Effects of Dantrolene on Rat Diaphragm Muscle during Postnatal Maturation

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Background: Dantrolene is the only known effective treatment for malignant hyperthermia. However, its effects on diaphragm muscle during postnatal maturation remain unknown.

Methods: The effects of dantrolene (10⁻⁸ to 10⁻⁴ M) were investigated in vitro on diaphragm muscle strips in adult rats and in postnatal rats aged 3, 10, and 17 days, and compared with those of ryanodine (10⁻⁸ to 10⁻⁶ M). The authors studied contraction and relaxation under isotonic and isometric conditions (29°C, Krebs-Henseleit solution, tetanic stimulation at 50 Hz). Data are mean ± SD.

Results: During postnatal maturation, the authors observed a progressive increase in active force developed per cross-sectional area (from 34 ± 25 to 69 ± 32 mN/mm²; P < 0.05) and maximum shortening velocity (from 2.9 ± 0.5 to 4.9 ± 1.4 Lmax/s; P < 0.05). Dantrolene induced a negative inotropic effect in diaphragm muscles in isotonic and isometric conditions in all groups, but this effect was significantly less marked in the 3-day-old rats compared with older rats. Dantrolene did not induce significant lusitropic effects during postnatal maturation.

Conclusion: Dantrolene induced less pronounced negative inotropic effects on the diaphragm in neonatal rats as compared with adult rats. Our study suggests that developmental changes in the pharmacologic response to dantrolene are more rapid than those of ryanodine.

IN skeletal and diaphragmatic muscles, postnatal maturation is associated with important ultrastructural changes, including changes in fiber type, distribution, and size; biochemical differentiation, including elimination of embryologic and neonatal myosin isoforms; changes in metabolic capacity; and progressive development of the sarcoplasmic reticulum (SR). These changes result in an improved diaphragm contractility. However, the precise mechanisms by which maturation induces changes in the contractile performance of diaphragm muscle remain a matter of debate.

Dantrolene, the only known effective treatment for malignant hyperthermia, is a postsynaptic skeletal muscle relaxant that inhibits calcium release during excitation–contraction coupling and reduces the myoplasmic free calcium concentration in a dose-dependent manner. The molecular basis of the action of dantrolene remains not completely understood but is generally presumed to involve either direct or indirect inhibitory effects on ryanodine receptor (RyR) Ca²⁺ channels of the SR. The effects of dantrolene on muscle during postnatal maturation remain unknown. In adults, dantrolene induces a major negative inotropic effect on skeletal and diaphragmatic muscles. During postnatal maturation, some quantitative and qualitative changes occur in the biochemical composition of the SR, especially changes regarding RyR, an isoform of RyR that is predominantly expressed in the diaphragm. Therefore, the extent of the inotropic effect of dantrolene on diaphragmatic muscle during postnatal development could be related to the state of maturation of this element in the contractile system, particularly that of RyR.

We therefore conducted an in vitro study on the effects of dantrolene on rat diaphragm muscle during postnatal maturation. We tested the hypothesis that maturation may modify the effects of dantrolene on diaphragm muscles.

Materials and Methods

Animals and Study Design

Care of the animals conformed to the recommendations of the Helsinki Declaration, and the study was performed in accordance with the regulations of the official edict of the French Ministry of Agriculture. After birth, rat pups were kept in cages with their mothers. Adult rats received rat chow and water ad libitum. A 12-h light–dark cycle was provided. Experiments were performed on Wistar rats aged 3 days (n = 18), 10 days (n = 18), 17 days (n = 20), and 10–12 weeks (adult, n = 21).

