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The Sarcoplasmic Reticulum Ca^{2+} Channel/Ryanodine Receptor: Modulation by Endogenous Effectors, Drugs and Disease States[a]

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I. Introduction

A. The ryanodine receptor

The ryanodine receptor (RyR)[c] corresponds to the sarcoplasmic reticulum (SR) Ca^{2+} channel (Inui et al., 1987; Imagawa et al., 1987). Its structure and function have been reviewed recently (Fleischer and Inui, 1989; Lai and Meissner, 1989; McPherson and Campbell, 1993a; Sorrentino and Volpe, 1993; Coronado et al., 1994; Meissner, 1994; Ogawa, 1994; Sorrentino, 1995; Wagenknecht and Radermacher, 1995; Marks, 1996; Sutko and Airey, 1996), and only a few issues will be recalled here.

The RyR binds specifically the plant alkaloid ryanodine, which is the reason for its name. In striated muscle, RyRs are located at the triadic junctions between SR terminal cisternae and sarcolemmal T-tubules (Fleischer et al., 1985) and correspond to the "feet" structures observed in electron microscope images within the triads. However, RyRs also have been identified in SR structures that do not lie in contiguity with the sarcolemma, such as corbular and expanded junctional SR, and in intracellular membranes of other cells and tissues, such as brain, smooth muscle, endothelium, liver, and fibroblasts (Franzini-Armstrong and Jorgensen, 1994; Meissner, 1994).

The RyR has been purified, cloned, and sequenced from a variety of species, and several isoforms have been identified. Mammalian tissues express three isoforms, known as RyR1, RyR2, and RyR3. They include about 5000 (4872 to 5037) amino acid residues and are encoded by three different genes. In humans, the three genes are located on chromosomes 19, 1, and 15, respectively. RyR1 and RyR2 are expressed predominatly in skeletal muscle and in cardiac muscle, respectively (Marks et al., 1989; Takeshima et al., 1989; Nakai et al., 1990; Otsu et al., 1990; Zorzato et al., 1990). RyR3 has a wide tissue distribution (Ledbetter et al., 1994; Giannini et al., 1995), although it has been originally identified in brain (Hakamata et al., 1992) and is sometimes called "brain isoform." All three isoforms are actually expressed in brain, and the major brain isoform does not appear to be RyR3, but rather RyR2 (Witcher et al., 1992; McPherson and Campbell, 1993b; Murayama and Ogawa, 1996b). Alternative splicing variants of RyR1 and RyR2 have been identified, but their functional relevance remains to be established (Sutko and Airey, 1996). Two RyR isoforms, known as α-RyR and β-RyR, have been identified in fish, amphibian, and avian skeletal muscle (Airey et al., 1990, 1993b; Olivares et al., 1991; Sutko et al., 1991; Lai et al., 1992; Murayama and Ogawa, 1992), and they are the homologues of mammalian RyR1 and RyR3, respectively (Oyamada et al., 1994; Ottini et al., 1996). The overall identity of the RyR isoforms is of the order of 66 to 67%.

The RyR monomer has a sedimentation coefficient of about 30S and a molecular weight of about 560 kDa. The functional receptor is thought to be a homotetramer, which has a quarterfoil shape and a size of 22 to 27 nm on each side (Inui et al., 1987; Lai et al., 1988; Wagenknecht et al., 1989). The center of the quarterfoil includes a pore, with a diameter of 1 to 2 nm, that most likely represents the Ca^{2+} channel. There is structural and functional evidence that the central channel is connected to four radial channels included in the peripheral portion of each monomer (Wagenknecht et al., 1989; Ding and Kasai, 1996). Near its cytoplasmic end, the channel appears to be blocked by a mass, sometimes referred to as the "plug," that might be involved in the modulation of channel conductance. The pore region corresponds to the carboxy-terminal portion of each RyR monomer and includes, according to different suggested models, four (Takeshima et al., 1989; Nakai et al., 1990; Hakamata et al., 1992) or 10 to 12 (Zorzato et al., 1990; Otsu et al., 1990) transmembrane segments. Results obtained with site-specific antibodies (Grunwald and Meissner, 1995) support the four-transmembrane segment model, whereas cryoelectron microscopy data (Serysheva et al., 1995; Wagenknecht and Radermacher, 1995) favor the 10-transmembrane segment model. The rest of the molecule forms a large extramembrane region that corresponds to the foot structure, has a hollow appearance, and includes at least two domains in each monomer (Serysheva et al., 1995). Recent observations suggest that the channel opening is associated with a 4° rotation of the transmembrane with respect to the cytosolic region (Orlova et al., 1996).

The channel included in the RyR is a cation-selective channel with low cationic selectivity and large unitary

[c] Abbreviations: RyR, ryanodine receptor; SR, sarcoplasmic reticulum; Po, channel open probability; K_D, dissociation constant; B_{max}, binding site density; ATPase, adenosine 5′-triphosphatase; EC_{50}, concentration giving half-maximal response; IC_{50}, concentration giving half-maximal inhibition; AMP-PCP, adenosine 5′-(β,γ-methylene)triphosphate; AMP, adenosine 5′-monophosphate; cAMP, cyclic AMP; ADP, adenosine 5′-diphosphate; CTP, cytosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; ITP, inosine 5′-triphosphate; UTP, uridine 5′-triphosphate; cADPR, cyclic ADP-ribose; NAD, nicotinamide-adenine dinucleotide; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; PKC, protein kinase C; CaMK, calmodulin-dependent protein kinase II; IP_3, inositol 1,4,5 trisphosphate; MBED, 9-methyl-7-bromoeudistomin D; DTT, dithiothreitol; DTDP, dithiodipyridine; SPDP, N-succinimidyl 3-(2-pyridyldithio)propionate; TMPyP, mesotetra-(4-N-methylpyridyl)-porphine tetraiodide; W7, N-(6-aminohexyl)-5-chloro-1-naphtalene sulfonamide; bisG10, 1,10-bis-guanidino-*n*-decane; mRNA, messenger ribonucleic acid; MH, malignant hyperthermia.

conductance. With Ca^{2+} as current carrier, the maximum conductance was equal to 80 pS for the cardiac channel, and to 172 pS for the skeletal muscle channel, with a dissociation constant ≈ 3 to 4 mM (Smith et al., 1988; Lindsay and Williams, 1991). The maximum conductance was higher with monocations as current carriers, e.g., about 0.6/1 nS with Na^+ and K^+, respectively (Smith et al., 1988; Liu et al., 1989). Although in single salt solutions, channel conductance was higher for monocations than for Ca^{2+}, in mixed salts, channel permeability was higher for Ca^{2+} than for monovalent cations. This finding has been interpreted on the basis of a model in which multiple ion-binding sites are arranged sequentially, assuming that Ca^{2+} binding is favored over monocation binding. In particular, a four-barrier, three-binding-site model might account for the experimental results (Tinker et al., 1992b; Tinker and Williams, 1992, 1993c).

The RyR mediates the efflux of Ca^{2+} from the SR or other intracellular stores. In striated muscle, it has a central role in excitation-contraction coupling, i.e., in the coupling between sarcolemmal depolarization and SR Ca^{2+} release.

There are at least two mechanisms that have been proposed to mediate excitation-contraction coupling. According to the direct-coupling model, SR Ca^{2+} release is produced by a direct interaction between the dihydropyridine and the RyRs. In particular, sarcolemmal depolarization is thought to produce a conformational change in the dihydropyridine receptor that is transmitted to the RyR and induces the release of Ca^{2+} from the SR (Rios and Pizarro, 1991; Rios et al., 1993; Schneider et al., 1994). In this model, the dihydropyridine receptor acts primarily as a voltage sensor rather than as a channel, because sarcolemmal calcium influx is not required for excitation-contraction coupling. Close contiguity between the ryanodine and dihydropyridine receptors has been shown by morphological studies (Block et al., 1988) and confirmed by biochemical investigations (Marty et al., 1994), although it is still uncertain whether other proteins that are closely associated with the dihydropyridine and RyRs (see below, II.A.7.) may play a role in the coupling process.

Alternatively, excitation-contraction coupling might be mediated by a process known as Ca^{2+}-induced Ca^{2+} release (Fabiato, 1983). Because the SR channel is activated by an increase in cytosolic [Ca^{2+}] (see below, II.A.1.a.), the sarcolemmal Ca^{2+} current, although insufficient to activate the contractile process directly, could induce further release of Ca^{2+} from the SR. This process may be favored by the existence of Ca^{2+} gradients in the cytosol, because Ca^{2+} ions entering the cell through the dihydropyridine receptor seem to have preferential access to the RyR, establishing a sort of "functional coupling" (Cannell et al., 1995; Sham et al., 1995).

The relative importance of these two mechanisms is still controversial. There is evidence that in skeletal muscle, the former (i.e., direct coupling) is sufficient to induce tension development, whereas in cardiac muscle, Ca^{2+} influx is necessary for contraction, and Ca^{2+}-induced Ca^{2+} release is thought to be the dominant mechanism (Näbauer et al., 1989; Callewaert, 1992; Stern, 1992; Meissner, 1994). Consistently, morphological data suggest closer association of dihydropyridine and RyRs in skeletal muscle than in cardiac muscle (Sun et al., 1995). However, a large fraction of skeletal muscle RyRs are not associated with dihydropyridine receptors (Franzini-Armstrong and Jorgensen, 1994), and it has been suggested that Ca^{2+}-induced Ca^{2+} release might also contribute to skeletal muscle activation (Anderson and Meissner, 1995; Yano et al., 1995b; Klein et al., 1996).

It is still uncertain whether the different modes of excitation-contraction coupling are related to differences in the RyR, in the dihydropyridine receptor, or in other components. Experiments performed in dysgenic myotubes with chimeric dihydropyridine receptors suggested that specific regions of the skeletal muscle dihydropyridine receptor (included between transmembrane repeats II and III) determine the appearance of skeletal-type excitation-contraction coupling (Tanabe et al., 1990). This conclusion has not been supported by the results of another study (Lu et al., 1994), in which peptides including the putative cytoplasmic loops between transmembrane repeats II and III of skeletal and cardiac dihydropyridine receptors were expressed in *Escherichia coli*, because both types of peptides activated the skeletal but not the cardiac RyR, suggesting that the type of excitation-contraction coupling was determined by the RyR.

B. Study of Ryanodine Receptor Modulation

In recent years, the RyR has emerged as the target of pharmacological interventions, and RyR alterations have been implicated in the pathogenesis of several diseases. These issues will be the object of the present review.

A brief description of the techniques used in the study of RyR modulation may be useful. Special emphasis will be given to the assay of Ca^{2+} release, to single channel studies, and to [^{3}H]ryanodine binding experiments. Results obtained with indirect techniques will also be mentioned whenever they add valuable information.

1. Ca^{2+} release studies. In Ca^{2+} release experiments, SR vesicles are loaded with labeled or unlabeled Ca^{2+} by passive diffusion or by active uptake. Ca^{2+} release is then induced by exposing the SR to a release solution, and its kinetics are determined. If free Ca^{2+} concentration is assayed by metallochromic indicators, the increase in extravesicular Ca^{2+} concentration can be monitored after rapid mixing of the preparation with release buffer (stopped flow technique) (Yamamoto and Kasai, 1982; Kim et al., 1983; Nagasaki and Kasai, 1983; Ikemoto et al., 1989). If ^{45}Ca is used, residual vesicle radioactivity must be measured at different time points. This can be accomplished with rapid quenching (Meissner,

1984, 1988; Ikemoto et al., 1985; Meissner et al., 1986; Meissner and Henderson, 1987) or rapid filtration techniques (Dupont, 1984; Submilla and Inesi, 1987; Chiesi et al., 1988; Moutin and Dupont, 1988; Calviello and Chiesi, 1989).

The kinetics of Ca^{2+} release are interpolated by an exponential curve, so that the release rate is described by a rate constant that is independent from the extent of Ca^{2+} loading and from intravesicular Ca^{2+} concentration. Under physiological conditions, the rate constant of SR Ca^{2+} release is of the order of 20 to 60 sec^{-1}, corresponding to a half-life of 10 to 35 msec. An accurate evaluation of SR Ca^{2+} release requires, therefore, a temporal resolution in the millisecond range, which is usually achieved through automatized or semi-automatized devices.

2. Single-channel studies. SR vesicles or purified RyRs are incorporated into artificial lipid bilayers, which separate two ionic solutions. Incorporation of a channel in the bilayer is shown by the appearance of a current flowing between the two chambers (Coronado et al., 1992). The chamber to which channels are added is called *cis* chamber; the other one is called *trans* chamber. Channel incorporation is usually polar, so that the cytosolic face corresponds to the *cis* chamber. Current recordings show spontaneous openings and closures and are used to calculate the conductance of the channel and its open probability, that is, the fractional time during which the channel is open, henceforward designed as Po. Channel gating is described on the basis of mathematical models that assume the existence of one or more open state(s) and one or more closed state(s). Statistical techniques provide a detailed evaluation of channel gating. The usual approach (lifetime analysis) consists in determining the time constant of each state, which is linearly related to its mean lifetime (Ashley and Williams, 1990; Jackson, 1992). Increased current may be due to increased conductance of the open channel or to increased Po. The latter can be due either to increased lifetime of the open channel or to decreased lifetime of the closed channel, also referred to as increased frequency of channel opening.

Under physiological conditions, the behavior of the RyR has been described by models including two or three closed states and two open states (Smith et al., 1986b; Ashley and Williams, 1990). With higher temporal resolution, up to three open and five closed states have been described recently for the cardiac channel (Sitsapesan and Williams, 1994b). Further complexity has been introduced by the observation that steady-state recordings show sequences of bursts of either low or high Po, suggesting the existence of different gating modes, as already described for other ion channels (Armisen et al., 1996). Channel gating and conductance are temperature-dependent: at low temperature the conductance decreased, while the Po increased owing to increased lifetime of the open channel, and the net result was an increased Ca^{2+} current (Sitsapesan et al., 1991).

A limitation of this technique is the relatively short duration of the recordings (about 30 min), so that it may be difficult to study equilibrium effects.

3. [^{3}H]ryanodine binding. The production of radiolabeled ryanodine (Pessah et al., 1985; Sutko et al., 1986) introduced a new approach in the study of RyR structure and function. High affinity binding of [^{3}H]ryanodine to the RyR has been extensively characterized. In a variety of tissues, the dissociation constant (K_D) for [^{3}H]ryanodine was in the low nanomolar range. The Hill coefficient was $\approx$ 1, and the kinetic K_D, i.e., the ratio of the dissociation and association constants, was close to the equilibrium K_D (Pessah et al., 1985, 1986; Michalak et al., 1988; Lai et al., 1989; McGrew et al., 1989; Holmberg and Williams, 1990a; Carroll et al., 1991; Pessah and Zimanyi, 1991). High affinity [^{3}H]ryanodine binding was correlated to the functional state of the Ca^{2+} channel. Conditions that are associated with increased channel Po usually favored [^{3}H]ryanodine binding, suggesting that ryanodine binds to the open channel. However, exceptions to this rule have been described, and this issue will be further discussed in section II.C.

The RyR also shows low affinity binding sites. Pessah and Zimanyi (1991) identified four different binding sites, with K_D in the range of 1 to 4 nm, 30 to 50 nM, 500 to 800 nM and 2 to 4 μM, respectively. The Hill coefficient for the low affinity sites was < 1, suggesting that low affinity binding reflect a negative cooperative interaction between four identical sites. Consistent with this hypothesis, the association rate decreased at high [^{3}H]ryanodine concentration (Buck et al., 1992; Zucchi et al., 1995a). Complex findings were obtained with regard to ryanodine dissociation. Some investigators observed a higher dissociation rate at high [^{3}H]ryanodine concentration (Pessah and Zimanyi, 1991; Zucchi et al., 1995a), in accordance with negative cooperativity. However, the dissociation rate of nanomolar [^{3}H]ryanodine decreased in the presence of micromolar unlabeled ryanodine (McGrew et al., 1989; Lai et al., 1989; Chu et al., 1990a; Pessah and Zimanyi, 1991; Zimanyi et al., 1992). The latter finding, originally regarded as evidence of positive cooperativity (McGrew et al., 1989), has been interpreted by assuming that occupancy of low affinity sites lead to a slow, possibly irreversible change of the receptor (Pessah and Zimanyi, 1991; Zimanyi et al., 1992).

In some studies, the ratio of low-affinity to high-affinity binding sites was close to 3, in accordance with the tetrameric model of the RyR (Lai et al., 1989). However, in other reports, the ratio was substantially higher, in the range of 10 to 100 (Inui et al., 1987, 1988; McGrew et al., 1989; Pessah and Zimanyi, 1991; Buck et al., 1992; Zucchi et al., 1995a). Wang et al. (1993) obtained a ratio close to 1 using either heavy SR or purified RyR, whereas the ratio was close to 6 in a T-tubule prepara-

tion. These authors suggested that high-affinity and low-affinity binding involve different sites, and that the low-affinity site may not be exclusive to the RyR and also may exist on associated proteins.

It should be stressed that the investigations involving low affinity [^{3}H]ryanodine binding should be interpreted with great care, because an accurate determination of the binding site density (B_{max}) is technically difficult, and confidence intervals are always large.

4. Indirect studies. RyR function often has been evaluated indirectly. For instance, tension development by skinned cells or intact preparations after exposure to caffeine or after rapid cooling has been regarded as an index of SR Ca^{2+} release. Although such techniques may produce useful results, their limitations should be kept in mind. The contractile response can be affected by actions at the contractile protein level, and both caffeine and rapid cooling have multiple targets besides the RyR (Akera, 1990; Feher and Rebeyka, 1994). Similar considerations apply to the analysis of changes in intracellular Ca^{2+} concentration (Ca^{2+} transients), which are affected by other sarcolemmal or intracellular Ca^{2+} transport systems, and by Ca^{2+} binding to intracellular proteins.

Another indirect approach is the measurement of oxalate-supported Ca^{2+} uptake in the presence and in the absence of SR channel blockers. The rationale of this approach is that, because oxalate is accumulated into the SR, oxalate-supported Ca^{2+} provides a measurement of net SR Ca^{2+} uptake, even in crude preparations, and represents the difference between active Ca^{2+} transport by the Ca^{2+}-adenosine triphosphatase (ATPase) and passive Ca^{2+} efflux through the RyR (Feher and Lipford, 1985). Therefore, the stimulation of oxalate-supported Ca^{2+} uptake after RyR blockade can be considered as an index of SR Ca^{2+} efflux (Feher et al., 1989; Limbruno et al., 1989). A limitation of this approach is that the distribution of RyRs between the vesicles produced after SR fragmentation is not homogeneous (Jones and Cala, 1981; Feher and Lipford, 1985). Changes in such a distribution, possibly produced by different homogenization and fractionation procedures, or by altered physical-chemical properties of the SR membrane, might bias the results obtained with this technique. Besides, the assumption of negligibility of Ca^{2+} release by passive diffusion or by reversal of the ATPase reaction might not be justified, especially under pathological conditions.

II. Modulation of the Ryanodine Receptor

Many substances can modulate RyR function. In this section (section II.), endogenous (physiological) and exogenous (pharmacological) modulators are distinguished, and their mechanisms of action are discussed. For the sake of clarity, the former subsection also includes ions that are not physiological cell components, but whose action is related closely to that of endogenous ions. A comprehensive summary of the effects of the chief endogenous and pharmacological modulators on Ca^{2+} release, single-channel gating, and [^{3}H]ryanodine binding is provided in tables 1 and 2, respectively.

A. Endogenous Modulators

1. Ions.

a. Ca^{2+}. Ca^{2+} has major importance in the regulation of the RyR, and it is thought to be the "physiological" channel activator, because other ligands cannot activate the channel in the absence of Ca^{2+}, or they require Ca^{2+} for maximum effect. Ca^{2+} efflux studies have shown a bell-shaped relationship between Ca^{2+} release and extravesicular Ca^{2+} concentration. Ca^{2+} release was negligible at pCa < 9, reached a maximum around pCa $= 5$, and decreased at millimolar Ca^{2+} concentration (Kim et al., 1983; Kirino et al., 1983; Nagasaki and Kasai, 1983; Chamberlain et al., 1984a; Meissner, 1984; Meissner et al., 1986; Rousseau et al., 1986; Meissner and Henderson, 1987; Submilla and Inesi, 1987; Moutin and Dupont, 1988; Calviello and Chiesi, 1989; Donoso and Hidalgo, 1993). In order to explain the biphasic response to Ca^{2+}, it has been suggested that the RyR contains a high-affinity Ca^{2+} binding site, which stimulates Ca^{2+} release, and a low-affinity Ca^{2+} binding site, which inhibits Ca^{2+} release. At physiological concentrations of Mg^{2+} and adenine nucleotides, the activating action of Ca^{2+} was cooperative, with Hill coefficient close to 2 and $EC_{50} \approx 2$ μM. In the absence of Mg^{2+} and nucleotides, the EC_{50} was lower (0.5 μM), and the Hill coefficient was close to 1 (Meissner et al., 1986). The IC_{50} for Ca^{2+} inhibition of Ca^{2+} release has been estimated to be of the order of 120 to 150 μM, with a Hill coefficient > 1 (Nagasaki and Kasai, 1983; Meissner et al., 1986). Recent results have shown that the sensitivity to Ca^{2+}-induced Ca^{2+} release was about 10 times lower for RyR3 than for the other isoforms (Takeshima et al., 1995).

Indirect studies suggested that Ca^{2+} release may be modulated also by intraluminal Ca^{2+} (Ikemoto et al., 1989; Nelson and Nelson, 1990). Consistently, in rabbit SR, the rate of Ca^{2+} release increased with luminal Ca^{2+} concentration according to a hyperbolic relationship ($EC_{50} = 260$ μM), and similar results were obtained in frog SR, except that the relationship was sigmoidal (Donoso et al., 1995).

Single-channel recordings have confirmed the results of the release experiments. Channel Po was regulated by the concentration of Ca^{2+} in the *cis* chamber. The EC_{50} was in the micromolar range, so that channel Po was close to zero at nanomolar Ca^{2+} concentration, and maximum activation was observed at concentrations in excess of 100 μM. In the presence of Ca^{2+} alone, higher Po values were obtained in cardiac than in skeletal muscle, but full channel activation could not be produced in either tissue. Detailed analysis of single-channel data suggested the existence of at least two open states and two or three (in skeletal and cardiac muscle, respec-

TABLE 1
Endogenous effectors

	Ca^{2+} release	Single channel		[^{3}H]Ryanodine binding	
		γ	Po	Affinity ($1/K_D$)	B_{max}
Ca^{2+}	+[a]	0	+[a]	+[a]	+[a]
Mg^{2+}	−	0	−	−	−
H^+	−[b]	0[c]	−[b]	−[b]	−[b]
Fe^{2+}	−	nd	nd	−	
Inorganic phosphate	+[d]	0	+[d]	+[d]	0
Lactate	−	0	−	0	−
Adenine nucleotides	+	0	+	+	+
Adenosine	+[e]	0	+[e]	nd	nd
cADPR	+[f]	0	+[f]	+[f]	+[f]
Palmitoyl carnitine	+[d]	0	+[d]	(+)[d]	(+)[d]
Sphingosine	−[g]	nd	nd	−	−
Spermine	−	−	0	+[h]	0
Phosphorylation[i]					
Protein kinase A[j]	+	0	+	+	
Endogenous CaMK II	−	0	(−)	−	0
Calmodulin	+/−[k]	0	+/−[k]	+/−[k]	

Summary of the effects of endogenous effectors in Ca^{2+} release, single-channel and [^{3}H]ryanodine binding experiments. Only the most representative effectors are included in the table. In particular, substances whose action are controversial or incompletely characterized are omitted. Proteins interacting with the RyR also are omitted, except for calmodulin. See text for further details. Effects on K_D and B_{max} could not be distinguished in some cases.

[a] The dose-response curve is bell-shaped, and the activation decreases at high concentrations.

[b] At physiological pH, an acidification was inhibitory, but the dose-response curve is bell-shaped, and high pH may decrease Ca^{2+} release.

[c] Channel conductance was reduced by acidification of the *trans* chamber.

[d] The effect is probably selective for the skeletal muscle channel.

[e] The effect is probably selective for the cardiac channel.

[f] The effect of cyclic ADPR is controversial and probably it is not physiologically relevant in striated muscle (see text for a full discussion).

[g] Stimulation of Ca^{2+} release was observed at high (unphysiological) concentrations.

[h] Effect observed only at very high (unphysiological) concentrations.

[i] Only cardiac muscle effects are shown, because skeletal muscle effects are controversial.

[j] Protein kinase G and PKC had similar effects in release and binding experiments.

[k] Stimulatory effects were observed at submicromolar (< 0.1 μM) Ca^{2+}, and inhibitory effects were observed at micromolar or millimolar Ca^{2+}.

γ, channel conductance; +, increased; (+), increase has not been consistently reported; −, decreased; (−), decrease has not been consistently reported; 0, unchanged; nd, not determined.

tively) closed states of the channel. The major effect of Ca^{2+} was a decrease in the lifetime of the closed states and/or a shift from short-lived closures to long-lived closures, although increased lifetime of the open channel was occasionally observed (Rousseau et al., 1986; Smith et al., 1986b; Ma et al., 1988; Rousseau and Meissner, 1989; Ashley and Williams, 1990; Fill et al., 1990, 1991b; Lee et al., 1991; Bull and Marengo, 1993; Chu et al., 1993; Shomer et al., 1993; Ma and Zaho, 1994).

