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RYANODINE AND THEOPHYLLINE-INDUCED DEPLETION OF ENERGY STORES IN AMPHIBIAN MUSCLE

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Abstract—The effects of high and low levels of ryanodine on theophylline-induced energy depletion were studied in isolated frog sartorius muscle. Whereas low concentrations of ryanodine (1–10 μM) did not change high energy phosphate contents (PE) after 60 min, high levels (100 μM) reduced resting energy contents by 60% after 60 min. Subcontracture levels of theophylline (2 mM), in the presence of high ryanodine, produced an 80% PE depletion, suggesting possible additive or synergistic effects of these two agents. In contrast to theophylline-induced depletion, neither the ryanodine-induced depletion nor the theophylline-plus-ryanodine-induced depletion of PE seemed sensitive to inhibition by 1 mM procaine. This suggests that there may be differences in the mechanisms whereby methylxanthines and ryanodine deplete energy stores and evoke contractures in amphibian skeletal muscle.

Key words: ryanodine; theophylline; energy depletion; skeletal muscle; high energy stores; calcium homeostasis (balance)

Recent evidence [1–4] suggests that methylxanthines, such as caffeine and ryanodine, bind to a common receptor. It also seems clear that the ryanodine receptor is the SR† calcium release channel [5–8] as well as foot protein of the triad. A recent review by Ashley *et al.* [9] summarizes evidence in skeletal muscle to support this thesis, whereas a review by Rios *et al.* [10] characterizes the role of the ryanodine receptor in excitation–contraction coupling in skeletal muscle. Besides binding to a common receptor, both caffeine and ryanodine have numerous other effects in skeletal muscle such as stimulation of oxygen consumption [11, 12], stimulation of calcium influx [11, 13, 14] and calcium efflux [14–18], and contracture production [11, 12, 19–21]. Contracture production with both caffeine and ryanodine has been reported to occur with minimal change in membrane potential [12, 19]. These other effects could be due to binding of caffeine or ryanodine to sites other than the SR calcium release channel. Another possibility is that these are secondary effects resulting from a single event, the binding of ryanodine or caffeine to the SR calcium channel. This binding of ryanodine to its receptor changes the characteristics of the calcium release channel [1, 2, 9, 22], thus causing a concomitant rise in intracellular calcium $[Ca]_i$. Many of the actions of these two agents, whether at subcontracture or contracture doses, including those mentioned above, can, particularly in skeletal muscle, be linked through a common event, the SR release of calcium and the resulting increase in $[Ca]_i$.

Caffeine and theophylline are both methylxanthines. Whereas caffeine has been widely used as a tool to understand skeletal muscle function, particularly in frog skeletal muscle, there have been few studies of the action of theophylline in this preparation [23, 24]. While both have similar qualitative effects in a given muscle preparation, there are quantitative differences [23, 25]. One similarity is the energy depletion that subcontracture levels of both evoke in isolated frog sartorius muscle [23]. This depletion is probably associated with the small rises in $[Ca]_i$ induced by methylxanthines at these levels [10, 26].

Studies of the action of ryanodine in muscle have produced conflicting results particularly in regard to the effects on SR calcium channels and SR calcium stores [5, 22, 27]. Whereas low levels of ryanodine may have a stimulatory effect on calcium release from SR [2, 5, 9, 14], higher levels seem to produce an inhibitory effect [2, 5]. Also ryanodine, like caffeine, has effects that with time become irreversible [7, 12, 20, 21, 23, 28]. On the other hand, ryanodine has been reported to have little or no effect on energy stores of unstimulated or nondepolarized muscle [12, 29], and ryanodine-induced rigor seems to occur without complete depletion of energy stores [29]. With caffeine and theophylline, irreversible effects on sodium efflux and contracture production have been reported in frog skeletal muscle [23, 24].

In frog sartorius muscle, subcontracture but twitch potentiating levels of methylxanthines create a dose-dependent energy imbalance between energy production and demand, resulting in a dose- and time-dependent decrease in energy stores, specifically

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† Abbreviations: SR, sarcoplasmic reticulum; PE, potential energy or total high energy adenine nucleotides; CrP, creatine phosphate; G6P, glucose-6-phosphate; Cr_f, free creatine; and Cr_T, total creatine.

