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THE PHARMACOLOGY OF RYANODINE

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I. INTRODUCTION

Ryanodine is a neutral alkaloid isolated from the stem and root of the plant *Ryania speciosa* Vahl, a member of the family Flacourtiaceae native to Trinidad. Related and possibly identical compounds appear to be present in other members of the Flacourtiaceae family. Originally of interest because of its insecticidal properties, ryanodine was soon shown to have profound effects on muscle from a variety of vertebrates and invertebrates, and it is this aspect of its pharmacology which has attracted most attention in the past 15 years. The nature of these effects varies with species and type of muscle; the most striking actions are an apparently irreversible contracture in skeletal muscle of vertebrates and some arthropods, and a negative inotropic effect on mammalian cardiac muscle. Effects on some muscles have been demonstrated with concentrations as low as 10^{-9} M. Despite a growing body of information on its effects on intact muscle and subcellular systems derived from it, its mechanism of action remains obscure, and its elucidation must probably await more complete understanding of the factors regulating contraction, relaxation, and metabolic coupling. The extreme potency of the drug and apparently specific nature of its effects suggest that it may play a significant role in the functional analysis of these factors.

A short review in German of the salient features of the chemistry and pharmacology of ryanodine has recently appeared (141); references to most of the early work on Ryania extracts may be found in this publication, in an earlier review by Hill and Murtha (72), and in a series of reports presented at a symposium on Ryania extracts in 1961 (71). Interpretation of most of the work with crude extracts of the plants is equivocal because of the presence of pharmacologically

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active components other than ryanodine, and it will not be discussed in detail in this review.

II. CHEMISTRY

Ryanodine was first characterized after isolation and crystallization from the ground stem wood and root of *Ryania speciosa* Vahl by Rogers *et al.* (124) in 1948. Similar insecticidal properties are shown by a number of species of Ryania and subspecies of *Ryania speciosa* (46, 69). The pure alkaloid is about 700 times more potent in its insecticidal properties than the starting material, and several procedures have been described for its isolation (122). Ryanodine accounts for a substantial proportion of the total activity, but other alkaloids are almost certainly present (123). A rotenoid called tephrosin is present in several *Ryania* extracts (123), and apparently accounts for the antimitotic activity described by Cornman (22). Retardation by *Ryania* extracts of development of salamander embryos (15) may have a similar explanation. The presence of toxic principles other than ryanodine is also indicated by a report of sustained inhibition by *Ryania* of photosynthesis by McIntosh apple leaves, while pure ryanodine produces only a temporary effect (70). An inhibitor of creatine phosphoryltransferase, originally thought to be ryanodine itself (74), has since been shown to be distinct from this alkaloid and has been partially separated from it (73), although it is apparently closely related (132). In view of the enormous potential value of a specific inhibitor of this enzyme in muscle biochemistry, additional work directed toward its isolation and characterization is clearly indicated.

Ryanodine is a nonbasic alkaloid of empirical composition $C_{25}H_{35}NO_9$. The nitrogen atom is contained in a pyrrole-2-carboxylate moiety, which accounts for its lack of basicity, its intense ultraviolet absorption with a maximum at 268.5 nm, and its characteristic color reaction with *p*-dimethylaminobenzaldehyde in concentrated sulfuric acid. It is freely soluble in water, ethanol, chloroform, acetone, and ether, and is extractable from aqueous solution into amyl acetate (distribution coefficient 7.5).

Ryanodine is stable in neutral aqueous solution and will withstand prolonged boiling. It undergoes saponification under relatively mild basic conditions, yielding pyrrole-2-carboxylic acid and ryanodol, a highly condensed polyhydric alcohol with the empirical formula $C_{20}H_{32}O_8$. Regeneration of ryanodine by selective re-esterification of ryanodol has not been reported and is apparently not a feasible approach to isotopic labelling of the compound (121). The structures of ryanodol and ryanodine have been the subject of prolonged investigation (4–6, 17, 80, 90, 91, 123, 124, 126, 134, 139, 140, 147, 148, 152). The most recent structural postulate has been made by Wiesner *et al.* (148) and is shown in figure 1. An X-ray crystallographic study of ryanodol-*p*-bromobenzyl ether has recently been reported (134), that yields a structure identical with that proposed by Wiesner *et al.* (148) except for reversal of configuration of the carbon atom bearing the isopropyl group. However, the possibility of rearrangement during derivatization or saponification of ryanodine cannot be discounted, and the proposed structure is difficult to reconcile with data recently presented by Rapo-

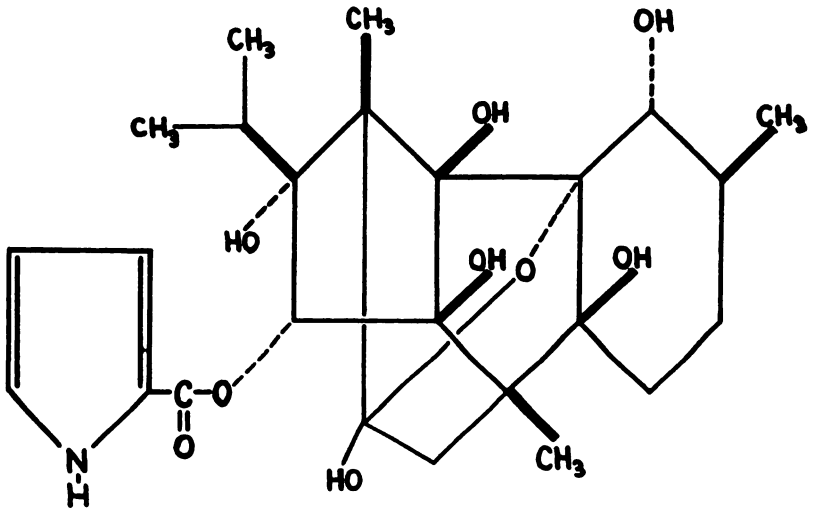

FIG. 1. Structural formula of ryanodine according to Wiesner *et al.* (148)

port and his collaborators on two periodate oxidation products of ryanodol (80, 152). The final structural elucidation must probably await X-ray crystallographic analysis of ryanodine itself or an unambiguous derivative.

III. PHARMACOLOGY

A. Intact animals

Procita (113) has described effects after the intravenous, intraperitoneal, or oral administration of ryanodine to a variety of unanesthetized mammals. The effects do not vary remarkably from species to species, but were observed in most detail in the dog. Intravenous administration of 50 μg/kg or more produced enophthalmos, followed by spastic rigidity, first of the hind limb musculature and then progressively spreading throughout the body. Vomiting, defecation, and profuse salivation were observed. Death resulted in 20 to 25 min from involvement of the respiratory muscles if the dose was large enough (>100 μg/kg); otherwise complete recovery ensued within 24 hr. Oral or intraperitoneal administration produced similar effects with a more variable time of onset. The lethal dose was in the range 20 to 300 μg/kg for dogs, cats, rabbits, guinea pigs, rats, mice, chickens, and bats, all of which showed marked muscular rigidity. Kuna and Heal (94), working with the powdered stem of *Ryania speciosa*, observed weakness, tremor, convulsions, and coma after lethal doses in conscious mammals, but did not report the rigidity characteristic of ryanodine; these findings suggest the presence of other pharmacologically active components. In the frog, ryanodine produced flaccidity at a dose of 5 mg/kg, followed by a progressive disappearance of "swallowing movements" and then the development of rigor (31).