The effect of high *cis* Ca^{2+} on channel Po is still controversial. In some experiments, Po did not saturate at high Ca^{2+} concentration (Smith et al., 1986b; Ashley and Williams, 1990), whereas other investigators reported inhibition of Po by millimolar *cis* Ca^{2+}, with IC_{50} ≈ 300 to 500 μM and Hill coefficient = 1.3 (Ma et al., 1988; Fill et al., 1990, 1991b; Bull and Marengo, 1993; Shomer et al., 1993; Ma and Zaho, 1994). Chu et al. (1993) suggested that channel inhibition at high Ca^{2+} is a property of skeletal, but not of cardiac, muscle. However, Laver et al. (1995) have reported recently that Ca^{2+} can inhibit both the skeletal and the cardiac channel, although with different affinity (IC_{50} = 0.7 mM and 15 mM, respectively). Ca^{2+} inhibition was the most labile characteristic of cardiac RyR, because it was lost in the presence of 500 mM *cis* Cs^+ and after 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate solubilization. The latter observations might account for the discrepancies mentioned above. In rat brain, three different gating patterns have been identified, suggesting that they may correspond to different RyR isoforms (Marengo et al., 1996): channels with high Po, not inhibited by Ca^{2+} (up to 500 μM); channels with high Po, inhibited by Ca^{2+} with IC_{50} = 152 μM; and channels with low Po and low IC_{50} (≈ 26 μM). In frog skeletal muscle, two populations of channels have been distinguished on the basis of inhibition or lack of inhibition by millimolar Ca^{2+} (Murayama and Ogawa, 1992; Bull and Marengo, 1993). In chicken skeletal muscle, β-RyR was more sensitive to inhibition by millimolar Ca^{2+} than was α-RyR (Percival et al., 1994).

Several studies have evaluated the effect of *trans* Ca^{2+} on channel gating. High (> 100 to 200 μM) *trans* Ca^{2+} was reported to decrease channel Po (Ma et al., 1988; Fill et al., 1990), but other investigators (Sitsapesan and Williams, 1994a, 1995a) observed that an increase in luminal Ca^{2+} favored channel activation by adenine nucleotides and sulmazole, and that millimolar *trans* Ca^{2+} was needed for a maximum effect. Tripathy and Meissner (1996) have observed recently that the effect of *trans* Ca^{2+} was voltage-dependent: at negative (*cis* minus *trans*) holding potentials, submillimolar (≤ 250 μM) *trans* Ca^{2+} increased channel Po, and the effect decreased at higher concentrations. At positive holding potentials, 5 to 10 mM *trans* Ca^{2+} was needed to activate the channel. On the basis of the response to other cations and to Ca^{2+} buffers, the authors concluded that luminal Ca^{2+} modulate the Po by diffusing through the channel and interacting with Ca^{2+}- activation and Ca^{2+}-inactivation sites located on the cytosolic side.

Binding experiments have shown that high affinity [^{3}H]ryanodine binding is strictly Ca^{2+}-dependent. In

TABLE 2
Pharmacological modulators

	Ca^{2+} release	Single channel		[^{3}H]Ryanodine binding	
		γ	Po	Affinity ($1/K_D$)	B_{max}
Ryanodine	+/−[a]	−[a]	+[a]		
Methylxanthines (e.g. caffeine)	+	0	+	+	+
MBED	+	0	+	+	+
4,6-dibromo-3-hyroxycarbazole	−	nd	nd	0	0
Sulmazole	+	0	+	+	0
Anthraquinones (e.g., doxorubicin)	+/−[a]	0[a]	+/−[a]	+	0/−[a]
Digoxin	+[b]	0	+[b]	nd	nd
Milrinone	+	0	+	+	
Suramin	+	0	+	+	+
Halothane	+	0	+	0	+[bc]
Enflurane	+	0	+	0	(+)[bd]
Isoflurane	+	0	(+)	0	(+)[ed]
4-chloro-*m*-cresol	+	0	+	+	0
δ-Hexachlorocyclohexane	+	nd	nd	(+)	−
FK-506	+	−	+	0	−
Rapamycin	+	−	+	0	−
Bastadin 5	+[e]	0	+[ef]	+[e]	+[ec]
Quinolidomicin A1	+	nd	nd	+	0
Heparin	+	0	+	0	0[g]
Ruthenium red	−[a]	0	−[a]	−[a]	−[a]
Aminoglycosides (e.g., neomycin)	−[a]	0	−[a]	−[a]	−[a]
FLA365	−	0	−	−	−
Dantrolene	−	0	+/−[h]	−	0
Tetracaine	−	0[i]	−	0	−
Procaine	−	0[i]	−	−	
Lidocaine	−	nd	nd	+	0
QX 314	−	−	0	+	0
Verapamil/gallopamil[j]	−	0	−	0	0[c]
Buthotus hottentota venom	nd	−	+	+	+
Imperatoxin-a	+[e]	0	+[e]	0	+[e]
Imperatoxin-i	−	0	−	−	
Miotoxin a	+	nd	nd	0	0
Helothermine	nd	0	−	−	
Ryanotoxin	+	−	+	+	0
Thimerosal	+	0	+/−[k]	0	−
Dithiodipyridine	+	nd	nd	−[l]	
Hydrogen peroxide	+/−[a]	0[a]	+/−[a]	+	+/−[a]
TMPyP	+	0	+	+	+
Disulfonic stilbene derivatives	+	0	+	nd	nd
Dicyclohexylcarbodiimide	−	nd	nd	−	
Fluorescin-5′-isothiocyanide	nd	−	+	0	−
Diethylpyrocarbonate	+	nd	nd	0	−

Summary of the effects of pharmacological modulators in Ca^{2+} release, single-channel and [^{3}H]ryanodine binding experiments. Only the most representative modulators are included in the table. In particular, substances whose action is controversial or incompletely characterized are omitted. See text for further details. Effects on K_D and B_{max} could not be distinguished in some cases.

[a] Prolonged exposure to high concentrations determined persistent channel inactivation with reduced ryanodine binding.

[b] An effect was produced selectively in cardiac muscle.

[c] Low affinity ryanodine binding was reduced.

[d] In cardiac tissue, low-affinity binding was increased.

[e] An effect was produced selectively in skeletal muscle.

[f] Both open and closed states were stabilized, the net effect being a slight increase in channel Po.

[g] Ryanodine binding may be increased at very high heparin concentrations.

[h] Controversial findings have been reported, and the effect might be biphasic.

[i] Conductance may be reduced at high concentrations.

[j] Ca^{2+} release and single-channel results were obtained with verapamil; modulation of low-affinity binding was studied with gallopamil.

[k] Channel Po was increased at low concentrations and decreased at high concentrations.

[l] Ryanodine binding was slightly stimulated at low concentrations.

γ, channel conductance; +, increased; (+), increase has not been consistently reported; −, decreased; 0, unchanged; nd, not determined.

skeletal muscle (Pessah et al., 1985, 1987; Michalak et al., 1988; Bull et al., 1989; Chu et al., 1990a; Holmberg and Williams, 1990a; Ogawa and Harafuji, 1990a,b; Zimanyi and Pessah, 1991a; Chu et al., 1993; Shomer et al., 1993; Fruen et al., 1994a), the Ca^{2+} dependence of [^{3}H]ryanodine binding was bell-shaped, with the peak in the 10 μM to 100 μM range. Micromolar Ca^{2+} favored [^{3}H]ryanodine binding by increasing both the affinity and the B_{max}. The K_{DCa}= dissociation constant for Ca^{2+} (K_{DCa}) was in the low micromolar range (in the absence of Mg^{2+} and adenine nucleotides), and the Hill coefficient for Ca^{2+}-stimulation was close to 2, suggesting a cooperative effect of Ca^{2+} on [^{3}H]ryanodine binding. Kinetic analysis revealed that Ca^{2+} increased the rate of [^{3}H]ryanodine association, whereas the dissociation rate was not affected (Chu et al., 1990a).

Similar results have been obtained in cardiac tissue (Pessah et al., 1985; Seifert and Casida, 1986; Alderson and Feher, 1987; Anderson et al., 1989; Holmberg and Williams, 1990a; Zimanyi and Pessah, 1991a), although cardiac [^{3}H]ryanodine binding was less sensitive to inhibition by high Ca^{2+} concentrations (Michalak et al., 1988; Chu et al., 1993; Fruen et al., 1994a). In brain microsomes, the Ca^{2+} sensitivity of ryanodine binding was higher than in striated muscle, (Zimanyi and Pessah, 1991b; Padua et al., 1994), and similar results were obtained with bullfrog β-RyR, which is thought to be homologue to mammalian RyR3 (Murayama and Ogawa, 1996b). In fish skeletal muscle, the chief functional difference between the α and β isoforms of the RyR was that the latter did not show any decrease of [^{3}H]ryanodine binding at high Ca^{2+} concentration (O'Brien et al., 1995).

Ca^{2+} might also affect the equilibrium between high-affinity and low-affinity binding sites. In purified RyR1, the decrease in [^{3}H]ryanodine binding observed at nanomolar Ca^{2+} concentration was associated with a compensatory increase in low affinity binding (Lai et al., 1989), but in cardiac microsomes, Ca^{2+} had no effect on low-affinity [^{3}H]ryanodine binding (Zucchi et al., 1995a).

Several investigators have attempted to identify the molecular sites involved in Ca^{2+} binding. On the basis of RyR1 primary structure, Takeshima et al. (1989) identified three putative Ca^{2+} binding sites at residues 4253 to 4264, 4407 to 4416, and 4489 to 4499, whereas Zorzato et al. (1990) predicted that residues 1873 to 1923 included a low-affinity Ca^{2+} binding site. Immunological studies helped to identify the epitopes involved in the Ca^{2+}-dependent modulation of RyR1. Fill et al. (1991a) showed that channel Po was decreased by antibodies reacting with epitopes in the regions 4445 to 4586 and 4760 to 4877. Treves et al. (1993) confirmed that antibodies interacting with epitopes in the region 4380 to 4625 blocked the Ca^{2+}-activating domain. By using fusion proteins and sequence-specific antibodies, Chen et al. (1994) suggested that the binding site involved in channel activation was located between residues 4489 and 4499. In RyR2, high-affinity Ca^{2+} binding has been tentatively attributed to regions 1336 to 1347 and 2010 to 2021, whereas in RyR3, Ca^{2+} binding might involve residues 3934 to 3945 (Nakai et al., 1990; Hakamata et al., 1992).

The stimulation of Ca^{2+} release by micromolar Ca^{2+} is the basis of the mechanism known as Ca^{2+}-induced Ca^{2+} release (Fabiato, 1983), whereas the physiological relevance of Ca^{2+}-dependent inactivation is a controversial issue. Because Ca^{2+}-induced Ca^{2+} release is a positive-feedback process, the existence of mechanisms able to terminate Ca^{2+} release is necessary. Fabiato (1985) originally suggested that Ca^{2+} release might be limited by Ca^{2+} binding to an inactivation site characterized by higher affinity and lower association rate than the Ca^{2+}-activation site. Subsequent investigations have shown that the time course of Ca^{2+} transients might be explained by such a model only assuming an IC_{50} in the range of 0.2 to 0.8 μM (Kwok and Best, 1991; Simon et al., 1991; Delbono, 1995; Garcia and Schneider, 1995), which is much lower than the IC_{50} observed in single-channel studies and release studies.

In other ionic channels, inactivation is a voltage-dependent phenomenon. The existence of voltage-dependent inactivation of the RyR is controversial. Sitsapesan et al. (1995b) and Percival et al. (1994) observed inactivation at positive holding potential, whereas a similar phenomenon occurred at negative holding potential in the experiments performed by Ma (1995). In any case, voltage-dependent inactivation is unlikely to have any physiological relevance, because the SR potential is close to zero during Ca^{2+} release, owing to the large SR conductance to K^+.

A peculiar response to Ca^{2+}, called adaptation, has been described in studies involving transient changes in Ca^{2+} concentration. The sudden increase in Ca^{2+} concentration produced by the photolysis of caged Ca^{2+} caused, in a few milliseconds, channel activation, which was greater than observed under steady-state conditions, but was followed by a spontaneous decrease in channel Po, even if Ca^{2+} concentration remained elevated. Contrary to classical inactivation, the ability to respond to a second Ca^{2+} stimulus was preserved (Györke and Fill, 1993; Györke et al., 1994; Yasui et al., 1994). A similar response has been described with different channel activators (Dettbarn et al., 1994b), and kinetic models of adaptation have been developed (Tang and Othmer, 1994; Cheng et al., 1995; Sachs et al., 1995). The rate constant of Po decay was in the range of seconds, so that the process was too slow to account for the physiological modulation of Ca^{2+}-induced Ca^{2+} release, but it has been suggested that adaptation may be faster in vivo, due to the presence of Mg^{2+} (Valdivia et al., 1995b). However, the results obtained with the photolysis of caged Ca^{2+} have not been reproduced in bilayer experiments after quick solution exchange. Sitsapesan et al. (1995b) could not show any adaptation process, whereas Laver and Curtis (1996) observed a time-dependent decrease in Po (rate constant: 0.5 to 15 sec), but they were unable to reactivate the channel by a new Ca^{2+} stimulus.

As an alternative to inactivation and adaptation, Ca^{2+}-induced Ca^{2+} release might be limited simply by the diffusion of Ca^{2+} away from the Ca^{2+}-activation sites (Stern, 1992). Recent observations have shown that the rate constant of such a process, tentatively named deactivation, was in the millisecond range (Schiefer et al., 1995), and, therefore, it was quick enough to account for the physiological modulation of Ca^{2+}-induced Ca^{2+} release.

b. Mg^{2+}. The effect of Mg^{2+} is quite the opposite of the effect of Ca^{2+}. In release experiments, Mg^{2+} inhibited Ca^{2+} -induced Ca^{2+} release (Kim et al., 1983; Kirino et al., 1983; Nagasaki and Kasai, 1983; Chamberlain et al., 1984a; Meissner, 1984; Meissner et al., 1986; Meissner and Henderson, 1987; Rousseau et al., 1986; Submilla and Inesi, 1987; Moutin and Dupont, 1988; Calviello and Chiesi, 1989). In skeletal muscle, the IC_{50} was of the order of 20 μM at 1 μM Ca, and of 70 to 200 μM at 10 μM Ca^{2+}, and the Hill coefficient was > 1, suggesting a cooperative effect (Meissner, 1984; Meissner et al., 1986; Moutin and Dupont, 1988). Cardiac RyR was less sensitive to Mg^{2+} inhibition, because IC_{50} values in excess of 300 μM were observed at 10 μM free Ca^{2+}, with Hill coefficient $\approx$ 1.5 (Meissner and Henderson, 1987). Adenine nucleotides made the channel less sensitive to Mg^{2+} inhibition. In skeletal muscle, Mg^{2+}-inhibition of Ca^{2+} release appeared to be modulated by depolarization, which decreased (by more than 10-fold) the Mg^{2+} affinity of the RyR, or at least of the RyRs functionally

coupled to dihydropyridine receptors (Lamb and Stephenson, 1994; Ritucci and Corbett, 1995).

In single-channel experiments, millimolar Mg^{2+} reduced channel Po by increasing the lifetime of the closed channel (Rousseau et al., 1986; Smith et al., 1986a,b, 1988; Hymel et al., 1988; Lai et al., 1988, 1992; Ma et al., 1988; Anderson et al., 1989; Holmberg and Williams, 1989, 1990a; Liu et al., 1989; Ashley and Williams, 1990; Ogawa and Harafuji, 1990a; Lindsay and Williams, 1991). In addition, in cardiac muscle, Mg^{2+} accelerated the kinetic of RyR adaptation (Valdivia et al., 1995b).

Mg^{2+} inhibited ryanodine binding by reducing the B_{max} and increasing the K_D (Pessah et al., 1985, 1986, 1987; Michalak et al., 1988; Chu et al., 1990a; Zimanyi and Pessah, 1991a). The latter effect was due to a reduced association rate, while the dissociation rate was unchanged. Mg^{2+} also affected the Ca^{2+} dependence of Ca^{2+} release by shifting the activation curve to the right. Ryanodine binding studies confirmed that the sensitivity to Mg^{2+} was higher in skeletal than in cardiac muscle (Pessah et al., 1985; Seifert and Casida, 1986; Michalak et al., 1988; Zimanyi and Pessah, 1991a). For instance, with 1 nM [^{3}H]ryanodine and optimal Ca^{2+} concentration, the IC_{50} was 0.45 mM versus 2 mM (Zimanyi and Pessah, 1991a). In brain tissue, the sensitivity to Mg^{2+} was even lower than in the heart (IC_{50} = 10.4 mM) (Zimanyi and Pessah, 1991b).

On a molecular level, it has been suggested that Mg^{2+} competitively displaces Ca^{2+} from its putative stimulatory site(s). There is also evidence that Mg^{2+} may interact with the low-affinity Ca^{2+} inhibitory site (Coronado et al., 1994).

c. H^+. Ca^{2+}-induced Ca^{2+} release and [^{3}H]ryanodine binding are pH-sensitive (Meissner, 1984; Sumbilla and Inesi, 1987; Ma et al., 1988; Michalak et al., 1988; Rousseau and Pinkos, 1990; Zimanyi and Pessah, 1991b; Donoso and Hidalgo, 1993). The optimal pH was usually around 7.2 to 8.0, but higher values were occasionally reported both in release (Meissner and Henderson, 1987) and in binding studies (Valdivia et al., 1990b). In single-channel experiments, the relationship between *cis* pH and Po was more complex, with two peaks at pH = 7.2 to 7.6, and at pH = 8.5. The effect of H^+ was highly cooperative, and it was due to a shift in the relative occurrence of short-lived versus long-lived openings (Ma et al., 1988; Ma and Zaho, 1994). On the other hand, acidification of the *trans* chamber led to reduction in channel conductance (Rousseau and Pinkos, 1990).

d. OTHER CATIONS. Fe^{2+} has been reported to inhibit Ca^{2+}-induced and doxorubicin-induced Ca^{2+} release (IC_{50} = 14 to 29 μM), whereas Fe^{3+} was ineffective (Kim et al., 1995). [^{3}H]ryanodine binding was also inhibited, due to decreased sensitivity to activation by Ca^{2+}. It was suggested that Fe^{2+} may compete with Ca^{2+} at the activator site of the channel complex, whereas lipid peroxidation did not appear to be involved

As to other cations, Ba^{2+}, Cd^{2+}, and La^{2+} inhibited [^{3}H]ryanodine binding (Kirino et al., 1983; Pessah et al., 1985; Seifert and Casida, 1986). A biphasic response was observed with Ln^{3+} and Tb^{3+}, which was attributed to interaction with the Ca^{2+}-activating and Ca^{2+}-inactivating sites (Hadad et al., 1994).

e. ANIONS. Inorganic phosphate can activate the skeletal muscle RyR (Fruen et al., 1994a,b). In skeletal muscle SR, millimolar phosphate favored [^{3}H]ryanodine binding, by decreasing the K_D, while the B_{max} was unchanged (EC_{50} = 4 mM). Phosphate affected the Ca^{2+}-dependence of ryanodine binding by increasing the IC_{50} for Ca^{2+}. Release experiments and bilayer recordings confirmed that millimolar phosphate increased the rate constant of Ca^{2+} release and channel Po. As to other anions, sulfate and arsenate were ineffective, whereas thiocyanate, iodide, nitrate, and vanadate had a similar effect, suggesting that these anions interact with a common and specific anion binding site. Although Fruen et al. (1994a) reported that phosphate and related anions were ineffective in cardiac tissue, other investigators described a stimulation of Ca^{2+} release by inorganic phosphate in saponin-treated rat cardiac trabeculae (Smith and Steele, 1992)

Perchlorate is a modulator of excitation-contraction coupling. At 10 to 100 mM concentration, perchlorate-induced SR Ca^{2+} release from skeletal muscle SR, increased channel Po in bilayer experiments, and increased the affinity of ryanodine binding in a Ca^{2+}-dependent way (Gallant et al., 1993; Ma et al., 1993; Fruen et al., 1994b; Yano et al., 1995a). The response to perchlorate was not affected by the thiol-reducing agent dithiothreitol, suggesting that sulfhydryl oxidation was not involved. It has been speculated that the effect of perchlorate may be due to its chaotropic action, i.e., to dissociation of protein complexes into subunits (Ma et al., 1993). Alternatively, because the action of perchlorate was similar to that of phosphate and other anions, perchlorate might act on the putative RyR anion binding site (Fruen et al., 1994b). Whereas high concentrations of perchlorate affected the RyR directly, lower (< 10 mM) concentrations activated Ca^{2+} release in a voltage-dependent way and required the presence of complete triads. Therefore, the latter effect has been attributed to interference with T tubule/junctional SR signal transmission, and its molecular mechanism is uncertain (Yano et al., 1995a).

Lactate affected skeletal muscle RyR independently of any pH change (Favero et al., 1995b). In particular, 10 to 20 mM lactate decreased single-channel Po and ryanodine binding. Unlike other anions, lactate decreased the B_{max} and did not affect the K_D.

Cl^- can modulate SR Ca^{2+} release. According to Sukhareva et al. (1994), the rate of Ca^{2+} release was maximum when Cl^- was included both in the luminal and in the release solution, although either luminal Cl^- alone or extravesicular Cl^- alone were stimulatory. On

the other hand, Allard and Rougier (1994), who included Cl^- in the intravesicular buffer, reported inhibition of Ca^{2+} release by extravesicular Cl^-. In single-channel experiments, Cl^- did not affect Ca^{2+} fluxes, and its effects on Ca^{2+} release were attributed to activation of a nonselective Cl^- channel localized in junctional SR. This channel was blocked by ruthenium red and clofibric acid (Sukhareva et al., 1994), and its physiological role has not been established.

f. IONIC STRENGTH AND OSMOLARITY. Buffers with high ionic strength stimulate [^{3}H]ryanodine binding by increasing the B_{max}. In skeletal muscle, the B_{max} increased by more than 10-fold to 12-fold in the presence of 1 M versus 100 mM NaCl or KCl (Michalak et al., 1988; Chu et al., 1990a; Ogawa and Harafuji, 1990b; Zimanyi and Pessah, 1991a,b; Padua et al., 1994). Because sucrose was as effective as KCl or NaCl, the effect should be attributed to osmolarity rather than to ionic strength (Ogawa and Harafuji, 1990b). The stimulation of ryanodine binding produced by Ca^{2+}, caffeine, or adenine nucleotides and the inhibition produced by millimolar Ca^{2+} and Mg^{2+} was much greater at low ionic strength than at high ionic strength. The sensitivity to ionic strength may be different in different RyR isoforms: in binding experiments, bullfrog skeletal muscle β-RyR showed a higher Ca^{2+} sensitivity than α-RyR at high (1 M NaCl) but not at low (0.17 M NaCl) ionic strength (Murayama and Ogawa, 1996a). Release experiments confirmed that a medium with high ionic strength modified the permeability of the channel, and neutral molecules such as glucose could not permeate through the SR Ca^{2+} channel, even in the open state, unless submolar concentrations of KCl were present (Kasai et al., 1992; Kasai and Kawasaki, 1993).

2. Nucleotides. Adenine nucleotides activate the RyR. Ca^{2+} release studies performed in skeletal muscle (Morii and Tonomura, 1983; Nagasaki and Kasai, 1983; Meissner, 1984; Meissner et al., 1986; Sumbilla and Inesi, 1987; Moutin and Dupont, 1988; Calviello and Chiesi, 1989; Wyskovsky et al., 1990; Donoso and Hidalgo, 1993) have shown that in the presence of adenine nucleotides, Ca^{2+} release occurred, even at nanomolar Ca^{2+} concentration and/or in the presence of Mg^{2+}. The Ca^{2+}-activation curve was shifted to the left, and the maximum rate of Ca^{2+} release was increased. In fact, full activation of Ca^{2+} release required the presence of both Ca^{2+} and nucleotides. The EC_{50} for adenine nucleotides was in the millimolar range at all Ca^{2+} concentrations, and the Hill coefficient was close to 2 (Meissner et al., 1986). In cardiac muscle, the effect of adenine nucleotides was qualitatively similar, although less remarkable (Rousseau et al., 1986; Meissner and Henderson, 1987). The order of potency was adenosine 5′-(β,γ-methylene)triphosphate (AMP-PCP) > cyclic AMP (cAMP) > adenosine diphosphate (ADP) > adenosine monophosphate (AMP), while nonadenine nucleotides, such as cytosine triphosphate (CTP), guanosine triphosphate (GTP), inosine triphosphate (ITP), and uridine triphosphate (UTP) were ineffective (Morii and Tonomura, 1983; Meissner, 1984). In cardiac muscle, adenosine and adenine were also effective (Meissner, 1984), whereas in skeletal muscle, Ca^{2+} release was produced by adenine but not by adenosine (Rousseau et al., 1988).

In single-channel experiments, millimolar concentrations of adenine nucleotides increased channel Po. In particular, adenine nucleotides increased the lifetime of the open channel and decreased the lifetime of the closed channel, without affecting the conductance of the open channel. Micromolar Ca^{2+} and millimolar adenine nucleotide together elicited persistent channel opening, with Po close to 1 (Smith et al., 1985, 1986b, 1988; Hymel et al., 1988; Lai et al., 1988, 1992; Rousseau et al., 1986; Anderson et al., 1989; Holmberg and Williams, 1989, 1990a; Liu et al., 1989; Rardon et al., 1989; Ashley and Williams, 1990; Lindsay and Williams, 1991). Activation of the sheep cardiac channel was produced also by millimolar adenosine (McGarry and Williams, 1994b).

Consistently, millimolar concentrations of adenine nucleotides, diadenosine polyphosphates, and adenine favored ryanodine binding by increasing the B_{max} and decreasing the K_D, the latter effect being accounted for by an increased association rate (Pessah et al., 1987; Michalak et al., 1988; Chu et al., 1990a; Ogawa and Harafuji, 1990a,b; Zimanyi and Pessah, 1991a; Zarka and Shoshan-Barmatz, 1993; Holden et al., 1996). The Ca^{2+}-sensitivity of ryanodine binding was either unchanged or slightly increased. Binding experiments confirmed that the sensitivity to adenine nucleotides was higher in skeletal than in cardiac muscle (Michalak et al., 1988; Zimanyi and Pessah, 1991a). Peculiar results were obtained in brain, where ryanodine binding was enhanced by 1 mM ATP and inhibited by higher ATP concentrations (Zimanyi and Pessah, 1991b).