After brief anesthesia with ether, a median laparotomy was performed, and a muscle strip from the ventral costal diaphragm was carefully dissected from the muscle in situ while the ribs and the central tendon were left intact, as previously reported. With this procedure, diaphragmatic fibers were parallel and of approximately equal length. This diaphragm strip was vertically sus-
pendent in a 200-ml jacketed reservoir with Krebs-Hense-leit bicarbonate buffer solution that contained 118 mM sodium chloride, 4.7 mM potassium chloride, 1.2 mM magnesium sulfate, 1.1 mM dipotassium hydrogen phosphate, 25 mM sodium hydrogen carbonate, 2.5 mM calcium chloride, and 4.5 mM glucose. The jacketed reservoir was maintained at 29°C with continuous monitoring of the solution temperature. The bathing solution was bubbled with 95% oxygen-5% carbon dioxide, resulting in a pH of 7.40. Preparations were field-stimulated with 1-ms rectangular pulses at a rate of 50 Hz for 300 ms, to induce a tetanic contraction (10 contractions per minute). After a 30-min stabilization period, at the initial muscle length at the apex of the length-active isometric tension curve (Lmax), diaphragm muscle strips recovered their optimal mechanical performance. At the end of the study, the cross-sectional area (millimeters squared) was calculated from the ratio of muscle weight to muscle length at Lmax assuming a muscle density of 1. Body weight was measured at the moment of killing.

All drugs were purchased from Sigma-Aldrich Chimie (L’Isle d’Abeau, Chesnes, France). Because dantrolene is poorly soluble in aqueous media, we used dimethylsulfoxide as a solvent, as previously reported. Blood therapeutic concentrations of dantrolene range from 0.3 to 6 μg/ml (10^-6 to 10^-5 M). Therefore, five concentrations (from 10^-8 to 10^-4 M) were tested in a cumulative manner, with a 10-min period between each concentration. In a preliminary study (data not shown), we observed that the effects of the highest concentration of dantrolene remained stable between 15 and 60 min and that dimethylsulfoxide alone had no significant effect, as previously reported in hamster diaphragmatic muscle.

Although expression of the RyR genes is modified during development, its pharmacologic consequences remain unknown. Therefore, in separated groups of diaphragmatic muscles, we also assessed the effect of ryanodine during postnatal maturation. Five concentrations of ryanodine (10^-8, 3.10^-8, 10^-7, 3.10^-7, and 10^-6 M) were tested in a cumulative manner, with a 10-min period between each concentration. These concentrations are in the range of concentrations used in another in vitro study, assessing the effect of ryanodine on neonatal and adult rat heart.

**Electromagnetic Lever System and Recording**

The electromagnetic lever system has been previously described. Briefly, the load applied to the muscle was determined by means of a servomechanism-controlled current through the coil of an electromagnet. Muscular shortening induced a displacement of the lever, which modulated the light intensity of a photoelectric transducer. The initial preload (resting force), which determined Lmax, was automatically maintained constant throughout the experiment. All analyses were made from digital records of force and length obtained with a computer, as previously described.

**Mechanical Parameters**

Mechanical parameters were calculated from three consecutive tetanic contractions preloaded at Lmax. The first contraction was isotonic and loaded with preload only to determine the maximum extent of shortening (ΔL), maximum shortening velocity (Vc), and maximum lengthening velocity (Vr). Tetanus 2 was loaded with preload and was abruptly clamped to zero-load immediately after the electrical stimulus to determine the maximum unloaded shortening velocity (Vmax). Tetanus 3 was fully isometric at Lmax to determine the maximum active force (AF) and the peak of the positive (+dF/dt) and negative (−dF/dt) force derivatives.

![Fig. 1. Mechanical parameters of contraction and relaxation.](image)

**Fig. 1. Mechanical parameters of contraction and relaxation.** (Top) Muscle shortening length (L/Lmax) plotted versus time. (Bottom) Force (mN/mm²) plotted versus time. Tetanus 1 was isotonic and loaded with preload only to determine the maximum extent of shortening (ΔL), maximum shortening velocity (Vc), and maximum lengthening velocity (Vr). Tetanus 2 was loaded with preload and was abruptly clamped to zero-load immediately after the electrical stimulus to determine the maximum unloaded shortening velocity (Vmax). Tetanus 3 was fully isometric at Lmax to determine the maximum active force (AF) and the peak of the positive (+dF/dt) and negative (−dF/dt) force derivatives.