ATP and CrP [23]. Ryanodine, even at contracture-producing levels, has been reported not to deplete energy stores [12, 29]. Local anesthetics, such as procaine, block or prevent many effects of low level methylxanthines. These include effects on oxygen consumption [11], internal alkalinization [18, 30], calcium transients [26] and energy depletion [23]. Local anesthetics are reported to block ryanodine-induced increases in oxygen consumption and ryanodine contractures [11, 14]. Thus, the first purpose of this work was to determine how low and high ryanodine concentrations, alone or in combination with theophylline, affect energy stores. The second purpose was to determine if low levels of procaine can prevent any of the depletion induced by ryanodine or ryanodine plus theophylline. Our results show that ryanodine induced energy depletion at high concentrations, an effect that was enhanced in the presence of theophylline. Our results also show that 1 mM procaine, which blocks the effects of caffeine and theophylline on energy depletion [23] at subcontracture levels, had little or no effect on the high ryanodine-induced energy depletion.

A preliminary report of these results has appeared elsewhere [31].

MATERIALS AND METHODS

All measurements were made on whole sartorius muscle isolated from the American leopard frog, *Rana pipiens*, essentially using methods previously described [23, 32]. All frogs were obtained from suppliers in Wisconsin and generally were used within 1–2 weeks of arrival.

Muscles were dissected, attached to stainless steel holders at rest length, and allowed to recover from dissection for at least 2 hr in normal frog Ringer solution at room temperature (21–22°C). The composition of the Ringer solution was (in mM): NaCl, 115; KCl, 2.5; $CaCl_2$, 1.8; Na_2PO_4, 2.15; and NaH_2PO_4, 0.85. Paired muscles were used in all experiments. In general, one member of the pair was transferred to a control solution while the other member of the pair was transferred to the experimental solution. Theophylline, ryanodine, and procaine were added in powder form to the Ringer solution. The pH of all solutions was 7.2 ± 0.1. All solutions contained curare at 1 μg/mL.

At the end of the incubation period, which was 60 min for all the experiments reported here, the muscles were frozen, weighed, and analyzed as described in detail by Connett and Hays [32]. G6P, ATP, CrP and Cr_f were all measured spectrophotometrically. All results are expressed as high energy phosphate contents (PE) where PE = CrP + 2 ATP. Because the contribution of ADP to the total available phosphate bond energy (PE) is small and total ADP is probably not changed significantly, it was not measured and included in our calculation of PE [23]. Since both CrP and Cr_f were measured in each muscle, all results are expressed on a Cr_T basis. This reduces the variability in measurements that occur when expressed on a weight (g) basis [32].

Results are presented as means ± SEM of N observations. Data were analyzed using a two-tailed

Table 1. Effect of low ryanodine on muscle energy stores and on theophylline-induced depletion of energy stores

Condition	N	Cr_f (μM/g)	G6P (μM/μM Cr_T)	ATP (μM/μM Cr_T)	CrP (μM/μM Cr_T)	PE (μM/μM Cr_T)	ΔPE (μM/μM Cr_T)
Normal Ringer	20	27.01 ± 3.54*	0.014 ± 0.004	0.131 ± 0.014	0.009 ± 0.031	0.771 ± 0.038	
(A) Normal Ringer	5	20.37 ± 4.36	0.017 ± 0.009	0.072 ± 0.023	0.532 ± 0.083	0.675 ± 0.056	−0.088 ± 0.113
4 μM Ryn		19.28 ± 3.65	0.019 ± 0.006	0.095 ± 0.036	0.574 ± 0.081	0.765 ± 0.046	
(B) Normal Ringer	5	15.67 ± 1.45	0.007 ± 0.004	0.191 ± 0.032	0.468 ± 0.050	0.852 ± 0.097	−0.016 ± 0.078
8 μM Ryn	5	23.10 ± 3.33	0.004 ± 0.000	0.160 ± 0.042	0.542 ± 0.051	0.862 ± 0.115	
(C) Normal Ringer	5	26.56 ± 4.00	0.024 ± 0.015	0.152 ± 0.022	0.549 ± 0.049	0.852 ± 0.074	0.192 ± 0.080
2 mM Theo + 4.8 μM Ryn		26.55 ± 2.36	0.026 ± 0.012	0.125 ± 0.016	0.410 ± 0.022	0.660 ± 0.026	
(D) Normal Ringer	5	43.44 ± 9.79	0.007 ± 0.002	0.109 ± 0.011	0.487 ± 0.072	0.706 ± 0.092	0.093 ± 0.122
2 mM Theo + 9.6 μM Ryn		30.86 ± 1.39	0.032 ± 0.008†	0.114 ± 0.007	0.383 ± 0.047	0.612 ± 0.061	

After a 2 hr recovery in normal Ringer solution, one member of the pair was transferred to normal Ringer while the other member of the pair was transferred to normal Ringer with either ryanodine (Ryn) for 60 min or ryanodine plus theophylline (Theo) for 60 min. ΔPE is calculated as control minus experimental PE value.

