Effects of ADP on sarcoplasmic reticulum function in mechanically skinned skeletal muscle fibres of the rat

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1. The sarcoplasmic reticulum (SR) Ca^{2+} content (expressed in terms of endogenous SR Ca^{2+} content under physiologically resting conditions and measured from caffeine-induced force responses) and the effective rates of the SR Ca^{2+} pump and SR Ca^{2+} leak (measured from the temporal changes in SR Ca^{2+} content) were determined in mechanically skinned skeletal muscle fibres of the rat at different [ADP] (< 0.10 μM to 1.04 mM).

2. The estimated SR Ca^{2+} pump rate at 200 nM Ca^{2+} did not change when [ADP] increased from below 0.10 μM to 10 μM but decreased by about 30% when [ADP] increased from 10 μM to 1.04 mM.

3. The rate constant of SR Ca^{2+} leak increased markedly with rising [ADP] when [Ca^{2+}] in solution was 200 nM (apparent dissociation constant $K_d^{ADP} = 64 \pm 27$ μM). Decreasing the [Ca^{2+}] in solution from 200 nM to < 10 nM significantly increased the leak rate constant at all [ADP]. The SR Ca^{2+} leak rate constant could be significantly reduced by blocking the SR Ca^{2+} pump with 2,5-di(*tert*-butyl)-1,4-hydroquinone (TBQ).

4. The decrease in the SR Ca^{2+} pump rate and the increase in the rate constant of SR Ca^{2+} leak when the [ADP] increased from < 0.10 μM to 1.04 mM caused a 4.4-fold decrease in SR Ca^{2+} loading ability at 200 nM Ca^{2+}.

5. The results can be fully explained by a mechanism whereby the presence of ADP causes a marked increase in the ADP-sensitive fraction of the phosphorylated pump protein, which can act as a Ca^{2+}–Ca^{2+} exchanger and demonstrates that ADP is an important modulator of SR function in skeletal muscle.

Skeletal muscle fatigue is a multi-factorial process where multiple steps in the excitation–contraction coupling can be affected by prolonged intermittent stimulation of muscle (Stephenson *et al.* 1995). A factor whose role in muscle fatigue is not clearly understood is the rise in ADP concentration from about 10 μM in skeletal muscle at rest to more than 200 μM after prolonged, intermittent activation of the muscle (Dawson *et al.* 1978; Nagesser *et al.* 1993). Moreover, using the data from Nagesser *et al.* (1993), Westerblad & Lännergren (1995) calculated that ADP could rise to as high as 3.0 mM during contraction, when the creatine phosphate stock is depleted due to the relatively low activity of myokinase.

At the sarcoplasmic reticulum (SR) level, unidirectional Ca^{2+} flux measurements under steady state conditions of Ca^{2+} loading in both cardiac and skeletal SR vesicles have shown that micromolar levels of ADP stimulate rapid exchange between intravesicular and extravesicular Ca^{2+} (Wass & Hasselbach, 1981; Soler *et al.* 1990) suggesting that under conditions of ADP elevation the SR Ca^{2+} pump could also operate as a Ca^{2+}–Ca^{2+} exchanger (Chiesi & Wen, 1983; Inesi & de Meis, 1989; Soler *et al.* 1990). It is important to point out that Ca^{2+} movements across the SR membrane via this Ca^{2+}–Ca^{2+} exchanging mechanism do not involve hydrolysis or synthesis of ATP and therefore the loss of Ca^{2+} from the SR via the pump acting as a Ca^{2+}–Ca^{2+} exchanger does not imply the reversal of the pump whereby ATP is synthesised. This ADP-dependent Ca^{2+}–Ca^{2+} exchanger in the SR membrane can have marked effects on the SR function during fatigue but there are no studies done under more physiological conditions examining the effect of ADP on the SR function in skeletal muscle and therefore it is currently not possible to predict what effect an increase in [ADP] will have on SR function.