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et al. (1993) Ireland). NCSS 6.0 software (Statistical Solutions Ltd, Cork, Ireland) was used for statistical analysis. Comparisons of several means were performed using repeated-measure analysis of variance. Comparisons of control values between groups were performed using analysis of variance. Statistical analysis was performed using NCSS 6.0 software (Statistical Solutions Ltd, Cork, Ireland).

Statistical Analysis

Data are expressed as mean ± SD. Comparisons of control values between groups were performed using analysis of variance. Comparisons of several means were performed using repeated-measure analysis of variance and Newman-Keuls test. All P values were two-tailed, and a P value < 0.05 was required to reject the null hypothesis. Statistical analysis was performed using NCSS 6.0 software (Statistical Solutions Ltd, Cork, Ireland).

Results

Physical characteristics and mechanical parameters of contraction, relaxation, and contraction–relaxation coupling in adult and postnatal rats in control conditions are shown in table 1. During postnatal maturation, we observed significant increases in body weight, diaphragm strip weight, section, and Lmax (table 1). We also observed significant increases in mechanical parameters testing inotropy in isotonic (Vmax, Vc) and isometric (AF, +dF/dt) conditions (table 1). Vr was not significantly modified by postnatal maturation, whereas −dF/dt was significantly lower in 3- and 10-day-old rats compared with adult rats. The ratio Vr/ΔL, which assessed lusitropy in isotonic conditions, was not significantly modified during postnatal maturation. In contrast, the ratio (−dF/dt)/AF, which assessed lusitropy under isometric conditions, was significantly higher in 10- and 17-day-old rats as compared with 3-day-old and adult rats (table 1).

Dantrolene induced a significant and concentration-dependent negative inotropic effect in diaphragm muscles under low (Vmax) and high (AF) loads in the four groups (fig. 2). However, this negative inotropic effect was significantly less marked in the 3-day-old rats as compared with older rats (fig. 2). Whatever the concentration, dantrolene did not induce any significant contraction. Dantrolene induced no significant changes in the Vr/ΔL ratio (fig. 3). Dantrolene also induced no significant changes in the ratio (−dF/dt)/AF except for the highest concentration at 30 M in the two groups of oldest rats (17-day-old and adults; fig. 3).

Ryanodine induced a significant and concentration-dependent negative inotropic effect in diaphragm muscles under low (Vmax) and high (AF) loads in the four groups (fig. 4). This negative inotropic effect was significantly more marked in the adult group under low and high loads as compared with the three other groups and was significantly less marked in the 3-day-old group under low loads (fig. 4).

To compare the developmental changes in the pharmacologic responses to dantrolene and ryanodine, we compared these two drugs at equipotent concentrations
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Fig. 2. Inotropic effects of dantrolene in isotonic (A) and isometric (B) conditions in diaphragm muscle during postnatal maturation. d3 = 3-day-old rats; d10 = 10-day-old rats; d17 = 17-day-old rats. $V_{\text{max}}$ = maximum unloaded shortening velocity; AF = isometric active force normalized per cross-sectional area. Data are mean ± SD. Significant differences between groups refer to $P < 0.05$.

Discussion

We studied the effects of dantrolene on the intrinsic contractility and relaxation of isolated rat diaphragm muscle during postnatal maturation. The main result of our study is that the negative inotropic effect of dantrolene was significantly less pronounced in the 3-day-old rats as compared with older rats. Moreover, the developmental changes in the pharmacologic response to dantrolene (i.e., a response comparable to that observed in the adult) appeared to occur more rapidly than those observed with ryanodine.

The effect of postnatal maturation on physical characteristics and mechanical parameters of contraction, relaxation, and contraction–relaxation coupling observed in our study (table 1) are similar to those previously described in other studies. In all groups, dantrolene induced a significant and concentration-dependent negative inotropic effect in diaphragm muscle. The effects observed in adult rats were similar to those previously described in other species and in various skeletal muscles, including diaphragm.

