MINI-REVIEW

Pharmacology of Calcium Release from Sarcoplasmic Reticulum

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Abstract

Calcium release from sarcoplasmic reticulum (SR) has been elicited in response to additions of many different agents. Activators of Ca^{2+} release are here tentatively classified as activators of a Ca^{2+}-induced Ca^{2+} release channel preferentially localized in SR terminal or as likely activators of other Ca^{2+} efflux pathways. Some of these pathways may be associated with several different mechanisms for SR Ca^{2+} release that have been postulated previously. Studies of various inhibitors of excitation–contraction coupling and of certain forms of SR Ca^{2+} release are summarized. The sensitivity of isolated SR to certain agents is unusually affected by experimental conditions. These effects can seriously undermine attempts to anticipate effects of the same pharmacological agents *in situ*. Finally, mention is made of a new preparation ("sarcoballs") designed to make the pharmacological study of SR Ca^{2+} release more accessible to electrophysiologists, and some concluding speculations on the future of SR pharmacology are offered.

Key Words: Ca^{2+} release; drug actions; excitation–contraction coupling; pharmacology; sarcoplasmic reticulum; skeletal muscle.

Introduction

The scope of this review is quite wide and many topics are touched upon only lightly. For further in-depth study, the reader is referred to other review articles that discuss drug effects on isolated sarcoplasmic reticulum (SR) (Herbette *et al.*, 1982; Martonosi, 1984) or muscle fibers (Bianchi, 1975;

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Caputo, 1983). In view of space limitations, apologies are offered to those investigators whose work has not been cited here; the intent has been to provide more of an overview than an exhaustive summary.

Studies on cardiac and smooth muscle SR in general are not included, but work with isolated skeletal muscle SR, skinned fibers, and intact fibers is discussed. In this regard, the reader should bear in mind that it is not always clear how an effect of a drug in one experimental system would translate into effects on another. Compared with effects on a simplified system like isolated SR, skinned fibers afford additional potential sites for drug action at the level of the contractile proteins, and intact muscle transverse(T)-tubule membrane charge movements (Schneider and Chandler, 1973) as well. Agents that inhibit the SR Ca^{2+} pump could result in SR Ca^{2+} depletion and indirect inhibition of Ca^{2+} release in fiber experiments. For the sake of simplicity, we first examine drugs that have been shown to affect the SR directly, beginning with agents that cause Ca^{2+} release. However, the reader is cautioned that experimental conditions *in vitro* may be quite different from those *in situ*.

Activators of SR Ca^{2+} Release

Agents that are known to release Ca^{2+} from SR are listed in Tables I and II. Release inducers may be categorized according to which efflux pathway they activate. In this respect, the only practical differentiation at the moment is whether the substance in question activates the Ca^{2+}-induced Ca^{2+} release channel (Imagawa *et al.*, 1987; Lai *et al.*, 1988) or some other less well characterized efflux pathway. The two simplest (though not infallible[2]) means of discriminating between these possibilities involve determining whether a given release can be inhibited by the Ca^{2+}-induced Ca^{2+} release channel blocker ruthenium red (Ohnishi 1979; Smith *et al.*, 1985) and whether Ca^{2+} is released only from SR terminal cisternae, where these particular channels are localized. The agents listed at the top of Table I have not all been tested for ruthenium red sensitivity, but those that have are ruthenium red insensitive.

Many different agents induce ruthenium red-insensitive Ca^{2+} release, as do some of the mechanisms discussed below. Several different Ca^{2+} efflux

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[2]Inhibitory effects of ruthenium red on a drug's ability to release Ca^{2+} could also be explained if the drug caused a small Ca^{2+} release through a ruthenium red-insensitive pathway. This release could then trigger a Ca^{2+}-induced Ca^{2+} release of larger magnitude that would be inhibited by ruthenium red. Such a situation would involve no *direct* effect of the releasing drug on the Ca^{2+}-induced Ca^{2+} release channel.

Table I. Some Agents that Induce Ca^{2+} Release from SR

Agent	Concentrations used	References
Releasers that might not involve the Ca^{2+}-induced Ca^{2+} release channel		
Acetic, maleic anhydride	0.1–1.1 mM	Shoshan-Barmatz (1986)
ADP	0.03–2 mM	Pick and Bassilian (1982)
N-Alcohols	0.001–10 M	Hara and Kasai (1977)
Arsenate	0.5–5 mM	Hasselbach et al. (1972), Alves and De Meis (1987)
Chloride	80–300 mM	Kasai and Miyamoto (1973)
Chlorpromazine	10–100 μM	Balzer et al. (1968), Palade (1985)
Co(III)–phenanthroline–ATP	0.05–0.3 mM	Haynes and Werber (1982)
Erythrosin B, rose bengal	0.9–17 μM	Watson and Haynes (1982)
Fatty acids and derivatives		
Arachidonic acid	0.01–0.25 mM	Cheah (1981)
Oleic acid, linoleic acid	0.01–0.25 mM	Cheah (1981), Messineo et al. (1984)
Palmitylcarnitine	2.5–50 μM	Pitts et al. (1978)
Palmityl co A	0.05–50 μM	Bindoli et al. (1983)
Hydroxide	0.1–1 mM	Duggan and Martonosi (1970), Nakamura and Schwartz (1970)
Imipramine	0.1–1 mM	Worsfold and Peter (1970), Volpe et al. (1984)
Lilly 18947	0.05–1 mM	Suarez-Kurtz and Paumgartten (1973)
Mersalyl, NEM	0.7–2 mM	Martonosi and Feretos (1964), Hasselbach and Seraydarian (1966)
Methylbenzimidate	3–16 mM	Shoshan-Barmatz (1987)
Phosphotungstic acid	0.3–3 mM	Agostini and hasselbach (1971)
Prostaglandin B_x	1–15 μg/ml	Ohnishi and Devlin (1979)
Quinine, quinidine	0.1–1 mM	Worsfold and Peter (1970), Harrow and Dhalla (1976)
Sr^{2+}, Ba^{2+}	0.1–1 mM	Mermier and Hasselbach (1976), Potreau and Raymond (1980)
Uranyl acetate	2–23 mM	Agostini and Hasselbach (1971)
Releasers that might involve the Ca^{2+}-induced Ca^{2+} release channel		
adenine nucleotides	0.1–2 mM	Millman and Azari (1977), Meissner (1984)
calmidazolium, trifluoperazine	0.1–0.5 mM	Wyskovsky et al. (1988)
compound 48/80	70 μg/ml	Wyskovsky et al. (1988)
heparin	0.5–10 μg/ml	Ritov et al. (1985)
Hoechst 33258	10–100 μM	Beeler (1988)
inositol 1,4,5-trisphosphate	2–20 μM	Volpe et al. (1985)
phthalocyanine dyes	1–100 μM	Abramson et al. (1988)
quercetin	10–300 μM	Shoshan et al. (1980), Kurebayashi and Ogawa (1984)
Zn^{2+}, Hg^{2+}	1–100 μM	Carvalho (1972), Abramson et al. (1983), Chiu et al. (1983)

