

Mechanism of theophylline-induced inotropic effects on foreshortened canine diaphragm

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Mechanism of theophylline-induced inotropic effects on foreshortened canine diaphragm. G. Gayan-Ramirez, S. Janssens, B. Himpens, M. Decramer. ©ERS Journals Ltd 1995.

ABSTRACT: The mechanisms of theophylline-induced inotropic effects at shorter diaphragm length have not yet been explored. We wondered whether the greater inotropic effects of the drug at shorter diaphragm length might result from an effect on intracellular calcium level.

Forty pairs of diaphragm bundles were stimulated at 70% of optimal length in the presence of either verapamil (10^{-5} M), calcium-free Krebs solution (buffered or not with 2 mM ethylene glycol tetra-acetic acid (EGTA)) or ryanodine (10^{-6} M). Theophylline (1 mM) was subsequently added to one muscle bundle and, after 15 min, twitches were repeated.

The twitch potentiation induced by theophylline ($37 \pm 21\%$) was unaffected by verapamil ($43 \pm 26\%$), or zero calcium ($39 \pm 18\%$) and virtually unchanged when the latter was buffered with EGTA. By contrast, theophylline failed to increase twitch tension after pretreatment with ryanodine, a blocker of the calcium release by the sarcoplasmic reticulum. This decreased twitch tension in control ($-5 \pm 11\%$) and experimental ($-14 \pm 12\%$) bundles and prolonged half-relaxation time as a result of impaired sarcoplasmic reticulum calcium reuptake.

We conclude that the inotropic effects of theophylline on twitch tension in foreshortened canine diaphragm bundles were not related to transmembrane calcium flux but were dependent on the release of calcium from the sarcoplasmic reticulum. This is consistent with an action of theophylline on the sarcoplasmic reticulum.

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In a previous *in vivo* study, we demonstrated that theophylline exerted more pronounced inotropic effects on foreshortened (70% of optimal length) canine diaphragm than on diaphragm placed at optimal length [1]. This difference was greater at low than at high stimulation frequencies and increased with increasing theophylline concentration. The preferential inotropic effect on foreshortened diaphragm was subsequently confirmed *in vitro* on canine diaphragm [2], showing, thereby, that the enhanced inotropic effects essentially resulted from an effect on the muscle itself and not from changes in rib cage or abdominal compliances, or diaphragm geometry.

The mechanism of theophylline-induced inotropic effects at shorter length has never been explored. To investigate this mechanism, the alterations in muscle mechanics with shortening need to be considered in order to demonstrate the potential action site of theophylline. Thus, the decrease in filament overlap between actin and myosin when muscle shortens [3] does not fully account for the decrease in tension generation observed at shorter muscle length. Indeed, due to muscle shortening, fibres are swollen, thereby compressing the T-tubular systems and "impeding exit electrolyte flow" as described previously [4]. Therefore, impairment of T-tubular conduction and/or hampering of calcium release from the

sarcoplasmic reticulum may occur because of insufficient signal transmission between T-tubular system and terminal cisternae [4, 5]. As a consequence, the action potential may not reach the central part of the muscle. This relates acute muscle shortening to calcium "deactivation" and probably explains that, at shorter muscle length, the central myofibrils of the muscle are not activated [6], the shortening velocity of central sarcomeres is decreased [7], and the rise in intracellular calcium is less [5].

Therefore, keeping in mind the results of the above-mentioned studies, we hypothesized that the greater inotropic effects of theophylline at shorter diaphragm length might result from an effect on intracellular calcium level. Consequently, each pathway able to enhance cytoplasmic calcium was selectively blocked and the effects of theophylline on twitch tension of foreshortened canine diaphragm examined *in vitro* under each condition.

