Cardiac excitation–contraction coupling

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Of the ions involved in the intricate workings of the heart, calcium is considered perhaps the most important. It is crucial to the very process that enables the chambers of the heart to contract and relax, a process called excitation–contraction coupling. It is important to understand in quantitative detail exactly how calcium is moved around the various organelles of the myocyte in order to bring about excitation–contraction coupling if we are to understand the basic physiology of heart function. Furthermore, spatial microdomains within the cell are important in localizing the molecular players that orchestrate cardiac function.

Cardiac excitation–contraction coupling is the process from electrical excitation of the myocyte to contraction of the heart (which propels blood out). The ubiquitous second messenger Ca^{2+} is essential in cardiac electrical activity and is the direct activator of the myofilaments, which cause contraction[1]. Myocyte mishandling of Ca^{2+} is a central cause of both contractile dysfunction and arrhythmias in pathophysiological conditions[2].

During the cardiac action potential, Ca^{2+} enters the cell through depolarization-activated Ca^{2+} channels as inward Ca^{2+} current (I_{Ca}), which contributes to the action potential plateau (Fig. 1). Ca^{2+} entry triggers Ca^{2+} release from the sarcoplasmic reticulum (SR). The combination of Ca^{2+} influx and release raises the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), allowing Ca^{2+} to bind to the myofilament protein troponin C, which then switches on the contractile machinery. For relaxation to occur $[Ca^{2+}]_i$ must decline, allowing Ca^{2+} to dissociate from troponin. This requires Ca^{2+} transport out of the cytosol by four pathways involving SR Ca^{2+}-ATPase, sarcolemmal Na^+/Ca^{2+} exchange, sarcolemmal Ca^{2+}-ATPase or mitochondrial Ca^{2+} uniport. Here I discuss the key Ca^{2+} transport systems in cardiac myocytes, how they interact dynamically and how they are regulated. The increasingly important area of local molecular signalling in microdomains will also be addressed.

The role of calcium in contraction and flux balance

Although Ca^{2+} is the switch that activates the myofilaments (the end effectors of excitation–contraction coupling), contraction is graded and depends on $[Ca^{2+}]_i$ and other factors. Figure 2a shows the amount of total cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_{Tot} = [Ca^{2+}]_i$ plus bound Ca^{2+}) that must be supplied to and removed from the cytosol during each cardiac beat. Half-maximal activation of contraction requires roughly 70 μmol of Ca^{2+} per litre of cytosol, which would raise $[Ca^{2+}]_i$ to 600 nM. This ratio of bound:free Ca^{2+} indicates that there is powerful cytosolic Ca^{2+} buffering (~100:1)[1].

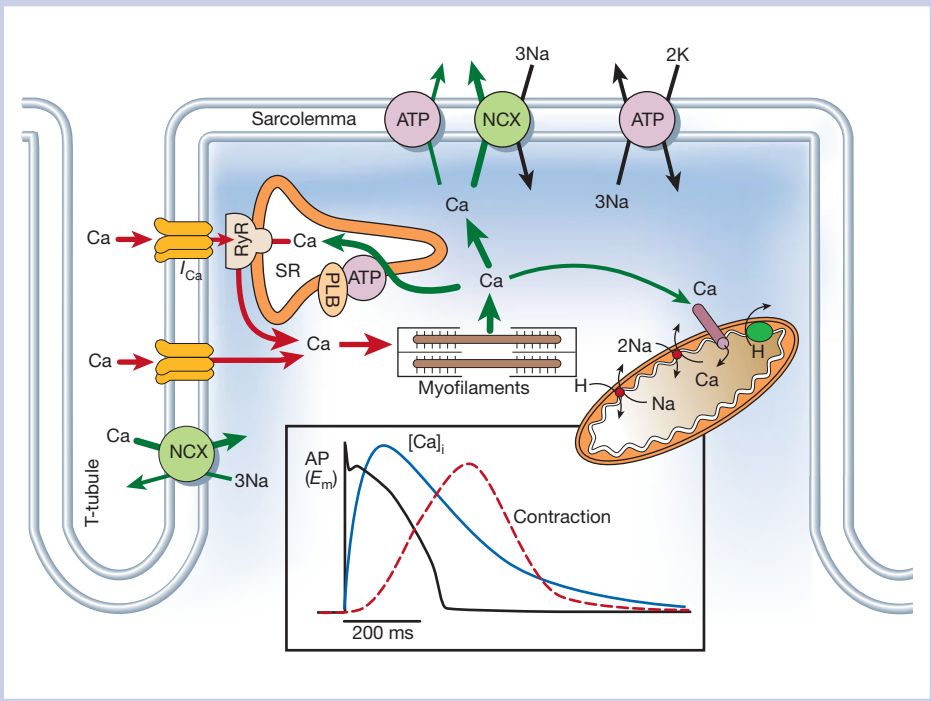

Figure 1 Ca^{2+} transport in ventricular myocytes. Inset shows the time course of an action potential, Ca^{2+} transient and contraction measured in a rabbit ventricular myocyte at 37 °C. NCX, Na^+/Ca^{2+} exchange; ATP, ATPase; PLB, phospholamban; SR, sarcoplasmic reticulum.

The development of contraction force depends on $[Ca^{2+}]_i$ and $[Ca^{2+}]_{Tot}$ in highly nonlinear relations, as a result of strong myofilament cooperativity with respect to $[Ca^{2+}]_i$ (refs 1,3,4). Moreover, the physiological contraction generates both isometric force (or ventricular pressure) and rapid shortening (to eject blood). There are two main ways to change the strength of cardiac contraction: by altering the amplitude or duration of the Ca^{2+} transient, and by altering the sensitivity of the myofilaments to Ca^{2+}. Myofilament Ca^{2+} sensitivity is enhanced dynamically by stretching the myofilaments (as the heart fills with blood), resulting in a stronger contraction. This is due, in part, to the transverse filament lattice compression that occurs on stretch, which enhances the actin–myosin interaction[5]. This lateral compression is an important autoregulatory mechanism by which the heart adjusts to altered diastolic filling (the classic Frank–Starling response).