In this study, we used the freshly dissected mechanically skinned muscle fibre preparation in conjunction with solutions where the concentration of ADP was varied and we examined the effect of ADP on the SR function. In this preparation the SR remains intact and physiological

Table 1. Composition of solutions

Solution	Ca^{2+} (μM)	$EGTA_{total}$ (mM)	HDTA* (mM)	CP (mM)	ADP (mM)	Free Mg^{2+} (mM)
Max activating	30	50	—	10	—	1.0
High relaxing	< 0.001	50	—	10	—	1.0
Low relaxing$_1$	0.05	0.05	50	10	—	1.0
Low relaxing$_2$	0.05	0.05	60	—	—	1.0
Load$_1$	0.2	1.0	50	10	—	1.0
Load$_2$	0.2	1.0	60	—	—	1.0
Load$_3$	0.2	0.5	50	10	—	1.0
Release	0.002	1.0	50	10	—	0.02
Leak$_1$	0.002	1.0	50	10	—	1.0
Leak$_2$	0.002	1.0	60	—	—	1.0
Wash$_1$	0.002	1.0	50	10	—	1.0
Wash$_2$	0.002	1.0	60	—	—	1.0
Wash$_3$	0.005	0.5	50	10	—	1.0
ADP stock	—	—	30	—	20	1.0

All solutions were adjusted to pH 7.10 at 23 ± 1 °C and contained (mM): K^+, 126; Na^+, 37; total ATP, 8.0; Hepes, 90; NaN_3, 1.0. The Release solution also contained 30 mM caffeine (added as a solid). Solutions Load$_2$ and Leak$_2$, Low relaxing$_2$ and Wash$_2$ could have their ADP level altered by adding various amounts of ADP stock solution (see below). Total Ca^{2+} and Mg^{2+} in solutions varied to provide the indicated free Ca^{2+} and free Mg^{2+} concentrations. Note that under our conditions EGTA, CaEGTA, HDTA and CP exist almost exclusively as divalent anions. *HDTA, hexamethylene diamine tetraacetate.

conditions can be accurately mimicked. The results show that ADP elevation in the micromolar range markedly reduces the ability of the SR to store Ca^{2+} at physiological myoplasmic Ca^{2+} levels by increasing the passive leak of Ca^{2+} from the SR via the pump Ca^{2+}–Ca^{2+} exchanger mechanism and by decreasing the rate of the SR Ca^{2+} pump and can explain a number of previously not understood features occurring in the fatigued muscle.

METHODS

Dissection, preparation of fibres and apparatus

Male rats (Long-Evans, 3 months old) were killed by halothane overdose (2% v/v) in accordance with permits issued by La Trobe University Animal Ethics Committee. The extensor digitorum longus (EDL) muscles were quickly removed, well blotted on filter paper (Whatman No. 1), and then placed in a dish containing paraffin oil (Ajax Chemicals, Sydney, Australia), above a layer of Sylgard 184 (Dow Chemicals, Midland, MI, USA). Single muscle fibres were then isolated, mechanically skinned with fine forceps (jeweller's forceps No. 5) under a dissecting microscope and mounted on a force transducer (AME875, SensoNor, Horton, Norway), whilst under oil as previously described (Fink *et al.* 1986). The length (L) and diameter (D) were measured using a dissecting microscope as described previously (Lamb & Stephenson, 1990). The preparation was then stretched to 120% of its slack resting length to facilitate measurement of force production in any segment of the preparation and was finally placed into a 2 ml Perspex bath containing a potassium hexamethylene diamine tetraacetate (HDTA) relaxing solution, mimicking the myoplasmic environment (see below). The dish with paraffin oil containing the muscle was placed on an ice pack to keep it cool between dissections of mechanically skinned fibre segments. The apparatus used in these experiments and the procedure of changing solutions have been described in detail earlier (Stephenson & Williams, 1981).

Solutions

Solutions were prepared as described by Stephenson & Williams (1981). Table 1 shows the composition of solutions used in this study. The osmolarity of all solutions was 290 ± 10 mosmol kg^{-1} as measured with a vapour pressure osmometer (5500 Wescor, Logan, UT, USA), and unless otherwise stated they contained (mM): K^+, 126; Na^+, 37; total ATP, 8.0; MgATP, 7.0; Hepes, 90; free Mg^{2+}, 1.0; NaN_3, 1.0. Maximum Ca^{2+}-activated force was determined by exposing a fibre to Max activating solution (referred to as 'max' in Fig. 4). The Release solution contained 30 mM caffeine and a low ionised Mg^{2+} concentration (0.02 mM) to facilitate the rapid and thorough release of SR Ca^{2+} (Fryer & Stephenson, 1996). The concentration of Ca^{2+} in the Load solutions was determined with a Ca^{2+} electrode (Orion, Boston, MA, USA). HDTA and 2,5-di(*tert*-butyl)-1,4-hydroquinone (TBQ) were obtained from Fluka (Buchs, Switzerland) and all other chemicals were obtained from Sigma (St Louis, MO, USA) unless stated otherwise. All experiments were conducted at room temperature (22 ± 2 °C).