* Values are means ± 1 SEM.

† Significantly different at $P \leq 0.05$.

t-test; values at the $P \leq 0.05$ level were considered statistically significant.

RESULTS

For the low ryanodine experiments, we chose ryanodine levels just below 10 μM because Jenden and Fairhurst [12] have reported maximal effects in vertebrate muscle within 30–60 min, and other investigators, such as Sutko *et al.* [27] and Nasri-Sebdani *et al.* [28], have reported tension depression at these levels. Thus, a depressant effect on the depletion of theophylline was possible. Table 1 is a composite of results obtained in paired experiments with low ryanodine concentrations. Low ryanodine alone had no effect on any of the parameters measured in muscles kept for 60 min in ryanodine-containing solution compared with muscles kept for 60 min in normal frog Ringer solution (Table 1, A and B). In another set of paired experiments (Table 1, C and D), experimental muscles were exposed to 2 mM theophylline plus either 4.8 or 9.6 μM ryanodine for 60 min while control muscles remained in normal Ringer for 60 min. While in both cases PE and CrP were reduced by theophylline in the presence of ryanodine, the decreases in this experiment were not significant. In one case (Table 1D), G6P was increased significantly, a typical result with theophylline exposure [23]. This increase in G6P was the only significant difference from normal that was observed in the paired data.

Because low ryanodine by itself neither had any detectable effect on energy stores nor prevented theophylline-induced depletion in 60 min, additional paired experiments were performed at higher (100 μM) ryanodine levels (see Table 2). In the first paired experiment (Table 2A), a 60% reduction in PE occurred after 1 hr in 100 μM ryanodine. In this experiment, ryanodine also produced significant decreases in ATP, CrP and ΔPE. In addition, significant increases occurred in G6P and Cr_T. In a second paired experiment (Table 2B) in which both members of the pair were exposed to 100 μM ryanodine, 2 mM theophylline reduced ATP, CrP and PE even further. Muscles treated with ryanodine alone had significantly higher Cr_T than the ryanodine-plus-theophylline-treated muscles, even though the average PE was lower in the latter muscles. Finally, a large significant increase in G6P was observed in the theophylline-exposed muscles.

In a third paired experiment, we examined the ability of 100 μM ryanodine to affect theophylline depletion of energy stores (Table 2C). Only PE and ATP were reduced significantly. On the other hand, the Cr_T was unchanged. These results suggest that the effects of high ryanodine and theophylline are additive and perhaps synergistic.

In another paired experiment (Table 2D), we tested the ability of 1 mM procaine to prevent ryanodine-induced depletion of energy stores. In the presence of procaine, energy stores were depleted less. Both ATP and PE were higher in muscles exposed to 1 mM procaine, suggesting that some part of the ryanodine-induced depletion is sensitive to procaine. However, the effect was small. Table 2E1 shows that 1 mM procaine had no effect on the ryanodine-plus-theophylline-induced depletion of energy stores. Because members of three pairs of muscles were lost during the experiment, it was repeated at a later date (Table 2E2). Although there were some differences in absolute values in this experiment (Table 2E2) compared with the earlier experiment (Table 2E1), there was no difference in any of the parameters measured. These data suggest that ryanodine plus theophylline depletion of energy contents cannot be prevented by 1 mM procaine.

Because some absolute differences seemed to exist in the results of the paired experiments, a composite table was created that combined the similarly treated muscles from all paired experiments with high ryanodine (Table 3). In addition, results of experiments on some normal muscles from other non-ryanodine experiments performed at the same time and on the same batches of frogs are shown. Table 3B clearly shows that 100 μM ryanodine alone reduced high energy phosphate contents by at least 60% and that all parameters measured were changed significantly after 1 hr in 100 μM ryanodine. Theophylline 2 mM (Table 3C) also reduced energy stores, with PE decreased by about 50%. Although G6P and Cr_T were increased, the changes were not significant. These PE values are remarkably close to the 2 mM theophylline PE values of Hays and Connett [23].