Myofilament Ca^{2+} sensitivity is reduced by acidosis, and by elevated phosphate and Mg^{2+} concentrations (all three of which occur during ischaemia). Myofilament Ca^{2+} sensitivity is also reduced by β-adrenergic activation (see below), but enhanced by caffeine and certain inotropic drugs. The rapid kinetics of the Ca^{2+} transient prevent the myofilaments from fully equilibrating with $[Ca^{2+}]_i$ during a normal twitch (especially in the rising phase). Thus, although contractile strength is indicative of underlying Ca^{2+} transients, there is a dynamic interplay between Ca^{2+} and myofilaments during excitation–contraction coupling.

Ca^{2+} must be removed from the cytosol to lower $[Ca^{2+}]_i$ and allow relaxation. This is achieved by several routes, the quantitative importance of which varies between species (ref. 6; and Fig. 2b). In rabbit ventricular myocytes, the SR Ca^{2+}-ATPase pump removes 70% of the activator Ca^{2+}, and Na^+/Ca^{2+} exchange removes 28%, leaving only about 1% each to be removed by the sarcolemmal Ca^{2+}-ATPase and mitochondrial Ca^{2+} uniporter (the last two are collectively referred to as 'the slow systems'). The amount of Ca^{2+} that leaves the cytosol by entering mitochondria is inconsequential with respect to excitation–contraction coupling, but slow cumulative changes in intra-mitochondrial $[Ca^{2+}]$ can stimulate key dehydrogenases that increase the production of NADH (nicotinamide adenine dinucleotide) and ATP to match increased energetic demands[7].

The activity of SR Ca^{2+}-ATPase is higher in rat ventricle than in rabbit ventricle (because of a greater concentration of pump molecules)[8], and Ca^{2+} removal through Na^+/Ca^{2+} exchange is lower, resulting in a balance of 92% for SR Ca^{2+}-ATPase, 7% for Na^+/Ca^{2+} exchange and 1% for the slow systems (Fig. 2b). Analysis in mouse ventricle is quantitatively like rat[9], whereas the balance of Ca^{2+} fluxes in ferret, dog, cat, guinea-pig and human ventricle are more like rabbit[1]. Thus, mouse and rat ventricle (which also show very spike-like action potentials) poorly mimic human with respect to the quantitative balance of cellular Ca^{2+} flux. Moreover, during heart failure in humans and rabbits, functional expression of SR Ca^{2+}-ATPase is reduced and Na^+/Ca^{2+} exchange is increased[10], such that these systems contribute more equally to the decline in $[Ca^{2+}]_i$ (refs 1,2). These changes counterbalance each other with respect to twitch relaxation and $[Ca^{2+}]_i$ decline, leaving it unaltered. But both changes tend to reduce Ca^{2+} content in the SR, limiting SR Ca^{2+} release, and this may be a central cause of systolic contractile deficit in heart failure.

The amount of Ca^{2+} extruded from the cell during twitch relaxation must be the same as the amount of Ca^{2+} entry for each beat, otherwise the cell would gain or lose Ca^{2+} (and would not be in steady state). Indeed, complementary measurements of Ca^{2+} influx and SR Ca^{2+} release during a twitch confirm this expectation in rabbit and rat[1,11]. This provides a quantitative framework of dynamic Ca^{2+} fluxes in ventricular myocytes.

Calcium current

Myocytes exhibit two classes of voltage-dependent Ca^{2+} channels (L- and T-type)[1] and the large electrochemical $[Ca^{2+}]$ gradient also drives Ca^{2+} into resting myocytes (at ~1 μmol l^{-1} cytosol s^{-1}) by unknown pathways[12]. As T-type I_{Ca} is negligible in most ventricular myocytes, I_{Ca} generally refers to the L-type here. I_{Ca} is activated by depolarization, but Ca^{2+}-dependent inactivation at the cytosolic side limits the amount of Ca^{2+} entry during the action potential. This Ca^{2+}-dependent inactivation is a local effect and is mediated by calmodulin bound to the carboxy terminus of the Ca^{2+} channel[13,14].

L-type Ca^{2+} channels (dihydropyridine receptors; DHPRs) are located primarily at sarcolemmal–SR junctions where the SR Ca^{2+} release channels (or ryanodine receptors; RyRs) exist[15]. During excitation–contraction coupling, SR Ca^{2+} release also contributes to Ca^{2+}-dependent inactivation of I_{Ca} (refs 16,17). Indeed, the total Ca^{2+} influx through I_{Ca} is reduced by about 50% when SR Ca^{2+} release occurs (from 12 to 6 μmol Ca^{2+} l^{-1} cytosol)[18]. Thus, SR Ca^{2+}

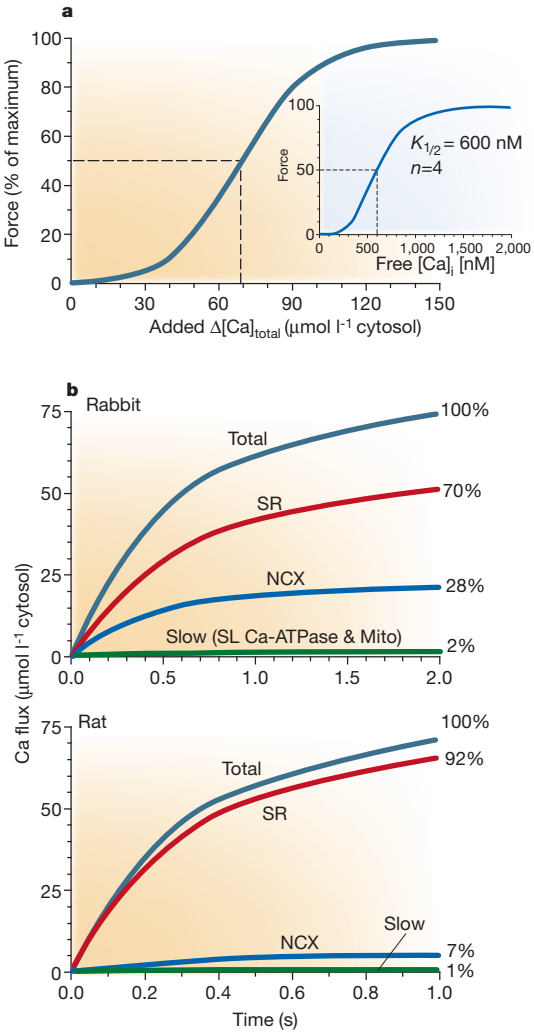

Figure 2 Quantitative Ca^{2+} fluxes during excitation–contraction coupling. **a**, Amount of Ca^{2+} required for contractile activation, assuming a diastolic intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of 150 nM and cytosolic Ca^{2+} buffers including troponin C (Ca^{2+} and Ca^{2+}/Mg^{2+} sites), myosin, SR Ca^{2+}-ATPase, calmodulin, ATP, creatine phosphate and sarcolemmal sites[1]. Inset shows force as a function of $[Ca^{2+}]_i$ (that is, force is equal to $100/(1 + \{600/[Ca^{2+}]_i\}^4)$; ref. 1). **b**, Integrated Ca^{2+} fluxes during twitch relaxation in rabbit and rat ventricular myocytes. Curves are based on $[Ca^{2+}]_i$ and the $[Ca^{2+}]_i$ dependence of transport rates measured for each system. Percentages are relative contributions to Ca^{2+} removal[4]. SL, sarcolemmal; SR, SR Ca^{2+}-ATPase; Mito, mitochondrial Ca^{2+} uniporter.

