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Modification of Ryanodine Toxicity by Dantrolene and Halothane in a Model of Malignant Hyperthermia

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Ryanodine toxicity in animals has been suggested to constitute a model of malignant hyperthermia. Dantrolene is known to block the development of malignant hyperthermia triggered by halothane in susceptible swine. The authors studied the influences of dantrolene and halothane on the effects of ryanodine *in vitro* in isolated rat diaphragm muscle segments, and *in vivo* in mice, to explore the validity of this model. In the diaphragm experiments, dantrolene was found to block or delay the development of contractures produced by ryanodine and to delay the potentiation of ryanodine-induced contractures caused by halothane. In mice, ryanodine at various dosages was injected and animals surviving after one hour were examined. Such survivors appeared grossly to be normal, and may constitute a model for the malignant hyperthermia patient. They were found to be susceptible to halothane and to succinylcholine, being killed by treatment with these two agents at dosages that were not lethal to control mice. Pretreatment of mice for 48 hours with orally administered dantrolene, followed by injection of ryanodine and then halothane anesthesia, decreased the lethality of ryanodine but did not reduce the number of deaths caused by the subsequent exposure to halothane. That the effects of ryanodine *in vitro* and *in vivo* are diminished and potentiated by dantrolene and halothane, respectively, would suggest that the ryanodine toxicity model of malignant hyperthermia may have validity and is worthy of further study. A prediction from this model is that the terminal cisternae of skeletal muscle sarcoplasmic reticulum may be altered in MH. (Key words: Anesthetics, volatile: halothane. Complications: malignant hyperthermia. Hyperthermia: malignant pyrexia. Neuromuscular relaxants: dantrolene; succinylcholine. Toxicity: ryanodine.)

THE ALKALOID ryanodine produces irreversible contracture in vertebrate skeletal muscle, together with a negative inotropic response in mammalian cardiac muscle.[1–3] Although relatively little is known of the mechanism of action of this agent at the molecular level, there is evidence from studies with both skeletal and cardiac muscle that ryanodine affects the sarcoplasmic reticulum and alters calcium uptake or release mechanisms.[4–7] An altered intracellular calcium metabolism in skeletal muscle which is further critically affected by the administration of halothane and other agents has been suggested to be central to the malignant hyperthermia syndrome (MH).[8] Similarities between MH and some of the effects of ryanodine led to the proposal by Casson and Downes[9] that ryanodine toxicity might constitute a useful laboratory model for the study of MH. They reported that halothane markedly increased the rate of contracture produced by ryanodine in isolated skeletal muscle preparations of frogs and mice. However, their experiments showed that the skeletal muscle relaxant dantrolene did not diminish the ryanodine–halothane-induced contractures when studied in frog muscle. Since pretreatment with dantrolene has been shown to block the halothane-induced muscle rigidity and hyperthermia in MH pigs,[10] the validity of the ryanodine model of MH would be strengthened if it could be shown that dantrolene diminished the effects of ryanodine plus halothane.

Although the mechanism of action of dantrolene in producing muscle relaxation is not completely understood, this drug has been shown to decrease the rate of calcium release from the isolated sarcoplasmic reticulum,[11,12] whereas, in contrast, ryanodine increases calcium release[6] from a reticulum preparation; thus, the two drugs may exert opposing actions at morphologically related sites. In experiments exploring the mechanism of action of dantrolene on isolated mammalian muscle, Morgan and Bryant[13] have shown that this drug is without effect below 18 C. Thus, since Casson and Downes' work with ryanodine and dantrolene in frog muscle[9] was performed at 15 C, it is possible that this temperature dependency explains their negative findings. Experimental work in MH has been limited by the infrequent occurrence of this syndrome in man and by the difficulties in procurement and maintenance of MH-susceptible swine. Thus, we were interested in exploring further the validity of the ryanodine toxicity model of MH in mammalian muscle. Our results show that dantrolene can block or delay the effects of ryanodine on skeletal muscle and thus lend further credence to the ryanodine toxicity model of MH. We also demonstrate limitations in this model.