Table II. Activators of Ca^{2+}-Induced Ca^{2+} Release Channels

Releasing agent	Max rate of release[a] (μmol Ca/mg min) TC[c]	LSR[d]	Appox. K_i for RR[a,b] (nM)	Other References
10 mM 1 Et, 2 Me benzimidazole	3.1	0	2.9	Chapman (1979)
10 mM caffeine	4.3	0	3.0	Weber (1968), Ogawa (1970)
5 mM chloroform	1.4	0	3.8	Bianchi (1975)
				Ogawa and Kurebayashi (1982), Ohnishi *et al.* (1983), Beeler and Gable (1985)
1 mM halothane	3.5	0	9.7	
1 mM menthol	2.9	0.4	2.2	Bindoli and Fleischer (1983)
1 mM DTNB	3.2	0	7.7	Ogawa (1970), Takishima *et al.* (1979)
300 μM thymol	9.5	1.4	7.7	Shoshan *et al.* (1983)
300 μM tetraphenylboron	9.5	0.3	6.2	Watras *et al.* (1983), Shoshan and MacLennan (1981)
300 μM quercetin	7.8	0.9	12	
100 μM W-7	3.8	0.2	2.6	Wyskovsky *et al.* (1988)
100 μM ketoconazole	4.0	0.6	2.3	Cheah (1982)
100 μM clotrimazole	3.9	0.3	2.5	
30 μM ryanodine	3.5	0	11	Fairhurst and Hasselbach (1970), Meissner (1986*a*), Nelson (1987), Zorzato *et al.* (1985*a*), Harris and Doroshow (1985)
20 μM doxorubicin	1.7	0	11	
15 μM miconazole	1.5	0.4	4.5	Katz *et al.* (1977), Ohnishi (1979)
2 μM free Ca^{2+}	1.6	0	7.3	Bindoli and Fleischer (1983)
1 μM pCMB	4.7	0.2	17	Abramson *et al.* (1983), Salama and Abramson (1984)
0.5 μM AgNO$_3$	9.6	0	3.7	

[a] Data derived from Palade (1987*b*).
[b] RR, ruthenium red.
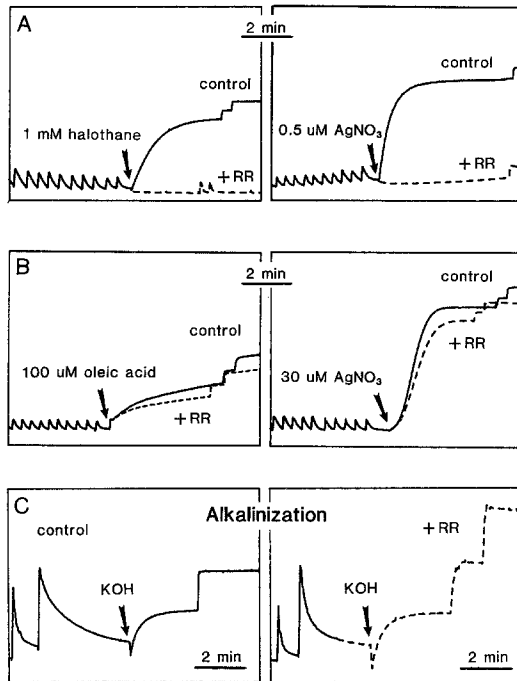[c] TC, terminal cisternae.
[d] LSR, light sarcoplasmic reticulum.

Fig. 1. Effects of certain uptake inhibitors on Ca movements across isolated SR. (A) Isolated terminal cisternae [42-μg fraction R4 (Saito *et al.*, 1984)] were preloaded with ten 12.5 nmol CaCl$_2$ additions at 35°C in a medium containing 88 mM KCl, 16 mM KMOPS, 7.5 mM Na$_4$P$_2$O$_7$, 1 mM MgATP, 5 mM Na$_2$ phosphocreatine, and 2 μg/ml creatine phosphokinase, pH 7.0, with 0.25 mM antipyrylazo III present as a Ca sensor. Absorbance readings were performed at A$_{710}$-A$_{790}$ to monitor Ca present outside of the vesicles. After each addition, the absorbance is high, but it decreases as the Ca is taken up into the vesicles. At the large arrows, 1 mM halothane or 0.5 μM AgNO$_3$ is added, resulting in significant rises in absorbance indicative of Ca release. Note that subsequent CaCl$_2$ additions reveal significant inhibition of Ca uptake ability. The experiment was also repeated with 1 μM ruthenium red (dashed traces) added after preloading to assess the contribution of Ca^{2+}-induced Ca^{2+} release channels to the releases. Upward deflections toward the end of each trace represent further 12.5 nmol CaCl$_2$ additions to recalibrate the dye in the presence of added drugs. (B) Isolated light SR [42-μg fraction R2 (Saito *et al.*, 1984)] was preloaded with CaCl$_2$ in the same manner as described above for terminal cisternae, but exposed instead to 100 μM oleic acid or to 30 μM AgNO$_3$ (arrows). Note that, in these cases, 1 μM ruthenium red had much less effect. In this same system, halothane or 0.5 μM AgNO$_3$ would not cause Ca release (not shown). The initial upward deflection that accompanies oleic acid addition is an artifact unrelated to Ca^{2+} release. (C) Isolated light SR (200 μg) was preloaded with two 12.5 nmol CaCl$_2$ additions at 25°C in 150 mM KCl, 20 mM Tris maleate, 1 mM MgATP, 0.25 mM Na$_4$P$_2$O$_7$, 5 mM Na$_2$ phosphocreatine, 20 μg/ml creatine phosphokinase, and 0.25 mM antipyrylazo III, pH 6.5. At the arrow 35 μl 0.2 M KOH was added, raising the pH to 7.5, causing an immediate downward deflection in the trace (unreleated to Ca movements) and a larger upward rise indicative of Ca release. (Right) The experiment was repeated in the presence of 300 nM ruthenium red added where the trace becomes dashed.

pathways may be involved, some possibly channels. Examples of both ruthenium red-sensitive and -insensitive releases are presented in Fig. 1. Among the releases discussed below, only those induced by inositol 1,4,5-trisphosphate (IP$_3$). Ca^{2+}, or sulfhydryl (SH) reagents have been reported to be ruthenium red sensitive.

Mechanisms of Ca^{2+} Release

Releases in response to additions of hydroxide ion (alkalinization), chloride ion (depolarization), heavy metals (SH oxidation), IP$_3$, and the Ca^{2+} ion have all been proposed for physiological roles. Fatty-acid-induced and spontaneous Ca^{2+} releases may be important under pathological conditions, particularly in the heart.

Alkalinization causes Ca^{2+} release from all SR subfractions, and in light, SR is insensitive to ruthenium red (Fig. 1; Palade, unpublished). While no pH changes large enough to trigger such release (Shoshan *et al.*, 1981) appear to occur in the bulk myoplasm (Palade and Vergara, 1982; Baylor *et al.*, 1982a), we know of no study ruling out the possibility of sufficient pH changes in the restricted diffusional space of the triadic gap, where any Ca^{2+} release is likely to be initiated.

