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Differential effects of *tert*-butyl-benzohydroquinone, a putative SR Ca^{2+} pump inhibitor, on isometric relaxation during the staircase in the rabbit and rat ventricle

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1 The effects of 2,5 di-(*tert*-butyl)-1,4-benzohydroquinone (TBQ), a putative inhibitor of the sarcoplasmic reticulum Ca^{2+} pump, on mechanical relaxation and contraction-relaxation coupling have been studied at different frequencies (0.5-3 Hz) in isometrically contracting isolated right ventricular preparations of rabbit and rat at 37°C. Two types of mechanical responses have been studied: the twitch tension and the force transient (rewarming spike, RSp) following a rapid cooling contracture (RCC, an index of sarcoplasmic reticulum Ca^{2+} content) on return to 37°C.

2 The coupling between contraction and relaxation was assessed by two methods: (a) by evaluation of the variation of the slope relating the maximal rate of tension fall to twitch peak tension; (b) by modelling the twitch according to the following equation: $TwT\ (t) = C \times (t/A)^B \times \exp(1-(t/A^B)$ where TwT(t) is the time course of isometric tension, t is time, C and A are an inotropic and a chronotropic index respectively and B, a contraction-relaxation coupling index (Nwasokwa, 1993).

3 In the rabbit ventricle, 30 μM TBQ did not prevent the frequency-induced shortening of the twitch time to half-relaxation ($t_{1/2}$) and of the time constant (τ) describing the final part of the RSp relaxation (τ decreased from 140 ms (0.5 Hz) to 133 ms (3 Hz) in control and from 253 ms (0.5 Hz) to 197 ms (3 Hz) after exposure to TBQ). By contrast, at a given frequency, the prolongation of relaxation induced by TBQ was proportional to its inotropic effect (unchanged slopes and B values) but TBQ did not prevent the acceleration of relaxation observed at high frequencies: B increased from 2.02 (0.5 Hz) to a peak value of 2.18 (1 Hz) in control and from 1.88 (0.5 Hz) to a maximum of 2.48 (2 Hz) after TBQ exposure. TBQ significantly attenuated the decay of RCCs elicited after increasingly longer periods of muscle quiescence as normally observed in control conditions.

4 In the rat ventricle, TBQ depressed relaxation more than expected on the basis of its negative inotropic effects (B decreased from 2.16 to 1.84 at 0.5 Hz and from 2.15 to 1.66 at 3 Hz). TBQ also slowed the rate of RSp relaxation (τ increased from 95 ms to 168 ms at 0.5 Hz, and from 109 ms to 149 ms at 3 Hz) and increased twitch $t_{1/2}$. By contrast with the results obtained in the rabbit ventricle, B, τ and $t_{1/2}$ were frequency-insensitive whether or not TBQ was present.

5 TBQ exerts negative inotropic effects consistent with inhibition of the SR Ca^{2+} pump. In the rabbit ventricle, the TBQ-induced potentiation of relaxation acceleration at high pacing frequencies suggests the involvement of counteracting Ca^{2+}-mediated mechanisms probably via Ca^{2+}-calmodulin-activated kinases. In the rat ventricle, TBQ did not have any differential effect on relaxation depending on the frequency, probably because the extent of the negative staircase was small in the present experimental conditions.

Keywords: 2,5 Di-(*tert*-butyl)-1,4-benzohydroquinone, rabbit and rat, ventricle; relaxation; contraction-relaxation coupling; modelling; rapid cooling contractures; force-frequency relationship

Introduction

The force-frequency relationship is a fundamental property of the mammalian heart. The staircase phenomenon occurs in physiological conditions, like exercise, and in pathological conditions, like tachyarrhythmias. In most mammalian species, the staircase is positive i.e. tension rises with increasing frequencies, with the notable exception of the rat ventricle, which exhibits a negative staircase. The cellular mechanism involved in this inotropic response is still the topic of numerous studies (Lewartowski & Pytkowski, 1987; Bers, 1991) which have pointed out that Ca^{2+} uptake by the sarcoplasmic reticulum (SR) is essential for the occurrence of the staircase (Bers, 1991). Definite experimental evidence for this hypothesis has lagged because of the absence of specific SR Ca^{2+} pump inhibitors. The recent introduction of such compounds (Inesi & Sagara, 1994) like thapsigargin (TG), cyclopiazonic acid (CPA) or 2,5 di-(*tert*-butyl)-1,4-benzohydroquinone (TBQ) has enabled testing of this hypothesis. Thus CPA (10 μM) and TBQ (30 μM) attenuate the positive staircase of the guinea-pig atrium (Yard *et al.*, 1994) and the rabbit ventricle (Baudet *et al.*, 1996) respectively. Similarly, TBQ blunted the negative staircase in the rat ventricle (Baudet *et al.*, 1995).

The wealth of data concerning the inotropic effect of high pacing frequencies strongly contrasts with the paucity of knowledge about the cellular mechanisms involved in mechanical relaxation. In fact, the occurrence of the positive staircase relies on an increased fraction of Ca^{2+} released from the SR (Frampton *et al.*, 1991; Siri *et al.*, 1991) which is itself due to progressive Ca^{2+} loading of the SR with increasing frequencies (Frampton *et al.*, 1991; Gwathmey & Morgan, 1993). Therefore, the amount of Ca^{2+} that has to be sequestered by the SR increases whereas the time available between beats decreases; logically, relaxation should be hampered. However, in isolated ventricular preparations (Gwathmey & Morgan, 1993; Hasenfuss & Just, 1994) and *in situ* hearts (Freeman *et al.*, 1987; Kambayashi *et al.*, 1992), relaxation is either unchanged or actually *faster*. Hitherto this paradoxical

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improvement of relaxation has not been satisfactorily explained. One hypothesis is that SR Ca^{2+} pump activity is increased although the exact mechanisms remain obscure (Schouten, 1990). A second mechanism could involve the contraction-relaxation coupling (CRC) process (Chemla *et al.*, 1986; Eichhorn *et al.*, 1992). Indeed, isolated muscle studies have shown that the various parameters characterizing contraction and relaxation were correlated (Tamiya *et al.*, 1977; Chemla *et al.*, 1986). In fact, the higher the Ca^{2+} transient, the faster its rate of decline (Bers & Berlin, 1995). It is therefore conceivable that an increased contraction is somehow coupled to a faster relaxation, by a mechanism that could involve an acceleration of SR Ca^{2+} uptake. To our knowledge, the importance of the CRC process in the positive staircase has not been studied.