Methods

Isolated rat diaphragm experiments explored the influence of pre-exposure to dantrolene *in vitro* on ryanodine-induced contractions in non-stimulated tissues. Diaphragm preparations were made from 90–120-g male Wistar rats, using four segments per

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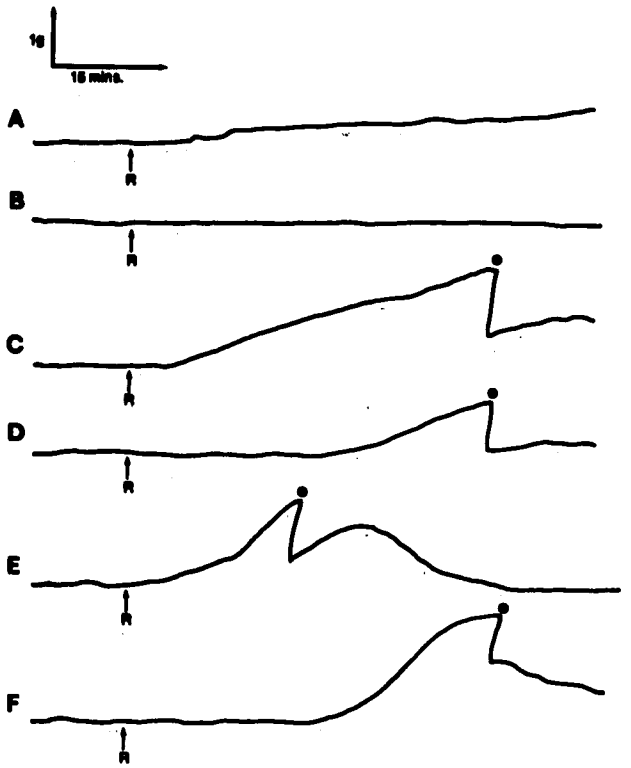

FIG. 1. Antagonism by dantrolene of the effects of ryanodine in a typical rat diaphragm preparation. Diaphragm segments were equilibrated for 45 min in Krebs medium, then for a further 15 min in Krebs medium containing 5.7×10^{-5} M dantrolene, or medium alone. The ryanodine concentrations used were 10^{-6} M (Experiments A and B); 3×10^{-6} M (Experiments C and D), and 10^{-5} M (Experiments E and F), the drug being added where indicated by arrows. Dantrolene was present in Experiments B, D and F. The sensitivity of the recorder was decreased by a factor of 2 where indicated by a dot.

diaphragm, and were suspended in 50 ml of Krebs medium gassed with a mixture of 95 per cent O_2–5 per cent CO_2 at 37 C and connected via FTO3C strain gauges to a Grass model 7 polygraph at an initial tension of 1 g. The tissues were equilibrated for 45 min prior to experimentation. In some experiments the muscles were further equilibrated for 15 min with medium containing dantrolene (or fresh medium, in controls). Dantrolene was added at the saturating concentration (5.7×10^{-5} M) by replacing the bath medium with Krebs medium in which an excess of dantrolene had been stirred at room temperature and then filtered off; lower concentrations of dantrolene were produced by dilution with medium. The concentration of dantrolene was determined by taking an aliquot of a dantrolene medium and diluting with 0.1 N NaOH, then measuring absorbance at a wavelength of 390 nanometers and comparing it with a standard curve using known amounts of dantrolene in 0.1 N NaOH. Ryanodine§ was added (subsequent to dantrolene when both drugs were present) from a concentrated solution to give final concentrations of 10^{-6} to 10^{-5} M. A halothane–oxygen mixture was generated in a Foregger model Compact 50 anesthesia machine, using an oxygen flow rate of 1 l/min and a halothane setting of 2.5 per cent, and was introduced into tissue baths via 26-gauge tubing. Equipment for measuring the actual content of dissolved halothane in the medium was not available in this laboratory, so the halothane concentration referred to in the text is that delivered from the anesthesia machine. By comparison with data in the literature,[14] however, it would appear that the dissolved halothane concentration was approximately 0.125 mg/ml at equilibrium. All experiments were repeated three or four times. Representative tracings are shown in figures 1 and 2. From these experiments it was found that approximately 3.6×10^{-6} M dantrolene was necessary to block the effects of 10^{-6} M ryanodine.

