

Calcium-Induced Calcium Release in Skeletal Muscle

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Endo M. Calcium-Induced Calcium Release in Skeletal Muscle. *Physiol Rev* 89: 1153–1176, 2009; doi:10.1152/physrev.00040.2008.—Calcium-induced calcium release (CICR) was first discovered in skeletal muscle. CICR is defined as Ca²⁺ release by the action of Ca²⁺ alone without the simultaneous action of other activating processes. CICR is biphasically dependent on Ca²⁺ concentration; is inhibited by Mg²⁺, procaine, and tetracaine; and is potentiated by ATP, other adenine compounds, and caffeine. With depolarization of the sarcoplasmic reticulum (SR), a potential change of the SR membrane in which the luminal side becomes more negative, CICR is activated for several seconds and is then inactivated. All three types of ryanodine receptors (RyRs) show CICR activity. At least one RyR, RyR1, also shows non-CICR Ca²⁺ release, such as that triggered by the t-tubule voltage sensor, by clofibrate, and by SR depolarization. Maximum rates of CICR, at the optimal Ca²⁺ concentration in the presence of physiological levels of ATP and Mg²⁺ determined in skinned fibers and fragmented SR, are much lower than the rate of physiological Ca²⁺ release. The primary event of physiological Ca²⁺ release, the Ca²⁺ spark, is the simultaneous opening of multiple channels, the coordinating mechanism of which does not appear to be CICR because of the low probability of CICR opening under physiological conditions. The coordination may require Ca²⁺, but in that case, some other stimulus or stimuli must be provided simultaneously, which is not CICR by definition. Thus CICR does not appear to contribute significantly to physiological Ca²⁺ release. On the other hand, CICR appears to play a key role in caffeine contracture and malignant hyperthermia. The potentiation of voltage-activated Ca²⁺ release by caffeine, however, does not seem to occur through secondary CICR, although the site where caffeine potentiates voltage-activated Ca²⁺ release might be the same site where caffeine potentiates CICR.

I. INTRODUCTION

The calcium ion (Ca²⁺) plays a crucial role in the regulation of cellular function as a major intracellular messenger (21). Convincing evidence for this role of Ca²⁺

was first obtained in skeletal muscle in the 1960s largely through the pioneering work of S. Ebashi (see review in Ref. 44).

The Ca²⁺ concentration in the cytoplasm is normally, i.e., in the unstimulated state, kept very low, ~0.1 μM.

When cells are stimulated, Ca^{2+} is mobilized from its source or sources into the cytoplasm, and as a result, local or global cytoplasmic Ca^{2+} concentrations become high enough to evoke cellular responses. The source of Ca^{2+} is either the extracellular medium or the membrane-bound intracellular Ca^{2+} pool, or both. In both sources, the Ca^{2+} concentration is at a millimolar level, four orders of magnitude higher than that in the cytoplasm. The membranes that separate the source(s) and the cytoplasm have Ca^{2+} channels, which, when stimulated, open to allow Ca^{2+} to flow rapidly from the source(s) into the cytoplasm along a large electrochemical potential gradient. The separating membranes also have Ca^{2+} -transporting systems that extrude Ca^{2+} from the cytoplasm to the source(s) against an electrochemical potential gradient to halt the response. Different types of Ca^{2+} channels are present in the plasma membrane and in the membrane of the intracellular Ca^{2+} store, and also among different cells, and so are the active transport systems of Ca^{2+} .

In striated muscles, the major source of Ca^{2+} is the intracellular Ca^{2+} store, not the extracellular medium. The main intracellular Ca^{2+} store is the endoplasmic reticulum (ER)/sarcoplasmic reticulum (SR). This was first demonstrated in skeletal muscle as well: a microsome fraction from skeletal muscle cells was identified as fragments of SR, which were found to strongly accumulate Ca^{2+} in the presence of ATP (45, 90). Therefore, studies on the mechanism of Ca^{2+} mobilization from intracellular stores naturally began in skeletal muscle, and two groups independently and nearly simultaneously discovered that Ca^{2+} itself causes Ca^{2+} release (57, 58, 72, 73). The phenomenon, called calcium-induced calcium release (CICR), was the first possibly physiological mechanism of the Ca^{2+} mobilization from the intracellular store to be proposed. The discovery of CICR preceded the early detection of the primary Ca^{2+} release mechanism in skeletal muscle as a change in the voltage sensor of the t-tubule membrane (228) and was much earlier than the discovery of inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release (254).

CICR is considered to be the physiological mechanism of Ca^{2+} release in cardiac muscle. It is generally agreed that an influx of Ca^{2+} through L-type voltage-dependent Ca^{2+} channels on the surface and the t-tubule membrane of myocytes activated by an action potential triggers Ca^{2+} release from the SR by the CICR mechanism to cause cardiac contraction (13, 20, 62, 226, 252). In skeletal muscle where CICR was first discovered, however, the primary mechanism of physiological Ca^{2+} release is not CICR, but direct protein-protein interaction between the voltage sensor of the t-tubule membrane, the dihydropyridine receptor (DHPR), and the Ca^{2+} release channel of the SR membrane, the ryanodine receptor (RyR) (221, 227). Whether CICR secondarily participates in physiological Ca^{2+} release is a controversial issue. The main object of this review is to discuss the significance of

CICR in skeletal muscle from physiological, pathophysiological, and pharmacological viewpoints.

II. HISTORY

Ford and Podolsky (73, 74) showed that, in the presence of a low free Mg^{2+} concentration ($\sim 25 \mu\text{M}$), the rate of tension development of a skinned muscle fiber activated by 0.1 mM Ca^{2+} is very low if the SR of the fiber is empty, but is very high if the SR has been preloaded with Ca^{2+} . They attributed the rapid tension development of the preloaded fiber to Ca^{2+} release from the SR, triggered by the applied Ca^{2+} . Although the rapid development of tension might also be due to the absence of an effective Ca^{2+} sink (as the storage organelle had been loaded already), its partial relaxation after 20 s suggests that the rapid contraction is due to Ca^{2+} released from the SR. Ford and Podolsky (73, 74) further showed that a change in electrical potential across the internal membrane, evoked by the replacement of propionate, the main anion in the medium, by chloride, produces only a brief contraction of the preloaded fiber in the presence of 1 mM Mg^{2+} , but produces a much larger and longer contraction when the Mg^{2+} concentration is kept low. They concluded that the change in electrical potential of the internal membrane causes a small quantity of Ca^{2+} to be released regardless of the Mg^{2+} concentration, but in the presence of low Mg^{2+} , the initially released Ca^{2+} releases further Ca^{2+} in a regenerative manner. Ford and Podolsky (75) then showed that 10^{-4} M Ca^{2+} evokes a large efflux of ^{45}Ca from the SR with a much smaller influx, causing a net efflux of Ca^{2+} from SR.

Endo et al. (58) observed that a low concentration of caffeine applied to skinned fibers in a medium containing a low concentration of EGTA ($\sim 50 \mu\text{M}$) induces regular oscillatory contractions of a very low frequency (one contraction in many minutes). The near-maximal magnitude of the repetitive contractions suggested the presence of a positive-feedback mechanism, and further investigation of the mechanism revealed that Ca^{2+} itself induces the release of Ca^{2+} from the SR. Thus, in the presence of 2 mM caffeine, a Ca^{2+} concentration of 1 μM , which is below the threshold for contraction by a direct effect on the contractile system, induces a transient contraction of skinned fibers when the SR has been preloaded. This contraction was proven to be due to Ca^{2+} release, because the amount of Ca^{2+} remaining in the SR after contraction is smaller. CICR in the absence of caffeine was also shown under appropriate conditions (58).

A few years later, Fabiato and Fabiato (62) demonstrated regenerative Ca^{2+} release in cardiac skinned fibers. In the following decades, various properties of CICR were revealed (see reviews in Refs. 49, 52, 60, 70, 162, 169). Early studies showed that in the presence of a

reversed Ca^{2+} concentration gradient, influx of Ca^{2+} into the SR is increased or decreased by potentiators or inhibitors of CICR, respectively, suggesting that CICR occurs through an increase in the permeability of the SR membrane to Ca^{2+} (127). In fact, in the mid 1980s, through the specific and unique action of ryanodine (71), the RyR, the Ca^{2+} release channel, was isolated from the SR of skeletal muscle, purified (105, 107, 143, 246), and sequenced (262, 284). The RyR was then shown to exhibit all the properties of CICR (96, 105, 143). The vast majority of the studies of RyR and CICR have been performed since then, and much information has been accumulated (see reviews in Refs. 11, 33, 66, 70, 78, 167, 169, 170, 198, 244, 248, 257, 281), although many problems remain unanswered.

In the early 1990s, it was found that the Ca^{2+} -releasing action of IP_3 is also enhanced by Ca^{2+} (14, 69, 97), and consequently, in the presence of a constant concentration of IP_3 , an increase in Ca^{2+} concentration causes a further release of Ca^{2+} from ER/SR through the IP_3 receptor. Thus the IP_3 receptor system exhibits an apparent CICR (12, 98–100, 176, 250, 264, 274).

III. CALCIUM-INDUCED CALCIUM RELEASE AND PROPERTIES OF RYANODINE RECEPTORS

Three isoforms of RyR (RyR1, RyR2, and RyR3) have been identified in mammals (see reviews in Refs. 247, 249). The first isoform to be detected was RyR1, the primary isoform in skeletal muscle, while RyR2 is the cardiac isoform. Mammalian skeletal muscles contain, in addition to RyR1, a small amount of RyR3, whose ratio to RyR1 varies among different muscles (30, 82, 153, 187). Skeletal muscles of nonmammalian vertebrates, such as chickens, frogs, and fish, mostly have an equal amount of two different isoforms of RyR, called RyR α and RyR β . In contrast, rapidly contracting muscles such as extraocular and swim-bladder muscles have only one isoform, RyR α (1, 144, 186, 196, 205). RyR α and RyR β are homologs of RyR1 and RyR3, respectively (206, 207).

All the isoforms of RyR exhibit CICR, i.e., Ca^{2+} activates their opening. However, Ca^{2+} release with characteristics different from those of CICR can also be evoked via RyRs. Therefore, it should be realized that the properties of CICR are not equal to those of RyR, but only a part of them.

A. Definition of CICR

CICR is, as its name indicates, a phenomenon whereby an increase in Ca^{2+} concentration at the cytoplasmic surface of the intracellular Ca^{2+} store induces a release of Ca^{2+} . The molecular mechanisms of CICR may not be unique, as action potentials in the surface membrane could be produced by either voltage-dependent

Na^+ or Ca^{2+} channels. As long as the open probability of the Ca^{2+} release channel increases with Ca^{2+} concentration in the cytoplasm, an increase in the cytoplasmic Ca^{2+} concentration should cause an increase in Ca^{2+} efflux from the Ca^{2+} store, namely, CICR. Both RyRs and IP_3 receptors (IP_3 Rs) have such a property; therefore, both kinds of Ca^{2+} release channel exhibit CICR behavior.

An important difference between RyR and IP_3 R is that whereas Ca^{2+} alone, without the help of any other agents or stimuli, can cause Ca^{2+} release through RyR (50, 245), it cannot do so through IP_3 R. In the case of IP_3 R, Ca^{2+} can cause Ca^{2+} release only in the presence of IP_3 (see reviews in Refs. 76, 98). Because of these findings, CICR is generally considered as an exclusive property of RyR, but not of IP_3 R, even though IP_3 R exhibits the apparent CICR behavior in the presence of IP_3 .