A better understanding of cellular mechanisms involved in faster mechanical relaxation at high frequencies is not only of fundamental importance but has also clinical relevance. In fact, slowing of isovolumic relaxation is one symptom of diastolic dysfunction encountered in pressure-overload hypertrophy, heart failure or idiopathic cardiomyopathies (Hasenfuss & Just, 1994). Although, at rest, slowing of relaxation may be asymptomatic, diastolic dysfunction may be revealed by exercise or atrial pacing, either experimental or pathological (tachyarrhythmias) (Feldman *et al.*, 1988; Fujii *et al.*, 1988; 1993). Experiments have shown that despite a preserved or blunted positive staircase (Fujii *et al.*, 1988; Schwinger *et al.*, 1992; Liu *et al.*, 1993; Hasenfuss *et al.*, 1994; Schmidt *et al.*, 1994), relaxation was impaired as assessed by the prolongation of the time constant of the final phase of relaxation and the elevation of end-diastolic pressure (Fujii *et al.*, 1988; Liu *et al.*, 1993). The worsening of relaxation abnormalities in the diseased heart is frequently explained by reduced Ca^{2+} uptake capacities of the SR, because of a decreased expression of the SR Ca^{2+} pump protein (Hasenfuss & Just, 1994).

Consequently, the present work has relied on acute exposure of isolated rabbit and rat ventricular muscles to TBQ, a putative SR Ca^{2+} pump inhibitor. With this compound, several points have been addressed. Firstly, the Ca^{2+} uptake inhibiting activity of TBQ has been studied by rapidly cooling the preparations to test SR Ca^{2+} content (Bridge, 1986; Bers & Bridge, 1989; Baudet *et al.*, 1993) and from the relaxation of the tension spike following the rewarming of the preparation (Bers & Bridge, 1989; Baudet *et al.*, 1993). Secondly, the effect of TBQ on the relaxation of the isometric twitch during the staircase has been investigated. Specifically, we have studied the importance of the CRC process by using a classical approach that studies the correlation between twitch tension and the rate of relaxation (Péry-Man *et al.*, 1993a,b). This study has also been an opportunity to test the validity of a model based on a global analysis of the twitch (Nwasokwa, 1993; 1994). Our data have shown profound differences between the rabbit and the rat right ventricle, in the intrinsic effect of TBQ on mechanical relaxation and its frequency-dependence.

Methods

Most of the methods concerning muscle handling, superfusion and temperature jumps have been detailed in the companion paper (Baudet *et al.*, 1996) and will be briefly recounted here.

Muscle handling and superfusion

Thin papillary muscles or trabeculae were excised from the right ventricle of anaesthetized New Zealand white rabbits or Wistar rats, mounted in 20 mM butane-dione monoxime (BDM)-containing Tyrode and equilibrated at L_{max} for 30 min at 0.5 Hz. Switching between Tyrode solution at either 0°C or 37°C (to trigger rapid cooling contractures (RCCs) and rewarming spikes) was performed at the bath inlet with solenoid pinch valves.

Solutions

The modified Tyrode solution used for muscle dissection and superfusion contained (mM): NaCl 125, KCl 6, $MgCl_2$ 1, $CaCl_2$ 2, glucose 5, Na pyruvate 10, ascorbic acid 1, HEPES 10, bupranolol 0.0003. pH was adjusted at pH 7.4 at 37°C with NaOH and solutions were gassed with 100% O_2. TBQ, prepared as a stock solution in dimethylsulphoxide (DMSO) at 10^{-1} M, was used at a working concentration of 30 μM (Baudet *et al.*, 1996).

Experimental protocol

Rabbit preparations: after equilibration, the twitch staircase phenomenon was studied at 0.5, 1, 2 and 3 Hz and back at 0.5 Hz. RCCs were elicited in place of a steady-state twitch at a given frequency. On readmission of Tyrode solution at 37°C, a rewarming spike (RSp) occurred and the stimulation was resumed at a new frequency until a new steady-state was reached. The protocol of post-rest RCCs allows indirect investigation of the role of SR Ca^{2+} uptake and Ca^{2+} efflux through the sarcolemmal Na^+/Ca^{2+} in handling Ca^{2+} leaking from the SR during rest (Bers, 1991). For this purpose, an RCC was triggered after periods of muscle quiescence between 2 and 600 s. After completion of both the force-frequency and the post-rest RCCs protocol in control conditions, 30 μM TBQ was applied until a new twitch tension was reached. Then a similar protocol was carried out in the presence of TBQ.

Rat preparations: for twitches (both in control and TBQ), the protocol was similar to the rabbit. However, RCCs elicited in rat ventricular muscle are a complex contractile response that encompasses a twitch and an RCC, yielding a 'spiky' response in which relaxation occurs during the cold (Busselen *et al.*, 1991). Therefore, by contrast with rabbit RCCs which reach a steady amplitude within 20–25 s, peak amplitude of rat 'RCCs' cannot be considered as reflecting only SR Ca^{2+} content. However, RSp relaxation was analysed to study the mechanisms involved in reducing Ca^{2+} previously released during the cooling period.

