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Effects of Azumolene on Ryanodine Binding to Sarcoplasmic Reticulum of Normal and Malignant Hyperthermia Susceptible Swine Skeletal Muscles

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Dantrolene is a primary specific therapeutic drug for prevention and treatment of malignant hyperthermia symptoms. The mechanisms underlying the therapeutic effects of the drug are not well understood. The present study aimed at the characterization of the effects of azumolene, a water soluble dantrolene analogue, on ryanodine binding to sarcoplasmic reticulum (SR) from normal and malignant hyperthermia susceptible (MHS) swine muscles. Characteristics of [^{3}H]ryanodine binding were clearly different between the two types of SR. Kinetic analysis of [^{3}H]ryanodine binding to SR in the presence of 2 μM Ca^{2+} showed that association constant ($K_{ryanodine}$) is significantly higher in MHS than normal muscle SR (2.83 vs. 1.32×10^7 M^{-1}), whereas the maximal ryanodine binding capacity (B_{max}) is similar between the two types of SR. Addition of azumolene (e.g. 400μM) did not significantly alter both $K_{ryanodine}$ and B_{max} of [^{3}H]ryanodine binding in both types of SR, indicating that the azumolene effect was not on the ryanodine binding sites. Addition of caffeine activated [^{3}H] ryanodine binding in both types of SR, and caffeine sensitivity was significantly higher in MHS muscle SR than normal muscle SR ($K_{caffeine}$: 3.24 vs. 0.82×10^2 M^{-1}). Addition of azumolene (e.g. 400 μM) decreased $K_{caffeine}$ without significant change in B_{max} in both types of SR suggesting that azumolene competes with caffeine binding site(s). These results suggest that malignant hyperthermia symptoms are caused at least in part by greater sensitivity of the MHS muscle SR to the Ca^{2+} release drug(s), and that azumolene can reverse the symptoms by reducing the drug affinity to Ca^{2+} release channels.

Malignant hyperthermia (MH) is a pharmaco-genetic disorder caused by abnormal intracellular Ca^{2+} movements in muscle cells during exposure to volatile anaesthetics, such as halothane and enfluorane (Britt et al., 1969; Ryan and Papper, 1970; Nelson et al., 1972; Gronert, 1980). Primary cause for malignant hyperthermia symptoms reflected by high fever, muscle rigidity, and muscle acidosis has been postulated to be abnormal Ca^{2+} release from the MH susceptible (MHS) muscle SR (Endo et al., 1983; Nelson, 1983; Ohnishi et al., 1983; Kim et al., 1984; Michelson et al., 1986). Evidence has suggested that abnormally high rates of Ca^{2+} release from MHS muscle SR (Kim et al., 1984) could be due to an abnormality in Ca^{2+} release channel (Michelson et al., 1988; Michelson et al., 1989; Knudson et al., 1990).

Dantrolene, a skeletal muscle relaxant (Britt, 1984) has been used to prevent and treat MH episodes. However, use of the drug for *in vitro* studies has been limited due to its low solubility in water, especially at neutral pH (Britt, 1984). Partial inhibition of halothane-induced Ca^{2+} release from MHS SR by dantrolene-Na was reported previously (Ohnishi et al., 1983). Azumolene, 1-[[[5-(4-bromophenyl)-2-oxazolyl] methylene]amino]-2,4-imidazolidinedione, is a water soluble dantrolene analogue having the useful greater water solubility than dantrolene (Fletcher et al., 1988; Leslie and Part, 1989; Dershwitz and Sreter, 1990). However, no study has yet been carried out on the effects of azumolene on Ca^{2+} release channel in the isolated pig skeletal SR vesicles. Inhibition of doxorubicin-induced Ca^{2+} release from cardiac SR by azumolene was reported previously (Tian et al., 1991).

The aim of the present study was to investigate the effects of azumolene on [^{3}H]ryanodine binding to SR to localize the site of drug action. MHS SR was found to have abnormally high affinity to caffeine and azumolene competitively inhibits caffeine binding.