Ryanodine exerts profound effects on the cardiovascular system in doses

of the order of 10 to 100 μg/kg (89, 118, 119). Although the most obvious effect is hypotension, resulting from the negative inotropic action on the heart (see below), the mean aortic pressure does not fall as much as the cardiac output; this indicates an increased peripheral resistance (89). A more detailed study of peripheral vascular resistance by Procita and co-workers (118, 119) in anesthetized cats revealed dramatic decreases in the rate of venous return from the hindlimb, spleen, and head and neck after ryanodine, while the kidney was considerably more resistant to the drug and blood flow from the superior mesenteric vein was unchanged. Blood flow in the hindlimb was reduced within a few minutes of ryanodine (>5 μg/kg) given intravenously, but was delayed when it was administered by the intra-arterial route, a result that precludes a direct effect on the vascular smooth muscle. The blood flow response was not seen in acute spinal cats and was unaffected by either ligation of the adrenal glands or high section of cranial nerves IX, X, XI, and XII. It was concluded that the vasoconstriction induced by ryanodine was neurally mediated and was central in origin. Although the former conclusion appears justified, evidence of a direct action of ryanodine on the central nervous system is lacking and participation of reflex pathways has not been excluded. Further work will be required to define the target organ from which these vascular effects are exerted.

Because of the commercial use of *Ryania* extractives for their insecticidal properties, first reported by Pepper and Carruth (106), a large number of reports has appeared in which the sensitivity of various species of insects has been investigated (for references, see 31, 68, 110). Sensitive species show a progressive flaccid paralysis, in marked contrast to the hyperactivity characteristic of DDT intoxication. Perhaps the most striking effect observed in intact insects is the dramatic increase in oxygen consumption occurring after injection of Ryania extractives (5 μg total dose) into cockroaches (*Periplanata americana*). A rise averaging 220% was observed during the period 2 to 2.5 hr after injection; the QO_2 returned to normal levels 24 hr later and subsequently decreased further (66). A similar increase has been reported in some isolated vertebrate muscle and will be discussed later.

B. Whole muscle

1. Skeletal muscle. Vertebrate skeletal muscle characteristically responds to ryanodine with a slow irreversible contracture, which is usually preceded by a latent period. Under isotonic conditions, shortening has been reported to proceed to as little as 14% of rest length, and it is then associated with the extrusion of large amounts of water (120). In isometric preparations, extensive rupture of muscle fibers can readily be observed, and the tension decays rapidly after its peak. Typically, 10^{-5} to 10^{-6} M ryanodine produces a maximal effect in most vertebrate skeletal muscle within 30 to 60 min, but several factors exert a major influence on the apparent sensitivity of the muscle and the rate at which it responds.

Several investigators have reported that cooling a muscle delays the response dramatically (32, 59, 120). Ramsey observed a Q_{10} of 8.36 for the onset of short-

ening in frog sartorius muscles in response to 2×10^{-5} M ryanodine (120); and a preliminary equilibration of frog sartorius to ryanodine close to 0°C followed by elevation of the temperature has been used as a method for obtaining a more uniform response. Since the muscle does not respond until the temperature is raised, the equilibration allows time for diffusion of the drug throughout the muscle so that all the fibers are exposed to a uniform concentration at the time the response is observed. In a more detailed study of the influence of temperature on the kinetics of the response of rat diaphragm preparations to ryanodine (59), an even greater temperature dependence was seen. Arrhenius plots of the half-time for contracture against temperature did not yield straight lines, and the temperature coefficient was greater at the upper end of the range studied (16 to 40°C). A limiting Q_{10} of over 20 was observed in this region. If a maximal twitch response was elicited every 10 sec by electrical stimulation, the contracture was accelerated, the effect being most marked at the lowest temperature (5.6 times at 16.1°C, 1.7 times at 40°C). As expected, the contracture was faster in response to a larger concentration of ryanodine, but the relationship was not a linear one.

Several investigators have reported acceleration of the ryanodine response by activity of the muscle (14, 59, 77, 78, 107, 112, 113, 128, 131). This effect may be seen most readily under conditions in which the spontaneous contracture is very slow or indefinitely delayed, by studying either an isolated muscle at a low temperature or a muscle *in situ* in an anesthetized animal. Procita (112) has found that in cats anesthetized with pentobarbital, no sustained contracture of the tibialis anticus muscle was observed after intra-arterial injection of ryanodine, regardless of the dose, unless the muscle was stimulated directly or indirectly. In this case a progressive contracture was seen after intra-arterial administration of 50 μg or more. Even in unanesthetized animals, denervated muscles show no rigidity after a lethal dose of ryanodine (111). The accelerating effects of indirect, but not of direct, stimulation are prevented by neuromuscular blocking agents. Similar results were obtained by Blum *et al.* (14) on both frog rectus abdominis and rat phrenic nerve-diaphragm preparations, in both of which the latent period before the onset of ryanodine contracture can be considerably shortened by electrical stimulation. Neuromuscular blocking agents do not, however, prevent the spontaneous contracture in isolated muscle preparations (14). Smith and Eldred (131) obtained evidence that ryanodine produces a contracture in intrafusal fibers in gastrocnemius and tenuissimus of the cat when these are stimulated through fusimotor fibers, and used this procedure in analyzing the mechanism by which succinylcholine accelerates afferent spindle discharge. Stimulation of muscle by drugs is equally effective in accelerating the response to ryanodine. Pick and Tullius (107) found that acetylcholine, potassium, and neostigmine markedly accelerated the response of the frog rectus abdominis to ryanodine, and Blum *et al.* (14) confirmed these observations. Elevation of the K^+ concentration above 2.5 mM markedly accelerated the response of the frog rectus abdominis, an effect which could be reversed by increasing the magnesium concentration. Bianchi (11) reported that in the frog

sartorius muscle, the normally transient contracture produced by 80 mM KCl can be converted by ryanodine (10^{-4} M) to a biphasic response of which the first component corresponds to the normal transient K^+ contracture; the second is larger and sustained and apparently represents an accelerated response to ryanodine.

Both Procita (112) and Blum *et al.* (14, 85) pointed out that contractile responses to various types of stimulation are unaffected by ryanodine until maximal contracture ensues; on the other hand, relaxation becomes progressively less complete. It was therefore reasonable to interpret the effects of ryanodine as resulting from specific interference with some process intimately concerned with relaxation. Effects of the drug on relaxation were examined in more detail by Seraydarian *et al.* (128) in isolated frog sartorius preparations. Although cooling the muscle to 2 to 4°C indefinitely prevents the contractural response to ryanodine, it was still possible to demonstrate extreme slowing of relaxation by ryanodine (2×10^{-4} M) after a twitch, while twitch height and rate of contraction were unaffected. A similar but less extreme slowing of relaxation can be produced by prolonged stimulation of an untreated muscle under anaerobic conditions (127). In both cases the slowing of relaxation could be prevented by iodoacetate (10^{-3} M) for as long as the muscle continued to contract, and this fact suggests that the ryanodine-sensitive step may be normally dependent on an oxidative mechanism. This conclusion was confirmed by the finding that the rate of relaxation returned to normal when the ryanodine-treated muscle was allowed to recover for 60 min in oxygenated Ringer solution containing ryanodine.

