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The effect of chelerythrine on depolarization-induced force responses in skinned fast skeletal muscle fibres of the rat

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1 We examined the effect of the protein kinase C (PKC) inhibitor chelerythrine on depolarization-induced force responses (DIFRs) and sarcoplasmic reticulum (SR) function in single, mechanically skinned skeletal muscle fibres of the rat.

2 In this study, the DIFRs in the skinned fibres normally underwent an irreversible loss of excitation-contraction coupling (ECC) after 10–15 responses. Chelerythrine (12 μM) was shown to restore ECC in these fibres. Restored force responses were similar in peak (control $50.8 \pm 6.4\%$, chelerythrine $56.9 \pm 12.4\%$ of maximum force, $P = 0.42$, $n = 21$), but significantly broadened compared to initial control responses (full-width at half maximum, control; 3.7 ± 0.3 s, chelerythrine; 13.3 ± 1.1 s, $P < 0.001$). Early exposure to chelerythrine prevented run-down of DIFRs. Chelerythrine also induced spontaneous force responses in some fibres.

3 The PKC inhibitors calphostin C and staurosporine did not restore ECC, and the PKC activator phorbol 12-myristate 13-acetate did not promote loss of ECC in the skinned fibres.

4 Chelerythrine significantly increased SR Ca^{2+} loading by $8.4 \pm 1.7\%$ ($P = 0.02$, $n = 9$) and SR Ca^{2+} release by at least $14.1 \pm 2.7\%$ ($P = 0.004$, $n = 11$) in the skinned fibres.

5 Chelerythrine had no significant effect on maximum force production or the $[Ca^{2+}]$ producing half maximal activation of the myofilaments. However, chelerythrine did have a small effect on the slope of the force-Ca^{2+} relationship ($P = 0.02$, $n = 10$).

6 Chelerythrine reverses the use-dependent loss of excitation-contraction coupling in skinend skeletal muscle fibres by a PKC independent pathway. Chelerythrine may be an important pharmacological probe for examining the mechanisms of contraction-induced muscle injury.

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Keywords: Chelerythrine; Ca^{2+}; skeltal muscle; protein kinase C; excitation-contraction coupling; redox; benzophenanthridine

Abbreviations: DIFRs, depolarization-induced force responses; ECC, excitation contraction coupling; EDL, *extensor digitorum longus*; PKC, protein kinase C; PMA, phorbol 12-myristyl 13-acetate; SR, sarcoplasmic reticulum

Introduction

In skeletal muscle, a complex series of events link electrical excitation of the sarcolemma to muscle contraction, a process known as excitation-contraction coupling (ECC) (Sandow, 1952). The action potential generated at the motor end plate region travels along the sarcolemma and into the transverse-tubular system. This results in depolarization of voltage sensors on the transverse-tubular membrane, which in turn, activates Ca^{2+} release channels located on the terminal cisternae of the sarcoplasmic reticulum (SR). The activation of the Ca^{2+} release channels by the voltage sensors is thought to involve protein-to-protein interactions (Leong & MacLennan, 1998). Ca^{2+} release from the SR *via* the Ca^{2+} release channels elevates myoplasmic $[Ca^{2+}]$ and triggers cross bridge cycling and contraction (Zot & Potter, 1987).

When skeletal muscle is repeatedly and intensely activated, it undergoes fatigue, which is a gradual decline in the ability to generate force. Usually, after a suitable rest period, the force returns to pre-fatigue levels (Fitts, 1994; Westerblad *et al*., 1998). However, eccentric contractions and/or repetitive exercise at low frequencies of stimulation ($\approx$20 Hz) can lead to a long lasting fatigue. This type of fatigue is thought to result mainly from disruption of the linkages between the voltage sensors and the SR Ca^{2+} release channels in the triadic region of the skeletal muscle fibre (Westerblad *et al*., 2000; Warren *et al*., 2001). The disruption to the triadic region is thought to be the result of raised intracellular Ca^{2+} levels. Increased intracellular Ca^{2+} accumulation induced by exposing fibres to Ca^{2+} ionophores or low concentrations of detergents has been shown to lead to muscle damage in mouse soleus muscle preparations (Jones *et al*., 1984). Exposure of contracting fast-twitch skeletal muscle fibres of the mouse to caffeine or the SR Ca^{2+} pump inhibitor TBQ (both of which increase intracellular Ca^{2+} beyond normal levels) has been shown to induce long-lasting fatigue (Chin & Allen, 1996).

A similar process is also thought to be responsible for the gradual reduction in the size of depolarization-induced force responses (DIFRs) elicited in mechanically skinned skeletal muscle fibres (Lamb *et al*., 1995; Lamb & Cellini, 1999). In these fibres, the transverse-tubular system seals after skinning and DIFRs can be elicited by replacing K^+-based bath

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solution with one containing predominantly Na^+ (Lamb & Stephenson, 1990). Typically, the DIFRs in skinned skeletal muscle fibres exhibit a rapid 'run-down' in size after 10–15 responses. This run-down is thought to be related to Ca^{2+}-induced damage, as exposure of the fibres to raised intracellular Ca^{2+} also abolishes depolarization-induced force in these fibres (Lamb *et al*., 1995). It has been hypothesized that activation of the uncoupling process plays an important protective role in preventing further skeletal muscle fibre damage (Stephenson *et al*., 1999).

The mechanism responsible for the uncoupling process that occurs during long lasting fatigue in intact fibres and run-down of DIFRs in skinned fibres is presently unknown. Studies using the calpain inhibitors calpeptin and leupeptin have shown that Ca^{2+}-activated neutral proteases are unlikely to be primarily involved in this process (Lamb *et al*., 1995; Chin & Allen, 1996).