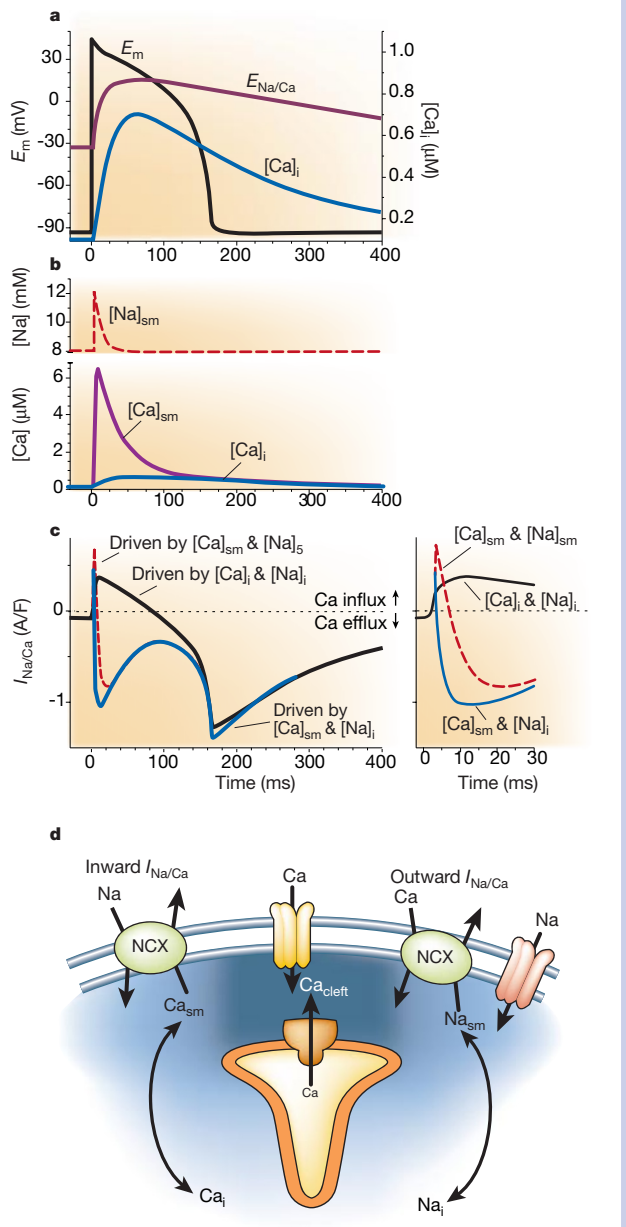

Figure 3 Na^+/Ca^{2+} exchange during an action potential. **a**, Typical action potential (E_m), Ca^{2+} transient ([Ca^{2+}]$_i$), and calculated $I_{Na/Ca}$ reversal potential ($E_{Na/Ca}$). **b**, Curves illustrating how submembrane [Na^+]$_i$ and [Ca^{2+}]$_i$ ([Na^+]$_{sm}$ and [Ca^{2+}]$_{sm}$) might change during the action potential owing to local diffusion limitations (note that [Ca^{2+}]$_{sm}$ may be lower than that in the cleft, [Ca^{2+}]$_{cleft}$, as shown in **d**). **c**, $I_{Na/Ca}$ calculated by the equation given in ref. 25 as a function of E_m and the indicated concentrations of Ca^{2+} and Na^+. Right panel is expanded in time. **d**, Geometry of junctional and submembrane spaces.

release and I_{Ca} create local negative feedbacks on Ca^{2+} influx. When there is high Ca^{2+} influx or release, further influx of Ca^{2+} is turned off.

The effect of SR Ca^{2+} release on inactivation of I_{Ca} provides a unique bioassay for local cleft [Ca^{2+}]$_i$ (which may exceed 50 μM during excitation–contraction coupling[19]). That is, the rapid inactivation of I_{Ca} allows measurement of the SR Ca^{2+} release time course (which peaks in 2–3 ms at 35 °C)[18]. This confirms the very brief delay expected between I_{Ca} and SR Ca^{2+} release during excitation–contraction coupling[20]. Thus, the location of Ca^{2+} channels allows electrophysiological signals that provide spatial information that cannot be assessed by optical fluorescence imaging (owing to the small cleft size).

Calcium influx and efflux

Na^+/Ca^{2+} exchange is reversible, with a stoichiometry of three Na^+ ions to one Ca^{2+} ion (but see refs 21,22) that produces an ionic current ($I_{Na/Ca}$). Na^+/Ca^{2+} exchange can extrude Ca^{2+} (as an inward $I_{Na/Ca}$) or bring Ca^{2+} into the cell (as outward $I_{Na/Ca}$). $I_{Na/Ca}$ exhibits a reversal potential that is analogous to those of ion channels ($E_{Na/Ca} = 3E_{Na} - 2E_{Ca}$, where E_{Na} and E_{Ca} are equilibrium potentials for Na^+ and Ca^{2+}). In simpler terms, high [Ca^{2+}]$_i$ favours Ca^{2+} efflux (inward $I_{Na/Ca}$), whereas positive membrane potential (E_m) and high [Na^+]$_i$ favour outward $I_{Na/Ca}$. Figure 3a shows a typical ventricular action potential, Ca^{2+} transient, and inferred $E_{Na/Ca}$. At rest $E_m < E_{Na/Ca}$, so Ca^{2+} extrusion is favoured (inward $I_{Na/Ca}$; Fig. 3c, black curve). Early in the action potential the E_m exceeds $E_{Na/Ca}$, which tends to drive Ca^{2+} entry by outward $I_{Na/Ca}$ (until $E_m = E_{Na/Ca}$ during repolarization). Note that $E_{Na/Ca}$ changes because [Ca^{2+}]$_i$ (and thus E_{Ca}) changes. On repolarization of the action potential, the negative E_m and high [Ca^{2+}]$_i$ drive a large inward $I_{Na/Ca}$ and this reflects Ca^{2+} extrusion from the cell.

