Journal of Zhejiang University SCIENCE B
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

A new linearly-combined bi-exponential model for kinetic analysis of the isometric relaxation process of *Bufo gastrocnemius* under electric stimulation in vitro*

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Received July 15, 2007; revision accepted Oct. 22, 2007

Abstract: There was a slow-relaxing tail of skeletal muscles in vitro upon the inhibition of Ca^{2+}-pump by cyclopiazonic acid (CPA). Herein, a new linearly-combined bi-exponential model to resolve this slow-relaxing tail from the fast-relaxing phase was investigated for kinetic analysis of the isometric relaxation process of *Bufo gastrocnemius* in vitro, in comparison to the single exponential model and the classical bi-exponential model. During repetitive stimulations at a 2-s interval by square pulses of a 2-ms duration at 12 V direct currency (DC), the isometric tension of *Bufo gastrocnemius* was recorded at 100 Hz. The relaxation curve with tensions falling from 90% of the peak to the 15th datum before next stimulation was analyzed by three exponential models using a program in MATLAB 6.5. Both the goodness of fit and the distribution of the residuals for the best fitting supported the comparable validity of this new bi-exponential model for kinetic analysis of the relaxation process of the control muscles. After CPA treatment, however, this new bi-exponential model showed an obvious statistical superiority for kinetic analysis of the muscle relaxation process, and it gave the estimated rest tension consistent to that by experimentation, whereas both the classical bi-exponential model and the single exponential model gave biased rest tensions. Moreover, after the treatment of muscles by CPA, both the single exponential model and the classical bi-exponential model yielded lowered relaxation rates, nevertheless, this new bi-exponential model had relaxation rates of negligible changes except much higher rest tensions. These results suggest that this novel linearly-combined bi-exponential model is desirable for kinetic analysis of the relaxation process of muscles with altered Ca^{2+}-pumping activity.

Key words: *Bufo gastrocnemius*, Ca^{2+}-pump, Cyclopiazonic acid (CPA), Linearly-combined bi-exponential model, Muscle relaxation rate, Residual distribution, Rest tension

doi:10.1631/jzus.2007.B0867 **Document code:** A **CLC number:** Q44; R96

INTRODUCTION

The isometric tension of a muscle usually relaxes exponentially and kinetic analysis of the relaxation process of a muscle with diverse models gives the relaxation rate to characterize muscle activity (Baudet and Noireaud, 1999; Laporte *et al*., 2004; Liao *et al*., 2008; Matsubara *et al*., 1995; Mizuno *et al*., 2000; 2006; 2007; Tamiya *et al*., 1995). There are two types of models commonly used for kinetic analysis of the muscle relaxation process. One is the logistic exponential model, which is composed of exponential function but mathematically non-exponential, and gives the relaxation rate of complicated physiological meanings (Matsubara *et al*., 1995; Mizuno *et al*., 2000; 2006; 2007). The other type includes both the single exponential model that gives an exponential relaxation rate and the classical bi-exponential model that

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* Project supported by the National Natural Science Foundation of China (No. 30472139) and the Education Commission for the First Batch of Excellent Young Teachers in Universities of Chongqing City, China

gives relaxation rate of complicated meanings (Baudet and Noireaud, 1999; Tamiya *et al*., 1995; Même *et al*., 1998). Ca^{2+}-pump, a membrane Ca^{2+}-Mg^{2+}-ATPase responsible for sequestering Ca^{2+} into sarcoplasmic reticulum, is a putative determinant of muscle relaxation (Creazzo *et al*., 2004; Dobrunz *et al*., 1995; Morgan *et al*., 1997; Tesi *et al*., 2002). Cyclopiazonic acid (CPA) is a specific inhibitor of sarcoplasmic reticulum Ca^{2+}-pump (Plenge-Tellechea *et al*., 1997; Seidler *et al*., 1989). By fitting the single exponential model to the relaxation process of muscle upon CPA action, there was a reduction of relaxation rate while accompanied by an elevation of rest tension (Baudet and Noireaud, 1999; Tamiya *et al*., 1995; Même *et al*., 1998). Therefore, besides rest tension, relaxation rates were also commonly used to characterize muscle activities and the pharmacological or toxicological actions of some agents on muscle contractility.