Insect skeletal muscle has not been extensively studied in its response to ryanodine, but the published studies (31, 58, 61, 86, 138) indicate a similarity between insect skeletal muscle and mammalian cardiac muscle in that the predominant effect is a progressive decrease in the contractile response to electrical stimulation rather than contracture. Nevertheless, slowing of relaxation under certain circumstances has been observed in isolated flexor tibiae of *Romalea microptera* (58, 61) and extensor tibiae of *Schistocerca gregaria* (138), and frequent stimulation may then lead to summation of successive responses as it does in frog sartorius (128) and rat diaphragm (14). The incomplete ventricular relaxation and increased diastolic pressure recently observed after ryanodine in open-chest cats (98) suggest that interference with relaxation may be a uniform component of the response to ryanodine, whatever its gross effect may be.

There is ample evidence that ryanodine contracture in vertebrate skeletal muscle is not mediated by an effect on the excitability of the muscle cell. Although small changes in resting potential have been reported (88, 138), the contracture develops independently of these changes (88) and resting potentials are normal even at the peak of the response, provided that rupture of the cells is prevented by allowing shortening to occur (14). Contracture in response to ryanodine has been demonstrated in a muscle depolarized with KCl (11), after treatment of a muscle with 0.2 M butanol or in isotonic urea (120), and even in glycerol-extracted muscle (35, 60, 86) in which the cell membrane is generally thought to have been destroyed. However, the situation is not so clear-cut in

mammalian cardiac muscle (see below) or in insect muscle, in both of which the electrical changes found after exposure to ryanodine (51, 130, 138) may possibly play a role in the mechanical events that ensue.

Usherwood (138) has published a detailed description of some electrical changes, measured with intracellular microelectrodes, which occur in insect muscle (*Periplanata americana* and *Schistocerca gregaria*) after exposure to ryanodine. These include increases in resting resistance, length constant, and time constant; a decreased voltage and current threshold for depolarizing current pulses, and conversion of the graded electrical response initially seen in some fibers to an all-or-none response. The last effect can be duplicated by substitution of alkaline earth ions for sodium (145) or by treatment with tetraethylammonium or choline (82). These changes were interpreted as evidence of decreased potassium conductance. Complex changes in neuromuscular transmission were also described, the response to stimulation of the fast axon (nerve 5 of *Schistocerca*) being more sensitive to ryanodine than the response from slow axon (nerve 36) stimulation. Becht and Dresden (9) and Becht (8) had earlier reported that fast muscles of *Periplanata* were more sensitive to ryanodine than slow muscles, and suggested this as an explanation of the sluggish behavior of these insects after sublethal doses of ryanodine (31). The electrical changes reported by Usherwood (138) were found after the application of ryanodine in concentrations of 10^{-3} to 10^{-7} M, while mechanical effects were observed at concentrations as low as 10^{-9} M. Even with high concentrations, mechanical effects were frequently observed before electrical changes became apparent. These observations were interpreted to imply that the drug is acting independently on the two mechanisms. However, it is also possible that the electrical effects are secondary to actions on the contractile system or its metabolic support, since they were never observed to occur alone.

Many agents and procedures produce an irreversible contracture or rigor in skeletal muscle by interfering with metabolism in such a way as to cause exhaustion of creatine phosphate and eventually ATP (144). It is therefore not unreasonable to seek a metabolic defect in ryanodine-treated muscle, and a major metabolic alteration was discovered early in its investigation. G. A. Edwards first reported in 1947 an increase in oxygen uptake in the fiddler crab in response to extracts of Ryania (30). In the following year, Edwards *et al.* (31) and Hassett (66) described increases of up to 9.6 times control levels of QO_2 after injection of ryanodine in cockroaches and other insects. L. E. Edwards and Flinker (32) and Ramsey and Arrighi (120) found even more dramatic increases in QO_2 in isolated frog sartorius muscles, but observed that the oxygen consumption began to fall again when the muscle started to shorten, reaching control levels when the length was about 30% of the rest length. Ramsey (120) claimed that shortening could be prevented also by "high oxygen tensions and optimum diffusion conditions," in which case the oxygen consumption was as high as 24 times resting level. Anoxia, on the other hand, facilitates the contracture following ryanodine both *in vivo* (112) and *in vitro* (14); metabolic inhibitors including cyanide, azide, and 2,4-dinitrophenol also accelerate the response. These data support the view that the

ryanodine-sensitive step may be normally dependent on an oxidative mechanism, but offer no clue as to why the drug increases oxygen consumption so enormously. In a later paper, Edwards and Flinker found that the increase in QO_2 is blocked by azide (2×10^{-3} M), iodoacetate (10^{-2} M), or methylene blue (10^{-3} M) (33), but the relation between QO_2 and muscle length was not reported in these experiments. In contrast to the results described above, Whalen *et al.* (146) found that ryanodine (6×10^{-6} M) produced a small ($< 20\%$) although significant increase in oxygen consumption in rat diaphragm preparations, and this effect did not depend to a significant degree on whether the muscle was allowed to shorten or developed tension. However, it was also found that unlike some species of frog (44), in rat diaphragm the resting QO_2 did not markedly depend on stretch; it was suggested that a common mechanism may underlie the QO_2 response to stretch and to ryanodine. That an increased QO_2 is not a necessary component of the disturbance produced by ryanodine is indicated by the observations of Bodenstein (15) on salamander embryos, which like insects are immobilized and unresponsive to stimulation on exposure to ryanodine, but show a marked reduction in oxygen uptake, which returns to normal as the embryos recover. Finally, it has been shown that an increased QO_2 is associated with contractures produced by a number of agents including caffeine (45); this leads to the suggestion that elevated oxygen consumption is determined by the state of contracture rather than by the particular agent inducing it.

It seems most reasonable to interpret the effect of ryanodine on oxygen consumption as resulting from the operation of homeostatic control mechanisms that may be brought into play because of impairment or uncoupling by the drug of some important energy-yielding or energy-utilizing process. There appears to be no direct evidence of interference with glycolysis or oxidative metabolism by ryanodine. Pick and Tullius (107) reported that glycogen and pyruvic acid levels in frog muscle were unchanged when rigor had been induced by injection of ryanodine (5 μg) into the anterior lymph sac. Seraydarian *et al.* (128) found no change in lactate levels in isolated frog sartorius muscles after exposure to ryanodine (2×10^{-4} M), and, in contrast to iodoacetate (97), ryanodine did not significantly lower the rate of lactate accumulation during stimulation under anaerobic conditions. Glycerol kinase (128), triose phosphate dehydrogenase, creatine phosphokinase (61), and myokinase (87) have all been reported to be unaffected by the alkaloid, and oxygen consumption and oxidative phosphorylation by mitochondria from rat liver, brain (19), and muscle (39) are unchanged. Finally, creatine phosphate and ATP levels in frog sartorius muscles were unchanged by ryanodine in the resting state at 2°C, or after 10 min of stimulation under anaerobic conditions at the highest rate that allows complete relaxation. When a maximal contractural response to ryanodine was induced by stimulation at room temperature, creatine phosphate was reduced to about 10% while the ATP concentration was maintained at 65 to 90% of control levels (128). This contrasts with the severe depletion of both creatine phosphate and ATP that is characteristic of iodoacetate rigor (83) or rigor mortis (10). Guinea pig atria had normal contents of creatine phosphate and (ATP + ADP) after exposure to ryano-

dine (10^{-6} M) for long enough (24 min) to reduce the force of contraction to 10%, although more prolonged exposure resulted in significant depletion of both components (52). From this accumulation of negative data, it appears unlikely that the net effects of ryanodine on vertebrate skeletal and cardiac muscle are mediated by obstruction of energy-yielding metabolic pathways.