In this review, CICR is defined as Ca^{2+} release evoked by the action of Ca^{2+} alone. Therefore, even if Ca^{2+} is necessary for evoking Ca^{2+} release, if Ca^{2+} is not, by itself, sufficient to evoke Ca^{2+} release and some other factor or factors must also be present, such Ca^{2+} release is not considered as CICR. By this definition, the apparent CICR of IP_3 -induced Ca^{2+} release described above (with a fixed concentration of IP_3) is CICR. However, Ca^{2+} release via an IP_3 R in an IP_3 -free medium evoked by the simultaneous application of Ca^{2+} and IP_3 is not CICR, because Ca^{2+} alone cannot evoke Ca^{2+} release. Thus the Ca^{2+} -induced conformational change of IP_3 R is sufficient to open the channel if an additional IP_3 -induced conformational change is present, but not when the additional change is absent. To reiterate, CICR is Ca^{2+} release when a Ca^{2+} -induced conformational change alone is sufficient to evoke Ca^{2+} release.

B. Important Properties of CICR

1. Ca^{2+} concentration dependence

In the early days of CICR research, Endo (47) reported that to evoke net Ca^{2+} release by the application of Ca^{2+} to a skinned fiber immersed in a physiological medium, a Ca^{2+} concentration higher than 100 μM was necessary. In these experiments, however, Ca^{2+} was applied to the entire fiber so that both the RyR channels and the Ca^{2+} pump protein molecules of SR were simultaneously stimulated; the results obtained reflected the net movement of Ca^{2+} , the algebraic sum of release and uptake. Under physiological conditions, such a simultaneous global appearance of Ca^{2+} is unlikely to occur, and the reported concentration greater than 100 μM may have no direct physiological significance.

In subsequent studies, the properties of CICR in the absence of Ca^{2+} pump activity were determined in skinned fibers, and micromolar concentrations of Ca^{2+} were found to induce CICR. The rate of CICR is a bell-

shaped function of Ca^{2+} concentration: whereas micromolar concentrations of Ca^{2+} activate Ca^{2+} release, millimolar concentrations of Ca^{2+} inhibit Ca^{2+} release. This biphasic dependence on Ca^{2+} concentration was first demonstrated in skinned fibers of skeletal muscle (50, 59, 182) and then in fragmented SR (FSR) (122, 124, 168, 191). A similar biphasic dependence on *cis* (cytoplasmic) Ca^{2+} is shown by the open probability of the purified Ca^{2+} release channel RyR, incorporated into a lipid bilayer, and by [^3H]ryanodine binding to RyR (19, 26, 31, 113, 182, 197, 199, 212, 213, 236), consistent with the notion that CICR takes place via the RyR. The biphasic effect of Ca^{2+} suggests the presence of two Ca^{2+} receptor sites on RyRs: a high-affinity activating site (CICR A-site) and a low-affinity inhibitory site (CICR I-site) (169, 198).

2. Effects of Mg^{2+}

The inhibition of CICR by Mg^{2+} had already been documented when CICR was discovered (73, 74). Later, Mg^{2+} was found to inhibit CICR in two different modes. Mg^{2+} shifts the relation between the CICR rate and the Ca^{2+} concentration to the right and downward, i.e., Mg^{2+} reduces the Ca^{2+} sensitivity of CICR and reduces the maximum rate of release (50, 124, 148, 171, 172, 182, 191, 213). These effects suggest that Mg^{2+} competitively antagonizes Ca^{2+} at the CICR A-site but collaborates with Ca^{2+} to inhibit CICR via the CICR I-site.

3. Potentiators

ATP and caffeine are the two most important potentiators of CICR.

A) ATP AND ADENINE COMPOUNDS. ATP and other adenine nucleotides, as well as adenosine and adenine, potentiate CICR without altering the dependence of CICR on the Ca^{2+} concentration. This was also first shown in skinned fibers and then in FSR (50, 54, 55, 110, 118, 168, 171, 181, 182, 191). The order of potency was ATP \sim adenosine 5'-(β , γ -methylene)triphosphate (AMPPCP) $>$ ADP \sim cAMP $>$ AMP $>$ adenine $>$ adenosine (54, 118, 168, 181). The probability of a single channel of RyR being open and the [^3H]ryanodine binding to the RyR activated by Ca^{2+} are markedly increased by ATP and its analogs in the same manner (32, 96, 143, 144, 182, 199, 213). An interesting finding was that adenine and adenosine inhibit CICR in the presence of ATP. This inhibition occurs because the strong potentiation of CICR by ATP is replaced by the much weaker potentiation by adenine or adenosine (42, 52, 110, 150, 169).

ATP and AMPPCP, but not AMP or adenine, induce Ca^{2+} release even in the absence of Ca^{2+} if Mg^{2+} is also absent (32, 52, 110, 245). Therefore, ATP and AMPPCP appear to be not mere CICR potentiators but Ca^{2+} -releasing agents themselves. However, the Ca^{2+} -releasing actions of ATP and AMPPCP in the absence of Ca^{2+} are

completely inhibited by Mg^{2+} (52, 95, 171).¹ Laver et al. (151) have shown that the magnitude of inhibition by Mg^{2+} in the presence of a wide range of Ca^{2+} concentrations, from its near-complete absence, pCa 9, to pCa 5, can be fitted using a model that assumes a single site activated by Ca^{2+} and inhibited by Mg^{2+} , i.e., the CICR A-site. Since Ca^{2+} bound to the A-site is minimal at pCa 9, the inhibitory effect of Mg^{2+} in this case is thought to be exerted not by preventing the binding of Ca^{2+} to the A-site, but by an effect of Mg^{2+} bound to the site (151). In other words, Mg^{2+} is not merely a competitive antagonist but an inverse agonist. For this reason, the Ca^{2+} -releasing action of ATP by itself (in the absence of Ca^{2+}) does not appear to be an action of ATP unrelated to CICR but is the result of the enhancement of CICR, because the A-site must be in its uninhibited state. In other words, if Mg^{2+} is present, the effects of ATP and related compounds on Ca^{2+} release are exerted only in the presence of Ca^{2+} .

B) CAFFEINE. Caffeine also potentiates CICR, but unlike ATP, it increases the Ca^{2+} sensitivity of CICR in addition to the maximum response at the optimal Ca^{2+} concentration. Again, these effects were first demonstrated in skinned fibers and in FSR and were then also shown in the open probability of RyR channels in a lipid bilayer and [^3H]ryanodine binding to RyR activated by Ca^{2+} (48, 50, 124, 168, 171, 182, 191, 199, 213, 223). In amphibian skeletal muscle, caffeine, even at concentrations greater than 10 mM, causes neither Ca^{2+} release nor [^3H]ryanodine binding in the virtual absence of Ca^{2+} (50, 199). In mammalian skeletal and cardiac muscle, however, a high concentration of caffeine induces Ca^{2+} release via RyR in the virtual absence of Ca^{2+} (0.08–2 nM), provided that Mg^{2+} is absent (223, 243). Although the active site of Mg^{2+} in this case has not been identified as the CICR A-site, as in the case of ATP, the Ca^{2+} -releasing action of caffeine in the absence of Ca^{2+} also appears to be the result of the potentiation of CICR, requiring an uninhibited A-site. However, these findings do not rule out the possibility that caffeine has pharmacological actions on RyR channels other than the potentiation of CICR, as discussed in section v.

4. Inhibitors

The inhibitory effect on CICR of Mg^{2+} and that of adenine and adenosine in the presence of ATP have already been described. In addition to Mg^{2+} , monovalent cations competitively antagonize Ca^{2+} at the CICR A-site (169).

¹ In the case of the high-activity RyR channels, Mg^{2+} is reported to not completely inhibit Ca^{2+} release by ATP in the absence of Ca^{2+} (32). However, because the high-activity channels may not be in the physiological state as discussed in sect. III B6c, this result was not taken into consideration.

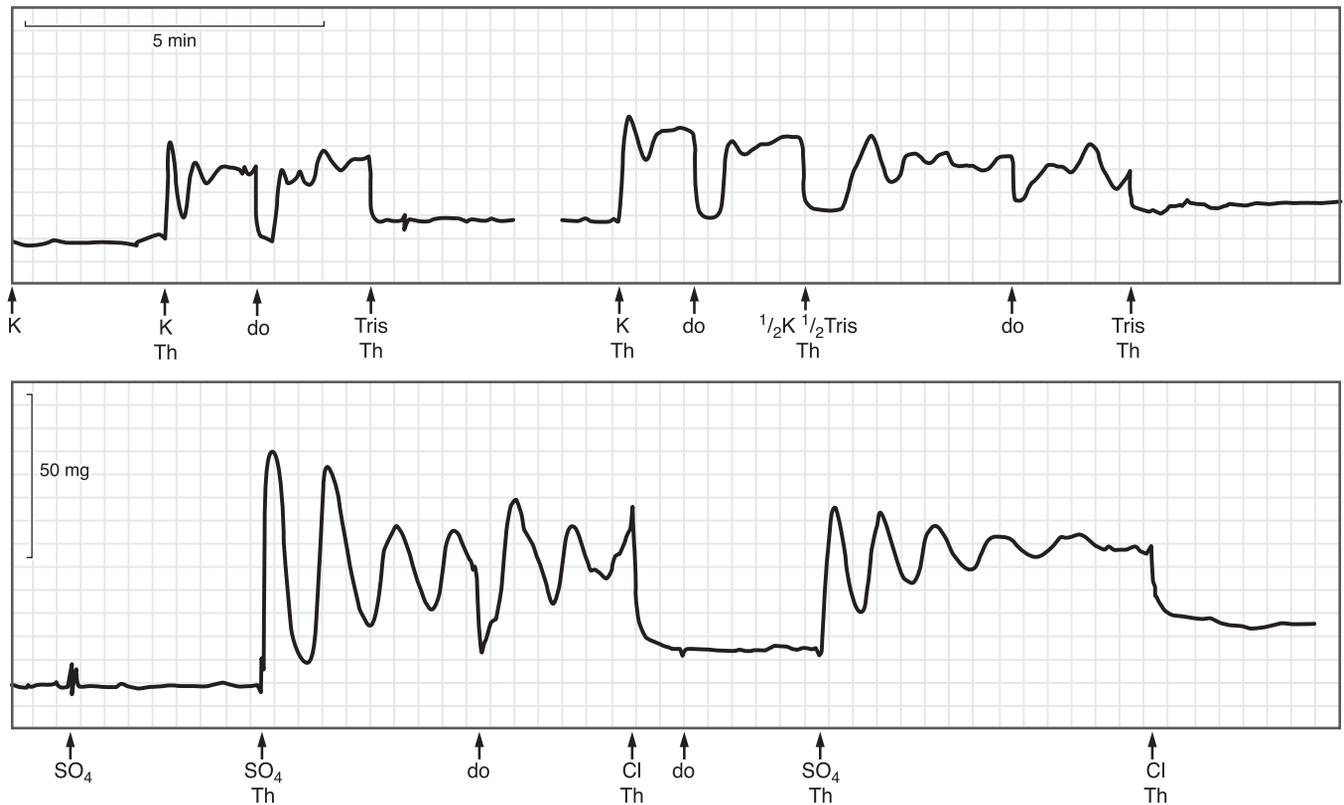


FIG. 1. Inhibitory effect of depolarization of the SR membrane on thymol-activated calcium-induced calcium release (CICR) in skinned skeletal muscle fibers of the frog. The isometric tension of skinned fibers was recorded in a relaxing solution containing K or Tris as the main cation and SO_4 or Cl as the main anion with $50 \mu\text{M}$ EGTA. The constituents of the relaxing solution are the same as those reported in Ref. 58. Oscillatory tension was induced by $4 \mu\text{M}$ thymol in a K_2SO_4 medium (KTh or SO_4Th). When the solution surrounding the skinned fiber was changed to a depolarizing solution by the replacement of K by Tris (TrisTh), or SO_4 by Cl (ClTh), in the continued presence of thymol, oscillatory contractions stopped, possibly after a brief initial activation. Solution exchange without ionic replacement (do) had no effects except transient relaxation due to the removal of Ca^{2+} , which had been released, by the new relaxing solution. Solution exchange with replacement of half K ($1/2\text{K}1/2\text{TrisTh}$) produced partial inhibition. This result was previously communicated (57).