Methods

Experimental set-up

Sixteen mongrel dogs (14.9 ± 1.8 kg) were anaesthetized with pentobarbital sodium (Nembutal, initial dose

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25 mg·kg⁻¹ *i.v.*) and mechanically ventilated (Hospal 251). According to Ethics Committee guidelines, animals did not experience any suffering. The level of anaesthesia was regulated to abolish the corneal reflex. After a mid-line laparotomy, a segment of costal diaphragm (width ranging 2–3 cm) was removed and immediately immersed in a cooled oxygenated Krebs solution for further dissection of two small bundles. For each dog, six pairs of diaphragm bundles were obtained. These bundles were then placed within the external chamber of a jacketed tissue bath filled with normal Krebs solution maintained at 37°C and perfused with 95% O₂ and 5% CO₂, pH 7.3–7.4. The Krebs solution contained in mM: 137 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, 1 KH₂PO₄, 12 NaHCO₃, and 6.5 glucose. One end of the bundle was attached to a rigid support whilst the other was fastened to an isometric force transducer mounted to a micrometer. Two large platinum stimulating electrodes were placed on both sides of the muscle bundle.

General procedure

After a 15 min thermoequilibration period, each bundle was placed at optimal length (L_o), defined as the length at which peak twitch force was obtained. Stimulation was delivered through a Harvard 50-5016 stimulator (Edenbridge, Kent, UK) which was connected in series to a power amplifier made from power one model HS24-4.8 developed by the computer technology resources centre, University of Virginia, USA (R.J. Evans, 1983).

Maximal stimulation was achieved at approximately 34 V. The voltage was then increased by 20% to ensure supramaximal stimulation. Isometric force was measured with a Maywood force transducer (Maywood Ltd, Hampshire, UK). The signal was amplified and recorded on computer *via* analog-to-digital conversion (DT 2801-A) using Labdat (Labdat/Anadat, RHT-Infodat, Montreal, Canada). Signal analysis was performed with anadat.

Two twitches and one tetanic stimulation (100 Hz applied during 450 ms with square waves of 0.5 ms) were recorded at L_o. Subsequently, both bundles were shortened to 70% L_o and stimulated twice at 1 Hz after 2 min. For twitches, time to peak tension (TPT) and half-relaxation time (1/2RT) were also measured.

Different protocols were then followed (tables 1 and 2) in which two bundles were studied simultaneously, eight pairs for each protocol.

Experimental protocols

The present study first examined the effects of a single dose of theophylline on twitch tension. Theophylline was used at a concentration of 1 mM, a concentration previously shown to produce clear inotropic effects on foreshortened diaphragm [2]. When used, theophylline was added to one bundle within a given pair 15 min before monitoring its influence on twitch tension (table 1). At the same time, the other bundle received normal Krebs solution to serve as time-matched control (table 1).

Table 1. – Summary of experimental protocols with calcium-free Krebs solution (0Ca²⁺) buffered or not with EGTA and verapamil

Incubation time min	Krebs/theophylline		0Ca ²⁺ /theophylline		0Ca ²⁺ +EGTA/theophylline		Verapamil/theophylline	
	15	Krebs	Krebs	0Ca ²⁺	0Ca ²⁺	0Ca ²⁺ +EGTA	0Ca ²⁺ +EGTA	Verapamil
15	Krebs 2P _t	Theophylline 2P _t	0Ca ²⁺ 2P _t	Theophylline 2P _t	0Ca ²⁺ +EGTA 2P _t	Theophylline 2P _t	Verapamil 2P _t	Theophylline 2P _t

Table 2. – Summary of experimental protocols with ryanodine

Incubation time min	Ryanodine/theophylline		Incubation time min	Krebs/ryanodine	
	40	Ryanodine 2P _t		Ryanodine 2P _t	15
15	Ryanodine 2P _t	Theophylline 2P _t	40	Krebs 2P _t	Ryanodine 2P _t

Pt: twitch tension.

It should be noted that, in this protocol, bundles were first incubated for 15 min with normal Krebs solution (table 1) to synchronize timing with the following protocols.

