has to buffer Ca^{2+} ions more dynamically. Since the structure and orientation of the C-terminal are not known, which is required for the understanding of the differential sequestering properties of both the isoforms, it opens up the scope to explore the same.

Polymerization of CASQ

With the further increase in calcium concentration the monomers polymerizes. Dimer formation is stabilized by two different contacts; the "front to front" and "back to back [10]." The front to front contact is established by the insertion of N-terminal of one monomer into the other and vice versa. Herein the two N-terminals of two monomers interact with each other and are stabilized with salt bridging interactions.[19] Salt bridging triads formed by Glu55-Lys49-Glu-59 and Lys45-Asp13-Tyr9 contribute to the front-to-front interaction and help stabilize the polymer. These salt bridges in combination with the other contacts, between the CASQ monomers, are mostly in between the residues of Domain I and Domain III of each monomer (Fig. 14). The front to front interactions arises with the insertion of the N-terminal part of one monomer into a hydrophobic cleft in the adjacent monomer. The second type of interaction is the back to back interaction. The back to back interaction is supposed to be C-terminal mediated, however since the structure of C-terminal and its orientation is yet to be deduced, therefore, there is a little knowledge on the same. Although there are different reports which give an idea on how the back to back interaction could be like, but without the crystal structure nothing could be said for certain. Earlier it was suggested that the back-to-back contact is stabilized by salt bridging interactions between Domain II and Domain I, the salt bridging interactions were supposed to be between Glu215-Lys86, Glu215-Lys24 and Glu169-Lys85 [10]. These studies were made prior to the isolation of CASQ1 hexameric crystal, and all the speculations related to the back to back interaction appeared wrong. Although the crystal structure did not contain the consecutive aspartate stretch of the C-terminal, Sanchez *et al*., showed that there was no interaction between the Domain II and Domain I, but there was a slight interaction between Domain III and Domain I [64]. Moreover, the back to back interactions were seen to be stabilized by Ca^{2+} mediated interactions. Whether it is the front to front interaction or the back to back interaction which is initiated first is debatable. [10] However, the residues which are involved in both the front to front and back to back interactions are highly conserved further indicating the structural resemblance at the polymeric level among the CASQs of various species.

The exact $[Ca]^{2+}$ required for each one of these interactions are yet to be deduced [10]. If either the C-terminal or the N-terminal is removed

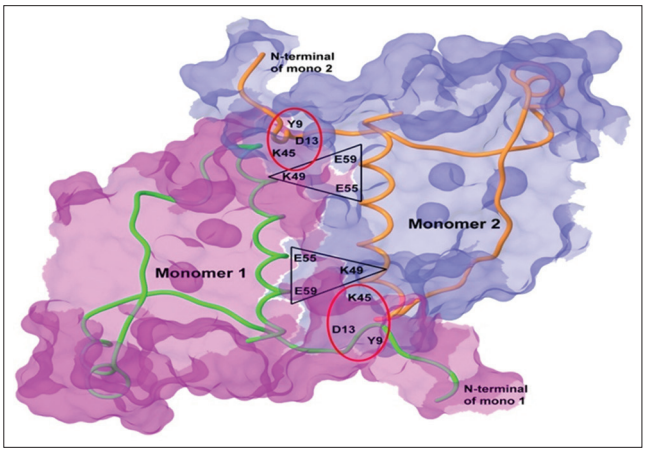

Fig. 14: Salt bridges linking the two monomers stabilizing the front to front contact in calsequestrin [11]