Ryanodine plus theophylline (Table 3D) caused even further depletion of ATP, CrP, and PE so that after 1 hr only about 15% of the total energy stores remained, and the muscles were significantly energy depleted. Although Cr_T was increased significantly when compared with that of normal muscles, the increase was significantly less than the large increase seen with ryanodine alone (Table 3B).

The composite data indicate no effect of 1 mM procaine on ryanodine-induced energy depletion either when high ryanodine was present alone (Table 3, B and F) or in combination with theophylline (Table 3, D and E). The only significant change was the higher Cr_T in muscles treated with procaine plus ryanodine (Table 3F) compared with muscle treated with ryanodine alone (Table 3B). Theophylline in the presence of ryanodine and procaine still could produce significant increases in G6P and decreases in ATP (Table 3E). CrP and PE were reduced but not significantly. Thus, in the presence of high ryanodine and theophylline, energy depletion was not prevented by 1 mM procaine. High ryanodine is interfering, in some way, with normal theophylline/procaine interactions.

DISCUSSION

Prior to 1969, several studies on the action of ryanodine in muscle, particularly in frog skeletal muscle, were carried out, as summarized in a review by Jenden and Fairhurst [12]. Because of conflicting results and particularly the irreversible effects of ryanodine, studies using ryanodine were few until the mid 1980s. Throughout most of the 1980s seemingly conflicting results were obtained in skeletal muscle and in skeletal versus cardiac and smooth muscle and non-muscle tissues regarding the mechanism of action of ryanodine [8, 27]. Particularly

Table 2. Effect of 100 μM ryanodine on energy stores and on theophylline-induced depletion of energy stores: Paired experiments

Condition	N	Cr_I (μM/g)	G6P (μM/μM Cr_I)	ATP (μM/μM Cr_I)	CrP (μM/μM Cr_T)	PE (μM/μM Cr_I)	ΔPE (μM/μM Cr_T)
(A) Normal Ringer	4	16.17 ± 1.98*	0.005 ± 0.000	0.284 ± 0.041	0.478 ± 0.057	1.046 ± 0.093	0.596 ± 0.113†
100 μM Ryn	5	25.96 ± 2.59†	0.010 ± 0.000†	0.142 ± 0.022†	0.176 ± 0.023†	0.461 ± 0.032†	
(B) 100 μM Ryn	6	32.81 ± 0.58	0.013 ± 0.000	0.120 ± 0.003	0.232 ± 0.021	0.473 ± 0.017	0.386 ± 0.022†
100 μM Ryn + 2 mM Theo	6	27.76 ± 0.68†	0.065 ± 0.006†	0.017 ± 0.004†	0.053 ± 0.015†	0.087 ± 0.021†	
(C) 2 mM Theo	6	23.68 ± 3.86	0.131 ± 0.108	0.154 ± 0.019	0.239 ± 0.084	0.548 ± 0.103	0.411 ± 0.102†
2 mM Theo + 100 μM Ryn	6	24.91 ± 1.77	0.075 ± 0.009	0.022 ± 0.004†	0.093 ± 0.008	0.138 ± 0.006	
(D) 100 μM Ryn	5	32.12 ± 1.81	0.024 ± 0.007	0.073 ± 0.004	0.098 ± 0.009	0.244 ± 0.005	−0.187 ± 0.077
100 μM Ryn + 1 mM Prn	5	34.53 ± 1.07	0.010 ± 0.002	0.117 ± 0.004†	0.146 ± 0.025	0.381 ± 0.029	
(E1) 100 μM Ryn + 2 mM Theo	4	30.60 ± 2.45	0.099 ± 0.009	0.022 ± 0.007	0.067 ± 0.007	0.108 ± 0.019	0.003 ± 0.021
100 μM Ryn + 2 mM Theo + 1 mM Prn	3	30.78 ± 3.39	0.102 ± 0.017	0.024 ± 0.009	0.057 ± 0.005	0.107 ± 0.014	
(E2) 100 μM Ryn + 2 mM Theo	6	21.41 ± 2.24	0.038 ± 0.010	0.047 ± 0.011	0.177 ± 0.025	0.270 ± 0.043	0.040 ± 0.053
100 μM Ryn + 2 mM Theo + 1 mM Prn	6	22.98 ± 1.48	0.038 ± 0.011	0.036 ± 0.011	0.158 ± 0.020	0.229 ± 0.040	

After a 2-hr recovery in normal Ringer solution, one member of the pair was transferred to control solution for 60 min while the other member of the pair was transferred to experimental solution with ryanodine (Ryn), theophylline (Theo) and procaine (Prn) as indicated for 60 min. ΔPE is calculated as control minus experimental PE value.

