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MINI-REVIEW

Kinetic Analysis of Excitation–Contraction Coupling

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Abstract

Recent studies of isolated muscle membrane have enabled induction and monitoring of rapid Ca^{2+} release from sarcoplasmic reticulum (SR)[5] *in vitro* by a variety of methods. On the other hand, various proteins that may be directly or indirectly involved in the Ca^{2+} release mechanism have begun to be unveiled. In this mini-review, we attempt to deduce the molecular mechanism by which Ca^{2+} release is induced, regulated, and performed, by combining the updated information of the Ca^{2+} release kinetics with the accumulated knowledge about the key molecular components.

Key Words: Excitation–contraction coupling; sarcoplasmic reticulum; transverse-tubular system; signal transmission; calcium release kinetics.

Introduction

Traditionally, most of the important information concerning the mechanism of excitation–contraction (E–C) coupling was derived from studies with either intact or skinned musble fiber preparations (Endo, 1977). However,

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[5] Abbreviations used: AMP-PCP, adenosine 5′-(β, γ-methylenetriphosphate); $C_{1/2}$, concentration a half-maximal activation or inhibition; Con-A, concanavalin A; DACM, *N*-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide; DCCD, dicyclohexylcarbodiimide; SR, sarcoplasmic reticulum; DHP, dihydropyridine; E–C, excitation–contraction; EP, phosphorylated intermediate of the enzyme; IP_3, inositol 1,4,5-trisphosphate; JFM, junctional face membrane; M_r, molecular weight; T-tubule, transverse-tubular system.

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the fiber system is too complex for an analysis of various steps of E–C coupling at a molecular level. On the other hand, isolated and purified protein systems are very useful for a clear-cut demonstration of functional roles played by particular proteins, as recently demonstrated in the single-channel measurements of the ryanodine-receptor protein incorporated into planar lipid bilayers (Imagawa *et al.*, 1987; Lai *et al.*, 1988; Hymel *et al.*, 1988). However, these systems are not always suitable for studies of more complex problems involving protein–protein and membrane–membrane interactions. An intermediate between the above two systems, namely, isolated and purified membrane systems that retain physiological functions, appears to be more appropriate to use in analyzing relatively complex problems at a molecular level (cf., Inesi and Malan, 1976; Martonosi, 1984).

The most effective strategy toward the elucidation of the E–C coupling mechanism using such isolated membrane systems would be first to resolve various elementary steps involved in the process through kinetic and chemical analyses, then to identify the molecular components involved in these events, and finally to establish the modes by which various key proteins play their roles in the individual steps of the signal–release coupling. The recent advance in the kinetic and biochemical studies of Ca^{2+} release from isolated SR is, to a large extent, in accord with this strategy. In this article, we first review the kinetic characteristics of various types of *in vitro* Ca^{2+} release described in the recent literature, and then discuss the deduced kinetic properties that are useful for an analysis of physiologically meaningful Ca^{2+} release. Finally, new information concerning several protein components of the triadic junctional membranes is discussed.

Kinetic Characteristics of Various Types of Ca^{2+} Release from SR *in vitro*

General Comments

Ca^{2+} release can be produced from the isolated SR vesicles under a variety of conditions as outlined below. To determine whether Ca^{2+} release induced in the *in vitro* conditions has any physiological significance, at least two criteria should be met. Firstly, the rate of Ca^{2+} release should be sufficiently high to account for rapid tension development in muscle *in vivo*. Secondly, the Ca^{2+} release process should be reversible in order to mimic the transient tension development and decay in an intact muscle, and also to ensure that the release that is produced is not due to the irreversible breakdown of the membrane permeability barrier. Some, but not all, types of Ca^{2+} release described in the literature meet these criteria.During the relatively short history of research of Ca^{2+} release *in vitro*, a variety of types of Ca^{2+} release have been reported in the literature. They are distinguishable in

Table I. Kinetic Characteristics of Various Types of Ca^{2+} release from Sarcoplasmic Reticulum Vesicles.

Type of Ca^{2+} release	Inductor	$C_{1/2}$	Activators	k, s^{-1}	A, nmol/mg	Ca dependence pCa	Inhibitors[b] $C_{1/2}$	Ref.[c]
Ca^{2+} induced Ca^{2+} release	exter Ca^{2+}	–	–	0.7	5	7–4	RR 01–0.8 μM	1–8
			ATP 1 mM	15	35	8–4	Mg^{2+} 0.2 mM	
			AMP-PCP				Tetracaine 0.1 mM	
			cAMP				Procaine 1 mM	
			AMP					
			ADP					
Drug-induced Ca^{2+} release	Caffeine	0.5 mM	–	0.6	25	8–4	RR 0.1 μM	1, 3, 6
			ATP (mM)	18	30	10–4	Mg^{2+} 0.15 mM	
							Tetracaine 0.1 mM	
	Quercetin	3.3 μM	–	0.6	25	6–4	RR 0.1 μM	1
							Mg^{2+} 0.15 mM	
							Tetracaine 0.1 mM	
							Procaine 1 mM	
	Halothane	150 μM	–	–	–	6–4	RR 0.6 μM	9, 10
			ATP (mM)				Mg^{2+} (5 mM)	
	Doxorubiin	3 μM	–	0.21	15	–	–	11
	BED	–	–	0.0125	–	–	RR 2 μM	12
Depolarization-induced Ca^{2+} release	Ionic replacement	–	–	k_1 60–140	A_1 10–15	6–5	Procaine 1 mM	7, 13, 14
			ATP	k_2 0.8–1.5	A_2 56		Tetracaine 0.1 mM	
			ATP	70	40	–	RR 0.1–0.8 μM	
SH modification	Silver ion	10 μM	–	5	–	–	RR (5 μM)	3, 15, 16
			ATP	15	–	–	Mg^{2+} (1–10 mM)	
			Mg^{2+} (0–1 mM)	–	–	–	Tetracaine (0.25–1 mM)	
							Procaine (1.5–10 mM)	
	PDS	1 mM	ATP	–	–	6–3	RR (10 μM)	17
							Procaine (25 mM)	
							Mg^{2+} (5 mM)	
	Alcian blue	3 μM	–	0.13	54	–	RR 0.1 μM	18
							Mg^{2+} 1 mM	
							Reducing agents	
Spontaneous release during active Ca^{2+} uptake ($+P_i$).	–	–	–	0.033	–	–	RR 0.8 μM (card)	19