In this study we examined the effects of the benzophenanthridine drug chelerythrine on DIFRs and SR function in skinned skeletal muscle fibres of the *extensor digitorum longus* (EDL) muscle of the rat. Chelerythrine is a drug that has been reported to have a broad range of biological effects within cells, including inhibition of liver alanine aminotransferase (Walterová *et al*., 1981) and taxol-mediated polymerization of rat brain tubulin (Wolff & Knipling, 1993). Chelerythrine also has anti-inflammatory (Lenfield *et al*., 1981), antiplatelet (Ko *et al*., 1990) and antitumour activity (Larsen *et al*., 1994).

Many of these actions have been attributed to chelerythrine inhibiting protein kinase C (PKC). Chelerythrine was initially reported to potently and specifically inhibit PKC from rat brain with an IC_{50} of 0.66 μM (Herbert *et al*., 1990). However, Lee *et al*. (1998) recently reported that chelerythrine did not inhibit PKC activity under experimental conditions published previously (Herbert *et al*., 1990), and proposed that a PKC-independent mechanism may be involved in the biological actions mediated by chelerythrine. Recently, the inhibitory effect of chelerythrine on acetylcholine-induced current in PC12 cells (Shi & Wang, 1999), and the activation of apoptosis by chelerythrine in Hela cells (Yu *et al*., 2000) and cardiac myocytes (Yamamoto *et al*., 2001) have all been shown to be independent of PKC inhibition.

The results of this study indicate that the presence of chelerythrine can cause reversal of the use-dependent run-down of DIFRs in the skinned skeletal muscle fibres of the rat. Chelerythrine was also found to have marked effects on the function of the sarcoplasmic reticulum.

Methods

Skeletal muscle fibres were isolated from the *extensor digitorum longus* (EDL) muscles of Wistar rats ($\geqslant$400 g), killed by exposure to a gas mixture of 80% CO_2 and 20% O_2. All experimental procedures and methods undertaken in this study were approved by the University of Western Australia Animal Ethics Committee. The single muscle fibres were dissected and mechanically skinned in paraffin oil. Isometric force was measured with a sensitive force transducer (SI Heidelberg). Data were acquired using a PowerLab data acquisition system (ADInstruments). To maximise the force production, the fibres were stretched from slack length by 20% to bring the sarcomere length to $\approx$2.8–3.0 μm (Lamb & Stephenson, 1990). Chelerythrine (SIGMA) was dissolved in pure dimethylsulphoxide. The final dimethylsulphoxide concentration in all the test and control solutions was 0.1%.

Contractile apparatus

The effect of chelerythrine on the sensitivity of the contractile apparatus to Ca^{2+} and maximal force production in the EDL fibres was determined by exposing fibres to a series of solutions with a range of different known free Ca^{2+} concentrations in the presence of 12 μM chelerythrine. The strongly Ca^{2+}-buffered solutions were prepared by mixing specific proportions of $EGTA^{2-}$ (Sol. A) and CaEGTA (Sol. B) solutions (Lamb & Stephenson, 1990. Sol. A contained (mM): K^+ 117, Na^+ 36, ATP (total) 8, free Mg^{2+} 1, creatine phosphate 10, EGTA (total), 50, N-2-Hydroxyethyl-piperazine-N′-2-ethanesulphonic acid (HEPES) 90, NaN_3 1 at pH 7.10$\pm$0.01. Sol. B was similar to Sol. A, with the exception that the [$EGTA^{2-}$] and [CaEGTA] of Sol. B was 0.3 mM and 49.7 mM respectively. The free [Ca^{2+}] of the solutions was calculated using a K_{app} for EGTA of 4.78×10^6 (Fink *et al*., 1986). Maximal force was determined by exposure of the fibre to Sol. B (free [Ca^{2+}] approximately 3.5×10^{-5} M). Force was returned to baseline between force measurements by a brief exposure to Sol. A. The plateaus of the force responses elicited by exposure to solutions of increasing free [Ca^{2+}] were expressed as a percentage of maximum Ca^{2+}-activated force and plotted as a function of pCa. The data were fitted with sigmoidal curves using the curve fitting software package, GraphPad Prizm (GraphPad Software Inc.). The slope and pCa_{50} values (pCa value corresponding to 50% of maximum force) of the individual curves derived from data from each fibre were determined for both chelerythrine and control data, and the values were compared statistically. Note that the data shown in Figure 1 show curves fitted to the mean data.

Depolarization-induced force responses

The fibres were maintained in a potassium hexamethylene-diamine-tetraacetate (potassium-HDTA) solution (mM): K^+ 125, Na^+ 36, $HDTA^{2-}$ 50, ATP (total) 8, Mg^{2+} (total) 8.6, creatine phosphate 10, EGTA (total) 0.03, HEPES 90, NaN_3 1

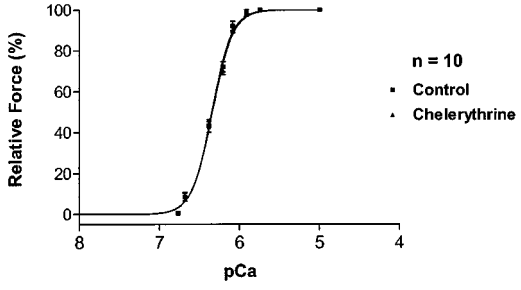

Figure 1 The effect of 12 μM chelerythrine on the relationship between Ca^{2+} and force in skinned EDL fibres of the rat. Sigmoidal curves were fitted to the mean force measurements (as a percentage of maximum force) made in a set of highly buffered Ca^{2+} solutions of different known free Ca^{2+} concentration, in the absence and presence of chelerythrine.

at pH 7.10±0.01 (Lamb & Stephenson, 1990). The free Mg^{2+} concentration was 1 mM. NaN_3 was added to inhibit mitochondrial Ca^{2+} fluxes. The sealed *t*-system was repolarized by exposure to the K^+-HDTA solution for 1 min. DIFRs were elicited by exposing the preparation to a Na^+-HDTA solution, which was similar in composition to the K^+-HDTA solution except that Na^+ had been substituted for K^+. The K^+ and Na^+ solutions used with rat fibres were isoosmotic. The K^+-HDTA and Na^+-HDTA solutions were weakly buffered to a pCa of approximately 6.7–7.0 using Sol. A.