This simple expectation is complicated by elevations in local submembrane [Ca^{2+}]$_i$ and [Na^+]$_i$ ([Ca^{2+}]$_{sm}$ and [Na^+]$_{sm}$), which are caused by rapid Na^+ and Ca^{2+} fluxes (through I_{Na}, I_{Ca} and SR Ca^{2+} release)[23,24]. Figure 3b shows possible time courses for [Ca^{2+}]$_{sm}$ and [Na^+]$_{sm}$ that may be sensed by the Na^+/Ca^{2+} exchanger during normal excitation–contraction coupling[25]. Although [Ca^{2+}]$_{sm}$ may not get as high as cleft [Ca^{2+}]$_i$ during I_{Ca} and SR Ca^{2+} release, the high [Ca^{2+}]$_{sm}$ causes $I_{Na/Ca}$ to become inward very early in the rise of the action potential, such that very little Ca^{2+} enters through $I_{Na/Ca}$ ($\ll 1$ μM). An I_{Na}-induced rise in [Na^+]$_{sm}$ during the action potential might delay the reversal of $I_{Na/Ca}$ to inward (Fig 3c, red curve), but outward $I_{Na/Ca}$ would still last for only 4 ms or less (with Ca^{2+} entry still less than 1 μM). Thus, under physiological conditions Na^+/Ca^{2+} exchange works mainly in the Ca^{2+} extrusion mode, driven mostly by the Ca^{2+} transient. The positive E_m during the action potential plateau can, however, limit Ca^{2+} extrusion. This emphasizes again the importance of considering local versus bulk ion concentration (Fig. 3d) as discussed above for inactivation of I_{Ca}.

Although Na^+/Ca^{2+} exchange may normally work mainly in the Ca^{2+} efflux mode, the amount of Ca^{2+} influx through $I_{Na/Ca}$ can be increased greatly if [Na^+]$_i$ is elevated (for example, by digitalis glycosides that block Na^+/K^+-ATPase), if SR Ca^{2+} release and/or I_{Ca} is inhibited, or if action potential duration is prolonged[26].

Role of the sarcoplasmic reticulum

A high load of Ca^{2+} in the SR directly increases the amount of Ca^{2+} available for release, but also greatly enhances the fraction of SR Ca^{2+} that is released for a given I_{Ca} trigger[27,28]. This is due, at least in part, to a stimulatory effect of high intra-SR free [Ca^{2+}] ([Ca^{2+}]$_{SR}$) on the open probability of RyRs[29,30]. This increased RyR sensitivity to [Ca^{2+}]$_i$ at high [Ca^{2+}]$_{SR}$ means that what is often referred to as 'spontaneous SR Ca^{2+} release' at high cellular SR Ca^{2+} content (> 100 μmol l^{-1} cytosol) might be considered mechanistically to be triggered by high [Ca^{2+}]$_{SR}$ (sometimes in synergy with high [Ca^{2+}]$_i$). This is the basis of 'aftercontractions', transient inward current and delayed after depolarizations that can trigger arrhythmias[1].

At moderately low SR Ca^{2+} content, I_{Ca} can fail to induce Ca^{2+} release from the SR[27,28]. This may help the SR to reload if it becomes relatively depleted. Indeed, low SR Ca^{2+} release allows more Ca^{2+} influx through I_{Ca} (less inactivation) and Na^+/Ca^{2+} exchange (less shift towards Ca^{2+} extrusion). A decline in SR Ca^{2+} load, even locally, may contribute dynamically to the turn-off of Ca^{2+} release from the SR during excitation–contraction coupling. SR Ca^{2+} content can be raised by increasing Ca^{2+} influx, decreasing Ca^{2+} efflux, or enhancing Ca^{2+} uptake into the SR (for example, by

adrenergic stimulation or an increase in stimulation frequency, action potential duration, I_{Ca} or $[Na^+]_i$).

Phospholamban is an endogenous inhibitor of the SR Ca^{2+}-ATPase. Phosphorylation of phospholamban by cyclic-AMP-dependent or calmodulin-dependent protein kinases (PKA or CaMKII) relieves this inhibition, allowing faster twitch relaxation and decline of $[Ca^{2+}]_i$. Because the SR Ca^{2+}-ATPase competes better with Na^+/Ca^{2+} exchange, phosphorylation of phospholamban also enhances Ca^{2+} content in the SR. Indeed, targeted gene knockout of phospholamban results in animals with hyperdynamic hearts, with little apparent negative consequence[31].

The sarcoplasmic reticulum release complex

The RyR is both the SR Ca^{2+} release channel and a scaffolding protein that localizes numerous key regulatory proteins to the junctional complex. These include calmodulin (which can exert Ca^{2+}-dependent modulation of RyR function)[32], FK-506 binding protein (FKBP 12.6; which may stabilize RyR gating and also couple the gating of both individual and adjacent RyR tetramers)[33], PKA (which can alter RyR and I_{Ca} gating), phosphatases 1 and 2A (ref. 34) and sorcin (which binds to RyR and DHPR)[35]. RyRs are also coupled to other proteins at the luminal SR surface (triadin, junctin and calsequestrin)[36]. These proteins participate in both intra-SR Ca^{2+} buffering and modulation of the Ca^{2+} release process. RyRs are arranged in large organized arrays (up to 200 nm in diameter with more than 100 RyRs) at the junctions between the SR and sarcolemma beneath DHPRs (on the surface and in T-tubules)[37]. These arrays constitute a large functional Ca^{2+} release complex at the junction (or couplon).