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Materials and Methods

Materials

[^{3}H]Ryanodine was obtained from New England Nuclear Co. Caffeine was purchased from Sigma, ryanodine from Calbiochem. Co. All other reagents used were of analytical grade.

Preparation of sarcoplasmic reticulum

Care and screening of pigs for MHS trait were carried out as described previously (Kim et al., 1984). A junctional SR fraction from pig back muscle (primarily fast twitch muscle) was prepared by differential centrifugation as described previously (Kim et al., 1983, 1988). Briefly, about 200 g muscle was homogenized in a Waring blender with 4 volume of a solution having 2.5 mM NaOH/20 mM MOPS (pH 6.8) for 2 min. The suspension was centrifuged at 10,000 g for 3 min in GSA rotor (Sorvall). The supernatant was filtered through 8 layers of cheesecloth and Whatman filter paper (No. 4). The filtrate was centrifuged at 17,000 g for 30 min. The pellets were suspended in a solution containing 0.15 M KCl, 20 mM MOPS (pH 6.8), 0.1 mM phenylmethylsulfonyl fluoride, 10 mg/liter aprotinin, 0.8 mg/liter antipain, 2 mg/liter trypsin inhibitor, and 0.3 M sucrose (Kim and Ikemoto, 1986) and centrifuged at 17,000 g for 35 min. The final pellets were suspended in the same sucrose buffer and the protein concentration was adjusted to 20-30 mg/ml. The samples immediately frozen in liquid nitrogen and stored at -80℃ were used within 3-4 weeks.

[^{3}H]Ryanodine binding assay

SR vesicles (0.4 mg/ml) were incubated with various concentrations of [^{3}H]ryanodine (54.7 Ci/mmol), unless otherwise specified, in a standard binding buffer containing 0.15 M KCl, 20 mM MOPS (pH 6.8), 2 mM EGTA, 1.97 mM $CaCl_2$ ($[Ca^{2+}]_{free}$=2 μM) or 1.47 mM $CaCl_2$ ($[Ca^{2+}]$=0.1 μM), for 2 h at 37℃ (Lee et al., 1991; Tian et al., 1991). The incubation mixtures were filtered (Millipore, 0.45 μM), rinsed with the same binding buffer (3×2.5 ml), dried, and counted. Specific binding of ryanodine was defined as the difference between total [^{3}H]ryanodine binding and nonspecific binding measured in the presence of 20 μM unlabelled ryanodine. Nonspecific binding was approximately 1-5% of the total binding.

Miscellaneous

$[Ca^{2+}]_{free}$ in the Ca-EGTA solutions was calculated using a computer program using constants described previously (Ikemoto, 1974). Protein concentrations were determined by Lowry method using bovine serum albumin as a standard (Lowry et al., 1953).

Results and Discussion

In agreement with the previous reports (Michelson et al., 1988), the association constant of [^{3}H]ryanodine binding ($K_{ryanodine}$) to the MHS muscle SR was significantly higher than that of the normal SR without appreciable difference in the maximal binding of ryanodine (B_{max}) (Fig. 1 and Table 1). Azumolene, even at very high concentrations (e.g. 400 μM) that completely inhibited caffeine-induced Ca^{2+} release (Kim and Ikemoto, unpublished data), produced little or no effect on either $K_{ryanodine}$ or B_{max} (Table 1). This indicates that azumolene acts at site(s) different from the ryanodine binding site.

The concept that the affinity of the SR membrane for caffeine is much higher in MHS muscle than in normal muscle is supported by several lines of evidence. First, caffeine-induced contracture of the biopsied muscles occurs at much lower concentrations of caffeine in the MHS muscle compared with the normal muscle (Nelson and Flewellen, 1983). Second, in accord with a report by Michelson et al. (1989), the caffeine-activated [^{3}H]ryanodine binding to the MHS muscle SR was seen at significantly lower concentrations of caffeine compared with the normal muscle SR (Table 2). Third, caffeine-induced increase in the opening probability of Ca^{2+} channels occurs at much lower concentrations of caffeine when MHS muscle SR was incorported into planar lipid bilayers compared with the normal SR (Ehrlich and Kim, unpublished data).