High Ca^{2+}/rigor experiments

In experiments to examine the effect of chelerythrine on the uncoupling of ECC induced by exposure to elevated intracellular Ca^{2+}, the fibres were firstly exposed to a 'rigor solution' (K^+-HDTA solution with 100 μM EGTA and all ATP and phosphocreatine replaced with HDTA) for 1 min and then transferred to the identical rigor solution with 400 μM Ca^{2+} (free [Ca^{2+}]: ~70 μM) for 30 s. Force responses upon depolarization were then measured under control conditions and in the presence of chelerythrine. In a similar experiment, chelerythrine was also added to the Ca^{2+}/rigor solution and control DIFRs were measured and compared to control responses made before exposure to this modified Ca^{2+}/rigor solution.

Effect of chelerythrine on SR function: caffeine-induced SR Ca^{2+} release, SR Ca^{2+} loading and SR Ca^{2+} leak experiments

In experiments designed to investigate the effect of chelerythrine on caffeine-induced Ca^{2+} release from the SR, the fibres were firstly depleted of Ca^{2+} by exposure to a 'Ca^{2+} release/depletion solution' (Table 1) for 2 mins to maximally release Ca^{2+} from the SR, and prevent SR Ca^{2+} re-accumulation. The fibre was then reloaded with Ca^{2+} for 20 s by exposure to a Ca^{2+} load solution (1 : 1 mixture of Sol. A and Sol. B. pCa 6.55). The Ca^{2+} load solutions were highly [Ca^{2+}]-buffered to ensure uniform loading throughout the fibre and to guarantee adequate clamping of the pCa of the solutions to a constant, known level, identical in both the test and control solutions. Loading was rapidly terminated at the end of each loading period by a brief (≈1–2 s) exposure to Sol. A. The fibre was then washed in a K^+-HDTA solution to remove excess EGTA, and exposed to a 'submaximal Ca^{2+} release solution' (Table 1) and the force response measured (Bakker *et al*., 1998). The peak values of force responses elicited after re-exposure to a caffeine solution containing 12 μM chelerythrine were compared to the peak force responses elicited in an identical caffeine solution without added chelerythrine. Before exposure to the caffeine solution the fibres were incubated for 30 s in a K^+-HDTA solution containing 0.75 mM EGTA (with and without chelerythrine) to allow time for the EGTA and/or chelerythrine/EGTA to equilibrate within the fibre.

In the experiments carried out to determine the effect of chelerythrine on SR Ca^{2+} loading, the SR of the fibre was firstly depleted of all releasable Ca^{2+} by exposure to the Ca^{2+} release/depletion solution (Table 1), and then reloaded submaximally by exposure to the Ca^{2+} load solution for 10 s. The fibre was then re-exposed to the Ca^{2+} release/depletion solution, and the integral of the force response elicited was used as an indicator of the amount of Ca^{2+} loaded during the loading period (Bakker *et al*., 1996). Depletion measurements made after loading in the presence of 12 μM chelerythrine were compared to control measurements made before and after loading with the drug to minimise errors associated with any deterioration in the size of the control responses. Before exposure to the load solution containing chelerythrine, the fibres were exposed to a K^+-HDTA solution containing chelerythrine for 30 s to allow time for chelerythrine to equilibrate within the fibre.

The experiments used to examine the effect of chelerythrine on leak of Ca^{2+} from the SR of the skinned fibres were similar to the SR Ca^{2+} release experiments described in the previous section. The SR of the fibres was firstly depleted of Ca^{2+} and then the SR was reloaded with Ca^{2+} for a specific time. The fibres were then exposed to a 'Ca^{2+} leak solution' (Table 1) for 70 s and transferred to a Ca^{2+} release/depletion solution. The normal SR Ca^{2+} leak was demonstrated by comparing the force responses made with or without exposure to the Ca^{2+} leak solution (Bakker *et al*., 1996). The effect of chelerythrine on SR Ca^{2+} leak was examined by comparing the responses elicited after exposure to the Ca^{2+} leak solution in the presence and absence of 12 μM chelerythrine.

Resting membrane potential measurement

The resting membrane potential was monitored in fibres of intact EDL muscles before and after incubation of 12 μM chelerythrine for 30 min, using a glass microelectrode filled with 3 M KCl in conjunction with a NeuroProbe Amplifier system (Model 1600, A-M Systems, Inc).

In this study, all experiments were conducted at room temperature (21–22°C). All data are expressed as mean± s.e.mean. Unless otherwise stated, all force responses in the presence of chelerythrine were converted to a percentage of the control response and compared using a two-tail, one sample Student's *t*-test. This particular statistical test was undertaken to remove the variability in the control data that was specifically due to the normal difference in the size of fibres found in the EDL muscle. All statistical analysis was undertaken using the statistics software package GraphPad INSTAT.

Table 1 The modified K^+-HDTA solutions used in the examination of SR function (mM)

Solutions	*Total Mg^{2+}*	*Caffeine*	*Total EGTA*
Ca^{2+} release/depletion solution	0.1275	30	0.75
Submaximal Ca^{2+} release solution	7.65	20	0.75
Ca^{2+} leak solution	8.5	–	0.875

Results

The effect of chelerythrine on the contractile apparatus of rat EDL fibres

In this study, force was used as an indicator of Ca^{2+} fluxes from the SR. Therefore, it was important to firstly rule out

the possibility that chelerythrine had direct effects on the contractile apparatus of the skinned fibres. In this study, the presence of 12 μM chelerythrine had no significant effect on either the maximum force production (97.5±3.3% of control, $P=0.11$, $n=10$) or the mean pCa_{50} value (chelerythrine; 6.147±0.017, control; 6.142±0.015, paired Student's t-test, $P=0.41$, $n=10$) in the skinned EDL fibres. The mean slope of the curves underwent a very small but significant decrease from 3.71±0.22 under control conditions to 3.52±0.17 in the presence of chelerythrine (paired Student's t-test, $P=0.02$, $n=10$) (Figure 1). These results indicate that chelerythrine does not have any effects on the contractile apparatus that would hamper interpretation of the force responses measured in this study.