[a] BED, bromo-eudistomin D; card, cardiac SR; exter, extravesicular; PDS, 4, 4′-dithiodipyridine; RR, ruthenium red.
[b] The concentrations of "inhibitors" shown in the parentheses are the examples of an effective concentration rather than the $C_{1/2}$.
[c] 1, Kim *et al.*, 1983; 2, Meissner *et al.*, 1986; 3, Moutin and Dupont, 1988; 4, Meissner and Henderson, 1987; 5, Meissner, 1984; 6, Ohnishi, 1979; 7, Antoniu *et al.*, 1985; 8, Kirino *et al.*, 1983; 9, Ohnishi, 1987; 10, Beeler and Gable, 1985; 11, Zorzato *et al.*, 1985; 12, Nakamura *et al.*, 1986; 13, Ikemoto *et al.*, 1984; 14, Ikemoto *et al.*, 1985; 15, Abramson *et al.*, 1983; 16, Salama and Abramson, 1984; 17, Nagura *et al.*, 1988; 18, Abramson *et al.*, 1988; and 19, Palade *et al.*, 1983.

terms of the methods of induction and their kinetic characteristics. Representative types of these are depicted in Table I. Most researchers of Ca^{2+} release *in vitro* have analyzed the kinetics of various types of release, assuming that the release time course follows a single or double exponential: $A_1\{1 - \exp(-k_1)t\}$ in the case when an increase of $[Ca^{2+}]_0$ is monitored, and $A_1 \exp(-k_1 t)$ when a decrease of $[Ca^{2+}]_i$ is monitored. To express kinetic properties of various types of reported Ca^{2+} release, therefore, the A and k values are used as listed in Table I. Several other important pieces of information concerning, e.g., activators and inhibitors are also included in Table I. Some of the Ca^{2+} release types described in the text (e.g., Ca^{2+} release induced by inositol 1,4,5-triphosphate (IP_3), chemical modification of amino groups, and a pH jump) are not listed in Table I because of virtual lack of kinetic data.

Ca^{2+}-Induced Ca^{2+} Release

Ca^{2+} release induced by an abrupt change in the extravesicular $[Ca^{2+}]$ with or without addition of potentiating agents, the so-called Ca^{2+}-induced Ca^{2+} release, has been investigated most extensively. The kinetic properties characteristic of this type of release are summarized as follows. Release is activated at a low $[Ca^{2+}]_0$ range with an apparent affinity of 10^5 M^{-1}, and further increase of $[Ca^{2+}]_0$ inhibits release (Kim *et al.*, 1983; Kirino *et al.*, 1983; Meissner *et al.*, 1986). An increase of $[Mg^{2+}]$ to physiological levels (e.g., 1 mM) suppresses Ca^{2+} release (Kirino *et al.*, 1983; Meissner and Henderson, 1987) with concomitant reduction of the Ca^{2+} affinity (Meissner, 1984; Meissner *et al.*, 1986). The size of Ca^{2+} release (A), but not the rate constant (k), increases as the extent of Ca^{2+} loading increases (Kim *et al.*, 1983). ATP and nonhydrolyzable ATP analogs [e.g., adenosine 5′-(β,γ-methylenetriphosphate (AMP-PCP)] produce considerable increases of both the size and the rate constant (Table I). All of the above characteristics of Ca^{2+}-induced Ca^{2+} release *in vitro* are very similar to those in the skinned fiber preparations (cf., Endo, 1977). This suggests that the basic molecular mechanism of Ca^{2+}-induced Ca^{2+} release operating *in situ* is maintained in the isolated SR vesicles.

In many Ca^{2+}-induced release experiments described above, release was initiated by adding an appropriate amount of Ca^{2+} at the steady state of ATP-dependent Ca^{2+} uptake to produce an abrupt increase of the $[Ca^{2+}]_0$. Essentially, the same type of Ca^{2+} release could be induced by changing $[Ca^{2+}]_0$ to appropriate levels after passively loading the SR vesicles with mM Ca^{2+}. For example, the solution in which the vesicles have been incubated with several mM Ca^{2+} was replaced with an EGTA solution containing release-blocking agents such as several mM Mg^{2+}, and the extravesicular solution

was replaced again with a solution containing an appropriate EGTA-Ca^{2+} buffer to create an abrupt increase in the $[Ca^{2+}]_0$ (Sumbilla and Inesi, 1987). In other passive loading-release experiments, Ca^{2+} release was induced by an abrupt decrease of the $[Ca^{2+}]_0$ from several mM to the order of μM (Moutin and Dupont, 1988). The kinetic properties of the resultant Ca^{2+} release appear to be not much different, depending upon the directions of a Ca^{2+} jump (up or down). However, according to the precise studies by Fabiato (1985*a–c*) on the skinned cardiac muscle fibers, the modes of creating the Ca^{2+} jump such as the rate and the amplitude of the jump as well as the final $[Ca^{2+}]_0$ affect the kinetic characteristics of the induced Ca^{2+} release. It seems important to examine whether these factors, especially (a) the direction and (b) speed, affect the kinetics of release in the isolated membrane system.