However, there was a slow-relaxing tail during isometric relaxation of *Bufo gastrocnemius* in vitro after the CPA treatment. The ignorance of the contribution of this slow-relaxing tail to the overall relaxation kinetics may result in some bias in the estimated relaxation rate, but none of the models in current use for kinetic analysis of muscle relaxation process considers this alteration of muscle relaxation kinetics by either CPA or other agents altering muscle calcium transportation. Herein, in comparison to common exponential models, a new linearly-combined bi-exponential model that resolved the slow-relaxing tail from the fast-relaxing phase was investigated for kinetic analysis of the isometric relaxation process of *Bufo gastrocnemius* in vitro after the inhibition of Ca^{2+}-pump by CPA.

MATERIALS AND METHODS

Chemicals

Cyclopiazonic acid (CPA) from Sigma (USA) was dissolved in dimethyl sulfoxide (DMSO). Other chemicals of analytic grade were used directly.

Experimental procedure

Experiments with animals were in accordance with the ethical requirement of the University and were performed at (25±1) °C. *Gastrocnemius* from healthy, active *Bufo gargarizans* (50~100 g) was prepared as usual (Liao *et al*., 1999; 2008). Distant tendon of the muscle was fastened on a hook at the bottom of a 20-ml plastic syringe containing 20 ml Ringer's solution, and the other side was vertically linked to a tension transducer. A silver electrode was placed at the bottom of the syringe in touch with the distant tendon of muscle for the stimulation. BL-Century system (Chengdu Technology and Market Corp. Ltd., China; http://www.tme.com.cn) was used for the stimulation and the record of tension. By stimulation at (7.2±1.3) V direct currency (DC) (n=7) with square pulse of a 2-ms duration at a 2-s interval, peak tension of each contraction usually reached its maximum after several stimulations. Therefore, square pulses at a 2-s interval with 2-ms duration at 12 V DC were used to stimulate the muscles. Baseline of tension was adjusted to 1.30 g after the fixation of the muscle (muscle weight was (0.68±0.12) g, n=7). After this adjustment, Ringer's solution plus CPA as the treatment (final DMSO was 1%) or Ringer's solution plus 1% DMSO as the control replaced the solution in the syringe. Immediately after this replacement of solutions, dynamic tensions for 10 stimulations were recorded to serve as the reference contractions. Then the muscle was incubated in the solution for 30 min before being directly stimulated again at a 2-s interval to record dynamic tensions within 7.0 min.

Analysis of relaxation process

The three exponential models are as follows:

$$F = F_b + F_m \times e^{-b \times t}, \quad (1)$$

$$F = a - b \times e^{-c \times e^{-d \times t}}, \quad (2)$$

$$F = F_b + a \times e^{-b \times t} + c \times e^{-d \times t}. \quad (3)$$

In Eq.(1), the single exponential model, b was the relaxation rate, t was the relaxation time, F_b was the rest tension, i.e., the tension after complete relaxation of the muscle at indefinite time, F_m was the peak tension, and F was the instantaneous tension, respectively. Unless stated otherwise, data with tensions falling from 90% to 10% of the peak were analyzed by Eq.(1) using the constraints of F_m>0 and b>0 (Baudet and Noireaud, 1999). In Eq.(2), the classical bi-exponential model, $a-b$ was the rest tension and d was the relaxation rate with the constraints

of $a>0$, $b>0$, $d>0$ and $c>1\times10^{-6}$ (presetting $c>1\times10^{-6}$ gave better fitting than that with $c>1.0$) (Tamiya *et al*., 1995). In Eq.(3), the linearly-combined bi-exponential model, b was the relaxation rate for the fast-relaxing phase while d was that for the slow-relaxing tail with F_m equal to $a+c$ using the constraints of $b>d$, $d>0$, $a>0$ and $c>0$, respectively, besides the same meaning of F_b as in Eq.(1). Relaxation curves of tensions falling from 90% of the peak to the 15th datum before next contraction were analyzed with Eq.(2) and Eq.(3) unless stated otherwise. The experimental rest tension was the lowest averaged tension of adjacent five recorded data.