In-vivo studies with mice were performed to detect any additional mortality induced by halothane in animals surviving after treatment with ryanodine and also to determine whether pretreatment with dantrolene offered any protection from the effects of ryanodine and halothane. Male white Swiss-Webster mice, 20–25 g, in groups of 20 or more, received intraperitoneal injections of 10–135 μg/kg ryanodine in saline solution, then were placed in an open box for observation, and the number of deaths recorded an hour after injection. Separate experiments had shown that mice surviving for this long would survive indefinitely. Those animals that survived ryanodine treatment appeared to be grossly normal, manifesting normal grooming and feeding behavior. An hour after ryanodine injection, survivors were transferred to closed transparent plastic chambers approximately 20 × 40 × 10 cm, through which the oxygen–halothane mixture was passed, delivered from Foregger machine at an oxygen flow rate of 2 l/min and a halothane setting of 2.5 per cent. The chambers and connecting tubing were saturated with halothane by passing the gas mixture through for 15 min prior to the introduction of the animals. After 45 min of exposure to halothane, the number of deaths was recorded. In one experiment exploring the duration of the ryanodine-primed state, two groups of 20 mice surviving an LD_{50} dose of ryanodine were anesthetized beginning two and a half and five hours after the injection of ryanodine, rather than

§ Obtained from S. G. Penick, 100 Church St., New York, New York 10007.

after the standard one-hour interval. In all experiments a few mice that had been injected only with saline solution but then also exposed to the anesthetic were included. In a total of 50 such control mice no deaths were produced by halothane alone. In some experiments succinylcholine was administered intraperitoneally at a dose of 0.75 mg/kg, two times, 10 min apart, to mice surviving one hour after the injection of ryanodine. With this dose of succinylcholine the animals could still move about, and artificial ventilation was not needed. In dantrolene-pretreatment experiments, mice in groups of 40 or more were given via gastric tube either saline solution or a suspension of dantrolene in approximately 0.1 ml saline solution, at a dose of 20 mg/kg, administered five times at 12-hour intervals over a 48-hour period. This dosage exceeds human dose regimens but was chosen to maximize any effect of dantrolene. At the end of the 48-hour period the mice received injections of ryanodine as indicated in table 2. Then, after a further hour, the number of deaths was recorded and the survivors anesthetized with the halothane–oxygen mixture as described above; after 45 min the number of deaths was again counted. Statistical comparisons of the various data were made using the two-tailed test described by Goldstein[15] in which $P < 0.05$ was taken to indicate a significant difference.

In attempts to detect a hyperthermic state produced by ryanodine, six rats and six mice were placed in animal holders, then rectal thermistor probes were inserted and body temperatures measured with a Tele-Thermometer (Yellow Springs Instrument Co.). These animals were anesthetized with 2.5 per cent halothane–oxygen, then injected intraperitoneally with 135 μg/kg ryanodine and also with 0.75 mg/kg succinylcholine. In another group of six rats, cardiac activity was monitored by electrocardiograms during treatment with 135 μg/kg ryanodine and subsequent halothane anesthesia.

Results

In the isolated rat diaphragm segments, the rate of development of contracture increased as the concentration of ryanodine was increased from 10^{-6} M through 3×10^{-6} to 10^{-5} M (fig. 1). When muscles were preexposed to medium containing a saturating concentration (5.7×10^{-5} M) of dantrolene, the contracture normally produced by 10^{-6} M ryanodine was completely inhibited. As the ryanodine concentration was increased to 3×10^{-6} M and to 10^{-5} M, dantrolene was found to produce a delay in the response to ryanodine, the delay decreasing as the ryanodine concentration increased, although the final extent of contracture was not appreciably altered.