Procaine and tetracaine inhibit CICR (49, 74, 125, 190, 214, 263, 275, 280) and are frequently used to detect CICR activity. Although the inhibition of physiological Ca^{2+} release in frog fibers by procaine is weak (132) or undetectable (263),² inhibition by tetracaine is fairly strong. In fact, the K contracture of single amphibian skeletal muscle fibers is not inhibited by 10 mM procaine (263) but is strongly inhibited by 1.0 mM tetracaine (158).

Ruthenium red and high concentrations of ryanodine are well-known inhibitors of RyR channels and, of course, abolish CICR.

5. Effects of voltage across the SR membrane

For the sake of convenience, a change in potential across the SR membrane in the direction that makes the cytoplasmic side more positive (luminal side more negative)

² In intact mammalian fibers, procaine strongly inhibits physiological Ca^{2+} release (81), but in peeled fibers, procaine was reported to not inhibit Ca^{2+} release induced by t-tubule depolarization (39).

is defined in this article as "SR depolarization" by analogy with the potential change at the surface membrane.

CICR is inhibited by SR depolarization lasting more than a few seconds. Figure 1 shows that repetitive cyclic contractions evoked by thymol-activated CICR are suppressed by SR depolarization induced by ion exchanges of either the cation (from more-permeable K to less-permeable Tris), or the anion (from less-permeable sulfate to more-permeable chloride) and are reactivated by the reversed ion exchange.

When a voltage step causing SR depolarization is applied to an isolated bilayer-reconstituted RyR in the presence of *cis*- Ca^{2+} , the channels are initially activated if they have been inactivated before the step (159, 242, 279), or their activity level is increased (149) or is sometimes unchanged (25, 212). After a few seconds, however, the channels are completely inactivated (25, 149, 159, 212, 242, 279) except in the case of low-activity channels (149). If Ca^{2+} is applied after the voltage step, the channels are initially activated and then completely inactivated (242). The inactivated

channels are not reactivated unless the voltage is reversed (25). These results are consistent with the results of skinned fiber experiments shown in Figure 1.

Thus CICR is affected by the membrane potential of the SR. This effect suggests that if Ca^{2+} is applied with a potential change of the SR membrane, the response could be different from that without the membrane potential change. The former, if it is different from the latter, is non-CICR release of Ca^{2+} , while the latter is, by definition, CICR.

6. Maximum rate of CICR under physiological conditions

Although Ca^{2+} can open RyR channels to evoke CICR, it cannot fully open the channels under physiological conditions; the open probability attained is much less than 1.0. Few quantitative reports on CICR activity under physiological conditions, i.e., in the presence of physiological modifiers of CICR, Mg^{2+} and ATP, have been published, but all the maximum rates of CICR in skeletal muscles reported to date are low.

A) SKINNED FIBER. In skinned fibers, when Ca^{2+} pump activity is halted by the absence of ATP, the time course of Ca^{2+} decay in the SR as a result of CICR induced by a fixed concentration of free Ca^{2+} (buffered with a high concentration of EGTA) follows first-order kinetics (53, 95, 182, 203). This indicates that Ca^{2+} permeability, i.e., the magnitude of RyR channel opening, in the SR membrane is maintained constant during stimulation by a fixed cytoplasmic Ca^{2+} concentration. Although Ca^{2+} binding by EGTA is slow, because the maximum rate of CICR in skeletal muscle (the probability of opening of Ca^{2+} -bound RyR channels) is very low as described later, EGTA seems to have sufficient time to buffer the Ca^{2+} concentration to make the measurements obtained reliable.

Horiuti (95) has determined the rate of CICR in skinned fibers of amphibian skeletal muscle in the presence of 1.5 mM free Mg^{2+} and 1 mM AMPPCP, and obtained 9 min^{-1} , or 0.015%/ms, as an approximately maximal rate at pCa 4.2 both at 21–22 and 1.5–3°C.

Murayama et al. (182) have measured the rates of CICR in skinned fibers of the frog at 16°C in wide ranges of Ca^{2+} , Mg^{2+} , and AMPPCP concentrations. On the basis of their results, they determined the affinities of CICR A-site and I-site to Ca^{2+} and Mg^{2+} , respectively. The absence of influence of AMPPCP on these affinities is confirmed. Murayama et al. (182) also examined the dependence of the maximum rate of CICR on AMPPCP concentration. On the basis of the values obtained, they estimated the maximum rate of CICR under physiological conditions, in the presence of 1 mM free Mg^{2+} and 4 mM ATP, assuming that the CICR-potentiating action of AMPPCP is equal to that of ATP. The value they obtained was $<10 \text{ min}^{-1}$, or 0.017%/ms, which was in good agreement with

Horiuti's result. They also showed that even in the absence of Mg^{2+} , the maximum rate of CICR was 35–50 min^{-1} , or 0.058–0.083%/ms.

The values obtained in mammalian muscles have been similar. For example, Ohta et al. (203) determined that the rate of CICR in pig skeletal muscle in the presence of 1.5 mM free Mg^{2+} and 1 mM AMPPCP is $\sim 12 \text{ min}^{-1}$, or 0.02%/ms, as the maximum rate of release at pCa 4.52 at 20°C.

B) FSR. The rate of CICR under a physiological ionic milieu has also been determined in FSR (171) and isolated triad preparations (40) of rabbit skeletal muscle. Unfortunately, corresponding data for amphibian skeletal muscle are not available. The values obtained in rabbit skeletal muscle are 1–3/s, or 0.1–0.3%/ms, which are several times greater than those obtained in skinned fibers, but are still low.

C) SINGLE RYR CHANNELS INCORPORATED INTO LIPID BILAYER. Many studies on RyR incorporated into lipid bilayer have been published, but few studies have been performed with physiological concentrations of Mg^{2+} and ATP and sufficiently high Ca^{2+} concentrations. Some data are compiled in Table 1 together with comparable data on cardiac muscle.

RyR1 and RyR α channels show two classes of channel activity with distinct open probabilities, high and low, whereas RyR3 and RyR β always show similar activities (31, 184, 212). At present, which open probability is closer to the physiological value is unknown. Although in many cases, lower activities are a result of deterioration during experimental manipulation, sometimes higher activity could be a result of manipulation. Indeed, Murayama and Ogawa (188) have found that whereas purified RyR α and RyR β treated with 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) show similar CICR activities, CICR of RyR α under conditions more closely resembling the physiological state is strongly suppressed to a few percent of that of RyR β . Murayama and Ogawa (189) have reported that bovine RyR1 is also "stabilized" in the physiological state but RyR3 is not, as in amphibian RyRs. Murayama et al. (185, 189) have suggested that a change in the interdomain interaction due to CHAPS treatment is the main factor for the "destabilization," but in the case of RyR1 (not RyR α), the removal of FKBP12 also contributes. These findings suggest that RyR1 in the low-activity group is in a more physiological condition. If this is the case, the maximum open probability of CICR of RyR1 channels under physiological conditions would be significantly lower than that of cardiac muscle (Table 1).

The open probabilities of single channels can be converted into rate constants of Ca^{2+} release from SR in living or skinned fibers by assuming the Ca^{2+} current through the open channel as 0.5–1 pA as Kettlun et al. (121) estimated, and the concentration of RyR channels in

TABLE 1. Open probability of RyRs incorporated into lipid bilayer in the presence of quasi-physiological Mg^{2+} and ATP

RyR	Animal	Preparation	Ca ²⁺ , μ M	Mg ²⁺ , mM	ATP, mM	Main Ionic Composition (<i>cis/trans</i>), mM	P_o	Year Published	Reference Nos.
Skeletal	Rabbit	Triad	10	1	1	250 CsCl, 10 HEPES-Tris/50 CsCl, 10 HEPES-Tris	0.06	1990	267
	Chicken α	Purified channel	10	0.5	5	210 KCl, 50 HEPES/(symmetrical)	0.02	1994	212
	Chicken β	Purified channel	10	0.5	5	210 KCl, 50 HEPES/(symmetrical)	0.14	1994	212
	Rabbit	SR vesicle	10–200	1	1	250 HEPES, 120 Tris/250 HEPES, 53 Ca(OH) ₂	0.01	1997	31
	Rabbit	SR vesicle	10	0.6	2	250 CsCl, 10 TES/250 CsCl, 10 TES, 1 Ca	0.56 \pm 0.14 0.289 \pm 0.006	1999	43
	Rabbit	SR vesicle	100 10	1 2	2	250 CsCl/250 CsCl, 1 Ca	0.052 \pm 0.018 ~0.35 ~0.18	2004	151
Cardiac	Rabbit, pig	SR vesicle	10	1	1	300 CsMs, 10 HEPES/(symmetrical)	0.318	1995	157
	Canine	SR vesicle	5.5	2.6	0.5	250 HEPES, 115 Tris/250 HEPES, 53 Ca	0.45–0.75	1995	89
	Canine	SR vesicle	10	1	1	250 HEPES, 120 Tris/250 HEPES, 53 Ca(OH) ₂	0.74 \pm 0.10	1997	31
	Canine	SR vesicle	60	1	3	350 CsMs, 20 HEPES/(symmetrical)	0.19 \pm 0.04	1998	87
	Canine	Purified channel	10 100	0.7	5	250 KCl, 20 HEPES/(symmetrical)	0.3 0.8–0.9	1998	275
	Canine	SR vesicle	6	0.9	3	350 CsMs, 20 HEPES/350 CsMs, 20 HEPES, 5 Ca	0.4	2004	88
			Purified channel				0.15		

muscle fiber as 0.55 μ M.³ If 0.5 pA is adopted as the Ca²⁺ current through the open RyR channel, with a RyR concentration of 0.55 μ M, an open probability (P_o) value of 0.01 corresponds to 8.6 μ M/ms,⁴ which is 0.42%/ms if the total Ca²⁺ content of the SR is assumed to be 2 mmol/l muscle. Thus the maximum rate of CICR of single channels in a lipid bilayer in a physiological medium appears to be much higher than that of skinned fibers or FSR.

If the results of “stabilization” mentioned above are taken into account, the CICR observed in skinned fibers of amphibian skeletal muscle may be largely the property of RyR β , whereas CICR of mammalian muscle should be ascribed to RyR1, because the endowment of RyR3 in mammalian skeletal muscle is at most a few percent (187, 198, 247), and the magnitude of stabilization of RyR1 is reported to be ~15% (189). The rate of CICR in skinned fibers and FSR of mammalian skeletal

muscle, 0.02–0.3%/ms (see sect. III B6, A and B), is substantially lower than even the lower values of P_o in Table 1, 0.01–0.06, corresponding to 0.42–2.5%/ms. The rate of CICR in skinned fibers of amphibian skeletal muscle is also orders of magnitude lower than P_o of RyR β in Table 1, which are data from chicken muscle.