Programming, smoothing and statistic analysis

Trust-region algorithm in MATLAB 6.5 was used to analyze the relaxation process. Fluctuation of tension before stimulation, the noise of recorded tension, was (0.017±0.001) g (n=11). The starting point of contraction was that followed by three continuous data with tension increment >0.06 g (Fig.1). There were 20 points inserted between two adjacent recorded data using the *SPLINE* function, to refine the datum having exactly the desired percentage of tension relative to its peak during relaxation. The minimal fluctuations of the estimated parameters and the minimum of standard deviations of the estimated parameters for adjacent five contractions were taken as the noises of parameters during repetitive stimulation. With the thrice noise critique, the peaks and valleys with half-height width not more than two contractions for the response of derived parameters during repetitive stimulation were filtered out before these derived parameters were smoothed using the *SMOOTH* function in MATLAB 6.5 for every five contractions. Indexes of the reference contractions were those of the averages of last five contractions from data recorded for 10 stimulations just before CPA treatment. All indexes were in mean±*SD* and compared by *t*-test with $P<0.05$ as the confidence limit.

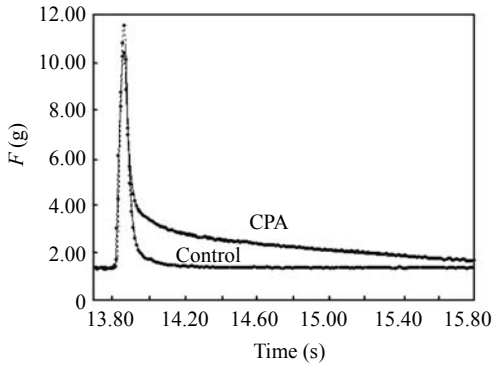

Fig.1 Altered relaxation kinetics of the muscle after the treatment by 10 μmol/L cyclopiazonic acid (CPA)

RESULTS

Differences among three exponential models for fitting the relaxation curve

In comparison to the control, there was an obvious slow-relaxing tail of the muscle after CPA treatment (Fig.1). For the control muscles after 10 stimulations, the residuals for the best fitting of Eq.(2) to the whole relaxation curve exhibited the narrowest fluctuation around zero in the fast-relaxing stage while they linearly, but very slowly, deviated from zero with the progress of the relaxation in the slow-relaxing tail (Fig.2). Eq.(3) and Eq.(1) showed

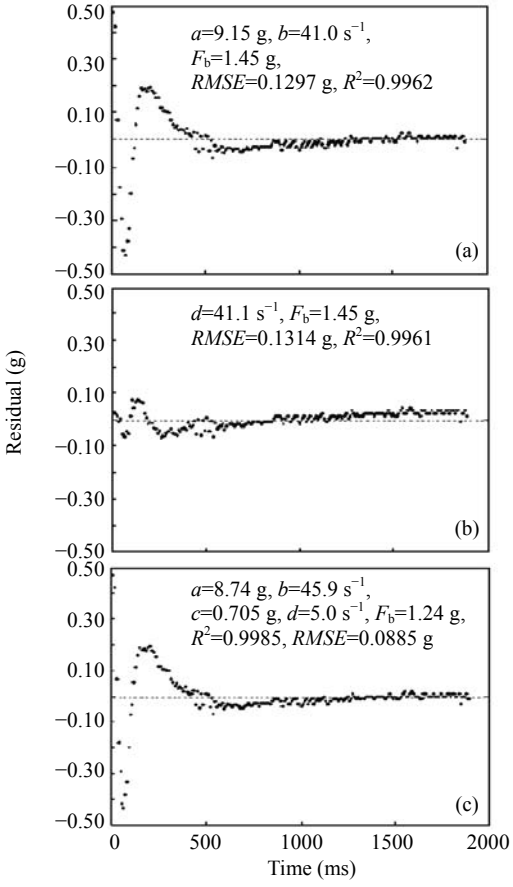

Fig.2 Residuals for the best fitting to the relaxation curve of the control muscle. (a) Eq.(1); (b) Eq.(2); (c) Eq.(3)

Data were those of tensions falling from 90% of the peak to the 15th datum before next contraction as presented in Fig.1. The dashed straight line in the middle represented zero residuals

no significant differences between their residuals for the best fitting to the same relaxation curves of the control muscles, in both the fast-relaxing phase and the slow-relaxing tail. For most control muscles, three exponential models gave relaxation rates of negligible differences.