Adenine nucleotides appear to interact with a molecular site that is different from, although interacting with, the Ca^{2+}-binding and Mg^{2+}-binding site. Sequence analysis identified two putative nucleotide binding sites in RyR1 and two to four similar sites in RyR2 and RyR3. All these sites contain the nucleotide binding motif GXGXXG. Using a photoaffinity analog of ATP, Zarka and Shoshan-Barmatz (1993) labeled the putative adenine nucleotide binding site of RyR1, observing a molar ratio of 1:1 with the tetrameric RyR, but the presence of lower-affinity sites could not be definitely excluded.

3. Cyclic adenosine diphosphate-ribose. Cyclic ADP-ribose (cADPR) is an endogenous metabolite of nicotinamide-adenine dinucleotide (NAD), which is thought to act as a second-messenger in several tissues (Clapper et al., 1987; Lee et al., 1989). In sea urchin eggs, nanomolar cADPR induced Ca^{2+} release from intracellular stores. Its action was independent from inositol 1,4,5-trisphosphate, was inhibited by ruthenium red and endogenous polyamines, and was potentiated by Ca^{2+}, ryanodine,

and caffeine (Galione et al., 1991, 1993a,b; Galione and White, 1994; Lee et al., 1993; Chini et al., 1995). On the basis of these observations, it has been suggested that cADPR activates the RyR. However, other findings have questioned this conclusion. In sea urchin eggs, Ca^{2+} release showed peculiar properties, because it was dependent on the presence of calmodulin (Lee et al., 1994, 1995; Tanaka and Tashjian, 1995), and it was not activated by ATP (Graeff et al., 1995). In addition, the cADPR derivative 8-amino-cADPR antagonized cADPR-induced, but not ryanodine-induced, Ca^{2+} release (Walseth and Lee, 1993). Finally, photoaffinity labeling studies showed that cADPR binds to two proteins of 100 kDa and 140 kDa (Walseth et al., 1993), and it is not known whether such proteins interact with the RyR or rather represent a novel type of Ca^{2+} channel. It should be stressed that RyR expression has not been extensively studied in sea urchin eggs. Antibodies raised against RyR1 identified a 380-kDa protein that has not been further characterized (McPherson et al., 1992). More recently, Ca^{2+}-sensitive and caffeine-sensitive [^{3}H]ryanodine binding has been described in a preliminary report, but no modulation by cADPR and ATP has been detected (Lokuta et al., 1996).

cADPR induced intracellular Ca^{2+} release in many other tissues and cell types, including neurons, pituitary cells, pancreatic β cells, pancreatic and lacrimal acinar cells, vascular smooth muscle, heart and skeletal muscle preparations, lymphoma cells, and plant vacuoles (Koshiyama et al., 1991; Currie et al., 1992; Meszaros et al., 1993; Morrissette et al., 1993; Takasawa et al., 1993; White et al., 1993; Hua et al., 1994; Thorn et al., 1994; Allen et al., 1995; Bourguignon et al., 1995; Gromada et al., 1995; Kannan et al., 1996). Evidence of RyR modulation has been reported by some investigators. In lymphoma cells, 1 μM cADPR increased the affinity for [^{3}H]ryanodine by five-fold (Bourguignon et al., 1995). In cardiac SR, 1 to 2 μM cADPR increased [^{3}H]ryanodine binding and single-channel Po, but only at submicromolar (10 to 100 nM) Ca^{2+} concentrations. A similar action was observed in brain, but not in skeletal muscle microsomes (Meszaros et al., 1993). However, the latter results have not been confirmed by other investigators. Fruen et al. (1994c) did not observe any effect of cADPR on [^{3}H]ryanodine binding nor any change in single-channel Po, either in heart or in skeletal muscle. Sitsapesan and coworkers (1994, 1995b) reported activation of cardiac (1994) and skeletal muscle (Sitsapesan and Williams, 1995b) ryanodine-sensitive channels by $\geq$ 1 μM cADPR, but this action occurred only at high (micromolar) Ca^{2+} concentration, was shared by ADPR and β-NAD^+, and was not detected in the presence of physiological concentrations of ATP and Mg^{2+}. These authors concluded that cADPR interacts with the adenine nucleotide binding site. They also stressed that the physiological tissue concentration of cADPR, that is, 20 to 600 nM, according to Walseth et al. (1991), is too low to exert any effect, even in the absence of Mg^{2+} and ATP. Morrissette et al. (1993) also reported that 1 to 17 μM cADPR induced Ca^{2+} release from skeletal muscle SR, but no change in single-channel Po was detected. The latter finding might be explained by the low intraluminal (*trans*) Ca^{2+} concentration used in that study, because it has been shown that RyR activation by adenine nucleotides requires $>$ 40 μM *trans* Ca^{2+} (Sitsapesan and Williams, 1995a).

Therefore, although it is clear that cADPR can mobilize intracellular Ca^{2+} in many cell types, its mechanism and site of action are still poorly understood (Sitsapesan et al., 1995a). Direct action on RyR1 or RyR2 seems unlikely, at least under physiological conditions. This conclusion is supported by the recent observation that flash photolysis of caged cADPR (up to 100 μM) did not induce nor modulate SR Ca^{2+} release in cardiomyocytes, whereas it triggered Ca^{2+} release in sea urchin eggs (Guo et al., 1996a).

4. Lipid derivatives. In skeletal muscle, but not in cardiac muscle, palmitoyl carnitine and other long-chain ($>$C14) acyl carnitines induced SR Ca^{2+} release (El-Hayek et al., 1993; Dumonteil et al., 1994). The stimulation of Ca^{2+} release was slower than that produced by Ca^{2+} or ATP and had a lag of about 100 to 150 msec. Consistently, palmitoyl carnitine increased ryanodine binding at all Ca^{2+} concentrations (1 μM to 1 mM). In mammalian muscle, palmitoyl carnitine increased the B_{max} without affecting the K_D (El-Hayek et al., 1993), whereas in avian muscle, Dumonteil et al. (1994) reported increased affinity with unchanged B_{max}. In bilayer experiments, channel Po increased, due to an increased ratio of long-lived versus short-lived openings. These actions occurred at concentrations ranging from 5 to 100 μM (EC_{50} = 10 to 15 μM), and their physiological or pathophysiological implications are uncertain, because the plasma palmitoyl carnitine concentration is of the order of 2 to 4 μM, but the cytosolic concentration might be higher (Dumonteil et al., 1994).

In skeletal muscle SR, Ca^{2+} release was induced also by medium-chain (C12-C16) acyl-CoAs. Although El-Hayek et al. (1993) reported that palmitoyl-CoA was ineffective, this finding was not confirmed by other investigators (Dumonteil et al., 1994; Fulceri et al., 1994). In particular, Fulceri et al. (1994) observed that palmitoyl-CoA induced Ca^{2+} release with EC_{50} = 6 μM and increased the affinity for [^{3}H]ryanodine without affecting the B_{max}.

Free fatty acids, namely palmitic, stearic, arachidic, oleic, and linoleic acid, have been reported to induce Ca^{2+} release (Cheah, 1981; Messineo et al., 1984). However, in other studies, these results could not be reproduced (El-Hayek et al., 1993) or were attributed to reversal of the Ca^{2+}-ATPase reaction (Cardoso and De Meis, 1993). Peculiar results have been obtained with arachidonic acid. Arachidonic acid (50 μM) induced SR Ca^{2+} release in skeletal and cardiac muscle (Damron

and Bond, 1993; Dettbarn and Palade, 1993; El-Hayek et al., 1993), but its action was not inhibited by ruthenium red (Dettbarn and Palade, 1993). In a recent cardiac muscle study (Uehara et al., 1996b) arachidonic acid inhibited ryanodine binding by increasing the K_D and reducing the B_{max} ($IC_{50} \approx 12$ μM), but single-channel Po was unaffected.

Sphingosine, a long-chain amino-alcohol that is a component of sphingolipids, inhibited Ca^{2+}-induced, caffeine-induced, and doxorubicin-induced Ca^{2+} release from skeletal and cardiac SR (Sabbadini et al., 1992; Dettbarn et al., 1994a; McDonough et al., 1994). In addition, sphingosine inhibited ryanodine binding, by reducing the B_{max} and increasing the K_D. The IC_{50} was of the order of 0.5 to 1 μM in skeletal muscle, and of 2 to 4 μM in cardiac muscle. The action of sphingosine did not involve protein kinase modulation, and sphingomyelin or sphingosylphosphoryl-choline were ineffective. However, at high concentrations, both sphingosine (30 to 50 μM) and sphingosylphosphoryl-choline (10 to 75 μM) induced Ca^{2+} release in skeletal muscle and in brain microsomes (Sabbadini et al., 1992; Dettbarn et al., 1995). Such stimulatory action was only partly inhibited by ruthenium red, suggesting that it might largely represent a nonspecific effect on the lipid bilayer. Because the T-tubule membrane contains sphingomielinase, an enzyme involved in sphingosine production (Sabbadini et al., 1992), and because the average cellular concentration of free sphingosine is of the order of 0.4 μM (Dettbarn et al., 1994a), RyR modulation by sphingosine might have physiological importance.

5. Endogenous polyamines. Palade (1987c) first reported that caffeine-induced and thymol-induced Ca^{2+} release were inhibited by endogenous polyamines such as spermine, spermidine, and putrescine. The IC_{50} for spermine was in the 10 to 100 μM range, whereas spermidine and putrescine, which contain fewer amino groups, were less effective.

In single-channel experiments (Uehara et al., 1996a), spermine and other polyamines did not affect channel Po, but decreased channel conductance in a voltage-dependent way, because the block was relieved at large positive (*cis* minus *trans*) potentials. These results suggest that polyamines enter the channel and compete with current-carrying ions in the permeation pathway. Under physiological conditions (close to 0 mV), spermine EC_{50} was < 100 μM. Because endogenous spermine concentrations are in the range of 0.15 to 0.8 μmol/g wet weight (Koenig et al., 1987; Busselen, 1991; Zarka and Shoshan-Barmatz, 1992), modulation of RyR activity might have physiological relevance.

In binding studies (Zarka and Shoshan-Barmatz, 1992), endogenous polyamines increased the affinity for ryanodine, by affecting both the association and the dissociation rate, without any change in the B_{max}. However, this action occurred only at low ionic strength and at very high (unphysiological) concentrations, because the EC_{50} was 3.5 mM for spermine and 40 mM for spermidine and putrescine.

6. Phosphorylation. The RyR is the substrate of several protein kinases, namely cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), protein kinase C (PKC), and calmodulin-dependent protein kinase II (CaMK). There is evidence that junctional SR contains membrane-bound CaMK (Chu et al., 1990b).

In cardiac SR, Takasago et al. (1989, 1991) observed that PKA, PKG, and PKC incorporated about 1 mole of phosphate per mole of high-affinity ryanodine binding site, suggesting the existence of a single phosphorylation site per tetramer, which was presumably the same for the three kinases. Phosphate incorporation by CaMK involved different sites and was about four times higher, suggesting the existence of four phosphorylation sites per tetramer, although only one of such sites appeared to be available to endogenous CaMK (Witcher et al., 1991). Hohenegger and Suko (1993) and Strand et al. (1993) observed a different stoichiometry, namely about 2 moles of phosphate per tetramer with CaMK, 1 mole per tetramer with PKA, and 0.3 moles per tetramer with PKG. When comparing these results, it should be considered that differences in the phosphorylation state of the RyR in the native tissue could account, at least in part, for the reported discrepancies.

From the functional point of view, phosphorylation of cardiac RyR by PKA, PKG, or PKC favored Ca^{2+} release and produced a 15 to 25% increase in ryanodine binding (Takasago et al., 1991). In single-channel experiments, RyR2 phosphorylation by PKA increased the responsiveness of the channel to Ca^{2+} and accelerated the kinetics of adaptation (Valdivia et al., 1995b). RyR2 phosphorylation was enhanced by β-stimulation (Yoshida et al., 1992), which was associated with increased Ca^{2+} release (Patel et al., 1995). CaMK produced different effects, namely decreased affinity for ryanodine (Takasago et al., 1991; Lokuta et al., 1995) and decreased channel Po, due to reduced lifetime of the open channel and increased lifetime of the closed channel. These effects were reversed by acidic phosphatase, which was able to increase Po and ryanodine binding also in native cardiac channels, suggesting that channel phosphorylation occurred in vivo. At variance with these findings, Witcher et al. (1991) observed increased Po after treatment of cardiac RyR with CaMK. Hain et al. (1995) provided evidence that CaMK may have multiple actions. In fact, phosphorylation by PKA or by exogenous CaMK made the channel insensitive to inhibition by Mg^{2+}, whereas phosphorylation by endogenous CaMK, presumably at a different site, produced channel inhibition. The latter was reversible upon exposure to potato acid phosphatase or to protein phosphatase 1.

On the whole, phosphorylation is likely to play an important role in the physiological modulation of RyR2. The consequences of phosphorylation should be consid-

ered when interpreting the results of many in vitro studies, because the phosphorylation state of the native channel is usually unknown, but it can significantly affect channel properties as assessed in vitro.

The sites of action of the different kinases are not well characterized. On the basis of sequence analysis, up to four serine residues and two threonine residues have been identified as possible phosphorylation sites. In particular, phosphorylation of serine 2809 has been involved in channel activation (Witcher et al., 1991).

The effects of RyR1 phosphorylation are controversial. It was reported that PKA, PKG, and CaMK phosphorylated serine residue 2843 (homologous to serine 2809 of RyR2), with stoichiometry ranging from 0.3 to 0.9 moles per receptor monomer (Suko et al., 1993), and that CaMK also phosphorylated other residues, including a 60-kDa protein later identified as an isoform of phosphoglucomutase (Kim and Ikemoto, 1986; Kim et al., 1988; Leddy et al., 1993). Other investigators observed only minimal phosphorylation of RyR1 by PKA or CaMK and questioned its physiological relevance (Strand et al., 1993). Functional studies have also provided controversial results, because either channel activation (Gechtman et al., 1991; Herrmann-Frank and Varsanyi, 1993), or channel inactivation (Wang and Best, 1992) or no effect (Chu et al., 1990b) have been reported. According to Hain et al. (1994), phosphorylation by PKA or exogenous CaMK removed Mg^{2+}-inhibition, whereas phosphorylation by endogenous CaMK blocked the channel, as observed in cardiac muscle.

7. Ryanodine receptor-protein interactions.

a. DIHYDROPYRIDINE RECEPTOR. Interaction between the dihydropyridine and RyRs has major importance in excitation-contraction coupling. This issue has been dealt with above (section I.A.), and has been extensively reviewed elsewhere (Rios and Pizarro, 1991; Franzini-Armstrong and Jorgensen, 1994; Schneider, 1994).

b. CALMODULIN. Calmodulin is a ubiquitous Ca^{2+} binding protein. Seiler et al. (1984) first observed that calmodulin was associated with high molecular weight proteins in the SR, later identified as the RyR. Calmodulin inhibited Ca^{2+}-induced, caffeine-induced, and AMP-induced Ca^{2+} release from cardiac and skeletal muscle SR, with $IC_{50} \approx 0.1$ to 0.2 μM. Inhibition of Ca^{2+} release occurred only at free Ca^{2+} concentrations > 0.1 μM, suggesting that Ca^{2+}-calmodulin was the inhibitory species, but the action was observed in the absence of ATP, showing that calmodulin-dependent kinases were not involved. The inhibition was not complete, and it was reversible (Meissner, 1986a; Meissner and Henderson, 1987; Plank et al., 1988; Fuentes et al., 1994).

In single-channel experiments performed with skeletal or cardiac channels, 2 μM calmodulin reversibly decreased channel Po. The action was Ca^{2+}-dependent and ATP-independent. Whereas Smith et al. (1989) reported decreased mean open time, Fuentes et al. (1994) reported that calmodulin decreased the frequency of open events. Tripathy et al. (1995) described two effects, i.e., stabilization of the closed channel in the absence of Mg^{2+}-ATP and reduced lifetime of the open channel in the presence of Mg^{2+}-ATP.

Consistently, in brain microsomes and in skeletal muscle, [^{3}H]ryanodine binding was inhibited by calmodulin, with $IC_{50} = 0.1$ μM (McPherson and Campbell, 1993b; Fuentes et al., 1994).

More recently, a biphasic action has been described (Tripathy et al., 1995). While calmodulin inhibited the skeletal muscle channel at micromolar or millimolar Ca^{2+} concentration, at submicromolar Ca^{2+} (< 0.1 μM) concentration ryanodine binding increased ($EC_{50} = 28$ nM, with Hill coefficient close to 1), Ca^{2+} release was stimulated, and single-channel recordings showed an increased Po.

Multiple calmodulin binding sites have been described, on the basis of sequence analysis, electron microscopy imaging, and labeling studies. Two to six sites per monomer have been tentatively identified, partially overlapping with Ca^{2+}-binding sites and ruthenium red binding sites (Takeshima et al., 1989; Zorzato et al., 1990; Chen and MacLennan, 1994; Menegazzi et al., 1994; Wagenknecht et al., 1994; Yang et al., 1994; Tripathy et al., 1995). Calmodulin binding depended on the Ca^{2+} concentration: according to Tripathy et al. (1995), four calmodulin molecules per RyR monomer were bound at $Ca^{2+} < 0.1$ μM, versus 1 molecule per monomer at micromolar Ca^{2+}. Calmodulin binding was affected by the pH (maximum at 6.5 to 7.2) and ionic strength (maximum at 0.1 to 0.25 M), whereas Mg^{2+} decreased the number of high-affinity calmodulin binding sites (Yang et al., 1994). One of the calmodulin binding sites includes a sequence that can be phosphorylated by PKA, and calmodulin binding appears to inhibit this phosphorylation (Guerrini et al., 1995).

Because the intracellular concentration of calmodulin is close to 2 μM, its actions on the RyR are likely to have physiological importance.

c. FK BINDING PROTEINS. A 12-kDa FK-506 binding protein known as FKBP12 is the cytosolic receptor for the immunosuppressant drug FK-506. The complex FKBP12/FK-506 is a potent inhibitor of calcineurin, a calmodulin-dependent Ca^{2+}-activated protein phosphatase involved in the activation of T-lymphocytes. In skeletal muscle, FKBP12 is closely associated with RyR1, with a molar ratio of 4:1, suggesting that one FKBP12 molecule is associated with each RyR monomer (Collins, 1991; Jayaraman et al., 1992). In cardiac muscle, RyR2 is associated with the same molar ratio to another recently identified FK binding protein that is closely related to FKBP12 and has been named FKBP12.6 (Timerman et al., 1994; Sewell et al., 1994; Lam et al., 1995).

Observations performed after FKBP12/RyR dissociation (Timerman et al., 1993; Mayrleitner et al., 1994) and after coexpression of FKBP12 and RyR in insect cells (Brillantes et al., 1994) have shown that FKBP12

stabilizes the RyR. In particular, FKBP12 prevented the appearance of subconductance states occasionally observed with purified RyRs (Smith et al., 1988; Ma, 1993; Ma et al., 1988; Liu et al., 1989), increased the threshold to caffeine activation, and produced longer opening events after caffeine activation. Furthermore, FKBP12 blocked the flow of "reverse" (*cis* to *trans*) current, without affecting the physiological (*trans* to *cis*) current flow (Chen et al., 1994; Ma et al., 1995). The stabilizing action of FKBP12 was independent from its peptidyl prolyl isomerase activity (Timerman et al., 1995).

Cryoelectron microscopy has identified FKBPs binding sites, which are located about 10 nm from the transmembrane baseplate assembly that contains the ion channel (Wagenknecht et al., 1996).

d. CALSEQUESTRIN. Calsequestrin is the primary Ca^{2+}-binding protein of the SR. It has been suggested that calsequestrin interacts with the RyR, and, in particular, that RyR activation induces the release of Ca^{2+} from calsequestrin (Ikemoto et al., 1989, 1991; McPherson and Campbell, 1993a). Consistent with this hypothesis, calsequestrin increased the Po of single skeletal muscle channels if added to the *trans* (luminal) chamber (Kawasaki and Kasai, 1994).

e. TRIADIN. Triadin is a 95-kDa SR membrane protein that exists as a disulfide-linked homopolymer and binds the RyR with ≈ 1:1 molar ratio. There is evidence that RyR/triadin interaction is also mediated by disulfide bonds (Caswell et al., 1991; Knudson et al., 1993a,b). Triadin was initially thought to be specific for skeletal muscle, but its presence has been demonstrated subsequently also in myocardium (Brandt et al., 1993; Peng et al., 1994). Anti-triadin antibodies inhibited the slow phase of Ca^{2+} release (Brandt et al., 1992), and it has been speculated that triadin mediates the interaction between the dihydropyridine and RyRs (Brandt et al., 1990; Kim et al., 1990). Immunolabeling studies have confirmed that triadin is closely associated with dihydropyridine and RyRs since its earliest appearance during skeletal muscle development (Carl et al., 1995). It has also been proposed that triadin may provide a functional connection between RyR and calsequestin (McPherson and Campbell, 1993a; Guo and Campbell, 1995; Guo et al., 1996b).

f. OTHER PROTEINS. Other proteins are associated with the RyR. Annexin VI (Diaz-Munoz et al., 1990), a minor SR Ca^{2+}-binding protein, and S-100 (Fano et al., 1989), a cytoplasmic Ca^{2+}-binding protein, have been reported to facilitate Ca^{2+} release. Ankyrin, a cytoskeletal protein, binds to all RyR isoforms. In lymphoma cells, ankyrin inhibited [^{3}H]ryanodine binding and blocked the inhibitory effect of ryanodine on Ca^{2+} release (Bourguignon et al., 1995). Sorcin, a 22-kDa binding protein initially identified in multidrug-resistant cells, has been identified in many tissues, including the heart, where immunoelectron microscopy and immunoprecipitation techniques have shown a close association with the RyR (Meyers et al., 1995). Phosphorylation of the 150-kDa protein sarcalumenin and of the 160-kDa histidine-rich Ca^{2+}-binding protein by casein kinase II has been associated with inhibition of ryanodine binding in skeletal muscle SR (Orr and Shoshan-Barmatz, 1996; Shoshan-Barmatz et al., 1996). Protamine inhibited thymol-induced Ca^{2+} release (IC_{50} = 1.5 nM), whereas several histones had a biphasic action, because they inhibited Ca^{2+} release at low concentrations and stimulated Ca^{2+} release at higher concentrations (Palade, 1987c). Several glycolytic enzymes (glyceraldehyde 3-phosphate dehydrogenase, aldolase, and phosphoglucomutase) have been identified in heavy SR membranes, but it is still unknown whether they have any specific function at this level (Meissner, 1984).

Proteolytic enzymes affect SR function, although their physiological role, if any, is unknown. Trypsin incubation had a biphasic effect in lipid bilayer experiments: channel Po initially increased, but channel activity was subsequently lost. High-affinity ryanodine binding decreased, while low-affinity binding increased after prolonged incubation with trypsin (Chu et al., 1988; Shoshan-Barmatz and Zarka, 1988; Trimm et al., 1988; Anderson et al., 1989; Meissner et al., 1989). Calpains also affect RyR function. Calpain I is associated with skeletal muscle RyR, and its activation produced two fragments with apparent molecular mass of 375 kDa and 150 kDa (Shoshan-Barmatz et al., 1994). The proteolytic fragments remained associated, but RyR cleavage stimulated Ca^{2+} efflux, although ryanodine binding was not modified. Cleavage by calpain I was prevented by 1 to 5 mM ATP and by high NaCl concentration. Similar findings have been obtained with calpain II. In particular, Rardon et al. (1990) have observed that incubation with calpain II had no effect on ryanodine binding but increased channel Po.

8. Other endogenous modulators. The second-messenger inositol 1,4,5 trisphosphate (IP_3) can release Ca^{2+} from intracellular stores in smooth muscle, neurons, and nonexcitable cells (Berridge, 1993). After the observation that IP_3 mobilized Ca^{2+} also from skeletal and cardiac SR (reviewed by Meissner, 1994), it was speculated that IP_3 might be involved in excitation-contraction coupling, possibly by modulating the RyR (Volpe et al., 1985; Nosek et al., 1986; Suarez-Isla et al., 1988, 1991; Kentish et al., 1990; Valdivia et al., 1990a, 1992a; Borgatta et al., 1991; Chu and Stefani, 1991). IP_3 interacts with a specific receptor, a homotetramer that includes a Ca^{2+} channel and shows significant homology with the RyR (Berridge, 1993; Mikoshiba, 1993). Because IP_3 receptors have been identified in striated muscle (Marks et al., 1990; Nakagawa et al., 1991; Kijima and Fleischer, 1992; Gorza et al., 1993; Kijima et al., 1993; Moschella and Marks, 1993; Go et al., 1995; Moschella et al., 1995), it seems likely that Ca^{2+} mobilization by IP_3 is mediated by the IP_3 receptor, rather than by the RyR.

It has been reported that nitric oxide can mobilize Ca^{2+} from ryanodine-sensitive stores in pancreatic β-cells (Willmott et al., 1995). However, in skeletal muscle preparations, nitric oxide donors inhibited SR Ca^{2+} release and decreased single-channel Po, due to a reduced number of channel openings. These effects were prevented by mercaptoethanol, suggesting that sulfhydryl oxidation was involved in the response to nitric oxide (Meszaros et al., 1996).

L-Thyroxine (250 μM) increased Ca^{2+} release, ryanodine binding, and single-channel Po in skeletal muscle (Connelly et al., 1994). In addition, thyroid hormones favored the expression of RyR genes (Arai et al., 1991).