Data analysis

Time course parameters: thermoprobe and transducer outputs were initally recorded on a strip chart recorder and later with Mac Lab (v. 3.3.5; AD Instruments, Phymep, France) running on a Macintosh Quadra 650. Eight twitches were sampled at 1 kHz and averaged; RCCs were sampled at 200 Hz. Twitch tension was expressed in mN mm^{-2} and classical indices of twitch time course were determined (time to peak tension: TTP, time to half-relaxation: $t_{1/2}$; maximal rates of tension rise: $+dT/dt_{max}$ and fall: $-dT/dt_{max}$). RCC amplitude, expressed in mN mm^{-2}, was measured as the difference between the plateau tension (reached in $\sim$20 s) and resting tension. Rewarming spike tension, in mN mm^{-2}, was the difference between peak and resting tensions and its relaxation was assessed by non-linearly fitting ('Sigmaplot' software; v. 4.1; Jandel Scientific, CA, U.S.A.) a mono-exponential function (described by a time constant τ) through data points between the time to maximal rate of relaxation ($-dT/dt_{max}$) and resting tension (Pagel *et al.*, 1994; Vittone *et al.*, 1994).

Contraction-relaxation coupling (CRC): a first approach to assess CRC in isometrically contracting muscles has consisted in studying the correlation between twitch tension and $-dT/dt_{max}$. Indeed, the slope of this relationship has been used as an index of the intrinsic relaxing capabilities of the muscle in isometric conditions (Tamiya *et al.*, 1977; Péry-Man *et al.*, 1993a,b). Any manoeuvre (application of TBQ or increase in the stimulation frequency) that decreases the slope of this correlation exerts an 'intrinsic' negative relaxant effect.

Nwasokwa's model: according to the three-parameters (A, B and C) model recently introduced by Nwasokwa (1993; 1994), any experimental intervention inducing a change in B indicates an intrinsic relaxant effect, i.e. the change in relaxation is greater than expected from modifications of contractility. This model was used to test whether changes in relaxation brought about by TBQ were entirely dependent on changes in contraction. For this purpose, a complete twitch time course was fitted non-linearly (Marquardt-Levenberg algorithm) according to

$$\mathrm{TwT}\ (t) = \mathrm{C} \times (t/\mathrm{A})^{\mathrm{B}} \times \exp(1 - (t/\mathrm{A})^{\mathrm{B}})$$

where TwT (t) is twitch developed tension at time t, C reflects the inotropic state (analogous to the peak tension), A, the chronotropic state (analogous to the time to peak tension) and B, a supposedly contractility-independent index of relaxation. By contrast with classical indices of contraction and relaxation ($+dT/dt_{max}$ and $-dT/dt_{max}$) determined at a single time point during the twitch, A, B and C contain information collected from the totality of the twitch.

Statistical analysis

Data are expressed as means ± s.e.mean and statistical analysis has been carried out as in the companion paper (Baudet *et al.*, 1996). The significance level was set at 5% level. If the two-way analysis of variance revealed a significant treatment effect, the P value for pairwise vertical comparisons was corrected with the Dunn-Sidãk procedure (Ludbrook, 1994). The slopes of the $-dT/dt_{max}$ *vs* twitch tension relationship were compared by a paired t test (Zar, 1984).

Results

Contraction-relaxation coupling in rabbit ventricular muscle

Figure 1 illustrates the relationship between twitch tension and $-dT/dt_{max}$ at various frequencies, both in the absence and in the presence of 30 μM TBQ.

There was a highly significant correlation between both parameters as already observed in isolated preparations from other mammalian species (Tamiya *et al.*, 1977; Péry-Man *et al.*, 1993a,b). By contrast, in control and during exposure to TBQ, there was no such correlation between twitch tension and $t_{1/2}$ (not shown). At a given frequency between 0.5 and 3 Hz, the slopes of the relationship were not different, whether or not TBQ was present. There was a trend for steepening of the slope with increasing frequencies but lack of data precluded any statistical analysis. With this approach, these results suggest that, in isometric conditions, TBQ does not exhibit any intrinsic negative relaxing effect whatever the frequency, i.e. changes in relaxation are entirely coupled to changes in contraction.

In Figure 2, the fit according to Nwasokwa's global model has been superimposed on the representative traces of the 0.5 and 2 Hz twitches, in control conditions and in the presence of 30 μM TBQ.

The fit was slightly shifted to the left at the beginning of relaxation whereas a rightward shift was observed at the end. This trend has been reported in the *in situ*, blood-perfused canine papillary muscle (Nwasokwa, 1993; 1994). A consistent observation was the general improvement of the fit in the presence of TBQ. Both parameters A and C were perfectly correlated with their counterparts, i.e. the time to peak tension (which decreases at higher frequencies; Baudet *et al.*, 1996) and peak twitch tension (not shown). Table 1 summarises the frequency-dependence of $t_{1/2}$ and B.

$t_{1/2}$ decreased regularly as frequency augmented, according to a pattern that was not modified by TBQ. B was significantly augmented with increasing frequencies ($P<0.0001$) suggesting

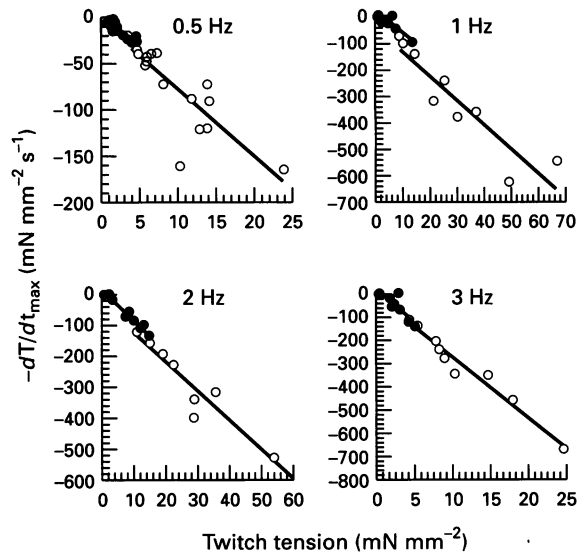

Figure 1 Relationship between maximum rate of tension fall ($-dT/dt_{max}$) and isometric twitch peak tension in rabbit ventricular preparations ($n=10$) stimulated at the indicated frequencies (○: control; ●: 30 μM TBQ). Data points were fitted by linear regression and the statistical differences in the slopes (S) at a given frequency were tested with a paired Student's t-test (NS: non significant). At 0.5 Hz, S = −7.11 s^{-1} (○, $r=0.839$, $P<0.0001$) and −6.08 s^{-1} (●, $r=0.911$, $P<0.0001$; slope difference: NS); at 1 Hz, S = 9.12 s^{-1} (○, $r=0.918$, $P=0.0005$) and −7.13 s^{-1} (●, $r=0.896$, $P=0.011$; slope difference: NS); at 2 Hz, S = −9.22 s^{-1} (○, $r=0.967$, $P<0.0001$) and −9.56 s^{-1} (●, $r=0.989$, $P<0.0001$; slope difference: NS); at 3 Hz, S = −10.7 s^{-1} (○, $r=0.982$, $P<0.0001$) and −11.6 s^{-1} (●, $r=0.861$, $P=0.028$; slope difference: NS). TBQ did not modify the slope of the relationship at any frequency.

