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Alterations in the force-frequency relationship by *tert*-butylbenzohydroquinone, a putative SR Ca^{2+} pump inhibitor, in rabbit and rat ventricular muscle

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1 The effects of 2,5 di-(*tert*-butyl)-1,4-benzohydroquinone (TBQ), a putative inhibitor of the sarcoplasmic reticulum (SR) Ca^{2+} pump, on twitch tension, time course and SR Ca^{2+} content have been studied at different stimulation frequencies (0.5–3 Hz) in isolated preparations from the rabbit and rat right ventricle, at 37°C.

2 At 0.5Hz, 30 μM TBQ induced a marked negative inotropic effect in both species (−57% in the rabbit and −68% in the rat) and decreased the rate of rise and fall of twitch tension. In parallel, SR Ca^{2+} content (assessed by rapid cooling contractures) was depressed in the rabbit by 42%. The force-frequency relationship (positive for the rabbit and negative for the rat) was significantly attenuated. In the rabbit, this alteration was shown to rely on insufficient SR Ca^{2+} reloading with increasing frequencies.

3 Exposure of TBQ-treated preparations to 8 mM extracellular Ca^{2+} or 5 μM isoprenaline were effective in reloading the SR with Ca^{2+} whereas 20 mM caffeine emptied this compartment.

4 In the rabbit ventricle, increase in stimulation frequency shortened control twitch time course by decreasing both the time to peak tension (TTP) and the time to half relaxation ($t_{1/2}$). TBQ did not differentially affect the pattern for $t_{1/2}$ but significantly attenuated the frequency-induced decrease of TTP.

5 In rabbit ventricular muscle, the action potential duration increased between 0.5 and 3 Hz whether or not TBQ was present. However, TBQ induced a small but significant additional action potential shortening.

6 TBQ decreased twitch tension in the rat ventricle between 0.5 and 3 Hz but the negative staircase was not differentially affected by the SR Ca^{2+} pump inhibitor. In control conditions and in the presence of 30 μM TBQ, $t_{1/2}$ was frequency-independent but TBQ consistently increased this parameter (by ~29%).

7 These data argue in favour of a specific and partial inhibition of the SR Ca^{2+} pump by 30 μM TBQ in the rabbit and rat ventricle and emphasise the importance of SR Ca^{2+} uptake in the force-frequency phenomenon.

Keywords: 2,5 di-(*tert*-butyl)-1,4-benzohydroquinone; rabbit and rat ventricle; inotropic effect; sarcoplasmic reticulum Ca^{2+} pump; rapid cooling contractures; force-frequency relationship

Introduction

The sarcoplasmic reticulum (SR) plays a dual role in the physiology of the cardiac cell: (a) it provides most of the calcium (Ca^{2+}) that triggers the contraction by releasing Ca^{2+} through the ryanodine-sensitive Ca^{2+} channel, via the Ca^{2+}-induced Ca^{2+} release mechanism and (b) it reduces myoplasmic Ca^{2+} through Ca^{2+} ATPase, a fraction of this Ca^{2+} being then released to activate the next contraction (Bers, 1991; Barry & Bridge, 1993). It has also been shown recently that Ca^{2+} crossing the sarcolemma, via the Ca^{2+} current $I_{Ca,L}$ during the action potential, was buffered by the SR but this effect was transient, as Ca^{2+} could be released during the *same* action potential (Janczewski & Lakatta, 1993). Therefore, the SR Ca^{2+} pump is crucial for both the contractile phase (by replenishing the SR with Ca^{2+}) and the mechanical relaxion (although, on a beat-to-beat basis, the sarcolemmal sodium/calcium (Na^+/Ca^{2+}) exchanger is also involved in this latter phase; Bers, 1991; Bassani *et al.*, 1994).

For a long time, the lack of specific inhibitors of SR Ca^{2+} ATPase has precluded detailed investigations of its role in the excitation-contraction (EC) coupling process. Ryanodine and caffeine, both Ca^{2+}-release channel opening drugs, have been used as surrogates, because their final effect on the SR is similar to the one expected from SR Ca^{2+} pump inhibitors, i.e. unloading of SR Ca^{2+}. However, neither caffeine nor ryanodine prevents Ca^{2+} uptake, so that the SR is still able to buffer, although very transiently, myoplasmic Ca^{2+} (Bers, 1991). Besides the physiological interest of SR Ca^{2+} ATPase inhibitors, their use could be a pharmacological model of experimental and clinical cardiac pathologies, such as hypertrophy or heart failure. In fact, defective SR Ca^{2+} uptake would account for the *in vitro* systolic and/or diastolic alterations encountered in preparations from these diseased hearts (Arai *et al.*, 1994). Therefore, Ca^{2+} pump inhibitors represent a unique opportunity to test whether this *sole* decrease of SR Ca^{2+} uptake accounts for all the contractile modifications, despite the fact that the *in vivo* pathophysiological responses are expected to be much more complex.

More specifically, the force-frequency relationship (staircase) is a fundamental property of cardiac muscle from which has emerged the concept that proper SR Ca^{2+} uptake was critical for the inotropic effect and also for adequate mechanical relaxation between beats (Lewartowski & Pytkowski, 1987). The absence of Ca^{2+} pump inhibitors has not allowed this hypothesis to be tested, despite its clinical interest, as it has

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been shown that the staircase was severely blunted or even negative in human heart failure which normally exhibits a positive force-frequency relationship (Hasenfuss *et al.*, 1994; Schmidt *et al.*, 1994).

The recent introduction of thapsigargin (TG) and cyclopiazonic acid (CPA) as selective Ca^{2+} pump inhibitors has brought new insights in the EC coupling process of cardiac myocytes (Barry & Bridge, 1993; Negretti *et al.*, 1993). In fact, it has been shown that both compounds had a negative inotropic effect in most animal species, which could be expected on the basis of a decreased SR Ca^{2+} content (Bassani *et al.*, 1993; 1994; Lewartowski *et al.*, 1994). However, paradoxical results have also been reported which showed that the shortening of TG- or CPA-treated ventricular myocytes from rabbit or guinea-pig had similar amplitude but a longer time course compared to control, despite an empty SR (Bassani *et al.*, 1994; Lewartowski *et al.*, 1994). This effect has been interpreted as the result of the suppression of the rapid SR buffering of incoming Ca^{2+} which could then directly activate the contractile proteins, although at a slower rate compared to the rate at which Ca^{2+} binds to the myofibrillar proteins when it is released from the SR (Janczewski & Lakatta, 1993). In multicellular preparations, the effects of these putative Ca^{2+} pump inhibitors have yielded more disappointing results as concentrations of TG or CPA at least 10 times higher (>50 μM) than in isolated cells have to be used to record significant, and sometimes modest, changes in inotropism and variable effects on SR Ca^{2+} content (Baudet *et al.*, 1993; Péry-Man *et al.*, 1993; Yard *et al.*, 1994). The use of high concentrations of hydrophobic molecules could provoke membrane partitioning, non-specific binding and possible non-selective effects (Baudet *et al.*, 1993; Du *et al.*, 1994; Yard *et al.*, 1994). Regarding the staircase phenomenon, Yard *et al.* (1994) have reported that application of 30 μM CPA attenuated the positive force-frequency relationship of the guinea-pig atrium, but these data may not be extrapolated to the ventricle, nor did this study investigate the Ca^{2+} load of the SR. Our own experience with TG and CPA in rabbit ventricle (Baudet *et al.*, 1993) has discouraged our investigation of the staircase phenomenon with these compounds.