from CASQ it fails to form the polymer [8]. Earlier it was thought that the N-terminal is responsible for binding to the membrane-bound proteins triadin and junctin. However, it was later understood that it wouldn't be possible for the N-terminal to have physiological interaction between the CASQ polymer and the anchoring proteins. Fig. 15 postulates, the steps involved in the anchoring of the CASQ with the neighboring proteins from its randomly distributed sequence. It was proposed that at 10 μM [Ca^{2+}] CASQ attains a compact state. With further increase to 100 μM [Ca^{2+}] causes it to dimerize and finally polymerize. When the luminal [Ca^{2+}] reaches 1mM, the polymer is stabilized and gets anchored to the SR membrane via the triadin and junctin. Any further increment in [Ca^{2+}] would result in dissociation of CASQ from triadin and junction [69]. The state of the CASQ polymer at this concentration, once dissociated from the junctional proteins, remains unknown [70,71]. It is proven that CASQ remains coupled with the RyR at 1mM [Ca^{2+}] and dissociates from the triadin and junctin only after it reaches above 10 mM [Ca^{2+}].

The role of the disordered C-terminal residues and its conformation is often arguable [73], it has been reported to be responsible for CASQ binding to the junctional proteins [74]. In a recent study, it has been suggested that how progressive alanine mutation in the C-terminus does affect the calcium binding capacity but does not significantly alter the CASQ1s association with trisk95/junctin. However when the entire C-terminal was mutated the calcium binding reduced further and the association with its functional proteins also reduced [75]. The major difference in the sequence of CASQ1 and CASQ2 is in their C-terminus both in their length and composition. This region is intrinsically disordered, and the feature is conserved from fishes to human. Although the disordered C-terminal does not contribute to the protein folding yet, the conformation of the C-terminal is highly sensitive to [Ca^{2+}]. Moreover when the C-terminals of the CASQ1 and CASQ2 are swapped, there is a reversal of polymerization kinetics [71]. This suggests that it's the C-terminal that is the governing feature for the difference in the polymerization.

CALCIUM BINDING IN CASQ

Ca^{2+} ion is the fifth most versatile element present in the earth's crust and the most abundant mineral in the human body. It impacts nearly every cellular life. In eukaryotes Ca^{2+} functions as a versatile and universal signal by interacting with more than hundreds of protein over a 10^6-fold range of affinities [76]. CASQ is a high capacity intermediate affinity calcium binding protein. Both of its isoforms has Ca^{2+} binding capacity between 800 and 1000 nMol mg^{-1}. Binding affinity (Kd) values suggest an intermediate affinity, although the exact value depends on the presence of other ions [77]. This change is binding affinity in the presence of other ions led to the investigations on its specificity. However, the specificity for Ca^{2+} is not much. Different cations competes for the Ca^{2+} with affinities; $La^{3+}>Zn^{2+}>Cd^{2+}>Mn^{2+}>Ca^{2+}>Mg^{2+}/Sr^{2+}>K^{+}$. Bal *et al*. suggested that although structural transitions could be brought