The effect of chelerythrine on DIFRs

In this study, around 10–15 DIFRs could be activated in the skinned muscle fibres before the force responses underwent the run-down process. Figure 2A shows a typical example of the DIFRs elicited in skinned rat EDL fibres in this study. In this fibre, DIFRs 3-12 remained relatively constant. After 20 depolarizations, force had diminished to zero. In this study, and our previous experience using the skinned fibre technique (Bakker *et al*., 1996; 1998; Bakker & Berg, 2002), no recovery of DIFRs was ever observed after full run-down had occurred (e.g. this study, last nine depolarizations, Figure 2A). In fibres in which force had run down to low levels, exposure to a weakly buffered Ca^{2+} load solution did not elicit force recovery (Figure 2B). However, the fibres did respond to caffeine with a large force response (77.4±8.2% of maximum force production, five fibres, Figure 2C), indicating that the run-down process found in the skinned fibres in this study was not due to decrease in SR Ca^{2+} content or inhibition of the SR Ca^{2+} release channels. These results indicate that the DIFRs and the pattern of force run-down observed in the fibres in this study are similar to those described by Lamb & Stephenson (1990).

When chelerythrine (12 μM) was applied before run-down had commenced, no run-down in the size of the force responses was observed (Figure 2D). The last two responses in chelerythrine were similar in peak to initial controls (peaks in chelerythrine; 91±7% of controls, $P=0.37$), but the full-width at half maximum was significantly increased to 299±45% of controls ($P=0.04$) ($n=3$). This result indicates that the presence of chelerythrine can also prevent the run-down process from occurring.

In separate experiments, two consecutive depolarizations in the presence of chelerythrine before rundown occurred, had no effect on the peak (111±10%, $P=0.07$) or full-width at half maximum (139±57%, $P=0.20$ ($n=5$) of DIFRs. Control DIFRs elicited after chelerythrine exposure were also similar to initial control responses, suggesting that 12 μM chelerythrine has little direct effect on depolarization-induced force production in normal, fully functional fibres (Figure 3).

When fibres were exposed to chelerythrine after run-down of depolarization-induced force had commenced, the mean peak of the DIFRs returned to control levels. In 21 of 25 cells in which DIFRs had eventually run-down to low levels (6.7±1.7% of maximum force) under control conditions, exposure to chelerythrine (12 μM) resulted in the reactivation of large DIFRs (56.9±4.1% of maximum force) that were of a similar size to the initial control responses (50.8±6.4% of maximum force, $P=0.42$) (Figure 4). No significant correlation between the final extent of force run-down under control conditions and the chelerythrine-induced force recovery level was observed ($r^2=0.005$, $P=0.85$, $n=21$). The responses in the presence of chelerythrine were however, markedly broadened compared to the control response (full-width at half maximum of the responses in chelerythrine, 13.3±1.1 s,

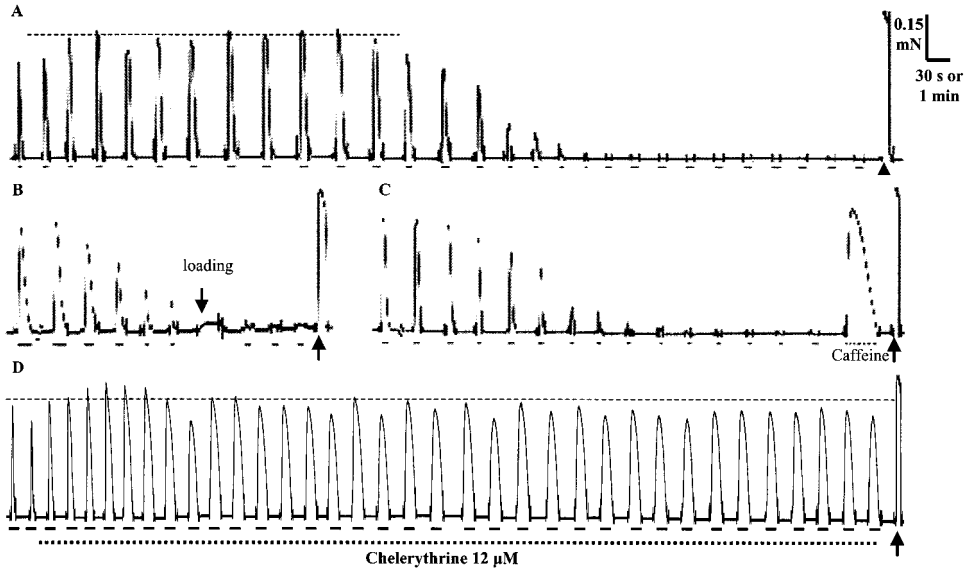

Figure 2 Typical examples of DIFRs elicited in skinned EDL fibres of the rat. (A) Normal use-dependent run-down of DIFRs in skinned EDL fibres of the rat; (B) SR Ca^{2+} loading had no effect on the force run-down of DIFRs; (C) After run-down caffeine (30 mM) was still able to elicit a large force response; (D) chelerythrine (12 μM) prevented the DIFRs run-down. The horizontal bars indicate when depolarization was applied to the fibre. The upright arrows indicate the maximum force responses elicited upon exposure to a 'maximum Ca^{2+} solution' (pCa 4.5). The diagnonal parallel lines in (B) and (C) separate the first control DIFR from later run-down of control DIFRs. Time scale: 30 s during depolarizations and 1 min elsewhere.

controls 3.7±0.3 s, $P<0.001$, paired *t*-test), suggesting that chelerythrine may also affect SR function. A lower concentration of chelerythrine (1.2 μM) had no effect of run-down of DIFRs in this study ($n=3$). In all cases, in fibres where DIFRs had been restored by exposure to chelerythrine after run-down, return of the fibres to control repolarization and depolarization solutions resulted in no activation of DIFRs, indicating that chelerythrine must be continually present to have its effects.