Recently, 2,5 di-(*tert*-butyl)-1,4-benzohydroquinone (TBQ) has been proposed as another Ca^{2+} pump inhibitor (for review, see Inesi & Sagara, 1994) but its effects on isolated cardiac muscle have not yet been reported. We have therefore studied the effects of TBQ on the force-frequency relationship and focused our experiments on contractility, SR Ca^{2+} content (assessed by rapid cooling contractures (RCCs); Bers, 1989; 1991) and action potential in rabbit isolated ventricular muscle, which exhibits a positive staircase. A comparison of some contractile responses has also been undertaken with rat ventricular preparations, because of their negative staircase and stronger dependence on SR Ca^{2+} handling for EC coupling (Bers, 1991).

Methods

Mechanical experiments

Muscle preparations Thin papillary muscles or trabeculae were dissected from the right ventricle of New Zealand white rabbits (length: 4.26 ± 0.22 mm; cross-sectional area: 0.289 ± 0.059 mm^2, $n=17$) or Wistar rats (length: 3.62 ± 0.54 mm; cross-sectional area: 0.240 ± 0.042 mm^2, $n=6$) have been used for this study. The animals were previously anaesthetized with sodium pentobarbitone (40 mg kg^{-1}, i.v.) for rabbits or with ether followed by exsanguination for rats. After excision, the heart was cannulated and the coronary system was flushed with a modified Tyrode solution (see composition below) to which was added 20 mM butane-dione monoxime (BDM) to uncouple excitation from contraction (Sellin & McArdle, 1994). After cardiac arrest and absence of blood in the effluent, the heart was placed in a dissecting dish, in oxygenated 20 mM BDM-Tyrode at 37°C. Two preparations were dissected and rapidly transferred to a 'mounting' bath containing 20 mM BDM-Tyrode. Each end of the muscle was snared in a loop of hair, sliding inside stainless steel tubes (200 μm i.d.). One tube was fixed on a rigid arm and the other, on a more compliant rod on which was glued a small copper disc, which served as a target for a displacement measurement system (Kaman KD 7000, Le Groupe Scientifique, France). This transducer assembly, which had a linear response up to 40 mN, was mounted on a micromanipulator, which allowed the rapid transfer of the preparation between the mounting bath and the adjacent superfusing bath.

Muscle diameter and length were measured by means of a micrometer in the binocular and the cross-sectional area was calculated assuming a cylindrical shape.

Solution superfusion The experimental solutions fed the superfusing bath by gravity at a flow rate of 60 ml min^{-1}. They were maintained at 37°C by water jacketing or at −4°C by jacketing with engine cooler. Solenoid pinch valves (NR Research, Bioblock, France), located at the bath inlet, were gated by a lab-built switcher, allowing synchronised solution changes. At this flow rate, the output of a thermoprobe (thermocouple T, response time at 63%: 10 ms) located at the vicinity of the muscle detected a $t_{90\%}$ change of temperature in less than 1 s. A peristaltic pump (Masterflex, Bioblock, France) allowed recirculation of the 'cold' solution and the solution coming out of the superfusion bath. Manifolds allowed this latter solution to be sent either to the waste or recirculated back to the flask. Preliminary experiments showed that recirculation did not alter muscle viability or the pattern of contractile responses nor did it accelerate rundown of the preparations.

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, *N*-2-hydroxyethyl-piperazine-N′-2-ethanesulphonic acid (HEPES) 10. pH was adjusted to 7.4 at 37°C with NaOH and solutions were gassed with 100% O_2. Bupranolol (3×10^{-7} M), a β-adrenoceptor antagonist, was added to avoid potential effects of released endogenous catecholamines.

A stock solution (10^{-1} M) of 2,5 di-(*tert*-butyl)-1,4-benzohydroquinone (TBQ) was made up in dimethyl sulphoxide (DMSO). It was kept in the cold room and diluted at the required concentration as needed. As quinones are hydrophobic substances, we tested their solubility in Tyrode solution; 10 μM TBQ provoked a slight flocculent precipitate; at 30 μM, the solution became faintly opalescent, but pH was unaltered; however, the actual concentration was less than nominal. Preliminary experiments ($n=3$) showed that the negative inotropic effect became prominent at concentrations above 10 μM. As 100 μM TBQ solutions were frankly opalescent and we were concerned about the possibility of non-specific effects of TBQ, all experiments reported in this paper were conducted with 30 μM TBQ. At this concentration, DMSO content was 0.03%, a concentration that is devoid of any inotropic effect. In some experiments, extracellular Ca^{2+} concentration was increased to 8 mM by adding $CaCl_2$ from a 1 M stock solution (BDH Analar grade, Poole, England) or 5 μM isoprenaline was added from a 10^{-2} M stock soloution with 1% ascorbic acid, kept from light and oxidation in a cold room. This concentration of isoprenaline was high enough to antagonize the action of bupranolol. In a few experiments, caffeine (20 mM) was added as a solid, directly into the Tyrode solution. All compounds were obtained from Sigma (France) except TBQ (Aldrich, France).

Experimental protocol

Muscle stabilization Muscles were field-stimulated with platinum electrodes located along the walls of the bath, at a basal frequency of 0.5 Hz. One interesting observation during washout of BDM was the fast recovery of twitch force and

equilibration. Thus, the first twitches reappeared after 3 to 5 s of superfusion with BDM-free Tyrode solution. The muscles were also very compliant so that stretching smoothly increased baseline tension. The muscle was then stretched to a length (L_{max}) at which developed tension (total minus resting tension) was maximal, which occurred within 30 min.

Force-frequency relationship After equilibration, a force-frequency relationship was constructed by increasing the basal pacing frequency from 0.5 to 3 Hz. At each frequency, twitch tension had to reach a steady amplitude before eliciting a RCC. In rabbits, a relevant observation was a rundown of the twitch elicited back at 0.5 Hz after the protocol. Thus, the 'return' 0.5 Hz twitch tension was decreased by ~40% compared to the initial 0.5 Hz for reasons that may be linked to transient calcium overload (Kitakaze *et al.*, 1988) induced by high frequencies and massive calcium release during RCCs. Therefore, initial and return 0.5 Hz tensions were averaged and tensions developed at 1, 2 and 3 Hz were normalised to this mean value. Afterwards, another set of experiments (post-rest RCCs, see accompanying paper) was performed, which provoked an additional rundown of twitch tension. TBQ was then applied so that the term 'pre-TBQ', in Figure 1 and in the text, refers to the level of tension developed at the end of the post-rest RCCs protocol. When steady-state twitches were developed in the presence of 30 μM TBQ, the same force-frequency protocol and same normalization procedure to the mean at 0.5 Hz was used.

The situation was somewhat different for RCCs in rat preparations. In fact, it is clear that RCCs elicited in rat ventricular muscle are a complex contractile response that encompasses a twitch and a RCC, yielding a spiky response whose relaxation occurs during the cold (Busselen *et al.*, 1991). Therefore, 'RCCs' in the rat cannot be considered as only reflecting SR Ca^{2+} content and were not therefore quantitatively analysed.