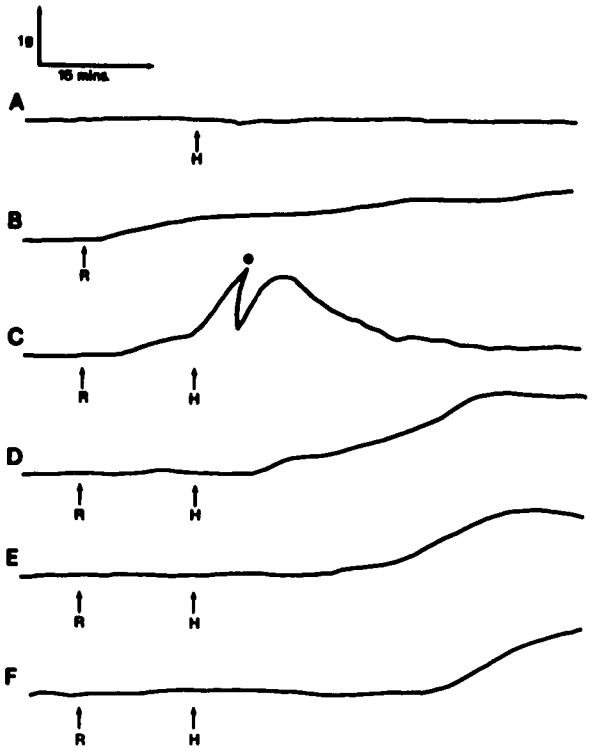

FIG. 2. Antagonism by dantrolene of halothane potentiation of ryanodine-induced contractures in a typical rat diaphragm preparation. Diaphragmatic segments were equilibrated in the presence or absence of different concentrations of dantrolene as described in figure 1. Ryanodine and 2.5 per cent halothane were introduced as indicated by arrows. Experiments A and B show responses to halothane alone and to ryanodine alone, respectively. In Experiment C both ryanodine and halothane were present. Tissues of Experiments D, E and F were equilibrated with 1.42×10^{-5} M, 2.85×10^{-5} M, and 5.7×10^{-5} M dantrolene, respectively, for 15 min prior to the addition of ryanodine, indicated by arrows. Addition of halothane is also indicated by arrows. A dot indicates that recorder sensitivity was decreased by a factor of 2.

In examining the action of halothane on the diaphragmatic segments, it was found that halothane alone had no effect, while ryanodine alone produced the expected slow contracture (fig. 2). The latter was markedly potentiated by the subsequent addition of halothane. Pre-exposure to dantrolene at concentrations of 1.42×10^{-5} M, 2.85×10^{-5} M, and 5.7×10^{-5} M completely blocked the response to 10^{-6} M ryanodine and produced delays in the responses to halothane that were dependent on dantrolene concentration.

In the mouse experiments, the percentage of deaths due to ryanodine alone decreased as the ryanodine dose was reduced, with only 5 per cent of the animals dying after receiving an alkaloid dose of 75 μg/kg (table 1). Those animals surviving the

TABLE 1. Mortality in Mice Produced by Ryanodine and by Subsequent Treatment of Survivors with Halothane*

Ryanodine Dose (μg/kg)	Ryanodine Treatment			Halothane Treatment		
	n	Deaths		n	Deaths	
		Number	Per Cent		Number	Per Cent
135	40	24	60	16	13	81
120	50	17	34	33	23	70
100	25	6	24	19	18	95
75	20	1	5	19	18	95
50	20	1	5	19	16	84
25	20	0	0	20	4	20
10	20	0	0	20	0	0
0	50	0	0	50	0	0

* Mice received intraperitoneal injections of ryanodine. The number of deaths was recorded an hour later, at which time anesthesia was initiated in the survivors, using an oxygen–halothane mixture as described in Methods. After 45 min of anesthesia, halothane was discontinued and the number of deaths recorded. The number of mice in each part of the experiment is indicated by n. Statistical analyses showed that halothane anesthesia following ryanodine in doses of 25 μg/kg or more significantly increased the incidence of deaths.

ryanodine treatment appeared to be grossly normal, manifesting normal grooming and feeding behavior. However, when halothane was administered to the survivors after ryanodine injections, halothane-associated deaths occurred, indicating halothane potentiation of ryanodine toxicity, although halothane produced no death in 50 control mice not given ryanodine. Mice dying after ryanodine plus halothane did not experience the ryanodine-associated gasping and asphyxiation seen in unanesthetized animals given lethal doses of ryanodine, nor did they appear to have the degree of rigor seen in the latter animals. In mice given even very low to subclinical doses of ryanodine, significant percentages of animals were killed by subsequent exposure to halothane. The duration of this ryanodine-induced state is short, since none of the mice surviving an LD_{50} dose of ryanodine was killed by halothane anesthesia initiated two and a half or five hours, rather than the usual one hour, after the injection of ryanodine (data not shown).