One possible reason for the discrepancy is that all single-channel experiments were carried out in a medium with a higher ionic strength than that used in the case of the skinned fiber experiments. High ionic strength increases the [³H]ryanodine binding of RyR channels (106). In addition, RyR channels in the lipid bilayer might somehow be further activated from the state in living or skinned fibers than “destabilization.”

C. Ca²⁺ Release Via RyR in the Absence of Ca²⁺

In the absence of Ca²⁺ and in the presence of a Mg²⁺ concentration sufficient to inhibit the CICR A-site, some stimuli can still evoke Ca²⁺ release via RyR. Such a Ca²⁺ release is not CICR by definition, and, in fact, it shows several characteristics different from those of CICR. At present, three kinds of stimulus are known to cause Ca²⁺ release in the absence of Ca²⁺ and presence of Mg²⁺.

1. Physiological Ca²⁺ release (depolarization of the t-tubule membrane)

Physiological Ca²⁺ release in skeletal muscle is caused by depolarization of the t-tubule membrane. De-

³ Pape et al. (211) estimated the concentration of RyR channels to be 0.27 μ M in reference to the myoplasm, assuming that one foot structure at the junctional region (77) corresponds to one channel. However, Felder and Franzini-Armstrong (64) later showed that the foot structures facing t-tubule membrane, coupled or uncoupled with DHPR, are all RyR α and that RyR β channels, the number of which is almost equal to that of RyR α , are located at the parajunctional region. Therefore, the concentration of RyR channels in amphibian skeletal muscle should be twice the previous estimation, 0.55 μ M. In mammalian muscles, the content of RyR3 is generally a few percent at most, but because each sarcomere has two t-tubules in mammals, unlike in amphibians, the estimated concentration could also be 0.55 μ M.

⁴ A concentration of 0.55 μ M/l muscle is equivalent to 3.3×10^{17} channels/l muscle. If P_o is 0.01, and each open channel carries 0.5 pA, which is $(0.5 \times 10^{-12} \text{ [C/s]}/2 \times 9.65 \times 10^4 \text{ [C/mol]}) = 0.26 \times 10^{-20} \text{ mol Ca}^{2+}/\text{ms}$, the rate of Ca²⁺ release is $8.58 \times 10^{-6} \text{ mol} \cdot \text{l muscle}^{-1} \cdot \text{ms}^{-1}$.

polarization-induced changes of the t-tubule voltage sensor, DHPR, are transmitted to RyR to cause Ca^{2+} release through protein-protein interaction (see reviews in Refs. 221, 227). Even in the presence of strong Ca^{2+} -chelating agents that can reduce the free Ca^{2+} concentration to 10^{-9} M or less, action potentials or depolarization by voltage-clamp pulses evokes the release of a large amount of Ca^{2+} (see sect. IV A). Because, in living muscle under physiological conditions, the cytoplasmic Mg^{2+} concentration is at least 1 mM (137, 273), which is sufficient to inhibit CICR A-site in the virtual absence of Ca^{2+} even in the presence of ATP (151), the primary physiological Ca^{2+} release evoked by DHPR is not CICR. That this release is not CICR was further confirmed in myotubes with RyR1 with the E4023A mutation. It had been reported that mutation of a key glutamate, which is conserved in all three RyRs (E4023 in the case of RyR1), to alanine practically abolished CICR (24, 65, 155). O'Brien et al. (195) demonstrated that whereas the E4023A mutation of RyR1 caused more than 100-fold suppression of CICR activity in bilayers in response to Ca^{2+} , it was accompanied by only an approximately fivefold reduction in Ca^{2+} release induced by surface membrane depolarization in myotubes.

Whether strong Ca^{2+} -chelating agents decrease the amount of Ca^{2+} released, in other words, whether physiological Ca^{2+} release involves a secondary CICR component, is a controversial issue that is discussed in the next section.

There are some pharmacological differences between physiological Ca^{2+} release and CICR. 1) Dantrolene sodium: in mammalian skeletal muscle, dantrolene sodium inhibits both physiological Ca^{2+} release and CICR to a similar extent at 37°C (134, 202). At room temperature, however, whereas CICR is inhibited only weakly or not inhibited at all by dantrolene sodium (79, 103, 133, 134, 180, 202–204), physiological Ca^{2+} release is inhibited to the same extent as that at 37°C (134). 2) Procaine: although procaine, an inhibitor of CICR, also inhibits physiological Ca^{2+} release in nonmammalian fibers to some extent (132), physiological Ca^{2+} release largely remains in the presence of 10–20 mM procaine (92, 263), which strongly inhibits CICR. 3) Adenine: the differential effects of adenine on physiological Ca^{2+} release and CICR are also noted, as described in section IV E.

2. Clofibrac acid

Sukhareva et al. (256) were the first to show that clofibrac acid induces Ca^{2+} release from the SR. Ikemoto and Endo (102) then demonstrated that clofibrac acid causes strong Ca^{2+} release (comparable to that induced by caffeine) from the SR through the RyR in mouse skeletal muscles in the absence of Ca^{2+} . The clofibrac acid-induced Ca^{2+} release in the absence of Ca^{2+} is inhibited by Mg^{2+} , but the inhibitory action of Mg^{2+} becomes sat-

urated at ~1 mM, and even in the presence of 10 mM Mg^{2+} , an appreciable Ca^{2+} release can be evoked (102). Unlike CICR, the clofibrac acid-induced Ca^{2+} release in the absence of Ca^{2+} is not inhibited by 10 mM procaine and is not potentiated by adenine nucleotide; on the contrary, it is inhibited by AMP (102). The persistence of Ca^{2+} release in the presence of high concentrations of Mg^{2+} and procaine has some resemblance to physiological Ca^{2+} release. The magnitude of the inhibitory effects of dantrolene derivatives on twitch responses and that on clofibrac acid-induced Ca^{2+} release show a good correlation ($r = 0.956$), whereas the same derivatives show only a weak inhibition of caffeine-induced Ca^{2+} release, with the magnitude of inhibition showing no correlation with twitch inhibition (103).

3. Ion substitution: its direct effect on the SR membrane

When another medium of different ionic composition is substituted for the medium in which skinned fibers or FSR is immersed, Ca^{2+} release can be evoked, if the new medium causes depolarization of the internal membrane systems, namely, resealed t-tubules (34, 251) and/or the SR.

Ion substitutions appear able to induce Ca^{2+} release through direct effects on the SR, probably SR depolarization. In anion substitutions such as chloride for the less-permeable methanesulfonate, part of the Ca^{2+} release may be due to swelling of the SR, but all the effects of ion substitutions cannot be attributed to swelling, because Ca^{2+} release is also induced when a more-permeable cation (such as potassium) is replaced by a less-permeable cation (such as choline, lithium, or sodium), or when the anion and the cation are both replaced to keep the KCl product constant (2, 52, 56, 177). A direct effect on the SR is supported by the fact that ion substitution can still evoke Ca^{2+} release in fibers skinned under conditions leading to loss of the membrane potential across the t-tubule membrane: in split fibers in which t-tubules remain open to the bathing medium, and/or when the Na pump is inhibited to prevent reestablishment of resealed t-tubule membrane potential (52, 56), or in saponin-skinned fibers, in which both the t-tubule membrane and the surface membrane must have lost the ability to hinder free diffusion (104).

The Ca^{2+} release induced by SR depolarization can be evoked in the absence of Ca^{2+} and in the presence of Mg^{2+} . In fact, unlike CICR, SR depolarization-induced Ca^{2+} release is unaffected by Mg^{2+} (263) and is not inhibited by procaine or tetracaine (2, 263).

RyR1 is responsible for SR depolarization-induced Ca^{2+} release, because such Ca^{2+} release is almost completely abolished in skinned skeletal muscle fibers of mice when RyR1 expression is blocked (104). Whether RyR3 is also responsible for SR depolarization-induced Ca^{2+} re-

lease is not known, but the weak SR depolarization-induced Ca^{2+} release in skinned fibers of RyR1 knock-out mice might occur through RyR3 (104).

There are no data with single channels of RyR comparable to those with the skinned fiber experiments on SR depolarization in the absence of Ca^{2+} and presence of Mg^{2+} . The initial activation following SR depolarization described earlier (see sect. *mB5*) may include a type of activation similar to that in skinned fibers.

D. Some Considerations Concerning the Activation of RyR Channels

The RyR Ca^{2+} -release channel is a complex protein tetramer of 550–560 kDa which has multiple sites where agonists or other stimuli can bind to open the channel. In fact, RyR1 and RyR α can be activated by at least two different mechanisms: CICR and t-tubule depolarization. SR depolarization-induced Ca^{2+} release is caused at least via RyR1 (and probably also via RyR α), as mentioned in the previous section. Clofibric acid-induced Ca^{2+} release in the absence of Ca^{2+} has been demonstrated in mouse skeletal muscle and, therefore, probably occurs through RyR1, because little RyR3 is present in this tissue. Thus at least RyR1 and, probably, RyR α can be activated by the three non-CICR stimuli. Local conformational change of RyR molecules during CICR may well be different from that of non-CICR Ca^{2+} release, although the same channel structure is probably opened in both types of release. Therefore, the pharmacology of CICR and that of non-CICR Ca^{2+} release may well differ.

Whether clofibric acid and SR depolarization can also activate RyR3 or RyR β in the absence of Ca^{2+} is unknown. However, because RyR1 and RyR α can be activated in more than one way, the possibility that RyR3 and RyR β can also be activated by means other than CICR should be considered.

Some agents specifically affect either CICR or non-CICR Ca^{2+} release, as described in section *mC*. However, other agents may affect both CICR and non-CICR Ca^{2+} release. Clofibric acid is one such agent. Crofibric acid causes non-CICR Ca^{2+} release as described earlier, but also potentiates CICR (102). Although clofibric acid-induced Ca^{2+} release, unlike CICR, is inhibited by AMP, and not by procaine, in the absence of Ca^{2+} (as described in sect. *mC2*), Ca^{2+} release enhanced by clofibric acid in the presence of a Ca^{2+} concentration that can evoke CICR is potentiated by AMP and inhibited by procaine (102). These two aspects of actions of clofibric acid could be the result of its binding either to one and the same site on RyR molecules or to two different sites. The possible multiple actions of caffeine are described in section *vA*.

IV. PHYSIOLOGICAL SIGNIFICANCE OF CALCIUM-INDUCED CALCIUM RELEASE IN SKELETAL MUSCLE

As previously described, the primary mechanism of physiological Ca^{2+} release in skeletal muscle is not CICR but protein-protein interaction between the t-tubule voltage sensor DHPR and RyR1 or RyR α . Whether Ca^{2+} released through the primary mechanism in turn activates CICR to contribute to the total amount of Ca^{2+} released physiologically is a matter of dispute.