Drug-Induced Ca^{2+} Release

Several drugs such as caffeine, quercetin, and halothane induce Ca^{2+} release (under both active and passive loading conditions) at low $[Ca^{2+}]_0$ where there is virtually no Ca^{2+}-induced Ca^{2+} release (cf., Table I; Kim *et al.*, 1983; Kirino *et al.*, 1983; Beeler and Gable, 1985; Ohnishi, 1987; Ogawa and Kurebayashi, 1982). In the presence of activating concentrations of Ca^{2+}, caffeine, quercetin, and halothane increase the size of Ca^{2+} release with concomitant increase of the Ca^{2+} affinity (Kirino *et al.*, 1983; Nagasaki and Kasai, 1983; Kurebayashi and Ogawa, 1984). However, the $[Ca^{2+}]_0$ dependence of the release induced by these drugs shows a bell-shaped profile similar to that of Ca^{2+}-induced release (Kim *et al.*, 1983). Furthermore, the modes of acceleration (e.g., by ATP or its analogs) and inhibition by various inhibitors of drug-induced release are essentially the same as those of Ca^{2+}-induced release. Thus, it appears that the drug-induced release is in fact a drug-accelerated form of the Ca^{2+}-induced release (cf., Endo, 1977). It is interesting that the V_{max} values of the release induced by caffeine and quercetin are about same, although their K_m values are significantly different (Kim *et al.*, 1983). Thus, it is likely that these release-inducing drugs share a common binding site, although some other explanations are possible.

Several other drugs such as doxorubicin (Zorzato *et al.*, 1985) and bromo-eudistomin D (Nakamura *et al.*, 1986) induce Ca^{2+} release similar to Ca^{2+}-induced Ca^{2+} release (Table I). Doxorubicin, bromo-eudistomin D, and well-known muscle contractants, such as caffeine, induce the twitch tension of an intact muscle fiber. The fact that many drugs that induce contraction in muscle fibers induce Ca^{2+} release from the isolated SR vesicles indicates that the drug-sensing mechanism built in the muscle membrane *in situ* is retained in the isolated vesicles.

Depolarization-Induced Ca^{2+} Release

Several methods of ionic replacement—viz. (a) replacement of an intravesicular impermeable anion with an extravesicular permeable anion, (b) replacement of an intravesicular permeable cation with an extravesicular impermeable cation, or (c) performance of both a and b—produce an abrupt change of the potential across the vesicular membrane to an intravesicular side-negative direction (chemical "depolarization"). However, the ionic replacement method tends to produce a nonspecific Ca^{2+} release due to an osmotic artifact (Meissner and McKinley, 1976). To circumvent such an osmotic artifact, many researchers of the Cl^--induced Ca^{2+} release in muscle fiber preparations kept the product of $[K]_0[Cl]_0$ constant before and after the ionic replacement (Mobley, 1979; Donaldson, 1985; Stephenson, 1985). In the case of isolated SR vesicles, one can devise an appropriate ion replacement protocol by monitoring light-scattering changes that serve as a sensitive monitor of the osmotic changes of vesicular size and shape (Kometani and Kasai, 1978). For instance, replacement of 0.15 M K gluconate with 0.15 M choline Cl (double replacement, see method c described above) eliminates the osmotic artifact as evidenced by the lack of light-scattering changes, and at the same time maximizes the membrane potential change (Ikemoto *et al.*, 1984, 1985).

Although earlier studies were carried out with an intention of producing Ca^{2+} release from SR by direct depolarization of the SR membrane (e.g., Kasai and Miyamoto, 1976*a*, *b*), it seems to be clear now that the ionic replacement-induced Ca^{2+} release is in fact triggered by the depolarization of the T-tubule membrane rather than that of the SR membrane *per se*, as evidenced by the following experiments. Using split mammalian muscle fiber preparations containing sealed T tubules, Donaldson (1985) demonstrated that the Cl^--induced release from SR was blocked when ouabain (the inhibitor of the $Na^+ - K^+$ ATPase) was trapped in the sealed T tubules. The ouabain trap has no effect on caffeine-induced Ca^{2+} release. Similarly, in an isolated microsomal preparation containing the triadic complex, Ikemoto *et al.* (1984) demonstrated that disruption of the T-tubule–SR linkage by a French press treatment (Caswell *et al.*, 1979) blocks ion replacement-induced release (replacement of K gluconate with choline Cl, see above). Upon reassociation of the transverse-tubular system (T-tubules) with SR by cacodylate treatment (Caswell *et al.*, 1979), however, the release activity was restored. On the other hand, Ca^{2+} release induced by caffeine or quercetin was not affected by the T-tubule dissociation and reassociation procedures (Ikemoto *et al.*, 1984). These results clearly indicate that the ion replacement-induced release is mediated via the attached T-tubules, whereas drug-induced release is produced by direct stimulation of the SR membrane by drugs (cf., Fig. 1).