Data analysis Thermoprobe and transducer outputs were initially recorded on a strip chart recorder (Recorder 320, W & W, Basel, Switzerland) (7 rabbit experiments) and later recorded with the softwares 'Chart' (temperature and RCCs) and 'Scope' (twitches) included in the Mac Lab package (v. 3.3.5; AD Instruments, Phymep, France) running on a Macintosh Quadra 650 (10 rabbits experiments and 6 rat experiments). Twitches were sampled at 1 kHz and RCCs were sampled at 200 Hz. Eight twitches were averaged to obtain a correct signal-to-noise ratio. Twitch tension was expressed in mN mm^{-2} and its time course was characterized by the time to peak tension (TTP) and time from peak tension to half-relaxation ($t_{½}$). The maximal rates of tension rise ($+dT/dt_{max}$) and fall ($-dT/dt_{max}$) were obtained by digital differentiation of the twitch, in the 'Scope' software. RCC amplitude (in mN mm^{-2}) was measured as the difference between the plateau tension (reached in ~20 s) and resting tension.

Electrophysiological experiments

Action potential (AP) measurements were performed in another subset of rabbit ventricular papillary muscle or trabeculae ($n=5$). Muscles were excised in BDM-Tyrode, placed in a Lucite chamber and superfused with BDM-free Tyrode (37°C) for 30 min. Solutions were pumped through the chamber at a rate of 12 ml min^{-1}, providing at least three changes in chamber content per minute. During this equilibration period, the muscle was stimulated at 0.5 Hz, using square-wave pulses 0.5–1 ms in duration and 20% above the threshold in intensity delivered through Teflon-coated bipolar silver wires. The preparation was impaled with a 3 M KCl-filled glass capillary microelectrode (tip diameter less than 1 μM, resistance of 10 to 25 MOhms). The muscle chamber was connected to ground through a 3 M KCl Ag-AgCl junction. The electrodes were coupled by a 3 M KCl interface to an Ag-AgCl electrode which led to an amplifer with a high input impedance and input capacity neutralisation (Biologic VF-102). Output was displayed on a digital storage oscilloscope (Gould 1604) and the 'Scope' option of the MacLab software. A single AP was captured at a sampling frequency of 2 kHz.

This paper describes experiments where a single continuous impalement could be obtained in the absence and in the presence of 30 μM TBQ. APs were then recorded from 0.5 to 3 Hz and back to 0.5 Hz. The maximum diastolic potential was measured as well as AP duration at 30 (APD_{30}), 60% (APD_{60}) and 90%(APD_{90}) of full repolarization.

Statistical analysis Data are expressed as means ± s.e.mean. Comparisons for a single parameter between control and TBQ-treated preparations (Figure 1a) were performed by use of a Student's two-tailed, paired t test. In this case, differences were considered significant at $P<0.05$. The effects of stimulation frequency on contractile tension, time course parameters or AP characteristics (Figure 4, 5 and 7) were tested with a two-way ANOVA for repeated measurements, with one within-factor (frequency) and one between-factor (control *vs* TBQ). If the F-test for the treatment group was significant, pairwise vertical comparisons based on Student's paired t test were performed at a given frequency. To avoid excessive type I errors, the P value was corrected with the Dunn-Sidāk procedure (Ludbrook, 1994) in which P', the modified P value is: $P'=1-(1-P)^k$. As four frequencies were studied, $k=4$. The slopes of the RCC *vs* twitch tension (Figure 3) relationship were compared by Student's paired t test (difference in regression slopes divided by the standard error of regression slopes) (Zar, 1984).

Results

Effects of TBQ (30 μM) on the twitch and RCC of rabbit ventricular muscle paced at 0.5 Hz

The amplitude of steady-state twitches and RCCs elicited in control conditions (0.5 Hz, 37°C, 2 mM Ca^{2+}) and after exposure to 30 μM TBQ are presented in Figure 1a.

The developed tension of twitches elicited just before the application of 30 μM TBQ ('pre TBQ') was smaller by 57% than the one at the end of the equilibration period ('initial'), indicating rundown of twitch tension, known to be fast at 37°C. In contrast, rundown of RCCs was very small (−14 ± 5%). Application of 30 μM TBQ induced a pronounced negative inotropic effect (−55 ± 5%, $n=17$, $P<0.01$) the time course of which is presented in Figure 1b in a representative muscle. At 37°C, steady-state depression of twitch tension was reached within 30 min, depending on muscle size. This negative inotropic effect was partly due to SR Ca^{2+} depletion as shown by the smaller amplitude of the steady-state RCC. Representative twitches and RCCs on an expanded time scale are shown in Figure 2.

The fact that the RCC was decreased by ~42% but not completely abolished by 30 μM TBQ also suggests that SR Ca^{2+} uptake depression was partial. Compared to the control twitch, the steady-state 30 μM TBQ twitch time course was prolonged, mostly because of a lengthening of the relaxation phase. Indeed, TTP was unchanged (182 ± 6 ms in control and 184 ± 5 ms in TBQ) but $t_{½}$ significantly increased from 108 ± 5 ms to 134 ± 6 ms ($P<0.05$). TBQ effects were not reversible.

During this series of experiments, as illustrated in Figure 1, we noticed some variability in twitch and RCC tensions. We therefore investigated whether both types of contractile responses were correlated and if TBQ altered this relationship. Figure 3 shows that there is indeed a strong correlation in control conditions and in the presence of 30 μM TBQ.

Nevertheless this compound decreases the steepness of the slope indicating that for the same RCC amplitude (and presumably, a similar SR Ca^{2+} load), twitch tension was lower in

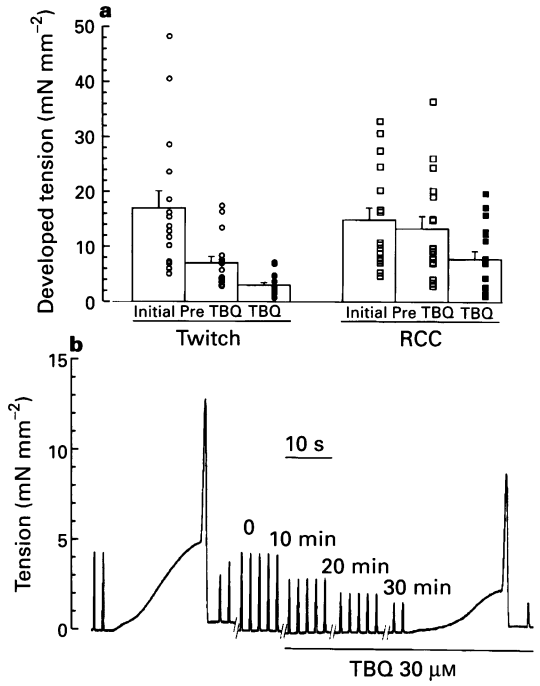

Figure 1 Effect of 30 μM TBQ on twitch and RCC tension in rabbit ventricular muscle. (a) Steady-state data ($n=17$). Individual data are represented by (□, ○) for control and (■, ●) for TBQ. Columns represent mean with s.e.mean. 'Initial' refers to the tension measured before the force-frequency protocol, 'Pre TBQ' refers to the tension level just before application of 30 μM TBQ and 'TBQ' to steady-state tension in the presence of 30 μM TBQ. TBQ significantly decreased twitch tension by 57% ($P<0.001$ *vs* pre TBQ; paired *t* test) and RCC by 42% ($P<0.0001$ *vs* pre TBQ; *t* test). (b) Time course of 30 μM TBQ negative inotropic effect in a representative muscle. The fast phasic responses rising from baseline tension are twitches. The RCC is the slow increase in diastolic tension induced by rapidly cooling the muscle to 0°C. The spike at the end of the RCC is induced by rewarming the preparation to 37°C (rewarming spike). The steady negative inotropic effect of TBQ was reached within 30 min.