* Values are means ± 1 SEM.

† Significantly different at $P \leq 0.05$.

Table 3. Effect of high ryanodine on muscle energy stores and on theophylline-induced depletion of energy stores: Composite data

Condition	N	Cr_I (μM/g)	G6P (μM/μM Cr_I)	ATP (μM/μM Cr_I)	CrP (μM/μM Cr_I)	PE (μM/μM Cr_T)
(A) Normal Ringer	10	19.63 ± 2.32*	0.001 ± 0.005	0.282 ± 0.034	0.507 ± 0.041	1.071 ± 0.054
(B) 100 μM Ryn	22	30.46 ± 1.22†	0.015 ± 0.003†	0.112 ± 0.010†	0.173 ± 0.018†	0.398 ± 0.029†
(C) 2 mM Theo	6	23.68 ± 3.86	0.131 ± 0.108	0.154 ± 0.019†	0.239 ± 0.084†	0.548 ± 0.103†
(D) 100 μM Ryn + 2 mM Theo	22	25.76 ± 1.11†‡	0.067 ± 0.006†‡	0.027 ± 0.004†‡	0.099 ± 0.013†‡	0.154 ± 0.020†‡
(E) 100 μM Ryn + 2 mM Theo + 1 mM Prn	9	25.58 ± 1.89§	0.059 ± 0.014§	0.032 ± 0.008§	0.125 ± 0.021	0.188 ± 0.033
(F) 100 μM Ryn + 1 mM Prn	5	34.53 ± 1.07‡	0.010 ± 0.002	0.117 ± 0.004	0.146 ± 0.025	0.381 ± 0.029

After a 2 hr recovery in normal Ringer solution, one member of the muscle pair was transferred to control solution for 60 min while the other member of the pair was exposed to experimental solution for 60 min. This table summarizes results from a number of different paired experiments. Abbreviations: Ryn, ryanodine, Theo, theophylline, and Prn, procaine.

* Values are means ± 1 SEM.

† Significantly different at $P \leq 0.05$ from normal muscles.

‡ Significantly different at $P \leq 0.05$ from ryanodine alone muscles.

§ Significantly different at $P \leq 0.05$ from ryanodine plus procaine muscles.

disturbing were reports that low ryanodine (nM) opened while higher levels (μM) closed ryanodine-sensitive calcium channels [5]. Only recently with the identification of the location and nature of the ryanodine receptor has a common mechanism of action begun to be elucidated, which may eventually resolve the earlier conflicting results and theories regarding the action of ryanodine [33]. In skeletal muscle, early investigators suggested that, even though ryanodine did not affect the SR Ca^{2+}-ATPase [34], ryanodine's effect was to prevent resequestration of calcium in the SR [12, 19, 34, 35]. Now it appears that the major action of ryanodine in skeletal muscle is on the mechanism of calcium release from the SR stores [5–8, 10, 36], and it may be possible to explain all previous data on the basis of a single mechanism where ryanodine slowly and irreversibly converts the calcium release channel from the fast high conductance state needed for normal excitation–contraction coupling to an open low conductance state that may eventually close [6, 8], particularly if ryanodine doses are high enough [8]. In whole cells and tissues, these effects may take hours depending on conditions [12, 34, 37] while occurring somewhat faster in isolated preparations or cell fractions [6–8]. Finally, as pointed out by Rousseau *et al.* [33], the conflicts in skeletal versus cardiac muscle may be a consequence of differences in the Ca^{2+} handling ability of the two tissues.

Low ryanodine

Low levels of ryanodine (less than 100 μM) open native and purified SR calcium release channels [8]. In frog skeletal muscle, they may also open calcium channels to the T-tubule allowing calcium to enter and trigger further calcium release [14]. Within 10–20 min oxygen consumption is increased at least 5- to 10-fold [11], and calcium influx with net calcium uptake by frog muscle fibers occurs at ryanodine levels below 100 μM [14]. In voltage-clamped frog muscle fibers, Nasri-Sebdani *et al.* [28] found that 0.1 to 10 μM ryanodine inhibited tension and slow outward potassium current. Although these effects may activate energy-utilizing processes, our results indicate that, below 100 μM, ryanodine produces no significant sustained energy imbalance and, after 1 hr, energy stores are the same as those of non-ryanodine-treated muscles. This is in keeping with the results of Seraydarian *et al.* [29] in unstimulated frog skeletal muscle at 2°.