that relaxation was more potentiated than contraction by high pacing rates. Furthermore the highly significant interaction ($P<0.0001$) indicated that TBQ potentiated the intrinsic relaxant effect induced by high pacing frequencies.

Both approaches of CRC suggest that high stimulation frequencies potentiate relaxation, independently of their positive inotropic effect and that TBQ potentiates this intrinsic relaxant effect.

Rewarming spike relaxation: influence of stimulation frequency and TBQ

Figure 3a illustrates the method used to analyse the relaxation of the RSp following an RCC elicited instead of a twitch at 0.5 Hz.

The time constant τ of the final phase of the RSp was consistently increased in the presence of TBQ, from 146 ± 4 ms ($n=77$) in control to 318 ± 14 ms ($n=88$; $P<0.0001$) whereas mean RCC amplitude decreased from 6.4 ± 0.7 mN mm^{-2} ($n=77$) in control to 3.6 ± 0.5 mN mm^{-2} after TBQ exposure ($n=88$; $P=0.0012$). Before concluding that the slowing of the RSp relaxation was indeed due to TBQ, we checked whether this effect could not simply be explained by the lower level of tension developed during the cold exposure in the presence of TBQ. Such a possible correlation between τ and maximal amplitude reached during the RCC was investigated (Figure 3b). A range of different RCC amplitudes was obtained with the post-rest RCCs protocol (see Methods section). There was no correlation between both parameters regardless of the presence of TBQ and for any given muscle, τ was consistently increased compared to control. Therefore, the slowed RSp relaxation in the presence of TBQ was independent of the level of the preceding RCC and can be interpreted in terms of slower myoplasmic Ca^{2+} reduction.

The positive staircase in the rabbit ventricle relies on increased SR Ca^{2+} content both in control conditions and in the

presence of TBQ although this compound blunted the staircase (Baudet *et al.*, 1995). If τ is indeed independent of the RCC amplitude, then increasing the frequency should not affect RSp relaxation except if higher pacing rates promote mechanisms that alter relaxation. Table 1 shows that TBQ slowed relaxation at all frequencies but did not differentially (no interaction effect) alter the acceleration of RSp relaxation with increasing pacing rates. Therefore, in rabbit ventricular muscle, high stimulation frequencies promote faster mechanical relaxation.

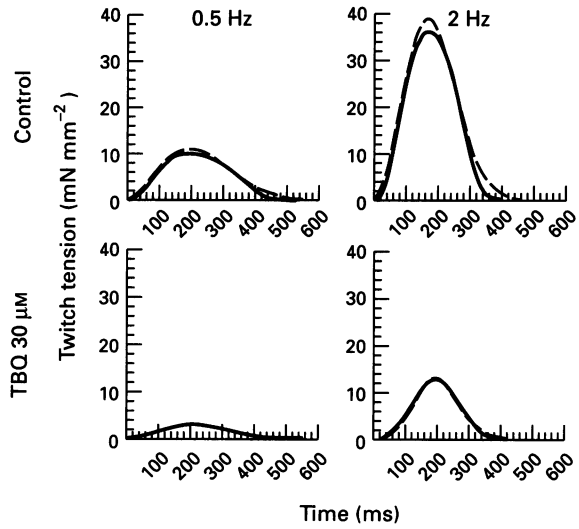

Figure 2 Frequency-dependence of TBQ-induced modifications of the twitch time course. (a) Representative twitch traces (continuous line) in control (upper panels) and in the presence of 30 μM TBQ (lower panels), at 0.5 Hz and 2 Hz. On each experimental trace is superimposed the fit (dashed line) according to Nwasokwa's equation (see Methods). The fitting parameters were: A = 194 ms, B = 1.77, C = 11.1 mN mm^{-2} (0.5 Hz, control) and A = 205 ms, B = 1.88, C = 2.8 mN mm^{-2} (0.5 Hz, TBQ); A = 168 ms, B = 2.11, C = 38.4 mN mm^{-2} (2 Hz, control) and A = 191 ms, B = 2.20, C = 12.8 mN mm^{-2} (2 Hz, TBQ). Correspondingly, TTP = 197 ms, peak tension = 10.2 mN mm^{-2} (0.5 Hz, control) and TTP = 212 ms, peak tension = 2.6 mN mm^{-2} (0.5 Hz, TBQ); TTP = 172 ms, peak tension = 35.7 mN mm^{-2} (2 Hz, control) and TTP = 197 ms, peak tension = 12.7 mN mm^{-2} (2 Hz, TBQ). Apart from its negative inotropic effect, 30 μM TBQ did not prevent the increase in parameter B at higher frequencies. Exposure to TBQ consistently improved the fit.

Post-rest RCCs

Figure 4a shows representative traces of RCCs elicited 2, 120 and 600 s of quiescence after a twitch at 0.5 Hz.

In control conditions, RCC amplitude decreases regularly with increasing periods of quiescence whereas this rest decay of RCCs appeared slower in the presence of 30 μM TBQ. Mean data are presented in Figure 4b. The absence of any TBQ effect was probably due to the high variability of RCC amplitudes in TBQ-treated muscles but the significant interaction term ($P<0.0001$) indicated that TBQ significantly attenuated the post-rest decay of RCCs observed in control conditions.