Evidence from a variety of sources has directed attention to ion translocation mechanisms as possible sites of action of ryanodine. In particular, calcium ions have long been assigned a central role in the mechanism linking membrane depolarization with contraction of the underlying myofibrils. The mechanisms regulating uptake and release of calcium are obvious sites of possible pharmacological interference (12). Most work with ryanodine along these lines has been with subcellular components prepared by differential centrifugation (see below), but the alternative approach of studying ion fluxes in intact isolated muscles has also been productive.

Ahmad and Lewis (2, 3) first showed that in frog sartorius muscles ryanodine (10 to 100 μg/ml) markedly increases both the influx and efflux of $^{47}Ca^{++}$, and increases the efflux of $^{42}K^{+}$ while reducing its influx. $^{24}Na^{+}$ uptake is also increased. A similar effect on Ca^{++} fluxes was observed with nicotine, succinylcholine, and neostigmine, and had earlier been observed after potassium depolarization and electrical stimulation (13, 129). It was suggested that the primary effect may be an increased Ca^{++} mobility; either this or rupture of the muscle fibers (14) might result in increased permeability of K^{+} and Na^{+}. Bianchi (11) reported an increased Ca^{++} efflux after ryanodine exposure, which reached its maximum at about the time rigor developed, but Ca^{++} uptake was unchanged. Effects on K^{+} and Na^{+} were similar to those described by Ahmad and Lewis (2, 3). Hajdu (54, 55) has also reported that ryanodine can increase the efflux of Ca^{++} from both skeletal and cardiac muscle.

Potassium contractures in frog sartorius are transient, and are associated with transient increases in calcium influx and efflux (13, 129). In a muscle exposed to ryanodine potassium produces an immediate sustained contracture and increase in calcium efflux; on the other hand, a ryanodine contracture in a control muscle or after the transient potassium contracture is characterized by a latent period (11). From these observations Bianchi speculated that "the action of ryanodine is to prevent the resequestration of calcium within the sarcoplasmic reticulum" (12), a concept for which direct evidence had been adduced several years earlier (40, 41).

2. Cardiac muscle. In contrast to the contracture and progressive failure of relaxation seen in vertebrate skeletal muscle, cardiac muscle responds to ryanodine with a progressive decline in contractile force (22, 49, 51–56, 75, 76, 78, 89, 98, 102, 130). Some insect hearts are affected similarly by crude *Ryania* extracts (1). A variety of effects on the electrical properties of the myocardium has also been described (51, 89, 130). The negative inotropic effect was first reported by Hillyard and Procita (76, 78) on spontaneously beating isolated kitten auricles and by Furchgott and de Gubareff (51, 52) on isolated electrically driven auricles of guinea pigs, rats, rabbits, and monkeys. Similar results have been ob-

tained in isolated, electrically driven rat ventricle strips (56) and toad ventricles (102); contractility of mouse heart cells in tissue culture is also suppressed (22) (very large concentrations were used in this study). In contrast, Furchgott has found that isolated strips of frog and turtle ventricle are not significantly affected by ryanodine (2×10^{-6} g/ml) for 1 hr (personal communication). The hypotensive effect of ryanodine is at least partly attributable to myocardial depression (89, 113, 116) and has also been observed in the dog heart-lung preparation (113).

The myocardium appears to be more sensitive to ryanodine than most vertebrate skeletal muscle, and the negative inotropic effect has been observed on mammalian atrium at concentrations as low as 5×10^{-9} M (76, 78). Mammalian ventricle is somewhat less sensitive (49). Like the action on skeletal muscle, the effect appears more rapidly at higher concentrations, and is evident in 3 to 4 min at 10^{-7} M. Nayler (102) found that 10^{-6} M ryanodine invariably decreased the force of contraction of electrically driven isolated toad ventricles by 80 to 100% in 60 min. The effect cannot be reversed or prevented by repeated washing after exposure to ryanodine; however, agents with marked positive inotropic effects may temporarily increase contractile force. These include catecholamines (76, 78), excess Ca^{++} (76, 78, 102, 130), cardiac glycosides (130), and caffeine (102). Hajdu and Leonard (56) reported that removal of potassium from the perfusing solution, or replacement of sodium with lithium or sucrose, reversed the inotropic effects of ryanodine on isolated rat ventricle strips, but they did not indicate whether the reversal was transient or long-lasting. Dramatic and apparently complete recovery of the toad ventricle after total disappearance of the contraction following 10^{-6} M ryanodine was described by Nayler (102). The recovery occurred after a 1-min exposure to 4 mM EDTA, and was preceded by a burst of spontaneous activity. Although it was not stated how long the recovery persisted, the records presented gave no indication that the effect was temporary. This reversal was interpreted to suggest that ryanodine is bound to the myocardium by a cation, that the cation-ryanodine complex is chelated by EDTA, and hence is removed from the myocardium. Washing skeletal muscle with EDTA after a maximal response to ryanodine has been reported to produce no reversal (14). There is no direct evidence regarding the ability of ryanodine to complex with divalent ions, but Ca^{++} and Mg^{++} do not influence the ultraviolet absorption spectrum of ryanodine, nor does ryanodine influence the enzymatic activity of creatine phosphoryl transferase under conditions in which the concentration of Ca^{++} or Mg^{++} is rate-limiting (87). Interpretation of this very interesting reversal is therefore an open question, and additional work along these lines is clearly required.

Although no extensive study of the influence of temperature has been made in the case of cardiac muscle, the available data indicate that the striking dependence observed with skeletal muscle is not seen in the myocardium. Hillyard and Procita (78) concluded that the effect of temperature on the rate of ryanodine-induced failure of spontaneously beating kitten auricles could be quantitatively accounted for by a change in the spontaneous frequency of contraction. In analogy with the established dependence of the ryanodine effect in skeletal muscle upon

induced activity, they found that temporary suppression of spontaneous contractions by acetylcholine (2×10^{-6} M) or KCl (1.68×10^{-2} M) delayed the negative inotropic response to ryanodine. However, the relationship between contractile force and frequency in cardiac muscle is a complex one, and this interpretation is almost certainly an oversimplification. Hajdu and Leonard (56) studied the force-frequency relationship in electrically driven strips of rat ventricle and found that prolonged washing of the preparation over a period of 3 to 5 hr caused a characteristic shift in which the contractile force is unchanged at high frequencies (up to 1/sec) but progressively decreases at low frequencies (1/min). Exposure to ryanodine (4×10^{-8} M) for 15 min produced a similar but even more marked shift. Furchgott (49) and Sleator *et al.* (130) reported that isolated guinea pig left atria which had been exposed to ryanodine (1 to 2×10^{-6} M for about 20 min, followed by washout) exhibited a much greater decrease in contractile strength with decrease in steady state frequency of contraction than did control preparations. If in this preparation a rest interval of several seconds is interposed in a regular series of contractions at 1/sec, the first contraction after the interval is much stronger than in the steady state. This "PR beat" (post rest) was particularly sensitive to ryanodine, and 30 min after a 15-min exposure to 4×10^{-6} M ryanodine, the PR beat was virtually abolished, although the steady state force was only moderately depressed. Furchgott (50) has provided additional data on the influence of ryanodine on the re-entry (PR) beat. When tested 1 hr after a 20-min exposure to ryanodine (2×10^{-6} g/ml), the relative amplitude of the re-entry beat became progressively smaller as the rest interval increased, and fell essentially to zero if the interval exceeded 5 to 10 sec.