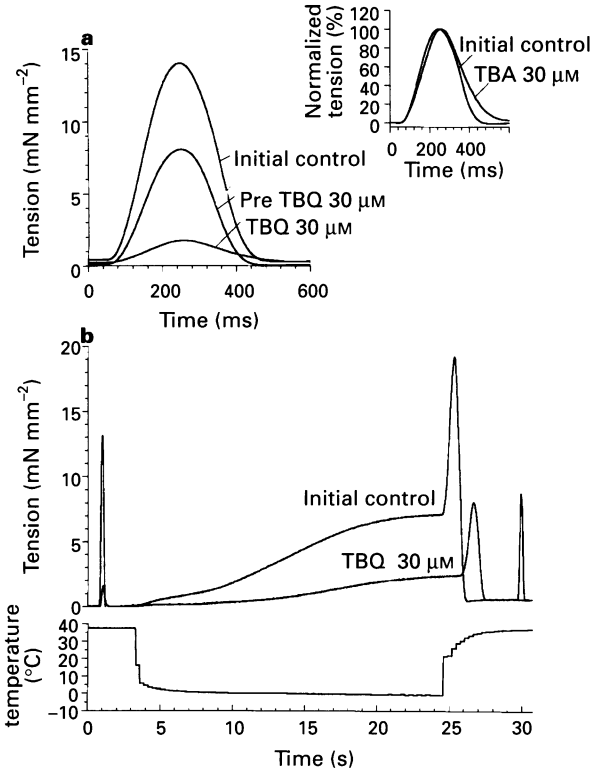

Figure 2 Representative traces of steady-state twitches (a) and RCCs (b) in control conditions and in the continuous presence of 30 μM TBQ, in rabbit ventricular muscle. (a) The strong negative inotropic effect of TBQ was accompanied by a prolongation of the twitch time course, mainly due to slower relaxation. This point is more clearly presented in the inset in which the initial and 30 μM TBQ twitches have been normalised to their respective maximal amplitude. Time to peak tension was unchanged. (b) *Upper panel*: representative effect of TBQ on the RCC elicited 2 s after a steady-state twitch. The steady RCC was decreased in this example by 70%. The rewarming spike in the presence of TBQ is smaller because of the lower RCC amplitude. *Lower panel*: time course of bath temperature changes (the trace applies for the initial control RCC) recorded with a miniature thermoprobe; 90% of the cooling step was reached within 1 s whereas the increase in resting tension was much delayed.

the presence of TBQ, suggesting that this quinone may affect twitch tension by other mechanisms unrelated to a lower SR Ca^{2+} content, possibly the action potential (see below).

Effects of TBQ (30 μM) on the frequency-dependence of twitch tension and time course in rabbit ventricular muscle

Twitch tension TBQ did not prevent the positive staircase usually observed in the rabbit ventricle (Figure 4)

In control conditions twitch tension increased sharply between 0.5 and 1 Hz and then reached a plateau (Figure 4a). After application of TBQ, the positive staircase was blunted but still present. The significant interaction indicated that TBQ differentially affected the positive staircase because of the smoother staircase between 0.5 and 2 Hz in its presence.

The positive staircase was accompanied by increases in $+dT/dt_{max}$ (Figure 4c) and $-dT/dt_{max}$ (Figure 4d) in the absence and in the presence of TBQ with a pattern similar to twitch tension. TBQ attenuated the frequency-induced acceleration of contraction and relaxation as shown by the significant interaction.

Increasing the stimulus frequency abbreviated twitch duration (Figures 4e and 4f). Control TTP and $t_{½}$ monotonically decreased by ~34%. This decrease in time course was significantly blunted by 30 μM TBQ with a stronger interaction for TTP ($P<0.0001$) than for $t_{½}$ ($P<0.02$). This was due to the less steeper decrease of TTP compared to the roughly parallel decrease in $t_{½}$.

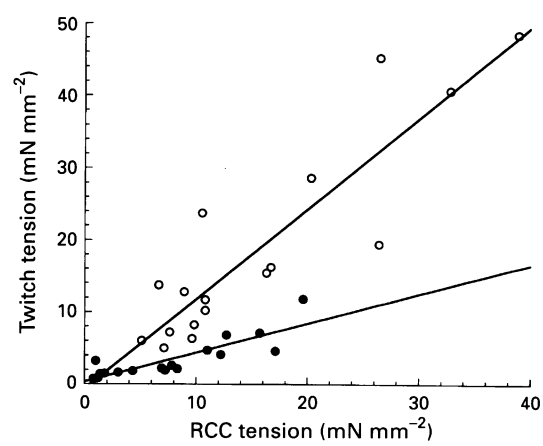

Figure 3 Relationship between the twitch elicited at 0.5 Hz and the steady-state RCC elicited in place of this steady-state twitch in the absence (○) and in the presence (●) of 30 μM TBQ in rabbit ventricular muscle ($n=17$). Measurements of twitches and RCCs in control conditions were performed at the end of the equilibration period. Twitches and RCCs were correlated both in control (slope: 1.247; $r=0.989$; $P<0.0001$) and in the presence of 30 μM TBQ (slope: 0.405; $r=0.857$; $P<0.0001$). TBQ treatment significantly decreased the slope value ($P<0.01$).

RCC The positive twitch staircase in the rabbit ventricle is paralleled by a frequency-induced increase in SR Ca^{2+} content (assessed by RCCs; Figure 4b and Bers, 1989) but 30 μM TBQ decreased SR Ca^{2+} load at all frequencies. The pattern of loading was slightly dissimilar (smoother increase) which accounts for the significantly different (although borderline) interaction term ($P=0.043$).

Although it would be tempting to attribute the smaller twitch tension at all frequencies in the presence of TBQ only to the smaller SR Ca^{2+} content, such a comparison is not valid