It is difficult to study the effects of membrane potential on SR Ca^{2+} movements because V_m cannot easily be controlled or measured (Beeler *et al.*, 1981; Koshita and Hotta, 1981). Inconsistent effects of ionophores for monovalent cations (Scarpa *et al.*, 1972; Koshita and Hotta, 1981) might well depend on the ionic gradients employed affecting the V_m. The Ca^{2+} release in response to Cl$^-$ addition has alternatively been ascribed to membrane depolarization (Kasai and Miyamoto, 1976) or to osmotic artifact (Meissner and McKinley, 1976). The absence of obvious electrical coupling between SR and T-tubule (Eisenberg, 1987), the high resting permeability to other ions (Meissner, 1983), and the lack of appreciable other ion gradients across the SR membrane (Somlyo *et al.*, 1977) render SR depolarization a rather unlikely physiological mechanism for inducing Ca^{2+} release.

While fatty acids and some of their derivatives induce Ca^{2+} releases that are relatively insensitive to ruthenium red (Fig. 1), their action on the membrane at concentrations promoting moderate efflux rates might be detergent-like. At the ratio of oleate to SR used here, oleate should have disrupted the physical continuity of the bilayer (Agostini and Drabikowski, 1969). Other fatty acids effective at lower concentrations might release Ca^{2+} in a different fashion. Fatty acids are known to increase during ischemia (Katz and Messineo, 1981) and might interfere with cardiac excitation–contraction (E–C) coupling.

Spontaneous Ca^{2+} releases from isolated SR are known (Van Winkle, 1976; Katz *et al.*, 1980; Palade *et al.*, 1983), but their participation in physiological E–C coupling is unlikely unless any channels mediating such releases are capable of being gated nonspontaneously. Spontaneous Ca^{2+} release may have a more important role in cardiac tissue, particularly in cases of Ca^{2+} overload (Kort *et al.*, 1985).

Sulfhydryl oxidation has recently been hypothesized as a physiological SR Ca^{2+} release mechanism (Trimm *et al.*, 1986). While heavy metals do indeed cause Ca^{2+} release both specifically from terminal cisternae (Fig. 1; Salama and Abramson, 1984) as well as other regions of the SR (Fig. 1; Gould *et al.*, 1987), the physiological relevance of SH oxidation depends on whether any endogenous SH oxidants can cause SR Ca^{2+} release (Trimm *et al.*, 1986). Since blockers (glutathione, dithiothreitol) of such releases (Brunder *et al.*, 1988) do not appreciably affect muscle E–C coupling *in situ*, a physiological role for SH oxidation in inducing Ca^{2+} release would seem quite remote.

In numerous tissues, IP_3 has been shown to release Ca^{2+} from non-mitochondrial internal stores presumed by many to involve the endoplasmic reticulum (Berridge and Irvine, 1984). While IP_3 has been demonstrated to cause Ca^{2+} release from SR in numerous skinned fiber studies (e.g., Vergara *et al.*, 1985), there is only one report of IP_3-induced Ca^{2+} release from isolated SR (Volpe *et al.*, 1985), together with one demonstration of IP_3-enhanced SR Ca^{2+} release channel activity in bilayers (Suasez-Isla *et al.*, 1988). Interestingly, while ruthenium red appears not to inhibit IP_3-induced Ca^{2+} release in other tissues (Berridge and Irvine, 1984), this polycationic dye has been reported to inhibit IP_3-induced Ca^{2+} release from SR (Volpe *et al.*, 1985; Suarez-Isla *et al.*, 1988), despite the fact that triads capable of Ca^{2+}-induced Ca^{2+} release do not respond to IP_3 (Palade, 1987c). In contrast to the rather uncertain role of IP_3 in skeletal muscle, IP_3 is far more likely to play a role in contraction of smooth muscle in response to certain stimuli (Hashimoto *et al.*, 1986; Smith, 1986; Walker *et al.*, 1987).

Ca^{2+}-induced Ca^{2+} release is critical for heart muscle function (Fabiato and Fabiato, 1977), but it is not widely held to be the *mechanism* of E–C coupling in skeletal muscle since muscle fibers twitch for long periods of time in the absence of extracellular Ca^{2+} (Armstrong *et al.*, 1972). Nevertheless, the possibility exists that the SR Ca^{2+} channels are opened by a voltage-dependent release of "trigger" Ca^{2+} from binding sites on the cytoplasmic portion of the T-tubule membrane (Bianchi and Bolton, 1967) or that the same SR Ca^{2+} channels are opened physiologically by a different activator. At present, there is no direct evidence that SR Ca^{2+}-induced Ca^{2+} release *channels* are involved in E–C coupling, although it is generally assumed that they are, largely on the basis of their localization and their capability to

support high Ca^{2+} flux rates. More conclusive evidence might come from pharmacological studies on muscle fibers still in preliminary stages (Brunder and Palade, 1988; Baylor *et al.*, 1988).

Activators of the Ca^{2+}-Induced Ca^{2+} Release Mechanism

The agents listed at the bottom of Table I and all those listed in Table II appear to open Ca^{2+}-induced Ca^{2+} release channels, since the releases are either ruthenium red sensitive or localized at the terminal cisternae. Several of these agents exert other actions on the SR at the same time, such as Ca^{2+} pump inhibition (e.g., quercetin; Shoshan and Mac-Lennan, 1981). Among the agents listed in Table II, only 1-ethyl, 2-methyl benzimidazole, chloroform, and menthol more than doubled the control rate of ruthenium red-insensitive unidirectional ^{45}Ca efflux (Palade, 1987*b*), a possible indication of activation of other Ca^{2+} efflux pathways. It is surprising that so many chemically diverse agents all activate the same channel (Palade, 1987*b*). It suggests that the Ca^{2+}-induced Ca^{2+} release channel must have multiple ligand receptor sites that will almost certainly prove difficult to analyze.

The only Ca^{2+}-induced Ca^{2+} release activators that merit further discussion are adenine nucleotides, caffeine, halothane, and ryanodine. Adenine nucleotides like ATP are endogenously present in muscle fibers. While the presence of ATP is suggestive of a possible physiological role, ATP is unlikely to be the physiological activator because its levels do not change appreciably, even during fatigue (Nassar-Gentina *et al.*, 1978). Since Ca^{2+} is not being released in resting muscle, ATP alone is not sufficient to open enough channels in the presence of physiological $[Mg^{2+}]$ to overcome the resequestration activity of the SR Ca^{2+} pump.

Caffeine is the only SR Ca^{2+} channel activator consumed by large segments of Western society. Fortunately, the caffeine content of a cup of coffee is only $\sim 100\,mg$. Given that caffeine readily crosses membranes and should have access to all body water, the concentration achieved from one cup of coffee would be $\sim 10\,\mu M$. This is quite a bit less than the 0.1–1.0-mM concentrations required for twitch potentiation or the still higher concentrations required to elicit muscle contracture. Its effects as a stimulant are exerted at the level of the nervous system, although it is unclear whether caffeine-sensitive internal stores inside neurons are involved.

Halothane (Fig. 1) is significant because it is used clinically as an inhalation anesthetic. A large number of cases have been recorded of malignant hyperthermia in which certain genetically predisposed patients have sustained contracture under halothane anesthesia and, consequently,

experienced an uncontrolled and fatal rise in body temperature (Moulds and Denborough, 1974; Nelson and Flewellen, 1979). The symptom is also known to occur in pigs genetically predisposed to malignant hyperthermia (Nelson et al., 1972). The Ca^{2+}-induced Ca^{2+} release channels from such pigs have been conclusively implicated as mediating larger than normal Ca^{2+} releases in response to halothane (Ohnishi et al., 1983; Kim et al., 1984).