Since direct measurements of caffeine binding were difficult due to its low affinity binding, we determined caffeine binding indirectly by monitoring the ability of caffeine to potentiate ryanodine binding (Fig. 2). For these experiments, the Ca^{2+} concentration was lowered to 0.09 μM to minimize the Ca^{2+} effects on the ryanodine binding, and values of ryanodine binding in the absence of caffeine were subtracted from those in the presence of caffeine to determine the caffeine-activated [^{3}H]Ryanodine binding. As shown in Fig. 2, the caffeine concentration-dependence of the caffeine-activated [^{3}H]Ryanodine binding was similar to that of caffeine-induced Ca^{2+}

Table 1. Effects of azumolene on ryanodine binding to normal and malignant hyperthermic SR

Types of SR	Addition	Binding parameters	
		$K_{ryanodine}$ ($\times 10^7$ M^{-1})	B_{max} (pmol/mg)
Normal	Control (4)	1.32 + 0.20	8.8 + 1.4
	+ 400 μM azumolene (3)	1.08 + 0.10	7.7 + 0.9
MH	Control (3)	2.83 + 0.40	9.9 + 0.8
	+ 400 μM azumolene (3)	2.79 + 0.45	9.2 + 0.5

All values are expressed as means + standard errors. Numbers in parentheses are the number of experiments from different animals. [^{3}H]Ryanodine binding to normal and MHS SR, and calculation of binding parameters were carried out as described in the legend to Fig. 1.

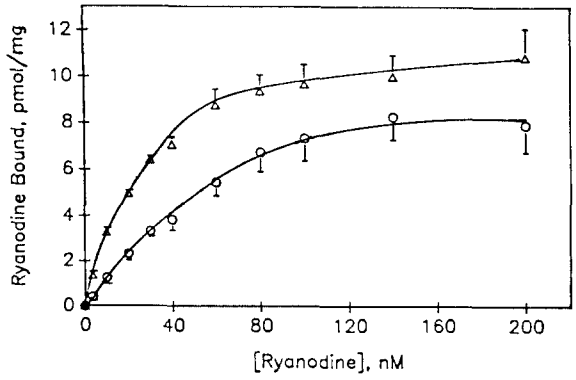

Fig. 1. Specific binding of [^{3}H]ryanodine to normal (circles) and MHS SR (triangles). SR vesicles (0.4 mg/ml) were incubated with various concentrations of [^{3}H]ryanodine in a solution containing 0.15 M KCl, 20 mM MOPS (pH 7.4), 6.25 mM EGTA, and 5 mM $CaCl_2$ ($[Ca^{2+}]_{free}$=2 μM) for 2 h at 37℃. Specific binding of ryanodine is defined as the difference between the total binding and the nonspecific binding measured in the presence of unlabelled ryanodine (see "Materials and Methods"). The curve fitting and calculation of the binding parameters were carried out by iterative computer fitting using the equation, $y=B_{max}.Kx/(1+Kx)$, where K is association constant, $A=B_{max}$ and $1/K=Kd$. The individual [^{3}H]ryanodine binding values are expressed as means + standard errors. Four normal and 3 MHS animals were used for the experiments. Note that the affinity for ryanodine ($K_{ryanodine}$) is significantly different between the two types of SR.