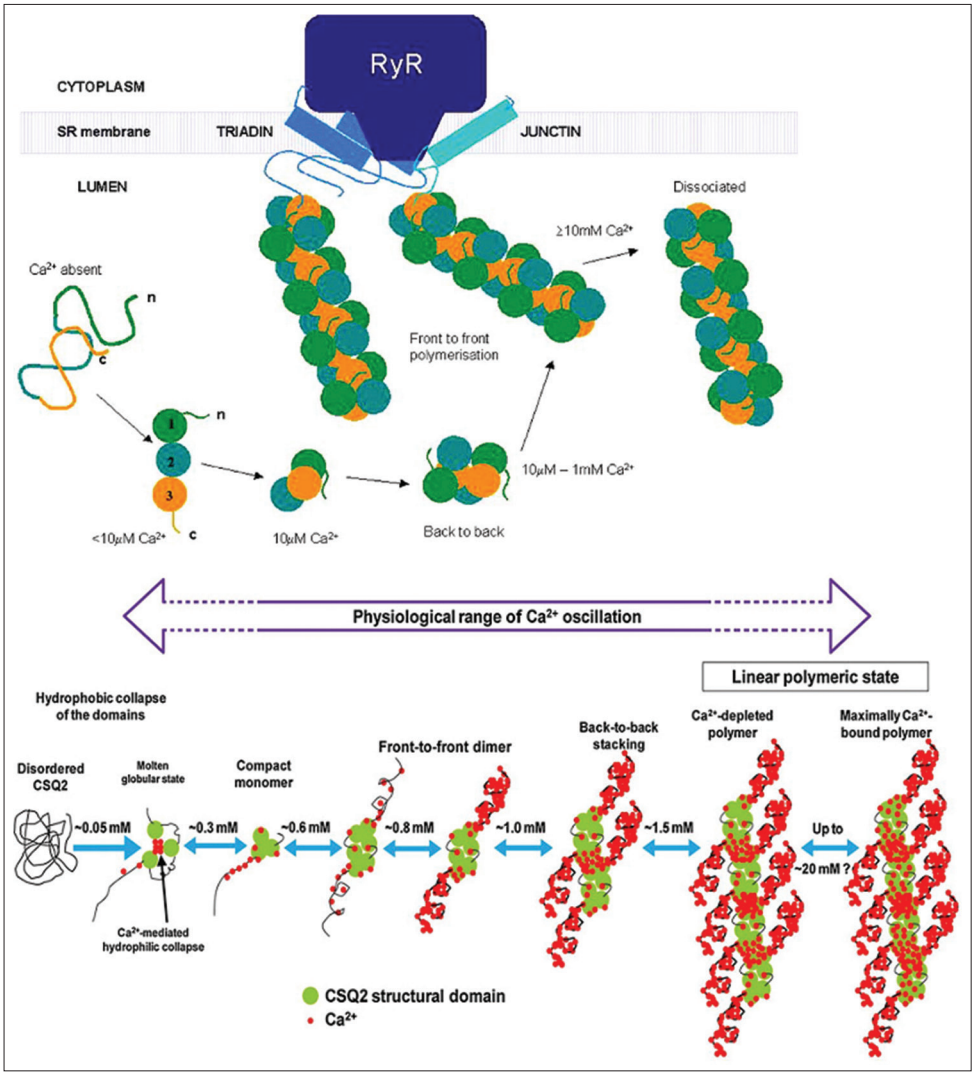

Fig. 15: Behavior of the calsequestrin 2 (CASQ 2) at different calcium concentration [72]. Indicating how a disordered CASQ 2 attains a polymeric state and further gets associated to the ryanodine receptors

about by many different cations but only specific cations can bring about the aggregation of the protein. Alkali metals, like Na^+ and K^+, can cause molecular compaction, however, they cannot support CASQ2 aggregation. Moreover, among the alkaline earth metals, only Ca^{2+}can promote CASQ2 to polymer formation. The numbers of Ca^{2+} binding sites is reported to vary between 18 and 50. Park *et al*., suggested that while CASQ1 binds 80 Ca^{2+} ions per monomer. CASQ2 binds to 60 Ca^{2+} ions [78]. Unlike other Ca^{2+} binding protein which has a specific motif for Ca^{2+} binding, like an "EF-hand" or "double clamp", CASQ requires only a pair of acidic residues thereby making the interaction weak and hence allow dynamic association and dissociation during high physiological demand.It was believed that Ca^{2+} binding is driven by a gain in entropy when water molecules surrounding the Ca^{2+} ion are liberated. The Ca^{2+} binding sites on the body of CASQ1 were first divided into high affinity and low affinity by Sanchez *et al*., [64]. While the low-affinity sites were identified as water bound Ca^{2+} the high-affinity sites were found to be buried in the core. These high-affinity sites are suggested to maintain the protein folding. Several low affinity and three high-affinity sites per found per monomer according to the biological studies. Moreover, the high-affinity sites were found in the interface of two monomers, these Ca^{2+} ions stabilizes the two monomeric units together. The high-affinity sites were found to have different Ca^{2+} geometries including, pentagonal bipyramidal, monocapped octahedral and trigonal pyramidal. Moreover, the C-terminus is thought to be responsible for most of the calcium binding site. With the deletion of the C-terminal, the calcium binding ability was seen to be reduced by 50%. However, a different number of acidic residues in the C-terminal does not correspond to the total number of Ca^{2+} bound to the protein. It has been recently reported that metal-containing macromolecules in which metals are misidentified are abundant in the PDB [79]. Therefore in earlier studies we used molecular dynamics as a tool to reveal the calcium binding sites upon the addition of the C-terminal in CASQ1. We had seen with the increase in calcium ions there was room for accommodating more number of calcium ions apart from just those which were obtained from the crystal structure. While the C-terminal attained saturation before the body of the protein did not gave an indication that the need for the stabilization of the dynamic C-terminal. Moreover, the differential binding affinity of calcium by the protein gives a scope for the protein to remain polymerized while buffer the low-affinity calcium ions. Furthermore, the C-terminal attains a compact form instead of linear structure (Fig. 16) which indicates the folding in the protein structure with the binding of calcium ions [80]. Although the PDB crystal structure of CASQ1 gives an insight about the calcium binding sites yet in depth studies is required for both CASQ1 and CASQ2 is required.