The possibility that CICR contributes to the physiological Ca^{2+} release in amphibian skeletal muscle was first proposed by Rios and Pizarro (220); they were motivated by the finding of Block et al. (15) that DHPR tetrads in the swim-bladder muscle of toadfish are associated with every other RyR tetramer, leaving the other half of Ca^{2+} release channels devoid of t-tubule voltage sensors. In amphibian skeletal muscle, as in most skeletal muscles of nonmammalian vertebrates, RyR α and RyR β are present in equal amounts. Therefore, reasonable assumptions were that, in amphibian skeletal muscle, the t-tubule voltage changes are transmitted to one type of RyR (later shown to be the RyR1 equivalent RyR α ; Refs. 260, 261) and that the “every other” foot structures lacking t-tubule voltage sensors are the other type of RyR (RyR β), which are activated by Ca^{2+} released through neighboring voltage-stimulated RyR α . The theory was further elaborated to a proposed model assuming release generators, couplons, composed of equal numbers of voltage-gated channels and Ca^{2+} -gated channels interacting through the local Ca^{2+} concentration (253). Later, Felder and Franzini-Armstrong (64) showed that the foot structures lacking t-tubule voltage sensors at the t-SR junctional region are also RyR1 or RyR α and that RyR3 and RyR β are located at lateral parajunctional regions immediately adjacent to the junctional region. However, the essential point in the model is that at least half of RyRs, RyR β , are not associated with t-tubule voltage sensors but are located at a position close to the voltage sensor-associated RyR α , and that point is not altered.

A. Effects of High-Affinity Ca Buffers

If CICR contributes secondarily to physiological Ca^{2+} release, a reduction in changes of myoplasmic Ca^{2+} with a rapidly reacting, high-affinity Ca buffer, such as BAPTA (265) or fura 2 (86), would decrease Ca^{2+} release. On the other hand, Ca^{2+} , in addition to activating Ca^{2+} release (CICR), also inhibits Ca^{2+} release during t-tubule depolarization (6, 173, 229, 241). When a train of action potentials are given, the peak rate of Ca^{2+} release associated with the second and subsequent action potentials is much less than that associated with the first action po-

tential. Similarly, when depolarizing square pulses are applied with the voltage-clamp technique, the rate of Ca^{2+} release initially increases to an early peak and then rapidly declines to a lower level that is maintained until the end of the pulse. The reduction in Ca^{2+} release has been attributed to the elevation of the Ca^{2+} concentration and is called "Ca inactivation of Ca^{2+} release." Therefore, the reduction in changes of myoplasmic Ca^{2+} with a rapidly reacting, high-affinity Ca buffer may increase Ca^{2+} release by reducing Ca inactivation of Ca^{2+} release. Experiments have been conducted to investigate this phenomenon, but different laboratories have obtained contradictory results.

Baylor and colleagues (8, 94), using intact fibers from *Rana temporaria*, found that 3–4 mM fura 2 increases both the amount and the peak rate of Ca^{2+} release induced by an action potential, consistent with a reduction in the Ca inactivation of Ca^{2+} release. On the other hand, Jacquemond et al. (112) and Csernoch et al. (35), using cut fibers from *Rana pipiens*, found that 2–3 mM fura 2 or 3–5 mM BAPTA decreases Ca^{2+} release with voltage-clamp depolarization: the early peak was completely eliminated, but the smaller steady level remained, suggesting a major contribution of CICR to the early peak.

To examine whether the apparent discrepancies were due to differences in preparations (for example, intact vs. cut fibers, and possible species differences) or to the type of stimulation (action potential vs. voltage clamp), Baylor, Chandler, and colleagues performed experiments with fura 2 using cut fibers for both action potentials (210) and voltage-clamp stimulation (116). In the latter study, possible differences between species were also examined. The experiments consistently showed that fura 2, at concentrations up to 3–4 mM, increased both the amount and the maximum rate of Ca^{2+} release by action potential, as well as both the peak and quasi-steady level rates of Ca^{2+} release by voltage-clamp stimulation, with a greater relative increase in the quasi-steady level rate than in the peak rate. These results are consistent with a reduction in the Ca inactivation of Ca^{2+} release.

Because these studies were carefully designed and comprehensive, the results appear to be reliable. Jong et al. (116) have noted that the discrepancy between their results and those of Jacquemond et al. (112) and Csernoch et al. (35) might be explained by differences in the physiological state of fibers. The discrepancy may be related to the finding by Jong et al. (116) that fibers with SR having a smaller Ca content may be more susceptible to the inhibitory effect of fura 2 on Ca^{2+} release. If Ca^{2+} has both an activating action and an inhibitory action on Ca^{2+} release, the net effect of a Ca buffer could be either a decrease or an increase in Ca^{2+} release, depending on the condition of the fiber. Even if Ca^{2+} has no activating effect, the situation will be the same if the Ca buffer has an inhibitory effect on Ca^{2+} release unrelated to Ca^{2+}

complexation (see below). Therefore, if we assume that Ca^{2+} release in fibers with SR having a smaller Ca content is accompanied by a lesser degree of Ca inactivation, a decrease rather than an increase of Ca^{2+} release by fura 2 might more easily occur.

At concentrations higher than 3–4 mM, however, the same group of authors has found that fura 2 decreases Ca^{2+} release both with action potentials (210) and voltage-clamp stimulation (116). These results might be consistent with the notion that CICR participates in physiological Ca^{2+} release, especially because λ_{Ca} , the characteristic distance that a Ca^{2+} is expected to diffuse before being captured by a Ca buffer (192), is calculated to be less than ~30 nm at fura 2 concentrations greater than 3–4 mM (115), which coincides with the 28 nm separating junctional and parajunctional rows of RyR (64). Yet, such a conclusion must be made with caution, as these authors pointed out that the results may also be due to the pharmacological effects of a high concentration of fura 2 unrelated to complexation with Ca^{2+} , because 1) with higher concentrations of fura 2, the initial rising phase of Ca^{2+} release is much slower than that in control, which should not occur if the effect of fura 2 is only the result of complexation with Ca^{2+} to reduce secondary CICR (116), and 2) the inhibitory effect of higher concentrations of fura 2 on Ca^{2+} release takes minutes to develop, whereas the result of complexation with Ca^{2+} should appear immediately (115). Even if the effect of higher concentrations of fura 2 is due to its complexation with Ca^{2+} , another possibility to be considered is that Ca^{2+} is certainly acting on secondary Ca^{2+} release, but in a non-CICR manner in collaboration with an additional stimulus.

B. Early Peak of Voltage-Activated Ca^{2+} Release and CICR

The possibility that the early peak of Ca^{2+} release during voltage-clamp depolarization is composed mainly of CICR, whereas the later quasi-steady level is the result of direct DHPR activation has been repeatedly considered since an early study by Jacquemond et al. (112), who reported that Ca buffer specifically inhibits the early peak. Shirokova et al. (231) has found that amphibian skeletal muscles, which have equal amounts of RyR β and RyR α , show a much more prominent early peak than do mammalian muscles, which have only a small amount of RyR β , and suggested that Ca^{2+} released from DHPR-activated RyR α channels may activate the CICR of RyR β to cause the more-prominent early peak in amphibian muscles (231).

However, as described in the previous section, careful studies on the effects of Ca buffer on physiological Ca^{2+} release did not confirm its specific inhibition of the

early peak. Studies have also shown that in cut fibers of amphibian skeletal muscle in the presence of 20 mM EGTA, which prevents Ca^{2+} released by a voltage-activated channel from reaching other Ca^{2+} channels more than a few hundred nanometers apart, Ca^{2+} release with a very small depolarization (four orders of magnitude smaller than maximal release) is still strongly voltage dependent and has the same steep slope as Ca^{2+} release with a stronger depolarization (209). This finding indicates that the steep voltage dependence of Ca^{2+} release at the peak in amphibian muscle fibers is the property of voltage-activated gating itself and does not require the Ca^{2+} -induced amplification.

The transition from the peak of Ca^{2+} release to the quasi-steady level is due to the Ca inactivation of Ca^{2+} release. Many researchers have assumed that Ca inactivation is due to inhibition of CICR, probably because Ca^{2+} was known to inhibit CICR. However, the inhibition of CICR by Ca^{2+} requires a high concentration, with a K_i of 0.4 mM, for example (182), whereas Ca inactivation of Ca^{2+} release still occurs in the presence of millimolar concentrations of fura 2 (115). Therefore, the Ca inactivation of Ca^{2+} release is unlikely to be the result of CICR inhibition. Furthermore, Jong et al. (115) have concluded that Ca inactivation affects voltage-gated channels, because, as a result of Ca inactivation, in addition to a reduction in the peak rate of Ca^{2+} release, the initial increase in Ca^{2+} release is delayed, which should not occur if Ca inactivation affects only the secondary release channels. Jong et al. (115) have also shown that a model to simulate Ca inactivation of Ca^{2+} release assuming a single uniform population of channels can reproduce all the experimental results. This model and the steep voltage dependence of unamplified Ca^{2+} release mentioned above do not rule out the participation of RyR β in physiological Ca^{2+} release and its activation by Ca^{2+} released by RyR α , but do indicate that if they occur, both kinds of channel must behave as a singly gated unit, i.e., RyR β must behave as a slave to the master RyR α . Even if Ca^{2+} is a mediator of the singly gated unit, the mechanism cannot be CICR because CICR is activated independently of voltage and because the secondarily induced CICR should deviate from the slave behavior as shown in section IV E.

Furthermore, it has been increasingly apparent that no clear separation can be made between the peak and the steady level. For example, Ca^{2+} sparks appear to underlie both the early peak and the steady level (130). Tetracaine and procaine, which are considered to be more or less specific inhibitors of CICR, appear to inhibit both phases (18, 215), although some studies have shown that tetracaine specifically inhibits the initial peak (216).

On the other hand, recent RyR3 transfection studies in mammalian skeletal muscle have shown that depolarization-activated Ca^{2+} release in transfected fibers is significantly larger with a much greater early peak than in

control fibers (154, 217). This finding indicates that transfected RyR3 molecules participate in the depolarization-induced Ca^{2+} release and contribute to the total amount of Ca^{2+} released. These studies suggest that Ca^{2+} release through DHPR-gated RyR1 channels is amplified by a mechanism that causes Ca^{2+} release via transfected RyR3. Because RyR1 and RyR3 have no direct contact (64), coupled gating would not be the mechanism, and a diffusible substance, such as Ca^{2+} , is certainly a likely possibility. However, CICR again cannot be the mechanism, even if Ca^{2+} is one of the essential factors for amplification, because the rate of CICR that can be achieved is too low to significantly increase the magnitude of Ca^{2+} release under physiological ionic conditions, as discussed in the next section.

C. Comparison of Maximum CICR Rates With the Rate of Physiological Ca^{2+} Release

Endo (51) first pointed out that the maximum rate of CICR in skeletal muscle, determined in skinned fibers under the physiological conditions, is at least one order of magnitude lower than the rate of action potential-induced Ca^{2+} release. In fact, the values of the maximum rate of CICR in skinned fibers or FSR, collected and presented in section III B 5, A and B, was 0.015–0.3%/ms, which is more than one order of magnitude lower than the rate of physiological Ca^{2+} release in living muscle, which is 2–5%/ms (35, 209, 210).

The concentrations of Mg $^{2+}$ or AMPPCP used in the CICR experiments of skinned fibers and FSR may not be strictly physiological, but the effects of the differences, if any, are minor and cannot explain the big difference.