Jenden and Fairhurst [12] suggested that 1–10 μM ryanodine produces maximal contracture effects in most vertebrate skeletal muscle within 30–60 min, while in frog sartorius at 20° Bianchi [11] determined that the threshold for ryanodine-induced contractures was 100 μM with a delay of onset of 30–40 min. We saw no change in energy stores and no contractures were noted in any of our experiments, all of which were carried out at room temperature. More recently, Baylor *et al.* [37] reported no contracture for 45 min in intact frog muscle bundles in a normal Ringer solution at 20° with 500 μM ryanodine. Also, while Fairhurst and Jenden [34] reported a half-time of 2 hr for 1 μM ryanodine to produce a maximal contracture in mammalian muscle at 37°, more recently Fryer *et al.* [20] reported that at 25° 5 μM ryanodine plus electrical stimulation at 8/min (0.033 Hz) required 40 min to produce a contracture in rat soleus and less time for contracture in rat extensor digitorum longus. Although our muscles were tied to rigid steel frames at close to rest length, muscles treated with ryanodine were generally as flaccid at the time of freezing as were muscles maintained in normal Ringer solution. In fact, in none of the experiments reported here were any significant contractures observed, including muscles with less than 15% of normal energy stores. Muscles were tied in a manner similar to that in experiments of Hays *et al.* [24] and Hays and Connett [23] where higher theophylline or caffeine often produced significant contractures. Thus, it appears that, at these lower levels of ryanodine and at room temperature, energy production can adequately match the increased energy utilization for at least 1 hr, preventing any significant depletion of energy stores. In addition, theophylline depletion of energy stores can still occur when ryanodine is present (Table 1), certainly indicating a lack of ryanodine antagonism of theophylline depletion. The absolute PE values in muscles exposed to 2 mM theophylline plus low ryanodine (Table 1) were not very different from the PE values obtained later with 2 mM theophylline alone (Table 3) or those reported previously [23]. One possible reason that the percent theophylline-induced reduction was smaller in these low ryanodine experiments is that the average PE values of normal muscle in these experiments were 20–25% lower than those usually seen [23, 32].

The significant difference in Cr_T in the 8 μM ryanodine experiment (Table 1B) does suggest an effect of this level of ryanodine on the muscle. The larger Cr_T in the ryanodine-treated muscles could be due to a water loss in ryanodine muscles compared with normal muscles. Jenden and Fairhurst [12] suggested that these levels of ryanodine produce large water losses in skeletal muscle. Dry–wet weight measurements of ryanodine-treated versus normal muscles suggest that this is not the case [38]. On the other hand, the five normal muscles in this experiment had the lowest average Cr_T for the four experiments presented in Table 1. Also in these five muscles there was little variation in Cr_T values, as indicated by the low SEM value. Note that the normal muscles in the D experiment had an average Cr_T almost three times higher with a much wider range of values as indicated by the much higher SEM. The significantly lower Cr_T in the theophylline/9.6 μM ryanodine muscles (Table 1D) was contrary to the effect usually seen, that is, Cr_T is usually higher in theophylline-exposed muscles. This may be due to the fact that the control muscles in this experiment had on average very high Cr_T with large variation, whereas the experimental muscles had Cr_T with little variation that were not significantly different from the composite value obtained by averaging Cr_T value for all control muscles in the low ryanodine experiments (Table 1). These differences could be due to variations in amounts of fluid adhering to muscles at the time of freezing or to differences in the amount of damaged fibers, but this type of variation should have occurred in both members of the pair. Expressing the metabolite values on a μM/

μM Cr_T basis usually corrects for these types of variations [32].

High ryanodine

In our experiments, high ryanodine certainly did not have a depressant effect on energy depletion nor was there any evidence of results consistent with a reduced SR calcium release. On the contrary, our results were consistent with elevated calcium release from the SR and an increased $[Ca]_i$. In fact, most cases of negative inotropy with ryanodine in skeletal muscle [27, 28] or other muscle types [27, 39–41] can be explained by depletion of calcium within the SR due to increased net loss of SR calcium, not a blockage of calcium release channels [8, 33]. A commonality of the effect of ryanodine in all types of muscle and even in non-muscle tissue seems appropriate and biologically sound. The commonality is the irreversible conversion of the regulated high conductance fast calcium release channel to a long-lived low conductance channel. This converted channel now becomes insensitive to regulation by substances such as calcium and ATP [2]. Thus, a chemical type of inactivation, perhaps reminiscent of voltage inactivation of ion channels, occurs.