Contraction-relaxation coupling in rat ventricular muscle

The coupling between contraction and relaxation in rat ventricular preparations ($n=6$) was investigated with the relationship between twitch tension and $-dT/dt_{max}$ (Table 2). Except the significantly shallower slope after TBQ exposure at 0.5 Hz, no such difference was found at higher frequencies. Moreover, there was no obvious trend for any frequency-induced changes in slopes.

As with the rabbit ventricle, in control and TBQ, there was no correlation between twitch tension and $t_{1/2}$ (not shown). Curve fitting according to Nwasokwa's model of twitches at different frequencies was qualitatively similar to the rabbit, i.e. exposure to TBQ improved the fit (not shown). Table 3 shows that increasing the stimulation frequency slightly shortened the relaxation phase (decreased $t_{1/2}$), an effect that was not altered by TBQ although $t_{1/2}$ was increased by the inhibitor at each frequency.

Nwasokwa's analysis showed that the pattern of modifications of B induced by high frequencies and TBQ clearly contrasted with the rabbit. Thus the negative inotropic effect of increasing frequencies was accompanied by proportional depression of relaxation, the latter being worsened by TBQ in a frequency-independent fashion.

Rewarming spike relaxation: influence of stimulation frequency and TBQ

As previously described in the rabbit ventricle, relaxation of the RSp of rat RCCs has been analysed in the same way as in the rabbit ventricle. The control τ value (88 ± 4 ms; $n=39$) was lower than in the rabbit (~150 ms). As RCCs in the rat spontaneously relax during the cold (Busselen *et al.*, 1991), the correlation between τ and a range of steady-state RCC amplitudes was studied by rewarming the preparation at different times during cold exposure. τ did not depend on previous RCC amplitude and TBQ significantly prolonged relaxation as τ increased to 168 ± 6 ms ($n=44$; $P<0.0001$) for almost similar levels of developed tension before rewarming (10.3 ± 1.5 mN mm^{-2} in control ($n=39$) and 7.9 ± 1.0 mN mm^{-2} after TBQ exposure, $n=44$; NS).

Table 1 Interaction of TBQ treatment and pacing frequency on relaxation indices of the twitch and the rewarming spike in rabbit right ventricular muscle ($n=9$)

Frequency (Hz)				*Two-way ANOVA* (P)		
0.5	1	2	3	*Frequency*	*Treatment*	*Interaction*
Twitch time to half-relaxation (ms)						
109 ± 7	95 ± 6	81 ± 5	72 ± 5			
119 ± 3	103 ± 4	89 ± 5	79 ± 3	$P<0.0001$	NS	NS
Parameter B						
2.01 ± 0.06	2.18 ± 0.04	2.14 ± 0.03	2.06 ± 0.04			
1.88 ± 0.05	2.26 ± 0.13	2.49 ± 0.16	2.28 ± 0.13	$P<0.0001$	NS	$P<0.0001$
Time constant of rewarming spike relaxation (ms)						
140 ± 11	132 ± 11	120 ± 12	133 ± 24			
253 ± 31	228 ± 31	175 ± 16	197 ± 27	$P=0.002$	$P<0.01$	NS

Table 3 shows that, within the range of frequencies studied (0.5–3 Hz), TBQ did not significantly affect τ nor was this parameter frequency-dependent. However, the borderline significant interaction ($P=0.044$) was probably due to the fact that, at high frequencies, τ slightly increased in control whereas it decreased in the presence of TBQ. Again the results contrast with observations from the rabbit ventricle.

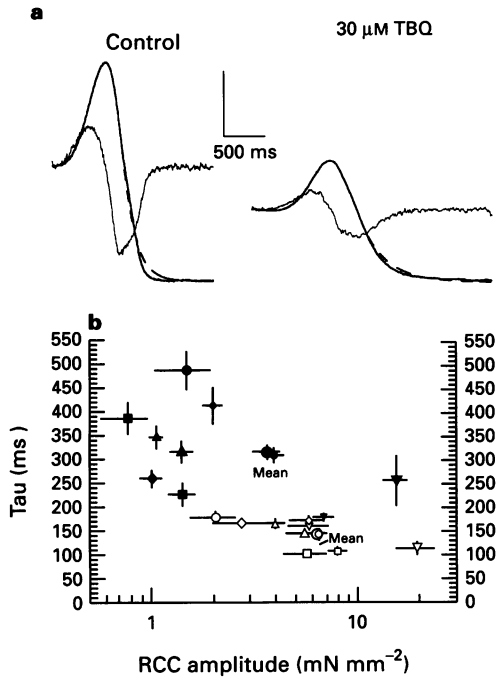

Figure 3 Effect of 30 μM TBQ on the relationship between rewarming spike (RSp) relaxation and the amplitude of the rapid cooling contracture (RCC) elicited in place of a steady-state 0.5 Hz twitch in rabbit ventricular preparations. (a) Representative traces (same muscle) of the experimental RSp (thick continuous line) and rate of tension change (thin noisy line). The part of the relaxation between the time of $-dT/dt_{max}$ and the baseline was fitted according to a monoexponential function (thick dashed line). The corresponding time constant τ is 145 ms in control and 304 ms after TBQ exposure. Vertical bar is 4 mN mm^{-2} or 75 mN mm^{-2} s^{-1}. (b) Relationship between steady-state RCC amplitude (note the logarithmic scale) and τ value of the corresponding RSp ($n=10$): (○) control; (●) 30 μM TBQ. Data (mean ± s.e.mean) are presented as different-sized symbols corresponding to individual muscles to assess the effect of TBQ in each experiment. The bigger circles ('mean') indicate the averaging of all data in a given group. Rewarming spike relaxation was slowed by TBQ (τ increased by 118%) but was independent of its preceding RCC amplitude.