This local functional unit concept is supported by observations of Ca^{2+} sparks or spontaneous local Ca^{2+} transients (Fig. 4). Ca^{2+} sparks reflect the nearly synchronous activation of a cluster of about 6–20 RyRs at a single junction, which is central to the generally accepted local control model of cardiac excitation–contraction coupling[38–41]. Ca^{2+} sparks are the fundamental units of SR Ca^{2+} release both at rest (rare, stochastic events) and also during excitation–contraction coupling. During excitation–contraction coupling, however, several thousand Ca^{2+} sparks in each cell are synchronized in time by the action potential, such that the local rises in $[Ca^{2+}]_i$ are completely overlapping in time and space (making the Ca^{2+} transient appear spatially uniform). Local Ca^{2+} release events can still be visualized during excitation–contraction coupling by either blocking more than 90% of I_{Ca} (to eliminate overlap of neighbouring events) or by trapping the released Ca^{2+} on exogenous buffers (preventing spatial overlap)[42–44].

Resting Ca^{2+} sparks are normally rare and isolated by the space between couplons. But when cellular and SR Ca^{2+} load rise, the exclusively local stochastic cluster behaviour is overcome and Ca^{2+} released at one junction can activate a neighbouring junction (owing to higher $[Ca^{2+}]_{SR}$ and $[Ca^{2+}]_i$) and lead to propagated Ca^{2+} waves and oscillations (Fig. 4d). The waves can propagate the length of a cell (at ~100 μm s^{-1}). When two waves collide they annihilate each other, indicative of RyR refractoriness in the wake of a wave of release.

Activating calcium release

The mechanisms underlying both activation (Fig. 5) and termination of Ca^{2+} release from the SR are controversial. By far the most widely accepted mechanism is Ca^{2+}-induced Ca^{2+}-release (CICR), in particular CICR mediated by the L-type Ca^{2+} channel current ($I_{Ca,L}$). It seems that the opening of one local L-type Ca^{2+} channel in each couplon (and 2–4 Ca^{2+} ions binding to the RyR) is sufficient to activate fully the release process at that couplon[1]. In a couplon, neighbouring RyRs are activated either by high local Ca^{2+} (> 10 μM) or coupled gating between RyRs. This renders an individual couplon all or none, but local $[Ca^{2+}]_i$ declines between couplons, which normally prevents wave propagation. Having more than one Ca^{2+} channel per couplon (10–25 DHPR/100 RyR) creates a safety margin to assure that each couplon will normally fire. Notably, only a fraction of the L-type Ca^{2+} channels and RyRs in a cell or couplon needs to open to produce the measured Ca^{2+} fluxes[1].

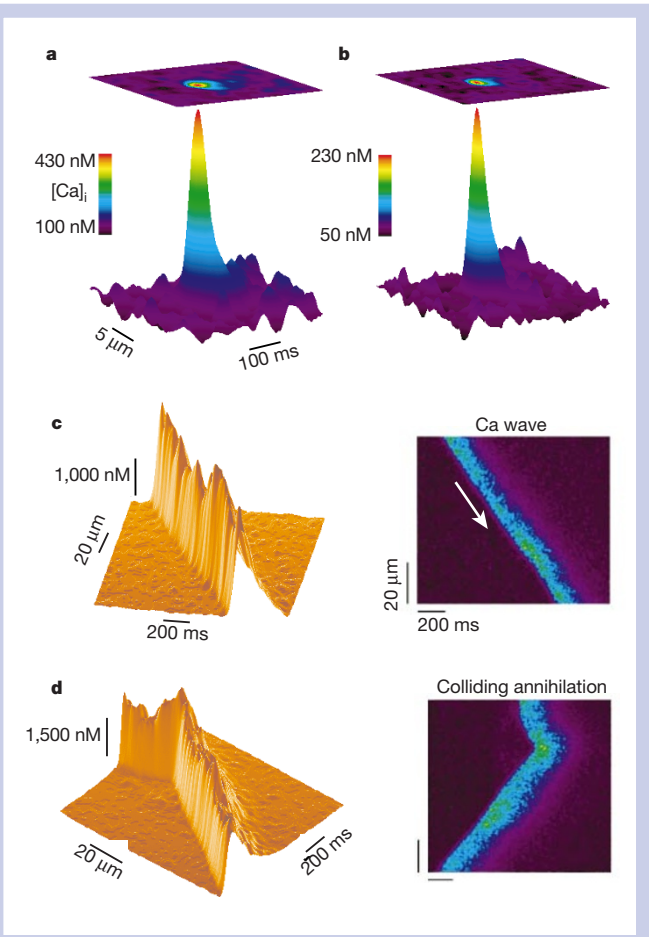

Figure 4 Confocal images of Ca^{2+} sparks and waves. **a**,**b**, Ca^{2+} sparks rise to a peak in about 10 ms, decline with a time constant of about 20 ms, and have a spatial spread of 2.5 μm (full-width at half-maximum). Ca^{2+} sparks were recorded in intact and permeabilized mouse ventricular myocytes, respectively. **c**,**d**, Ca^{2+} waves recorded in intact myocytes at high $[Ca^{2+}]_o$ (extracellular $[Ca^{2+}]$). Top panels in **a** and **b** and right panels in **c** and **d** are longitudinal line scan records (length versus time); the others are surface plots (length versus time versus $[Ca^{2+}]$). Local $[Ca^{2+}]_i$ is in pseudocolour in most panels, and the arrow indicates the direction of wave propagation at 93 μm s^{-1}. Figure prepared by Y. Li.

Overwhelming data support this general model as the main excitation–contraction coupling mechanism in heart, but contributions from other pathways have been also been proposed. T-type I_{Ca} ($I_{Ca,T}$) might work like $I_{Ca,L}$, but is nonfunctional in most ventricular myocytes. Even where present (in some Purkinje and atrial cells), a given $I_{Ca,T}$ is very much weaker than $I_{Ca,L}$ in triggering Ca^{2+} release[45,46]. Thus, T-type Ca^{2+} channels are not preferentially located in junctional regions, and might only be a very minor contributor to excitation–contraction coupling.