Ryanodine produces contractures in skeletal muscle, whereas in cardiac muscle it decreases the strength of contraction, producing a negative inotropic effect (Jenden and Fairhurst, 1969). Ryanodine enhances Ca^{2+} uptake by cardiac SR, presumably by blocking Ca^{2+}-induced Ca^{2+} release channels (Jones et al., 1979). Researchers were confronted with an agent that appeared to inhibit Ca^{2+} release from cardiac SR, yet caused Ca^{2+} release from skeletal SR, which discourged futher investigation until fairly recently. Then [^{3}H]ryanodine came to be used for binding studies to SR fractions (Pessah et al., 1985; Fleischer et al., 1985) and, subsequently, for solubilization, purification, and reconstitution of the Ca^{2+}-induced Ca^{2+} release channels to which it binds (Imagawa et al., 1987; Lai et al., 1988; Hymel et al., 1988). Ryanodine has thus proved a boon to the biochemical dissection of SR Ca^{2+} release. Recently the apparent discrepancy between skeletal and cardiac results has been resolved by the demonstration that a slow ryanodine-induced Ca^{2+} release from cardiac SR depletes the SR of Ca^{2+} for physiologically triggered Ca^{2+} release (Hansford and Lakatta, 1987).

Muscle Fiber Studies of Ca^{2+} Releasers

Not all of the substances that release Ca^{2+} from isolated SR have been demonstrated to cause Ca^{2+} release from SR inside muscle fibers. Among the agents listed in the Tables I and II that have been reported to cause SR Ca^{2+} release in muscle fibers, skinned or intact, are the following: quinine (Isaacson et al., 1970) quinidine (Su and Libao, 1984), N-ethylmaleimide (Kirsten and Kuperman, 1970b), parachloromercuribenzoate (Kirsten and Kuperman, 1970a), Ag^+ (Oba and Hotta, 1985), halothane (Moulds and Denborough, 1974; Su, 1980), chlorpromazine and imipramine (Andersson, 1972), Lilly 18947 (Suarez-Kurtz and Paumgartten, 1973), quercetin (Stephenson, 1985; Kurebayashi and Ogawa, 1986), caffeine (Lüttgau and Oetliker, 1968), tetraphenylboron (Shoshan et al., 1983), ryanodine (Jenden and Fairhurst, 1969), Ca^{2+} (Endo et al., 1970), IP_3 (Vergara et al., 1985), chloride (Costantin and Podolsky, 1965), and alkalinization (Shoshan et al., 1981).

A few substances have been reported to release Ca^{2+} from skinned fibers or intact muscle that have not been corroborated by isolated SR studies. These include certain protein-modifying reagents like trinitro benzene-

sulfonic acid (TNBS; Aoki *et al.*, 1986). Sr^{2+} can be taken up readily by the SR, competes with Ca^{2+} for uptake by the pump, and can cause Ca^{2+} release from isolated SR under some conditions (Mermier and Hasselbach, 1976). Ba^{2+} and Sr^{2+} entry have been reported to induce SR Ca^{2+} release in muscle fibers (Potreau and Raymond, 1980; Cognard and Raymond, 1985). However, no Ba^{2+}-induced Ca^{2+} release from isolated SR is seen under conditions where responsiveness to other releasing agents is maintained (Abramson *et al.*, 1983; Nagasaki and Kasai, 1984; Palade, 1987*a*). Ba^{2+} does not appear to be taken up by the SR or to interfere with Ca^{2+} uptake (Palade, 1987*a*), but rather seems to inhibit releases mediated by the Ca^{2+}-induced Ca^{2+} release channel (Palade, 1987*b*), which may contribute to a progressive decline in muscle fiber responsiveness (Potreau and Raymond, 1980).

The action of nicotine in inducing contractures (Weiss, 1966) has not been explored at the level of isolated SR and may instead involve Ca^{2+} entry into fibers through end-plate channels or via voltage-sensitive T-tubule Ca^{2+} channels activated by the end-plate depolarization. K^+ contracture responses also depend in part on extracellular Ca^{2+} (Stefani and Chiarandini, 1973) and are not addressed here.

Many substances have been shown to affect E–C coupling by potentiation of muscle twitches without causing contractures. While this is similar to the situation with low concentrations of caffeine (Lüttgau and Oetliker, 1968), there remain a multiplicity of possible sites of action for such agents. Twitch potentiation can result from direct effects on the SR, as with caffeine. It can also be caused by compounds that increase Ca^{2+} release by broadening the action potential or shifting the voltage dependence of the charge movements (Schneider and Chandler, 1973) believed to gate the SR Ca^{2+} channels. Diazepam (Khan and Edman, 1983) and certain heavy-metal cations are examples of twitch-potentiating agents acting by way of prolonging Ca^{2+} release by increasing action potential duration (Sandow and Isaacson, 1966), and perchlorate causes twitch potentiation by shifting the voltage dependence of charge movements (Gomolla *et al.*, 1983).

SR Ca^{2+} Uptake Inhibitors

Most of the drugs listed in Tables I and II were first identifed as Ca^{2+} uptake inhibitors and only later shown to increase Ca^{2+} efflux. Certain releasers both open channels and independently inhibit the SR Ca^{2+} ATPase activity directly (alcohols: Hara and Kasai, 1977; Ohnishi *et al.*, 1984; quercetin: Watras *et al.*, 1983; Kurebayshi and Ogawa, 1984; heavy metals: Fig. 1; Brunder *et al.*, 1988). It is also believed that certain agents can cause net Ca^{2+} release by causing the SR Ca^{2+} pump to run in reverse (e.g., ADP:

Table III. SR Ca^{2+} Uptake Inhibitors

Agent	Concentrations used	References
Ammonium molybdate	1–16 mM	Agostini and Hasselbach (1971)
Antioxidant phenols	0.1–50 μM	Sokolove et al. (1986)
Chaotropic anions	40–800 mM	The and Hasselbach (1975)
Cyclopiazonic acid	0.1–10 μM	Goeger et al. (1988)
Cytosolic proteins	25–400 μg/ml	Narayanan et al. (1983)
DFP	1 μM	Van der Kloot (1966)
Dichlorophenol–indophenol	0.15–0.6 mM	Azzone et al. (1966a)
Diethyl ether	2–10%	Inesi et al. (1967)
2,4-Dinitrophenol	0.001–5 mM	Van der Kloot (1966)
Doxorubicinol	0.5–10 μg/ml	Boucek et al. (1987)
Ergotamine, ergobasinine	10–50 μM	Azzone et al. (1986b)
Fluorescein isothiocyanate	0.5–10 μM	Pick and Karlish (1980)
Mn^{2+}	0.1 mM	Van der Kloot (1966)
Myotoxin A	0.1–1 mg/mg SR	Volpe et al. (1986)
Parathion	0.05 mM	Binder et al. (1976)
Physostigmine, neostigmine	0.05–1 mM	Van der Kloot (1966)
Propranolol, timolol	0.05–20 mM	Noack et al. (1978), Messineo and Katz (1979)
Suramin	1–40 μM	Layton and Azzi (1974)
TNBS, FDNB	0.1–2 mM	Vale and Carvalho (1980)
Tris	10–100 mM	Alonso et al. (1979)
Vanadate	0.1–0.3 mM	Pick (1982)