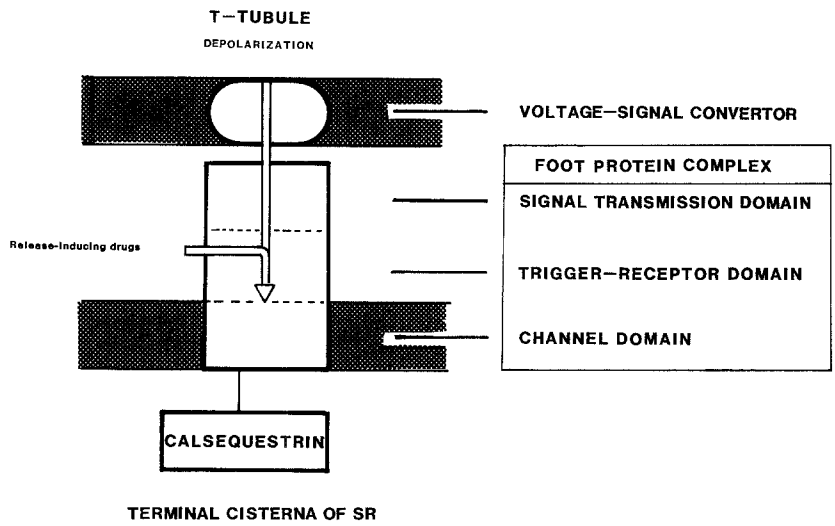

Fig. 1. Schematic illustration of the hypothetical mechanism for the activation and regulation of Ca^{2+} release from sarcoplasmic reticulum deduced from the recent kinetic and biochemical studies. The depolarization of the T-tubule membrane would generate some form of the transmittable signal by the putative voltage-signal convertor located in the T-tubule. The signal is propagated through the foot via the signal-transmission domain, and then to the trigger-receptor domain, and activates the Ca^{2+} release channel. On the other hand, the release-inducing drugs bind to the trigger-receptor domain and activate the channel. The intravesicular protein calsequestrin interacts with the JFM proteins (particularly the foot protein). This lets calsequestrin regulate the functions of the channels; conversely, the triggering signal applied to the channel appears to be transmitted to calsequestrin leading to the dissociation of the casequestrin-bound Ca.

Several pieces of evidence suggest that polarization of the T-tubule membrane (in a direction to make the inside of the T-tubule vesicles, viz., extracellular side, positive) due to the $Na^+ - K^+$ pump *prior to* the ionic replacement is required for the induction of Ca^{2+} release. For example, (a) Ouabain blocks Cl^--induced release as described above (Donaldson, 1985) and (b) permeabilization of the T-tubule membrane to Na^+ by monensin also results in blocking of release (Volpe and Stephenson, 1986). This also seems to be the case in the ion replacement-induced release in the isolated triad vesicles, since the induction of release has been successful only after ATP-dependent active Ca^{2+} loading (the conditions that enable the $Na^+ - K^+$ pump to operate), but has never occurred after passive Ca^{2+} loading (viz., in the absence of ATP).

In the absence of activating nucleotides, the time course of the depolarization-induced Ca^{2+} release *in vitro* consists of several distinguishable phases (Ikemoto *et al.*, 1984): (1) a latent period, (2) a rapid

(k = 60–145 sec^{-1}) release of a relatively small amount of Ca^{2+} (A = 10–15 nmol/mg; see Table I), and (3) a slow ($k \leqslant 1\ sec^{-1}$) release of a larger amount of Ca^{2+} (e.g., 50 nmol/mg). Subsequently, all of the Ca^{2+} released in phases 2 and 3 is reaccumulated. The rate constants of phase 2 are on the same order of magnitude as expected for Ca^{2+} release in an intact fiber. The amount of Ca^{2+} released in phase 2 (up to 15 nmol/mg) would be sufficient to produce an increase of cytosolic $[Ca^{2+}]_0$ at least by several micromolar units (cf., Ikemoto *et al.*, 1984). The kinetic characteristics of phase 3 are essentially the same as those of Ca^{2+}-induced release in the absence of activators (cf., Table I), and hence it presumably represents a secondary release induced by the Ca^{2+} released in phase 2.

The $[Ca^{2+}]_0$ dependence of activation and suppression of depolarization-induced release shows a bell-shaped curve similar to that of Ca^{2+}-induced release. However, there are several distinct differences. For example, (a) at $[Ca^{2+}]_0 \simeq 0$, there is virtually no Ca^{2+}-induced release, but there is a small but significant amount of depolarization-induced Ca^{2+} release. (b) In the $[Ca^{2+}]_0$ range where activation and suppression of release occur, the $[Ca^{2+}]_0$-dependence profile of depolarization-induced release is much sharper than that of Ca^{2+}-induced Ca^{2+} release.

Other Types of Ca^{2+} Release

The type of Ca^{2+} release induced by chemical modification of well-defined amino acid groups such as thiols [Salama and Abramson, 1984; Abramson and Salama, 1989 (this volume)] and amino groups (Shoshan-Barmatz, 1986) would be useful for investigating possible roles of particular amino acid side-chain groups of the proteins involved in the release mechanism.

Earlier studies by Katz *et al.* (1977*a–c*) have shown that the unidirectional Ca^{2+} efflux component develops spontaneously during ATP-dependent Ca^{2+} uptake in the presence of relatively high concentrations of inorganic phosphate. It has also been shown that the Ca^{2+} efflux component is activated by several mM $[Ca^{2+}]_i$ and several μM $[Ca^{2+}]_0$. Thus, what is generally called "spontaneous Ca^{2+} release" can be produced if both $[Ca^{2+}]_i$ and $[Ca^{2+}]_0$ are brought up to critical threshold levels by appropriate methods [e.g., repetitive addition of Ca^{2+} pulses in a titrating fashion (Palade *et al.*, 1983)]. The spontaneous Ca^{2+} release is potentiated by caffeine. Together with the similarity of its activating $[Ca^{2+}]_0$ to that of Ca^{2+}-induced Ca^{2+} release, spontaneous Ca^{2+} release seems to share a common underlying mechanism with Ca^{2+} and drug-induced Ca^{2+} release. However, the presence of high concentrations of P_i or pyrophosphate (e.g., 135 mM P_i) was reported to be

essential for this type of release. The reasons for the requirement for such unusual conditions remain to be clarified.

IP_3 might serve as a chemical messenger in the E–C coupling mechanism [Vergara *et al.*, 1985; Donaldson *et al.*, 1987; Hidalgo and Jaimovich, 1989 (this volume)]. The IP_3-mediated Ca^{2+} release has been investigated with isolated SR vesicles as well as muscle fiber preparations with rather controversial results. Judging from the fact that the success rate of the production of IP_3-induced Ca^{2+} release is much less in the isolated vesicles (Volpe *et al.*, 1985) than in the intact or skinned muscle fiber preparations, the IP_3-sensitive components might tend to be removed or denatured during the isolation of the vesicles.