Ca^{2+} influx through Na^+/Ca^{2+} exchange has also been proposed to trigger SR Ca^{2+} release in two different ways. First, Na^+ current can raise local $[Na^+]_{sm}$ (see Fig. 3b), causing Ca^{2+} entry through $I_{Na/Ca}$ to trigger SR Ca^{2+} release[24,47,48]; however, this interpretation has been challenged[49–51]. Na^+ channels might also be excluded from the junctional cleft[15], making this mechanism less plausible. Second, outward $I_{Na/Ca}$ is activated directly by depolarization (Fig. 3), and can trigger SR Ca^{2+} release and contraction, especially at very positive E_m and when $I_{Ca,L}$ is blocked[52,53]. But a given Ca^{2+} influx through $I_{Na/Ca}$ is much less effective and slower than $I_{Ca,L}$ in triggering SR Ca^{2+} release[54]; therefore, when both I_{Ca} and $I_{Na/Ca}$ triggers coexist CICR is controlled

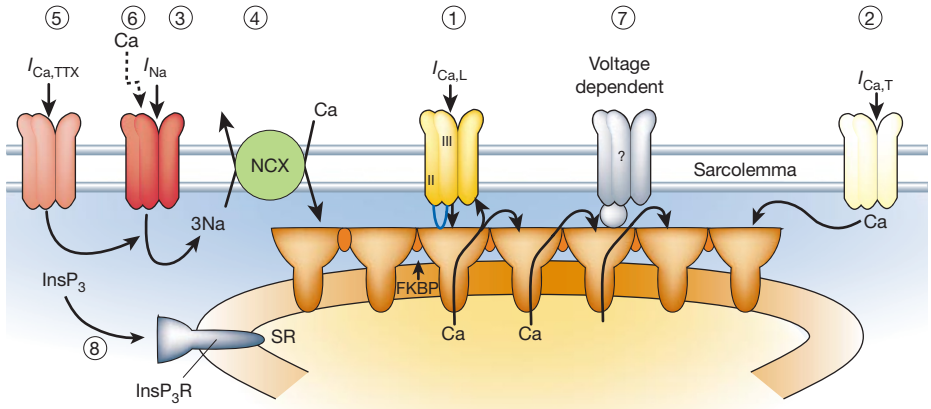

Figure 5 Candidate mechanisms for activation of Ca^{2+} release from the sarcoplasmic reticulum. Numbers refer to the order in which each is discussed in the text. The proposed mechanisms are as follows: 1, Ca^{2+}-induced Ca^{2+}-release (CICR) mediated by L-type Ca^{2+} channel current ($I_{Ca,L}$); 2, CICR mediated by T-type Ca^{2+} channel current ($I_{Ca,T}$); 3 and 4, CICR triggered by calcium influx through Na^+/Ca^{2+} exchange; 5, CICR triggered by calcium entry through tetrodotoxin (TTX)-sensitive Ca^{2+} current ($I_{Ca,TTX}$); 6, Ca^{2+} release mediated by 'slip mode conductance' in which Na^+ channels have altered preference for Ca^{2+}; 7, voltage-dependent Ca^{2+} release; and 8, Ca^{2+} release triggered by inositol (1,4,5) trisphosphate ($InsP_3$) through $InsP_3$ receptors. FKBP, FK-506 binding protein; NCX, Na^+/Ca^{2+} exchange.

almost completely by I_{Ca}. Na^+/Ca^{2+} exchanger molecules may be largely excluded from the junctional cleft[15]. Thus, although outward $I_{Na/Ca}$ can trigger SR Ca^{2+} release, its physiological role might be to raise $[Ca^{2+}]_{sm}$ during the latent time before a particular Ca^{2+} channel opening (or to buoy junctional $[Ca^{2+}]_i$).

Calcium entry through tetrodotoxin (TTX)-sensitive Ca^{2+} current ($I_{Ca,TTX}$) has been reported to occur in the absence of $[Na^+]_o$ (extracellular $[Na^+]$) and has been attributed to a distinct subpopulation of Na^+ channels[55,56]. Although $I_{Ca,TTX}$ might trigger CICR, it seems unlikely that appreciable Ca^{2+} entry occurs at physiological $[Na^+]_o$. Provocative data suggest that cardiac Na^+ channel selectivity is altered markedly by either β-adrenergic agonists or cardiac glycosides, making Na^+ channels prefer Ca^{2+} over Na^+ (termed 'slip-mode conductance')[57,58]. This TTX-sensitive Ca^{2+} entry might even trigger SR Ca^{2+} release. The huge change in Na^+ channel selectivity (slip-mode) induced by cAMP or ouabain has eluded detection by other groups and remains controversial[59–61]. It is unclear whether $I_{Ca,TTX}$ and slip-mode conductance are related, or if either really contributes to cardiac excitation–contraction coupling[39].

Despite overwhelming evidence that Ca^{2+} influx is essential for cardiac excitation–contraction coupling, a few studies have suggested a voltage-dependent Ca^{2+} release that does not require Ca^{2+} influx[62]. Several major concerns have strongly challenged this hypothesis, however, and at this point it is not convincing[1,39,63].

Inositol (1,4,5)-trisphosphate ($InsP_3$) can trigger Ca^{2+} release from smooth muscle SR and endoplasmic reticulum in many cell types, by means of $InsP_3$ receptors. There are $InsP_3$ receptors in ventricular myocytes (primarily isoform 2)[64,65]. Although high concentrations of $InsP_3$ can cause Ca^{2+} release in cardiac myocytes (particularly atrial cells, which have more $InsP_3$ receptors), the rate and extent of Ca^{2+} release are very much lower than for CICR, and action potentials are not known to stimulate $InsP_3$ production[66]. Moreover, cardiac α_1-adrenergic and muscarinic agonists increase production of $InsP_3$ and contractile force[67,68], but this inotropic effect is mediated mainly by protein kinase C rather than $InsP_3$ (refs 69,70).

These neurohumoral agents also stimulate hypertrophic gene transcription in a Ca^{2+}-dependent manner[71]. The $InsP_3$ produced may bind to perinuclear $InsP_3$ receptors (G. A. Mignery and D.M.B., unpublished observation), raising local $[Ca^{2+}]_i$ in this discrete microdomain, thereby activating calmodulin-dependent transcriptional regulation pathways (for example, CaMKII or calcineurin). This speculation is untested, but could represent a local control of excitation–transcription coupling, which is distinct from that involved in excitation–contraction coupling. On balance, $InsP_3$ has, at most, a minor modulatory role in cardiac excitation–contraction coupling, but may serve other spatially and functionally discrete roles.

In summary, cardiac SR Ca^{2+} release occurs mainly through CICR, and $I_{Ca,L}$ is the dominant Ca^{2+} source. The other mechanisms shown in Fig. 5 may slightly modulate Ca^{2+} release or be redundant back-up systems. A central limitation for some mechanisms is that the Ca^{2+} flux is not as spatially focused on the RyR as it is for $I_{Ca,L}$.