Hasselbach, 1978) or become uncoupled (e.g., arsenate: Hasselbach *et al.*, 1972). Other uptake inhibitors are listed in Table III. Most have not been tested for their ability to cause SR Ca^{2+} release. Vanadate and the phenolic antioxidants (Sokolove *et al.*, 1986) seem most likely to exert direct effects on the SR Ca^{2+} pump. Mn^{2+} has comparatively small inhibitory effects (Mermier and Hasselbach, 1976) and has been shown to substitute for Mg^{2+} as a cofactor for the SR Ca^{2+} pump (Chiesi and Inesi, 1980). Other agents listed may eventually prove to be releasers. In this context, TNBS (Aoki *et al.*, 1986), prenylamine (Kirsten and Lustig, 1977), and physostigime (Pagala and Sandow, 1976) have been reported to cause muscle fiber contractures, while lower concentrations of physostigimine reduced SR Ca^{2+} release *in situ* (Szucs *et al.*, 1983).

Inhibitors of Excitation–Contraction Coupling

A number of substances are known to inhibit E–C coupling in muscle. Many of these have not been tested on isolated SR, and results with several others have been equivocal. It should also be stressed that muscle fiber experiments could erroneously assign a site of action to a given agent. A Ca^{2+} uptake inhibitor or slow Ca^{2+} releaser could result in depletion of SR Ca^{2+} stores (as ryanodine does in cardiac muscle) and thus inhibit caffeine responses in a fiber. We suspect this to be the case with TMB-8.

TMB-8 has been reported to inhibit contraction produced either by direct electrical stimulation or by caffeine contracture (Malagodi and Chiou, 1974) and to inhibit caffeine-induced Ca^{2+} release from isolated SR without impairing Ca^{2+} uptake (Chiou and Malagodi, 1975). Higher concentrations have been reported to inhibit K contractures, but potentiate caffeine contractures, and to cause contractures in malignant hyperthermic pigs (Sullivan *et al.*, 1983). The sensitivity of twitches to TMB-8 appears greater than the sensitivity of caffeine contractures, implying that this compound may not be acting directly on the SR (Frank, 1986). Our own observations (Fig. 2) suggests that TMB-8 does not inhibit caffeine-induced Ca^{2+} release under conditions in which local anesthetics and polyamines are effective. Rather it seems to inhibit SR Ca^{2+} uptake in a slow time-dependent fashion (Creel and Palade, unpublished), which might explain some of the inhibition of caffeine responses in muscle fibers.

While D_2O has long been known to inhibit muscle twitches with less effect on tetanic tension (Sandow *et al.*, 1976), it clearly does not abolish SR Ca^{2+} release, as only small reductions in myoplasmic Ca^{2+} transients can be detected in its presence (Baylor *et al.*, 1982*b*). Distilled D_2O appears not to affect Ca^{2+} transport by isolated SR (Huxtable and
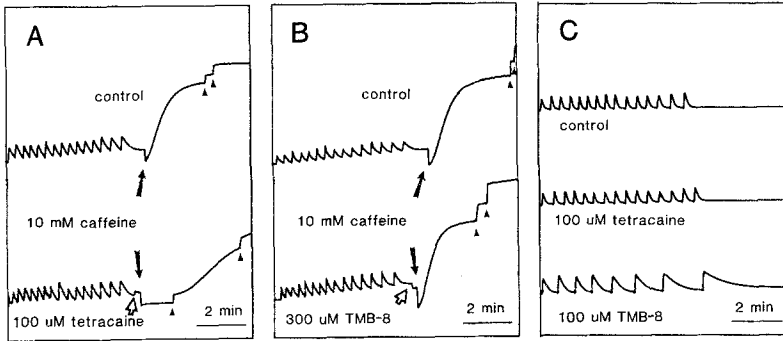
Fig. 2. Effects of TMB-8 and local anesthetics on caffeine-induced Ca^{2+} release. (A) Isolated triads [42 μg pyrophosphate variant triads (Mitchell *et al.*, 1983)] were preloaded with $CaCl_2$ as described for Fig. 1 (except with fifteen 12.5 nmol $CaCl_2$ additions) and subsequently exposed to 10 mM caffeine, generating an immediate artifactual downward deflection (unrelated to Ca movements) followed by a larger rise in the trace indicative of Ca^{2+} release. Addition of 100 μM tetracaine (open arrow) under the same conditions greatly inhibited caffeine-induced release, and further additions of Ca^{2+} (arrowheads) could only partially restore release rates. (B) Addition of 300 μM TMB-8 (open arrow) prior to caffeine neither caused release not inhibited the action of caffeine. (C) Light SR at room temperature (42 μg) was incubated with 100 μM TMB-8, 100 μM tetracaine, or neither (control) for 10 min and then Ca uptake was initiated at 23°C in the same medium used above, but with 6 mM K_2 oxalate substituted for the $Na_4P_2O_7$ in order to minimize Ca efflux. Note that only TMB-8 drastically impairs Ca pumping. These effects are much less dramatic with shorter-term exposures (not shown). Presumably, TMB-8 acts on a Ca pump or Ca efflux site situated on the lumenal SR surface while tetracaine acts on the Ca^{2+}-induced Ca^{2+} release channel at a site on the SR cytoplasmic surface.

Bressler, 1974), and more likely exhibits other sites of action (Allen *et al.*, 1984).

Formaldehyde is a very nonspecific agent that prolongs action potential durations (Dominguez and Hutter, 1969), but can abolish E–C coupling in response to membrane depolarization (Hutter, 1969). It is known to inhibit Ca^{2+} uptake by isolated SR (Sommer and Hasselbach, 1967).

Dantrolene has been shown to inhibit muscle twitches (Ellis and Bryant, 1972) and protect against malignant hyperthermia (Nelson and Flewellen, 1979). One site of action may be at the level of charge movements in the T-tubule membrane (Morgan and Bryant, 1977; Hui, 1983). Its actions on isolated SR have been examined on numerous occasions. Inhibitory effects on various forms of Ca^{2+} release have been reported by Van Winkle (1976), Francis (1978), Campbell and Shamoo (1980), Ohnishi *et al.* (1983), and Danko *et al.* (1985). Lack of effects has been reported as well (Herbette *et al.*, 1982; White *et al.*, 1983; Nelson, 1984). Evidently, experimental conditions can modulate the sensitivity of SR toward dantrolene, and dantrolene might not directly block the SR Ca^{2+} release channel itself (Danko *et al.*, 1985).

Methylenedioxyindenes have been reported to inhibit caffeine contractures (Rahwan and Gerald, 1981) and twitches (Burchfield *et al.*, 1982). These compounds do not inhibit Ca^{2+} uptake by isolated cardiac SR, nor do they inhibit a form of Ca^{2+} release induced by EGTA addition (Weishaar *et al.*, 1983).