The effects of calcium channel blockers and zero calcium on theophylline-induced twitch potentiation were subsequently evaluated in separate protocols. Verapamil (Isoptine 2.5 mg·mL⁻¹; Knoll, Germany) was used to block calcium entry, whereas ryanodine (produced from a 10⁻² M solution; Molecular Probes, Oregon, USA) inhibited the release of calcium from the sarcoplasmic reticulum. In these protocols, blocking concentrations of either verapamil 10⁻⁵ M or ryanodine 10⁻⁶ M were used. These agents were added to bundles 15 min (verapamil) or 40 min (ryanodine) before theophylline (tables 1 and 2). Note that the effects of ryanodine on twitch tension were examined before adding theophylline (table 2), as ryanodine was previously shown to depress twitch tension [8, 9].

In addition, a supplemental protocol was performed with ryanodine to ensure the nontoxicity of this agent. In this protocol, theophylline was first given and then ryanodine while, at the same time, Krebs solution alone was added to the other bundles to serve as time-matched controls (table 2).

Finally, for experiments performed in the absence of calcium, normal Krebs solution was replaced by a calcium-free Krebs solution 15 min prior to the addition of theophylline. The same experiments were repeated with a calcium-free Krebs solution buffered with 2 mM ethylene glycol-bis(β-amino-ethyl-ether) N, N, N', N'-tetra-acetic acid (EGTA), in order to minimize extracellular calcium (either still present in the solution or located in the transverse tubular system).

Data analysis

At the end of each experiment, the length, thickness and width of each bundle were measured at L_0 , and

Table 3. – Contractile properties of the bundles at optimal length (L_0) and at 70% L_0 under control conditions

	P_t N·cm ⁻²	TPT ms	1/2RT ms	P_0 N·cm ⁻²	P_t/P_0
L_0	7.6±1.7	43±9	62±9	23±3	0.34±0.06
70% L_0	3.0±1.2	30±8	47±9	-	-

Pooled values of twitch tension (P_t), time to peak tension (TPT), half-relaxation time (1/2RT), tetanic tension (P_0) and twitch to tetanus ratio (P_t/P_0). Notice that at 70% L_0 , P_0 was not measured so that P_t/P_0 cannot be calculated.

Table 4. – Time to peak tension (TPT) and half-relaxation time (1/2RT) of diaphragm bundles at 70% optimal length (70% L_0) for each protocol described in table 1

		Krebs/theophylline	0Ca ²⁺ /theophylline	0Ca ²⁺ +EGTA/theophylline	Verapamil/theophylline				
TPT	Before	31±6	31±10	26±8	34±19	29±10	26±12	29±13	34±13
	After	32±5	36±19	36±19	36±12	27±16	28±11	32±9	38±13
1/2RT	Before	46±6	48±8	46±7	51±7	42±10	45±11	52±14	51±6
	After	49±6	60±10*	49±7	52±6	45±8	50±18	57±15	56±10

Values are expressed in ms. *: p<0.01 vs respective control (before). For definitions see legend to tables 1 and 2.

bundles were weighed. Cross-sectional area (CSA) was calculated by dividing weight by specific density (1.056) and muscle length. Twitch and tetanic forces were expressed per unit cross-sectional area.

Data of theophylline-treated bundles were compared to data obtained with calcium blockers or zero calcium using two-way analysis of variance. A p-value of less than 0.05 was set as the level of significance. Differences between means were assessed by a subsequent Duncan test. Data reported in the text and figures are means±standard deviation (sd).

Results

Geometric and contractile properties of the bundles at L_0

Geometric properties of the bundles were similar at L_0 for each protocol. For all studies combined (n=96), length, thickness, weight and cross-sectional area obtained at L_0 averaged 4.3±0.8 cm, 1.4±0.4 mm, 0.15±0.05 g and 0.036±0.029 cm², respectively. Similarly, contractile properties of bundles were the same at L_0 in all protocols. Pooled values (n=96) of twitch tension (P_t), time to peak tension, half-relaxation time, tetanic tension, and twitch to tetanus ratio are summarized in table 3. These values are in the range of that reported at 37°C in dog diaphragm [10].