The physiological modulation of RyR gene expression has not been extensively studied. Apart from the effect of thyroid hormones, it has been observed that fibroblast growth factors inhibited the expression of RyR1 in a myogenic cell line (Marks et al., 1991), whereas in another cell line, RyR3 expression depended on transforming growth factor β (Giannini et al., 1992). Forskolin, an adenylate cyclase activator, decreased RyR1 mRNA levels in cultured myotubes, whereas PKC produced the opposite effect. Both forskolin and PKC appeared to act by modulating the stability of RyR gene transcripts rather than the transcription process itself (Ray et al., 1995). Recently, it has been reported that two novel transcription factors, designated as RYREF-1 and RYREF-2, are involved in the regulation of RyR1 gene transcription (Schmoelzl et al., 1996).

B. Pharmacological Modulators

Many exogenous substances have been reported to modulate RyR function. (for earlier reviews, see Herbette et al., 1982 and Palade et al., 1989). Their actions are often complex and/or not completely clarified. Even the basic distinction between agonists and antagonists is not easy, because several modulators may either stimulate or inhibit Ca^{2+} release, depending on concentration or incubation time. Therefore, we have decided to classify the pharmacological modulators on the basis of their chemical structure. Only compounds with reasonably well-defined structural or functional similiarity have been grouped together. A separate chapter includes agents that produce covalent modifications. In section II.C., we outline general mechanisms of RyR modulation and attempt to set a basis for functional classification. Unless otherwise specified, reference to therapeutic drug concentrations is based on Gilman et al. (1990).

1. Ryanoids. Ryanodine is an ester of pyrrole-α-carboxylic acid with ryandolol. Modulation of cardiac and skeletal muscle function by ryanodine has been known for many years (Jenden and Fairhurst, 1969). Evidence of an action on the SR Ca^{2+} release channel was first provided by the observation that ryanodine increased SR Ca^{2+} uptake without affecting active Ca^{2+} transport (Jones et al., 1979; Sutko et al., 1979). Subsequent investigations have shown that ryanodine has complex, antithetical effects on the RyR.

In release experiments (Meissner, 1986b; Hasselbach and Migala, 1987; Lattanzio et al., 1987; Carroll et al., 1991; Pessah and Zimanyi, 1991; Zimanyi and Pessah, 1991a; Kasai and Kawasaki, 1993), ryanodine favored Ca^{2+} release and allowed it to proceed even in the presence of a nonrelease medium. Cardiac muscle was more sensitive than skeletal muscle to ryanodine activation, and the EC_{50} was inversely related to free Ca^{2+} concentration, ranging from 40 nM to 50 μM. While the immediate effect of ryanodine was always channel activation, prolonged incubation with micromolar ryanodine eventually inhibited Ca^{2+} release. The incubation time required for inhibition to develop was inversely related to temperature and to ryanodine concentration, e.g., 1 h with 1 μM ryanodine or 5 to 10 min with 500 μM ryanodine. Channel blockade by micromolar ryanodine was not reversible, at least on the time scale of release experiments.

Single-channel recordings have provided a more direct insight into the action of ryanodine (Rousseau et al., 1987; Hymel et al., 1988; Anderson et al., 1989; Bull et al., 1989; Holmberg and Williams, 1989, 1990a; Lai et al., 1989; Chu et al., 1990a; Lindsay and Williams, 1991; Buck et al., 1992; Lindsay et al., 1994). At micromolar concentration, ryanodine locked the channel open, but in a state of subnormal conductance, corresponding to about 40 to 60% of the conductance of the open channel. The modified state was characterized by a remarkable (more than 20-fold) increase in open lifetime, associated with decreased closed lifetime. Contrary to the normal channel, the Po of the modified channel was voltage-dependent, and the relationship between Po and membrane potential was bell-shaped (Ma, 1993). This modified state was insensitive to activation by Ca^{2+} and ATP and was less sensitive than the normal channel to Mg^{2+} inhibition, H^+ inhibition, and ruthenium red inhibition. At higher ryanodine concentrations (in the millimolar range), such an action was followed by persistent channel blockade. Occasionally, further subnormal conductance states were transiently observed before complete blockade. Bull et al. (1989) and Buck et al. (1992) described multiple effects of increasing concentrations of ryanodine: 5 to 40 nM ryanodine increased channel Po and induced the occasional appearance of a subnormal state with $\approx$ ½ of the basal conductance; $>$ 50 nM ryanodine stabilized the channel in the one-half-conductance state that was not readily reversible; $>$ 70 μM ryanodine induced a transition to a lower conductance ($\approx$ 1/4 of the basal value), and $>$ 200 μM ryanodine caused complete blockade, which was not reversible on the time scale of the recordings.

The characteristics of ryanodine binding have been described above (section I.B.3.). The current interpretation of these results (Meissner, 1986b; Chu et al., 1990a; Carroll et al., 1991; Pessah and Zimanyi, 1991; Buck et

al., 1992) is that the interaction of ryanodine with its high-affinity site stabilizes the open state of the channel, but the conductance of the open channel is subnormal, possibly due to partial occlusion of the channel lumen by the ryanodine molecule. At higher concentrations, ryanodine binds to low-affinity sites, which causes further reduction in channel conductance, up to complete blockade. Although this issue has not been definitely established, low-affinity binding is probably due to negative cooperativity between identical binding sites of different monomers. After occupation of the low-affinity sites, the channel undergoes a slow transition to a state characterized by persistent channel inactivation, which is associated with decreased ryanodine binding. The latter effect is either irreversible or very slowly reversible, because prolonged incubation with > 100 nM ryanodine produced the complete loss of low-affinity binding and a concentration-dependent decrease in high-affinity binding that persisted for at least 48 h (Mack et al., 1992; Zimanyi et al., 1992). The decrease in ryanodine binding observed after prolonged exposure to micromolar ryanodine might be due to sulfhydryl oxidation, because it was prevented by the thiol reducing agent dithiothreitol. Covalent labeling of the RyR with a photo-activatable derivative of ryanodine suggested that channel inactivation was associated with the development of stable, virtually irreversible interactions between receptor monomers (Bidasee et al., 1995).

The comparison of single-channel and [^{3}H]ryanodine binding experiments reveals that the concentrations required to produce corresponding effects (e.g., stabilization of a $\approx 50\%$ subconductance state and high-affinity binding) were significantly higher in the former. The usual explanation of this discrepancy is that single-channel experiments are performed under nonequilibrium conditions, owing to the relatively short duration of the recordings, which leads to the use of high ryanodine concentrations to produce an immediate response. However, it cannot be excluded that RyR incorporation into lipid bilayers may cause the loss of proteins that modulate the response to ryanodine.

Indirect evidence suggested that ryanodine may also inhibit the transition between low-affinity and high-affinity states of an intracellular Ca^{2+} compartment, possibly calsequestrin (Gilchrist et al., 1992). However, in other indirect studies, no evidence of an intra-SR action of ryanodine was observed (Nelson and Nelson, 1990).

Ryanodine produced opposite functional effects in heart versus skeletal muscle (Jenden and Fairhurst, 1969; Sutko et al., 1985; Meissner, 1986b; Lewartowski et al., 1990; Northover, 1991). In the former, nanomolar ryanodine had a pronounced negative inotropic action, sometimes preceded by a transient positive inotropic response, whereas in the latter, contracture was produced. Such a difference has been attributed to differences in the regulation of intracellular Ca^{2+} homeostasis. The effect of nanomolar ryanodine is to lock the SR channel open, so that Ca^{2+} is released into the cytosol. In cardiomyocytes, Ca^{2+} is quickly extruded by the sarcolemmal Na^+/Ca^{2+} exchanger and Ca^{2+}-ATPase, so that the increase in cytosolic [Ca^{2+}] is short-lived; the final effect is depletion of the intracellular Ca^{2+} pool, which accounts for negative inotropism. In skeletal muscle, sarcolemmal Ca^{2+} extrusion is much slower, so that ryanodine determines a persistent increase in cytosolic [Ca^{2+}], which is responsible for the contracture.

Other natural ryanoids contain several substituents at C-8, C-9, and C-10 of the cyclohexane ring. The most important one in amount and biological activity is 9,21-didehydroryanodine, which differs from ryanodine by the absence of two H atoms. Didehydroryanodine had the same functional effects as ryanodine, and competitively inhibited [^{3}H]ryanodine binding (Pessah et al., 1985, 1986; Carroll et al., 1991). It should be stressed that several commercial ryanodine preparations contain up to 50% didehydroryanodine, so that many reports actually describe the effects of a mixture of ryanodine and didehydroryanodine.

Many other biologically active ryanoids have been synthesized by derivatization of the cyclohexane ring or of the hydroxyls at C-2, C-4, C-6, C-10, C-12 and by inversion, relocation, or substitution of the pyrrole nucleus (Waterhouse et al., 1987; Gerzon et al., 1993; Humerickhouse et al., 1993, 1994; Jefferies et al., 1993, 1996a, 1996b; Ruest and Deslongchamps, 1993; Welch et al., 1994, 1996). Although most derivatives were less active than ryanodine, some C_{10}-O_{eq} esters containing positively charged side groups (e.g., guanidinopropionyl- and β-alanil-ryanodine) were more potent than ryanodine in binding experiments. In single-channel experiments, biologically active derivatives induced the appearance of long-lived subconductance states, whose amplitude ranged from about 5% (C_{10}-O_{eq} guanidinopropionyl-ryanodine) to about 70% (ryanodol) of the control state (Tinker et al., 1996).

The evaluation of synthetic ryanoids has shown that different molecular features are responsible for the multiple pharmacological actions of ryanodine. In particular, the synthesis of C_{10}-O_{eq} esters yielded compounds with pure agonist activity (e.g., β-alanil-ryanodine) since channel blockade was not observed at concentrations as high as 300 μM (Humerickhouse et al., 1994; Bidasee et al., 1995).

The molecular location of ryanoid binding sites is still unknown. Results obtained with proteolytic fragments suggest that ryanodine binding involves the foot region of the protein. More precisely, in RyR1 both low-affinity and high affinity binding sites are located between Arg 4475 and the carboxyl terminus (Callaway et al., 1994). Photoaffinity labeling with an azido derivative of ryanodine confirmed the carboxy-terminal location of the binding site(s) (Witcher et al., 1994). On the basis of the response to different ryanoids, Welch et al. (1994, 1996)

concluded that ryanodine binds to the receptor with the pyrrole and isopropyl groups buried deep inside a cleft of the protein, while the 9-portion and 10-portion remain at the mouth of the binding site and extend into the solvent.

2. Purine derivatives and related compounds. This group includes substances that have a similar sterical structure, based on a purine, carboline, carbazole, or imidazopyridine ring and are likely to act on the same molecular site.

a. METHYLXANTHINES. It has been known for many years that caffeine favors SR Ca^{2+} release (Endo, 1977; Herbette et al., 1982; Fabiato, 1983; Kim et al., 1983; Nagasaki and Kasai, 1983; Meissner, 1984; Meissner et al., 1986; Meissner and Henderson, 1987; Palade, 1987a; Pessah et al., 1987; Moutin and Dupont, 1988; Rousseau et al., 1988; Akera et al., 1990; Wyskovsky et al., 1990; Lee et al., 1991). Caffeine increased the Ca^{2+}-sensitivity of the process, so that significant Ca^{2+} release was produced even at nanomolar [Ca^{2+}]. A remarkable stimulation was obtained at suboptimal (low micromolar) [Ca^{2+}], whereas at optimal [Ca^{2+}], the stimulation was minimal. The sensitivity to caffeine was higher in cardiac than in skeletal SR, and the EC_{50} was of the order of 0.2 to 0.5 mM. The stimulatory actions of caffeine and adenine nucleotides were additive, and the response to caffeine was inhibited by Mg^{2+} and ruthenium red.

These results have been confirmed in single-channel experiments (Rousseau et al., 1988; Rousseau and Meissner, 1989; Sitsapesan and Williams, 1990; Hernandez-Cruz et al., 1995). In skeletal and cardiac muscle, caffeine increased channel Po without any conductance change. At low concentrations (0.5 to 2 mM), caffeine had a Ca^{2+}-sensitizing action: channel activation required the presence of submicromolar Ca^{2+}, and the increase in Po was due to a reduced lifetime of the closed states. Higher concentrations (> 5 to 10 mM) activated the channel also at picomolar Ca^{2+}, and increased the lifetime of the open channel, which was associated with the appearance of an additional long-lived open state. Channels activated by caffeine were still characteristically modified by ryanodine, ATP, Mg^{2+}, and ruthenium red.

In binding experiments (Pessah et al., 1987; Chu et al., 1990a; Holmberg and Williams, 1990a; Ogawa and Harafuji, 1990a,b; Zimanyi and Pessah, 1991b; Hernandez-Cruz et al., 1995), caffeine (1 to 30 mM) favored ryanodine binding by increasing the affinity and Ca^{2+} sensitivity of the binding reaction. The maximum binding at optimal Ca^{2+} concentration was only slightly increased. Kinetic experiments showed that caffeine increased the association rate.

In general, the effect of caffeine was similar to the effect of adenine nucleotides. However, several lines of evidence suggest that caffeine and adenine nucleotides act on different, although possibly interacting, sites: (*a*) caffeine and adenine nucleotides had synergic effects on channel gating and on ryanodine binding (Ogawa and Harafuji, 1990b); (*b*) adenine and adenosine inhibited the response to ATP analogues, yet they stimulated the response to caffeine (Rousseau et al., 1988; McGarry and Williams, 1994b); (*c*) the stimulation of ryanodine binding produced at optimal Ca^{2+} concentration was greater with adenine nucleotide than with caffeine (Pessah et al., 1987); (*d*) contrary to adenine nucleotides, caffeine did not affect the lifetime of the open channel, except possibly at very high concentrations (Sitsapesan and Williams, 1990); (*e*) the sensitivity to caffeine was higher in cardiac muscle, whereas the sensitivity to adenine nucleotide was higher in skeletal muscle (Zimanyi and Pessah, 1991a).

Theophylline (Seifert and Casida, 1986) and other methylxanthines shared the action of caffeine. Rousseau et al. (1988) reported the following order of effectiveness in release experiments: 1,7-dimethylxanthine > 3,7-dimethylxanthine (theobromine) ≈ 1,3-dimethylxanthine (theophylline) > 1,3,7-trimethylxanthine (caffeine) ≈ 3,9-dimethylxanthine, whereas 1,9-dimethylxanthine and 1,3,9-trimethylxanthine were minimally effective. The integrity of the imidazole ring was necessary for activity, because 1,3-dimethyluracil was ineffective.

Although most useful in the experimental setting, it is unlikely that RyR modulation will be important in the therapeutic response to methylxanthines, because their plasma concentration (e.g., about 55 μM for theophylline) is lower than the effective concentration range, as determined in vitro.

b. CARBOLINE DERIVATIVES AND CARBAZOLE DERIVATIVES. Derivatives of eudistomin D, a natural product with a β-carboline skeleton isolated from the Caribbean tunicate *Eudistoma olivaceum*, induced SR Ca^{2+} release. One of the most active derivatives was 9-methyl-7-bromoeudistomin D (MBED), which produced the same effects as caffeine on SR Ca^{2+} release and ryanodine binding and was about 1000 times more potent (Seino et al., 1991). Many other α-carboline and β-carboline derivatives showed similar effects, and their potency was increased by C-5 or C-7 halogenation or by N-9 methylation (Takahashi et al., 1995a). Because the spacial structure of MBED is similar to that of caffeine, it has been postulated that MBED interacts with the caffeine binding site in the RyR (Seino et al., 1991). This hypothesis was supported by binding experiments performed with [^{3}H]MBED, because a specific binding site was identified and [^{3}H]MBED binding was competitively inhibited by caffeine. Ca^{2+}, Mg^{2+}, and ryanodine did not affect [^{3}H]MBED binding, whereas AMP-PCP had a stimulatory effect (Fang et al., 1993). These results support the concept that caffeine and adenine nucleotide binding sites are separated and partially interacting. [^{3}H]MBED binding was inhibited by several channel blockers, such as procaine, tetracaine, spermine and dantrolene, whereas it was slightly stimulated by

ruthenium red. In skeletal muscle, the density of [^{3}H]MBED binding sites was almost equal to that of [^{3}H]ryanodine binding sites, but in brain, the former was more than 100-fold higher (Yoshikawa et al., 1995). It is unclear whether these observations reflect the presence of MBED-binding proteins different from the RyR, or rather of isolated RyR monomers, that are unable to bind [^{3}H]ryanodine.

Several carbazole derivatives shared the Ca^{2+}-releasing action of carboline derivatives such as MBED. However, derivatives with a carbazole skeleton and bromine at C-6 had an inhibitory action (Takahashi et al., 1995a). In particular 4,6-dibromo-3-hydroxycarbazole was a potent inhibitor of SR Ca^{2+} release (IC_{50} = 58 μM). This substance maintained its inhibitory action also at high (> 30 μM) Ca^{2+} concentration, and it did not affect ryanodine binding (Takahashi et al., 1995b).

c. SULMAZOLE. Sulmazole is an imidazopyridine compound, namely 2-(2-methoxy-4-(methylsulfinyl)-phenyl)-1H-imidazo(4,5-β)pyridine. In single-channel experiments performed with sheep cardiac RyR, sulmazole increased the frequency and duration of open events with an EC_{50} of about 400 μM (Williams and Holmberg, 1990; Sitsapesan et al., 1991; McGarry and Williams, 1994a). Sulmazole acted by both Ca^{2+}-dependent and Ca^{2+}-independent mechanisms, because it induced channel opening even in the absence of Ca^{2+} (60 pM free Ca^{2+}); in addition, it increased the Ca^{2+}-sensitivity of channel activation. Channels activated by sulmazole were still inhibited by Mg^{2+} and ruthenium red. The Hill coefficient for Ca^{2+}-independent channel activation was close to 1, whereas a Hill coefficient close to 2 was obtained in the presence of Ca^{2+}, suggesting that one sulmazole molecule can bind to the closed conformation of the channel, whereas Ca^{2+} is required for the binding of a second molecule.

In binding experiments (Holmberg and Williams, 1990a; McGarry and Williams, 1994a), sulmazole increased ryanodine binding, by increasing the K_D, with EC_{50} and Hill coefficient comparable to those observed in single-channel experiments.

The action of sulmazole was not stereospecific and was shared by the enantiomer isomazole. Analogs lacking the methylsulfinyl oxygen were also effective and were about 10-fold more potent than sulmazole and isomazole (McGarry and Williams, 1994a).

The pharmacological actions of sulmazole resemble those of caffeine. Because the tridimensional structures of the imidazopyridine ring of sulmazole and of the purine ring of caffeine are quite similar, it is likely that they act on the same molecular site. In fact, sulmazole shares other actions with caffeine, such as sensitization of contractile proteins to Ca^{2+}, inhibition of type III phosphodiesterase, inhibition of sarcolemmal Na^+/K^+ ATPase, and A1-adenosine antagonism (see McGarry and Williams, 1994a for reference).

3. Anthraquinones. Zorzato et al. (1985) first reported that the anthraquinone compound doxorubicin, an antineoplastic drug also known as adriamycin, induced Ca^{2+} release from skeletal muscle SR, with $EC_{50} \approx 5$ μM. The same action was produced by other anthraquinones such as mitoxantrone, daunorubicin, rubidazone, and doxorubicinol and occurred also in cardiac muscle (Palade, 1987b; Abramson et al., 1988a; Kim et al., 1989; Pessah et al., 1990; Tian et al., 1991). In some experiments, the response to doxorubicin was bell-shaped, because the stimulation of Ca^{2+} efflux decreased at concentrations > 30 μM (Pessah et al., 1992b). Naphtoquinones such as menadione and plumbagin were minimally effective, and benzoquinones were ineffective (Abramson et al., 1988a). In the presence of physiological Mg^{2+} concentration, the action of anthraquinones was sharply dependent on [Ca^{2+}], showing that these substances increased the Ca^{2+}-sensitivity of Ca^{2+} release.

In single-channel experiments, doxorubicin increased cardiac channel Po (Nagasaki and Fleischer, 1989; Holmberg and Williams, 1990b; Ondrias et al., 1990). At low concentrations (1 to 10 μM), doxorubicin decreased the lifetime of the closed channel; at higher concentrations (25 μM), it also increased the lifetime of the open channel. Ondrias et al. (1990) reported a biphasic, time-dependent effect, because doxorubicin (2.5 to 10 μM) initially increased channel Po and subsequently inactivated the channel. The degree of channel activation was concentration-dependent, whereas the time needed to inactivate the channel was concentration-independent and averaged 8 min.

In binding experiments, doxorubicin decreased the K_D for ryanodine by increasing the association constant, and it did not modify the B_{max} ($EC_{50} \approx 20$ to 30 μM at 1 μM Ca^{2+}) (Abramson et al., 1988a; Kim et al., 1989; Pessah et al., 1990, 1992b; Holmberg and Williams, 1990b; Zimanyi and Pessah, 1991b). These effects are similar to those of caffeine, and it was speculated that caffeine and doxorubicin compete for the same binding site on the RyR (Pessah et al., 1990). However, kinetic analysis suggested that anthraquinone and caffeine bind to different, although interacting, sites (Abramson et al., 1988a).

Although the acute effect of doxorubicin was quickly reversible, long-term (1 to 7 days) treatment increased the sensitivity of rat cardiac preparations to anthraquinones, because the increase in Ca^{2+} release and ryanodine binding observed after the acute administration of doxorubicin was much higher than in the control animals (Pessah et al., 1990). However, if chronic treatment was further prolonged, RyR inactivation eventually ensued: after 4 weeks (total dose 9 mg/kg), the B_{max} for ryanodine was reduced, with unchanged K_D, and Ca^{2+} release was impaired (Pessah et al., 1992b). Similar findings were obtained in a rabbit model of chronic doxorubicin toxicity (Dodd et al., 1993). After 6 to 9 weeks (total dose: 12 to 18 mg/kg), a significant reduction in the

B_{max} for ryanodine was observed in heart, but not in skeletal muscle. The K_D was unchanged, and the K_{DCa} was slightly increased. Decreased RyR density was confirmed by Western blot analysis of cardiac homogenates, using specific antibodies.

Significant differences between anthraquinones have been observed. In cardiac tissue, mitoxantrone and doxorubicin had different actions on ryanodine binding (Kim et al., 1994b). Both compounds decreased the K_D for ryanodine, but, whereas doxorubicin increased the association rate and did not affect the dissociation rate, mitoxantrone decreased the dissociation rate. Contrary to doxorubicin, mitoxantrone did not increase the Ca^{2+} sensitivity of ryanodine binding, and, under optimal conditions (30 μM Ca^{2+} and no Mg^{2+}), ryanodine binding was actually inhibited. In single-channel experiments, high concentrations of mitoxantrone induced the formation of a low-conductance state (Holmberg and Williams, 1990b).

It is likely that interference with RyR function by anthraquinones may produce a transient cytosolic Ca^{2+} overload, followed by an impairment of the SR Ca^{2+} release capability. These phenomena are thought to be of major importance in determining the toxic effects of these substances, both in experimental preparations and in the clinical setting. The acute effects of anthracyclins include a transient positive inotropic response followed by a sustained negative inotropic action, prolonged time to peak-twitch-tension, and decreased relaxation rate, whereas chronic doxorubicin toxicity is characterized by the progressive development of a cardiomyopathy leading to congestive heart failure (Hagane et al., 1988; Doroshow, 1991; Boucek et al., 1993; Wang and Korth, 1995). On the other hand, the arrhythmias that are often the only clinical evidence of acute toxicity have been related to interference with delayed rectifier K^+ current (Wang and Korth, 1995).

4. Digitalis glycosides. At therapeutic concentration (1 nM), digoxin increased the rate of Ca^{2+}-induced Ca^{2+} release from cardiac SR (McGarry and Williams, 1993). Digoxin could not induce Ca^{2+} release at subactivating (picomolar) Ca^{2+} concentrations, and its action was inhibited by Mg^{2+}. In single-channel experiments, digoxin increased channel Po, owing to decreased lifetime of the closed channel. High concentrations (10 to 20 nM) produced an additional effect, i.e., increased lifetime of the open channel. Digoxin appeared to sensitize the SR channel to Ca^{2+}, because channel-gating was not modified at picomolar Ca^{2+} concentration. Equimolar digitoxin had the same effect as digoxin, whereas ouabain was effective only at higher concentrations (10 nM). Spironolactone and chlormadinone, which inhibit digoxin binding to Na^+/K^+ ATPase, were uneffective.

This activation of RyR2 occurred at a therapeutic concentration, was clearly distinct from Na^+/K^+ ATPase inhibition, and might contribute to the inotropic action of digoxin and digitoxin. Such action was similar to that of caffeine and sulmazole, but digitalis glycosides had no effect on RyR1.

The antiarrhythmic agent R56865 (N-[1-[4-(fluorophenoxy)-butyl]4-piperidinyl]-N-methyl-2-benzothiazolamine) inhibited [^{3}H]digoxin binding to SR membranes (but not to the Na^+/K^+-ATPase) and the response of single SR channels to digoxin. However, in the absence of digoxin, it had no direct effect on SR Ca^{2+} release nor on ryanodine binding (McGarry et al., 1995). This property might contribute to the antiarrhythmic action of R56865, even if this substance acts also on other targets, i.e., Na^+ current, Na^+-activated K^+ current, and, possibly, T-type sarcolemmal Ca^{2+} current (Ichikawa et al., 1994; McGarry et al., 1995).

5. Milrinone and other bipyridine derivatives. Both indirect (Malecot et al., 1986) and direct (Holmberg and Williams, 1991) evidence showed that milrinone (1,6,-dihydro-2-methyl-6-oxo-(3,4-bipyridine)-5-carbonitrile), a bipyridine derivative used as an inotropic agent, can activate cardiac RyR. At 10 μM Ca^{2+} concentration, 100 μM to 2 mM milrinone increased channel Po, by reducing closed channel lifetime. At higher (100 μM) Ca^{2+} concentration, the duration of channel opening increased, which was reflected in lifetime analysis as the appearance of a third, long-lived, open state. In binding experiments, 100 μM to 2 mM milrinone increased [^{3}H]ryanodine binding at a concentration of 5 nM in the presence of 5 μM Ca^{2+}, whereas no stimulation was produced at optimal (100 μM) Ca^{2+} concentration, suggesting that milrinone increased the Ca^{2+} sensitivity of the binding reaction.