Dihydropyridines have long been known to decrease cardiac contractility by inhibiting Ca^{2+} influx through surface membrane Ca^{2+} channels (Fleckenstein, 1983). Effects of this and other classes of surface membrane Ca^{2+} channel blockers are much less striking in skeletal muscle (Gonzales-Serratos *et al.*, 1982; McCleskey, 1985). Nevertheless, under certain conditions, skeletal muscle fibers do appear sensitive to some of these inhibitors, perhaps by interfering with the T-tubule membrane charge movements believed to gate the SR Ca^{2+} release channels (Rios and Brum, 1987). It is clear that SR Ca^{2+} release channels are distinct from the surface membrane/ T-tubule Ca^{2+} channels. Even high concentrations of nifedipine have only relatively minor effects on Ca^{2+} release from isolated SR (Palade, 1987*b*), and the agonist dihydropyridine BAY-k 8644 does not cause SR Ca^{2+} release (Zorzato *et al.*, 1985*b*). Dihydropyridines also have only modest effects on SR Ca^{2+} uptake (Colvin *et al.*, 1982).

Release Inhibitors

Release inhibitors, if specific enough for a given form of Ca^{2+} release, could be particularly useful in determining what forms of Ca^{2+} release are physiologically important. Unfortunately, most reports discuss inhibition of only one form of Ca^{2+} release, and thus inhibitor specificity is not easily assessed in most cases.

Various sugars have been shown to inhibit Cl^--induced Ca^{2+} releases from both skinned fibers and isolated SR (Endo, 1977(Martonosi, 1984). The mechanism of action of sugars in inhibiting these and other releases has been presumed to be due to osmotic shrinking of the vesicles, which would inhibit osmotic swelling and/or rupture (Martonosi, 1984). Solutions made hypertonic with sucrose additions also inhibit muscle fiber contractions. These hypertonic solution effects are due to intracellular ionic strength effects on the contractile apparatus (Gordon *et al.*, 1973), as opposed to a direct effect of the sugars.

Dithiothreitol (Trimm *et al.*, 1986) and glutathione (Brunder *et al.*, 1988) are effective inhibitors of heavy-metal-induced Ca^{2+} release. These agents are selective in inhibiting only Ca^{2+} releases induced by heavy metals. They do not inhibit releases that occur spontaneously or in response to caffeine, OH^-, or Cl^- addition (Brunder *et al.*, 1988; Palade and Dettbarn, unpublished).

Mn^{2+} has been reported to block Cl^- induced Ca^{2+} release from skinned fibers (Saida and Suzuki, 1981) and spontaneous Ca^{2+} release from isolated SR (Mitchell et al., 1984). There are no reports of its effects on other forms of Ca^{2+} release.

Dicyclohexylcarbodiimide is an effective antagonist of several different forms of Ca^{2+} release (Shoshan et al., 1981; Yamamoto and Kasai, 1982; Argaman and Shoshan-Barmatz, 1988). However, the clear lack of specificity of this carboxyl-modifying reagent limits its utility. It interacts with many different SR proteins and inhibits Ca^{2+} uptake as well as release (Argaman and Shoshan-Barmatz, 1988).

Pump Stimulators?

A stimulator of the SR Ca^{2+} pump could also reduce the rate or extent of net Ca^{2+} release. Few drugs have ever been reported to stimulate the SR Ca^{2+} pump directly, aside from phospholamban-type regulation in cardiac or in slow skeletal SR (Kirchberger et al., 1974), which is outside the scope of this review. Some reports have suggested that Ca^{2+} release inhibitors may also directly stimulate the pump (Watras, 1985; Mészáros and Ikemoto, 1985), but such demonstrations are difficult to assess (see Palade, 1987a). In any case, other explanations of their inhibitory effects on Ca^{2+} release are known. Recently, claims have been made that palmitate (Messineo et al., 1984) and gingerol (Kobayashi et al., 1987) stimulate the pump. Effects of these compounds on skeletal muscle fibers have not been reported.

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

The classic inhibitors for Ca^{2+}-induced Ca^{2+} release are local anesthetics (Johnson and Inesi, 1969) and elevated Mg^{2+} (Nagasaki and Kasai, 1981). Local anesthetics were first extensively studied in muscle fibers. Most tertiary amine anesthetics (Bianchi and Bolton, 1967) and ethylaminobenzoates like benzocaine (Freidman et al., 1974) inhibit caffeine contractures. Local anesthetics also inhibit contraction produced by depolarizing the membrane electrically (Heistracher and Hunt, 1969; Almers 1977). While local anesthetics have also been shown to inhibit a variety of Ca^{2+} releases from isolated SR (Fig. 2; Volpe et al., 1983; Antoniu et al., 1987; Palade, 1987b), relatively high concentrations are required. These agents are known to affect many other processes at these and even lower concentrations (Seeman, 1972), which decreases their utility for studies of E–C coupling. Additionally, they have been reported to inhibit a portion of charge movements (Hui, 1983; Vergara

and Caputo, 1983) and to affect contractile "inactivation" (Caputo and Bolaños, 1987), suggesting multiple sites of action on E–C coupling. At high concentrations (Johnson and Inesi, 1969) or at high pH (Bianchi, 1975), local anesthetics may even cause Ca^{2+} release from SR.

Magnesium has also been reported to inhibit Ca^{2+}-induced Ca^{2+} release from both skinned fibers (Endo, 1977) and isolated SR (Nagasaki and Kasai, 1981). Estimates of the free $[Mg^{2+}]$ in myoplasm were even used to argue against a role for Ca^{2+}-induced Ca^{2+} release in E–C coupling (Endo, 1977), although this analysis may have underestimated the stimulatory effect of adenine nucleotides (Meissner, 1984).

More recently, adenine has been shown to inhibit this nucleotide-activated channel (Ishizuka and Endo, 1983). Ba^{2+} and 9-aminoacridine also inhibit Ca^{2+}-induced Ca^{2+} release (Palade, 1987b), but probably have other actions as well. Beeler (1988) has reported effects of two structurally similar dyes, one that causes Ca^{2+} release (Hoechst 33258) and another that inhibits release (Hoechst 33342). It has also been suggested that calmodulin may inhibit Ca^{2+} releases through this channel, both from the releasing effects of many calmodulin antagonists (Chiesi and Carafoli, 1982; Hasselbach *et al.*, 1986; Wyskovsky *et al.*, 1988) as well as direct inhibitory effects of calmodulin, which do not appear to involve any calmodulin-dependent phosphorylation reaction (Meissner, 1986a; Plank *et al.*, 1988). The most widely utilized blocker of Ca^{2+}-induced Ca^{2+} release is the polycationic dye ruthenium red (Ohnishi, 1979; Antoniu *et al.*, 1985). Ruthenium red fails to inhibit other forms of Ca^{2+} release from light SR, such as those elicited by alkalinization, Cl^- addition, ADP, arsenate (Dettbarn and Palade, unpublished), high concentrations of Ag^+ (Brunder *et al.*, 1988), or spontaneously (Volpe *et al.*, 1983). Analogs such as hexammine cobalt (III) chloride and hexammine ruthenium (III) chloride are also effective blockers, though less potent (Abramson *et al.*, 1988) and possibly less specific for this particular form of Ca^{2+} release (Dettbarn and Palade, unpublished).