There were transient contractures of *Bufo gastrocnemius* during the treatment by 40 μmol/L CPA if the muscle was not fixed, but this situation was seldom observed in the control muscles. To the slow-relaxing tail of the muscle after CPA treatment, the residuals for the best fitting of Eq.(3) showed the narrowest random fluctuation around zero whereas those for the best fitting of other two exponential models exhibited much larger linear deviations from zero with the progress of relaxation (Fig.3). To the fast-relaxing phase of muscles after CPA treatment, the residuals for the best fitting of both Eq.(1) and Eq.(2) also exhibited larger systematic deviations from zero than those with Eq.(3). These differences, however, grew smaller and smaller and finally faded out after repetitive stimulations for about 3 min. Usually, Eq.(1) and Eq.(2) gave relaxation rates of negligible difference while Eq.(3) gave a higher relaxation rate to the same relaxation curve of the muscle after the CPA treatment.

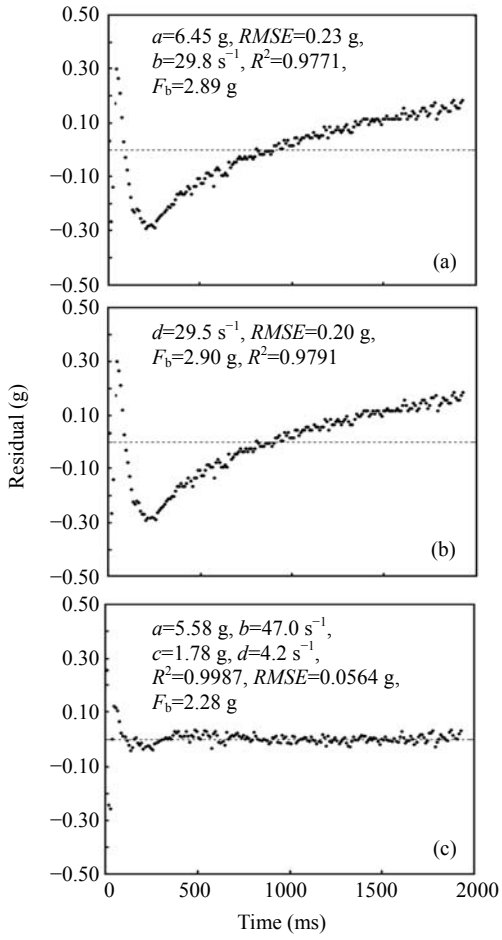

Fig.3 Residuals for the best fitting to the relaxation curve after cyclopiazonic acid (CPA) treatment. (a) Eq.(1); (b) Eq.(2); (c) Eq.(3)

Data were those of tensions falling from 90% of the peak to the 15th datum before next contraction as presented in Fig.1

There were differences in the goodness of fit among three models. For the best fitting to the relaxation process of the control muscles, the determination coefficients of three models were usually >0.994. During the repetitive stimulations within 3 min, the goodness of fit, such as Akaike's information criterion (AIC), Bayesian information criterion (BIC), root mean square of errors (RMSE), indicated that Eq.(3) was comparable to Eq.(2) for kinetic analysis of the relaxation process of the control muscles while Eq.(1) was usually the worst (Fig.2). However, after the stimulation of the control muscles for more than 3 min, usually Eq.(3) was slightly better than Eq.(2) while Eq.(2) was slightly better than Eq.(1). To the relaxation process of the muscle treated by CPA, the determination coefficients with Eq.(3) were usually >0.995 while those with Eq.(1) and Eq.(2) were sometimes <0.960. In the initial 3 min during the repetitive stimulations of the muscles after the treatment by 10 μmol/L CPA, Eq.(3) was the best to fit to the relaxation curves and there was no difference in the goodness of fit between Eq.(1) and Eq.(2) for the fitting to the same relaxation curves (Fig.3). There were similar differences at higher CPA. Nevertheless, Eq.(1) and Eq.(2) produced the estimated rest tension with positive biases, whereas Eq.(3) produced the estimated rest tension consistent to the experimental ones, for both the control and treatment.