Chelerythrine failed to reactivate force responses after run-down in four fibres producing substantial initial control DIFRs (48.2±8.1% of maximum force). In one fibre, force had run-down to zero, while in the other three fibres, force had run-down to only 17.4±5.7% of maximum force before the fibres were exposed to chelerythrine, indicating that excitation-contraction coupling was still partially functional in three of the fibres immediately before exposure to chelerythrine. In addition, when using the skinned fibre technique there are always a certain percentage of fibres in which DIFRs cannot be elicited, i.e. excitation-contraction coupling has already been in some way uncoupled. Exposure of these fibres to chelerythrine resulted in successful DIFRs in only three of 17 fibres. These results suggest that in some cases the run-down process may be irreversible.

Exposure to a depolarization solution was not always essential for force activation in the presence of chelerythrine. In some cases, functional fibres placed in the K^+-based repolarization solution for approximately 2–5 min in the presence of chelerythrine (12 μM), exhibited one or more large spontaneous force responses (38.8±3.2 of maximum force, $n=8$) (Figure 5) or even continual rhythmic force responses (see last two responses in Figure 5 for an abbreviated example). At a higher concentration of chelerythrine (24 μM), the spontaneous force responses were more prevalent.

In order to determine whether chelerythrine was acting *via* the inhibition of PKC, the PKC inhibitors staurosporine and calphostin C were also examined for their effects on the run-down process. Both staurosporine (100 nM) ($n=5$) (Figure 6A) and calphostin C (250 nM, not shown) ($n=3$) failed to reactivate any depolarization-induced force after force had run-down to low levels. However, later depolarization of the same fibres in chelerythrine resulted in large DIFRs (Figure 6A). Alternatively if PKC was involved in this uncoupling process, the presence of phorbol 12-myristyl 13-acetate (PMA), a potent activator of PKC, should result in the activation of the run-down process in fully functional fibres (i.e. before the run-down process occurred). However, the application of 1 μM PMA had no significant effect on the peak (128±31% of control, $P=0.17$) or the full-width at half maximum (82±31% of control, $n=4$, $P=0.32$) of DIFRs elicited in fully functional fibres (Figure 6B). These results suggest that chelerythrine is able to reverse the run-down of DIFRs in skinned skeletal muscle fibres in this study *via* a pathway other than PKC.

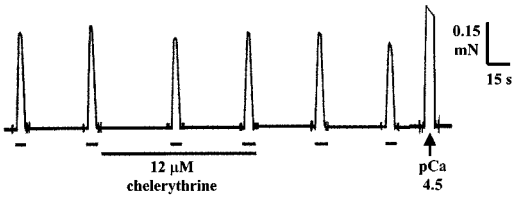

Figure 3 The effect of chelerythrine on DIFRs before run-down had commenced. Incubation of the fibres with 12 μM chelerythrine did not significantly affect the peak or full-width at half maximum of the DIFRs in the skinned EDL fibres of the rat. The short horizontal bars indicate when depolarization was applied.

In this study, chelerythrine had no significant effect on the mean resting membrane potential of randomly chosen intact EDL fibres (before chelerythrine; −69±2 mV, after 12 μM chelerythrine; −65±3 mV, unpaired Student's *t*-test, $P=0.25$, $n=10$), suggesting that chelerythrine is not preventing run-down of DIFRs through effects on the membrane potential.

The effect of chelerythrine on fibres exposed to damaging levels of intracellular Ca^{2+}

As chelerythrine was able to reverse the run-down process, we examined whether chelerythrine also protected against the effects of raised Ca^{2+} levels on the excitation contraction coupling mechanism. In these experiments, after two initial control DIFRs were elicited, the fibre was exposed to a 'rigor solution (see Methods) for 1.5 min to induce a rigor response. DIFRs elicited following rigor were not markedly different to initial controls (Figure 7). The same fibres were then exposed to a rigor/high Ca^{2+} solution (free $[Ca^{2+}]$: ~ 70 μM) for 30 s. Following this rigor/Ca^{2+} treatment, the DIFRs elicited under control conditions were completely eliminated, but DIFRs could still be elicited in the presence of chelerythrine (Figure 7). In a similar experiment (not shown), chelerythrine was also added to the rigor and Ca^{2+}/rigor solutions. Control DIFRs (but not DIFRs in chelerythrine) were still abolished by exposure to the modified rigor and Ca^{2+}/rigor solutions. These results indicate that while excitation-contraction coupling can recover in the presence of chelerythrine after Ca^{2+}-induced damage, chelerythrine itself is not protective against Ca^{2+}-induced damage.

The effect of chelerythrine on sarcoplasmic reticulum function

In this study, chelerythrine was found to increase the width of DIFRs, and induce spontaneous release of Ca^{2+} from the SR in some fibres, suggesting that chelerythrine may also have direct effects on SR function in skeletal muscle. Therefore, we also examined the effect of chelerythrine on SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} leak in the skinned fibres.