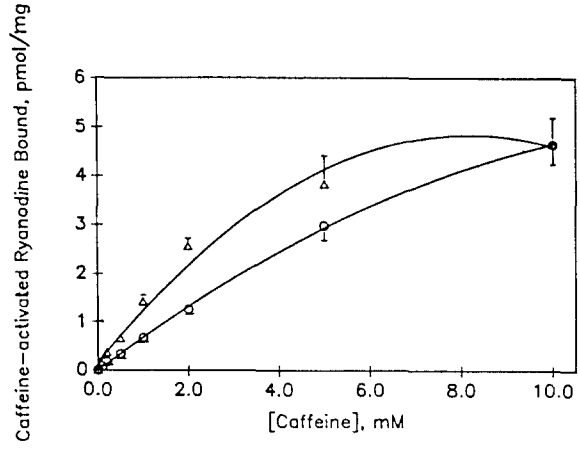

Fig. 2. Caffeine concentration dependence of ryanodine binding to normal (circles) and MHS (triangles) SR. [^{3}H]Ryanodine binding and calculation of the binding parameters were carried out as described in the legend to Fig. 1 except that concentrations of free calcium and [^{3}H]Ryanodine were fixed at 0.09 μM and 50 nM, respectively. [^{3}H]Ryanodine binding activated by caffeine was defined as the difference between the total binding and the binding in the absence of caffeine. The individual [^{3}H]ryanodine binding values are expressed as means + standard errors. Four normal and 5 MHS animals were used for the experiments.

release (Michelson et al., 1989). The association constant for caffeine binding ($K_{caffeine}$) as determined from the caffeine concentration-dependence of the caffeine-activated [^{3}H]ryanodine binding was significantly higher in the MHS muscle SR than in normal muscle SR (3.24×10^2 versus 8.2×10^1 M^{-1}) (Table 2).

As shown in Table 2, azumolene decreased the association constant for caffeine ($K_{caffeine}$) without appreciable change in B_{max}, indicating that azumolene may compete with caffeine at the identical site(s), as shown in the dog cardiac muscle (Tian et al., 1991). The findings that azumolene competes with caffeine, but that it has virtually no effect on the ryanodine binding (Tables 1 and 2) suggest that ryanodine binds to a site(s) that differ from the site(s) to which azumolene and caffeine bind competitively. Therefore, the reversal of the MH symptoms by dantrolene or azumolene could be produced by the competitive removal of Ca^{2+} release inducing drugs (e.g. caffeine or halothane) from the drug-binding site(s). This notion is consistent with our previous observation that dantrolene produces a selective inhibition of drug-induced Ca^{2+} release, although it has either no effect or complex effects on other types of Ca^{2+} release (Danko et al., 1985).

In conclusion, the inhibition of drug-activated ryanodine binding by azumolene, a water soluble analogue of dantrolene, appears to result from binding of azumolene to the drug-binding site(s). Ryanodine binds to site(s) that differ from the azumolene/caffeine binding sites(s). The binding affinity of the Ca^{2+} release activators (e.g. ryanodine and caffeine) or Ca^{2+} release inhibitors (e.g. azumolene) appears to be higher in the MHS SR than normal muscle SR. In view of the evidence that there is an abnormality in the gene expressing the ryanodine receptor in MHS muscle (MacLennan et al., 1990; Fujii et al., 1991), it is tempting to hypothesize that alterations in the primary structure of the ryanodine receptor are in the region where azumolene and the Ca^{2+} triggering agents bind.

Table 2. Effects of azumolene on caffeine concentration-dependence of ryanodine binding to normal and malignant hyperthermic SR

Types of SR	Addition	Binding parameters	
		$K_{caffeine}$ (caffeine, 10^2 M^{-1})	B_{max} (ryanodine, pmol/mg)
Normal	Control (4)	0.82 + 0.10	10.6 + 1.7
	+400 μM azumolene (4)	0.50 + 0.003*	9.3 + 0.9
MH	Control (5)	3.24 + 0.60	6.7 + 0.9
	+400 μM azumolene (5)	2.05 + 0.43	8.3 + 1.5

All values are expressed as means + standard errors. *P<0.05 (from the Student's unpaired t-test). Numbers in parentheses are number of experiments from different animals. [^{3}H]Ryanodine binding and calculation of the binding parameters were carried out as described in the legend to Fig. 2. [^{3}H]Ryanodine binding activated by caffeine was defined as the difference between the total binding and the binding in the absence of caffeine.

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