Creatine build-up

The freshly mechanically skinned fibres are known to retain a high creatine phosphokinase activity associated with the myosin A-band (Saks *et al.* 1978). Therefore, when CP is present in solution in millimolar concentrations and in the absence of added creatine, the level of ADP which can accumulate in the fibre is expected to remain very low at all times due to the equilibrium reaction between ADP and CP which is displaced towards the formation of ATP (ADP + CP $\rightleftharpoons$ ATP + creatine, K (equilibrium constant) = 260, Chase & Kushmerick, 1995). The average creatine concentration in the preparations is estimated at less than 33 μM under our conditions (10 mM CP, 8 mM ATP, $pCa = -\log_{10}[Ca^{2+}] = 6.7$, see below) and this corresponds to < 0.10 μM ADP (< 8 mM × 33 μM/(260 × 10 mM)). The maximum contribution to [creatine] of 10 mM CP in solutions is 30 μM because the total [P_i] in these solutions was 30 μM (Fryer *et al.* 1995), indicating that at most 30 μM CP could have broken down into P_i and creatine. Furthermore, the average build-up of creatine during SR

Ca^{2+} loading is less than 3 μM. This latter value was estimated from the total ATPase activity in the fibre at pCa 6.7 in solutions containing 10 mM CP and 8 mM ATP.

First, the SR Ca^{2+} pump rate under these conditions was estimated in this study (see Results) to be 80 % of endogenous SR Ca^{2+} min^{-1} which is equivalent to 0.007 mM ATP s^{-1} assuming that two Ca^{2+} ions are transported per molecule of ATP hydrolysed and an endogenous SR [Ca^{2+}] of 1.1 mM (Fryer & Stephenson, 1996). Second, the myofibrillar ATPase rate at pCa 6.7 is close to background levels (0.018 mM ATP s^{-1}, Stephenson *et al.* 1989), because the contractile apparatus is in a relaxed state. Since each ATP molecule hydrolysed will generate one molecule of creatine, the rate of creatine production ($Creatine_{rate}$), can be assumed to be 0.025 mM s^{-1} under our conditions. In a typical fibre preparation of 20 μm radius, r, the average concentration of creatine built up in the fibre will be 3 μM given by the following expression:

$$[\text{Creatine}] = \text{Creatine}_{\text{rate}} \times r^2/8\delta, \quad (1)$$

where δ is the diffusion coefficient for creatine (about 4×10^{-6} cm^2 s^{-1}; Kushmerick & Podolsky, 1969).

ADP build-up

When CP is removed from solutions, the level of ADP is expected to increase markedly during SR Ca^{2+} loading, first because of contamination of ATP with ADP, and second because of the ADP build-up as a consequence of ATP hydrolysis in the fibre. The contribution of 8 mM ATP to P_i in solutions was 40 μM for solutions used in this work (as determined by the molybdate technique reported by Chifflet *et al* (1988), and modified by Patterson *et al* (2000)). Therefore, the contribution of [ADP] cannot exceed 40 μM. The build-up of ADP as a consequence of ATP hydrolysis in the fibre can be further estimated by a similar procedure to that indicated for creatine accumulation. Assuming that each ATP molecule hydrolysed will generate one molecule of ADP and that the diffusion coefficient for ADP is similar to that for ATP (1.2×10^{-6} cm^2 s^{-1}; Kushmerick & Podolsky, 1969), the average build-up of ADP in a 20 μm diameter fibre is 1.6 μM. Therefore, the total [ADP] in the fibre during SR loading experiments will be less than 42 μM. For the purpose of this study it will be assumed that the total [ADP] in preparations during SR Ca^{2+} loading in solution without CP was 40 μM. The concentration of ADP in preparations loaded in CP-free solutions with added ADP was also corrected by 40 μM. In these experiments we did not add a myokinase inhibitor because [ATP] was high in all experiments, and therefore the concentration of ADP which would have been converted to ATP and AMP was estimated to be less than 0.7 μM in preparations bathed in solutions without CP and without added ADP ($2ADP \rightleftharpoons ATP + AMP$, $K = 1.25$; Lehninger, 1970, p. 308).