MOLECULES WITH KNOWN AFFINITY FOR CASQ2

Although not much work has been performed in this aspect, different classes of molecules, with different biological significance, has been suggested to bind to CASQ2 and affect its polymerization pattern, based on docking, X-ray crystallography and other biological studies [20,81,82]. While some of the molecules were reported to bind to CASQ2 and cause destabilization of the polymeric state [20], other were reported to induce polymerization [82]. Anthracyclines like doxorubicin and daunorubicin as well as their metabolites doxorubicinol and daunorubicinol and phenothiazines like promethazine, thioridazine and chlorpromazine (Fig. 17) are reported to induce the release of SR bound Ca^{2+} and possible mechanism suggested for this alteration, is their affinity for CASQ2 [83-86]. Moreover, the same molecule has been suggested to induce Ca^{2+} at micromolar concentration as well as inhibit Ca^{2+} release at nanomolar concentrations [83].

Cocaine is also reported to bind to CASQ2.[82] It has been suggested that in the absence of Ca^{2+}, 75% of the CASQ2 molecules acquire the monomeric state and 25% the dimeric state while in the presence of 1 mmol Ca^{2+} most of the molecules transitioned to dimeric state. With the addition of cocaine in Ca^{2+} free solution the formation of dimeric and polymeric structures were observed, however in the presence of Ca^{2+}, calcium-mediated oligomerization were perturbed. The accumulation of these molecules is however a major concern [86]. Based on molecular site prediction and docking studies Subra *et al*., predicted the binding sites in the CASQ1 monomer. They identified three different sites for the binding of different classes of molecules [20] and suggested that molecules with large aromatic groups to be strong binders whereas molecules with loose side chains to be weak binders (Fig. 18) [20]. However, it has been reported that binding of these molecules to the CASQ2 inhibit the polymerization and therefore are cardiotoxic.