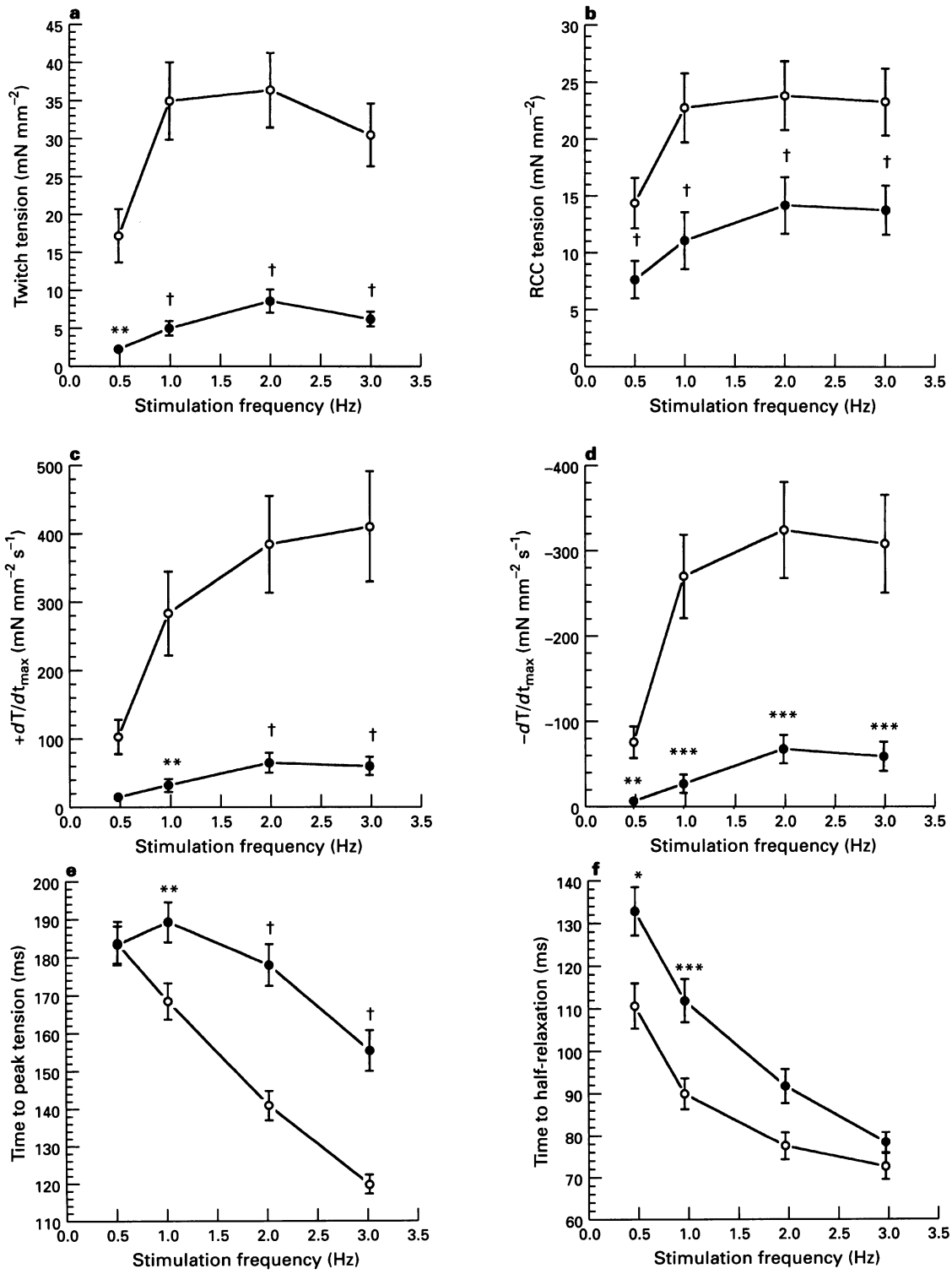

Figure 4 Effects of 30 μM TBQ on the frequency-induced changes in twitch (a) and RCC (b) tension, maximal rate of tension rise (c), maximal rate of tension decline (d), time to peak tension (e) and time to half-relaxation (f) in rabbit ventricular muscle ($n=17$, except for (c) and (d) where $n=10$). (○) Control; (●) in the presence of 30 μM TBQ. Data are mean ± s.e.mean. TBQ attenuated the frequency-induced acceleration of the twitch time course and increase in SR Ca^{2+} loading (assessed by RCCs) normally observed in control conditions. It delayed the decrease in time to peak tension but did not prevent the shortening of the relaxation phase. Two-way ANOVA revealed a significant interaction ($P<0.001$ for (a), (b), (c), (d), (e) and $P=0.0125$ for (f)). *$P<0.05$; **$P<0.02$; ***$P<0.01$; †$P<0.001$ *vs* control.

mainly because of the different mechanisms that trigger and modulate twitches (source of activating Ca^{2+}, different release mechanisms and Ca^{2+}/tension relationship at 37°C and 0°C; Harrison & Bers, 1990). It can simply be concluded that a fraction of the blunted positive twitch staircase relies on smaller Ca^{2+} reloading capacity of higher frequencies in the presence of 30 μM TBQ. However, other mechanisms could not be excluded such as modification of the AP characteristics.

Effects of TBQ (30 μM) on the frequency-dependence of AP characteristics in rabbit ventricular muscle

Figure 5 summarises the effects of frequency and 30 μM TBQ on membrane diastolic potential and AP duration at various levels of repolarization.

Increasing the stimulus frequency between 0.5 and 3 Hz depolarized the membrane (Figure 5a), a pattern that was not modified by 30 μM TBQ. Moreover, on return to 0.5 Hz, the diastolic membrane potential was not significantly different from the initial 0.5 Hz value. The major prolongation of the AP durations intervened between 0.5 and 1 Hz, an effect which may be involved in the strong positive inotropic effect in this frequency range (see Figure 4a). Representative APs at 0.5 and 3 Hz, both in the absence and in the presence of 30 μM TBQ, are displayed in Figure 5e.

TBQ shortened AP duration but this effect started to be significant from APD_{30} but remained small ($P=0.048$ for APD_{30}, 0.036 for APD_{60} and 0.042 for APD_{90}). Importantly, the absence of a significant interaction indicated that TBQ did not alter the frequency-induced prolongation of APDs. Therefore, although APD shortening may be involved in the negative inotropic effect of TBQ and progressive SR Ca^{2+} unloading, it is unlikely that this accounts quantitatively for the dramatic twitch decrease.

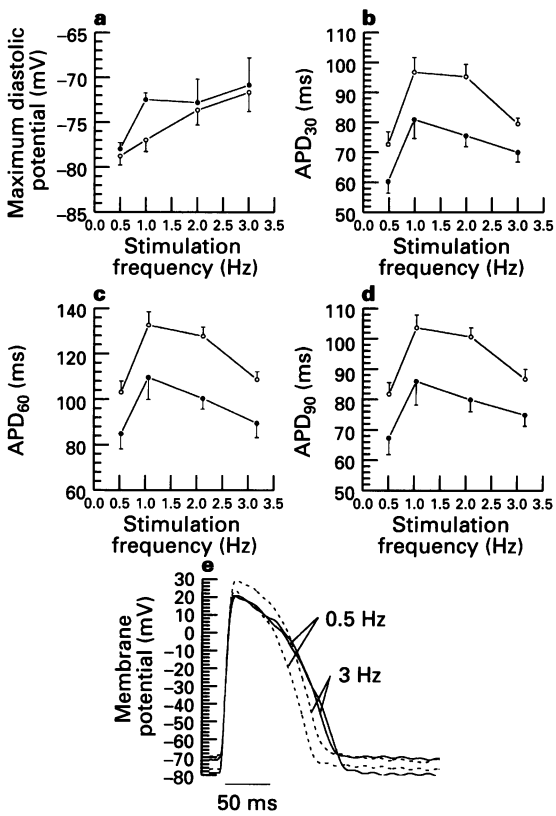

Figure 5 Effects of 30 μM TBQ on the frequency-induced changes in action potential (AP) characteristics in rabbit ventricular muscle ($n=5$): maximum diastolic potential (a), action potential duration at 30% (b), 60% (c) and 90% (d) repolarization. (○) Control; (●) in the presence of 30 μM TBQ. Data are mean ± s.e.mean. (e) Representative traces of the TBQ-induced alteration of the AP at 0.5 and 3 Hz in control (continuous line) and after 30 μM TBQ exposure (dashed line). TBQ did not change the maximum diastolic membrane potential but shortened AP duration. Two-way ANOVA showed a small but significant overall TBQ effect ($P=0.048$ (APD_{30}), 0.034 (APD_{60}), 0.036 (APD_{90})) but no significant interaction.