Quite recently our laboratory has determined that other polycationic substances are capable of inhibiting Ca^{2+} releases mediated by the Ca^{2+}-induced Ca^{2+} release channel (Palade, 1987c). The ability of certain of these substances to inhibit such Ca^{2+} release has recently been confirmed, yet other polyamines, notably protamine and spermine, were found to cause Ca^{2+} release under different experimental conditions (Cifuentes *et al.*, 1988). Similarly, ryanodine has been shown to activate or inhibit this channel, depending on the experimental conditions (Meissner, 1986b; Nelson, 1987). Our own experiments indicate that Ca^{2+} precipitation inside the SR by submillimolar concentrations of pyrophosphate can prevent protamine-induced Ca^{2+} release (Fig. 3). Pyrophosphate appears to need time, presumably to lower the free Ca^{2+} inside the SR, to exert this effect; it does not seem
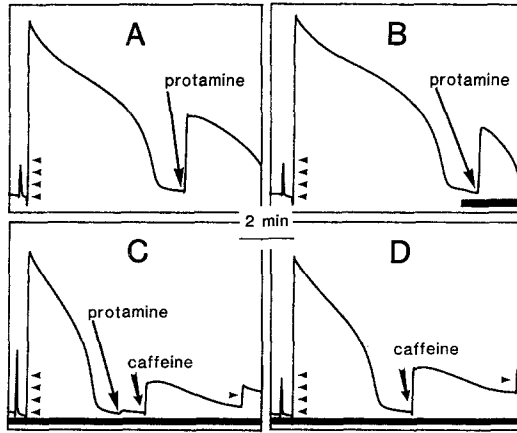
Fig. 3. Protamine effects on isolated SR depend upon whether Ca-precipitating anions are present. (A) Intermediate SR [800 μg protein, fraction R3 (Saito *et al.*, 1984)] was added to a cuvette containing 1 ml 100 mM KCl, 20 mM KMOPS, 0.25 mM antipyrylazo III, 0.5 mM MgATP, 5 mM Na$_2$ phosphocreatine, and 20 μg creatine phosphokinase, pH 6.8. After uptake of contaminating Ca^{2+} was completed, 50 nmol CaCl$_2$ was added (four arrowheads), resulting in an opening of some Ca-induced Ca release channels, which delayed uptake of Ca^{2+} until the channels became inactivated (Morii *et al.*, 1985). At the arrow, 1 μg/ml protamine was added, resulting in a transient release of Ca^{2+}. (B) The addition of 100 μM pyrophosphate at the beginning of the broad horizontal bar, after Ca has been preloaded, is unable to prevent protamine-induced Ca release. (C) The experiment was repeated with 100 μM pyrophosphate present throughout the experiment (see broad horizontal bar), demonstrating no release and only slight inhibition of subsequent caffeine-induced Ca release compared with the control trace in D.

simply to inactivate protamine outside the vesicles. Under these conditions, protamine neither causes release nor greatly inhibits caffeine-induced Ca^{2+} release. Thus, depending on the experimental conditions, protamine (and spermine) can either cause, inhibit, or do little to affect release.

We wished to determine whether polyamines could be used to block Ca^{2+}-induced Ca^{2+} release in muscle fibers in order to determine its role in E–C coupling. Using muscle fibers, we found that the sensitivity of physiological SR Ca^{2+} release to all polyamines, but particularly protamine and spermine, is much less than our isolated SR experiments (Palade, 1987c) had indicated (Fig. 4). These muscle fiber experiments with protamine and spermine seemed also to indicate relative inability either to produce Ca^{2+} release *or to inhibit effects of caffeine* (not shown). Thus, it is still quite possible that SR Ca^{2+}-induced Ca^{2+} release channels are involved in E–C coupling physiologically. Since isolated frog SR displays nearly the same responses as rabbit SR (not shown), we do not believe species differences account for the discrepancies between isolated SR and muscle fiber results. We are still trying to determine what factors modulate the polyamine sensitivity
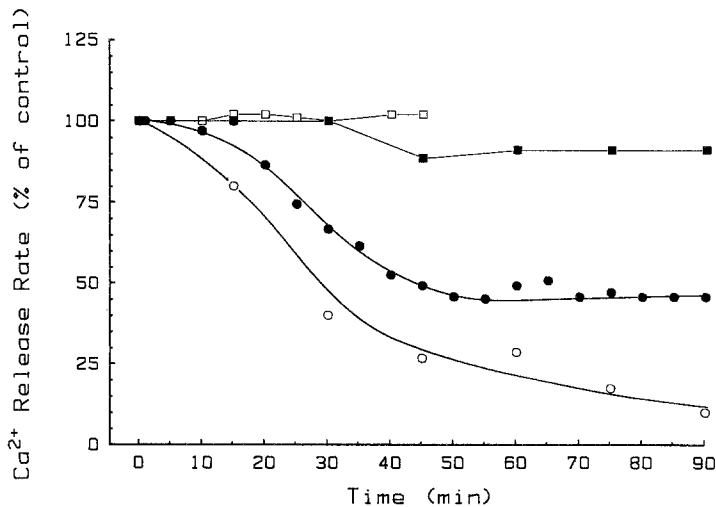
Fig. 4. Time course of drug-induced effects when polyamine inhibitors are applied to the cut ends of muscle fibers. Frog muscle fibers mounted in a triple Vaseline gap chamber (Hille and Campbell, 1976) were voltage clamped and held at $-90\,\text{mV}$ in Ringer solution ($115\,\text{mM}$ NaCl, $2.5\,\text{mM}$ KCl, $1.8\,\text{mM}$ CaCl$_2$ and $3\,\text{mM}$ MOPS, pH 7.2) containing $1\,\mu\text{M}$ tetrodotoxin to block unclamped T-tubular action potentials and possible drug effects on them. The internal solution bathing the other three pools consisted of $120\,\text{mM}$ K aspartate, $3\,\text{mM}$ MgATP, $5\,\text{mM}$ Na$_2$ phosphocreatine, $3\,\text{mM}$ Tris maleate, and $0.1\,\text{mM}$ EGTA with added Ca to buffer at pCa 7.0 and pH 7.1. At zero time, the solution bathing the E pool end was changed to one containing $200\,\mu\text{g}/\text{ml}$ protamine (filled squares), $1\,\text{mM}$ spermine (open squares), $50\,\mu\text{g}/\text{ml}$ gentamicin (filled circles), or $100\,\mu\text{M}$ neomycin (open circles). The parameter measured was the minimum stimulus duration, the shortest possible stimulus pulse to $+100\,\text{mV}$ that elicited a barely detectable fiber movement. Following arguments mentioned in Almers (1977), this minimum stimulus duration should be inversely proportional to the rate of SR Ca release.

of the Ca^{2+}-induced Ca^{2+} release channel, but our findings to date suggest a clear need for caution in attempting to extrapolate pharmacological effects on isolated SR to physiological conditions *in situ*. Evidently, such extrapolations must take into account not only other possible sites of action (SR Ca^{2+} uptake, charge movements, and contractile proteins), but also whether *in vitro* and *in situ* conditions are sufficiently similar. This makes it less likely that endogenous polyamines like spermine are physiological modulators of SR Ca^{2+} release in skeletal muscle, although they might be in cardiac muscle (Koenig *et al.*, 1988).