Caffeine-induced responses

Caffeine-induced force responses can be used to estimate the relative amount of Ca^{2+} in the SR by referring to the relative area under the caffeine-induced force response (Endo & Iino, 1980; Launikonis & Stephenson, 1999). Before commencement of repeated cycles of Ca^{2+} release and reloading the skinned fibre was initially equilibrated for 2 min in Low relaxing$_1$ solution where the SR remained loaded at the endogenous level. The endogenous Ca^{2+} content of the SR was then determined from the area under the caffeine-induced SR Ca^{2+} release response. First, the skinned fibre was placed in Wash$_1$ solution for 30 s and then rapid Ca^{2+} release was triggered by transferring the preparation to Release solution containing 30 mM caffeine. The presence of 1.0 mM EGTA in this release solution ensured that the level of Ca^{2+} during caffeine-induced release did not maximally activate the contractile apparatus, which is necessary to avoid deterioration of the preparation. The area under the response upon first depletion of the SR was used to compare the level of loading of the preparation relative to the endogenous level of SR Ca^{2+}. The fibre was left in the Release solution for 2 min to ensure complete Ca^{2+} depletion (Fryer & Stephenson, 1996), before washing for 30 s in a Wash solution. Thereafter the SR was reloaded with Ca^{2+} in one of the Load$_{1-3}$ solutions under different conditions (see below), and following various protocols the SR Ca^{2+} was released in the Release solution with caffeine before the cycle was repeated. Force was continuously recorded on a chart recorder and the relative area under the caffeine-induced force response was measured using the gravimetric method (Fink & Stephenson, 1987).

The areas under the appropriate caffeine-induced force responses could be used to estimate the amount of Ca^{2+} in the SR providing that the areas and the SR Ca^{2+} content are directly proportional to each other (Launikonis & Stephenson, 1997). Figure 1 shows results obtained from six mechanically skinned rat fibres loaded for different durations in Load$_1$ solution and then exposed to Release solution. The data points for individual fibres have been divided by the area obtained under the standard loading conditions (2 min) for a particular fibre. This allowed for analysis of data points from different fibres. The results in Fig. 1 show that under our conditions there is approximately a linear relationship of the area under the caffeine-induced force response and the loading time. Launikonis & Stephenson (1997) used a correction procedure to ensure direct proportionality between areas under the caffeine-induced force responses and the SR Ca^{2+} content. This procedure shifts the origin of the x–y-axes to the point where the line of best fit intersects the y-axis. In Fig. 1, the line of best fit intersects the y-axis at −32.7 % of the area corresponding to the standard loading conditions and the correction would entail adding 32.7 % to all area values to ensure direct proportionality between areas under the caffeine-induced force responses and SR Ca^{2+} content. We used this value to correct the relevant areas and to calculate the relative changes in SR Ca^{2+} content for these conditions (Fig. 2).

The amount of endogenous Ca^{2+} released from the SR immediately after skinning produced a caffeine-induced response that was similar (113 ± 26 %, $n = 5$) to the caffeine-induced response after the SR was loaded with Ca^{2+} for 2 min under our standard conditions (Load$_1$ solution). It is therefore reasonable to conclude that the Ca^{2+} loading of the SR under standard conditions was close to that occurring *in*

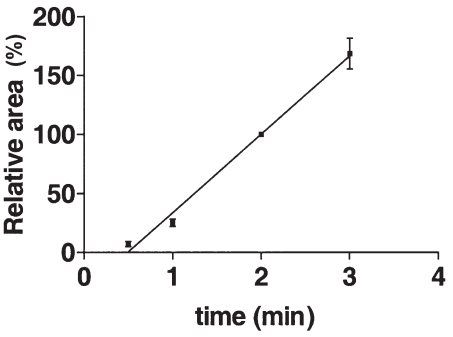

Figure 1. Relationship between mean areas under the caffeine-induced force responses and duration of SR Ca^{2+} loading

Areas under the caffeine-induced force responses for the individual fibres ($n = 6$), when the preparations were moved from Wash$_1$ to Release solution, were normalised to the area obtained under standard loading conditions (2 min) for that particular fibre and are shown as means ± S.E.M.; 100 % corresponds to 90 % endogenous level SR Ca^{2+} (see Methods). The data were fitted by linear regression.

vivo (see also Launikonis & Stephenson, 1997). For the purpose of this study the SR Ca^{2+} loading under standard conditions was assumed to represent 90 % of the endogenous SR Ca^{2+} content.