In the SR Ca^{2+} release experiments, the fibres were firstly loaded with Ca^{2+} under control conditions. Then the effect of chelerythrine on SR Ca^{2+} release was examined, by comparing force responses elicited upon exposure to a submaximal Ca^{2+} release solution in the presence and absence of chelerythrine (see Methods, also Table 1). Chelerythrine (12 μM) significantly increased the peak of the caffeine-induced force responses by 36.4±9.8% ($P=0.0004$, $n=11$) compared to initial control responses, and by 14.1±2.7% ($P=0.004$, $n=11$) when compared to the mean of the control responses made both before and after SR Ca^{2+} release responses in the presence of chelerythrine (Figure 8A). These results indicate that chelerythrine has a marked

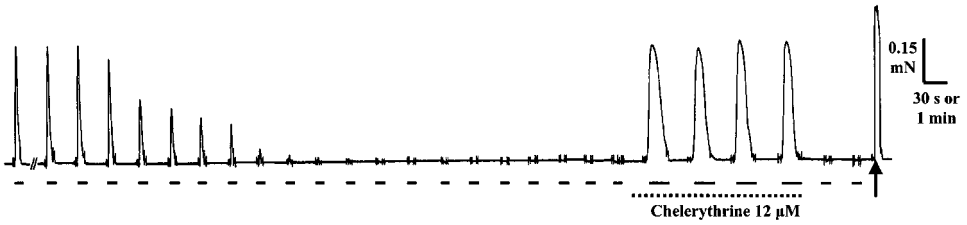

Figure 4 Effect of chelerythrine (12 μM) on DIFRs after complete run-down of control DIFRs. The horizontal bars indicate when depolarization was applied. The upright arrows indicate the maximum force responses elicited upon exposure to a 'maximum Ca^{2+} solution' (pCa 4.5). Time scale: 30 s during depolarizations and 1 min elsewhere.

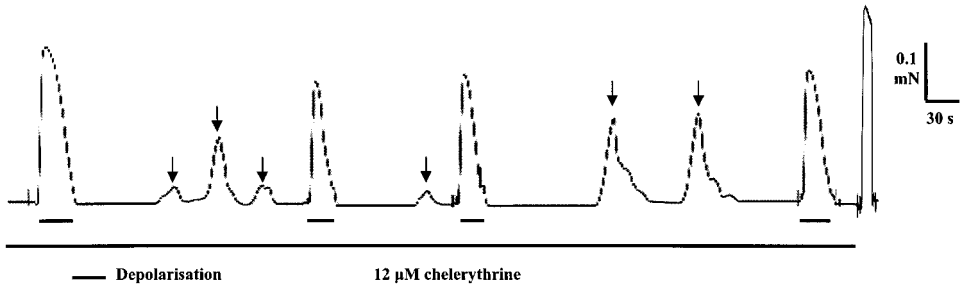

Figure 5 Spontaneous force responses induced by chelerythrine in the K^+ repolarization solution. The arrows indicated the spontaneous force responses induced by chelerythrine.

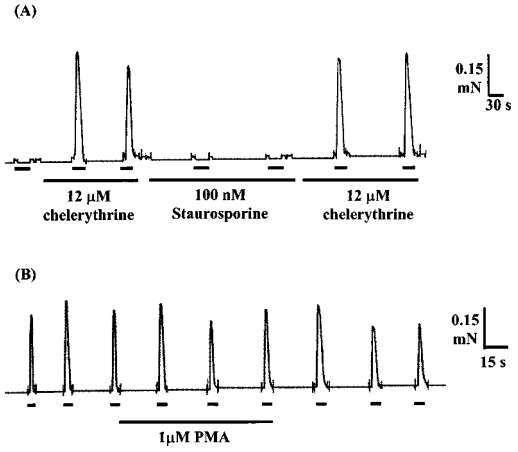

Figure 6 Effects of the PKC inhibitor staurosporine (A) and the PKC activator PMA (B) on DIFRs. Staurosporine (100 nM) did not mimic the effect of chelerythrine in the skinned fibres. PMA did not induce premature run-down of DIFRs in the skinned fibres. The short horizontal bars indicate when depolarization was applied.

stimulatory effect on SR Ca^{2+} release in the skinned fibres, and this effect appears to persist after removal of chelerythrine.

In the SR Ca^{2+} loading experiments, the SR of skinned fibres that had previously been completely depleted of Ca^{2+}, were partly reloaded with Ca^{2+} (in the presence or absence of 12 μM chelerythrine) for a specific period of time, and then the fibres were exposed to a Ca^{2+} release/depletion solution. The integral of the subsequent force responses was taken as a measure of the amount of Ca^{2+} loaded during exposure to the loading solution. Using this protocol, we found that chelerythrine significantly increased SR Ca^{2+} loading to $108.4 \pm 1.7\%$ ($P=0.02$, $n=9$) of control levels (mean of control measurements made before and afer exposure to chelerythrine) in skinned EDL fibres (Figure 8B). A comparison of the SR loading in the presence of chelerythrine and the initial control measurement only, yielded a similar difference ($109.5 \pm 1.9\%$, $P=0.01$).

The increased SR Ca^{2+} accumulation activated by chelerythrine could also be due to decreased SR Ca^{2+} leak (Bakker *et al.*, 1996). However, measurements of SR Ca^{2+} leak in the presence and absence of chelerythrine (see Methods) were not significantly different (chelerythrine leak; $105.1 \pm 2.3\%$ of controls, $P=0.054$, $n=12$) (not shown), indicating that the increased SR Ca^{2+} accumulation in the presence of chelerythrine reported in this study is the result of increased SR Ca^{2+} uptake.

Discussion

The most important finding of this study was that the benzophenanthridine drug chelerythrine rapidly reversed the use-dependent run-down of DIFRs that occurs in skinned fast-twitch skeletal muscle fibres of the rat. To our knowledge this is the first report of a substance that can reverse what until now was though to be an irreversible process. The use-dependent run-down in force in skinned fibres bears many similarities to the loss of excitation-contraction coupling that occurs after contraction-induced muscle injury in intact muscle. In both, the main focus of the disruption has been narrowed down to events that occur between the voltage sensors and the SR Ca^{2+} release channels (Warren *et al.*, 2001).