Because peak plasma milrinone concentration is nearly one order of magnitude lower than the concentrations used in this study (Prielipp et al., 1996), it is uncertain whether RyR modulation may contribute to its inotropic action, which usually is attributed to phosphodiesterase inhibition. It is worth noting that other phosphodiesterase inhibitors such as caffeine and sulmazole are also RyR modulators. By contrast, enoximone, another phosphodiesterase inhibitor used as an inotropic agent, did not affect RyR function (Holmberg and Williams, 1991).

Other dipyridine derivatives influence SR Ca^{2+} release. For instance, in skeletal muscle, 1,1′-diheptyl-4,4′-bipyridinium bromide inhibited SR Ca^{2+} release induced by polylysine, Ag^+, or caffeine, and decreased [^{3}H]ryanodine binding, with IC_{50} of the order of 2.5 to 5 μg/ml (Kang et al., 1994). By contrast, diethyl bipyridinium, dibenzyl bipyridinium, and dimethyl bipyridinium had no effect on skeletal muscle SR (Kang et al., 1994).

6. Suramin. Suramin (*sym*-bis(*m*-aminobenzoyl-*m*-amino-*p*-methylbenzoyl-1-naphthyl-amino-4,6,8,-trisulfonate)carbamide) is a polysulphonated naphtylurea originally developed for the treatment of trypanosomiasis and used as an anticancer agent. Suramin is also an antagonist of ATP at P_2 purinergic receptors. In skeletal

muscle, suramin inhibited Ca^{2+}-ATPase activity, induced SR Ca^{2+} release, and increased ryanodine binding (Emmick et al., 1994). RyR actions occurred at 10 μM to 1 mM concentration. The effect on ryanodine binding was similar to that of adenine nucleotides, and the response to suramin and AMP-PCP was not additive, suggesting that suramin interacted with the adenine nucleotide binding site. In single-channel experiments, micromolar suramin increased the Po of the sheep cardiac channel by stabilizing the open states (Sitsapesan and Williams, 1996).

7. Halogenated hydrocarbons and phenols. Several halogenated compounds affect SR Ca^{2+} release. The most extensively studied are volatile anesthetics such as halothane (2-bromo-2-chloro-1,1,1-trifluoroethane), enflurane (2-chloro-1-(difluoromethoxy)-1,1,2-trifluoroethane), and its isomer isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoroethane). More recently, hexachlorocyclohexane and chlorinated phenol derivatives have been reported to affect RyR function. These substances have been grouped for their structural similarity, although no common mechanism of action has been identified.

a. VOLATILE ANESTHETICS. In skeletal muscle and cardiac SR preparations, halothane increased SR Ca^{2+} release at gas concentrations ranging from about 0.002% to 3.8% (v/v) (Kim et al., 1984; Beeler and Gable, 1985; Ohnishi, 1987; Nelson and Sweo, 1988; Herland et al., 1990; Carrier et al., 1991; Frazer and Lynch, 1992; Louis et al., 1992; Beltran et al., 1996). The response to halothane was Ca^{2+}-dependent and pH- dependent. At a pH of 7.4, halothane increased the rate of Ca^{2+} release at all Ca^{2+} concentrations, so that the normal Ca^{2+} dependence of Ca^{2+} release was retained. At a pH of 7.1, halothane shifted the Ca^{2+}-dependence of Ca^{2+} release to the left, but no stimulation was observed at $pCa < 5$. At a pH of 6.8, no significant Ca^{2+} release occurred in the absence of halothane, at any Ca^{2+} concentration, and the stimulation produced by halothane was Ca^{2+}-independent (Beltran et al., 1996). The response to halothane was not affected by adenine nucleotides, and it was inhibited by ruthenium red (Beeler and Gable, 1985; Herland et al., 1990; Frazer and Lynch, 1992). Similar effects have been observed with isoflurane and enflurane (2.5 to 4%).

In single-channel experiments performed with RyRs obtained from cardiac muscle (Connelly and Coronado, 1994) or frog skeletal muscle (Bull and Marengo, 1994), halothane increased channel Po without affecting channel conductance. Lifetime analysis showed increased duration of open events and decreased lifetime of the closed channel. Channel activation was reversible and Ca^{2+}-dependent: the maximum effect occurred at 1 to 10 μM Ca^{2+}, whereas Ca^{2+} efflux was only slightly increased at 100 μM Ca^{2+}, and no activation was observed at 0.1 μM Ca^{2+}. The EC_{50} was inversely related to Ca^{2+} concentration and ranged from 13 μM to 184 μM (aqueous phase concentration), approximately corresponding to 0.03 to 0.45% gas concentration. Reduction of pH from 7.4 to 7.1 caused maximum channel activation to occur at lower *cis* Ca^{2+} concentration (Beltran et al., 1996). Enflurane (1.6%) was also effective, but no effect was observed with 1.4% isoflurane. Similar results have been obtained in whole-cell patch-clamp experiments performed in ventricular myocytes (Pancrazio and Lynch, 1994). In contrast with these findings, Nelson (1992) reported that in normal human skeletal muscle, channel Po was not affected by halothane, although increased Po was observed in about half of the channels obtained from patients predisposed to malignant hyperthermia (see III.C.). It should be noted that the drug concentrations used in this study were quite low (2.2 to 17.6 μM), which might explain the failure to activate the normal channel.

Volatile anesthetics increased ryanodine binding by shifting its Ca^{2+} dependence to the left. This action was tissue-specific, because halothane and enflurane (1.5 to 2%) increased ryanodine binding in cardiac but not in skeletal muscle, whereas the opposite was observed with isoflurane (Connelly et al., 1992). More precisely, in cardiac preparations 0.75 to 1.5%, halothane increased the density of high-affinity binding sites, whereas the K_D was unchanged, and low-affinity binding was reduced (Lynch and Frazer, 1994). Enflurane (3.5%) and isoflurane (2.5%) had different effects, because high-affinity binding was unchanged or decreased, and low-affinity binding was stimulated.

The interaction of volatile anesthetics with the RyR occurred at doses that are lower than their minimum effective alveolar concentration (that is, about 0.75% for halothane, 1.68% for enflurane, and 1.15% for isoflurane) and should therefore have clinical importance, contributing to the negative inotropic effect and to the transient vasoconstrictor action produced by these drugs. However, volatile anesthetics can modulate other systems involved in Ca^{2+} homeostasis, i.e., SR Ca^{2+}-ATPase, Na^+/Ca^+ exchange, sarcolemmal Ca^{2+} channel, and contractile proteins, which might also contribute to their clinical effects (Su and Zhang, 1989; Su et al., 1994; Herland et al., 1990; Komai and Rusy, 1990; Marijic et al., 1990; Puttick and Terrar, 1993; Stadnika et al., 1993; Kakuyama et al., 1994; Tsuchida et al., 1994; Wheeler et al., 1994; Akata and Boyle, 1995; Boyle and Maher, 1995; Vogel et al., 1995).

Indirect studies showed that other chloromethane, chloroethane, and chloroethylene derivatives can induce SR Ca^{2+} release. Because sarcolemmal Ca^{2+} fluxes were also affected, it has been speculated that the response to halogenated hydrocarbons may reflect a nonspecific membrane action (Hoffmann et al., 1994).

Nonvolatile general anesthetics such as ketamine (Kongsayareepong et al., 1993; Connelly et al., 1995) and propofol (Puttick and Terrar, 1993; Cook and Housmans, 1994; Fruen et al., 1995) did not affect SR Ca^{2+} release, although they inhibited ryanodine binding at concentra-

tions much higher than their therapeutic range (Connelly et al., 1995; Fruen et al., 1995). Thiopental inhibited postrest contraction in rabbit papillary muscle, which was interpreted as indirect evidence of decreased SR Ca^{2+} release (Komai and Rusy, 1994).

b. PHENOL DERIVATIVES. Chlorocresol (chloro-methylphenol) induced Ca^{2+} release from skeletal muscle terminal cisternae (Zorzato et al., 1993), and its action was inhibited by ruthenium red. The most effective isomer was 4-chloro-*m*-cresol (EC_{50} = 300 μM), and 4-chloro-*o*-cresol was much less potent. The presence of the methyl group was important, because *p*-chlorophenol was effective only at millimolar concentrations, and the presence of chloride was critical, because *o*-cresol was ineffective. In skeletal muscle terminal cisternae and in several nonexcitable cell lines, Ca^{2+} release was induced also by 100 to 500 μM 4-chloro-*m*-ethylphenol (Larini et al., 1995).

In binding experiments, 4-chloro-*m*-cresol stimulated ryanodine binding by decreasing the K_D with unchanged B_{max} (EC_{50} = 112 μM). In bilayer experiments, it increased channel Po by prolonging the lifetime of the open channel (Herrmann-Frank et al., 1996). With regard to the latter action, the sensitivity to 4-chloro-*m*-cresol was higher when it was included in the *trans* (luminal) chamber (EC_{50} = 50 μM versus 150 μM).

Several polychlorinated biphenyls increased ryanodine binding and Ca^{2+} release both in skeletal muscle and in cardiac SR (Wong and Pessah, 1996). Compounds with chloride substitutions in the ortho-position were most active (EC_{50} $\approx$ 1 μM). Polychlorinated biphenyl-activated Ca^{2+} release was inhibited by ryanodine and ruthenium red, but it was quite resistant to Mg^{2+} inhibition.

Nonhalogenated phenol derivatives also showed some activity. In particular, several 4-alkylphenols (ethylphenol to nonylphenol) have been reported to cause SR Ca^{2+} release, and the effectiveness was proportional to the length of the alkyl chain (Beeler and Gable, 1993a). Thymol (5-methyl-2-isopropyl-1-phenol) and menthol (hexahydro-thymol) have also been used to induce SR Ca^{2+} release (Herbette et al., 1982; Palade, 1987b).

c. HEXACHLOROCYCLOHEXANE. Hexachlorocyclohexane, particularly the δ isomer, produced Ca^{2+} release from cardiac SR, with EC_{50} = 22 μM (Pessah et al., 1992a). δ-hexachlorocyclohexane had peculiar properties, because its action was not inhibited by ruthenium red and was associated with decreased ryanodine binding. In equilibrium experiments, δ-hexachlorocyclohexane decreased the B_{max} for ryanodine, either in heart, skeletal muscle, or brain (IC_{50} = 37, 123, and 100 μM, respectively: Hill coefficient $\approx$ 2). The K_D showed biphasic changes, because it decreased at low (20 to 30 μM) concentrations, and was unchanged at higher concentrations. Pretreatment with δ-hexachlorocyclohexane produced complex results: ryanodine binding increased in membranes pretreated with 25 μM δ-hexachlorocyclohexane, but decreased in membranes pretreated with 50 μM δ-hexachlorocyclohexane. These results have been interpreted on the basis of the hypothesis that δ-hexachlorocyclohexane may produce sequential changes in the RyR, whose final result is an irreversible alteration of the channel structure, leading to increased Ca^{2+} efflux and preventing the binding of ryanodine and ruthenium red.

8. Macrocyclic compounds.

a. IMMUNOSUPPRESSANT MACROLIDES. As mentioned above (II.A.7.c.), the macrolide immunosuppressant FK-506, also known as tacrolimus, can induce the dissociation of FKBPs from the RyR and can modify RyR gating. In skeletal muscle SR, 3 to 20 μM FK-506 induced Ca^{2+} release by increasing channel Po and determining the appearance of a long-lived subconductance state (Ahern et al., 1994; Brillantes et al., 1994; Mayerleitner et al., 1994). The subconductance state corresponded to $\approx$ 30% of the maximum conductance, and it was different from the ryanodine-induced subconductance state. In the presence of FK-506, channel inactivation by millimolar Ca^{2+} was alleviated. These effects appear to be associated with inhibition of ryanodine binding, because, in liver microsomes, FK-506 decreased ryanodine binding, due to reduced B_{max} with unchanged K_D (Kraus-Friedmann and Feng, 1994). Interference with RyR function might be involved in some toxic effects of FK-506, particularly in the development of myocardial hypertrophy and failure, which has been observed in pediatric transplant patients (Atkison et al., 1995).

Rapamycin is another macrolide immunosuppressant that can dissociate FKBPs from the RyR. In cardiac muscle, 0.2 to 12 μM rapamycin increased single-channel Po and decreased channel conductance (Kaftan et al., 1996). These effects were sequential: within 2 to 10 min from the addition of rapamycin, Po increased, owing to increased lifetime of the open channel; with prolonged exposure (> 10 min), channel conductance decreased. It has been speculated that the former effect is the consequence of drug binding to FKBPs, whereas changes in channel conductance occur after FKPB/RyR dissociation. The involvement of FKBPs in the response to rapamycin was confirmed by the observation that no effect was produced on channels expressed in insect cells and lacking FKBPs (Brillantes et al., 1994). As observed with FK-506, 20 μM of rapamycin inhibited ryanodine binding to cardiac microsomes by decreasing the B_{max} (Kaftan et al., 1996).

b. BASTADINS. Bastadins are macrocyclic bromotyrosine derivatives isolated from the sponge *Ianthella basta*. Bastadins are selective modulators of RyR1 (Mack et al., 1994), and behave either as pure agonists (e.g., bastadin 5 and bastadin 7) or as partial agonists (e.g., bastadin 19). In the presence of > 100 μM Ca^{2+}, bastadin 5 increased SR Ca^{2+} release, and its action was inhibited by ruthenium red. In single-channel experiments, bastadin 5 showed peculiar properties, since it

remarkably (by 50- to 180-fold) increased the lifetime of either the open or the closed states. The final effect was a slight increase in channel Po and a remarkable reduction in the frequency of opening and closure events. Bastadin 5 (1 to 5 μM) decreased the K_D and increased the B_{max} of the high-affinity ryanodine binding site, which was associated with a reduction in low affinity binding. The Ca^{2+}-dependence of ryanodine binding was preserved, but detailed analysis showed that the affinity of the Ca^{2+}-inhibitory site decreased five-fold. In addition, the IC_{50} for Mg^{2+} increased.

The action of bastadins was additive with that of caffeine and adenine nucleotides, suggesting a different site of action. Because the response to bastadins was inhibited by FK-506, it was concluded that bastadins interact with FKBP12 and stabilize the RyR homotetramer.

c. QUINOLIDOMICIN A1. Quinolidomicin A1 is a 60-membered macrolide isolated from the actinomycete *Micromonospora* species, which shows cytotoxic effects in a murine leukemia cell line. In skeletal muscle SR (Ohkura et al., 1996), it induced Ca^{2+} release with $EC_{50} \approx 20$ μM. The Ca^{2+} dependence of Ca^{2+} release was not modified; the stimulation was additive with that produced by caffeine, and it was only partly inhibited by Mg^{2+}, procaine, and ruthenium red. In binding experiments, quinolidomicin A1 increased the affinity for ryanodine, whereas the B_{max} was unchanged. Although these observations suggest that quinolidomicin A1 may modulate the RyR, its precise site and mechanism of action have not been clarified and need further investigation.

9. Heparin. An early study showed that 0.5 to 10 μg/ml heparin induced Ca^{2+} release from skeletal muscle SR by a ruthenium red-sensitive pathway (Ritov et al., 1985). Single-channel studies showed that heparin increased channel Po, with $EC_{50} = 0.23$ μg/ml and Hill coefficient ≈ 4 (Bezprozvanny et al., 1993). The action was Ca^{2+}-dependent, because channel activation was observed at 80 nM $[Ca^{2+}]$ but not at $[Ca^{2+}] < 20$ nM, and it was shared by other polyanions, such as pentosan polysulfate and polyvinyl sulfate. These results have been interpreted on the basis of the hypothesis that heparin and related polyanions can increase the local Ca^{2+} concentration near the regulatory sites of the RyR.

Ryanodine binding has not been determined in the above-mentioned studies. In brain microsomes, comparable concentrations of heparin did not affect ryanodine binding (Zimanyi and Pessah, 1991b). In skeletal muscle, heparin caused a slight stimulation of ryanodine binding only at substantially higher concentrations (0.1 to 1 mg/ml) (Ogawa and Harafuji, 1990a).

10. Polyamines. Several RyR modulators have one feature in common: the presence of several amino groups in their structures. These substances include: inorganic polyamines such as ruthenium red, aminoglycoside antibiotics, synthetic polyamines, endogenous polyamines, and cationic proteins. The effects of endogenous polyamines and of cationic proteins have been reviewed above (see II.A.5. and II.A.7.f.).

a. RUTHENIUM RED. Ruthenium red is a polycationic dye with a structure that includes 14 amino groups. Ruthenium red has been shown to inhibit SR Ca^{2+} release, both in cardiac muscle and in skeletal muscle. In release experiments, effective concentrations ranged from 1 nM to 20 μM (Kim et al., 1983; Chamberlain et al., 1984b; Antoniu et al., 1985; Chu et al., 1986; Meissner et al., 1986; Meissner and Henderson, 1987; Palade, 1987a,c; Chiesi et al., 1988; Moutin and Dupont, 1988; Baylor et al., 1989; Calviello and Chiesi, 1989; Holmberg and Williams, 1989; Wyskovsky et al., 1990; Zimanyi and Pessah, 1991a,b). In skeletal muscle, the IC_{50} was of the order of 19 to 90 nM, whereas in cardiac muscle, it was slightly higher; the inhibition was incomplete, because complete blockade required the addition of either Mg^{2+} or FLA365.

In bilayer experiments, micromolar ruthenium red dramatically decreased channel Po, producing a long-term closure that was substantially irreversible on the time scale of the recordings (Smith et al., 1985, 1986b, 1988; Rousseau et al., 1986; Hymel et al., 1988; Ma et al., 1988; Anderson et al., 1989; Liu et al., 1989; Ashley and Williams, 1990; Lindsay and Williams, 1991; Buck et al., 1992). Further information was obtained by studying the effect of ruthenium red on ryanodine-modified channels (Ma, 1993). The action of ruthenium red was asymmetrical and voltage-dependent. When added to the *cis* (cytosolic) chamber, ruthenium red decreased channel Po without affecting its conductance. At low concentration (0.5 μM), the chief effect was a decreased lifetime of the open channel, whereas at higher concentration (1.2 μM), the lifetime of the closed channel increased (more precisely, the percentage of long-lived versus short-lived closures increased). This action was voltage-dependent (i.e., it was more evident at higher holding potentials) and cooperative, with a Hill coefficient close to 2. When added to the *trans* (luminal) chamber, ruthenium red decreased channel current in the *trans* to *cis* direction (but not in the reverse direction) without any change in Po.

In binding experiments, ruthenium red inhibited ryanodine binding, with a Hill coefficient close to 1. Decreased B_{max} and increased K_D were observed, and the latter effect was due to a slower association rate (Pessah et al., 1985,1986; Michalak et al., 1988; Chu et al., 1990a; Holmberg and Williams, 1990a; Ogawa and Harafuji, 1990a; Zimanyi and Pessah, 1991a; Mack et al., 1992). The IC_{50} was in the range of 10 to 40 nM, both in cardiac and in skeletal muscle, whereas it was higher (600 nM) in brain (Zimanyi and Pessah, 1991b). In addition, high (>1 μM) concentrations of ruthenium red slowed ryanodine dissociation, suggesting the occurrence of a persistent receptor alteration, similar to that produced by micromolar ryanodine. In fact, prolonged

incubation with ruthenium red gave a 50% decrease in the number of ryanodine binding sites (Mack et al., 1992).

Binding studies performed on cloned segments of RyR1 have shown extensive overlapping among Ca^{2+} binding sites, calmodulin binding sites, and ruthenium red binding sites. In particular, ruthenium red binding sites have been localized at residues 1861 to 2094 and 3657 to 3776 (Chen and MacLennan, 1994). On the basis of multiple inhibition studies, Mack et al. (1992) have suggested that ruthenium red interacts with a subregion of the ryanodine binding site, which also binds other polycations and is distinct from the FLA365 binding site (see II.B.11.). On the basis of single-channel results, it has been concluded that the binding site is located within the membrane field, probably close to the pore of the channel, and that ruthenium red cannot permeate through the open channel (Ma, 1993).

Other inorganic polyamines structurally related to ruthenium red inhibited SR Ca^{2+} release. Tetramine palladium and tetramine platinum had similar effects in single-channel and in binding experiments (Ma, 1993). In addition, phthalocyanine-induced Ca^{2+} release was blocked by 50 μM hexamminecobalt chloride or hexammineruthenium chloride (Abramson et al., 1988b).

b. AMINOGLYCOSIDES. Aminoglycoside antibiotics inhibited Ca^{2+} release induced by Ca^{2+}, caffeine, thymol, or tetraphenylboron, with the following order of potency: neomycin > gentamicin > streptomycin ≥ clindamycin ≥ kanamycin ≥ tobramycin. In skeletal muscle, the IC_{50} for neomycin and gentamicin was of the order of 50 to 200 nM, whereas the IC_{50} for the other aminoglycosides was in the micromolar range (Meissner and Henderson, 1987; Palade, 1987c; Chiesi et al., 1988; Calviello and Chiesi, 1989; Wyskovsky et al., 1990; Mack et al., 1992; Zimanyi et al., 1992).

In bilayer experiments, neomycin (5 μM) decreased channel Po. The addition of ryanodine after neomycin produced no effect, whereas the addition of 10 μM neomycin to a ryanodine-modified channel decreased the mean open time (Wang et al., 1996).

Neomycin inhibited high-affinity ryanodine binding by increasing the K_D as a consequence of a slower association rate. The IC_{50} was lower in skeletal than in cardiac muscle (0.32 to 0.38 versus 5 to 37 μM), and the Hill coefficient was < 1. The Ca^{2+} dependence of ryanodine binding was unaffected (Zimanyi and Pessah, 1991a; Mack et al., 1992; Wang et al., 1996). At high concentrations (≥ 0.6 μM), neomycin had additional actions, because it decreased the B_{max} and slowed ryanodine dissociation. Furthermore, prolonged incubation with 0.3 μM neomycin produced a 20% decrease in ryanodine binding (Mack et al., 1992). It was concluded that high concentrations of neomycin irreversibly inactivate the RyR, as observed with high concentrations of ryanodine or ruthenium red. Ruthenium red and neomycin are likely to react with the same molecular site. Assays performed after trypsin treatment confirmed that ryanodine and neomycin binding sites were located in a 76-kDa fragment, corresponding to the carboxyl terminal part of the protein (up to Arg 4475) (Wang et al., 1996).

In skeletal muscle, neomycin inhibited depolarization-induced Ca^{2+} release. This action was voltage-dependent and occurred at concentrations (1 to 100 nM) much lower than those necessary to inhibit caffeine-induced or polylysine-induced Ca^{2+} release (Yano et al., 1994). The existence of a high-affinity neomycin binding site able to modulate T-tubule/SR interaction has been suggested, and [^{3}H]neomycin binding experiments have shown that this putative site is not localized in the T-tubule membrane, but rather in the RyR or in some other closely associated protein.

Because therapeutic aminoglycoside concentrations are in the micromolar range, it is possible that RyR modulation might be involved in the toxic effects. However, this issue has not been specifically investigated.

c. OTHER ORGANIC POLYAMINES. Palade (1987c) reported that synthetic polyamines such as polylysine and polyarginine inhibited thymol-induced Ca^{2+} release with an estimated IC_{50} of 1 to 6 μg/ml (corresponding to about 0.5 to 1.5 μM). In a later study, Cifuentes et al. (1989) observed a biphasic effect: polylysine induced Ca^{2+} release at low concentration (IC_{50} = 0.3 μM), but inhibited Ca^{2+}-induced Ca^{2+} release at higher concentration (3 μM). El-Hayek et al. (1995c) have described a bell-shaped dose-response curve for the stimulation of Ca^{2+} release by polylysine, with the peak at 100 nM. Polylysine-induced Ca^{2+} release showed the same Ca^{2+} dependence as did caffeine-induced Ca^{2+} release and was inhibited by 10 μM ruthenium red. Stimulation of ryanodine binding by 1 μM polylysine has been observed (Lu et al., 1994).

A radiolabeled polylysine derivative bound to the RyR, and the binding was not inhibited by ruthenium red. Fluorescent labeling studies showed that polylysine-induced Ca^{2+} release was preceded by a conformational change in the junctional foot protein (El-Hayek et al., 1995c) and revealed that neomycin did not affect polylysine binding, suggesting that the polylysine binding site was distinguished from the neomycin/ruthenium red binding site (Kang et al., 1992).

11. FLA365. [2,6-dichloro-4-(dimethylamino)phenyl]-isopropylamine, commonly known as FLA365, is a synthetic compound that proved to be effective in modulating SR Ca^{2+} release. FLA365 inhibited Ca^{2+}-induced Ca^{2+} release from skeletal muscle SR (Calviello and Chiesi, 1989). At an activating free Ca^{2+} concentration of 2 μM, the IC_{50} was 3.4 μM, and full inhibition was achieved with 50 μM FLA365. However, when free Ca^{2+} concentration was increased to 30 μM, full inhibition of Ca^{2+} release could not be produced by FLA365 alone, but required the association of neomycin or ruthenium red. Similar results were obtained in indirect cardiac muscle studies (Chiesi et al., 1988), because Ca^{2+} up-

take was minimally affected by FLA365 alone, and the association of neomycin or ruthenium red was required to produce a full stimulation.