Modifications of SR Ca^{2+} load

The partial inhibition of SR Ca^{2+} uptake by 30 μM TBQ could be due, apart from too low a concentration, to an incapacity of the RCC to be completely abolished. This was tested by treating the preparations with 20 mM caffeine, a manoeuvre known to unload the SR totally (Bers, 1991). Exposure to 20 mM caffeine for 20 min increased twitch tension but completely abolished the steady-state RCC (not illustrated). After caffeine washout, the RCC could be elicited again.

The SR Ca^{2+} uptake activity reserve was tested with manoeuvres intended to increase intracellular Ca^{2+} and SR Ca^{2+} load, or to accelerate SR Ca^{2+} uptake. For this purpose, the TBQ-treated preparations were either exposed to 8 mM extracellular Ca^{2+} (Figure 6) or 5 μM isoprenaline.

In both cases, twitch force as well as the RCC reaugmented. The effect of such manoeuvres agrees with the notion that SR Ca^{2+} uptake inhibition by 30 μM TBQ is indeed incomplete.

Effect of TBQ (30 μM) on the force frequency-dependence of twitch characteristics in rat ventricular muscle

In rat ventricular muscle, contractile activation and relaxation relies heavily on proper SR Ca^{2+} handling and so TBQ might be expected to have a strong negative inotropic effect in this species. Furthermore, rat ventricle exhibits a negative staircase which is reversed to positive by ryanodine (Borzak *et al.*, 1991). Species comparisons therefore may help to determine whether TBQ also acts as an SR Ca^{2+} channel opener. Figure 7 illustrates the effects of TBQ on twitch tension and time course.

Figure 7a is a representative effect of 30 μM TBQ on twitch tension at 0.5 and 3 Hz. TBQ strongly inhibits twitch tension at all frequencies and prolonged the twitch at both frequencies, mostly by increasing the duration of relaxation. Figure 7b shows that the negative staircase in control condition remained so in the presence of 30 μM TBQ. Statistical analysis showed an overall significant effect of treatment but no interaction, meaning that TBQ did not differentially affect the pattern of negative staircase. Figure 7c and 7d shows that both the rate of tension development and relaxation were slowed, consistent with the negative inotropic effect of TBQ. TBQ did not affect the staircase pattern, nor did it prevent the frequency-induced changes. However, TBQ showed the rate of relaxation at all frequencies but 0.5 Hz.

TBQ had no significant effect on TTP averaged on all frequencies, nor did it prevent the decrease of TTP with increasing frequencies observed in control. The interaction was

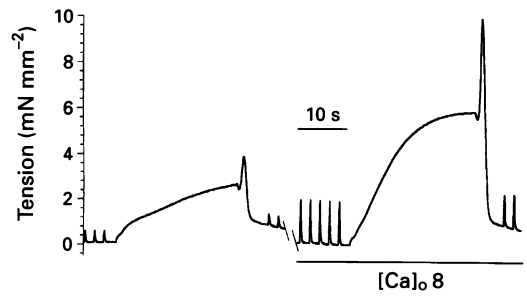

Figure 6 Representative effect of SR Ca^{2+} reloading by exposure of a 30 μM TBQ-treated rabbit papillary muscle to 8 mM extracellular Ca^{2+}. Twitch tension as well as SR Ca^{2+} load increased as assessed by the threefold increase in steady RCC tension.

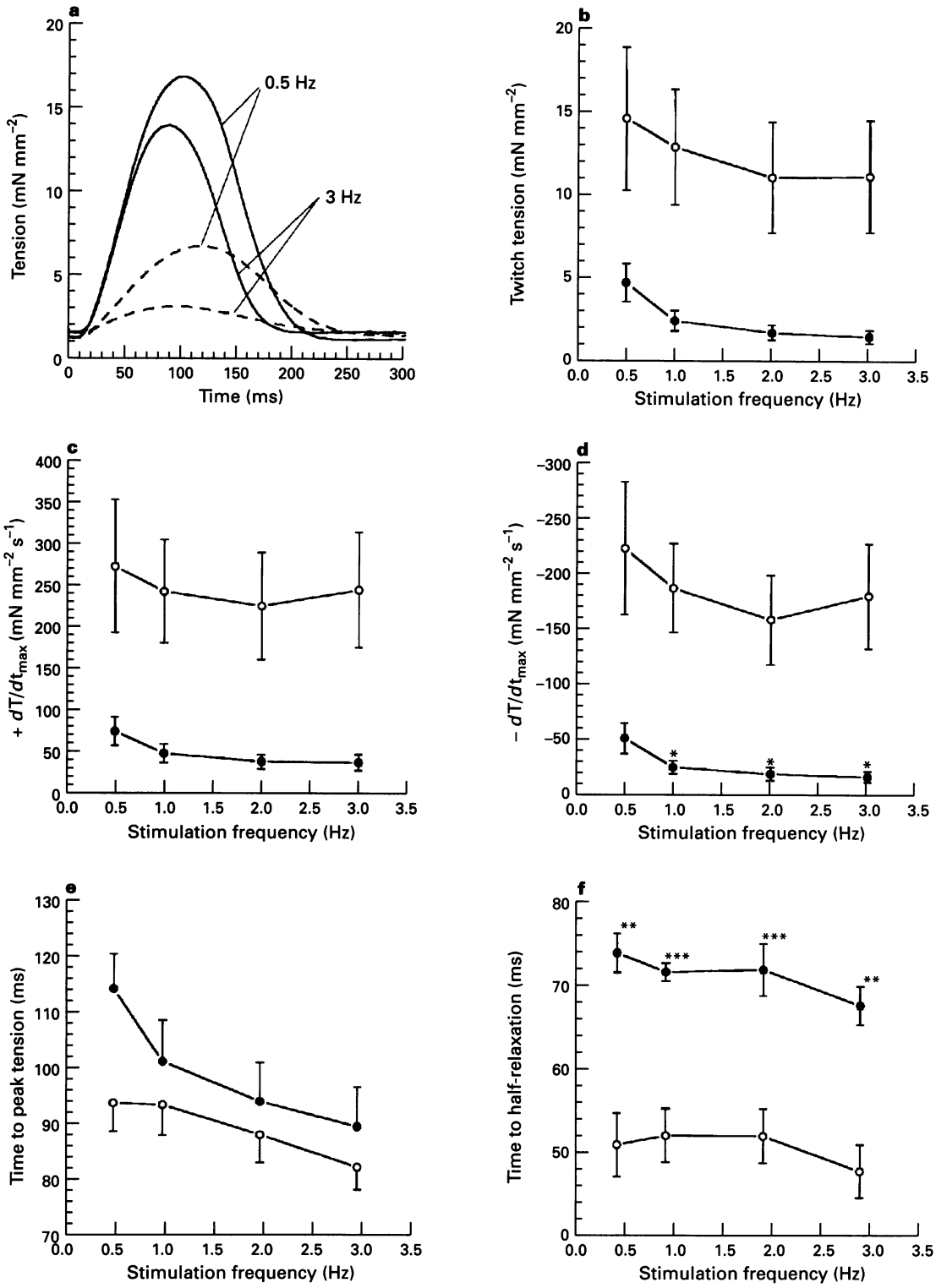

Figure 7 Effects of 30 μM TBQ in rat ventricular muscle ($n=6$) on the frequency-induced changes in twitch tension and time course: representative traces at 0.5 and 3 Hz (a), changes in twitch tension (b), maximal rate of tension rise (c), maximal rate of tension decline (d), time to peak tension (e) and time to half-relaxation (f). (○) Control; (●) in the presence of 30 μM TBQ. Data are mean ± s.e.mean. TBQ did not prevent the negative staircase characteristics of the rat ventricle, nor did it reverse the relationship to a positive staircase. Two-way ANOVA showed an overall significant effect of TBQ for (b) ($P=0.02$), (c) ($P=0.017$), (d) ($P=0.006$) and (f) ($P=0.0004$). Time to peak tension (e) was not altered by TBQ but this parameter was the only one in which a significant interaction occurred ($P<0.001$). *$P<0.05$; **$P<0.02$; ***$P<0.01$ *vs* control.