Mechanisms of inotropic effects at 70% L_0

Twitch characteristics. Under control conditions (before addition of drugs), the pooled values of P_t , TPT and 1/2RT obtained at 70% L_0 are summarized in table 3 (n=96). In a given protocol, no statistically significant differences in twitch characteristics were observed between the pairs except for the protocol evaluating the effect of theophylline (1 mM). Indeed, in the latter (n=16), P_t was higher in the time-matched control bundles than in theophylline-treated bundles (3.9±0.7 vs 2.9±1.0 N·cm⁻²; p<0.01) but TPT and 1/2RT were similar in the two groups (table 4).

Effects of theophylline (1 mM) on twitch tension. In time-matched controls, P_t tended to decrease by 12±9%, while P_t of theophylline-treated bundles increased significantly by 37±21% (p<0.01) (fig. 1). TPT tended to increase after theophylline and 1/2RT was significantly

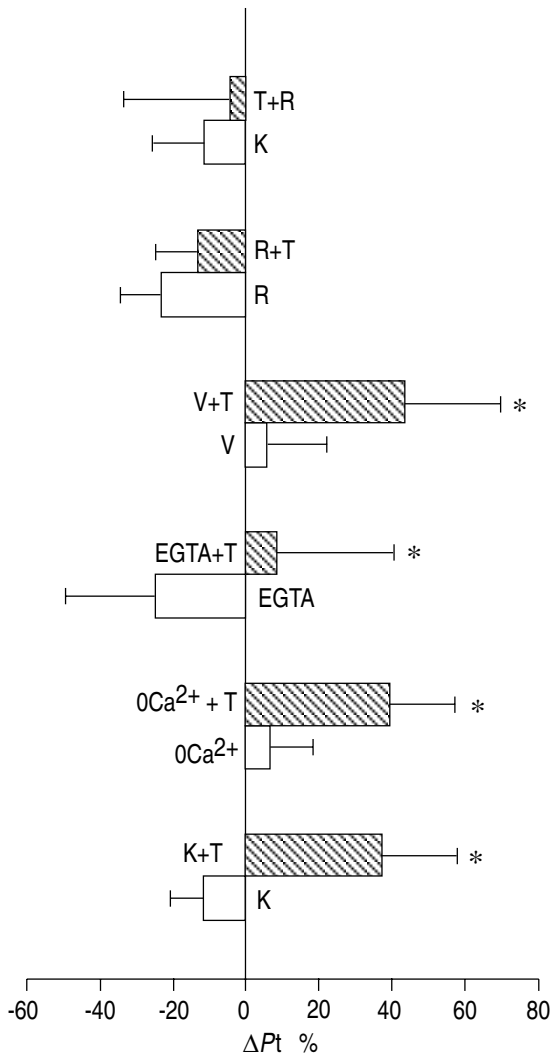


Fig. 1. — Effects of calcium blockers and calcium-free Krebs solution on theophylline-induced twitch potentiation in foreshortened canine diaphragm bundles. Changes in P_t (ΔP_t) are expressed as percentage of initial P_t . P_t : twitch tension; K: normal Krebs solution; T: theophylline 1 mM; $0Ca^{2+}$: calcium-free Krebs solution; EGTA: calcium-free Krebs solution buffered with 2 mM ethylene glycol-bis (β -amino-ethyl ether) N, N, N', N'-tetra acetic acid (EGTA); V: verapamil 10^{-5} M; R: ryanodine 10^{-6} M. For each protocol, eight pairs of diaphragm bundles were studied; *: $p < 0.05$ experimental (hatched bars) vs control (open bars) bundles.

prolonged ($p < 0.01$) (table 4). In the control bundles, TPT and $1/2RT$ remained unchanged (table 4).