Binding experiments (Mack et al., 1992) showed that FLA365 competitively inhibited ryanodine binding at its high-affinity site and that the IC_{50} was similar in skeletal and in cardiac muscle (1.4 and 4.4 μM at 0.6 nM [^{3}H]ryanodine). FLA365 decreased the affinity for ryanodine, without any change in the B_{max}. Contrary to what happened with ruthenium red and neomycin, prolonged incubation with FLA365 produced no persistent change in ryanodine binding. The effect of FLA365 was not modified by either ruthenium red or neomycin, and FLA365 had no effect on ruthenium red inhibition of ryanodine binding. It was concluded that FLA365 interacts with the RyR on a site that is separate from the ruthenium red/neomycin site. The additive effect of FLA365 and ruthenium red/neomycin suggested that these two sites may correspond to subregions of a larger ryanodine binding site (Mack et al., 1992).

12. Dantrolene. Dantrolene is the hydantoin derivative 1-((5-(*p*-nitrophenyl) furfurylidine)amino)hydantoin sodium. It acts as a postsynaptic muscle relaxant and is the chief drug used in the prevention and in the treatment of malignant hyperthermia (see III.C.). In skeletal muscle SR preparations, 10 to 90 μM dantrolene inhibited Ca^{2+} release (Van Winkle, 1976; Herbette et al., 1982; Otha et al., 1990). Because therapeutic dantrolene concentration is in the range of 10 μM (Flewellen et al., 1983), inhibition of Ca^{2+} release is likely to be responsible for its clinical effects. Dantrolene reduced the maximum rate of Ca^{2+} release without affecting the Ca^{2+}-sensitivity of the process, and its action was much more evident in the presence of caffeine and adenine nucleotides than in their absence. Doxorubicin-induced Ca^{2+} release was inhibited by azumolene (100 to 400 μM), a water-soluble analog of dantrolene (Tian et al., 1991). The response to dantrolene was temperature-dependent, because Ca^{2+} release was inhibited at 38°C but not at 20°C (Otha et al., 1990). Inhibition of SR Ca^{2+} release was also observed in cardiac muscle, but the sensitivity to dantrolene was lower than in skeletal muscle.

Suarez-Isla et al. (1986) reported that 50 μM dantrolene decreased the Po of a skeletal muscle SR channel. Although the channel was activated by caffeine, it was not inhibited by ruthenium red, and its conductance was much smaller than usually observed for RyRs (maximum Ca^{2+} conductance = 7.9 pS). Therefore, the nature and the physiological role of such channel are not clear. Studies involving typical SR channels have produced conflicting results. Bull and Marengo (1994) reported that halothane-induced activation of frog skeletal muscle RyR was not affected by $\leq$ 100 μM dantrolene. Single-channel studies using porcine and human RyR1 (Nelson et al., 1996) showed a biphasic response to dantrolene: nanomolar (0.5 to 2 nM) dantrolene increased channel Po, owing to increased lifetime of the open channel, whereas 1 to 5 μM dantrolene produced channel inactivation, which was preceded by a transient period of increased activity and was accounted for by an increased lifetime of the closed channel. Similar results were obtained with purified porcine RyR1. The reasons for these discrepancies are not clear and might be related to differences in species, in temperature (about 20°C versus 25°C), or in the stimulus used to activate the channel (Ca^{2+} and halothane versus Ca^{2+} alone).

Ryanodine binding was inhibited by micromolar dantrolene and azumolene, due to reduced affinity with unchanged B_{max} (Pessah et al., 1986; Ogawa and Harafuji, 1990a; Tian et al., 1991). Doxorubicin-stimulated binding was much more inhibited than Ca^{2+}-stimulated or caffeine-stimulated binding (Tian et al., 1991; El-Hayek et al., 1992).

Experiments performed with [^{3}H]dantrolene (Parness and Palnitkar, 1995) have shown that dantrolene binding sites and ryanodine binding sites have a parallel distribution in skeletal muscle membrane fractions. Although high concentrations of dantrolene and azumolene inhibited dihydropyridine binding (El-Hayek et al., 1992), dantrolene binding was not detectable in sarcolemmal or T-tubule membranes. Dantrolene binding did not require Ca^{2+}, and it was inhibited by high ionic strength buffers and by millimolar Mg^{2+}. Recently, selective solubilization techniques have allowed the separation of [^{3}H]ryanodine and [^{3}H]dantrolene binding sites, suggesting that dantrolene does not interact directly with the RyR (Palnitkar and Parness, 1996).

13. Local anesthetics. Procaine and tetracaine inhibited SR Ca^{2+} release induced by Ca^{2+}, caffeine, or quercetin, with IC_{50} of the order of 1 to 2 mM and 0.1 mM, respectively (Endo, 1977; Morii and Tonomura, 1983; Volpe et al., 1983; Xu et al., 1993; Chamberlain et al., 1984b; Meissner, 1984; Antoniu et al., 1985; Palade, 1987a; Lee et al., 1991; Klein et al., 1992; Garcia and Schneider, 1995). A similar action has been observed with other local anesthetics, such as the tertiary amines etidocaine, bupivacaine, prilocaine, lidocaine, and mepivacaine, the quaternary amines QX 572 and QX 314, and the neutral anesthetic benzocaine (Volpe et al., 1983).

In contrast with these findings, early studies had suggested that local anesthetics induced muscle contraction, inhibited the SR Ca^{2+} pump, and stimulated SR Ca^{2+} release (see Herbette et al., 1982; Volpe et al., 1983 for review). In particular, dibucaine was reported to stimulate Ca^{2+}-induced Ca^{2+} release (Yagi and Endo, 1980) or to have a biphasic action, i.e., inhibition of Ca^{2+} efflux at low concentration and stimulation of Ca^{2+} efflux at higher concentration (Nash-Adler et al., 1980). In another study, millimolar tetracaine induced Ca^{2+} release from skeletal muscle SR vesicles. However, the involvement of the RyR was unlikely, because the effect was much more evident in mixed SR vesicles than in RyR-enriched heavy SR vesicles (Shoshan-Barmatz,

1988). Reports of Ca^{2+} release induced by high concentrations of local anesthetics have been tentatively attributed to a nonspecific increase in membrane permeability (Herbette et al., 1982).

Single-channel studies have shown two different actions of local anesthetics. Tetracaine and procaine decreased channel Po by stabilizing a long-lived closed state of the channel, without affecting its unitary conductance (Xu et al., 1993; Zahradnikova and Palade, 1993). The Hill coefficient for Po modulation was ≥ 2, indicating a cooperative action, and the IC_{50} was comparable to that derived from Ca^{2+} release experiments, i.e., 0.1 to 0.6 mM for tetracaine and 4 mM for procaine in skeletal muscle, whereas in cardiac muscle, the IC_{50} for procaine was 0.3 mM. On the other hand, QX 314 elicited a voltage-dependent blockade of the skeletal muscle channel, characterized by reduced conductance with unchanged Po. Voltage-dependent blockade also was observed when millimolar concentrations of procaine or tetracaine were used in the presence of 2 μM ryanodine, which induced the formation of a low-conductance open state (Xu et al., 1993). In cardiac preparations, voltage-dependent blockade was produced by QX 222 (another quaternary amine) and by 3 mM procaine, whereas QX 314 induced the formation of a subconductance open state (Tinker and Williams, 1993a). The latter action resembled the effect of large tetra-alkylammonium cations and has been attributed to a sterical or electrostatic obstruction in the conduction pathway (Tinker et al., 1992a; Tinker and Williams, 1993b).

Voltage-dependent blockade characterized by reduced channel conductance without apparent changes in Po has been observed also with cocaine, which has local anesthetic properties (Tsushima et al., 1996). Because the apparent K_D was 38 mM at 0 mV, this action is unlikely to have clinical importance in overdose victims, in whom cocaine levels are in the micromolar range.

Ryanodine binding studies showed multiple actions of local anesthetics. Both in skeletal muscle (Shoshan-Barmatz and Zchut, 1993) and in brain microsomes (Martin et al., 1993), tetracaine inhibited ryanodine binding by decreasing the B_{max}, without changing the K_D (IC_{50} in the submillimolar range). The inhibition decreased at high ionic strength and was counteracted by ATP (Shoshan-Barmatz and Zchut, 1993), wheras it was not affected by caffeine (Martin et al., 1993). Interaction with the ATP binding site was suggested by the observation that tetracaine inhibited the binding of the photoreactive ATP analogue α-^{32}P-benzoyl-ATP. Procaine (10 mM) also decreased ryanodine binding (Ogawa and Harafuji, 1990a). Dibucaine had a biphasic action, because ryanodine binding was stimulated at low (< 0.3 mM) concentrations and inhibited at higher concentrations. Lidocaine and its analogue QX 314 stimulated ryanodine binding by decreasing the K_D, without any change in the B_{max}. In kinetic experiments, the increased affinity for ryanodine was accounted for by an increased association rate, while the dissociation rate was unaffected.

In summary, local anesthetics show at least two different actions. (*a*) Channel inhibition, due to decreased Po and associated with reduced ryanodine binding. Such action, produced by tetracaine and procaine, is likely to be mediated by a high-affinity binding site, corresponding to or interacting with the Ca^{2+}-binding and adenine nucleotide binding sites. (*b*) Voltage-dependent channel blockade, characterized by reduced channel conductance. This action occurred with lidocaine, with quaternary amines, and, possibly, with high concentrations of procaine and tetracaine; it was associated with increased affinity for ryanodine, and it is supposed to be mediated by a lower-affinity site, located close to the conductive pathway. Additional effects cannot be excluded. In particular, as mentioned above, high concentrations of local anesthetics might affect SR Ca^{2+} release by a nonspecific action on membrane permeability.

14. Phenylalkylamines. Phenylalkylamine Ca^{2+} antagonists, i.e., verapamil, gallopamil, and amipamil, are well known blockers of the sarcolemmal L-type Ca^{2+} channel (Spedding and Paoletti, 1992; McDonald et al., 1994). Several investigations have provided evidence that phenylalkylamines may affect excitation-contraction coupling directly, i.e., independently of the modulation of the sarcolemmal Ca^{2+} current (Zucchi, 1996).

Fernandez-Belda and Gomez-Fernandez (1987) studied the release of Ca^{2+} induced by tetraphenylboron in a skeletal muscle model and concluded that verapamil and diltiazem (a benzothiazepine Ca^{2+} antagonist) blocked the pathway responsible for Ca^{2+} release. The action occurred in the micromolar concentration range, and the K_D for verapamil was 8 μM. In similar experimental models, dihydropyridines were ineffective (Fernandez-Belda and Gomez-Fernandez, 1987; Ohkusa et al., 1991). Indirect studies also provided interesting results. Ca^{2+} antagonists have no major effect in skeletal muscle under physiological conditions. However, at low temperature (5 to 7°C), gallopamil was able to paralyze the frog skeletal muscle: after a conditioning K^+-induced contracture, further contraction was inhibited (Eisenberg et al., 1983), although a normal response was restored after membrane hyperpolarization (Berwe et al., 1987; Feldmeyer et al., 1990). Contrary to L-channel blockade, this action occurred at low temperature, but not at room temperature (Eisenberg et al., 1983). It was not reproduced by Cd^{2+}, an inorganic calcium channel blocker (Berwe et al., 1987), and it showed a bell-shaped dose-response relationship.

Indirect evidence of a SR action of gallopamil has been obtained also in cardiac muscle. Zucchi et al. (1992b) observed that low (0.01 to 0.3 μM) concentrations of gallopamil stimulated Ca^{2+} uptake. The stimulation was not additive with that produced by ryanodine, suggesting that gallopamil blocked Ca^{2+} release, rather than enhancing active Ca^{2+} transport. Consistent with

this result, Ca^{2+}-ATPase activity was not affected. The response to gallopamil was biphasic, because concentrations > 1 μM were ineffective, in accordance with the results of earlier studies, in which submicromolar concentrations had not been tested (Entman et al., 1972; Nayler and Szeto, 1972; Watanabe and Besch, 1974; Colvin et al., 1982; Wang et al., 1984; Movsesian et al., 1985). Similar results were obtained with verapamil and diltiazem, whereas nifedipine was ineffective (Zucchi et al., 1992a).

In a single-channel study, Valdivia et al. (1990b) observed that RyR1 Po decreased significantly in the presence of 50 μM verapamil, due to reduced lifetime of the open channel and reduced burst time.

Binding studies showed that gallopamil or verapamil did not affect high-affinity ryanodine binding either in skeletal muscle or in cardiac muscle (Pessah et al., 1985), except possibly in pathological states or under peculiar experimental conditions, because high verapamil concentrations (>100 μM) increased the affinity of ryanodine binding in the cardiomyopathic Syrian hamster (Finkel et al., 1993), and verapamil produced a moderate inhibition of ryanodine binding (< 20%) in skeletal muscle if the assay was performed at alkaline pH and at low ionic strength (Valdivia et al., 1990b). On the other hand, in cardiac microsomes, gallopamil inhibited low affinity ryanodine binding, with IC_{50} in the micromolar range (Zucchi et al., 1995a). Kinetic analysis showed that gallopamil slowed the dissociation rate of ryanodine from the low-affinity site(s) and potentiated the effect of micromolar ryanodine on the dissociation of 4 nM [^{3}H]ryanodine from the high-affinity site. It was concluded that gallopamil modulates the cooperativity of ryanodine binding and/or the transition to a receptor state characterized by slow ryanodine dissociation.

The molecular site of action of phenylalkylamines has not been determined. Because the dihydropyridine and RyRs remain associated, even in subcellular preparations (Marty et al., 1994), the action might be mediated by the former. However, the gating of solubilized SR calcium channels by verapamil (Valdivia et al., 1990b), and the observation that the ratio of phenylalkylamine to nitrendipine binding sites was higher in microsomal than in sarcolemmal preparations (Garcia et al., 1984), are difficult to reconcile with this hypothesis and instead suggest that phenylalkylamines interfere directly with the RyR. Oeken et al. (1986) observed that [^{3}H]verapamil binding was enriched in cardiac SR membranes and proposed the existence of an SR binding site for phenylalkylamines. However, this putative site appeared to be associated with free SR rather than junctional SR, and its relationship to the RyR is unknown.

In summary, the bulk of evidence suggests that, at equilibrium, gallopamil and verapamil are effective at micromolar or even submicromolar concentrations and that the dose-response relationship is bell-shaped. The latter finding has been interpreted in the context of a modulated receptor hypothesis, with speculation that low drug concentrations stabilize the inactive state of the SR channel, whereas higher concentrations stabilize also the active state (Fill and Best, 1989; Rios and Pizarro, 1991). The issue of the effective concentration range is critical with regard to the clinical relevance of RyR modulation. The plasma concentrations of verapamil and gallopamil in human patients, assuming conventional therapeutic dosages, are in the range of 0.1 to 0.5 μM and 0.02 to 0.1 μM, respectively (Brogden and Benfield, 1994), but phenylalkylamines enter myocardial cells (Hescheler et al., 1982), and their intracellular concentration might be higher than the plasma one (Pang and Sperelakis, 1983).

15. Peptides. A fraction enriched in peptides of estimated molecular weight of 5 to 8 kDa obtained from the venom of the scorpion *Buthotus hottentota* stimulated ryanodine binding in SR preparations obtained from skeletal muscle, heart or brain (Valdivia et al., 1991a). The chief effect was an increase of the B_{max}, with a slight reduction of the K_D, and the estimated dissociation constant of the peptide/RyR complex was 20 to 30 nM. Peptide-stimulated binding retained a normal Ca^{2+}-dependence, and the stimulation was synergic with that produced by caffeine. Single-channel experiments showed an increased open lifetime and the appearance of a subconductance state. Contrary to the action of ryanodine, these effects were quickly reversible.

Other RyR-modulating peptides have been purified from the venom of the scorpion *Pandinus imperator*. An 8.7-kDa peptide, imperatoxin-a, stimulated ryanodine binding by increasing the B_{max} ($EC_{50} \approx 6$ nM), whereas the K_D was unchanged, and its action was selective for the skeletal muscle isoform (Valdivia et al., 1992b; El-Hayek et al., 1995a). Similar results were obtained after RyR purification. As observed with caffeine, the stimulation of ryanodine binding was greater at low than at high Ca^{2+} concentration. In single-channel experiments, the response to imperatoxin-a was rapid and reversible, and consisted in increased channel Po due to decreased mean closed time.

Another *Pandinus imperator* venom 10.5 kDa peptide, designated imperatoxin-i, decreased ryanodine binding ($IC_{50} \approx 10$ nM) and single-channel Po both in skeletal muscle and in cardiac preparations (Valdivia et al., 1992b). Although its action was rather specific for the RyR, in skeletal muscle preparations the binding of the dihydropyridine PN 200-110 was also partially inhibited.

A 42-residue peptide purified from the venom of *Crotalus viridis viridis*, named myotoxin-a, increased SR Ca^{2+} release in skeletal muscle, at 0.1 to 10 μM concentration (Furukawa et al., 1994). The Ca^{2+}-dependence of its action resembled that of adenine nucleotides, and synergism with caffeine was observed. Myotoxin-induced Ca^{2+} release was stimulated by low concentrations of spermine, and it was inhibited by high concen-

trations of spermine and by Mg^{2+} and ruthenium red, whereas it was only slightly inhibited by procaine. Binding experiments showed that myotoxin-a did not affect ryanodine binding and that labeled myotoxin-a did not interact with the purified RyR, suggesting that its target was a regulatory protein different from the RyR (Okhura et al., 1995). Myotoxin-a binding was inhibited by spermine, K^+ and Na^+, but it was not affected by Ca^{2+}, Mg^{2+}, ruthenium red, procaine, and 9-methyl-7-bromoeudistomin D.

Helothermine is a 25-kDa cysteine-rich protein derived from the salivary secretion of the lizard *Heloderma horridum horridum* (Morrissette et al., 1995). In single-channel experiments, micromolar helothermine decreased the Po of skeletal muscle and cardiac channels, by increasing the lifetime of the closed channel and decreasing the lifetime of the open channel. Binding experiments showed that helothermine reversibly displaced ryanodine.

Ryanotoxin is a 11.4-kDa peptide that has been recently isolated from the venom of the scorpion *Buthotus judaicus* (Morrissette et al., 1996). Its action was similar to that of ryanodine, because micromolar ryanotoxin stimulated Ca^{2+} release from skeletal muscle SR, induced a state of reduced conductance in single-channel recordings, and increased the affinity for [^{3}H]ryanodine (EC_{50} = 0.16 μM), without affecting the B_{max}.

Indirect evidence for RyR modulation has been reported for other peptides. Mellitin (100 nM), the major component of bee venom, decreased the threshold of Ca^{2+} release in skeletal muscle SR preparations which were subjected to successive Ca^{2+} additions until Ca^{2+} release was induced (Fletcher et al., 1992). However, mellitin did not affect [^{3}H]ryanodine binding. Stimulation of SR Ca^{2+} release was produced also by *Naja naja kaouthia* cardiotoxin (Fletcher et al., 1991a).

16. Agents producing covalent modifications.

a. SULFHYDRYL REAGENTS. Heavy metals and mercurials induce Ca^{2+} release from heavy SR preparations (Abramson et al., 1983, 1995; Bindoli and Fleischer, 1983; Salama and Abramson, 1984; Trimm et al., 1986; Palade, 1987b; Brunder et al., 1988; Nagura et al., 1988; Tatsumi et al., 1988; Abramson and Salama, 1989; Prabhu and Salama, 1990a; Salama et al., 1992; Boraso and Williams, 1994; Tanaka and Tashjian, 1994). This effect was produced at micromolar concentrations (e.g., 4 μM Hg^{2+}, 10 to 50 μM Ag^+, 2 μM Cu^{2+}, 15 μM Cd^+, 20 μM Zn^{2+}, 5 to 50 μM mersalyl, and 5 to 50 μM *p*-chloromercuribenzoate), and it was inhibited by the thiol-reducing agent dithiothreitol (DTT), suggesting that it was mediated by sulfhydryl oxidation. In accordance with this hypothesis, classical sulfhydryl reagents also induced Ca^{2+} release, which was blocked by DTT or glutathione. Such agents include N-ethylmaleimide (1 to 30 mM), thimerosal (200 to 400 μM), 5-5′-dithiobis-(2-nitrobenzoic acid) (> 300 μM), and the highly specific thiol reagents 2,2′-dithiodipyridine (2,2′-DTDP) (> 2.5 μM), 4,4′-DTDP (> 2.5 μM) and N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (25 to 100 μM) (Bindoli and Fleischer, 1983; Palade, 1987b; Nagura et al., 1988; Zaidi et al., 1989a; Prabhu and Salama, 1990b; Abramson et al., 1995).

Ca^{2+} release induced by sulfhydryl reagents was blocked by ruthenium red, tetracaine, or procaine, and it was dependent on pH, ionic strength, and Mg^{2+} concentration. Adenine nucleotides inhibited the oxidation of SR thiols, but stimulated Ca^{2+} release once it was induced (Salama and Abramson, 1984; Nagura et al., 1988; Stuart and Abramson, 1988; Zaidi et al., 1989a; Prabhu and Salama, 1990a,b). These observations are consistent with the hypothesis that the target of sulfhydryl reagents is the RyR.

In some experiments, sulfhydryl reagent-induced Ca^{2+} release showed a fast and a slow component, and the latter was not blocked by ruthenium red. Because sulfhydryl reagents can inhibit the SR Ca^{2+}-ATPase, it has been suggested that the inactivated pump might act as a pathway for Ca^{2+} release (Gould et al., 1987). However, measurements performed after pump inhibition by vanadate have shown that the contribution of this mechanism to Ca^{2+} release was negligible (Brunder et al., 1988). Intriguing findings have been obtained with biotinylated SPDP. Biotinylation identified a 106-kDa protein (Zaidi et al., 1989b; Hilkert et al., 1992) that contained a Ca^{2+}-activated, Mg^{2+}-sensitive, and ruthenium red-sensitive channel, which did not appear to be a proteolytic fragment nor a subunit of the RyR, because polyclonal antibodies obtained against the 106-kDa protein did not cross-react with the RyR. The nature and functional role of this protein remain unclear.

Although sulfhydryl oxidation was associated usually with stimulation of Ca^{2+} release, some authors observed that high concentrations of heavy metals inhibited Ca^{2+} release (Prabhu and Salama, 1990a). A biphasic effect of thiol oxidation has been observed also in single-channel and ryanodine binding experiments. In single-channel recordings, 100 μM thimerosal increased channel Po, but with 1 mM thimerosal, channel activation was transient and was followed by a persistent inactivation (Abramson et al., 1995). In binding experiments, a slight increase in ryanodine binding was produced by low concentrations (0.1 to 0.3 μM) of 4,4′-DTDP or 2,2′-DTDP, but higher concentrations reduced ryanodine binding with IC_{50} of the order of 1.5 to 7.5 μM. Thimerosal and SPDP had a monophasic inhibitory action, with IC_{50} = 50 μM and 15 μM, respectively (Zaidi et al., 1989a; Abramson et al., 1995). In the case of thimerosal, it was observed that the K_D was not modified, whereas the B_{max} was reduced.

Other lines of evidence suggest that sulfhydryl oxidation can inactivate the RyR: prolonged exposure to micromolar ryanodine promoted channel inactivation and reduced ryanodine binding, which was prevented by DTT (Zimanyi et al., 1992); doxorubicin-induced stimulation of ryanodine binding was further increased by

DTT (Pessah et al., 1990), and in single-channel experiments, the inactivation caused by prolonged exposure to doxorubicin was prevented by DTT (Ondrias et al., 1990).

Other oxidizing agents can modulate SR Ca^{2+} release by interacting with thiol groups. H_2O_2 induced SR Ca^{2+} release, increased channel Po, and stimulated ryanodine binding (Boraso and Williams, 1994; Favero et al., 1995a). In single-channel experiments, H_2O_2 increased the lifetime of the open channel, decreased the lifetime of the closed channel, and allowed channel openings to occur even at subactivating Ca^{2+} concentration. Binding experiments showed higher affinity for ryanodine, increased B_{max}, and enhanced Ca^{2+}-sensitivity of the binding reaction. These responses were inhibited by DTT, suggesting the involvement of sulfhydryl oxidation. In bilayer and binding experiments, H_2O_2 was effective at 0.1 to 0.6 mM concentration, whereas millimolar concentrations were necessary in release experiments, probably because such experiments were not performed under equilibrium conditions. As observed with other sulfhydryl reagents, 10-fold to 100-fold higher concentrations of H_2O_2 caused an irreversible decrease in ryanodine binding and in channel Po (Favero et al., 1995a).

Free-radical-generating systems, such as xanthine-xanthine oxidase and iron-ascorbate, increased channel Po (Okabe et al., 1991; Stoyanovsky et al., 1994), but in these models, the involvement of sulfhyrdyl groups has not been demonstrated. Stimulation of Ca^{2+} release, which was inhibited by DTT or glutathione, has been observed with phthalocyanine dyes and alcian blue (Abramson et al., 1988b; Koshita et al., 1993).

The different responses to sulfhydryl reagents suggest that RyRs contain several classes of reactive sulfhydryls, possibly located in different domains, whose oxidation produces different functional consequences. Labeling with a coumarin maleimide identified highly reactive sulfhydryls on the RyR and on triadin (Liu et al., 1994). The accessibility of these groups was enhanced under conditions favoring channel closure and was decreased under conditions favoring channel opening. In addition, sulfhydryl oxidation stabilized a high molecular weight complex between RyR and triadin. On the basis of these findings, it has been speculated that sulfhydryl oxidation might be involved in the physiological mechanism of channel activation, possibly by modulating RyR-triadin interaction (Trimm et al., 1986; Abramson and Salama, 1989; Salama et al., 1992; Liu and Pessah, 1994). In contrast with this hypothesis, in permeabilized voltage-clamped frog skeletal muscle, excitation-contraction coupling was not affected by DTT nor by other reducing agents, and Ca^{2+} release induced by caffeine and other activators was unaffected also (Brunder et al., 1988).

b. OTHER COVALENT REAGENTS. The porphyrin derivative mesotetra-(4-N-methylpyridyl)-porphine tetraiodide (TMPyP), which is structurally similar to phthalocyanine dyes, stimulated Ca^{2+} release from skeletal muscle SR vesicles with EC_{50} = 18 μM (Abramson et al., 1993). The anionic and deiodinated phorphyrin tetrasodium-mesotetra-(4-sulfonatophenyl)-porphine dodecahydrate was also effective in inducing SR Ca^{2+} release. TMPyP-induced Ca^{2+} release retained the physiological modulation by Ca^{2+}, Mg^{2+}, and adenine nucleotide. It was inhibited by ruthenium red, but not by 1 mM DTT. In binding experiments, 30 μM TMPyP stimulated high-affinity ryanodine binding by increasing the B_{max} and by slightly reducing the K_D. Single-channel recordings showed increased Po, which was not affected by DTT. Therefore, it seems unlikely that sulfhydryl oxidation was involved in the response to TMPyP.