It may be argued that Ca^{2+} might also inhibit Ca^{2+} release at concentrations lower than those required to activate CICR so that CICR in skinned fibers, where Ca^{2+} reaches active sites slowly owing to diffusion delay, may have been inhibited before being activated by the hypothetical Ca^{2+} -induced inhibition, and “true” rates of CICR that should have been obtained if Ca^{2+} were applied rapidly were missed. However, in skeletal muscles, such a Ca^{2+} -induced inhibition of CICR at lower concentrations of Ca^{2+} has so far not been demonstrated in any preparations, skinned fibers, FSR, or an isolated channel incorporated into a lipid bilayer, although Fabiato (61) has reported preliminary results showing that CICR can be evoked with rapid application of Ca^{2+} but not with slow application of Ca^{2+} in skinned skeletal muscle fibers. Ca inactivation of Ca^{2+} release is effective at low Ca^{2+} concentrations, but the inactivation is exerted upon voltage-gated channels, as described in the previous section, and no evidence for Ca inactivation being due to CICR inhibition has been obtained. In fact, rapid application of Ca^{2+} at concentrations greater than 100 μM has been

accomplished in saponin-skinned frog fibers by Zhou et al. through photolysis of Ca nitrophenyl-EGTA with laser flashes (283), but detectable CICR was not evoked as its immediate effect. This result is consistent with early skinned fiber CICR experiments with solution exchange (see sect. III B1) (47).

The inhibition of CICR by Ca^{2+} is consistently demonstrated only with concentrations in the millimolar range. The buffering action of EGTA, the calcium-chelating agent used in skinned fiber experiments, is extremely weak, particularly at concentrations that evoke the maximal rate of CICR, and is slow as well. Therefore, RyR channels near an active channel might be inactivated by Ca^{2+} released from the active channel because the concentration of Ca^{2+} is likely higher there than in the ambient solution owing to the short diffusion distance. However, this inactivation should not significantly affect the maximum rate of release determined in a fiber, because at this rate, only $<0.1\%$ ⁵ of the RyR channels are open so that inactivated channels surrounding the active one are only a small fraction of all channels; most channels are unaffected by the active channel.

Thus, if we accept as valid the reported value for the maximum rate of CICR in skinned fibers, it is too small to support a considerable fraction of physiological Ca^{2+} release. A similar conclusion was reached by Ogawa and colleagues (182) on the basis of their measurement of CICR rates in skinned fibers. They also concluded that even in the absence of Mg^{2+} , their value for the maximum rate of CICR was much lower than the physiological rate of Ca^{2+} release (182) and was inconsistent with the model proposed by Lamb and Stephenson (147).

Comparisons between data from skinned and intact fibers must be carefully performed. Differences between Ca^{2+} sparks in cut fibers and those in intact fibers suggest that the activity of RyR channels is higher in cut fibers than in intact fibers (5, 23). However, because skinned fibers are probably farther removed from intact fibers than are cut fibers, the finding of higher activity of RyR channels in cut fibers increases, rather than decreases, the difference between rates of physiological Ca^{2+} release and those of CICR estimated from skinned fiber experiments.

D. Ca^{2+} Sparks

Ca^{2+} sparks, which are small, brief, and highly localized releases of Ca^{2+} , were first observed in cardiac myo-

cytes (27) and then in skeletal muscle fibers (128, 266). They occur spontaneously in frog skeletal muscle fibers in the resting state (128) and at higher frequencies during depolarization (128, 266). The frequency of Ca^{2+} sparks activated by depolarization steeply increases with voltage at ~ 4 mV per e -fold change (128), which is similar to the voltage dependence of the activation of Ca^{2+} release determined in whole fibers (6, 209). Unlike the spark frequency, the spatiotemporal properties of sparks are almost identical at rest and during depolarizations to different potentials (128, 130, 141). The pattern of occurrence of Ca^{2+} sparks after the start of a large depolarization, i.e., the latency histogram of Ca^{2+} sparks, is similar to the pattern of the rate of Ca^{2+} release after depolarization showing Ca inactivation of Ca^{2+} release (129, 130). These results suggest that the Ca^{2+} spark is the basic unit event of physiological Ca^{2+} release.

1. Coordination mechanisms

The range of the reported numbers of RyR channels involved in a Ca^{2+} spark is large (see review in Ref. 130), but it is certain that Ca^{2+} sparks are events involving the opening of multiple Ca^{2+} release channels (83, 93, 130, 222). The multiple channels appear to open almost simultaneously in a spark (142). The question arises, then, of how the opening of these channels is coordinated. A remarkable property of the coordination in Ca^{2+} sparks is its extremely high success rate. In the case of a voltage-activated spark, a voltage-gated channel is believed to open first, which is always followed immediately by the opening of one or more RyR channels. The notion that the coordination in Ca^{2+} sparks has a high success rate is based on the observation that the spatiotemporal pattern of Ca^{2+} sparks is always the same and that the opening of a single voltage-gated channel without coordination with other channels has not been observed in normal intact fibers (93).

In cut fibers, however, smaller Ca^{2+} releases such as “small event Ca^{2+} release” (234), “ridge” and “ember” (84) have been detected and are believed to represent the opening of voltage-gated channels not associated with the opening of Ca^{2+} -gated channels. The activity of RyR channels is greater in cut fibers than in intact fibers (5, 23). The presence and absence of small event Ca^{2+} release, however, may not be the difference between cut fibers and intact fibers, because small event Ca^{2+} release was not observed in other cut-fiber experiments (129, 130). In any case, Ca^{2+} sparks in cut fibers occurring over the small event Ca^{2+} releases also have a stereotypic morphology (84, 234), which again indicates that coordination has an extremely high success rate.

Coordination might involve coupled gating (163), but natural assumption is that coordination is mediated through Ca^{2+} . Under physiological conditions, however, the open probability of RyR1 and RyR3 in CICR is

⁵ The maximum rate of CICR is $\sim 0.02\%/ms$ (see sect. III B6), or $0.4 \mu M/ms$ if the Ca^{2+} content in the SR is assumed to be 2 mol/l muscle. If the Ca^{2+} current through RyR channels is assumed to be 0.5 pA (121), which is $0.26 \times 10^{-20} \text{ mol Ca}^{2+} \cdot ms^{-1} \cdot \text{channel}^{-1}$, then 1.54×10^{14} channels/l muscle, which is $2.6 \times 10^{-10} \text{ mol/l}$ muscle, are open. If the density of RyR channels is assumed to be $0.55 \mu M$ (see sect. III B6), then $\sim 0.05\%$ of channels are open.

far less than 100%, even in channels incorporated in a lipid bilayer, in which the open probability is much higher than in skinned fibers or FSR (see sect. III B6 and Table 1). Therefore, CICR cannot be the mechanism of coordination. Even if Ca^{2+} does play a role, some other factor(s) must also be involved. A possible factor is molecular interactions inside the SR lumen. A change in the membrane potential of the SR associated with Ca^{2+} release through DHPR-gated channels, although probably small (7), might also affect the conformation of neighboring RyRs.

On the other hand, the initiation of spontaneous Ca^{2+} sparks might be due to CICR because their frequency is dependent on the Ca^{2+} concentration (128, 283) and can be fitted by Ca^{2+} activation and competitive Mg^{2+} inhibition schemes of CICR (283). However, the mechanism of coordination of spontaneous sparks also remains unclear.

Voltage-activated sparks, as well as spontaneous Ca^{2+} sparks, terminate spontaneously, probably owing to Ca inactivation (5, 130, 219). However, the results of Lacampagne et al. (140) suggest that voltage-activated sparks could be prematurely terminated if the surface membrane is repolarized during their rising phase. Thus, not only the initial voltage-gated channel opening in Ca^{2+} sparks but also the opening of coordinated channels is under the control of the t-tubule voltage sensor. This control is inconsistent with CICR being the coordination mechanism, because such a voltage sensor control is not believed to be involved in CICR. In fact, CICR has been shown not to be controlled by the voltage sensor (see sect. IV E).

The fact that the otherwise stereotypic size of the Ca^{2+} sparks is increased by caffeine, a CICR potentiator (84), and inhibited by Mg^{2+} (84) and tetracaine (93), CICR inhibitors, is consistent with the CICR theory of coordination in Ca^{2+} sparks, but does not prove it.

2. Ca^{2+} sparks and RyR3

Adult mammalian skeletal muscle fibers usually do not show spontaneous Ca^{2+} sparks (29, 232), except when the sarcolemma is permeabilized, (36, 126, 282) or in certain pathological states, e.g., mitochondrial degradation or osmotic shock (108, 109, 269), nor do they show sparks in response to depolarization (36, 232). Because sparks have been recorded in myotubes expressing either RyR1 or RyR3 alone (28, 29, 270), it is certain that both RyR1 and RyR3 can produce Ca^{2+} sparks. However, because Ca^{2+} sparks are rarely seen in adult mammalian skeletal muscles, in which the expression of RyR3 is minimal, whereas Ca^{2+} sparks are evoked in amphibian skeletal muscles, in which RyR β and RyR α are present in equal amounts, it was proposed that sparks are produced by RyR3 (or RyR β), and the ability of RyR1 to evoke Ca^{2+}

sparks is somehow suppressed in intact adult skeletal muscle fibers (154, 217, 235). Indeed, Pouvreau et al. (217) have shown that in adult mouse skeletal muscles expressing exogenous RyR3 after transfection with RyR3 cDNA, abundant Ca^{2+} sparks are evoked both spontaneously and via depolarization, but are not evoked in muscles, in which RyR1 cDNA was transfected. In a similar study, Legrand et al. (154) did not detect "sparklike" Ca^{2+} release events in RyR3-transfected muscles, but found repetitive spontaneous elevations of Ca^{2+} in the RyR3-transfected region. The difference between the results of RyR3 expression studies of the two groups may be due to differences in transfection methods and resulting differences in molecular architecture between the transfected RyR3 and the junctional RyR1 molecules (154, 218). It seems likely that Ca^{2+} sparks in intact fibers require RyR3 channels as Pouvreau et al. (217) have shown. Although CICR does not appear to be involved in the production of Ca^{2+} sparks as discussed above, the RyR3 requirement is reminiscent of the findings of Ogawa and colleagues that CICR in RyR1 or RyR α , but not RyR3 or RyR β , is somehow "stabilized" in the physiological state (185, 188, 189).

The increase in depolarization-induced Ca^{2+} release in RyR3-transfected fibers was discussed in section IV B.

E. Some Pharmacological Observations

Although the rate of CICR is much lower than the rate of physiological Ca^{2+} release, the usually undetectable CICR becomes detectable when it is potentiated by caffeine. Thus Simon et al. (240) and Klein et al. (131) have shown that in the presence of 0.5 mM caffeine, Ca^{2+} release does not immediately cease after the termination of voltage-clamp pulse, unlike in the absence of caffeine, but continued for some time. The voltage-uncontrolled Ca^{2+} release was clearly demonstrated (without any calculation of release rate): $[\text{Ca}^{2+}]$ continued to increase for several tens of milliseconds after repolarization (240). The magnitude of voltage-uncontrolled Ca^{2+} release is on the order of 1 $\mu\text{M}/\text{ms}$ (131) that CICR can achieve in the presence of caffeine, and this release is, indeed, inhibited by the CICR inhibitor procaine (132). A similar voltage-uncontrolled Ca^{2+} release was not observed by Shirokova and Rios (233). However, in these experiments, voltage was applied in the presence of 5 or 10 mM caffeine and 10 mM EGTA. Therefore, CICR had already been activated before the pulse, and a small increase in the CICR rate after a voltage-clamp pulse (due to depolarization-induced increase in cytoplasmic $[\text{Ca}^{2+}]$, which was small because of the presence of EGTA) may well have been obscured when the Ca^{2+} release rate was calculated. Struk and Melzer (255) also stated that they could not confirm the voltage-uncontrolled Ca^{2+} release. However,

in Figure 7B of their paper (255), 10 ms after the end of the voltage-clamp pulse, a small but clear residual Ca^{2+} release that departed from the main time course of the turn off of Ca^{2+} release is seen with 0.1 mM EGTA and caffeine, but not with 15 mM EGTA and caffeine, or with 0.1 mM EGTA (control).