Effects of zero calcium on theophylline-induced effects at 70% L_0 . Compared to values obtained before treatment, P_t of the bundles treated with both zero calcium and theophylline increased significantly by $39 \pm 18\%$ ($p < 0.001$), while P_t of the bundles with zero calcium remained stable (fig. 1). TPT and $1/2RT$ were unaffected by any treatment (table 4).

In bundles incubated with zero calcium buffered with 2 mM EGTA, P_t decreased significantly by $25 \pm 25\%$ ($p < 0.05$), such that after EGTA pretreatment the increase in P_t of theophylline-treated bundles was only $8 \pm 32\%$ (fig. 1). The difference between the P_t of theophylline-treated bundles and that of time-matched controls, however, remained in the range of the expected effect of theophylline on P_t . TPT and $1/2RT$ were unaltered under these conditions (table 4).

Effects of calcium entry blockade on theophylline inotropic effects at 70% L_0 . Whilst P_t significantly increased by $43 \pm 26\%$ after incubation with verapamil (10^{-5} M) and theophylline ($p < 0.01$), it remained unchanged after treatment with verapamil alone (fig. 1). TPT and $1/2RT$ remained unchanged whatever the treatment (table 4).

Effects of calcium release blockade on theophylline inotropic effects at 70% L_0 . In all bundles, ryanodine tended to induce a slight decrease in P_t of $13 \pm 18\%$ for bundles treated only with ryanodine during the whole protocol, and of $10 \pm 18\%$ for bundles subsequently treated with theophylline. This decrease did not reach statistical significance and was associated with a prolonged $1/2RT$ (table 5) as observed previously [8, 9]. In addition, the passive tension was progressively increased after ryanodine treatment in both groups (from 52 ± 59 to 72 ± 65 and from 38 ± 42 to 56 ± 35 mN, respectively). The subsequent addition of theophylline failed to increase P_t , which remained depressed (fig. 1). Baseline tension further increased to 67 ± 45 mN after theophylline, whilst it remained similar in the other bundles. $1/2RT$ remained longer in both groups (table 5). Figure 2a shows an example of twitch profiles obtained in a theophylline-treated bundle before any treatment and after ryanodine and subsequent addition of theophylline.

Table 5. — Time to peak tension (TPT) and half-relaxation time ($1/2RT$) of diaphragm bundles at 70% optimal length (70% L_0) for each protocol with ryanodine described in table 2

		Ryanodine/theophylline		Krebs/ryanodine	
TPT	Before	28±6	30±5	29±7	31±5
	After	27±6 (R)	28±5 (R)	29±12 (K)	34±5 (T)
		25±7 (R)	29±9 (R+T)	32±7 (K)	28±5 (T+R)
$1/2RT$	Before	46±8	43±8	45±8	49±10
	After	53±6 (R)	56±8* (R)	48±7 (K)	53±6 (T)
		54±9 (R)	51±10* (R+T)	51±10 (K)	54±15 (T+R)

Values are expressed in ms. *: $p < 0.01$ vs respective control (before). R: ryanodine 10^{-6} M; T: theophylline 1 mM; K: normal Krebs solution.

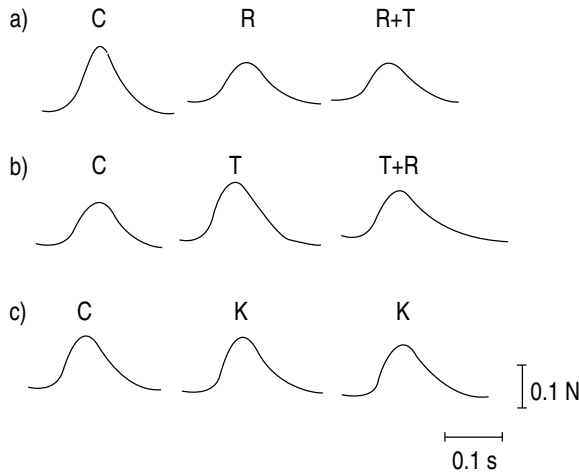


Fig. 2. — Representative tracing showing: a) the twitch depressant effect of ryanodine (R) blocking the inotropic effect of theophylline (R+T); b) the reversal of theophylline-induced rise (T) in twitch tension by ryanodine (T+R); and c) twitches of time-matched control bundle. This bundle was treated with normal Krebs solution (K) given at the equivalent time-points. C: control twitch obtained before treatment.