CONCLUSION

Cardiac arrhythmias are prevalent among humans, across all age ranges and can be caused even without any structural defect of the heart. The three major mechanisms responsible for cardiac arrhythmias are automaticity, triggered activity and reentry. The present line of treatment of cardiac arrhythmia is generally classified into four classes, based on Singh Vaughan Williams classification. It comprises of; (i) Na^+ channel blockers, (ii) K^+ channel blockers, (iii) beta blockers and (iv) CCBs. Calcium ions from the trigger to the EC coupling and thereby controlling the rhythm of the heart. The CCBs generally targets the entry and exit of the calcium ions across the voltage-gated membrane. There are three chemical classes of CCBs (Fig. 10): (i) phenylalkylamines (e.g. verapamil) (ii) benzothiazepines (e.g. diltiazem) and (iii) dihydropyridines (e.g. amlodipine, felodipine, lercanidipine, nicardipine, nifedipine, nimodipine) [38]. The most common of which is the dihydropyridines which targets the L-type calcium channels. The general problem with the CCBs is its toxicity and resistance and hence opens up an avenue to explore a new target. The newer line of treatment approaches the machineries related to intracellular calcium handling in the cardiac myocyte [31]. The calcium ion regulation depends on both the entry and exit of calcium ions and also on the sequestration and release of calcium. Targeting the sequestration and release properties instead of the entry and exit channels could be an interesting area of research. CASQ is the major calcium buffering glycoprotein present in the SR and is responsible for the sequestration of calcium ions. Mutations and ablations in CASQ have been associated with arrhythmia which is caused by the change in calcium buffering ability and thereby to its polymeric structure. CASQ2 attains its polymeric structure upon binding to calcium ions. The exploration of structural details is still under progress. The structure of the monomer and the front to front dimer is studied in details. However due to the disordered C-terminal there is a little knowledge about the back to back interactions and orientation of the C-terminal. It's

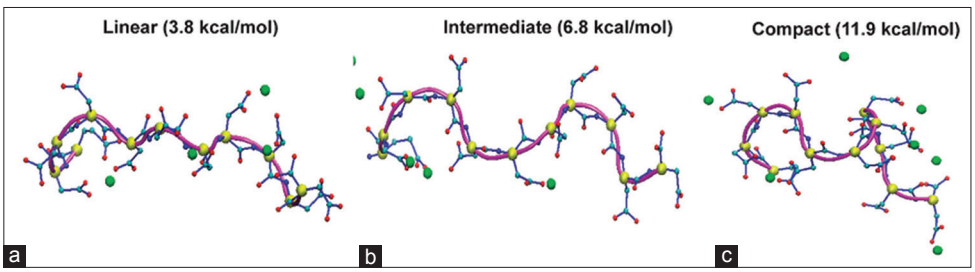

Fig. 16: The C-terminal of calsequestrin 1 assumes compact conformation upon binding of Ca^{2+} ions with increase in $[Ca]^{2+}$ [80]. (a) At 20 $[Ca]^{2+}$ the C-terminal has a linear structure, (b) at 60 $[Ca]^{2+}$ the C-terminal starts folding, (c) At 80 $[Ca]^{2+}$ attains a compact structure