Discussion

The interaction between TBQ application and changes in the stimulation frequency on SR Ca^{2+} content and contraction-relaxation coupling in isometric conditions has been studied in rabbit and rat right ventricular muscle. Analysis of post-rest RCCs and RSp relaxation adds to previous experimental evidence (Baudet *et al.*, 1996) showing that TBQ decreases the SR Ca^{2+} load by inhibiting the SR Ca^{2+} ATPase. In the rabbit ventricle, both approaches to coupling between contraction and relaxation showed that the TBQ-induced prolongation of relaxation was entirely coupled to its inotropic effect. Moreover, Nwasokwa's analysis suggests that increasing the stimulation frequency may accelerate relaxation more than expected from its positive inotropic effect and that TBQ potentiates this improvement. In the rat ventricle, results were less straightforward depending on the method used to analyse

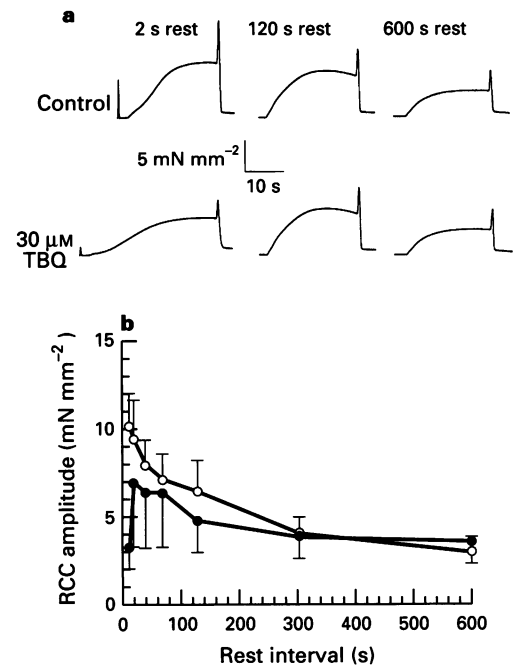

Figure 4 Effects of 30 μM TBQ on post-rest RCCs in rabbit ventricular preparations. (a) Experimental records (same muscle) of RCCs elicited in control (upper traces) and after 30 μM TBQ exposure (lower traces) after the indicated periods of quiescence. (b) Average data (mean ± s.e.mean) of the absolute amplitude of post-rest RCCs in control (○, $n=10$) and 30 μM TBQ (●, $n=6$) Two-way ANOVA revealed no TBQ effect but a highly significant effect of rest ($P<0.0001$) and interaction ($P=0.0163$).

Table 2 Interaction of TBQ treatment and pacing frequency on relaxation indices of the twitch and the rewarming spike in rat right ventricular muscle ($n=6$)

Frequency (Hz)				*Two-way ANOVA* (P)		
0.5	1	2	3	*Frequency*	*Treatment*	*Interaction*
Twitch time to half-relaxation (ms)						
51 ± 4	52 ± 3	52 ± 3	48 ± 3			
73 ± 3	73 ± 1	72 ± 3	68 ± 2	$P<0.05$	$P<0.001$	NS
Parameter B						
2.01 ± 0.06	2.18 ± 0.04	2.14 ± 0.03	2.06 ± 0.04			
1.88 ± 0.05	2.26 ± 0.13	2.49 ± 0.16	2.28 ± 0.13	NS	$P<0.001$	NS
Time constant of rewarming spike relaxation (ms)						
95 ± 9	98 ± 23	100 ± 17	109 ± 16			
168 ± 26	167 ± 27	145 ± 23	149 ± 23	NS	NS	$P<0.05$

Table 3 Effects of frequency and TBQ on contraction-relaxation coupling in rat right ventricular muscle ($n=6$)

	Control			*TBQ* (30 μM)			
Frequency (Hz)	*Slope* (s^{-1})	r	P	*Slope* (s^{-1})	r	P	P'
0.5	−13.8	0.979	0.003	−8.96	0.786	<0.0001	0.0005
1	−11.6	0.990	0.006	−8.44	0.934	0.001	NS
2	−12.0	0.978	0.003	−13.0	0.987	0.001	NS
3	−14.1	0.996	0.002	−12.0	0.968	<0.0001	NS

Slope: slope of the correlation between the maximal rate of relaxation ($-dT/dt_{max}$) and twitch tension. *r*: correlation coefficient. *P*: level of statistical significance of the regression at a given frequency. *P'*: level of statistical significance for comparisons of the slopes.

the CRC process. Thus the relationship between tension and $-dT/dt_{max}$ suggests that the TBQ-induced slowing of relaxation is entirely coupled to its negative inotropic effects, except at 0.5 Hz whereas Nwasokwa's analysis indicates that relaxation was more prolonged that twitch tension was decreased.

Additional evidence for TBQ-induced inhibition of SR Ca^{2+} uptake

The experimental approaches used in the present study support our previous work (Baudet *et al.*, 1996) which shows that TBQ is an effective inhibitor of the SR Ca^{2+} pump. Thus the rest decay of RCCs in rabbit ventricular muscle, currently accounted for by the progressive Ca^{2+} unloading of the SR due to SR Ca^{2+} leak, is abolished by TBQ, similarly to previous observations with other SR Ca^{2+} uptake inhibitors like TG or CPA (Baudet *et al.*, 1993). In their presence, the fraction of Ca^{2+} that leaks during quiescence cannot be pumped back as efficiently inside the SR and is therefore extruded in the extracellular medium via the sarcolemmal Na^+/Ca^{2+} exchanger (Bassani & Bers, 1994). A second insight into the mode of action of TBQ arises from the analysis of the RSp. Despite the complex relationship between intracellular Ca^{2+} and tension in intact muscle (Backx *et al.*, 1995), the rate of RSp relaxation has been considered as an indirect means to assess the respective involvement of the Ca^{2+} pump and the Na^+/Ca^{2+} exchanger in intracellular Ca^{2+} reduction (Bers & Bridge, 1989). In fact RCCs induce a sort of 'Ca^{2+}-clamp' situation such that during rewarming, a better correspondence between the Ca^{2+} and the tension signals may allow more straightforward inferences of Ca^{2+} regulation from tension (Naqvi & MacLeod, 1994).