Within 1 hr 100 μM ryanodine produced a large and significant depletion of high energy phosphate stores accomplished by a 50% decrease in ATP stores and a 65–70% decrease in CrP stores. Thus, high ryanodine produces a large enough energy imbalance to cause significant depletion of stores. This imbalance may also be occurring at lower ryanodine levels (4–10 μM) but is either not large enough to produce significant depletion after 1 hr or is of such a short duration as to be compensated for by the end of 1 hr. This latter possibility would certainly be supported by the observation of Bianchi [42] of complete reversal of ryanodine stimulation of oxygen consumption usually within 30 min over a range of ryanodine levels from 0.01 nM to 10 μM. It is clear that ryanodine upsets cellular calcium homeostasis. As a result, more cellular energy must be expended to restore and maintain normal calcium levels; thus an increase in energy utilization occurs [42]. As energy demands increase, so should energy production. As long as production and utilization are matched, energy stores are maintained even though many metabolically dependent parameters, such as O_2 consumption, calcium influx, calcium efflux, and calcium concentrations, are increased. It may even seem for a while that a new steady state or balance has been achieved, and high energy metabolites, such as ATP, CrP, and PE, are normal or unchanged.

In isolated cellular fraction preparations, the binding of ryanodine to its receptor is very slow (the time constant of association is about 40 min with 5 nM ryanodine [7] and apparently irreversible [7, 8, 20]). Within intact cells and tissues the consequences of ryanodine binding should be even slower. As ryanodine binds to its receptor, the calcium channel of the receptor is converted irreversibly to a low conductance state [6–8], producing a slow leakage of calcium out of the SR. Ryanodine binding is not favored by the submicromolar resting calcium levels [6], but as calcium levels increase, ryanodine binding to its receptors increases [5, 6, 43]. Too high calcium inhibits ryanodine binding [6–8]. It seems that a slowly developing positive feedback occurs [21, 42, 43]. Thus, with time energy requirements increase in intact muscle cells and whole muscle preparations. At normal high intracellular ATP levels, this positive feedback system is reinforced and amplified by methylxanthines which, by raising $[Ca]_i$ and alkalinizing pH_i, stimulate ryanodine binding even more [2, 4]. The fact that Bianchi [11] observed a large stimulation of oxygen consumption in whole muscle preparations after less than 10–20 min of exposure to ryanodine concentrations as low as 0.01 nM compromises this interpretation unless the stimulation of oxygen consumption is a consequence of a much faster action than is postulated above.

Methylxanthines apparently bind to the ryanodine receptor/calcium release channel [1, 4, 5, 33]. Caffeine stimulates ryanodine binding [4], possibly by opening the channel itself or secondarily via a caffeine-stimulated calcium-induced calcium release [26]. Even at subcontracture levels, methylxanthines may open up some calcium release channels [1, 22] or at least make the SR calcium release easier [10]. Caffeine concentrations as low as 0.5 mM produce calcium release and transients in frog skeletal muscle [26, 44]. Since ryanodine binds more readily to the calcium-induced open state of the SR release channel [7], the effect of caffeine may be synergistic to that of ryanodine. A synergistic release of calcium by caffeine and ryanodine has been reported in other tissues [45]. Caffeine, like electrical stimulation, opens or promotes calcium-induced calcium release but much more slowly, particularly at subcontracture levels. Electrical stimulation just makes the whole process faster. The levels of ATP, which induces the closing of native and ryanodine-modified channels [1], fall, and the channel becomes even more leaky, amplifying the positive feedback. As ATP levels fall, positive feedback could be accelerated even more by decreased ATP [2, 5, 21] and other effectors such as increased hydrogen ion [5], increased ADP [46] and perhaps low total adenines [2].

Although Seraydarian *et al.* [29] found no significant changes in ATP and CrP in muscles treated with 200 μM ryanodine for 30 or 60 min at 10°, we found large depletions with 100 μM ryanodine after 1 hr at room temperature. Because of the slowness of the effects of ryanodine even in isolated SR [4, 7], in whole muscle 30 or 60 min at 10° may not have been long enough to produce sufficient energy imbalance to cause significant changes in ATP and CrP. Our results clearly show a ryanodine-induced decrease in energy stores in frog sartorius muscle after a 1 hr exposure to 100 μM ryanodine, suggesting that under our conditions the imbalance is large enough to deplete energy stores. Yet adequate levels of ATP are present to prevent rigor contractures. On the other hand, the contractures observed by Baylor *et al.* [37] in frog skeletal muscle at 20° with 500 μM ryanodine are most likely rigor contractures similar to those seen with high caffeine [23]. The fact that subcontracture levels of theophylline enhance this depletion is not surprising,

since subcontracture levels of caffeine increase calcium release in muscle and nonmuscle tissue [26, 44, 45] and could thus enhance the effects of ryanodine. In any case, theophylline enhances the effects of ryanodine and vice versa.