In diaphragm, as well as in other muscles, postnatal maturation is associated with important ultrastructural changes, including changes in fiber type, distribution, and size; biochemical differentiation, including elimination of embryologic and neonatal myosin isoforms; changes in metabolic capacity; and progressive development of the SR. These changes result in a progressive improvement in diaphragmatic contractility, leading to an increase in the tension developed and an enhanced rate of muscle shortening, and a decrease in the duration of contraction and half-relaxation time. Some investigators have proposed that these changes could be mainly related to the postnatal transitions in myosin heavy chain (MHC) isoform expression. Indeed, the progressive increase in velocity and force seems strongly associated with the progressive decrease in MHC–neonatal isoform expression and the progressive increase in MHC-2X and MHC-2B isoform expression. In contrast, other studies suggest that there is only a low correlation between MHC isoform expression (especially MHC IIB) and changes in diaphragmatic velocity during maturation, supporting the hypothesis that factors in addition to the postnatal transitions in MHC isoform expression are involved in regulating diaphragmatic increase in velocity.
Other investigators have proposed that the increase in velocity and force could be related to an increase in total number of cross bridges and in peak total rate of energy release.\textsuperscript{19}

The negative inotropic effect of dantrolene was significantly less marked in 3-day-old rats as compared with older rats. The effect of dantrolene is thought to be related to an inhibition of calcium release from the SR by either direct or indirect interaction with the RyR.\textsuperscript{8,10,11}

In adult skeletal and diaphragmatic muscles, the type 1 isoform of RyR is essential in triggering contraction. Expression of RyR1 requires approximately 3–4 weeks to reach the high levels that are maintained throughout adult life.\textsuperscript{27} Another isoform, RyR3, is predominantly expressed during fetal and neonatal development and has been shown to play a physiologic role in excitation-contraction coupling of neonatal skeletal muscles.\textsuperscript{15,28} RyR3 is already expressed during fetal development, but its expression is maximum during the neonatal phase (2–15 days) in the rat.\textsuperscript{13} Moreover, RyR3 is more expressed in the diaphragm than in other skeletal muscles.\textsuperscript{13} Therefore, our results may suggest that RyR3, which is predominantly expressed in the neonatal phase, is less susceptible to the action of dantrolene than RyR1. Because dantrolene may also act on multiple other sites such as triadin and FKBP12 proteins,\textsuperscript{8} we cannot rule out the hypothesis that maturation of these proteins and changes in their binding to RyR could also be involved in the decreased susceptibility of neonatal rats to dantrolene. We therefore analyzed the pharmacologic response to ryanodine during diaphragmatic muscle maturation, because ryanodine is a highly specific inhibitor of RyR.\textsuperscript{29} We observed that developmental changes in the response to dantrolene were more rapid than those of ryanodine (fig. 5). This is an indirect argument that suggests that dantrolene does not act only directly on the RyR. Further studies are required to elucidate this point.

Dantrolene did not modify isotonic relaxation (fig. 3), suggesting that it did not modify the calcium reuptake by the SR. This result agrees with those previously reported in hamster diaphragm muscle.\textsuperscript{12} Dantrolene did not significantly modify isometric relaxation, except at the highest concentration (fig. 3). This result agrees with previous results in skeletal muscles.\textsuperscript{12,30} Therefore, our results suggest that dantrolene up to $10^{-4}$ M, did not alter myofilament calcium sensitivity. At $10^{-4}$ M, dantrolene induced a significant decrease in the ratio $\left(\frac{\Delta F}{\Delta t}\right)/AF$, indicating a negative lusitropic effect under high load and suggesting an increase in myofilament calcium sensitivity. It should be pointed out that this effect was not observed in 3-day-old rats (fig. 3B). In any case, our results indicate that dantrolene induced very weak lusitropic effects that are not markedly modified during development.