Caffeine-induced potentiation of twitch may be explained by the secondary Ca^{2+} release by CICR induced by physiologically released Ca^{2+} . I have calculated the amount of extra Ca^{2+} release with an action potential in the presence of caffeine due to CICR, using the time course of Ca^{2+} concentration at the triad during action potential calculated by Baylor and Hollingworth (9) and the Ca^{2+} dependence of CICR determined by Horiuti (95) in the presence and absence of 1.2 mM caffeine at 22°C. The extra amount of Ca^{2+} released by CICR in the presence of 1.2 mM caffeine was ~6% of the amount released without caffeine, which might explain the twitch potentiation, whereas in the absence of caffeine, the extra amount was only 0.86%. Twitches are inhibited by adenine, a CICR inhibitor in the presence of ATP (see sect. III B3), only if the twitch is potentiated by caffeine, whereas adenine does not inhibit twitches in the absence of caffeine, or when the twitch is potentiated by nitrated Ringer solution (111).

The effect of caffeine will be discussed further in the next section.

F. Conclusions

The main conclusions in this chapter are recapitulated.

Secondary CICR, which is Ca^{2+} release induced by the direct action of Ca^{2+} released through a voltage-activated RyR channel on neighboring channels, does not seem to significantly contribute to the physiological Ca^{2+} release, because the rate of Ca^{2+} release by CICR under physiological conditions is too low.

The basic events of physiological Ca^{2+} release, voltage-activated Ca^{2+} sparks, consist of the coordinated activities of multiple channels. The coordination might involve coupled gating (163) between RyR1 channels associated with DHPR tetrads and unassociated channels. However, coupled gating is believed to be unlikely in the case of RyR3 or RyR β on morphological grounds (64). CICR is unlikely to be the mechanism of coordination because its P_o is too low to support the reliable production of Ca^{2+} sparks. Ca^{2+} might still play an essential role in the coordination of RyR channels in Ca^{2+} sparks and/or physiological Ca^{2+} release in a non-CICR manner. However, in that case, some other factor or factors must operate at the same time.

V. PHARMACOLOGICAL AND PATHOPHYSIOLOGICAL SIGNIFICANCE OF CALCIUM-INDUCED CALCIUM RELEASE IN SKELETAL MUSCLE

A. Effects of Caffeine

1. Major effects of caffeine on skeletal muscle

Caffeine at high concentrations can induce contracture of skeletal muscle without changing membrane potentials (3). At lower concentrations, it can potentiate twitches without changing action potentials (225) and can shift the relation between membrane potentials and activation to a more negative potential in potassium contracture (158) and in voltage-clamp experiments without changing the charge movement of t-tubule voltage sensor (131, 138). Caffeine can also induce "sarcomeric oscillation" (139, 161).

2. Contracture induced by caffeine

The contracture-inducing action of caffeine may be explained by its potentiating effect on CICR. Horiuti (95), to explain "rapid cooling contracture," measured the rates of CICR and Ca^{2+} uptake in skinned fibers of the frog and *Xenopus* at room or low temperature, in the presence or absence of a low concentration of caffeine, and compared the values.

Rapid cooling contracture is a phenomenon whereby a frog muscle, when immersed in a Ringer solution containing a subthreshold concentration of caffeine at room temperature and rapidly cooled to <5°C, can undergo vigorous contracture with no changes in membrane potential (224). The contracture has been shown to be due to Ca^{2+} release from the SR (136). Thus the rapid cooling contracture appears to simply be a caffeine contracture at low temperature, at which the threshold of caffeine contracture is lower.

With the simplifying assumption that Ca^{2+} movement in living muscle fibers is determined only by the activity of the Ca^{2+} pump and the Ca^{2+} release channel of the SR, Horiuti (95) calculated the rates of Ca^{2+} uptake and Ca^{2+} release in living muscle fibers, where total exchangeable Ca^{2+} is constant, using the Ca^{2+} concentration dependence of the rates of Ca^{2+} uptake and Ca^{2+} release determined experimentally in skinned fibers (95). The results are shown in Figure 2. U and R are Ca^{2+} uptake and Ca^{2+} release rates, respectively. The subscripts "h" and "l" indicate high (22°C) and low (2°C) temperatures, respectively, in the presence of 1.2 mM caffeine, and the subscript "0" indicates the absence of caffeine at 2°C. At 22°C, curve R_h crosses U_h only at a point a , which is the stable point: if the cytoplasmic Ca^{2+} concentration is increased to greater than (or decreased to lower than)

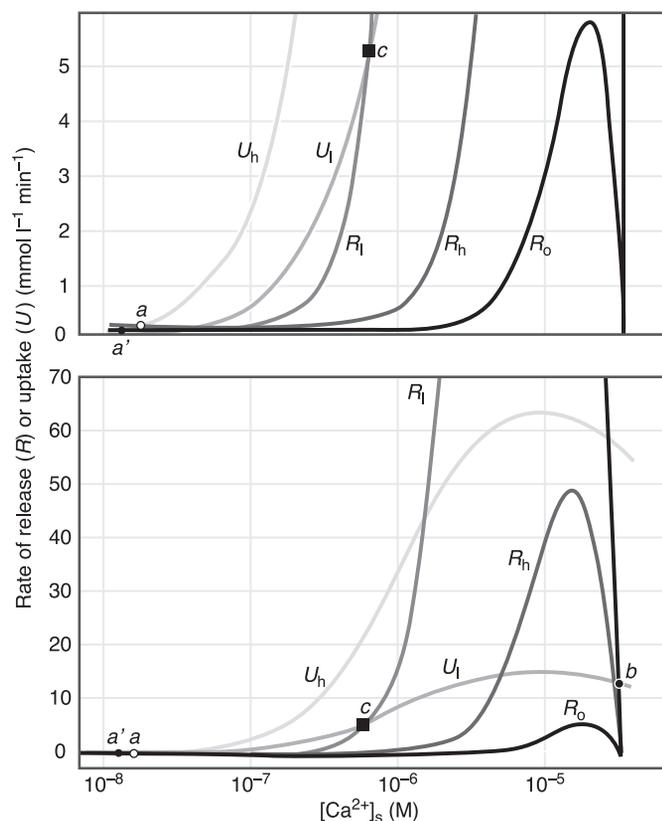


FIG. 2. Rates of Ca^{2+} release from (R) and Ca^{2+} uptake into (U) the SR at various sarcoplasmic concentrations of free Ca^{2+} ($[\text{Ca}^{2+}]_s$) in a model fiber. The suffixes "h" and "l" indicate the rates at 22 and 2°C, respectively, in the presence of 1.2 mM caffeine in both cases. R_o is R at 2°C but in the absence of caffeine. The top half is an enlargement of the bottom part of the bottom half. For further explanation, see the text. [From Horiuti (95).]

this point, it would tend to be restored to the point a , because Ca^{2+} uptake by SR is more (or less) active than Ca^{2+} release. Because the Ca^{2+} concentration at point a is sufficiently low, the fiber will not contract despite the presence of 1.2 mM caffeine. At 2°C, however, curves R_l and U_l cross at three points a' , b , and c . While a' and b are stable points, c is an unstable point: if the cytoplasmic Ca^{2+} concentration somehow increases to greater than the critical point c , it would continue to increase, because the release rate is higher than the uptake rate, and contracture will occur. In the absence of caffeine at 2°C, curve R_o crosses U_l near point a' , but this is the only crossover point so that contracture will not occur without caffeine.

Thus the results shown in Figure 2 may essentially explain how rapid cooling contracture occurs. However, according to Figure 2, for contracture to be evoked, a small initial Ca^{2+} release is necessary to increase the cytoplasmic Ca^{2+} concentration from point a to above c . Horiuti (95) speculated that because the Ca^{2+} pump is less efficient when the SR is loaded (271), Ca^{2+} uptake rates in Figure 2 determined with an empty SR may be

overestimated. If the uptake activity is decreased by a factor greater than 6, contracture occurs spontaneously. Another discrepancy between the model and actual muscle fibers is that contracture is slower with the model than in experiments. As a possible mechanism, Horiuti (95) speculated that mechanical stress due to rapid solution exchange might stimulate the SR to cause some Ca^{2+} release.

The discussion presented above is rather crude, and a complete understanding of the rapid cooling contracture is yet to be reached. However, the analysis shown in Figure 2 suggests that caffeine contracture can be explained in general, at least qualitatively. Because a caffeine concentration of 1.2 mM is much less than the saturation point for potentiating CICR, with an increase in caffeine concentration, both R_l and R_h in Figure 2 are shifted upward and to the left so that the crossover point can move to a Ca^{2+} concentration lower than the resting Ca^{2+} level, which allows spontaneous contracture.

In the presence of a low concentration of caffeine, CICR should qualitatively increase and the stable crossover points of U and R , whether detectable or not, are shifted toward higher Ca^{2+} concentrations. In accordance with this consideration, an increase in the resting cytoplasmic Ca^{2+} concentration caused by a low concentration of caffeine was demonstrated (131, 135, 138), and shown to be reversed by the CICR inhibitors procaine and adenine (135), although such an increase in the resting cytoplasmic concentration was not found in another report (37).

Consistent with the notion that the caffeine contracture is caused by potentiation of CICR, contracture is suppressed by inhibitors of CICR. Local anesthetics such as procaine and tetracaine have been known for many years to inhibit caffeine contracture (2, 63, 158, 230). Adenine, an inhibitor of CICR in the presence of ATP, strongly inhibits the caffeine contracture in living skeletal muscle fibers (110). Dantrolene reliably inhibits CICR at 37°C but does so only very weakly or not at all at room temperature, as described in section III C1. In fact, at 37°C, dantrolene inhibits the caffeine contracture of mouse skeletal muscle to an extent comparable to its inhibitory effect on K contracture, but at 20°C, it does not inhibit caffeine contracture, whereas its inhibitory effect on K contracture is similar to that at 37°C (134).

Even if caffeine contracture is basically understood as a consequence of CICR potentiation, some aspects of the phenomenon still remain to be elucidated. For example, the spontaneous relaxation and oscillatory behavior of the caffeine contracture of intact skeletal muscle fibers demonstrated by Lüttgau and Oetliker (158) have not been fully explained. The sarcomeric oscillation reported by Nastuk and colleagues (139, 161) in frog muscles immersed in Ringer solution containing 1 mM caffeine is also not fully understood.

3. Potentiation of voltage-activated Ca^{2+} release by caffeine

The twitch-potentiating effect of caffeine might be explained by secondary Ca^{2+} release through a caffeine-potentiated CICR mechanism, as described in section IV E. However, the shift of the relation between membrane potential and Ca^{2+} release (131, 138, 158) seems impossible to explain by means of CICR. The increase by caffeine of the rate of Ca^{2+} release at any voltage is too large (too rapid) to be explained by secondary CICR, even if it is potentiated by caffeine. This is most clear at the potential near the normal threshold. For example, Figure 2 of the report by Klein et al. (131) shows that whereas a depolarization to -50 mV evokes no detectable Ca^{2+} release without caffeine, in the presence of caffeine, this depolarization evokes a clear Ca^{2+} release, with a peak rate of $\sim 20\%$ of the maximum, which apparently cannot be explained by secondary release. Furthermore, in the E4023A mutant of RyR1, in which CICR is almost completely abolished with P_o being < 0.0001 at $100 \mu\text{M}$ Ca^{2+} and 2 mM ATP even in the presence of 2 mM caffeine, caffeine still strongly enhances voltage-activated Ca^{2+} release (195). Therefore, the only possible conclusion is that caffeine also potentiates voltage-activated Ca^{2+} release independently of CICR. This is not surprising because voltage-activated Ca^{2+} release and CICR can occur through the same RyR channel; therefore, a caffeine-induced conformational change in RyR molecules to potentiate CICR may also have a positive effect on voltage-activated Ca^{2+} release. However, the possibility that caffeine exerts its effect via a site of action on RyR molecules independent of CICR potentiation cannot be ruled out.