A rapid increase of pH by 0.2 pH units or more, but not a decrease of pH, produces Ca^{2+} release from SR as evidenced by the tension development of skinned muscle fiber (Shoshan *et al.*, 1981). Treatment of the SR membrane with dicyclohexylcarbodiimide (DCCD) inhibits the pH-change-induced Ca^{2+} release, and the major site of DCCD incorporation is a low M_r proteolipid of SR.

Reversibility of Various Types of Ca^{2+} Release

As described in *General Comments*, one of the important criteria to screen physiologically meaningful Ca^{2+} release is the reversibility of release. Depolarization-induced release is reversible in that all Ca^{2+} released is reaccumulated actively as described above. Similarly, Ca^{2+}-and drug-induced Ca^{2+} release are also reversible. However, the other types of Ca^{2+} release are virtually irreversible.

Only the depolarization-induced (T-tubule-mediated) release shows a physiological rate constant (k) in the absence of activators, while the rate constant of Ca^{2+} and drug-induced Ca^{2+} release reaches about the same level after maximal activation (Table I; for further discussion, see below). However, the rate constant of Ca^{2+} reaccumulation is much lower than that of the rapid decay of the transiently developed twitch tension in muscle fiber, in any types of reversible Ca^{2+} release *in vitro*.

Activators and Inhibitors of Various Types of Ca^{2+} Release

The presence of millimolar concentrations of ATP or its analogs during the Ca^{2+} release reaction produces a considerable increase in the rate constant (k) of Ca^{2+}-induced release and caffeine-induced release with little increase in the amount of release (A). Conversely, in the case of depolarization-induced Ca^{2+} release, ATP produces a considerable increase in the A value with little change in the k value (Table I). As the consequence

of such effects, at maximally activating concentrations of ATP, the kinetic properties (both A and k values) of the two different classes of Ca^{2+} release (Ca^{2+}- and drug-induced release, and depolarization-induced release) become indistinguishable (cf., Table I). The other types of Ca^{2+} release, such as Ag^{+}-induced release, are also activated by millimolar concentrations of ATP, suggesting that different types of Ca^{2+} release described above share some common mechanism so far as the activation by ATP is concerned. The activation by ATP occurs immediately after mixing, and nonhydrolyzable ATP analogs [e.g., AMP-PCP (Meissner, 1986)] are equally effective, indicating that nucleotide binding *per se* rather than hydrolysis is a mechanism for the observed activation.

It is surprising that various types of Ca^{2+} release, which are quite different in their kinetic characteristics and in the conditions of release induction, are inhibited by many Ca^{2+} release inhibitors in about the same concentration range. We would call this category of inhibitors a *common inhibitor*. For instance, the dose-dependence curve of the ruthenium-red-inhibition of Ca^{2+}-induced release and that of depolarization-induced release are almost completely identical (Antoniu *et al.*, 1985), in spite of the fact that they belong to two discretely different classes of Ca^{2+} release and that their kinetic properties are quite different in the absence of activating nucleotides (see above). The other types of Ca^{2+} release are also inhibited by about the same concentration of ruthenium red (Table I; Palade, 1987).

There is the second category of release inhibitors, viz., those that inhibit particular types of Ca^{2+} release without effect on the other types (*selective inhibitor*). Dantrolene, for example, exerts selective effects on different types of Ca^{2+} release: caffeine-induced release, inhibition; Ca^{2+}-induced release, no effect; and depolarization-induced release, both activation and inhibition depending upon the time of drug application (Danko *et al.*, 1985).

The third category may include the reagents that work as activators at low concentrations, but become inhibitors at higher concentrations. For instance, ryanodine (Lattanzio *et al.*, 1984) and polylysine (Cifuentes *et al.*, 1988) belong to this category.

Beside the inhibiting chemical reagents described above, there appear to be several types of built-in release-inhibition mechanism as follows. Calmodulin-dependent phosphorylation of a 60-kDa protein component of the SR membrane inhibits Ca^{2+} release, but, upon dephosphorylation, release activity is restored (Kim and Ikemoto, 1986; Campbell and MacLennan, 1982). Meissner (1986) and Plank *et al.* (1988) reported that calmodulin alone inhibits Ca^{2+} release from SR. In view of an earlier report that the 400-kDa foot protein is a calmodulin-binding protein (Seiler *et al.*, 1984), the phosphorylation-independent inhibition might be produced by calmodulin interaction with the 400-kDa protein.

The "Common Channel" Concept

As discussed above, a variety of types of Ca^{2+} release share some common properties, such as (a) similar $[Ca^{2+}]_0$ dependence of activation and suppression, (b) approximate identity of the Ca^{2+} release kinetics when maximally activated (e.g., by ATP), and (c) the presence of a common inhibitor such as ruthenium red. On the other hand, these release types are sharply distinguishable in terms of the kinetic properties in the absence of activating ATP and the requirement for T-tubules. The most appropriate hypothesis to account for these observations is a "common channel" concept implying that these types of Ca^{2+} release are mediated by a common release channel. However, the activation of the channel is controlled via different triggering mechanisms, resulting in different kinetic properties of the induced release. Binding of the "common inhibitor" to the channel blocks different types of release with the same $C_{1/2}$.