In the protocol evaluating a possible toxic effect of ryanodine, theophylline significantly increased P_t by $31 \pm 27\%$ ($p < 0.01$), while TPT and $1/2RT$ remained unchanged (table 5). Moreover, passive tension was slightly increased after theophylline (from 28 ± 40 to 34 ± 43 mN). At the same time, P_t of the Krebs-treated bundles did not change, neither did TPT, $1/2RT$ (table 5) nor baseline or passive tension (46 ± 46 vs 44 ± 45 mN).

The subsequent addition of ryanodine reversed the theophylline-induced increase in P_t (fig. 1). This effect was associated with a further and greatly increased passive tension (from 34 ± 43 to 67 ± 57 mN). At the same time, P_t of the time-matched control bundles tended to decrease by $12 \pm 14\%$ (fig. 1), with no significant changes in TPT and $1/2RT$ (table 5). Figure 2b and c depicts an example of a twitch tracing recorded in a theophylline-treated bundle (fig. 2b) before and after theophylline and subsequent addition of ryanodine, and in a time-matched control bundle (fig. 2c). Note that in the example, the increased passive tension with theophylline (fig. 2b) appears similar to that induced by ryanodine (fig. 2a) but, in general, this effect was smaller and more variable with theophylline.

Discussion

This *in vitro* study demonstrated that in foreshortened canine diaphragm strips, theophylline-induced inotropic effects on twitch tension (P_t) were unaffected when transmembrane calcium fluxes were blocked either by verapamil (10^{-5} M) or by removing calcium from the extracellular level, even when buffered with EGTA (2 mM). In contrast, these inotropic effects were dependent on calcium release from sarcoplasmic reticulum as theophylline effects were abolished after ryanodine (10^{-6} M) pretreatment.

The model used in the present study was quite stable, as demonstrated by the results obtained in time-matched

control experiments. Indeed, compared to initial values, the decrease in P_t over time averaged $-12 \pm 9\%$ after 45 min (for all the protocols except those with ryanodine) and $-12 \pm 14\%$ after 70 min (for ryanodine protocols).

Several attempts have been made to investigate the mechanism by which theophylline exerts its inotropic effects. These studies have suggested that theophylline induced a hyperpolarization of the cell membrane [11, 12], or interacted with intracellular [13–15] or transmembrane [15, 16] calcium transfer but not with cyclic adenosine monophosphate (cAMP) [15]. However, it should be noticed that all these experiments pertain to studies performed at optimal diaphragm length in rodents [11–16]. But, it is well-established that muscle mechanics at optimal length and at shorter length are different. Indeed, whilst tension generation decreases with muscle shortening due to a reduction in overlap between actin and myosin filaments [3] and due to calcium "deactivation" [4, 5], tension generation at optimal muscle length is maximal because filament geometry is optimal and calcium activation normal.

In our study, we attempted to investigate the mechanism involved in the preferential effect of theophylline at shorter length. This mechanism has never previously been explored. Thus, in view of previous reports implicating calcium deactivation in acute muscle shortening [4, 5], we examined the effects of theophylline on P_t of foreshortened diaphragm in the presence of different calcium inhibitors to block separately each pathway able to enhance intracellular calcium level.