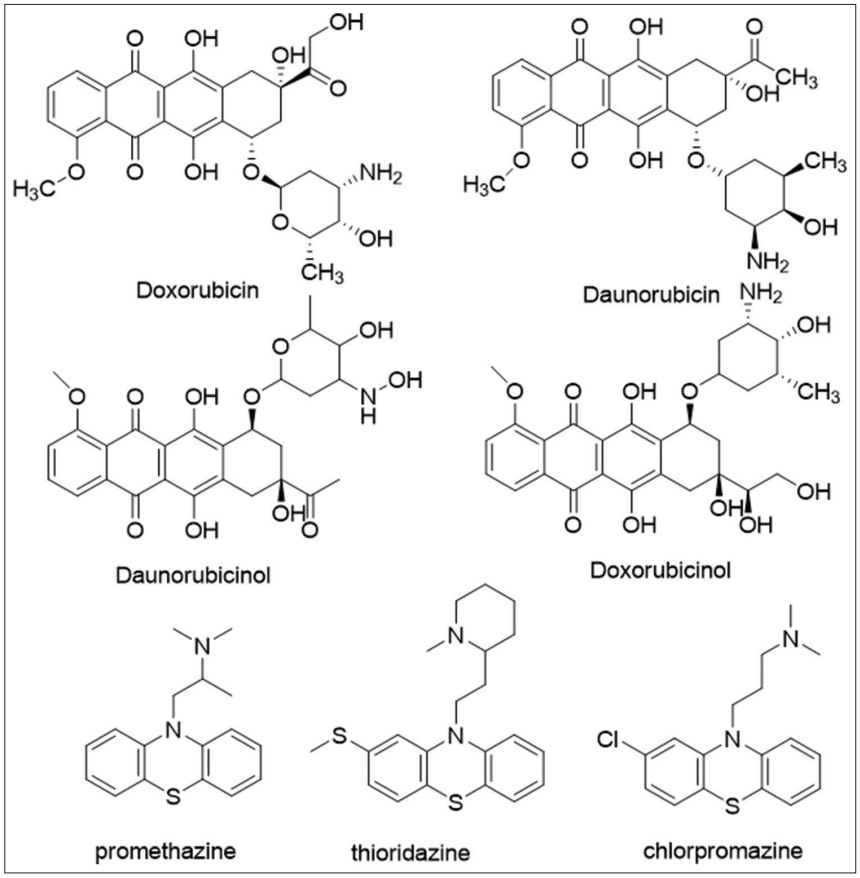

Fig. 17: Examples of antracyclines and phenothiazines having affinity for calsequestrin 2

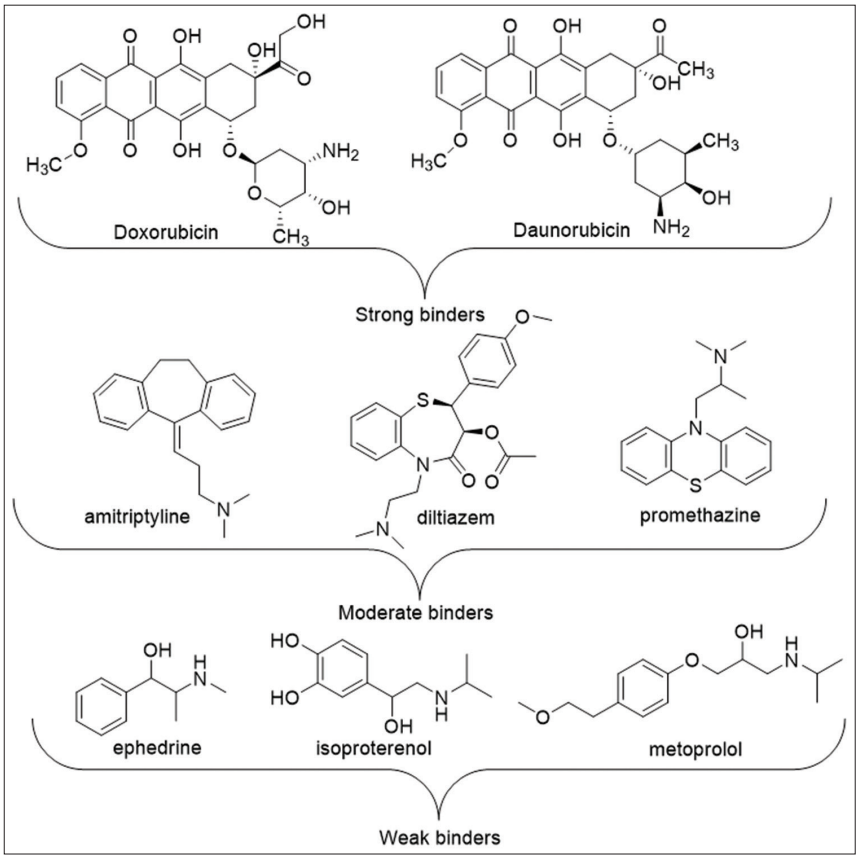

Fig. 18: Known drugs with variable binding affinity

necessary to understand the structure to explore the calcium binding sites in order to modulate the sequestering properties of CASQ2. Certain molecules are reported to bind CASQ2 monomer, although no definite class has been identified for the same. The strong binders are reportedly the molecules with large aromatic cores and weak binders with a flexible chain. Although the binding of these molecules are confined only up to the monomer level and not to dimer or tetramer level, which could be a necessity to understand the mode of action in altering the calcium release properties

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