Molecular Components Involved in Various Steps of the Ca^{2+} Release Mechanism

As emerged from the above considerations, a variety of types of Ca^{2+} release *in vitro* are mediated by a common Ca^{2+} release channel. One of the intrinsic proteins of SR with a M_r of 400 k was identified as a constituent of the feet structure [Caswell and Brandt, 1989 (this volume)]. The most remarkable progress in the recent research on Ca^{2+} release *in vitro* concerns further characterization of this protein as reviewed in detail in the other chapters of this volume (Lai and Meissner, 1989). Briefly, the 400-kDa protein has several important properties as follows. (a) Electron-microscopic structure of the isolated 400-kDa protein complex [an assembly of four structural units, each of which presumably represents the 400-kDa polypeptide (Imagawa *et al.*, 1987; Lai *et al.*, 1988; Hymel *et al.*, 1988)] is essentially identical to that of an individual foot (Ferguson *et al.*, 1984). We designate the foot consisting of four 400-kDa subunits the "foot protein complex" (see Fig. 1). (b) This is a specific ryanodine-binding protein, and hence the site of action of previously described effects on the SR Ca^{2+} release by ryanodine ["binds to the opened channel and locks the channel at the opened state" (Fleischer *et al.*, 1985)] is localized in this protein. (c) The purified 400-kDa protein incorporated into planar lipid bilayers (presumably in a form of the four-subunit complex) exhibits "single channel" conductance behavior, and the opening frequency is regulated to some extent by the Ca^{2+} release effectors such as ATP, ryanodine, $[Ca^{+}]$, and Ruthenium red (Smith *et al.*, 1985; Imagawa *et al.*, 1987; Hymel *et al.*, 1988; Lai *et al.*, 1988). Thus, single Ca^{2+} channels

observed in the SR membrane (Smith *et al.*, 1985; Suarez-Isla, 1986) are now localized in the 400-kDa protein. These findings (a–c altogether) have provided so far the most convincing evidence that the SR Ca^{2+} release channel resides in the foot protein complex, presumably in the domain of the foot that traverses the SR membrane (i.e., the channel domain of the foot; see Fig. 1).

However, the conditions used for the single-channel conductance measurements of the lipid-incorporated foot protein [e.g., 50 mM Ca^{2+} at the *trans* (luminal) side, and 125 mM Tris at the *cis* (cytoplasmic) side] are rather unusual in comparison with the standard conditions used for the induction of Ca^{2+} release from SR vesicles. Furthermore, the single channel is characterized by rather random opening and closing at a high frequency. On the other hand, Ca^{2+} release from SR vesicles is characterized by exponential kinetics as described above. At the moment, it is rather difficult to conceive how the random opening–closing behavior of the single channels is manifested in the exponential Ca^{2+} release kinetics in the membrane system. Ca^{2+} release experiments with the use of some intermediate systems such as reconstituted vesicles containing the 400-kDa foot protein would be helpful in solving this problem. The possibility that such a big difference in the kinetics between both systems may be due to the difference in the assay conditions described above also remains to be investigated.

Several different mechanisms have been proposed for the functional coupling between the T-tubule and SR, such as involvement of electromechanocoupling (Schneider and Chandler, 1973; Chandler *et al.*, 1976) and of some chemical mediators such as Ca^{2+} (Fabiato, 1982) and IP_3 [Hidalgo and Jaimovich, 1989 (this volume)]. These models would suggest different types of approaches to the molecular components involved in the process. At the moment, however, the most plausible approach would be to locate the hypothetical routes for triggering of Ca^{2+} release deduced from kinetic studies in the anatomically and chemically well-characterized elements of the triadic complex. As schematically illustrated in Fig. 1, the two major routes for triggering Ca^{2+} release would be (a) a pathway that is initiated by the depolarization of the T-tubule membrane and activates the channel via the putative signal transmission and trigger-receptor domains, and (b) a pathway that involves the binding of release-inducing drugs to the putative trigger-receptor domain and activates the channel.

We would propose that the trigger-receptor domain is located within the foot protein complex for the following reasons. Polylysine at low concentrations (e.g., $C_{1/2} = 0.2\,\mu M$) induces Ca^{2+} release with characteristics similar to those of Ca^{2+} and drug-induced release, by specifically reacting with the 400-kDa protein (Cifuentes *et al.*, 1988). The finding that doxorubicin induces Ca^{2+} release and that the 400-kDa protein is one of the doxorubicin-binding

SR components (Zorzato *et al.*, 1985, 1986) is also consistent with this idea. Caffeine increases the frequency as well as the duration of the opening of the single channel in the bilayer-incorporated 400-kDa protein (Rousseau *et al.*, 1984). Furthermore, two types antibodies raised against the 400-kDa protein have discrete effects on Ca^{2+} release, one activating Ca^{2+} release (Rosemblatt *et al.*, 1988) and the other inhibiting release (Zorzato and Volpe, 1988).

The existence of another domain in the foot protein complex (viz., a signal transmission domain) is suggested from the finding that concanavalin A (Con-A) reacts rather specifically with the 400-kDa protein and inhibits depolarization-induced (T-tubule-mediated) release without producing any effect on Ca^{2+} and drug-induced Ca^{2+} release (Cifuentes *et al.*, 1987). Thus, an appropriate location of this domain would be in a region of the foot somewhere between the T-tubule and the trigger-receptor domain described above (cf., Fig. 1).

The above facts suggest that the foot performs not only the functions attributable to the Ca^{2+} release channel, but also at least part of the functions attributable to the T-tubule–SR communication pathway and to the drug-induced channel activation pathway. One of the next important steps for analysis of the signal transmission pathway would be the identification of the regions of the foot representing the putative domains described above by, for example, localizing appropriate domain-specific probes (selective inhibitor of the T-tubule-mediated Ca^{2+} release, covalently reacting derivatives of release-inducing drugs, etc.) within the proteolytic subfragments derived from the 400-kDa polypeptide.