Terminating calcium release

Ca^{2+}-induced Ca^{2+}-release is inherently a positive-feedback mechanism, but its turn-off is essential for diastolic refilling of the heart. So what turns release off? Three possibilities include local depletion of SR Ca^{2+}, RyR inactivation (or adaptation), and stochastic attrition[44,72,73]. Stochastic attrition means that if the L-type Ca^{2+} channels and all RyRs in a junction happen to be closed simultaneously (as channels gate stochastically), then local $[Ca^{2+}]_i$ will fall very rapidly and interrupt the otherwise regenerative release. This might work for 1 DHPR and 1–2 RyRs, but with more realistic numbers of channels it becomes too unlikely that they will all close at once. The coupled gating of RyRs[74] might overcome this limitation, especially if most RyRs in a spark site are really coupled that way (but this remains to be tested).

Local depletion of SR Ca^{2+} cannot explain completely the turn-off of release, because very long lasting Ca^{2+} sparks are observed that do not decline with time (>200 ms)[38,75]. Thus, diffusion from other regions of the SR can limit local Ca^{2+} depletion in the SR. During a global Ca^{2+} transient, however, the whole $[Ca^{2+}]_{SR}$ declines. Because $[Ca^{2+}]_{SR}$ modulates RyR gating, $[Ca^{2+}]_{SR}$ depletion might contribute to shutting-off global SR Ca^{2+} release during a twitch. But, as stated above, this cannot explain fully why Ca^{2+} sparks turn off or why SR Ca^{2+} release terminates.

Two types of RyR inactivation have been reported (and both depend on $[Ca^{2+}]_i$). One is an absorbing inactivation (as in Na^+ channels), in which the RyR is unavailable for reopening until it recovers[44,76–78]. The second type is called adaptation, in which the RyR

after activating relaxes to a lower open probability, but can still be reactivated by a higher $[Ca^{2+}]_i$ (refs 79,80). Whether only one of these is functionally relevant remains controversial, and few cellular studies have addressed this unequivocally. But there is clearly some refractoriness in cellular and local events of SR Ca^{2+} release[44,75]. Recovery of RyR availability occurs with two time constants: one fast (100–300 ms) and one very slow (several seconds). Inactivation of RyRs may be important in minimizing inappropriate SR Ca^{2+} release events between heartbeats. In summary, it seems that both RyR inactivation and partial luminal depletion of SR Ca^{2+} (to reduce RyR opening) both contribute to the turn-off of release. Coupled gating of RyRs (so many gate as one) may also mean that a variant of stochastic attrition also contributes.

Modulation of calcium by sympathetic activation

Physiological sympathetic stimulation of the heart through β-adrenergic receptors increases developed contractions (inotropy) and accelerates relaxation (lusitropy) and $[Ca^{2+}]_i$ decline (Fig. 6). β-Adrenergic receptor stimulation activates a GTP-binding protein (G_S), which stimulates adenylyl cyclase to produce cAMP, which in turn activates PKA. This kinase phosphorylates several proteins related to excitation–contraction coupling (phospholamban, L-type Ca^{2+} channels, RyR, troponin I and myosin binding protein C).

The lusitropic effect of PKA is mediated by phosphorylation of phospholamban and troponin I, which speed up SR Ca^{2+} re-uptake and dissociation of Ca^{2+} from the myofilaments, respectively. But phosphorylation of phospholamban is by far the dominant mechanism for both the lusitropic effect and accelerating the decline in $[Ca^{2+}]_i$ (ref. 81). The faster SR Ca^{2+} uptake also contributes to increasing the SR Ca^{2+} content. The inotropic effect of PKA activation is mediated by the combination of increased I_{Ca} and greater availability of SR Ca^{2+}. This synergistic combination greatly enhances Ca^{2+} transient amplitude, and more than offsets the reduction in myofilament Ca^{2+} sensitivity (caused by troponin I phosphorylation, which by itself would reduce force). The depressant of PKA on the myofilaments seem to be completely attributable to phosphorylation of troponin I (versus myosin-binding protein C), because substitution of troponin I with a non-phosphorylatable troponin I abolishes the myofilament effects of PKA[82].

PKA can also modulate the open probability of RyR channels. In isolated single-channel recordings, PKA increased initial RyR opening during an abrupt $[Ca^{2+}]_i$ rise, but decreased the steady-state open probability at a given $[Ca^{2+}]_i$ (ref. 80). In contrast, Marx *et al.*[33] found that PKA enhanced the steady-state open probability of single RyRs in bilayers, and attributed this to the displacement of FKBP-12.6 from the RyR. Moreover, they found that RyRs were hyperphosphorylated in heart failure, which could cause a diastolic leak of SR Ca^{2+} and contribute to the reduced SR Ca^{2+} content in heart failure (see above). But in more intact cellular systems, no effect of PKA-dependent RyR phosphorylation could be detected on resting SR Ca^{2+} leak (as Ca^{2+} sparks) in the absence of phospholamban (with unchanged SR Ca^{2+} load)[83]. Phosphorylation of RyRs may also alter the intrinsic responsiveness of SR Ca^{2+} release to an I_{Ca} trigger signal, but results concerning this have been mixed, showing an increase, decrease and lack of change[84–86]. Thus, whether PKA-dependent phosphorylation alters RyR behaviour during rest or excitation–contraction coupling remains controversial. This process is particularly challenging to measure in intact cells, because increases in I_{Ca} and in SR Ca^{2+} uptake make isolation of intrinsic RyR effects difficult.

Eisner *et al.*[87] have also argued that, because of autoregulation, altered systolic gating properties of RyRs in intact cells alone exert only transitory effects on Ca^{2+} transient amplitude. That is, abrupt increases in RyR opening or fractional SR Ca^{2+} release cause greater Ca^{2+} extrusion through Na^+/Ca^{2+} exchange at the first beat, thereby decreasing SR Ca^{2+} available for the next beat. In the steady state, this lower SR Ca^{2+} content offsets the increased fractional SR Ca^{2+} release such that Ca^{2+} transients are almost unchanged. However, enhanced diastolic leak of SR Ca^{2+} might still contribute to reduced SR Ca^{2+} load and systolic function in heart failure.