Although, as mentioned earlier, twitch potentiation by caffeine might be explained by secondary CICR, the effect of caffeine on voltage-activated Ca^{2+} release should also contribute at least qualitatively. The relative contributions of each of these two factors are unclear. That adenine, a CICR inhibitor in living muscle, can specifically suppress the increase in twitch tension induced by caffeine does not discriminate between the two factors, because both effects of caffeine (and of adenine) may well be the result of the binding of caffeine (or adenine) to one and the same site on RyR.

B. Malignant Hyperthermia

Unlike physiological contraction, CICR may play a crucial role in contractions in certain pathophysiological states such as malignant hyperthermia (MH).

MH is an inherited pharmacogenetic disorder triggered by volatile anesthetics, such as halothane, first reported by Denborough and Lovell (38) (for review and books, see Refs. 16, 85, 175, 200). The main manifestations of MH are a rapid and sustained rise in body temperature

that can exceed 43°C , generalized muscular contracture, and a severe metabolic acidosis, all of which might result from an increased Ca^{2+} concentration in muscle cells. If not properly treated, MH can rapidly lead to severe tissue damage and, eventually, death. A similar disorder occurs in pigs. In some cases, MH is associated with central core disease, a congenital myopathy with mutations in RyR1 (156).

Kalow et al. (119) were first to show that the skeletal muscle of patients with MH is more sensitive to the contracture-producing action of caffeine, applied either alone or with halothane. Ellis and Harriman (46) then showed that halothane can induce contracture at lower concentrations in the muscles of patients with MH than in healthy muscles. These findings led to the development of the "caffeine halothane contracture test," which is widely used to diagnose MH. Takagi et al. (258, 259) confirmed that the muscles of patients with MH are more sensitive to halothane and showed that the halothane-induced contracture is due to potentiation of CICR by the anesthetic. Because caffeine, of course, also potentiates CICR (48), these findings suggest that CICR in the muscles of humans or pigs with MH are more easily activated than are muscles of healthy individuals. Indeed, such easier activation has been demonstrated in skinned fibers from both humans and pigs: the Ca^{2+} sensitivity of CICR and the maximum rate of Ca^{2+} release at an optimal Ca^{2+} concentration are significantly higher in MH muscles (59, 120, 203). Halothane at anesthetic concentrations enhances both the Ca^{2+} sensitivity of CICR and the maximum rate of CICR at an optimal Ca^{2+} concentration, in the muscles of both MH-susceptible and healthy humans or pigs (59, 203). Other volatile anesthetics have also been shown to potentiate CICR (41, 164).

Enhancement of CICR in patients with MH or in MH-susceptible pigs has also been demonstrated in FSR (22, 67, 123, 174, 193, 201), [^3H]ryanodine binding to SR vesicles (91, 174, 267), and the single-channel activity of RyR1 (68, 174, 194, 236, 237, 238). Although some studies failed to find increased Ca^{2+} sensitivity of CICR (68, 236, 238), the maximum rate of Ca^{2+} release was increased and, therefore, the absence of the increased Ca^{2+} sensitivity does not rule out the possibility of the essential involvement of CICR in MH, as discussed below. The possibility that Mg^{2+} inhibition of Ca^{2+} release plays a crucial role in MH (41, 145, 152) can also be regarded as emphasizing an aspect of CICR.

On the basis of experimental data obtained in skinned fibers from a patient with MH and healthy subjects, the rates of CICR and Ca^{2+} uptake by SR at various concentrations of Ca^{2+} with or without halothane were calculated and compared in the normal muscles and the muscles from patients with MH (59). As shown in Figure 3, although the rates of CICR, at any but the lowest Ca^{2+} concentrations, are much lower than the Ca^{2+} uptake

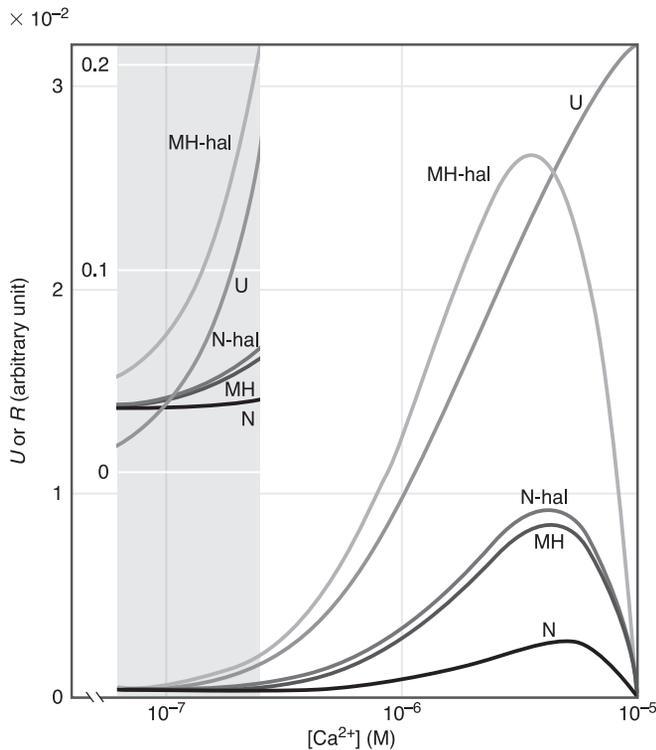


FIG. 3. Dependence of the rates of Ca^{2+} release and the rate of Ca^{2+} uptake on the free Ca^{2+} concentration in a model system. U , rate of Ca^{2+} uptake common to normal and malignant hyperthermia (MH) muscles, in the absence and presence of halothane. N and MH indicate a healthy person and a patient with MH, respectively. The suffix “-hal” indicates the presence of 0.01% (vol/vol) halothane. For further explanation, see the text. [From Endo et al. (59).]

rates in normal muscle with or without halothane and in MH muscle without halothane, in MH muscle with halothane, the rates of CICR exceed the rates of Ca^{2+} uptake at all Ca^{2+} concentrations. These results indicate that Ca^{2+} release occurs spontaneously in MH muscle with halothane but not in MH muscle without halothane or in normal muscle with or without halothane. Here, the differences in Ca^{2+} sensitivity and the maximum rate of Ca^{2+} release between MH muscles and healthy muscles were only 1.8 and 2.0 times, respectively, and the Ca^{2+} sensitivity and the maximum rate of Ca^{2+} release with halothane were both 2.0 times higher than those without halothane in muscles of both patients with MH and healthy subjects. Differences of this magnitude can clearly distinguish between MH muscles and normal muscles as well as between the presence and absence of halothane, in regard to whether muscles contract or not.

The essential point in the above theory is whether, in the physiological ionic milieu, the halothane-activated rate of CICR exceeds the rate of Ca^{2+} uptake by SR. Therefore, for example, even with a smaller increase in the Ca^{2+} sensitivity of CICR in MH muscles than in normal muscles, if the increase in the magnitude of Ca^{2+} release is sufficiently large for the Ca^{2+} release rate to

exceed the Ca^{2+} uptake rate, the MH response, i.e., halothane-induced contracture, would occur.

In the calculation of Figure 3, certain values were not experimentally obtained but were estimated on the basis of several assumptions. 1) ATP was assumed to equally influence CICR of MH and normal muscles with or without halothane. 2) The affinity of the Ca^{2+} pump for Ca^{2+} and the magnitude of the increase in the maximum rate of Ca^{2+} release by halothane were assumed to be within reasonable ranges. 3) The assumed maximum rate of Ca^{2+} pump activity relative to the Ca^{2+} leakage rate was chosen so that the resting myoplasmic Ca^{2+} concentration of normal muscle would be $0.1 \mu\text{M}$. If these assumptions are valid, Figure 3 probably explains in essence halothane-induced contracture of skeletal muscle in MH. In other words, MH could be understood in principle as a disorder of CICR.

The molecular mechanisms of the disorder of CICR to bring about MH could be diverse. Studies of large numbers of families of MH patients have demonstrated a linkage between MH and RyR1 (160, 165). Although only a single mutation of RyR1 is known to cause MH in pigs (80), many RyR1 mutations have been identified in patients with MH (117, 156, 166). These mutations are found in three restricted regions of RyR: the NH_2 -terminal, the central, and the COOH -terminal regions. In a series of studies, Ikemoto and his colleagues proposed a “domain-switch” hypothesis as follows: the NH_2 -terminal and central domains of RyR interact with each other to stabilize the channel in a “zipped” state at rest. When stimulated, the interdomain contact is weakened leading to an “un-zipped” state, which is recognized by the channel as an activation signal (101, 146, 239, 277, 278). Certain MH mutations are believed to cause partial domain unzipping and to destabilize the channel (4, 183). However, mutations in the COOH -terminal region, which is probably not directly related to the domain switch, also cause MH (17, 179, 208).

Some mutations of the $\alpha_{1\text{s}}$ -subunit of the DHPR have also been reported to cause MH (117, 178). By using dysgenic ($\alpha_{1\text{s}}$ -deficient) myotubes, $\alpha_{1\text{s}}$ has been shown to function as a negative allosteric modulator of release-channel activation by caffeine, and an MH mutation of $\alpha_{1\text{s}}$ disrupts the negative regulatory effect (272). This finding indicates that regardless of the molecular mechanisms, intramolecular for RyR1 or extramolecular as in the case of DHPR, if the CICR is sufficiently enhanced in the presence of volatile anesthetics, MH can occur.

Figure 3 further suggests that, in principle, MH might even occur in persons with normal RyR and DHPR molecules, if the activity of the Ca^{2+} pump were sufficiently impaired; however, to date no such mutation or impairment has been discovered.

VI. CONCLUDING REMARKS

CICR was first discovered in skeletal muscle, where physiological Ca^{2+} release occurs in a quasi-all-or-none manner. Yet, CICR, with its inherently regenerative nature, does not seem to be used in skeletal muscle, but instead, utilized in cardiac muscle that must regulate Ca^{2+} release in a finely graded manner to meet its physiological requirements. The low probability of opening of RyR channels in CICR in skeletal muscle under physiological conditions, together with the Ca inactivation of Ca^{2+} release, might be nature's way of avoiding unnecessary and excessive Ca^{2+} release in skeletal muscle, which requires a large amount of Ca^{2+} to function.

RyR1 channels open in multiple modes, including t-tubule voltage sensor-activated Ca^{2+} release and CICR. The details of changes in the molecular structure of RyR1 channels during different modes of opening are an important subject for future studies.

The most important subjects in the study of excitation-contraction coupling of skeletal muscle are the mechanism of coordination of multiple channels in Ca^{2+} sparks, the mechanism of communication between RyR1 and transfected RyR3 in mammalian muscles, and possible communication between RyR α and RyR β in amphibian muscles, following t-tubule depolarization, because neither appear to be CICR. These mechanisms may be the same, if Ca^{2+} sparks are the unit events in the physiological Ca^{2+} release.

ACKNOWLEDGMENTS

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