Actions of CPA on skeletal muscles analyzed by different models

For the fitting of Eq.(3) to the relaxation curves of muscles after CPA treatment, the amplitudes of the slower phase accounted for 10% to 50% of those for the fast-relaxing phase in the initial 10 stimulations (Fig.4). The amplitudes of the slower phase showed larger variations, but the peak tensions, the sum of

amplitudes for both the slower phase and the fast phase in Eq.(3), showed much smoothed changes during the repetitive stimulations (Fig.4). Usually, the relaxation rates for the slower phase were below 20% of those for the fast-relaxing phase in the initial 10 stimulations, but after the repetitive stimulations for about 3 min, they became indistinguishable from those for the fast-relaxing phase (Fig.4). During repetitive stimulations within 7 min, the relaxation rates and the amplitudes of the slow-relaxing phase after CPA treatment showed so large fluctuations that they were not further analyzed in detail.

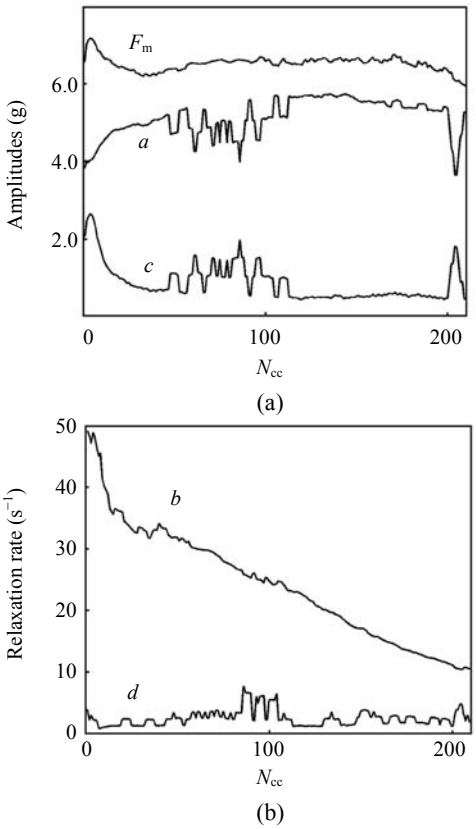

Fig.4 Changes of parameters of two phases in Eq.(3) for muscles treated by 10 μmol/L cyclopiazonic acid (CPA). (a) Amplitudes; (b) Relaxation rates

Symbols were the same as described in the MATERIALS and METHODS section. N_{cc} was the number of contractions at 2-s interval

There was no difference in the relaxation rates of the same reference contractions among the three models. For the control muscles, Eq.(3) usually gave two relaxation rates of no differences, besides their consistence to those estimated by Eq.(1). Moreover, for the control muscles, relaxation rates by three models usually decreased monotonically and there were no differences among the trends for the changes of relaxation rates during the repetitive stimulations within 7 min. In the first 3 min of the repetitive stimulations of muscles after CPA treatment, usually there was a peak of relaxation rate with Eq.(1) while the relaxation rates by Eq.(3) or Eq.(2) essentially decreased monotonically after about 10 stimulations (Fig.5). Moreover, there was a concentration-depended decrease of the initial and the maximal relaxation rates estimated by Eq.(1) while there was only the reduction of the initial relaxation rate by Eq.(2). By Eq.(3), however, neither the initial relaxation rate nor the maximal relaxation rate of muscles showed a significant change after CPA treatment (Table 1).

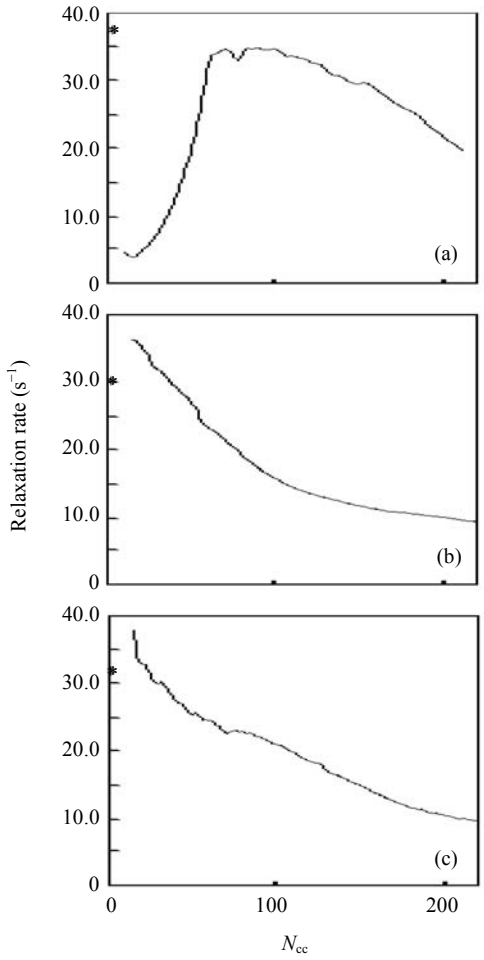

Fig.5 Typical changes of muscle relaxation rates after the treatment by 10 μmol/L cyclopiazonic acid (CPA). (a) Eq.(1); (b) Eq.(2); (c) Eq.(3)