significant which is accounted for by the sharp decrease in TTP between 0.5 and 1 Hz. There was a significant but borderline effect ($P=0.042$) of frequency whether or not TBQ was present, because $t_{½}$ decreased between 2 and 3 Hz. TBQ strongly slowed the relaxation phase at all frequencies, but did not modify the general pattern of $t_{½}$ evolution. Therefore, the strong negative inotropic effect of TBQ coupled to a markedly slowed relaxation is strongly in favour of SR Ca^{2+} uptake depression and the importance of proper SR Ca^{2+} handling for the maintenance of rat ventricular contractility.

Discussion

The main results obtained in this study can be summarised as follows: (a) TBQ (30 μM) induces a negative inotropic effect in rabbit and rat isolated ventricular muscle; (b) experimental evidence suggests that a decreased SR Ca^{2+} content, rather than a slight shortening of the action potential accounts in part for this effect; (c) TBQ prolongs twitch time course by increasing the contraction phase in the rabbit and relaxation in the rat; (d) TBQ (30 μM) blunted the positive twitch staircase in the rabbit and negative staircase in the rat. In the rabbit ventricle, TBQ (30 μM) also blunted SR Ca^{2+} reloading normally occurring at high frequencies; (e) TBQ decreases the rate of relaxation but does not worsen it at increasing frequencies in the rabbit. Our observations are consistent with partial inhibition of SR Ca^{2+} uptake by TBQ at 30 μM and importance of SR Ca^{2+} uptake for occurrence of the force-frequency relationship.

Specificity of TBQ

Molecules belonging to the quinone family are known to possess many physiological and also toxic activities (Nagakawa & Moldéus, 1992). Among these compounds, TBQ seems to be devoid of toxicity and has been introduced as a fairly specific inhibitor of the endoplasmic reticulum/SR Ca^{2+}-ATPase (review by Inesi & Sagara, 1994). In muscular preparations, TBQ has similar biological effects to those encountered with other Ca^{2+} pump inhibitors such as TG or CPA (Llopis *et al.*, 1991; Westerblad & Allen, 1994).

In the present study carried out in multicellular preparations, most of our data fit with the hypothesis of a selective but partial depression of SR Ca^{2+} uptake. However, in non-muscular preparations, TBQ inhibits $I_{Ca,L}$ (Nelson *et al.*, 1994) which may account for the observed APD shortening and progressive SR Ca^{2+} unloading. However, the negative inotropic effect of Ca^{2+} channel inhibitors in rabbit ventricular muscle is not accompanied by changes in twitch time course (Morcos *et al.*, 1992) whereas TBQ significantly prolonged it. TBQ effects on AP duration are more probably explained by a smaller amount of SR released Ca^{2+} that would decrease the Na^+/Ca^{2+} exchange current, a depolarizing current in rabbit ventricular cells (Bers, 1991). Shortening of the AP by TBQ may however provide less time for trans-sarcolemmal Ca^{2+} entry, decreasing in turn the efficacy of the trigger signal and thereby accounting for the decrease of the slope relating RCC and twitch tension.

Experimental evidence for partial TBQ-induced depression of SR Ca^{2+} uptake

In rabbit ventricular muscle, several results argue in favour of partial inhibition of SR Ca^{2+} uptake by 30 μM TBQ. For instance, RCCs were still present in basal conditions and manoeuvres intended to increase SR Ca^{2+} load (high stimulation frequencies, elevated extracellular Ca^{2+} or isoprenaline) were all effective in increasing RCC amplitude in TBQ-treated preparations. Therefore, in terms of enzymatic activity, 30 μM TBQ provokes a reduction in the overall pumping rate (decreased V_{max} or V_{pump}) which leaves a proportion of pump sites active at normal rates and allows a significant pumping reserve.

Another argument relies on the observation that in the rabbit and rat ventricle, TBQ, like CPA or TG, exerts a negative inotropic effect that was steady. We did not observe any reversal of the negative inotropic effect and we are not aware of such an effect with other inhibitors in multicellular preparations, whereas it has been reported in isolated guinea-pig (Lewartowski *et al.*, 1994) or rabbit (Bassani *et al.*, 1993; 1994) ventricular myocytes. As such a reversal is currently explained by abolition of trans-sarcolemmal Ca^{2+} buffering by the SR, it is likely that a fraction of pumps unblocked by 30 μM TBQ is still able to buffer incoming Ca^{2+}. Unfortunately, solubility problems and possible non-specific effects related to membrane partition of hydrophobic compounds (Baudet *et al.*, 1993; Du *et al.*, 1994) precludes complete SR Ca^{2+} pump inhibition and investigation of the reversal phenomenon in multicellular preparations.

Interestingly, in the rabbit ventricle, twitch relaxation was not markedly slowed by 30 μM TBQ as also observed by Yard *et al.* (1994) in 30 μM CPA-treated guinea-pig atrium and by Péry-Man *et al.* (1993) in 10 μM CPA-treated rat papillary muscle. However, Backx *et al.* (1995) reported a marked slowing of relaxation by 100 μM CPA in rat ventricular trabeculae whereas the Ca^{2+} transient had almost entirely returned to baseline. By contrast, the rate of relengthening was prolonged in rat ventricular trabeculae exposed to 10 μM CPA (Péry-Man *et al.*, 1993) or in 5 μM TG-treated cardiomyocytes (Bassani *et al.*, 1994). Overall these data support the concept that Ca^{2+} unbinding from troponin C rather than the rate of myoplasmic Ca^{2+} reduction may represent the limiting factor of isometric twitch relaxation (Brutsaert & Sys, 1989).

Force-frequency relationship in the rabbit ventricle

The positive staircase in rabbit ventricular muscle (and most other mammalian species) has been attributed to an increase in the duration of membrane depolarization per unit of time, therefore increasing Ca^{2+} influx through Ca^{2+} channels and increasing Ca^{2+} entry (or decreasing Ca^{2+} efflux) by the Na^+/Ca^{2+} exchanger because of the elevated intracellular Na^+ (Frampton *et al.*, 1991; Schouten *et al.*, 1991). SR Ca^{2+} uptake also increases by activation of the type II Ca^{2+}-calmodulin kinase (Bassani *et al.*, 1995). As a consequence, SR Ca^{2+} load increases accounting for the positive inotropism. Therefore, SR Ca^{2+} pump inhibitors should blunt the staircase phenomenon which was indeed observed in the presence of TBQ and in guinea-pig atrial muscle treated with CPA (Yard *et al.*, 1994). Moreover blunting of the positive RCC staircase strengthens the importance of SR Ca^{2+} uptake for the force-frequency relationship.