The theophylline depletion observed here is similar to that reported previously [23]. A major difference between ryanodine- and theophylline-induced depletion seems to be the effects on G6P levels. In this study theophylline produced a very large increase in G6P that was not significant because of the unusually large variation in this set of muscles. Ryanodine produced a smaller yet significant increase in G6P that was on the magnitude of that observed by Hays and Connett [23] for 2 mM theophylline. Dose-dependent increases in G6P prevented by procaine are commonly seen with theophylline [23] and cyanide [11]. In the latter case, measurement of G6P can be used to monitor the inhibition of glycolysis. The small increase in G6P with high ryanodine could indicate an inhibitory effect of ryanodine on glycolysis that could also contribute to the energy imbalance.

While procaine blocks theophylline-induced depletion of energy stores [23] as well as many other contracture and subcontracture-induced effects [18, 19], 1 mM procaine had little or no effect on ryanodine-induced energy depletion. Local anesthetics have been shown to block ryanodine-induced as well as caffeine-induced stimulation of oxygen consumption and contractures [11, 22]. G6P levels are not as high as with ryanodine alone and are not statistically different from normal muscle values. The reason for the significantly larger Cr_T value in the procaine-plus-ryanodine-exposed muscles is unknown. As indicated earlier, it probably is not the result of decreased muscle volume. Dry–wet weight ratios have not been measured for procaine-plus-ryanodine-treated muscles. The inability of 1 mM procaine to prevent ryanodine-induced energy depletion was seen in both the presence and the absence of theophylline. Bianchi [11] blocked ryanodine-induced stimulation of oxygen consumption with 2.5 mM benzocaine. Perhaps, unlike theophylline or caffeine depletion, ryanodine depletion is more resistant to local anesthetics, and procaine levels higher than 1 mM would be needed to prevent ryanodine depletion. Further experiments would be needed to resolve this discrepancy. While methylxanthines and ryanodine and other modulators act on the same receptor molecule but not the same site [3], there are suggestions that local anesthetics also act on the same receptor but not at the same site as methylxanthines or ryanodine. Klein *et al.* [26] reported that procaine non-specifically blocks the SR calcium release channel, while Garcia *et al.* [47], working with frog muscles, concluded that ryanodine and tetracaine may act on the same process but not at the same site. Even though energy depletion is greater when both theophylline and ryanodine are present, the fact that procaine blocks the depletion of theophylline but not that of ryanodine supports different sites of action for methylxanthines and ryanodine [3, 33].

In whole cells and tissues, it is likely that the effects of ryanodine may not be limited to effects on the SR calcium release channel alone. Increasing $[Ca]_i$ also affects energy production. Lee *et al.* [48] suggested that, in frog muscle, the following are also consequences of increasing cytoplasmic calcium: activation of enzymes increasing muscle metabolism, stimulation of protease activity (causing structural damage), energy uncoupling and structural damage of mitochondria. Calcium buffers may be overwhelmed [49, 50] although over the range of 5 nM to 2 μM ryanodine is reported to have no effect on calcium movements across mitochondrial membranes [12] nor to directly affect mitochondrial respiration [51]. Its stimulatory effect on oxygen uptake is most likely secondary to increased cytoplasmic calcium levels [52].

It is clear that ryanodine can affect energy stores in amphibian muscle and that ryanodine contractures can be a consequence of energy depletion rigor. Subcontracture levels of the methylxanthines caffeine and theophylline also deplete energy stores in amphibian muscle. Methylxanthines facilitate ryanodine depletion and vice versa. Thus, the combined effects of these two agents on energy balance are additive, not antagonistic. The ability of procaine to prevent methylxanthine- but not ryanodine-induced depletion in amphibian muscle is disturbing but could be a consequence of the need for the SR calcium release channels to be opened before ryanodine binding occurs [33]. Methylxanthines open the channel, but local anesthetics such as procaine usually can prevent the channel from being opened by theophylline. In the presence of ryanodine, though, the action of procaine may be prevented. How this occurs requires further investigation.

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