Disulfonic stilbene derivatives are amino-group modifiers and affect the gating of several ion channels. In bilayer experiments, 100 μM 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid and 4-acetoamido-4′-isothiocyanostilbene-2,2′-disulfonic acid induced the appearance of an irreversibly open state with unitary conductance. This action was observed both in skeletal muscle (Kawasaki and Kasai, 1989) and in cardiac (Zahradnikova and Zahradnik, 1993) preparations, and it was attributed to a covalent modification. However, in some experiments, the instantaneous appearance of a ruthenium red-inhibitable open state suggested that channel activation could also be induced by noncovalent interactions.

Dicyclohexylcarbodiimide is a hydrophobic carboxyl-reacting compound. At concentrations ≥ 25 μM (EC_{50} = 0.3 mM), it decreased ryanodine binding and inhibited Ca^{2+} release induced by alkalynization, triphenylboron, and sulfhydryl reagents (Shoshan et al., 1981; Yamamoto and Kasai, 1982; Chiesi, 1984; Shoshan-Barmatz, 1987; Argaman and Shoshan-Barmatz, 1988; Michalak et al., 1988). Lower concentrations (10 μM) did not affect channel activation by Ca^{2+}, but prevented the inactivation produced by Mg^{2+}, ruthenium red, or high Ca^{2+} concentration (Martinez-Azorin et al., 1993). Labeling experiments showed minimal dicyclohexylcarbodiimide incorporation in high molecular weight proteins, suggesting that it might not act directly on the RyR (Argaman and Shoshan-Barmatz, 1988).

Fluorescin-5′-isothiocyanate, which reacts with lysine ϵ-amino groups, inhibited ryanodine binding (IC_{50} = 20 μM) without affecting the affinity for ryanodine, whereas in single-channel experiments, it increased channel Po and induced the appearance of subconductance states (Orr et al., 1993). Acetic or maleic anhydride, also thought to react with lysine amino groups, induced rapid Ca^{2+} release (Shoshan-Barmatz, 1986).

Like fluorescin-5′-isothiocyanate, the histidyl reagent diethylpyrocarbonate (0.1 to 1 mM) decreased ryanodine binding (reduced B_{max} with unchanged K_D), and yet favored Ca^{2+} release (Aoki and Oba, 1989; Shoshan-Barmatz and Weil, 1994). Ca^{2+} release was induced also by ethoxyformic anhydride, another histidine-modifying

reagent (Stuart et al., 1992). It has been suggested that these amino acid reagents may react with residues located in the ryanodine binding site, thus preventing ryanodine binding and, at the same time, reproducing the effect of nanomolar ryanodine, i.e., channel activation.

The photo-oxidation of rose bengal generates singlet oxygen. This experimental system produced SR Ca^{2+} release and increased channel Po (Holmberg et al., 1991; Stuart et al., 1992; Xiong et al., 1992). The response to rose bengal had some peculiarities, because the stimulation of Ca^{2+} release was not inhibited by Mg^{2+} and was associated with reduced ryanodine binding (Stuart et al., 1992). In another study, prolonged exposure to rose bengal eventually produced channel inactivation (Holmberg et al., 1991). The molecular target of rose bengal photo-oxidation is probably a histidyl residue.

17. Others. High concentrations of propranolol inhibited Ca^{2+} accumulation and yet stimulated Ca^{2+}-ATPase activity, which was interpreted as evidence of an activation of Ca^{2+} efflux (Shoshan-Barmatz, 1988). Recently, it has been reported (Zchut et al., 1996) that 1 to 2 mM propranolol increased the B_{max} for ryanodine in skeletal muscle junctional SR, without any change in the K_D. However, this effect was not observed with purified RyR1, and at low ionic strength (0.2 M NaCl), ryanodine binding was inhibited. Under these experimental conditions, propranolol caused a partial loss of the Ca^{2+} dependence and pH dependence of ryanodine binding, and its effect was antagonized by ATP. Single-channel experiments confirmed that 0.2 mM propranolol elicited complete channel blockade. The subsequent addition of ATP reactivated the channel but induced the appearance of a low conductance state. Therefore, propranolol appears to have complex actions that have not been completely clarified. In any case, RyR modulation was observed only at very high concentrations, and it should not have any clinical relevance.

The observation that calmodulin inhibited Ca^{2+} release (see II.A.7.b.) promoted the investigation of calmodulin antagonists. Meissner (1986a) reported that trifluoperazone, calmidazolium, and compound 48/80 did not affect SR Ca^{2+} release when used at concentrations able to inhibit calmodulin-mediated interactions in other systems (respectively, 25 μM, 5 to 25 μM, and 5 to 25 μg/ml). However, Ca^{2+} release was stimulated at higher concentrations, i.e., 40 to 500 μM trifluoperazone, 100 μM calmidazolium, and 20 to 70 μg/ml compound 48/80 (Chamberlain et al., 1984b; Wyskovsky et al., 1988; Vale, 1990). Ca^{2+} release was inhibited by Mg^{2+} and ruthenium red, suggesting that it was not due to a nonspecific increase in membrane permeability. A biphasic action was observed with N-(6-aminohexyl)-5-chloro-1-naphtalene sulfonamide (W7): 10 μM W7 reduced Po, but 100 μM W7 reactivated the channel (Smith et al., 1989). Chlorpromazine (160 to 200 μM) was also effective, whereas other calmodulin antagonists such as bepridil and felodipine were not (Bindoli and Fleischer, 1983; Palade, 1987b; Wyskovsky et al., 1988). On the whole, although some calmodulin antagonists may affect SR Ca^{2+} release, calmodulin antagonism does not appear to be involved in such an action.

BisG10 (1,10-bis-guanidino-*n*-decane) is a blocker of the SR K^+ channel. In skeletal muscle, 70 to 360 μM bisG10 inhibited Ca^{2+}-induced and caffeine-induced Ca^{2+} release (Allard et al., 1992). This action was not accounted for by inhibition of K^+ counter-transport, suggesting direct interaction with the RyR, which was confirmed by the observation that bisG10 inhibited ryanodine binding. The inhibition was characterized by reduced B_{max} with unchanged K_D.

Inhibition of Ca^{2+} release by the K^+-ATP channel opener pinacidil was suggested by its effect on the ryanodine-sensitive outward current in vascular smooth muscle (Xiong et al., 1991). However, in skeletal muscle, the K^+-ATP opener cromakalim and the K^+-ATP blocker glibenclamide had no effect on caffeine-induced Ca^{2+} release (Ishida et al., 1992). In the same preparation, aspecific K^+ blockers such as 4-aminopyridine and tetraethylammonium chloride induced SR Ca^{2+} release, but this was attributed to SR depolarization. Therefore, there is no clear evidence of any direct action of these K^+ blockers on the RyR.

Micromolar concentrations of the triazine dyes cibacrom blue F3A-G (reactive blue 3) and reactive red 120 induced Ca^{2+} release from skeletal muscle SR vesicles and increased channel Po in bilayer experiments (Xu et al., 1989). Benzimidazole dyes such as hoechst 33342 and hoechst 33258 inhibited the SR channel incorporated into lipid bilayers. However, in Ca^{2+} release experiments, these two compounds had opposite effects, because the former inhibited and the latter stimulated Ca^{2+} release (Beeler and Gable, 1993b).

Several other substances have been used to induce SR Ca^{2+} release, but their action has not been fully characterized. These include quercetin (Kim et al., 1983; Antoniu et al., 1985; Palade, 1987b), originally used as Ca^{2+}-ATPase inhibitor; the fungicides miconazole, clotrimazole and ketonazole (Palade, 1987b); and the alcohols 1-heptanol and 1-octanol (Ma et al., 1988). Other compounds have been suggested to modulate the RyR on the basis of indirect studies, usually relying on the measurement of tension development, which still need confirmation by direct methods. These include the putative activators DPI 201-106 (Kihara et al., 1989), KT-362 (Kodama and Shibata, 1991), and cyclosporin A (Banijamali et al., 1993), the putative inhibitors TMB-8 (Himmel and Ravens, 1990), 2,3-butanedione monoxime (Gwathmey et al., 1991), and methylenedioxyndenes (Rahwan, 1985).

Ca^{2+} release can be induced in skeletal muscle SR by replacing permeable cations with impermeable ones, or by replacing impermeable anions with permeable ones. This phenomenon occurs only when the connection be-

tween the junctional SR and the sarcolemmal T-tubule is preserved. After T-tubule dissociation, Ca^{2+} release was still induced by caffeine or other drugs, but not by ion replacement (Ikemoto et al., 1984), suggesting that the latter acted indirectly by depolarization of the T-tubule membrane.

C. Overview of the Mechanisms of Ryanodine Receptor Modulation

The investigations reviewed above (II.A. and II.B.) have shown that the RyR is the target of multiple agents having complex pharmacological actions. The assessment of a putative modulator requires dose-response studies of its effect on Ca^{2+} release, single-channel gating, ryanodine binding (by equilibrium and kinetic experiments), and of its interaction with other modulators. Such a thorough evaluation has not been performed for most modulators. However, some general patterns of RyR modulation can be tentatively identified. The proposed classification, the major features of which are outlined in table 3, is based on pharmacological actions, rather than chemical structure of agents, because some agents show multiple actions, possibly mediated by interaction with different molecular sites.

A special position (class I) is reserved to ryanodine-like action, both for historical reasons and for the peculiar properties of ryanodine, which affects both channel conductance and channel gating, leading to a biphasic effect on Ca^{2+} release, as described in detail above (B.1.). Agents that modulate channel gating but do not affect channel conductance are included in classes II-V, whereas agents that decrease channel conductance without affecting the Po are included in class VI. An additional group (class VII) is defined by a peculiar action, namely delayed persistent channel inactivation.

Class II action (typical activation) is characterized by channel activation due to increased Po (increased lifetime of the open channel and/or decreased lifetime of the closed channel) with unchanged channel conductance and increased affinity for [^{3}H]ryanodine. Several patterns can be distinguished. Ca^{2+}-activation deserves special emphasis because Ca^{2+} is the physiological activator of the RyR. Increased Po is mainly due to decreased lifetime of the closed channel, and in binding experiments, both the density of ryanodine binding sites and the affinity for ryanodine are increased, the latter due to an increased association rate. Another pattern is characterized by Ca^{2+}-sensitization, i.e., substantial stimulation of Ca^{2+} release at suboptimal Ca^{2+} concentration, with minimal stimulation at optimal Ca^{2+} concentration. In single-channel experiments, the main effect is a decreased lifetime of the closed channel, although, at higher drug concentration, the lifetime of the open channel may be increased. In binding experiments, the affinity for ryanodine is increased, while the B_{max} is either unchanged or slightly increased. Such modes of action are shown by caffeine, 9-methyl-7-bromoeudistomin D, sulmazole, doxorubicin, and possibly by digoxin. The third pattern is characterized by (*a*) substantial stimulation of Ca^{2+} release at high Ca^{2+} concentrations; (*b*) parallel effect on open and closed states, with increased lifetime of the open channel and decreased lifetime of the closed channel; and (*c*) greater increase in the B_{max} for ryanodine. This is the effect of adenine nucleotides, of suramin, and, possibly, of some oxidizing agents, e.g., H_2O_2.

These substances appear to interact at closely related molecular sites. On the basis of indirect evidence, Pessah et al. (1987) suggested the existence of distinct but interacting sites for Ca^{2+}-Mg^{2+}, adenine nucleotides,

TABLE 3
Ryanodine receptor modulation: Functional classification

	Ca^{2+} Release	γ	Po	K_D	Examples
Class I	increased or decreased[a]	decreased	increased	—	ryanodine
Class II	increased	unchanged	increased[b]	decreased	Ca^{2+} caffeine, MBED, sulmazole, doxorubicin adenine nucleotides, suramin
Class III	increased	unchanged	different from class II		halothane, heparin, bastadins
Class IV	decreased	unchanged	decreased[c]	increased	Mg^{2+}, ruthenium red, aminoglycosides, FLA 365
Class V	decreased	unchanged	different from class IV		procaine, tetracaine phenylalkylamines dantrolene
Class VI	decreased	decreased	unchanged	variable	spermine, QX 314
Class VII	——— delayed persistent channel inactivation[d] ———				doxorubicin, ruthenium red, neomycin

[a] Ryanodine has a double effect in release experiments, as detailed in the text.
[b] Due to decreased lifetime of the closed channel and/or increased lifetime of the open channel.
[c] Due to increased lifetime of the closed channel and/or decreased lifetime of the open channel.
[d] This action is produced after prolonged incubation with several agents whose immediate effect belongs to a different class.
γ, channel conductance; K_D, [^{3}H]ryanodine dissociation constant.

and caffeine. Additional sites are likely to exist. For instance, although doxorubicin and caffeine have a similar action, they do not interact with the same site (Abramson et al., 1988a).

Class III action (atypical activation) is defined as stimulation of Ca^{2+} release with unchanged channel conductance, which does not fulfill the criteria set for class II action. Such behavior is shown, for instance, by halothane, which does not modify the affinity for ryanodine, although it seems to increase the number of ryanodine binding sites, and by heparin, which does not affect ryanodine binding except possibly at very high concentrations. Class III agents should also include hexachlorocyclohexane and rose bengal, which inhibit ryanodine binding, and bastadins, which stabilize both the open and the closed states of the channel. On a molecular level, different mechanisms of action may produce an atypical activation, e.g., halothane might have a nonspecific membrane action, heparin might increase local Ca^{2+} concentration close to the Ca^{2+} activation site, and bastadins might interact with FK binding proteins.

Class IV action (typical inhibition), is the opposite of class II action, i.e., channel inhibition due to reduced Po with unchanged conductance and decreased affinity for [^{3}H]ryanodine. These effects are produced by the "physiological" inhibitor Mg^{2+} and by millimolar Ca^{2+}. Other typical inhibitors include ruthenium red, aminoglycosides, and FLA365. Ca^{2+} and Mg^{2+} act at divalent cations sites that seem to overlap at least in part with the polyamine binding sites. It has been suggested that the polyamine binding site and the FLA365 binding site may represent different subregions of the ryanodine binding site (Mack et al., 1992). However, these conclusions are largely speculative, and the concept that all polyamines interact with the same site has been challenged (Kang et al., 1992).

Class V action (atypical inhibition) is defined as inhibition of Ca^{2+} release with unchanged channel conductance, which does not fulfill the criteria set for class IV action. For instance, low concentrations of procaine and tetracaine decrease ryanodine binding but do not modify the affinity for ryanodine, whereas phenylalkylamines affect low-affinity rather than high-affinity ryanodine binding. Dantrolene action should also be considered as atypical, because it shows peculiar properties, namely preferential inhibition of doxorubicin-induced versus Ca^{2+}-induced Ca^{2+} release, and complex, probably biphasic effects on single-channel gating.

Class VI action (channel blockade) is characterized by reduced conductance of the open channel with unchanged Po, as produced, for instance, by spermine and quaternary amines. Ryanodine binding is usually stimulated, and these agents are likely to interact with site(s) located close to the conduction pathway of the channel pore.

Prolonged incubation with micromolar ryanodine or other modulators determines a persistent loss of channel function associated with reduced ryanodine binding. This action has been observed with substances that show different acute effects, such as doxorubicin, ruthenium red, and neomycin, and it might be mediated by sulfhydryl oxidation, because, in several experimental models, it was prevented by disulfide-reducing agents. As discussed above (II.B.16.a.), it is likely that a similar process may account for the reduction in ryanodine binding observed after prolonged incubation with sulfhydryl-oxidizing compounds. Because the available evidence suggests that the molecular mechanisms involved in delayed channel inactivation are different from those that are responsible for acute effects, we suggest that the former be considered as a separate and independent (class VII) action.

An important issue is the observation that some modulators have a selective action on a specific RyR isoform. RyR1 selectivity has been described with palmitoyl carnitine, bastadins, and imperatoxin-a, whereas digoxin and possibly adenosine are selective for RyR2. However, several modulators have not been tested comparatively in different tissues, and their selectivity is unknown.

III. The Ryanodine Receptor in Disease

A. Myocardial Ischemia and Reperfusion

Myocardial ischemia is associated with important modifications of intracellular Ca^{2+} homeostasis (Lee et al., 1987; Steenbergen et al., 1987). Whereas Ca^{2+} transients undergo complex time-dependent changes, mean cytosolic Ca^{2+} increases progressively during the first seconds or minutes of ischemia (Lee and Allen, 1992). Cytosolic Ca^{2+} overload contributes to the development of cellular injury by multiple mechanisms, such as the activation of Ca^{2+}-dependent proteases and phospholipases (Opie, 1989; Silverman and Stern, 1994). Postischemic reperfusion may lead to either a progressive normalization of intracellular Ca^{2+} homeostasis, which is associated with functional recovery, or an exacerbation of Ca^{2+} overload, which is associated with the development of irreversible cellular injury. Due to the importance of Ca^{2+} in the pathophysiology of ischemic injury, the effects of ischemia and reperfusion on the systems involved in Ca^{2+} homeostasis have raised special interest. The effect of myocardial ischemia on SR function has been reviewed recently by Mubagwa (1995).

The function of RyR2 in experimental models of ischemia and reperfusion has been evaluated initially on the basis of indirect methods, namely the effect of channel blockers such as ryanodine or ruthenium red on oxalate-supported Ca^{2+} uptake. The results have not been unequivocal: whereas Ca^{2+} uptake, as measured in the absence of inhibitors, was consistently reduced, the extent of the stimulation produced by ryanodine or ruthenium red was either decreased (Limbruno et al., 1989),

unchanged (Rehr et al., 1991; Kaplan et al., 1992; Zucchi et al., 1994), or increased (Feher et al., 1989; Abdelmeguid and Feher, 1994; Wu and Feher, 1995). Davis et al. (1992) reported decreased response to ruthenium red and increased response to ryanodine in homogenates obtained from ischemic rat hearts. In the isolated rat heart preparation studied by Tani et al. (1996), the stimulation of Ca^{2+} uptake produced by ryanodine was increased after prolonged (40 min) ischemia, but it was reduced after brief (10 min) hypoxia. Reasons for these discrepancies remain to be defined. It has been claimed that the use of purified SR fractions may introduce a bias, because the preparative procedures result in the loss of more than 90% of the Ca^{2+} uptake activity and might select vesicles that are not representative of the whole SR (Feher et al., 1980; Rapundalo et al., 1986). However, different results were obtained, even in unfractionated homogenates. Another potential bias is related to SR disruption during tissue homogenization. As mentioned above (I.B.4.), only part of the SR vesicles formed after homogenization contain RyRs, and channel-containing vesicles cannot accumulate Ca^{2+} unless the channel is blocked (Jones and Cala, 1981; Feher and Lipford, 1985). Therefore, a different distribution of RyRs between SR vesicles, possibly produced by different homogenization and fractionation procedures, or by altered physical-chemical properties of the SR membrane, might bias the results of the Ca^{2+} uptake studies.

This potential bias can be overcome with the use of intact or skinned cells. In skinned cardiomyocytes subjected to simulated ischemia, the stimulation of Ca^{2+} uptake produced by ruthenium red and procaine decreased at $pCa < 6$ (Hohl et al., 1992). In another study, tension development after exposure to caffeine, which is another indirect index of SR Ca^{2+} release, was decreased in human myocardium subjected to surgical ischemia (Luciani et al., 1993).

Ca^{2+} release experiments performed with quick filtration techniques showed that the rate constant of Ca^{2+}-induced Ca^{2+} release decreased after ischemia or ischemia-reperfusion (Zucchi et al., 1995b), whereas the Ca^{2+}-dependence of Ca^{2+} release and its modulation by Mg^{2+}, H^+, and ruthenium red were preserved (Darling et al., 1992; Zucchi et al., 1995b). These changes appeared after a few minutes of ischemia. In particular, in the isolated working rat heart, the rate of Ca^{2+} release decreased after three cycles of 3-min ischemia and 3-min reperfusion, whereas three cycles of 1-min ischemia and 3-min reperfusion were ineffective. RyR dysfunction disappeared after 3 to 4 h of reperfusion.

Darling et al. (1992) reported that ryanodine binding decreased in heavy SR vesicles obtained from dog ventricular myocardium subjected to prolonged (60 min) ischemia, but the implications of this finding were not clear, because of potential contamination of ischemic SR vesicles by myofibrillar proteins. Reduced ryanodine binding has been observed also in microsomes and crude homogenates obtained from isolated rat hearts subjected to ischemia or ischemia-reperfusion (Zucchi et al., 1994, 1995b). Significant reduction occurred after 10 min of ischemia, i.e., in the absence of irreversible tissue injury, and no further decrease was observed after prolonged (up to 30 min) ischemia. Maximal binding was reduced by 15 to 25%, while the K_D and K_{DCa} were unchanged. Valdivia et al. (1995a) have obtained recently similar results with SR vesicles obtained from pig myocardium subjected to 10 min of ischemia and 120 min of reperfusion, whereas no change in ryanodine binding has been observed by Wu and Feher (1995), who used Langendorff-perfused rat hearts subjected to 15 min of ischemia. Because the latter was a nonworking model, the extent of ischemic injury may have been lower than in the other studies.

In single-channel experiments, Holmberg and Williams (1992) did not observe significant differences between channels obtained from ischemic versus control sheep myocardium. In a similar study, Valdivia et al. (1995a) used RyRs obtained from pig ventricular myocardium subjected to 10 min of ischemia and 120 min of reperfusion, and observed that ischemia-reperfusion reduced channel Po.

On the whole, the bulk of evidence suggests that myocardial ischemia is associated with a moderate and persistent reduction in the number of active SR channels. The molecular mechanism of this action remains to be determined. In a preliminary report, it has been shown that the decrease of Ca^{2+} release observed after ischemia-reperfusion can be prevented or reversed by DTT, suggesting that sulfhydryl oxidation might play a role (Ronca-Testoni et al., 1996).

It is not easy to predict the pathophysiological consequences of these findings. The cytosolic Ca^{2+} overload produced during ischemia largely represents a redistribution of intracellular Ca^{2+} (Kleber and Oetliker, 1992), which is due to inhibition of the SR and sarcolemmal Ca^{2+}-ATPases because of reduced ATP phosphorylation potential, in the face of persisting SR Ca^{2+} release. A moderate reduction in RyR density cannot prevent Ca^{2+} overload, although it might possibly affect its time course. During reperfusion, persistent abnormalities in SR function might affect the capability of the cell to cope with increased cytosolic Ca^{2+}, although other processes, such as recovery of Ca^{2+}-ATPase function and reversal of Na^+/Ca^{2+} exchange, are likely to have equal or greater importance (Tani, 1990; Silverman and Stern, 1994).

RyR dysfunction might be involved in the pathogenesis of some peculiar postischemic syndromes, namely myocardial stunning and ischemic preconditioning. Stunning refers to persistent contractile dysfunction occurring after ischemia and reperfusion, in the absence of any necrosis (Braunwald and Kloner, 1982; Bolli, 1990; Hearse, 1991; Kusuoka and Marban, 1992). The contractile impairment lasts hours or days and is eventually

reversible. Although the molecular mechanisms responsible for myocardial stunning are not completely clear, reduced Ca^{2+} sensitivity of the contractile proteins (Kusuoka and Marban, 1992; Atar et al., 1995; Gao et al., 1995) and reduced SR Ca^{2+} release represent the most likely causes. The latter phenomenon has been attributed to depletion of the SR Ca^{2+} pool, owing to reduced activity of the SR Ca^{2+}-ATPase (Krause et al., 1989; Limbruno et al., 1989; Krause, 1991; Krause and Rozanski, 1991; Zucchi et al., 1996), but RyR alteration might also play an important role.

Ischemic preconditioning is a term used to describe the increased resistance to ischemic injury caused by previous exposure to one or more brief periods of ischemia-reperfusion (Murry et al., 1986). Several effectors have been implicated in the pathophysiology of ischemic preconditioning (Marber and Yellon, 1996), and myocardial protection appears to be related to changes in Ca^{2+} homeostasis, because the development of cystosolic Ca^{2+} overload is delayed in the preconditioned myocardium (Steenbergen et al., 1993a,b). A reduction in the number of active SR channels might contribute to such a delay. The observation that the time course of RyR changes parallels the time course of myocardial protection (Zucchi et al., 1995b) supports this hypothesis, which deserves further investigation.

B. Cardiac Hypertrophy and Failure

In pressure overload-induced cardiac hypertrophy of rat, guinea pig, and ferret, RyR density was reduced, if expressed per mg of protein, whereas the affinity for ryanodine was unchanged (Naudin et al., 1991; Sainte Beuve et al., 1992; Kim et al., 1994a; Rannou et al., 1995). It has been calculated that RyR density per unit of SR surface was reduced by about 50%, whereas the density of dihydropyridine receptors was unchanged. Consistently, the expression of RyR2 messenger ribonucleic acid (mRNA) decreased in rat and in rabbit pressure-overload hypertrophy (Matsui et al., 1995; Rannou et al., 1996). Arai et al. (1996) described a biphasic response, because RyR2 mRNA increased in mild hypertrophy and decreased to subnormal levels as the severity of hypertrophy increased. Although the functional consequences of these observations remain to be determined, it has been speculated that RyR2 down-regulation may be involved in the decrease of Ca^{2+} transients observed in the hypertrophic myocardium (Sainte Beuve et al., 1992; Carré et al., 1993). Qualitative abnormalities in channel function have been described. In particular, Kim et al. (1994a) reported that the stimulation of ryanodine binding and of Ca^{2+} release produced by caffeine and doxorubicin was greater in hypertrophied than in control hearts.