In a later study, Hajdu (54, 55) concluded that ryanodine specifically interferes with the mechanism responsible for the "reverse staircase" phenomenon originally described by Woodworth (153) for cardiac muscle, but apparently demonstrable also in skeletal muscle (24, 54, 55). This phenomenon is characterized by an increase in contractile force after a period of rest, and its abolition by ryanodine was found to be associated in both cardiac and skeletal muscle of several species with an efflux of calcium (54, 55). Grossman and Furchgott (53) had earlier reported that calcium content and exchange in guinea pig atria were unaffected by ryanodine. The reasons for this discrepancy are obscure.

Sleator *et al.* (130) pointed out that the mechanical effects of ryanodine were exactly opposite to those of strophanthin K or excess Ca^{++}, either of which could reverse the ryanodine effects if these had not progressed too far. This opposition extended beyond observations of contractile strength. Action potentials of atrial cells recorded with intracellular electrodes after exposure to ryanodine showed a characteristic prolongation of the plateau phase, which was associated with the decline in contractile force. Strophanthin K and excess Ca^{++} both cause a decrease in duration of the action potential. Nayler (102) also showed a temporary reversal of the inotropic effects of ryanodine by an increase in stimulus frequency or Ca^{++} concentration. Hajdu and Leonard (56) drew attention to the opposite nature of the effects of strophanthin and ryanodine on rat ventricle strips, but found that although ryanodine (0.02 μg/ml) abolished the response to strophan-

thin, the reverse was not true. It is interesting to note that ryanodine apparently does not reverse the ouabain-induced inhibition of sodium efflux from erythrocytes (79).

The antagonism between ryanodine and cardiac glycosides led Hajdu and Leonard (56) to investigate the possibility that ryanodine might reverse the effects of glycosides on cardiac rhythm. It was found that ryanodine (10 to 15 μg/kg infused intravenously over a period of about 5 min) caused the disappearance of ventricular arrhythmias which had been induced by the previous administration of digitoxin. When ryanodine was given first, lethal doses of digitoxin failed to cause ventricular arrhythmias; however, in both series of experiments, sinoatrial activity was depressed and ectopic rhythms appeared. Since these effects were also seen after either drug alone and were abolished by atropine in some animals, it was suggested that they result from a synergistic effect mediated by vagal stimulation. A more detailed study was undertaken by Kahn *et al.* (89), who confirmed the basic finding that ryanodine, in doses ranging from 2.4 to 18.6 μg/kg, invariably abolished ectopic ventricular rhythms induced in anesthetized dogs by intravenous digoxin. Again, conversion to sinus rhythm was generally transient, and supraventricular tachycardias often supervened. In contrast to Hadju and Leonard's results (56), sinus bradycardia and intermittent A-V block produced by ryanodine alone or by both drugs were unaffected by up to 40 μg of atropine per kg. Because of the suppression of ventricular arrhythmias induced by digitalis, and the complete absence of ventricular escape beats during the long periods of sinoatrial arrest after ryanodine alone, it was concluded that ryanodine exerts a specific suppressive action on ventricular pacemakers. Doses of 10 to 15 μg of ryanodine per kg reduce cardiac output, mean aortic pressure, and left ventricular work and elevate systemic vascular resistance. This was interpreted as evidence of the negative inotropic effect, which is well established *in vitro* but had not before been fully documented *in vivo*. It was suggested that the toxicity of ryanodine in the relevant dose range precludes its use for the treatment of digitalis intoxication (89).

3. Smooth muscle. Relatively little information is available concerning the effects of ryanodine on vertebrate smooth muscle. Hillyard (75) and Hillyard and Procita (77) studied the effects on isolated rabbit duodenal strips; this preparation was selected because of the spontaneous rhythmic contractions it normally exhibits. At concentrations in excess of 10^{-7} M, ryanodine gradually increased the tone without completely abolishing the rhythmic contractions. As in the case of skeletal muscle, the rate at which the effect appeared was greater when the drug concentration was high, or when the spontaneous tone of the preparation was enhanced by caffeine (0.1 mg/ml); conversely, the increase in tone produced by ryanodine was delayed when the spontaneous tone or activity of the preparation was reduced by a Ca^{++}-deficient solution, elevated Mg^{++} concentrations (8.4×10^{-3} M), or cyanide (10^{-4} M). Dinitrophenol (5×10^{-5} M), which had little effect by itself, also delayed the ryanodine response, but in each case the typical response developed when the bathing solution was replaced with fresh Tyrode solution containing no ryanodine. As in the case of skeletal muscle, washing the

preparation after development of the contracture did not produce a reversal of the effect; unlike vertebrate skeletal muscle, however, normal tone could be restored to rabbit duodenal strips by the addition of agents that relax intestinal smooth muscle, including epinephrine (1 μg/ml), papaverine (20 μg/ml), procyclidine, and pyrilamine. When the relaxant was removed, the tone again increased to the level seen after ryanodine alone.

Similar results were obtained by Haslett and Jenden (61) on isolated intestinal preparations from rat and guinea pig, but ryanodine had no effect on guinea pig or rat uterus, even when it was repeatedly stimulated to contract.

C. Miscellaneous effects

Reports by Cornman (22) and Bodenstein (15) of antimitotic activity suggested that ryanodine might interfere with contractile mechanisms other than muscle. Growth and motility of several microorganisms were unaffected by the drug (14, 58, 61), and motility of spermatozoa was also unchanged. It subsequently appeared that the antimitotic activity reported by Cornman and Bodenstein might be due to a rotenoid present in Ryania extracts (123).

Subsequent investigation showed that many types of muscle show no mechanical response to ryanodine, including some vertebrate smooth muscle (58, 61); others respond like mammalian cardiac muscle with a progressive contractile failure. There was no obvious correlation between the morphological or functional characteristics of the muscle and its response to ryanodine, although muscles responding to ryanodine with rigor had in common relatively high phosphagen levels, regardless of the type of phosphagen they contain. Both creatine- and arginine-ATP-transphosphorylase were unaffected by ryanodine (61), and the significance of the correlation is obscure.

Two studies have been published describing inhibition of spontaneous rhythmic contraction of cardiac and skeletal muscle cells in tissue culture (22, 108), where no neural elements are present.

D. Subcellular components of muscle

Although it was postulated many years ago (31) that ryanodine exerted its effect specifically on the contractile process in striated muscle, no direct evidence was cited, and subsequent work failed to reveal any interference with the ATPase activity of myosin B (14, 107), the light-scattering response of myosin B to ATP, or shortening of glycerol-treated psoas fibers induced by ATP (14). However, in psoas fibers extracted in glycerol for a few days instead of the usual period of $\geq$3 months ATP induces a brief contraction followed by relaxation (16). After ATP-induced contraction and relaxation of these briefly extracted fibers, ryanodine induces a maximal contracture (60, 86); this finding suggests that ryanodine interferes specifically with a glycerol-sensitive mechanism by which relaxation is effected. It seemed probable that this might be the "relaxing factor system" (see 28 and 142 for excellent reviews), a particulate fraction of muscle homogenate that operates through the sequestration of Ca^{++} by an ATP-driven transport system (25, 26, 29, 64). Progressive inactivation of the calcium trans-

port system during glycerol extraction of rabbit psoas fibers and cardiac slices has been demonstrated (34, 43), and the site of uptake has been shown by electron microscopy to be in the sarcoplasmic reticulum (23, 63, 105). Calcium transport by particulate components of muscle became a prime candidate as the site of action of ryanodine, and it was soon shown to be disrupted by the alkaloid (40).