Many of the foot-protein-reacting functional probes (e.g., Con-A and doxorubicin) cross-react with a 160-kDa glycoprotein. This suggests that the 160-kDa protein might be another integral component of the foot. The finding that the 160-kDa protein binds to the 400-kDa protein (Kawamoto *et al.*, 1986) supports this idea. Alternately, it might be a proteolytic fragment of the 400-kDa protein [Lai *et al.*, 1988; for the opposing view, see Volpe *et al.*, 1989 (this volume)]. One of the intrinsic (hydrophobic) protein components of SR with an M_r of 32 k has highly reactive $-SH$ groups, and the fluorescence intensity of *N*-(7-dimethylamino-4-methyl-3-coumarinyl)-maleimide (DACM) incorporated into this protein changes approximately in parallel to the time course of Ca^{2+} release induced by drugs as well as by T-tubule depolarization (Morii *et al.*, 1986). This suggests that the 32-kDa protein might be a constituent of the Ca^{2+} release channel or a protein located in close contact to the channel. Recently it was found that the 400-kDa protein also has thiol groups that are highly reactive with DACM (Ikemoto *et al.*, 1989). Thus, it might well be that the 32-kDa protein is a proteolytic cleavage product derived from the 400-kDa protein.

Very little is known about (a) how the depolarization of the T-tubule membrane is transformed into the type of signal that is transmittable through such a pathway as described above, and (b) the chemical or physical nature of the transduced signal. However, it is anticipated that a hypothetical voltage-signal converter (Fig. 1) plays a role in the T-tubule-to-SR signal transmission mechanism. The concept that the dihydropyridine (DHP) receptor protein complex located in the T-tubule membrane may play a role as a voltage sensor has emerged from several pieces of evidence as follows. Nifedipine blocks a nonlinear charge movement and Ca^{2+} transient in an intact fiber (Rios and Blum, 1987). Several features of its polypeptide structure characteristic of voltage-dependent ion channels (e.g., the putative voltage-sensor region similar to that of the acetylcholine-receptor Na^+ channel) are conserved in the alpha-1 subunit of the T-tubule DHP receptor (Tanabe *et al.*, 1987). Attempts to block the T-tubule-mediated Ca^{2+} release by DHPs in an isolated vesicular system have led to inconsistent results (Ikemoto *et al.*, unpublished result), which in view of a sharp membrane potential dependence of the DHP inhibition (Rios and Blum, 1987) is probably due to the difficulty of adjusting the membrane potential exactly to an optimal level in the isolated vesicles. There are several other proteins in the T-tubule–foot junctional region, such as the 80-kDa "anchoring" protein, glyceroaldehydephosphate dehydrogenase, and aldolase [cf., Caswell and Brandt, 1989 (this volume)]. A monoclonal antibody raised against a 27.5-kDa protein component of the T-tubule, which apparently is a separate entity from the low M_r subunit of the DHP receptor complex, inhibits depolarization-induced release in the isolated triad preparation (Rosemblatt and Ikemoto, unpublished result). According to a recent report (Chadwick *et al.*, 1988), another T-tubule protein with M_r of 71 k is cross-linkable with the 400-kDa foot protein, suggesting that this protein may be involved in the T-tubule–foot linkage. Thus, there are a number of proteins that can be key proteins involved in the structural and functional coupling between the T-tubule and the foot. We also speculate that the putative voltage-signal converter described above might be composed of not only the DHP receptor subunits, but also some non-DHP receptor proteins described above.

The recent findings as follows suggest that the Ca^{2+} release channel is regulated not only from the cytoplasmic side, but also from the luminal side by calsequestrin. Selective removal of calsequestrin from SR by treatment with cholate at high concentrations of NaCl (e.g., 0.5 M) led to the vesicles that are capable of ATP-dependent Ca^{2+} uptake, but incapable of caffeine-induced Ca^{2+} release. Upon reassociation of calsequestrin with the membrane, the Ca^{2+} release activity was restored, suggesting that calsequestrin plays a crucial role in Ca^{2+} release (Ronjat and Ikemoto, 1989). Recent

studies on the isolated junctional face membrane (JFM)–calsequestrin complex and on the SR vesicles (Ikemoto *et al.*, 1989) have enabled some new insights into the mechanism by which calsequestrin is involved in the Ca^{2+} release mechanism. Using the isolated JFM–calsequestrin complex, it was shown that the fluorescence intensity of DACM incorporated in the JFM proteins (the 400-kDa foot protein and the 32-kDa protein) changes in parallel to Ca^{2+} binding to the JFM-attached calsequestrin (Ikemoto *et al.*, 1989). Since calsequestrin undergoes rather extensive conformational changes upon Ca^{2+} binding (Ikemoto *et al.*, 1974; Ostwald and MacLennan, 1974; Aaron *et al.*, 1984), and since no DACM is incorporated into calsequestrin, the above experiment indicates that the Ca^{2+}-induced conformational changes of calsequestrin in turn control the conformational states of the JFM proteins. In the native SR vesicles, it is actually seen that the kinetic properties of drug-induced Ca^{2+} release vary with Ca^{2+} binding to calsequestrin under both active and passive Ca^{2+}-loading conditions (Ikemoto *et al.*, 1988).

Using the JFM–calsequestrin complex, the addition of caffeine to the complex, but not to dissociated calsequestrin, was shown to produce dissociation of a portion of the Ca bound to calsequestrin (Ikemoto *et al.*, 1989). This suggests that the triggering signal applied to the JFM (e.g., the trigger-receptor domain of the foot protein complex; see above) is transmitted to calsequestrin that is attached to the luminal side of the JFM, and leads to release of the bound Ca. In the native vesicles, this mechanism would end up with the generation of an extremely high $[Ca^{2+}]_i$ pool at the luminal side of the channel. Under such conditions, it would not be unreasonable to expect that the local $[Ca^{2+}]_i$ reaches a very high level (e.g., 50 mM, the concentration used at the *trans* side in the single-channel conductance measurements; see above).

In view of the reports that calsequestrin binds to the purified foot protein (Kawamoto *et al.*, 1986) and a 26-kDa protein component of the JFM (Mitchell *et al.*, 1988), calsequestrin may regulate the Ca^{2+} channel functions by either direct interaction with the foot protein or indirect interaction with it via the 26-kDa protein.