Interestingly, there were similarities between our results and those of Yard *et al.* (1994) who used CPA-treated guinea-pig atrium. In both studies, the frequency-induced TTP shortening was blunted by the SR Ca^{2+} pump inhibitor whereas TBQ or CPA did not prevent $t_{1/2}$ decrease. The involvement of SR Ca^{2+} uptake in twitch tension activation may rely on two mechanisms. On one hand, impairment of SR Ca^{2+} loading by TBQ will decrease the Ca^{2+} gradient across the SR membrane and prevent a fast rise of the intracellular Ca^{2+} transient. On the other hand, the Ca^{2+} transients will be decreased by TBQ but not be as curtailed by SR Ca^{2+} uptake as in control conditions, thereby favouring prolonged activation of the contractile proteins. Regarding the unchanged pattern of relaxation acceleration, it is likely that the partial inhibition of SR Ca^{2+} uptake by 30 μM TBQ leaves an important fraction of pumps able to be stimulated by elevated intracellular Ca^{2+} and Ca^{2+} efflux by the exchanger can still compensate for this deficiency. Clearly, provided that complete block of SR pumps could be achieved in multicellular preparations, relaxation should be hampered at high frequencies.

Force-frequency relationship in the rat ventricle

In rat multicellular preparations, the extent of negative inotropic effect provided by SR Ca^{2+} pump inhibitors is clearly dose-dependent as 10 μM CPA did not exhibit significant negative inotropic effects (Péry-Man *et al.*, 1993) whereas profound and reversible depression was obtained with 100 μM (Backx *et al.*, 1995). Despite the fact that RCCs are not interpretable in rat multicellular preparations, it seems safe to assume that depletion of SR Ca^{2+} by 30 μM TBQ accounts for the negative inotropic effect, although the extent of inhibition could not be estimated. In the rat ventricle, the negative staircase has been accounted for by progressive SR Ca^{2+} unloading as frequency increases (Busselen *et al.*, 1985; Frampton

et al., 1991). In fact, AP shortening at high frequencies (Mitchell *et al.*, 1985) will shift cytosolic Ca^{2+} reduction in favour of the Na^+/Ca^{2+} exchanger so that more Ca^{2+} will be extruded from the cell during a contraction (Shattock & Bers, 1989). An important observation is that TBQ did not reverse the negative staircase as does ryanodine (Borzak *et al.*, 1991). This indirectly indicates no evidence for an effect of TBQ on SR Ca^{2+} release.

By contrast with the rabbit ventricle, the twitch time course is relatively frequency-insensitive between 0.5 and 3 Hz and TBQ affects more the relaxation than the contraction phase. The relative insensitivity towards frequency is likely to be explained by the fact that, in our experimental conditions, the extent of the negative staircase is small, suggesting that the Ca^{2+} transient is roughly constant and should place an almost similar Ca^{2+} load on the various intracellular and membraneous Ca^{2+} binding sites. The absence of significant effect of TBQ on TTP has also been reported in CPA-treated rat cardiomyocytes in which TTP of the Fluo 3 signal was also unaffected (Wrzosek *et al.*, 1992). These data indicate that despite the lower SR Ca^{2+} content in the presence of TBQ, the rate of SR Ca^{2+} release and subsequent activation of the contractile proteins is not significantly curtailed by simultaneous SR Ca^{2+} uptake maybe because SR released Ca^{2+} may have a preferential access to the contractile proteins rather than to the SR Ca^{2+} ATPase. By contrast with the rabbit ventricle, the slowing of relaxation in the presence of TBQ at all frequencies shows that partial inhibition of SR Ca^{2+} uptake cannot be compensated for by other mechanisms like the Na^+/Ca^{2+} exchanger (Bassani *et al.*, 1994). Again, the absence of significant worsening of relaxation at increasing frequencies is probably accounted for by the small extent of the negative staircase.

Comparison of TBQ-induced changes in inotropism/relaxation and cardiac hypertrophy

Prolongation of isometric twitch tension and attenuation of the force-frequency relationship in the rabbit ventricle are reminiscent of alterations of similar parameters in the pressure-overloaded (Gwathmey & Morgan, 1993) or failing (Gwathmey *et al.*, 1991; Hasenfuss *et al.*, 1994; Schmidt *et al.*, 1994) human ventricle. Such alterations have been attributed to intracellular Ca^{2+} handling defects (Gwathmey *et al.*, 1991) mainly a reduced capacity of SR Ca^{2+} uptake (Studer *et al.*, 1994) because of a decreased expression of the SR Ca^{2+} pump protein (Arai *et al.*, 1994). Despite these functional similarities, the molecular mechanisms involved may differ. In fact TBQ blocks a fraction of *normal* pumps thereby reducing the maximal rate of Ca^{2+} uptake, similar to a decreased V_{max}. Although reduction of the total amount of pumps exists in diseased human hearts, there is no experimental evidence as to whether such pumps do normally function, especially in terms of K_m. For instance modification of their phospholipidic environment and/or the phosphorylation state of phospholamban may alter the affinity of the pumps for intracellular Ca^{2+}.

This work also shows that depressed systolic function induced by partial SR Ca^{2+} uptake inhibition is not necessarily coupled to profound alteration of relaxation. Similar results have been shown using invasive *in vivo* measurements in human subjects exhibiting ventricular hypertrophy (Liu *et al.*, 1993) and in isolated trabeculae from failing hearts studied in physiological conditions of pacing (1 Hz) and temperature (37°C). In particular no increase in resting tension was observed as we also noticed with TBQ. By contrast, contracture appeared in multicellular preparations at 30°C (Gwathmey *et al.*, 1991). Lower temperatures are likely to have differential effects on the SR Ca^{2+} pump and the Na^+/Ca^{2+} exchanger (Bers, 1991) and may also alter AP responses to increased frequencies (Kiyosue *et al.*, 1993).

In conclusion, TBQ exerts effects on inotropism and SR Ca^{2+} content that are consistent with inhibition of SR Ca^{2+} uptake, both in the rabbit and the rat ventricle. However, these systolic modifications are accompanied by marked alterations of relaxation in the rat but not in the rabbit ventricle. These data show the importance of SR Ca^{2+} uptake in reducing myoplasmic Ca^{2+} in the former species whereas the Na^+/Ca^{2+} exchanger can compensate for this deficiency in the rabbit. Blunting of the force-frequency relationship by TBQ strongly argues for the importance of SR Ca^{2+} uptake activity for occurrence of this phenomenon and strengthens the idea that the defective staircase in heart failure may partly rely on defective SR Ca^{2+} pumping. TBQ seems to be a fairly specific SR Ca^{2+} pump inhibitor in multicellular cardiac preparations provided that only partial inhibition of SR Ca^{2+} uptake is required. However, solubility constraints and possible non-specific effects preclude the use of TBQ at higher concentrations to block fully SR Ca^{2+} uptake in isolated muscles. Finally, the coupling between inotropism and relaxation (Chemla *et al.*, 1986) has led us to analyse more precisely the part taken by the SR Ca^{2+} pump and the Na^+/Ca^{2+} exchanger in Ca^{2+} reduction. This, and a possible 'intrinsic' relaxant effect (Chemla *et al.*, 1986) of TBQ, are reported in the companion paper (Baudet *et al.*, 1996).

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