Succinylcholine has been shown to induce the MH syndrome in susceptible patients[16] and to trigger a hyperthermic response in one of three ryanodine–halothane-treated cats.[9] When 17 mice that had survived an LD_{50} dose of ryanodine received injections of succinylcholine, 14 died, whereas none of a control group of 15 mice died after receiving succinylcholine.

The effects of pretreatment of mice with orally administered dantrolene on the ryanodine and ryanodine-plus-halothane toxicities was studied. Dantrolene decreased the deaths produced by 135 μg/kg ryanodine from 60 to 14 per cent and, at 120 μg/kg ryanodine, from 34 to 6 per cent (table 2). However, when mice surviving the ryanodine treatments were anesthetized with halothane one hour after the ryanodine injections, dantrolene was found to produce not a decrease but instead a significant increase in the percentage of deaths.

In a search for a possible hyperthermic response of mice and rats treated with ryanodine, ryanodine plus halothane, or ryanodine plus halothane plus succinylcholine, no temperature increase was detected during these treatments, either in animals surviving or in those dying during the experiments. During halothane anesthesia both mice and rats became hypothermic by 2.5–3.5 degrees C, but such decreases in temperature were not affected by the injection of ryanodine. Electrocardiograms of rats dying during halothane anesthesia after ryanodine injection showed various marked arrhythmias.

Discussion

Some of our data support the ryanodine toxicity model for MH, in that dantrolene was found to provide significant protection from the effects of ryanodine, as seen both *in vivo* in mice as a decrease in the percentage of deaths and in isolated rat diaphragm preparations examined at 37 C, in which the dantrolene protection is inversely related to the dose of ryanodine. Ryanodine causes release of Ca^{++} from isolated skeletal muscle sarcoplasmic reticulum preparations,[6] whereas dantrolene reduces the rate of the release of Ca^{++} from such structures.[11,12] Thus, the antagonism by dantrolene, manifested by either complete inhibition or slowing of the rate of development of the contracture-producing effect of ryanodine on skeletal muscle, may reflect opposing influences of these two agents on the intracellular free Ca^{++} level, which determines the activity of the contractile protein complex.

It is interesting that mice surviving ryanodine dosages ranging from an LD_{60} to threshold dose for lethality are killed by subsequent exposure to halothane at a concentration that is not lethal to control mice. The mice surviving the ryanodine treatment appear grossly to be normal prior to the administration of halothane, and to that extent may constitute a model of the MH patient, who is also often apparently normal. The normal behavior of the mice in this state indicates that the effects of ryanodine on Ca^{++} metabolism are not attaining critical dimensions and/or are being effectively compensated for. In addition to their increased susceptibility to toxic effects of halothane, mice in this immediate post-ryanodine state also had an enhanced incidence of deaths when treated with succinylcholine, an agent that has also been shown to trigger the MH syndrome

TABLE 2. Effects of Pretreatment of Mice with Dantrolene on the Responses to Ryanodine and Halothane*

		Ryanodine Treatment			Halothane Treatment		
			Deaths			Deaths	
	Ryanodine (μg/kg)	n	Number	Per Cent	n	Number	Per Cent
Dantrolene pretreatment							
−	135	40	24	60	16	13	81
+	135	29	4	14†	25	25	100‡
−	120	50	17	34	33	23	70
+	120	31	2	6†	29	27	93‡

* Mice received dantrolene, administered as a suspension in saline solution via gastric tube at a dosage of 20 mg/kg, five times at 12-hour intervals over a 48-hour period. Ryanodine was then injected intraperitoneally, followed one hour later by exposure to halothane as described for the experiments of table 1.

† Less than controls, $P < 0.01$, by two-tailed statistical analyses.

‡ Dantrolene significantly increased the incidence of deaths in ryanodine-plus-halothane-treated animals, $P < 0.05$.

in susceptible patients.[16] Further examination of the mice in this ryanodine-primed state for other changes may strengthen this possible linkage to the MH state.