Interrelationship between Ca^{2+} Release and Ca^{2+} Uptake

There are virtually no Ca^{2+} pumps in the JFM region of the terminal cisternal SR where the feet are located, whereas a large number of the Ca^{2+} pump molecules are located in the regions of the nonjunctional terminal cisternal SR membrane and the longitudinal SR (Costello *et al.*, 1986). Thus,

the Ca^{2+} release channels and the Ca^{2+} pump molecules seem to be segregated from each other. However, several pieces of evidence suggest that the functions of the Ca^{2+}-ATPase are not independent from the Ca^{2+} release functions. The first evidence for this notion emerged from earlier observations of Fabiato (1982) with skinned cardiac muscle fibers that a "hyperpolarization" signal of the SR membrane, which may be ascribable to a rapid Ca^{2+} translocation by the Ca^{2+}-ATPase, preceded Ca^{2+}-induced Ca^{2+}-release. More direct evidence that the Ca^{2+}-ATPase enzyme undergoes a rapid conformational change in response to triggering of Ca^{2+} release has emerged from the studies with isolated terminal cisternal vesicles as described below. For instance, the levels of the phosphorylated intermediate (EP), the tryptophan fluorescence of the Ca^{2+} ATPase (Mészáros and Ikemoto, 1985*a*), and the fluorescence intensity of the conformational probe attached to the Ca^{2+} ATPase (4′,6-diamidino-2-phenylindole, DAPI; Mészáros *et al.*, 1987) changed transiently upon inducing Ca^{2+} release. Interestingly, the binding of a stoichiometrical amount of DAPI to the Ca^{2+}-ATPase with a high affinity ($K_{assoc} = 3.0 \times 10^5 M^{-1}$) led to an increase of the initial rate of Ca^{2+} uptake and the inhibition of both caffeine-induced and ionic replacement-induced Ca^{2+} release (Mészáros *et al.*, 1987). Furthermore, the Ca^{2+} release-inducer caffeine and the release-blocker ruthenium red have discrete effects on several functions intrinsic to the Ca^{2+}-ATPase, and the concentrations for half-maximal effects on these functions are about same as those on Ca^{2+} release (Mészáros and Ikemoto, 1985*b*). All of these facts suggest that an intimate functional interrelationship exists between the Ca^{2+} release and the Ca^{2+} pump.

The concept that emerged from earlier studies—that the Ca^{2+}-ATPase may play a principal role in the Ca^{2+} release mechanism (Katz *et al.*, 1980; Chiesi and Wen, 1983)—might offer the simplest explanation for these observations. However, such a possibility became less likely, although not excluded entirely, especially after the appearance of recent reports that the functions attributable to the Ca^{2+} release channel are localized in the foot; in other words, the release functions are localized in the region where there are virtually no Ca^{2+} pump molecules (see above). It is more likely, then, that the observed functional and pharmacological coupling between Ca^{2+} release and Ca^{2+} pump is mediated by a third mechanism. There are electron-microscopically discernible links of the calsequestrin network with both the JFM and nonjunctional membrane regions of the terminal cisterna (Franzini-Armstrong *et al.*, 1987). It is tentatively proposed that calsequestrin serves as a mediator of the release–pump communication. Since the release-triggering signal applied to the cytoplasmic side of the JFM appears to be transmitted to calsequestrin as described above, it is not unreasonable that such a signal is propagated further to the Ca^{2+} pump located in the nonjunctional membrane

region via the intravesicular calsequestrin network. The trigger-induced increase in the $[Ca^{2+}]_i$ (see above) in turn might affect the Ca^{2+} pump molecules. It is interesting to note in this context that the Ca^{2+}-ATPase of light SR vesicles or that of the calsequestrin-depleted heavy SR vesicles shows no conformational response to the Ca^{2+} release-inducing drugs (Mészáros and Ikemoto, 1987).

Another interesting observation in the context of the release–pump interrelationship is that the spontaneous Ca^{2+} release activity is low during the rapid phase of active Ca^{2+} transport, while, upon attenuation of the Ca^{2+} influx in the steady state of the Ca^{2+} uptake reaction, Ca^{2+} release appears to be activated. This suggested a hypothesis that Ca^{2+} release and Ca^{2+} transport might be regulated in a mutually exclusive fashion (Morii *et al.*, 1985). However, caffeine can trigger a small, but significant, Ca^{2+} release even in the initial phase of Ca^{2+} uptake. Therefore, the mutually exclusive regulation of pump and release is not necessarily valid for the trigger-induced Ca^{2+} release.

Concluding Remarks

The following conclusions and proposals may be deduced from the kinetic and biochemical data gathered through recent studies on Ca^{2+} release from isolated SR vesicles. Various types of Ca^{2+} release *in vitro* described in the literature appear to be mediated by a single class of Ca^{2+} release channel. The release channel is tentatively located in the membrane-spanning domain of the foot protein complex containing the 400-kDa protein subunits. On the other hand, there are at least two clearly distinguishable routes for triggering of Ca^{2+} release: (a) a remote-control pathway involving the transmission of the signal elicited by the depolarization of the T-tubule membrane, and (b) a shortcut pathway involving the activation of the channel via the neighboring trigger–receptor domain. The signal transmission and the trigger– receptor mechanisms described above may be at least partly located in the cytoplasmic portion of the foot protein complex. Ca^{2+} release is regulated not only from the cytoplasmic side of the channel, but also from the luminal side by calsequestrin. Furthermore, Ca^{2+} release and Ca^{2+} pumping are not independent processes, but they appear to be mutually regulated probably via a third mechanism. Further resolution of the complex molecular mechanisms involved in the regulation of the Ca^{2+} release channel, especially in the T-tubule-to-SR signal transmission pathway, is the major task in future studies of Ca^{2+} release from SR *in vitro*. Current studies are progressing rapidly in this direction.

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