Fitting the final part of the RSp signal with a mono-exponential function, from the time of $-dT/dt_{max}$ and estimating the relaxation rate by the time constant (τ) value, has been classically carried out in the analysis of ventricular relaxation in the *in vivo* or *ex-vivo* heart (Pagel *et al.*, 1994) and in isolated preparations (Vittone *et al.*, 1994). Although previous authors have used $t_{1/2}$ of the RSp (Bers & Bridge, 1989; Baudet *et al.*, 1993), this parameter is determined at a single time point which may be less informative than τ. The analysis of τ raised several interesting points. On one hand, in control conditions, relaxation of the RSp was faster in the rat than in the rabbit ventricle, a result which fits with the well-known faster rate of SR Ca^{2+} uptake in the rat ventricle (Hove-Madsen & Bers, 1993; Bassani *et al.*, 1994). On the other hand, τ was independent of RCC amplitude within a broad range as reported in previous studies assessing RSp relaxation with $t_{1/2}$ (Bers & Bridge, 1989; Baudet *et al.*, 1993). The independence of τ from RCC amplitude was surprising at first as a high RCC amplitude (and hence, intracellular Ca^{2+}) should have placed a heavier Ca^{2+} load on the various Ca^{2+} transporters, requiring more time for Ca^{2+} extrusion. However Ca^{2+} transport by the SR Ca^{2+} pump, the major mechanism for RSp relaxation in the rabbit (Bers & Bridge, 1989) is non-linear (Hill coefficient: 2; Bers & Berlin, 1995). Therefore, it is likely that during rewarming, intracellular Ca^{2+} reaches baseline well before tension. Data from cell shortening and fluorescence signals during rewarming in guinea-pig myocytes support this view (Naqvi & MacLeod, 1994). The prolongation of RSp relaxation and global improvement of the fit consistently observed after TBQ exposure suggests a better correspondence between intracellular Ca^{2+} and tension fall. This hypothesis is supported by data from TG-treated guinea-pig myocytes (Naqvi & MacLeod, 1994) or CPA-exposed rat ventricular trabeculae (Backx *et al.*, 1995). In these conditions, the relaxation phase would become more dependent on the degree of SR Ca^{2+} pump inhibition, which would account for the slowing of the RSp relaxation in both species.

Contraction-relaxation coupling

To date, no method of studying the relationship between contraction and relaxation has gained widespread acceptance (Brutsaert & Sys, 1989; Vittone *et al.*, 1994) because of the absence of in-depth testing of the models by specific experimental interventions that alter contraction and/or relaxation at discrete steps. For this reason, we preferred to analyse our data with two different approaches. Studies of the CRC process intend to determine whether changes in relaxation induced by protocols affecting inotropism are, in some way, proportional to variations in twitch tension (Tamiya *et al.*, 1977; Chemla *et al.*, 1986). If coupling varies, then interventions are said to exert an 'intrinsic' relaxant effect, i.e. changes in relaxation are greater than expected from modifications of contractility (Clergue *et al.*, 1990; Péry-Man *et al.*, 1993a,b). In the present work, CRC has been studied from the slope of the relationship between $-dT/dt_{max}$ and the corresponding peak isometric twitch tension. Variation of steepness are assumed to indicate an intrinsic relaxant effect (Tamiya *et al.*, 1977, Péry-Man *et al.*, 1993a,b). Thus, both in the rabbit and the rat ventricle, TBQ (30 μM) did not change the slope nor did 10 μM CPA in the rat ventricle (Péry-Man *et al.*, 1993a,b). These results are interpreted within the concept of Ca^{2+} detachment from the myofilaments being the limiting factor of isometric relaxation (Brutsaert & Sys, 1989). In fact, only compounds or protocols modifying myofilament Ca^{2+} sensitivity are expected to modify the slope (Péry-Man *et al.*, 1993b) although, to the best of our knowledge, this hypothesis has never been rigorously tested with myofilament Ca^{2+} (de) sensitizers. Another limitation to this interpretation is the fact that this approach relates two indices of contraction determined at single time points which may exclude some information gathered from the whole twitch. The recently introduced global model of Nwasokwa (1993, 1994) has not been extensively characterized but may implement the 'slope' method because it takes into account data from the whole twitch envelope. This model also shows good correspondence between calculated and experimentally determined indices of isolated muscle contractility like peak tension (parameter C), TTP (parameter A), $t_{1/2}$, $+dT/dt_{max}$ and $-dT/dt_{max}$ (Nwasokwa, 1993). In this model, B is considered as a coupling parameter: it increases with dobutamine (Nwasokwa, 1993) suggesting that acceleration of relaxation is greater than the positive inotropic effect, possibly indicating the well-known Ca^{2+} desensitization brought up by β-agonists (Bers, 1991). We are aware that one obvious limitation of the

present model is the difficulty of interpreting modifications of the parameters in terms of intracellular [Ca^{2+}] because of the highly non-linear relationship between Ca^{2+} and tension. Clearly, simultaneous measurement of tension and intracellular [Ca^{2+}] in multicellular preparations (Backx *et al.*, 1995) will test the validity of this model.

Nwasokwa's analysis of twitches from the rabbit and rat ventricle yielded opposite results: whereas in the rat, TBQ depressed relaxation more than expected on the sole basis of its negative inotropic effect, this was not observed in the rabbit. In the rabbit ventricle, but not in the rat, high frequencies potentiated more relaxation than tension. This improvement of relaxation was potentiated by TBQ suggesting an involvement of the SR Ca^{2+} handling in this phenomenon.