1. Calcium transport system. The mechanism whereby the sarcoplasmic reticulum accomplishes the removal of calcium is not fully understood, but the process is believed to occur in two steps. First, the calcium is bound to specific sites in the membrane of the reticulum in the presence of ATP, and second, a part of this initially bound calcium is transported into the lumen of the reticulum. The transport phase can be conveniently followed in the presence of oxalate, which provides a large "sink" for the transported calcium. Under these conditions ryanodine (40) alters the stoichiometry of calcium uptake and ATP hydrolysis in crude preparations of the sarcoplasmic reticulum of skeletal muscle, causing the breakdown of a larger amount of ATP for a given amount of calcium transported, and thereby effecting an apparent "uncoupling" of the calcium transport mechanism. Ryanodine also increases the associated Ca^{++}-activated ATPase activity but has no effect on the Mg^{++}-activated ATPase. Subsequently, Martonosi and Feretos (99) found no effect of 5×10^{-4} M ryanodine on the rate of Ca^{++} uptake by a rabbit skeletal muscle fraction isolated between 8,000 and 28,000 g, whereas Takauji *et al.* (135) showed an inhibition of Ca^{++} uptake rate by 10^{-3} M ryanodine, but little effect on the steady state uptake. Since the muscle fractions used by various workers differed widely, a study was made of the distribution of a ryanodine-sensitive Ca^{++} pump within fractions isolated from skeletal muscle. The fraction widely used in studies of calcium pump activity (obtained in the range 8,000 to 30,000 g) was much less sensitive to ryanodine at any concentration than the heavy particles isolated in the range 2,000 to 8,000 g (41), which are significantly inhibited by ryanodine at concentrations down to 10^{-6} M. Addition of ryanodine to these heavy particles produces a typical alteration in the kinetics of calcium uptake. Initially, a marked inhibition is seen, but after a prolonged interval spontaneous recovery occurs, until eventually the steady state calcium uptake approximates that of a control experiment (42). This recovery from ryanodine inhibition apparently is not due to destruction of the alkaloid or to accumulation of materials such as ADP during the prolonged incubation, and is still under investigation.

The heavy muscle fractions (2,000 to 8,000 g) exhibit appreciable cytochrome oxidase activity and undoubtedly contain mitochondria. It appeared possible that the presence of mitochondrial elements was related to the ryanodine sensitivity of the fraction, but the available evidence is opposed to this view. The calcium pumping activity and associated ATPase in the presence of oxalate are not affected by azide, 2,4-dinitrophenol, or oligomycin, agents which markedly modify these activities in muscle mitochondria (18). In the absence of oxalate, the rate of calcium accumulation is much lower, but it is now inhibited by azide and is not affected by ryanodine. This suggests that the mitochondrial component of the heavy muscle fraction accounts for much of the calcium accumula-

tion when oxalate is omitted, and is insensitive to ryanodine; in the presence of oxalate, reticular elements are activated and the mitochondrial contribution thus becomes only a very small part of the total pumping activity. Other workers have also concluded that the calcium-pump activity of heavy muscle fractions is not due to mitochondria. Martonosi and Feretos (99) suggested that calcium uptake by a 1,000 to 8,000 g muscle fraction is due to the presence of unbroken reticular elements, since uptake is not affected by antimycin or dicumarol; Weber *et al.* (143) reached a similar conclusion after finding that uptake by their 2,000 to 8,000 g fractions was insensitive to oligomycin-cyanide or dicumarol. Although the sensitivity of heavy fractions of muscle homogenates to ryanodine does not appear to be directly attributable to interference with a mitochondrial calcium pump, the possibility remained that mitochondria might influence the sensitivity of other particulate fractions, either directly or by conversion of ryanodine to an activated form. This possibility was examined by incubating ryanodine with mixtures of muscle mitochondria and lighter fractions (12,000 to 35,000 g), which by themselves show little or no ryanodine sensitivity. In none of these experiments was the sensitivity to ryanodine altered (42), and it may be concluded that mitochondria play no significant role in the mechanism by which ryanodine interferes with calcium transport by the heavy fraction of muscle.

The effect of ryanodine has also been examined on calcium accumulation by heavy fractions obtained from white muscle (psoas) and red muscle (diaphragm), and the preparations were found to be equally sensitive to the alkaloid (42). Since the relative proportions of mitochondria and sarcoplasmic reticulum are markedly different in these muscle types, these results could be interpreted as lending further support to the thesis that mitochondria are not involved in the ryanodine effect. Sreter and Gergely (133) found differences in morphology and inhibitor sensitivities of the calcium pump and ATPase between sarcoplasmic reticulum fractions isolated from red and white muscles, and suggested that the mechanism of relaxation in red muscle might be different than that of white muscle. On the other hand, these findings and those of Takauji *et al.* (136) and Harigaya *et al.* (57), that active calcium-pumping particles can indeed be isolated from red muscle, suggest that relaxation in both red and white muscle is brought about by similar mechanisms involving calcium translocation. Differences in myosin (7) have also been reported for red and white muscles, and against this background of differences between the two muscles, it is significant that similar effects of ryanodine are seen on calcium uptake by the red and white heavy fractions and also on isolated, intact preparations of the two muscles.

By electron microscopy, the heavy particulate fraction of skeletal muscle is quite heterogeneous, containing mitochondria and a variety of vesicular structures, including elements resembling triads and terminal vesicles. Subfractionation of the heavy particle fraction on a sucrose density gradient has been partially successful, since four or five subfractions can be separated, which differ from the parent fraction in calcium accumulation rates and ryanodine sensitivities (42). However, assays of cytochrome oxidase indicate incomplete redistribution of mitochondrial elements within the subfractions, and electron microscopy of the

subfractions has not yet provided a definitive correlation of morphological content and enzymic activity. Another factor that has limited the usefulness of density gradient subfractionation of the heavy particles is loss of calcium-pumping activity during the fractionation, with only some 50 to 70% of the initial activity being recovered after separation on gradients of cesium chloride, sucrose, or Dowex-treated sucrose; there is also a partial loss of ryanodine sensitivity in the recovered material after subfractionation. Both the decreased control activity and decreased sensitivity to ryanodine may be due to osmotic damage to vesicular structures, but the altered ryanodine sensitivity after subfractionation seems attributable in part also to a more direct effect of sucrose, which reduces the ryanodine inhibition of calcium uptake by unfractionated heavy muscle particles (42) when added during or after their preparation. Uchida *et al.* (137) showed that particulate fractions of muscle isolated between 10,000 and 40,000 g in sucrose had a higher calcium-pumping activity than preparations isolated in an electrolyte medium, and that these latter preparations contained contaminating actomyosin, which could be removed at high ionic strengths. The fact that the ryanodine sensitivity of 2,000 to 8,000 g fractions was unaltered by extraction with 0.6 M KCl to remove a possible actomyosin contaminant (40) suggests that the low ryanodine sensitivity of preparations isolated in sucrose is not related to the absence of contaminating actomyosin.