N_{cc} was the number of contractions at 2-s interval; * represented the averaged values of the reference contractions

For the control muscles, the estimated rest tension by Eq.(3) monotonically decreased after a negligible peak within 15 contractions, and the duration was usually <0.50 min when the estimated rest tension was higher than that of the reference contractions (Fig.6). After CPA treatment, the estimated rest tension by Eq.(3) increased quickly to a peak and then decreased so slowly that for most time the estimated rest tension was much higher than that of the reference contractions (Table 2). However, CPA had no effect on the dynamic change of either the peak tension or the power-out during the continued stimulations (data not given).

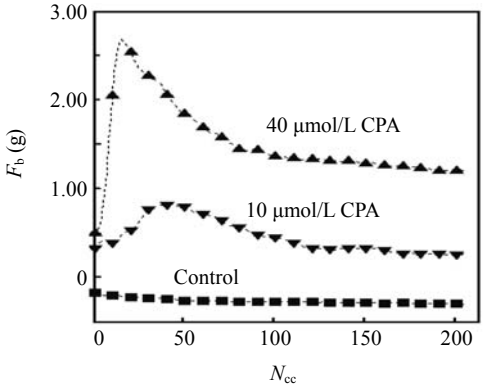

Fig.6 The effects of cyclopiazonic acid (CPA) on muscle rest tension estimated by Eq.(3)

Table 1 Effects of cyclopiazonic acid (CPA) on relaxation rate of muscles

		Relaxation rate								
		Eq.(1)			Eq.(2)			Eq.(3)		
		RR_{ref} (s^{-1})	RR_{ini}/RR_{ref}	RR_{max}/RR_{ref}	RR_{ref} (s^{-1})	RR_{ini}/RR_{ref}	RR_{max}/RR_{ref}	RR_{ref} (s^{-1})	RR_{ini}/RR_{ref}	RR_{max}/RR_{ref}
Control (n=14)		31±10	1.1±0.2	1.2±0.2	39±14	1.0±0.3	1.1±0.3	33±7	1.1±0.1	1.2±0.1
CPA (μmol/L)	10 (n=14)	31±12	0.8±0.4*	1.1±0.3	41±17	0.7±0.2*	1.0±0.4	36±14	1.1±0.3	1.2±0.4
	40 (n=10)	36±8	0.6±0.3**	0.9±0.2**	36±18	0.6±0.4*	1.1±0.5	36±6	1.2±0.3	1.4±0.4

*P<0.05, and **P<0.01 vs control, respectively; RR_{ini}: Initial value of relaxation rate; RR_{max}: Maximal relaxation rate during repetitive stimulations within 7.0 min; RR_{ref}: Relaxation rate of the reference contractions

Table 2 Effects of cyclopiazonic acid (CPA) on the estimated rest tension (F_b) of muscles

		Estimated rest tension (F_b)			
		$B_{max}-B_{ref}$ (g)	$B_{ini}-B_{ref}$ (g)	$B_{7\,min}-B_{ref}$ (g)	Duration (min)
Control (n=14)		0.0±0.4	−0.1±0.4	−0.3±0.5	0.14±0.09
CPA (μmol/L)	10 (n=14)	1.7±1.5*	0.3±0.4*	0.7±0.7*	6.0±1.8*
	40 (n=10)	3.2±1.5*#	1.3±0.7*#	1.7±1.3*#	6.1±1.9*

The estimated rest tension (F_b) was from Eq.(3). *P<0.05 and #P<0.01 vs that at 10 μmol/L CPA. $B_{7\,min}$: The rest tension estimated by Eq.(3) after repetitive stimulation for 7.0 min; B_{ini}: The initial value of the rest tension estimated by Eq.(3); B_{max}: The maximum of rest tension estimated by Eq.(3); B_{ref}: The averaged rest tension of the reference contractions estimated by Eq.(3); Duration: The duration within 7.0 min when the estimated rest tension was above B_{ref}