Local signalling is also important in the β-adrenergic receptor cascade. L-type Ca^{2+} channels co-assemble with β_2-adrenergic receptors, G_S, adenylyl cyclase, PKA and phosphatase 2A (at least in brain)[88]. The cardiac RyR serves as both a PKA target and a scaffolding protein (where PKA and phosphatases 1 and 2A are all bound to the RyR through anchoring proteins)[34]. The close physical proximity may be functionally essential[89]. The activation of β_1-adrenergic receptors in ventricular myocytes produces robust inotropic and lusitropic effects, paralleled by phosphorylation of Ca^{2+} channels,

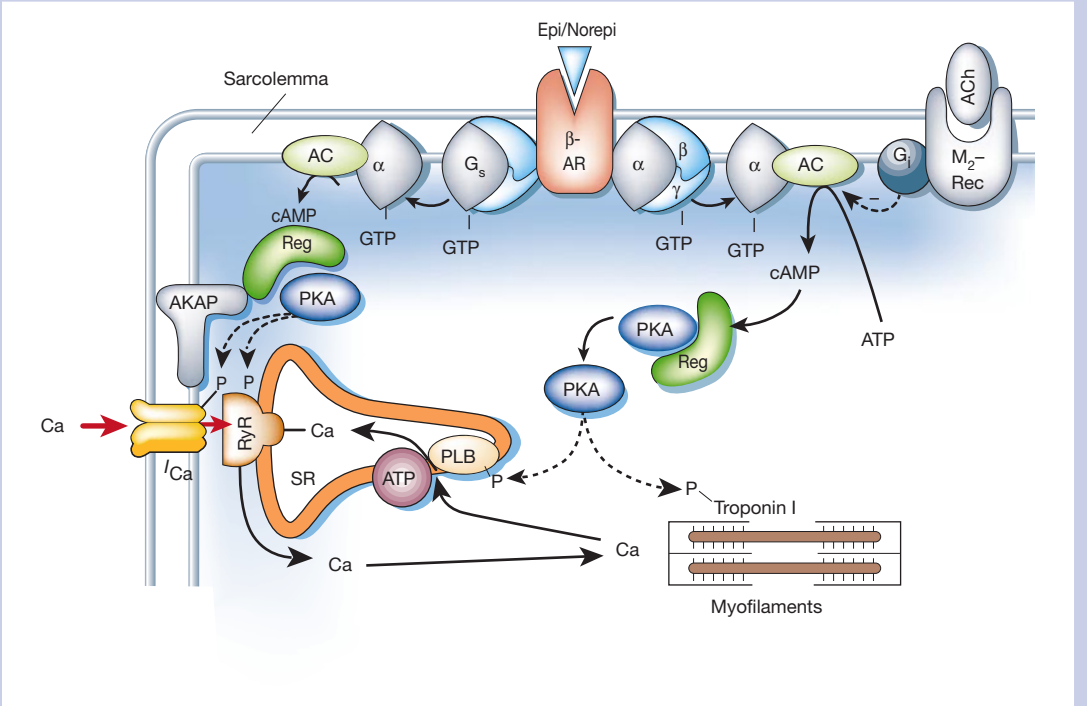

Figure 6 β-Adrenergic receptor activation and phosphorylation targets relevant to excitation–contraction coupling. AC, adenylyl cyclase; ACh, acetylcholine; AKAP, A kinase anchoring protein; β-AR, β-adrenergic receptor; M_2-Rec, M_2-muscarinic receptor; PLB, phospholamban; Reg, PKA regulatory subunit; SR, sarcoplasmic reticulum.

phospholamban and troponin I. By contrast, the activation of β_2-adrenergic receptors may be more restricted to I_{Ca} enhancement[90], and β_2-adrenergic receptors are located almost exclusively in specialized sarcolemmal invaginations called caveolae (versus β_1-adrenergic receptors, which are largely non-caveolar)[91].

Activation of other G-protein-coupled receptors that greatly stimulate cAMP production (for example, prostaglandin E, histamine, glucagon-like peptide-1) produce little or no inotropic effect (as compared with β_1-adrenergic receptors)[92]. Thus, the functionally important levels of cAMP, activated PKA, phosphatase and phosphodiesterase (which breaks down cAMP) are those very near to that of the target protein. The total cellular concentration of cAMP might be irrelevant to key regulatory pathways, except as an overflow from local cAMP-mediated signal transduction. However, if this is true, it is less clear how targeting would practically work for phospholamban and troponin I phosphorylation (as compared with I_{Ca} or RyR). This would require very high amounts of the various anchoring and signalling proteins, because troponin I and phospholamban are present at 50 μM or higher concentrations and are dispersed widely in the cell.

Relative receptor locations can also regulate this signalling cascade. For example, M_2-muscarinic receptor activation can either decrease or increase concentrations of cAMP levels, depending on whether they were produced by β_1- or β_2-adrenergic receptors, respectively[93]. This may be due in part to the relative exclusion of M_2-muscarinic receptors from caveolae. Thus, the location of receptors and their signalling cascade components can selectively determine function.

Implications for calcium handling

Calcium in cardiac myocyte is in a dynamic yet delicate balance, created by multiple interacting cellular systems that can be tuned by physiological modulators. It is also clear that we must think increasingly in terms of microdomains and local control, without losing perspective on the integrative framework in which these domains function. RyR and I_{Ca} are both responsible for, but also controlled by, the local cleft $[Ca^{2+}]_i$ (which may differ greatly from the $[Ca^{2+}]_{sm}$ that controls Na^+/Ca^{2+} exchange). Key regulatory pathways (for example, β-adrenergic receptors, calmodulin and possibly Ca^{2+}-dependent transcription) also exhibit local functional coupling in microdomains. These various signalling domains surely overlap spatially and functionally.

It will be important to develop new experimental tools to assess how the key signalling molecules (including Ca^{2+}) interact functionally and are targeted to the appropriate microdomains. Future studies will need to clarify how cells distinguish between Ca^{2+} involved in excitation–contraction coupling and transcriptional regulation. We may also have to start thinking more stochastically about local reactions. For example, at resting $[Ca^{2+}]_i$ there is less than one free Ca^{2+} ion in an entire junctional cleft, making the concept of collision probability more meaningful than concentration. Thus, much challenging work lies ahead if we are to understand the physiological functions of many of these processes *in situ*, particularly with respect to signalling in microdomains, as well as the pathophysiological and therapeutic implications. □

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