Firstly, theophylline's action was studied in the presence of a transmembrane block as it was proposed that diaphragmatic contraction at L_0 was dependent on extracellular calcium [17]. Indeed, removal of calcium from the extracellular milieu or administration of verapamil, a voltage-dependent calcium channel blocker, significantly depressed muscle strength [18]. Although theophylline-induced inotropic effects at L_0 have been demonstrated to depend on transmembrane calcium movement [15, 16, 18, 19], we could not observe a similar effect in foreshortened bundles. Indeed, calcium withdrawal from the extracellular milieu or the use of verapamil (10^{-5} M) did not affect P_t of control bundles or the rise in P_t induced by theophylline. Similar results were obtained in experiments where calcium-free solution was buffered with 2 mM of EGTA. Note that, in these experiments, EGTA itself induced a depressant effect on P_t of control bundles, a phenomenon observed previously [20] even with lower concentrations of EGTA [21]. Conversely, previous reports showed that the inhibitory effect of calcium withdrawal on muscle contraction resulted, in fact, from the absence of bound metal cations (like calcium) to the voltage sensor of the membrane [22, 23]; thereby abolishing its function in excitation-contraction coupling. This function was, however, restored when other metal cations (other than calcium) were added to the calcium-free solution. The absence of muscle contraction in calcium-free solution is, therefore, related more to inactivation of the voltage sensor in the absence of cations essential for its function rather than to the lack of a transmembrane calcium transient.

From our above-mentioned experiments, it follows that theophylline-induced inotropic effects on foreshortened diaphragm were not related to transmembrane calcium flux. Therefore, the action of theophylline was studied when calcium release from the sarcoplasmic reticulum was blocked with ryanodine (10^{-6} M). Indeed, the calcium release channel is abundantly present in the terminal cisternae of skeletal sarcoplasmic reticulum. It there forms a homo-tetrameric complex with the foot structure, which spans the 12 nm gap between the T-tubules and the sarcoplasmic membranes [24, 25]. Following depolarization, this channel releases calcium from the sarcoplasmic reticulum in a still unelucidated way. In a complex manner, ryanodine binds to this release calcium channel at different sites in a micromolar range and, after an initial activation phase, ryanodine inactivates the calcium release channel [26].

After ryanodine pretreatment, P_t was similarly depressed in control and experimental bundles and half-relaxation time similarly prolonged. Theophylline, subsequently added, failed to increase P_t . An even more dramatic depression of P_t after ryanodine treatment was previously observed in rat diaphragm [8]. In fact, this effect referred to ryanodine action on calcium channel conductance. Indeed, ryanodine progressively locked the sarcoplasmic reticulum calcium channels in an open subconductance state, such that P_t decreased as a result of a decrease in the amount of calcium available for release [27]. As a consequence, a passive leak of calcium occurs, thereby causing muscle contraction [9], a phenomenon also obtained in our study. Nevertheless, to ensure that a toxic effect of ryanodine would have masked theophylline-induced inotropic effects, in another set of experiments, theophylline was added before ryanodine (experimental bundles) and these data were compared to time-matched control bundles. Under these circumstances, the depression in P_t of experimental bundles and time matched control bundles was small and similar, thereby excluding a nonspecific effect of ryanodine.

The theophylline-induced inotropic effects on foreshortened diaphragm may be clinically relevant. Indeed, as respiratory muscles shorten with acute hyperinflation [28], they may become considerably more susceptible to the inotropic action of theophylline, such that inotropic actions may be present at considerably lower serum levels. Whether this actually occurs in these patients and whether or not it affects clinical outcome variables has not been studied. Clinical trials comparing the effects of theophylline in patients with or without acute hyperinflation appear warranted.

In conclusion, the present study demonstrated that the inotropic effects of theophylline on P_t of foreshortened canine diaphragm bundles were not related to extracellular calcium but were essentially dependent on the release of calcium from the sarcoplasmic reticulum. This effect could result either from a direct action of theophylline on sarcoplasmic calcium channels or from interaction with an upstream event in the excitation-contraction coupling chain of skeletal muscle.

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