A New Preparation Suitable for Pharmacological Studies

Some of the difficulties that we have experienced in extrapolating between isolated SR and muscle fiber experiments have led us to consider
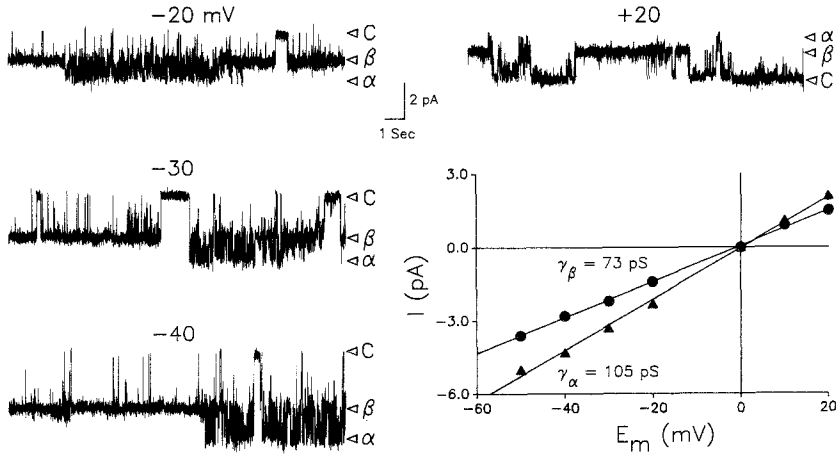
Fig. 5. Ca^{2+}-induced Ca^{2+} release channel activity recorded from an excised inside-out patch of sarcoball membrane from a mechanically skinned frog skeletal muscle fiber (Stein and Palade, 1988). The patch had 50 mM Ca(gluconate)$_2$ and 2.5 mM CaCl$_2$, pH 7.0, on both sides of the membrane, and the traces were recorded at the potentials indicated (bath = SR lumen, grounded). Note the two conductance levels labeled α and β, with full closures (C) from either level together with numerous transitions between α and β indicating that both conductance levels are mediated by the same channel protein.

alternative approaches to the study of SR Ca^{2+} release channels. Other authors in this volume (Meissner *et al.*, 1989) have described experiments involving electrical recording from SR Ca^{2+} release channels incorporated into a planar lipid bilayer. This method requires the preparation of purified vesicles as well as some electrophysiological skill. We have developed a skinned fiber preparation that should enable electrophysiologists to study SR channels more easily. The preparation enables patch clamping of exposed SR membrane ("sarcoballs") (Stein and Palade, 1988). It also avoids the needs for lengthy vesicle preparation procedures that might alter channel properties. Compared with isolated vesicle studies, it enables far easier control and assessment of effects of possible membrane potential changes.

Using sarcoballs, we have made recordings of the same caffeine, ATP, and ruthenium red-sensitive SR Ca^{2+} release channel studied by others. We have also found it to be more voltage sensitive than originally described in bilayer experiments (Smith *et al.*, 1985) and to display prominently a substate conductance (Fig. 5). Additionally, we have been studying a high-conductance chloride channel in these sarcoball membranes (Hals and Palade, 1988), and we find some similarities and yet also some salient differences with respect to SR Cl$^-$ channels incorporated into bilayers (Tanifuji *et al.*, 1987). Specifically, we see a very steeply voltage-sensitive high-conductance channel not described in bilayers (Fig. 6). We are tempted to conclude that significant
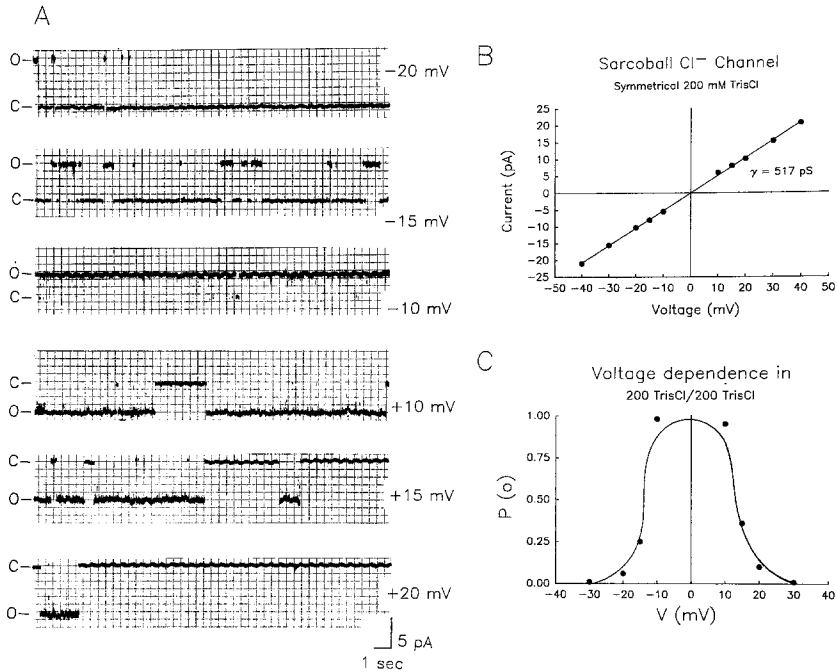
Fig. 6. Chloride channel recorded from an excised inside-out patch of sarcoball membrane. (A) The patch had 200 mM Tris Cl on both sides of the membrane and the traces were recorded at the potentials indicated. Open (O) and closed (C) current levels are indicated to the left. Note that the channel spends more time closed as the potential is moved away from 0 mV. (B) The current–voltage relationship reveals a γ of 517 pS. (C) A plot of the open-channel probability (P_0) versus voltage reveals a maximum near 0 mV.

alterations in certain channel properties may occur either during the process of preparing the isolated SR or in the process of incorporating the SR channels into the foreign lipid environment of a planar lipid bilayer. This would again suggest that any new findings on isolated SR should be checked with a more intact physiological system before any firm conclusions are drawn regarding mechanisms of action or physiological relevance.

The Future for SR Pharmacology

One of the most useful objectives of any pharmacology research is the identification of agents suitable for ligand-binding studies. This has already been accomplished with ryanodine, with the result that a sarcoplasmic reticulum Ca^{2+} release channel has been purified and reconstituted. Other agents have not yet proven as useful for furthering our understanding of E–C

coupling. Our ability to predict the future is limited, however, since five years ago few researchers could have anticipated the advances soon to be made possible by ryanodine.

The single most useful pharmacological approach to the problem of E–C coupling would involve the demonstration that the Ca^{2+}-induced Ca^{2+} release channel is in fact the one that the muscle fiber relies on. The same sort of pharmacological approach of applying specific inhibitors of other forms of Ca^{2+} release from SR could assess the roles in E–C coupling of the mechanisms and channels that they block. Finally, given how useful surface membrane Ca^{2+} channel blockers have proven in treating heart disease, it would seem quite likely that new pharmacological agents targeted at the SR could provide useful new drug therapies. In this regard, some of the agents listed here, most of limited utility to researchers, might eventually become useful therapeutic agents.

Acknowledgments

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