DISCUSSION

The physiological and statistical validities are the fundamental prerequisites for a kinetic model to analyze muscle relaxation process. Complicated physiological meanings of the relaxation rate by the logistic exponential model prevented its comparison to Eq.(3) in this study (Matsubara *et al*., 1995; Mizuno *et al*., 2000; 2006; 2007). The existence of the slow-relaxing tail supported its resolution from the fast-relaxing phase for kinetic analysis of the relaxation process of muscles after CPA treatment. Eq.(3) exhibited a comparable validity for kinetic analysis of the relaxation process of the control muscles. With muscles after CPA treatment, however, the differences in both the goodness of fit and the distribution of the residuals for the best fitting to the same relaxation curves supported the superiority of Eq.(3). Moreover, Eq.(3) can be simplified into Eq.(1) when relaxation rates for two phases were indistinguishable, which may account for the consistence among the relaxation rates of the control muscles by Eq.(3) and those by Eq.(1). Therefore, Eq.(3) may be statistically desirable for kinetic analysis of the relaxation process of muscle.

Skeletal muscle fiber was not strictly isometric

during relaxation (Mutungi and Ranatunga, 2000). Both the significant increase of rest tension and the transient contracture of muscle upon CPA action suggested that there was a small change of muscle length. However, the kinetics for the recovery of muscle length to its original length, a factor that may determine the relaxation kinetics of the slower phase, may be too complicated to give a characteristic relaxation rate. Furthermore, the analysis of muscle relaxation process with Eq.(1) and Eq.(2) yielded the lowered relaxation rates of muscles upon CPA action, which supported that CPA exerted a similar action on *Bufo gastrocnemius* as observed by others (Même *et al*., 1998). The relaxation rate for the faster phase in Eq.(3) had the same physiological meanings as that in Eq.(1). The maximal increase of rest tension of muscles after the treatment by 40 μmol/L CPA was close to half of the peak tension, and the relaxation rate by Eq.(3) for the slower tail was usually below 20% of that for the fast-relaxing phase in the initial stage of stimulation (Fig.5 and Tables 1 and 2). For most studies of CPA action on muscles, only the relaxation rates of the contractions during a few stimulations were measured, averaged and compared, during which the contribution of the slower phase was so significant that the biased relaxation rates from both Eq.(1) and Eq.(2) could not be excluded (Fig.4). Therefore, different actions of CPA on muscle relaxation may be due to different contribution of the slower phase to the overall relaxation kinetics of muscles after CPA treatment in different models.

CPA is a specific inhibitor of sarcoplasmic reticulum Ca^{2+}-pump (Plenge-Tellechea *et al*., 1997; Seidler *et al*., 1989). Under some special situations, slowed Ca^{2+}-uptaking did not alter the relaxation kinetics of muscle estimated by the single exponential model (Booth *et al*., 1997; Westerblad *et al*., 1997). There were species-dependent differences in a cellular mechanism of muscle relaxation (Bassani *et al*., 1994), and it was observed that Ca^{2+}-pumping was not the primary determinant of muscle relaxation under some special situations (Bassani *et al*., 1994; Belus *et al*., 2003; Poggesi *et al*., 2005). Using the same experimental condition, nonspecific damages to muscle by reactive oxygen species caused a significant increase of the rest tension, but Eq.(1) still stood for the analysis of muscle relaxation process (Liao *et al*., 2008). Therefore, the rest tension, rather than the relaxation rate, of muscle may be more reliable for characterizing the actions of agents to alter muscle Ca^{2+}-pumping activity.

This linearly-combined bi-exponential model is a general form of Eq.(1) with a statistical superiority for kinetic analysis of the muscle relaxation process, and the estimated relaxation rates have more definite physiological meanings in this model than those in the classical bi-exponential model. Therefore, the resolution of the slow-relaxing tail from the fast-relaxing phase for kinetic analysis of the isometric relaxation process of skeletal muscle may be desirable for characterizing muscle contractility and the actions of agents that may alter muscle Ca^{2+}-pumping activity.

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