In the rabbit ventricle, the proportional depression of contraction and relaxation by TBQ may be explained by the fact that the lower SR Ca^{2+} content in TBQ-treated muscle would lead to a lower amount of SR-released Ca^{2+}. The Ca^{2+} transporters involved in relaxation (Ca^{2+} pump and Na^+/Ca^{2+} exchanger) will not be as stimulated as in control conditions and will reduce myoplasmic Ca^{2+} at a slower rate, accounting for the slowing of relaxation. The absence of further depression of relaxation despite SR Ca^{2+} pump inhibition by TBQ is probably explained by the shift in Ca^{2+} regulation towards the Na^+/Ca^{2+} exchanger (Bassani *et al.*, 1994). By contrast, in the rat ventricle, such a compensation by the exchanger is not as efficient as in the rabbit ventricle (Bers, 1991; Bassani *et al.*, 1994) leading to more marked depression of relaxation compared to contractility, probably accounting for the decrease in B. Disproportionate depression of relaxation compared to contraction by TBQ in the rat ventricle contrasts with the absence of effect of CPA when assessed by the slope method (Péry-Man *et al.*, 1993a,b). These authors concluded that the proportional depression of contraction and relaxation supported the idea that CPA did not affect myofilament Ca^{2+} sensitivity. However, it is noteworthy that the negative inotropic effect induced by 10 μM CPA was small (13%) suggesting a modest inhibition of SR Ca^{2+} pumps, leaving an important reverse of unblocked pumps able to cope with SR-released Ca^{2+}.

Interestingly, in the rabbit but not in the rat ventricle, high frequencies promoted faster relaxation than expected from their sole effect on twitch tension, an effect that was potentiated by TBQ. This phenomenon is also observed when the twitch is assessed by $t_{1/2}$ in isolated muscle preparations from species exhibiting a positive staircase like the ferret ventricle (Gwathmey & Morgan, 1993), the guinea-pig atrium (Yard *et al.*, 1994) and the human ventricle (Hasenfuss & Just, 1994). In fact, increasing the pacing frequency loads the SR with Ca^{2+}, enhances SR-released Ca^{2+}, accounting for the positive inotropic effect (Bers, 1991; Baudet *et al.*, 1996). However, the decreased diastolic interval should leave less time for Ca^{2+} to be extruded and impairment of relaxation should occur whereas it actually improved. In the *in situ* contracting papillary muscles, B did not change between 1.7 and 2.5 Hz (Nwasokwa, 1994). Differences in preparations (*in situ vs in vitro* papillary muscle), neurohormonal input or narrower range of frequencies may explain such discrepancies.

The potentiation of the frequency-induced relaxant effect by TBQ suggests that SR Ca^{2+} handling is involved in this mechanism, probably involving SR Ca^{2+} uptake and Ca^{2+}-mediated phenomena. Experimental evidence has been recently provided by Bassani *et al.* (1995) who showed that an increase in time-averaged Ca^{2+} in isolated myocytes paced at 1 Hz at 22°C stimulated the Ca^{2+}-calmodulin (CaM) kinase II (CaMKII), accelerated SR Ca^{2+} uptake and hastened relaxation. The two latter effects were abolished by pretreatment with TG at a concentration known to inhibit fully all the Ca^{2+} pumps. By contrast, in our conditions, TBQ, at a concentration (30 μM) that did not inhibit all the pumps (Baudet *et al.*, 1996), potentiated the positive relaxant effect. This potentiation suggests that in control conditions, acceleration of relaxation at high frequencies may actually involve two counteracting mechanisms: a 'positively' relaxing (SR Ca^{2+} uptake activation) and a 'negatively' relaxing mechanism yet to be defined. We suggest that the latter could be Ca^{2+}-CaM-mediated phosphorylation of the myosin light chains (MLC) by the specific MLC kinase, known to increase myofilament Ca^{2+} sensitivity (Sweeney *et al.*, 1993) which would slow relaxation. Within this framework, we suggest the following mechanism for the TBQ-induced potentiation. Despite the lower amount of SR-released Ca^{2+} in TBQ-treated muscle, and consequent smaller activation of CaMKII and MLCK, the pumps unblocked by TBQ could still be activated by CaMKII, accounting for a remaining positive relaxant effect. Assuming that MLCK requires a higher Ca^{2+} threshold than CaMKII to be activated by the Ca^{2+}-CaM complex (which may be the case in the heart, Sweeney *et al.*, 1993), MLC phosphorylation will no longer counteract the remaining potentiation of SR Ca^{2+} uptake. Additional indirect evidence for long-lasting effects that may involve phosphorylations is provided by analysis of RSp relaxation at various frequencies. Indeed TBQ potentiated the acceleration of RSp relaxation with increasing frequencies. Cooling the muscle will prevent dephosphorylation of CaMKII and MLCK target sites and of the autophosphorylated CaMKII and MLCK, an already slow phenomenon at physiological temperatures (Braun & Schulman, 1995).

In the rat ventricle, the absence of an intrinsic relaxant effect by frequency is likely to be explained by the small extent of negative staircase, whether or not TBQ was present (Baudet *et al*, 1996). This suggests that the Ca^{2+}-mediated consequences of high stimulation frequencies proposed above for the rabbit ventricle do not apply in the rat.

Potential clinical relevance

In the pressure-overloaded and/or failing ventricle, the positive relaxant response of high pacing frequencies is blunted or even reversed to negative and often accompanied by increases in end-diastolic tension or pressure (Feldman *et al.*, 1988; Fujii *et al.*, 1993). Although these changes have been attributed to a reduction of SR Ca^{2+} uptake, our results with TBQ (which yields the same *functional* effects) and CPA in the guinea-pig atrium (Yard *et al.*, 1994) suggest that additional mechanisms based on deficient Ca^{2+}-calmodulin-mediated phosphorylations may also be deficient in these pathological occurrences. This latter consideration is supported by recent data reporting a decreased expression of CaM mRNA in the failing human heart (Jeck *et al.*, 1994).

In conclusion, TBQ has differential effects on the contraction-relaxation coupling process in the rabbit and the rat right ventricle. Despite evidence that TBQ inhibits SR Ca^{2+} uptake in both species, the fact that the slowing of relaxation remains proportional to the negative inotropic effect of TBQ is consistent with the view that Ca^{2+} efflux can still occur through the Na^+/Ca^{2+} exchanger. In the rat right ventricle, as such the compensation is not as efficient and may explain the disproportional slowing of relaxation compared to the TBQ-induced negative inotropic effect. The frequency-dependent improvement of relaxation in the rabbit ventricle may be mediated by Ca^{2+}-calmodulin-mediated processes.

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