Lamb *et al.* (1995) showed that experimental elevations in intracellular [Ca^{2+}] also abolished E-C coupling in skinned

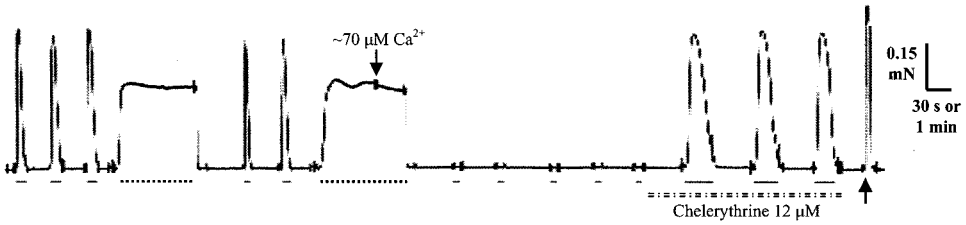

Figure 7 Exposure to a 'rigor solution' containing ~70 μM free [Ca^{2+}] for 30 s resulted in abolition of control DIFRs. DIFRs elicited in the presence of chelerythrine were not affected. Exposure to the rigor solution alone did not substantially affect the control DIFRs. The horizontal bars indicate when depolarization was applied. The dash lines indicate that the fibre was exposed to a rigor solution. The upright arrows indicate the maximum force responses elicited upon exposure to a 'maximum Ca^{2+} solution' (pCa 4.5). Time scale: 30 s during depolarizations and 1 min elsewhere.

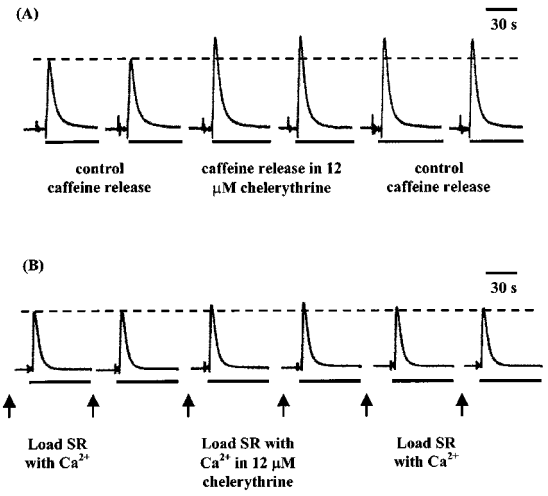

Figure 8 Effect of chelerythrine on caffeine-induced Ca^{2+} release (A) and SR Ca^{2+} loading (B) in skinned EDL fibres. (A) The fibres were loaded with Ca^{2+} for a fixed period of time (10 s) in a highly buffered Ca^{2+} solution (pCa 6.55) before exposure to a 'submaximal Ca^{2+} release solution'. Chelerythrine was only present in the caffeine release solution. Chelerylthrine increased the peak of the caffeine responses. (B) In this experiment chelerythrine was present only in Ca^{2+}-loading solution before the 3rd and 4th responses shown. SR Ca^{2+} loading in the presence of chelerythrine increased the integral of the caffeine responses.

skeletal muscle fibres, and proposed that uncoupling (and run-down of DIFRs) may result from the effects of high myoplasmic [Ca^{2+}] during repeated contraction rendering the voltage sensors, or some protein/s linking the voltage sensor to the ryanodine receptor, dysfunctional. The ryanodine receptor itself appears to be unaffected by the run-down process, as a normal caffeine response could be elicited after run-down had occurred (Lamb *et al*., 1995; this study). In this study, exposure to high free Ca^{2+} concentrations (~70 μM) under rigor conditions also rapidly abolished control DIFRs, but DIFRs elicited in the presence of chelerythrine persisted. Addition of chelerythrine to the high Ca^{2+} solution failed to protect control DIFRs from the Ca^{2+}-induced damage. Taken together these results indicate that chelerythrine acts to compensate for the uncoupling of ECC in some way, rather than preventing it occurring.

In a few fibres, exposure to chelerythrine did not restore excitation-contraction coupling in the skinned fibres. It is possible that these fibres may have experienced greater Ca^{2+}-induced effects than other fibres, resulting in large scale ultrastructural damage to the triadic region (Lamb *et al*., 1995) or high levels of membrane phospholipid degradation (Jackson *et al*., 1984; Duncan & Jackson, 1987). Even in the presence of chelerythrine, there was a gradual drop in the size of these responses (Figure 2D). These results suggest either that chelerythrine cannot completely inhibit run-down, or that more than one process is involved in triggering the loss of excitation-contraction coupling in the skinned fibres.

How chelerythrine reverses the run-down process is unknown, however it is unlikely to be *via* inhibition of PKC as two other potent PKC inhibitors, staurosporine and calphostin C, failed to reverse the uncoupling process. The PKC agonist PMA also failed to induce premature run-down of DIFRs in the skinned fibres. Furthermore, 1.2 μM chelerythrine, a concentration almost twice the dose reported for half maximal inhibition of protein kinase C (0.66 μM, Herbett *et al*., 1990) had no effect on the run-down process in the skinned fibres in this study. Therefore, this study provides another example of a non-PKC dependent action of chelerythrine.

It is possible that chelerythrine may act by phosphorylating proteins in the triadic junction. Chelerythrine has been shown to stimulate the phosphorylation of a ~20 K M_r protein present in the mitochondrial fraction of rat retina (Lombardini, 1995). However, this is unlikely as Lamb *et al*. (1995) found that the uncoupling process was not noticeably affected by the presence of ATP, protein kinase inhibitors, or phosphatase inhibitors.

A more likely possibility is that chelerythrine interacts with sulphhydryl groups located on the triadic proteins involved in the excitation-contraction coupling process. Past studies have shown that chelerythrine acts in many cases by inducing a reversible adduct formation between thiol groups on target proteins and the imminium bond of the chelerythrine molecule (Walterová *et al*., 1981; Wolff & Knipling, 1993). Skeletal muscle ryanodine receptors have large numbers of reactive sulphhydryl groups (Takeshima *et al*., 1989) and oxidation of reactive sulphhydryls on both L-type Ca^{2+} channels and ryanodine receptors have been shown to alter channel function (Campbell *et al*., 1996; Trimm *et al*., 1986; Zable *et al*., 1997). Therefore, chelerythrine may directly and reversibly bind to critical thiol groups on the RyRs or other proteins important for linking voltage sensor activation to ryanodine receptor and restore normal activity. Chelerythrine also enhanced SR Ca^{2+} pump function in the skinned fibres

in this study, and likewise, skeletal muscle SR Ca^{2+}-MgATPase molecules also have many reactive sulphhydryls that are thought to play a role in modulation of pump activity (Saito-Nakatsuka *et al*., 1987; Yamashita & Kawakita, 1987; Kawakita & Yamashita, 1987). This mechanism of action of chelerythrine would explain the diversity of effects of chelerythrine on excitation-contraction coupling in skinned fibres. Furthermore, the relatively temporary nature of most of the effects produced by chelerythrine in this study is also consistent with the reversible nature of the adduct formation that occurs between chelerythrine and its target molecules.