This priming effect of ryanodine is of short duration, less than two and a half hours in mice surviving a LD_{50} dose of ryanodine, which may indicate that the duration of this state is related to the ryanodine level in the tissues. However, although many mice succumb when halothane is administered during this ryanodine-primed state, these animals while dying do not have the same degree of rigidity or the gasping seen in mice dying after treatment with ryanodine alone, suggesting different causes of death in the two experimental conditions and thus a limitation of the ryanodine toxicity model of MH. Also, whereas dantrolene provides significant protection from the lethality of ryanodine itself, dantrolene does not provide any protection from the halothane-potentiated effects of ryanodine, but instead significantly increases the incidence of deaths.

These observations suggest that the lethality of ryanodine may be mediated via two sites of action. In non-anesthetized mice given a lethal dose of ryanodine, the primary site of action appears to be on the skeletal muscles, and death is due to asphyxiation, with the animals gasping for air as respiratory muscles undergo rigor. In anesthetized mice the picture is different. Procita[17] has shown that the effect of ryanodine on skeletal muscle is dependent on the activity of the muscle, and that ryanodine-induced skeletal muscle rigidity is minimal in anesthetized cats. The reason for this diminished effect of ryanodine is not known, but presumably involves the number of muscle activation–relaxation cycles and the associated Ca^{++} influx and efflux from the sarcoplasmic reticulum, which are modified by ryanodine.[3] However, in such anesthetized cats, the negative inotropic effect of ryanodine on cardiac muscle still occurs. This suggests that a second site of action of ryanodine is now more critically involved and was seen in the mice in the present study, in that deaths occurring during halothane administration subsequent to ryanodine treatment may have been due to an action on cardiac muscle. The effect of halothane may thus be to reduce the action of ryanodine on skeletal muscle by reducing skeletal muscle activity and respiratory rate during anesthesia, while potentiating the negative inotropic effect of the alkaloid on the heart, since halothane itself suppresses cardiac activity.[18] The differential effects of dantrolene, in protecting mice against ryanodine alone but not protecting against ryanodine plus halothane, might then be explained by the greater sensitivity of skeletal versus cardiac muscle to dantrolene.[19] Also, whereas the effects of dantrolene and ryanodine on skeletal muscle reticulum preparations are functionally opposite,[6,11] ryanodine appears to act on the heart by inhibiting the release of Ca^{++} from the cardiac sarcoplasmic reticulum,[7] an effect that would be potentiated by dantrolene if this drug acted similarly on the cardiac reticulum and on the skeletal muscle sarcoplasmic reticulum. Such a potentiation might also explain the increased incidence of deaths seen in dantrolene-pretreated mice given ryanodine and halothane (table 2).

Unlike the situation *in vivo* in mice, dantrolene in the isolated rat diaphragm experiments diminished the effects of both ryanodine alone and ryanodine plus halothane. Again, these effects could be explained if dantrolene reduced the release of Ca^{++} from the sarcoplasmic reticulum, since, within limits, this action would functionally counteract the release of Ca^{++} produced by ryanodine and halothane. The limitation in the *in-vivo* manifestation of the ryanodine toxicity model for MH thus may reside in the involvement of the heart as the more sensitive target organ during anesthesia following treatment with ryanodine. Also, although Casson and Downes[9]

found hyperthermia in one of three cats following administration of ryanodine, halothane, and succinylcholine, our inability to detect hyperthermia in ryanodine-treated mice and rats suggest that hyperthermia is not characteristic of ryanodine toxicity in these species. Even with these limitations of the ryanodine toxicity model, however, it would seem that additional studies of the mechanism of action of ryanodine might further our understanding of the MH state.

Recently, another laboratory model for MH has been described by Durbin and Rosenberg,[20] who showed that the injection of large amounts of caffeine into halothane-anesthetized rabbits produced increases in serum potassium and muscle rigidity, and a small (1.3 degrees C) rise in body temperature, together with cardiac arrythmias. Their model thus has some advantages over the ryanodine toxicity model. However, it seems probable that both models share a common mechanism involving excitation–contraction coupling, since caffeine and ryanodine act on the same subfraction of the sarcoplasmic reticulum[5] to interfere with Ca^{++} transport in skeletal muscle, and since this subfraction is also sensitive to dantrolene.[12] Since this reticulum subfraction is enriched in terminal cisternae,[5] one prediction that could be made from the ryanodine toxicity model is that the terminal cisternae may be altered in some way in MH.

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