\textbf{Fig. 4.} Inotropic effects of ryanodine in isotonic (A) and isometric (B) conditions in diaphragm muscle during postnatal maturation. $d_3$ = 3-day-old rats; $d_{10}$ = 10-day-old rats; $d_{17}$ = 17-day-old rats; $V_{\text{max}}$ = maximum unloaded shortening velocity; AF = isometric active force normalized per cross-sectional area. Data are mean ± SD. Significant differences between groups refer to $P < 0.05$.

\textbf{Fig. 5.} Comparison of the response to dantrolene (100 $\mu$M; A) and ryanodine (0.3 $\mu$M; B) expressed as percent of baseline active force (AF) during postnatal maturation. $d_3$ = 3-day-old rats; $d_{10}$ = 10-day-old rats; $d_{17}$ = 17-day-old rats. These concentrations induced comparable negative inotropic effect in the adult rat. Developmental changes in the pharmacologic response to dantrolene were more rapid than those of ryanodine. Data are mean ± SD. *$P < 0.05$ versus adult.
The effects of dantrolene on diaphragm contraction may have potential clinical consequences. Dantrolene decreases tidal volume,\textsuperscript{31} while minute ventilation is maintained by increasing respiratory rate.\textsuperscript{32} Dantrolene-induced muscle weakness can contribute to prolonged postoperative tracheal intubation.\textsuperscript{33,34} Our study demonstrates that dantrolene did not markedly alter diaphragmatic relaxation, whereas it induced significant negative inotropic effect in all age groups. The highly compliant infant rib cage places a greater functional demand on the diaphragm during ventilation than does the structurally stable rib cage in older children and adults, and a greater diaphragmatic contraction is needed to maintain a comparable tidal volume in an infant.\textsuperscript{35} Thus, dantrolene could more markedly contribute to respiratory distress in infants as compared with adults. However, this effect might be counterbalanced by the fact that neonatal diaphragm is less susceptible to dantrolene than adult diaphragm (fig. 2). Further in vivo studies are mandatory to assess the ventilatory consequences of dantrolene during postnatal maturation.

The following points must be considered in the assessment of the relevance of our results. First, this study was conducted at 29°C. Nevertheless, the stability of the preparation is not sufficient at 37°C. Only very low temperature (18–20°C) can markedly modify the negative inotropic effect of dantrolene.\textsuperscript{31} Second, our study did not enable us to detect a small increase in resting tension that has been recently reported at low concentrations of dantrolene.\textsuperscript{36} Nevertheless, this phenomenon is thought to involve high-affinity binding sites and thus to occur at very low concentrations ($10^{-9}$ M) of dantrolene, and is associated with a positive inotropic effect that was not observed in our study, even at the lowest concentration ($10^{-9}$ M). Lastly, maturation in the rat may differ from that in humans. The literature has provided few data to enable reliable extrapolation between rat and human diaphragm maturation. A 3-day-old rat has a ratio of body weight to adult body weight of approximately 2–3%, a 10-day-old rat a ratio of 5%, and a 17-day-old rat has a ratio of 8%. Considering the body weight growth in humans, 3- and 10-day-old rats appear equivalent to premature infants, and a 17-day-old rat appears equivalent to a newborn infant. Considering lung function, there is a close match between rat and human. Considering MHC isoform maturation, a 17-day-old rat appears equivalent to a human newborn.\textsuperscript{22–24,38} Considering fiber type proportion, a 3-day-old rat appears equivalent to a premature infant, and a 17-day-old rat appears equivalent to a human newborn.\textsuperscript{39}

In conclusion, in neonatal rats, dantrolene induced less pronounced negative inotropic effects on the diaphragm as compared with older rats, and did not induce significant lusitropic effects. Developmental changes in the pharmacologic response to dantrolene were more rapid than those of ryanodine. This is an indirect argument suggesting that dantrolene does not act only directly on the RyR.

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References

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