It is unlikely that orthodox redox reactions are responsible for the effects of chelerythrine as high concentrations of the reducing agents dithiothreitol and reduced glutathione had no perceivable effects on the size of the DIFRs in functioning fibres, nor prevented the run-down of the responses. However, the reducing agents did reverse the normal inhibitory effects of oxidation on excitation-contraction coupling in skinned fibres (Lamb *et al*., 1995; Posterino & Lamb, 1996).

Another possible mechanism of chelerythrine's action could be to increase Na^+/K^+-ATPase activity in the skinned fibres. Chelerythrine has been reported to activate the Na^+/K^+-ATPase in isolated rat hearts (Lundmark *et al*., 1999). If run-down is due to some degree to the steady depolarization of the sealed T-system, increased Na^+/K^+-ATPase activity in the presence of chelerythrine could help to prevent this process or increase the rate of repolarization and assist in recovery of ECC. In this study, chelerythrine was found to have no significant effect on the membrane potential in intact EDL fibres. However, in cardiac myocytes for example, Na^+/K^+-ATPase activity has been shown to contribute only a few mV to the membrane potential (Stimers *et al*., 1990; Levi, 1992) and therefore, any affect of chelerythrine on Na^+/K^+-ATPase activity may not shown up as a large change in membrane potential. It should be noted however, that in rat brain neurons chelerythrine has been reported to inhibit Na^+/K^+-ATPase activity (Cohen *et al*., 1978).

The results of this study also show that chelerythrine has a number of significant direct effects on the function of the SR in skinned skeletal muscle. Chelerythrine was found to significantly increase caffeine-induced Ca^{2+} release in the skinned fibres, a finding consistent with a recent report that sanguinarine, another benzophenathridine drug, induces Ca^{2+} release from SR vesicles at a similar concentration (10–20 μM) (Hu *et al*., 2000). It is unlikely, however, that the effects of chelerythrine on run-down are purely due to a caffeine-like effect on SR Ca^{2+} release. Firstly, the SR Ca^{2+} release channel is still functional after run-down in skinned fibres and therefore, enhancing Ca^{2+} release cannot repair the excitation-contraction coupling process on its own. Secondly, exposure to chelerythrine in the K^+-based repolarization solution for the usual 30 s had no immediate effect on force in most fibres, while exposure to chelerythrine in the depolarization solution immediately resulted in a large force response in a vast majority of fibres. However, it is possible that effects of chelerythrine on SR function help to promote the return of DIFRs induced by chelerythrine in the skinned fibres. Run-down of DIFRs in skinned fibres appears to result from a gradual uncoupling of T-tubular depolarization and SR Ca^{2+} release, leading to a reduction in the amount of depolarization-induced SR Ca^{2+} release. The enhancement of CICR (and SR Ca^{2+} loading) induced by chelerythrine may enable the fibres to react with a large force response (mainly *via* CICR) in response to the decreased depolarization-induced Ca^{2+} release that occurs during and after run-down.

Chelerythrine did induce large repeated, spontaneous force responses in a number of fibres, if fibres were exposed to chelerythrine for longer periods of time in the repolarization solution (2–5 mins). This indicates that prolonged exposure to chelerythrine can lead to SR Ca^{2+} release in fibres under conditions where the voltage sensors remain polarized. Chelerythrine was shown to significantly increase both the caffeine responses and SR Ca^{2+} uptake in the skinned fibres in this study. Caffeine is reported to trigger SR Ca^{2+} release in mammalian skeletal muscle by increasing Ca^{2+}-induced Ca^{2+} release (CICR) (Fryer & Neering, 1989; Gallant *et al*., 1995), which suggests that chelerythrine also enhances CICR in the skinned fibres used in this study. CICR has been clearly demonstrated in mechanically skinned fibres of the rat, however, this phenomenon was only observed in skinned fibres in which the SR had been over-loaded with Ca^{2+} (Lamb & Stephenson, 1990). Therefore, the spontaneous force response triggered by longer chelerythrine exposure in this study are likely to have occurred due to chelerythrine-induced SR Ca^{2+} uptake gradually leading to SR Ca^{2+} overload. SR Ca^{2+} overload, in combination with the enhancing effects of chelerythrine on the SR Ca^{2+} release channels, could then lead to a sudden spontaneous release of Ca^{2+} from the SR. It should be noted that in this study, the SR Ca^{2+} loading experiments were undertaken using a highly Ca^{2+} buffered SR Ca^{2+} load solutions where loading under control conditions occurred very quickly. During the depolarization experiments, the Ca^{2+} buffering was substantially less and the effect of chelerythrine on Ca^{2+} accumulation may be even greater.

This study shows that chelerythrine can reverse uncoupling of excitation-contraction coupling in skeletal muscle fibres after run-down or exposure to high Ca^{2+} concentrations. Chelerythrine may act at the level of the voltage sensors, or proteins linking the voltage sensors to the SR Ca^{2+} release channels, the same region thought to be responsible for contraction induced muscle injury in intact muscle (Westerblad *et al*., 2000; Warren *et al*., 2001). Chelerythrine could provide a valuable pharmacological tool for examining the mechanism of Ca^{2+}- and/or contraction-induced uncoupling of excitation-contraction coupling in skeletal muscle, which could provide new insights into the mechanisms of long-term muscle fatigue.

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