The cardiomyopathic Syrian hamster is a well-known model of hereditary cardiomyopathy, with a phenotype that is transmitted in autosomal-recessive fashion. The disease is characterized by progressive myocytolytic necrosis, which begins at 30 to 40 days of age and leads to fatal congestive heart failure at 10 to 12 months of age. In SR preparations obtained from 1-month-old to 2-month-old cardiomyopathic hamsters, the density of ryanodine binding sites was increased, and the affinity for ryanodine was unchanged (Finkel et al., 1992; Sapp and Howlett, 1995). This finding has been attributed to increased efficiency of junctional SR isolation (Tawada-Iwada et al., 1993), possibly due to decreased resistance of cell membranes to osmotic stress, owing to structural protein deficiency (Iwata et al., 1993; Roberds et al., 1993). In fact, in unfractionated homogenates and in crude membrane preparations, ryanodine binding was either unchanged (Tawada-Iwada et al., 1993) or decreased (Lachnit et al., 1994), and RyR2 mRNA was decreased also (Lachnit et al., 1994). RyR2 had abnormal functional properties in the cardiomyopathic hamster, because ryanodine binding showed an increased sensitivity to Ca^{2+} activation and doxorubicin activation (Lachnit et al., 1994). The implications of these findings are not clear. It has been speculated that myocyte necrosis be mediated by cytosolic Ca^{2+} overload, which might be favored by abnormal SR channel gating. However, in 110-day-old cardiomyopathic hamsters, total cellular Ca^{2+} was not increased, and junctional SR Ca^{2+} was decreased (Keller et al., 1995): the decrease was attributed to Ca^{2+}-ATPase dysfunction.

Similar findings have been obtained in other experimental models of heart failure. As mentioned above (II.B.3.), ryanodine binding was reduced in the cardiomyopathy produced by chronic doxorubicin administration (Pessah et al., 1992b; Dodd et al., 1993). In SR preparations obtained from dogs with heart failure that was induced by rapid pacing, the B_{max} for ryanodine was reduced, whereas the K_D was unchanged (Cory et al., 1993; Vatner et al., 1994). Reduced B_{max} for ryanodine was also observed in dogs with spontaneous dilated cardiomyopathy (Cory et al., 1993) and also after endotoxin administration, which is known to produce myocardial depression (Liu and Wu, 1991). These results should be interpreted with a note of caution, because SR fractions obtained from failing myocardium might not be comparable to control SR fractions.

Indirect studies have supported the concept of defective RyR2 function in experimental heart failure. After rapid pacing, the mechanical restitution response, post-extrasystolic potentiation, and ryanodine-induced stimulation of Ca^{2+} uptake were decreased (Cory et al., 1993, 1994; Vatner et al., 1994). In furazolidone-induced turkey cardiomyopathy, indirect studies produced complex results, which were interpreted as evidence of defective SR channel function, associated with a nonspecific (i.e., not related to the RyR) increase in SR permeability (O'Brien et al., 1991).

Rats with streptozocin-induced diabetes mellitus develop a form of cardiomyopathy. In the crude homogenate obtained from diabetic hearts, ryanodine binding

was reduced, due to reduced B_{max} with unchanged K_D (Yu et al., 1994). The functional relevance of this finding was confirmed in myocyte experiments, because the amplitude of caffeine-induced or rapid cooling-induced contracture was decreased.

Human studies have relied on samples obtained from the hearts of patients undergoing cardiac transplantation, with donor heart samples as controls. According to Brillantes et al. (1992), RyR2 mRNA was reduced in patients with end-stage heart failure due to ischemic cardiomyopathy, whereas no change was observed in patients with end-stage dilated cardiomyopathy. In other series, RyR2 mRNA was reduced in end-stage heart disease, irrespective of etiology (Arai et al., 1993; Go et al., 1995).

However, changes in gene transcription do not necessarily correspond to changes in protein expression. Although Go et al. (1995) have reported a reduction of ryanodine binding in a small series of four patients, in two larger series, no change in cardiac RyRs has been observed, on the basis of either immunological techniques (Meyer et al., 1995) or [^{3}H]ryanodine binding (Nimer et al., 1995; Schumacher et al., 1995).

Indirect studies have provided conflicting results. The stimulation of Ca^{2+} uptake produced by ryanodine was reduced in SR vesicles associated with end-stage ischemic or dilated cardiomyopathy (Nimer et al., 1995; O'Brien and Gwathmey, 1995). However, in skinned fibers obtained from dilated cardiomyopathy patients, the analysis of caffeine-induced tension development suggested that the rate and extent of Ca^{2+} release were normal, although the caffeine threshold was increased (D'Agnolo et al., 1992). The latter finding might imply abnormal channel gating, yet no gating abnormality has been reported in bilayer experiments using channels obtained from explanted hearts (Holmberg and Williams, 1989, 1992).

A serious limitation of the human studies is the use of severely diseased tissue. Although all investigators took care in avoiding scars and grossly abnormal tissue, the presence of fibrosis and/or myocyte degeneration was a potential bias that could be neither excluded nor quantified. Besides, most patients were receiving an intensive pharmacological treatment (e.g., digoxin, adrenergic agents, nonadrenergic inotropes), which may have affected RyR function of RyR gene expression.

In summary, the study of RyR changes in heart failure is at an early stage, and controversial findings have been reported. Reduced RyR2 density has been observed in different models of heart failure. In some cases, RyR2 mRNA was also reduced, suggesting that the down-regulation was due to reduced synthesis of the channel protein. Abnormal channel gating has been occasionally described, but this issue requires confirmation. The functional implications of these findings have not been established. Abnormal Ca^{2+} handling appears to be a key feature of heart failure, leading to prolongation of Ca^{2+} transients, reduced Ca^{2+} availability for the contractile process, and increased susceptibility to stimuli inducing Ca^{2+} overload (Gwathmey et al., 1987; Katz, 1990; Hano and Lakatta, 1991; Morgan, 1991). These phenomena are usually attributed to decreased Ca^{2+}-ATPase activity, but RyR2 down-regulation might contribute to reduce SR Ca^{2+} release. On the other hand, modulation of Ca^{2+} uptake and release might be regarded as a compensatory mechanism leading to lower energy expenditure.

C. Malignant Hyperthermia

Alterations in RyR1 have been implicated in the pathogenesis of malignant hyperthermia (MH), and this issue has been addressed in several recent reviews (Joffe et al., 1992; MacLennan and Phillips, 1992, 1995; Ohnishi and Ohnishi, 1993; Gronert and Antognini, 1994; Mickelson and Louis, 1996).

MH in an autosomal-inherited myopathy, characterized by sustained uncontrolled skeletal muscle contracture, hypermetabolism, hyperkalemia, hyperthermia, and cardiac arrhythmias. The syndrome is most often triggered by the administration of halogenated anesthetics such as halothane and/or of the depolarizing neuromuscular blocker succinylcholine, and it can be fatal unless immediately treated with dantrolene. It is one of the main causes of anesthesia-induced death, occurring in about 1/40,000 adult and 1/150,000 pediatric surgical cases. In pigs, physical stress due to sudden changes in ambient temperature, excitement, transport, or fighting is the major inducing factor for a MH-like syndrome, usually referred to as porcine stress syndrome.

The characteristic feature of MH is muscle contracture. Sustained contracture is due to abnormal myoplasmic Ca^{2+} elevation, which determines a massive activation of both aerobic and glycolytic metabolism, accounting for metabolic and respiratory acidosis and hyperkalemia. Higher resting Ca^{2+} levels have been observed in MH muscle fibers by using Ca^{2+} microelectrodes, but this result has not been confirmed with fluorescent Ca^{2+}-specific dyes (reviewed by Mickelson and Louis, 1996). In any case, the peculiar property of MH muscle is an abnormal sensitivity to stimuli that induce SR Ca^{2+} release. In particular, lower concentrations of halothane and caffeine are required in MH than in normal fibers to increase intracellular Ca^{2+} concentration and tension development. This is the basis of the clinical test used to assess MH susceptibility, which consists of determining the contracture threshold of muscle bundle biopsy specimens exposed to incremental doses of either caffeine, or halothane, or a combination of these agents. However, standardization of the test and precise definition of positive versus negative responses are still controversial issues. It has been observed recently that micromolar concentrations of 4-chloro-*m*-cresol induce contracture in MH-susceptible muscle and potentiate

the caffeine contracture response (Tegazzin et al., 1996). Because chlorocresol is a preservative added to commercial succinylcholine preparations, it might be responsible for the cases of "succinylcholine-induced" MH.

SR function has been extensively studied in MH-susceptible patients and in MH-susceptible animals (pigs). We will first deal with the animal model, and then with the human disease.

Many studies have demonstrated that active SR Ca^{2+} transport is normal in MH-susceptible pigs (reviewed by Mickelson and Louis, 1996). On the other hand, significant abnormalities in Ca^{2+}-induced Ca^{2+} release have been observed. In skinned fibers and in SR preparations, the rate and extent of Ca^{2+} release were higher than normal (Ohnishi, 1987; Ohnishi et al., 1983; Kim et al., 1984; Mickelson et al., 1987, 1988; Donaldson et al., 1989; Otha et al., 1989; Fill et al., 1990; Carrier et al., 1991; El-Hayek et al., 1995b). Although early studies suggested that the Ca^{2+} threshold for the activation of Ca^{2+} release was significantly lower in MH muscle (Nelson, 1983; Ohnishi et al., 1983; Fletcher et al., 1991b), quick release experiments showed a normal Ca^{2+}-dependence of SR Ca^{2+} release (Kim et al., 1984; Fill et al., 1990; Carrier et al., 1991). The sensitivity to H^+ inhibition of Ca^{2+} release was reduced in MH: at a pH of 6.5, MH-susceptible SR was still able to release Ca^{2+}, whereas Ca^{2+} release from normal SR was completely inhibited (Louis et al., 1992). No difference was observed with regard to the modulation by Mg^{2+}, adenine nucleotides, and fatty acids, (Carrier et al., 1991; Fletcher et al., 1991b), whereas some observations suggested an increased sensitivity to caffeine (Nelson, 1983; Ohnishi, 1987).

Single-channel studies (Fill et al., 1990; Shomer et al., 1993, 1994a; Nelson and Lin, 1995; Nelson et al., 1996) showed that the Ca^{2+} dependence of channel gating was abnormal. At optimal Ca^{2+} concentration, channel Po was either normal or slightly increased, but Ca^{2+}-inactivation was not observed, so that at pCa < 4 the Po was higher in MH than in control preparations. At low pH (6.8), the Po of MH channels was significantly higher at all Ca^{2+} concentrations in the range of 7 μM to 10 mM (Shomer et al., 1994b, 1995). In contrast with the release experiments, the sensitivity to caffeine was unchanged (Shomer et al., 1994b), whereas the sensitivity to activation by nanomolar dantrolene was reduced (Nelson et al., 1996).

Binding experiments, performed on native membranes or purified receptors, showed that the affinity for ryanodine was normal under optimal binding conditions, but it was higher than normal under suboptimal conditions, i.e., at low ionic strength or in the absence of adenine nucleotides (Mickelson et al., 1988, 1990; Carrier et al., 1991; Vita et al., 1991; Hawkes et al., 1992). Ca^{2+} stimulation of ryanodine binding was normal (Mickelson et al., 1988; Hawkes et al., 1992), whereas conflicting results were reported with regard to Ca^{2+} inhibition, because increased IC_{50} was observed by Mickelson et al. (1988), but not by Hawkes et al. (1992).

The relationship between these defects in RyR1 function and the occurrence of MH episodes has not been entirely understood. It has been suggested that a MH episode may represent the "final common pathway" resulting from the convergence of a number of small abnormalities in the presence of a triggering stimulus, such as the exposure to volatile anesthetics (Pessah et al., 1996).

Porcine MH syndrome is associated with a mutation in a gene originally designed as halothane gene (*hal*) or PSS gene, which is inherited in an autosomal-recessive manner, so that only homozygotes manifest the disease and respond positively to the halothane/caffeine challenge test (Mabry et al., 1981; Reik et al., 1983). Pigs hetorozygous for the MH allele are not susceptible to MH episodes, but their muscles show abnormal in vitro responses. RyRs from heterozygous pigs are heterotetramers comprising normal and abnormal subunits, with different gating kinetics, and more than one MH subunit per channel is required to determine an abnormal function (Shomer et al., 1995).

In pigs, the *hal* gene has been mapped to chromosome 6, through haplotype analysis of linked marker loci (Archibald and Imlah, 1985; Davies et al., 1988). The RyR1 gene has been mapped to the same region of chromosome 6, namely band 6q12 (Harbitz et al., 1990; Chowdhary et al., 1994). The comparison of RyR1 cDNA obtained from MH and normal pigs (Fujii et al., 1991) showed that only one of the observed polymorphisms, namely replacement of cytidine with thymidine at nucleotide 1843, affected the amino acid sequence, causing replacement of arginine with cysteine at residue 615 (Arg615Cys). Linkage between the inheritance of this mutation and MH susceptibility has been clearly demonstrated in all the breeds of pigs examined (Otsu et al., 1991; MacLennan and Phillips, 1992, 1995). Pig MH appears to have originated from a mutation occurring in a single animal, which was probably selected because it is associated with positive consequences with regard to pork production, namely increased lean meat content and decreased back fat.

Cell lines have been transfected with normal and mutant (Arg615Cys) RyR1 genes (Otsu et al., 1994; Treves et al., 1994). In the cells expressing the mutant gene, resting Ca^{2+} concentration was normal, but caffeine, halothane, and 4-chloro-*m*-cresol produced a larger increase in intracellular Ca^{2+} than in the controls. The molecular mechanism by which the Arg615Cys mutation alters channel function has not been clarified. Residue 615 is located in the large cytoplasmic foot structure, but its function is unknown. It is unlikely that this region may be involved in the ion conductance pathway, and sequence analysis does not suggest the presence of binding sites for any known RyR modulator.

Biochemical and physiological studies concerning human MH have not been so informative and conclusive as those performed in the pig, due to the limited amount of tissue obtained from muscle biopsies, to the quality of the tissue and to the heterogeneity of the human disease. No defect in SR Ca^{2+} uptake and storage has been demonstrated convincingly (Mickelson and Louis, 1996). On the other hand, an increased rate of SR Ca^{2+} release was observed in skinned fibers obtained from suspected patients suspected to have MH (Endo et al., 1983; Kawana et al., 1992), whereas the threshold for Ca^{2+}-induced Ca^{2+} release was unchanged (Fletcher et al., 1991b, 1993).

In single-channel recordings, Fill et al. (1991b) did not detect significant differences in conductance, Po, and Ca^{2+}-activation between normal and MH muscle, although caffeine sensitivity was higher in the latter. Nelson (1992) observed increased halothane sensitivity of MH channels: whereas 2.2 to 17.6 μM halothane did not affect channel Po in normal muscle, it increased channel Po in about 50% of MH patients. This author also reported that halothane-sensitive channels had a higher basal Po.

In binding experiments (Valdivia et al., 1991b), purified RyRs obtained from human MH muscle showed an increased affinity for ryanodine and an increased sensitivity to caffeine. The Ca^{2+} dependence of ryanodine binding was abnormal. The Ca^{2+} concentration required for half-maximal activation of binding was reduced, but no abnormality was observed with regard to inhibition by high Ca^{2+} concentration.

On the whole, the human studies confirmed the presence of RyR1 abnormalities, although the specific abnormalities were not identical with those observed in MH-susceptible pigs.

In humans, MH-susceptibility is inherited as an autosomal dominant trait. Association of the disease with a point mutation in the RyR1 gene in pigs stimulated similar investigations in humans (MacLennan et al., 1990). Human RyR1 gene has been mapped to region 19q13.1 in the proximal long arm of chromosome 19 (MacKenzie et al., 1990). After the identification of the Arg615Cys mutation in porcine MH, a homologous mutation (ArgG14Cys) was identified in humans (Gillard et al., 1991; Hogan et al., 1992), but cosegregation of this mutation with MH susceptibility was observed in only a small fraction (about 5%) of families with MH (Levitt et al., 1991; Iles et al., 1992; Ball et al., 1993; Fagerlund et al., 1994, 1995; Deufel et al., 1995; Moroni et al., 1995; Steinfath et al., 1995; Serfas et al., 1996; Wallace et al., 1996). Other RyR1 point mutations have been reported, and these probably account for additional forms of MH. Such mutations include Arg163Cys, Gly248Arg, Gly341Arg, Tyr522Ser, Gly2433Arg (Gillard et al., 1991, 1992; Keating et al., 1994; Phillips et al., 1994; Quane et al., 1993, 1994a,b). Interestingly, all the mutations associated with MH or with central core disease (see III.D.) produce amino acid changes clustered around two regions of the large foot domain of the RyR1 molcule. On the whole, association of chromosome 19-linked mutations with MH-susceptibility has been demonstrated in less than 50% of the families examined (Ball and Johnson, 1993; Pessah et al., 1996), although it should be noted that, in most studies, MH susceptibility was identified on the basis of the in vitro contracture test, and the threshold chosen to define the positive result influences the extent of the genetic linkage. For instance, if the threshold used to indicate a positive response is lowered, i.e., if weak positive responses are taken into consideration, then the percentage of families with MH showing RyR1 gene mutations increases substantially (MacKenzie et al., 1991). In addition, it cannot be excluded that subtle RyR1 abnormalities, insufficient to produce a clear in vitro contracture, may determine an abnormal reaction to anesthetics in vivo.

It has been speculated that the cases of MH susceptibility for which no linkage to the RyR1 gene has been demonstrated may be caused by mutations in other proteins involved in Ca^{2+} homeostasis, but no conclusive results have been obtained so far. Loci that have been tentatively associated with MH-susceptibility include (*a*) region 17q11.2-24 on chromosome 17, coding for the α subunit of the Na^+ channel and for the β and γ subunits of the dihydropyridine receptor; (*b*) a microsatellite close to the α_2/δ dihydropyridine receptor subunit genes on chromosome 7q; (*c*) region 1q31-q32 on chromosome 1, coding for dihydropyridine receptor α_1 subunit; and (*d*) locus 3q13.1 on chromosome 3 (for further review, see MacLennan and Sorrentino, 1995; Mickelson and Louis, 1996).

D. Other Skeletal Muscle Diseases

Central core disease is a skeletal muscle disease transmitted as an autosomal dominant trait and characterized by muscle hypotonia and weakness, in which muscle biopsy shows fibers with single or multiple discrete zones (cores) devoid of oxidative enzymes. The disease is usually nonprogressive and, in many cases, it is associated with predisposition to MH during general anesthesia. Genetic analysis has mapped central core disease to chromosome 19q13.1 (Kausch et al., 1991; Mulley et al., 1993; Schwemmle et al., 1993), i.e., to the same locus as MH susceptibility, suggesting that both diseases originate from mutations in the RyR1 gene and therefore may be allelic. This hypothesis has been confirmed by the identification of RyR1 gene mutations in families with central core disease, e.g., Arg163Cys, Ile403Met, Tyr522Ser, and Arg2433His (Quane et al., 1993, 1994a; Zhang et al., 1993b).

Myasthenia gravis is an acquired disease characterized by weakness and fatigability of skeletal muscles. The basic defect is a decrease in the number of available acetylcholine receptors at neuromuscular junctions, due to the presence of anti-acetylcholine receptor autoanti-

bodies. Thymus abnormalities are frequent in myasthenia gravis patients, with about 10% having thymic tumors (thymoma). Antibodies against RyR1 have been detected in about 50% of the patients with myasthenia gravis and thymoma, whereas they were absent in the cases not associated with thymoma. The presence of RyR1 antibodies was correlated with the severity of the disease, suggesting a pathogenetic role, but the effects of the antibodies on SR Ca^{2+} release have not been determined (Mygland et al., 1992a,b, 1993, 1994).

RyR1 alterations have been described in chicken dystrophy, an autosomal-recessive myogenic disease that primarily affects fast-twitch fibers. Whereas in normal muscle SR preparations the density of RyRs decreased in the first days after birth, in dystrophic muscle, such decrease did not occur, and the density of low-affinity binding sites (K_D in the range of 0.7 to 4.5 μM) increased progressively (Pessah and Schiedt, 1990). Qualitative abnormalities were also observed, because in dystrophic muscle, high-affinity ryanodine binding showed an increased sensitivity to stimulation by caffeine and to inhibition by high Ca^{2+} concentrations.

The so-called crooked neck dwarf mutation of embryonic chicken has also been associated with a RyR gene mutation. The normal α isoform of the RyR could not be detected in skeletal muscle, and extremely low levels of α-RyR immunoreactivity were observed with atypical distribution, suggesting the presence of an abnormal α-RyR protein (Airey et al., 1993a).

RyR1 knockout mice (known as $skrr^{m1}$) have been produced recently (Takeshima et al., 1994). No immunological evidence of RyR1 expression was obtained in homozygous mice carrying the mutation, and skeletal muscle from these mice did not show any contractile response to electrical stimulation. Mutant myocytes still released Ca^{2+} in response to caffeine, ryanodine, and adenine nucleotides, which was attributed to the residual expression of RyR3 (Takeshima et al., 1995). Mice homozygous for the mutation showed gross developmental defects in the musculoskeletal system and were not viable after birth, due to respiratory failure. At the subcellular level, the cytoplasmic "foot" domain of the RyR was missing, although junctions between SR cisternae and T-tubules were still formed, probably mediated by RyR-associated proteins (Takekura et al., 1995).

Muscle fatigue is a term used to describe the decline in force development during periods of repetitive contraction. Fatigue has been associated with reduced Ca^{2+} transients, determined at least in part by decreased SR Ca^{2+} release (Westerblad et al., 1991; Györke, 1993). Accumulation of lactate, H^+, and Mg^{2+} might contribute to the process (Favero et al., 1995b), but primary RyR1 changes are also involved, because SR vesicles isolated from fatigued muscle showed a depressed ryanodine binding and a reduced rate of Ca^{2+} release (Favero et al., 1993).

Changes in the neural control of muscle function affect RyR1 expression and function. In rat skeletal muscle, denervation caused a decrease in RyR1 mRNA, whereas muscle paralysis, obtained by chronic superfusion of the sciatic nerve with tetrodotoxin, produced the opposite effect (Ray et al., 1995). In the latter case, RyR1 up-regulation was confirmed by Western blot analysis. In rabbit skeletal muscle, the rate of doxorubicin-induced and caffeine-induced Ca^{2+} release decreased after denervation, which was paradoxically associated with an increased B_{max} for [^{3}H]ryanodine, suggesting the presence of abnormal channels, and/or the occurrence of an isoform shift (Zorzato et al., 1989). The mechanism of these effects is unknown, although it has been speculated that the cAMP cascade, PKC, and nerve-derived factors may be involved.

E. Smooth Muscle and Nonmuscle Diseases

Very little is known about the role of RyRs in pathological conditions not affecting striated muscle. Increased ryanodine binding due to increased B_{max} with unchanged K_D was observed in microsomal membranes obtained from the rabbit bladder in a model of partial outlet obstruction (Levin et al., 1994). Indirect evidence of reduced caffeine-sensitive or ryanodine-sensitive Ca^{2+} release was obtained in aortic smooth muscle derived from spontaneous hypertensive rats (Aiello and Grassi de Gende, 1995). Finally, protection from ischemic neural injury was described with dantrolene and trimethoxybenzoate derivatives, which was attributed to modulation of SR Ca^{2+} release (Zhang et al., 1993a; Chiou and Hong, 1995). As far as the role of RyR in disease is concerned, these findings should be regarded as preliminary and still need confirmation.

IV. Conclusions

The RyR was identified as the SR Ca^{2+} release channel about 10 years ago; since that time, its regulation has been intensively studied. The RyR is the largest of all the known ion channels, and its structural complexity is matched by the myriad of effects produced by endogenous and exogenous modulators. Different patterns of modulation have been described, although these have not been thoroughly categorized, and some modulators may produce multiple actions. In the present review, we propose a pharmacological classification of these modulatory agents, based on their effects on Ca^{2+} release, channel gating, and [^{3}H]ryanodine binding. This classification will need revision as further knowledge is acquired. The number of RyR modulators identified will certainly increase in the future. In fact, most drugs analyzed in this review have other target sites of action, and discovery of their actions at RyRs has been occasionally serendipitous. Efforts to develop specific RyR modulators have just begun.

The potential clinical implications of RyR modulation should not be overlooked. Because the RyR has a central

role in excitation-contraction coupling, selective RyR modulators have the potential to be highly effective tools for regulation of cardiac and muscular function. In addition, the role of the RyR in disease is becoming more and more clear. MH provides a unique example in which mutations in the RyR1 gene have been related to a specific clinical syndrome, in which RyR modulators, such as dantrolene, have proved to be effective and already are used in patient management. Recent investigations suggest that SR Ca^{2+} efflux may be of major importance in the pathogenesis of myocardial ischemic injury and that RyR modulation may be a new effective strategy in myocardial protection. There is preliminary evidence that RyR alterations are involved in the pathogenesis of heart failure, cardiomyopathies, and degenerative skeletal muscle diseases. The role of the RyR in the physiology and pathophysiology of nonmuscle tissues is a new research field that is likely to produce substantial results in the near future.

The pharmacological modulators that are available at present show serious limitations with regard to their clinical use. Many lack selectivity, which may mask or limit effects at RyRs; others show an irreversible action that is associated with serious toxic effects. However, it is reasonable to hope that these limitations can be overcome by future developments, and we believe that RyR modulation is going to become at least as important as sarcolemmal Ca^{2+} channel modulation.

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