It seems reasonable to suppose that the ryanodine-sensitive particles represent only one component of the sarcoplasmic reticulum, which is both pharmacologically and functionally heterogeneous. Certainly distinct substructures can be seen within the reticulum (104), and the terminal cisternae appear to possess a modified membrane structure in apposition to the transverse tubules (38). Convincing evidence of the functional heterogeneity of the reticulum has been presented in a recent radioautographic study of calcium movements in resting and tetanized muscle (150). However, identification of a specific site of action of ryanodine within the sarcoplasmic reticulum must await the development of more successful subfractionation procedures, or demonstration of a specific intracellular location of ryanodine. It would be reasonable to expect that the muscle cell membrane contains a calcium transport system (142), and Koketsu *et al.* (93) have shown that skeletal muscle cell membranes can bind calcium. However, little is known of the characteristics of any sarcolemmal transport mechanism, and the effect of ryanodine on such preparations has not been reported.

All of the above studies on the effect of ryanodine on calcium uptake have been concerned with the second phase of calcium uptake; namely, calcium transport across the sarcoplasmic membranes into the lumen. The initial phase of transport, that of calcium binding to the membranes, has been shown to occur rapidly and can best be demonstrated in the absence of oxalate, under which conditions the bound calcium represents the major fraction of the calcium taken up (84). Carvalho and Leo (21) have shown that the amount of calcium bound is increased in the presence of ATP and have termed this increment "active binding." Recent work in the authors' laboratory has shown that ryanodine strongly suppressed

this active binding, and that if ryanodine is added to the system after active binding has occurred, there is a rapid release of much of this bound calcium, similar to the release of bound calcium from reticular fractions produced by caffeine (21). Interference by ryanodine with the initial calcium binding step would undoubtedly lead to some type of inhibition of the subsequent step involving transport of calcium, and it is possible that the effect of the alkaloid on calcium uptake is attributable to interference with the initial binding. It has been suggested (28) that ATP induces active binding of calcium by inducing a conformational change in the reticular membranes resulting in an increased affinity for calcium and a more successful competition by Ca^{++} with Mg^{++} and K^{+} for binding sites (21), and it will be interesting to see by what means ryanodine exerts its effects in this somewhat simpler system.

Ebashi and Endo (28) have also suggested that calcium binding is one of the general features of the endoplasmic reticulum, irrespective of the cellular species, and Otsuka *et al.* (103) have shown that brain microsomes exhibit a slow but considerable ATP-dependent calcium binding. This mechanism of binding appears to be different from that of the heavy skeletal muscle fractions, however, since calcium binding by brain preparations is not affected by ryanodine (42).

2. Contractile proteins. Uncoupling of calcium transport or inhibition of active calcium binding would provide a sufficient explanation for most of the gross phenomena observed after application of the drug to skeletal muscles. There is, however, evidence for an independent effect exerted directly on the contractile proteins. Elison and Jenden (35) found that ryanodine enhanced the tension development induced by ATP in rabbit psoas fibers which had been extracted in glycerol for long enough (120 days) to destroy the calcium transport system, or which had been treated with desoxycholate, a procedure known to cause rapid inactivation of the calcium pump (34, 47). This enhancement was dependent on Ca^{++} concentration, which was buffered with EGTA in these experiments, and the ryanodine effect could be interpreted as sensitization to calcium ions. Failure of earlier experiments to demonstrate an effect of ryanodine on chronically extracted fibers (14) might be related to inadequate control of Ca^{++} concentration in the relevant range (10^{-7} to 10^{-5} M), since the importance of this ion as a regulator of actomyosin-ATP interaction was not fully recognized at the time. Similar experiments on natural actomyosin and myofibrils in the presence of a Ca^{++} buffer also indicated a direct effect of ryanodine (36), particularly when ATP is present in excess. It was suggested on the basis of this work that ryanodine might inhibit the dissociation of actomyosin into actin and myosin that occurs at high ATP levels (62).

Procita (114) had earlier described some experiments of a different type which led to a similar conclusion. Ryanodine (0.2 to 2 mg/kg) was injected into rabbits, and after the development of rigor the muscles were extracted by techniques that would extract myosin A or myosin B from normal muscle. A modified form of myosin B, termed myosin R, was extracted from the ryanodine-poisoned muscle, and fiber models (67) formed from myosin R developed greater tension when exposed to ATP than similar fibers prepared from myosin B obtained from

control muscles. Myosin R, obtained after only 30 min extraction of ryanodine-poisoned muscle, did not exhibit the reduced viscosity changes typically seen with myosin B upon exposure to ATP. Electrophoresis experiments indicated that addition of ATP to myosin R did not produce a simple dissociation of the macromolecule, and comparisons of the extractions of control and ryanodine-poisoned muscle for myosin A showed a lower amount of extractable myosin A in the poisoned muscle. These observations were interpreted as indicating that the actin and myosin A are much more tightly bound in myosin R than in myosin B, and that the pharmacological effect of ryanodine reflects its modification of the dissociability of actomyosin.

It is of interest to examine the stoichiometry of this interaction of ryanodine with the contractile protein complex, by using these data. A rabbit has approximately 400 g/kg of muscle (wet weight) and 1 g of muscle contains about 100 mg of myofibrillar protein. Assuming that the smallest unit of the contractile protein complex consists of some combination of actin, myosin, and a tropomyosin-like protein, this unit would have a molecular weight of about 700,000; there would thus be some 57 μmoles of the contractile complex per kg of rabbit, if all the myofibrillar protein consisted of contractile protein. The dose of ryanodine used by Procita was 0.2 to 2.0 mg/kg (0.4 to 4.0 μmoles/kg), and even if the ryanodine were distributed exclusively in muscle, it would appear that less than 7% of the contractile protein complex could be combined with ryanodine; even this value is too high since the sarcoplasmic reticulum is also affected by ryanodine (40) and presumably also binds the alkaloid. If this calculation represents a fair assessment of the stoichiometry it suggests that ryanodine produces these changes in the contractile proteins by acting catalytically rather than directly. An elevated sarcoplasmic Ca^{++} level resulting from interference with Ca^{++} sequestration could explain the lack of stoichiometry, but it is more difficult to account for the formation of a modified contractile protein (myosin R) which was interpreted as a stabilized "total preparation of actomyosin." Parallel experiments in which rigor was induced with iodoacetate failed to yield a similarly altered myosin B preparation, but it would be of interest to examine the extractability and properties of myosin after the injection of other contracture-producing drugs under similar conditions. In view of the fact that caffeine is thought to act by interference with calcium binding by the sarcoplasmic reticulum (125, 142) a similar study with this drug would be of particular importance.

Elison and Jenden (37) investigated the effect of ryanodine on actomyosin reconstituted *in vitro* from actin and highly purified myosin A. The ATPase activity was enhanced by ryanodine up to 3-fold if the actin was extracted at room temperature, but pure preparations of actin yielded actomyosin that was unaffected by the alkaloid. The enhancement showed a characteristic time course, increasing progressively for 10 to 30 min after addition of ryanodine. Maximal enhancement could be induced by preincubation of crude actin and myosin A with ryanodine in the absence of Mg ATP, but not by preincubation of any of the components of the system unless and until ryanodine was added; the usual slow enhancement then occurred. Calcium was also required in subsaturating amounts

for this effect to be seen. It was concluded that a factor extractable with actin is required for the enhancement of actomyosin ATPase by ryanodine and that the enhancement probably depends upon stabilization of the enzymatic complex by the drug. Several factors have been described that influence the physicochemical and enzymatic properties of actomyosin (see 28 for recent review) and the possible identity of one of these with the actin contaminant required for the ryanodine effect has not yet been established.

IV. CONCLUSIONS

Pharmacological potency of the order exhibited by ryanodine is usually associated with a highly specific mechanism of action. The great variety of effects observed and the differences between the responses in different muscles make it difficult to suggest a single underlying mechanism for this drug, or even to distinguish between its primary action and secondary phenomena that represent degenerative or compensatory changes in a self-regulating cellular system. From the evidence available, it seems probable that the increased oxygen consumption, and perhaps some changes in the electrical properties of the cell membrane, may fall into the latter category.

In the case of vertebrate skeletal muscle, the evidence favors the view that ryanodine specifically interferes with relaxation, which is generally thought to be effected by sequestration of calcium ions by the sarcoplasmic reticulum. Ryanodine has been shown to obstruct both active binding and active uptake of Ca^{++} by particulate fractions of muscle homogenates, and to result in an efflux of Ca^{++} from skeletal muscle. It seems reasonable to conclude that ryanodine interferes with intracellular Ca^{++} translocation mechanisms that normally lower the sarcoplasmic concentration and thereby effect relaxation. The efflux of calcium could be accounted for by a compensatory increase in the rate of active transport across the cell membrane, either directly or through the T-tubular lumen. Although such a sarcolemmal transport system has not been unequivocally demonstrated, its existence can be deduced from the very large electrochemical gradient for Ca^{++} which is normally maintained across the resting muscle cell membrane. The increased oxygen consumption observed after ryanodine treatment could result from an elevated metabolic requirement presented by 1) uncoupling of intracellular Ca^{++} translocation mechanisms, 2) activation of a sarcolemmal Ca^{++} transport system, and 3) increased myofibrillar ATPase activity resulting from elevated sarcoplasmic Ca^{++} concentrations. Changes in the electrical properties of muscle cell membranes could also be secondary to alterations in calcium balance, since calcium concentration is well known to have a profound influence on alkali metal ion permeability, threshold, and other electrical characteristics of the membrane.

Although this proposal provides a reasonable explanation for many of the effects observed after ryanodine treatment, other experimental data are more difficult to account for. Perhaps the most important of these are the difference between the responses shown by skeletal and cardiac muscle and the apparent lack of effect on some types of smooth muscle. Hajdu (54, 55) has drawn attention

to the apparently intimate relationship between the action of ryanodine and the "reverse staircase effect" in both cardiac and skeletal muscle, and has presented data (55) showing that skeletal muscle also responds to ryanodine with progressive contractile failure in a calcium-free solution. This suggests that the different effects normally produced by ryanodine in cardiac and skeletal muscle may depend upon a quantitative rather than a qualitative difference in the mechanisms underlying the contraction-relaxation cycle. One possibility consistent with the proposal made here is a difference in the relative importance of intracellular translocation and transmembrane flux of Ca^{++} in regulating the contraction relaxation cycle. It is well established that cardiac muscle is dependent on extracellular calcium for its contractility, while skeletal muscle may retain its ability to contract for prolonged periods in the absence of extracellular calcium. If extrusion of calcium from the cell is relatively more efficient and important in effecting relaxation in cardiac muscle while intracellular translocation of calcium is the principle factor terminating the active state in skeletal muscle, interference with intracellular Ca^{++} transport by ryanodine might be insufficient to cause a rise in sarcoplasmic Ca^{++} above the critical level for contraction in the myocardium, so that the net effect of the drug would be to cause a progressive depletion of calcium [or at least the "contraction pool" (53, 101)] and hence contractile failure. In skeletal muscle, on the other hand, outward transport through the cell membrane may be inadequate to remove the calcium as it is liberated from an intracellular site by stimulation, and inhibition of intracellular uptake mechanisms would result in a progressive failure to relax and eventually in contracture. The contracture should be inhibited either by reducing the rate of intracellular Ca^{++} liberation (less frequent stimulation and perhaps cooling) or by favoring the operation of an outward sarcolemmal transport system (reducing extracellular Ca^{++} concentration or improving conditions for oxidative metabolism).

This explanation predicts a decrease in cellular calcium levels in both cardiac and skeletal muscle. The available data are consistent with this in the case of skeletal muscle (11, 54, 55) but conflicting in cardiac muscle (53–55). However, it seems significant (102) that the effects of ryanodine on cardiac muscle are antagonized by caffeine, digitalis glycosides, increased stimulation rate and elevated extracellular Ca^{++} concentrations; all of these have been shown to result in Ca^{++} accumulation by the myocardium (92, 96, 100, 149, 151), although toxic amounts of the drugs or Ca^{++} may be required. As others have pointed out, only a fraction of the total calcium appears to be involved in excitation-contraction coupling in cardiac muscle (95), and changes in it may be difficult to detect (53). Flux studies in contracting cardiac muscle are also complicated by changes in the duration of the action potential induced by drugs (53, 130).

The total lack of response to ryanodine in certain types of smooth muscle (61) could be simply explained if in these muscles intracellular calcium transport systems were of negligible importance compared to outward transport across the cell membrane. Information on this point is lacking, but the small cell dimensions in these muscles and the relatively slow contraction and relaxation that they normally exhibit would seem to make an intracellular calcium transport system less necessary for their normal function.

Studies on particulate components of vertebrate skeletal muscle have demonstrated heterogeneity in the ryanodine sensitivity of different fractions (41). The sensitive heavy fraction may be derived from a specialized intracellular uptake site, while the insensitive lighter fractions could represent resealed fragments of T-tubules and cell membrane. Investigation of this possibility will require considerable refinement in particle fractionation techniques and detailed correlation of biochemical properties with ultrastructure.

Postulation of an intracellular calcium uptake mechanism as the only site of action of ryanodine leaves unexplained a series of experiments in which effects of the drug have been found in model contractile systems free of functional remnants of the sarcoplasmic reticulum (35, 36, 37), and these must tentatively be accepted as indicating a second discrete action of the drug. Ryanodine has been shown to influence both a contractile protein complex and calcium binding by a reticular fraction under conditions in which the other sensitive system is absent or has been inactivated (37). However, both actions are demonstrable only when Ca^{++} is present in subsaturating amounts, and are discernible as an increase in Mg^{++}-activated ATPase, which appears to be sensitized to calcium ions. It is tempting to speculate that a common molecular mechanism may underlie both effects. One possible mechanism which remains to be investigated is modification of the capacity or affinity of a key protein or proteins for complexing Ca^{++}. Troponin, a protein which in association with tropomyosin has been shown to cause a calcium-reversible inhibition of actomyosin (*i.e.*, to sensitize actomyosin to calcium) (28), has recently been reported to have a high capacity and affinity for calcium (27, 155), and binding of calcium to troponin in the myofibril may account for activation of myofibrillar contraction (48). This or a similar protein could serve as a sink in the intact sarcoplasmic reticulum [the granular material seen in terminal cisternae (104)?] in the same manner that oxalate is thought to function *in vitro*. Interference with the ability of such a protein to complex Ca^{++} could reduce the speed and efficiency of the reticular calcium pump and might also reduce the calcium requirement for activation of the contractile protein complex.

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