Load experiments

The SR's ability to load Ca^{2+} was investigated by loading the SR in the $Load_1$ solution in the presence of 200 nM [Ca^{2+}] and releasing SR Ca^{2+} in Release solution, after 30 s in $Wash_1$ solution or by loading in $Load_2$ solution with (0.1, 0.2, 1.0 mM ADP) or without additional ADP, followed by a 30 s wash in $Wash_1$ solution and release in Release solution. The area under the ensuing force response was an indication of the amount of Ca^{2+} the SR could load for that loading duration. The response to standard loading (2 min in $Load_1$ solution) and SR Ca^{2+} release in Release solution was monitored throughout an experiment to allow correction for the rather small deterioration (< 4 %) in SR function between standard loading controls. The correction procedure involved the normalisation of a particular response by the interpolated value of the response to standard loading. For all conditions used in this study, the SR was submaximally loaded with Ca^{2+} (< 35 %) as judged from the force responses in the Release solution after loading the same preparations in the presence of 200 nM and 1 μM Ca^{2+} for 10 min in the presence of 10 mM CP.

Leak experiments

To estimate the percentage of Ca^{2+} lost from the SR due to the passive leak of Ca^{2+} under different conditions, the following experimental procedure was used. The fibre was loaded for 2 min in $Load_3$ solution (Table 1), which has the same [Ca^{2+}] as the $Load_1$ solution, but a lower total [EGTA]. The preparation was then washed in $Wash_1$ solution for 30 s and then the SR Ca^{2+} content was released in Release solution (control). Thereafter, the preparation was washed in $Wash_3$ solution prior to re-loading for 2 min in $Load_3$ solution, transferred to $Leak_1$ solution (control) or $Leak_2$ solution (with or without added ADP for test) for 60 s, washed in $Wash_1$ solution for 30 s and the remaining SR Ca^{2+} was released in Release solution. The control was then repeated and the area (corrected for proportionality between area and SR Ca^{2+} content) under the test run was divided by the average of the corrected areas under the caffeine-induced force responses in the controls before and after the test run. This gives an estimate of the fraction of SR Ca^{2+} leaked over the 60 s in $Leak_2$ solution.

SR Ca^{2+} leak was also measured under conditions where the SR Ca^{2+} pump was blocked with 20 μM TBQ. TBQ stock solution was obtained by dissolving TBQ in pure DMSO (dimethyl-d_6 sulfoxide) at a concentration of 20 mM. $Wash_3$ solution contained 20 μM TBQ and the corresponding control $Wash_3$ solution contained the same concentration of DMSO as the $Wash_3$ solution with TBQ. In all TBQ experiments, solutions were made in double the required volume, mixed and split into two equal parts, thus ensuring the identical free [Ca^{2+}] in both sets of solutions. The fibre was depleted of endogenous Ca^{2+}, as described above, and then loaded with Ca^{2+} for 2 min in $Load_3$ solution prior to passively 'leaking' in $Leak_2$ solution for 120 s. The fibre was then placed in $Wash_2$ solution for 30 s, dipped in paraffin oil for 30 s to remove TBQ from the skinned fibre (see Bakker *et al.* 1996), washed in $Wash_3$ solution for 15 s, prior to depletion of Ca^{2+} in Release solution. The cycle was then repeated with or without TBQ in $Leak_2$ solution. The corrected area under the caffeine-induced force response was compared to a 2 min load control, the difference in size being due to the Ca^{2+} lost from the SR during the leak period.

Data analysis

Results are expressed as means ± S.E.M. and curve fitting and statistical analyses were performed using the scientific analysis program GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Effects of ADP on SR Ca^{2+} loading

In order to find out whether the ability of the SR to load Ca^{2+} was affected by ADP, experiments were conducted where the SR was loaded submaximally with Ca^{2+} for a variety of loading times (0.5, 1, 2, 3 and 10 min) and under conditions where [ADP] was varied between < 0.10 μM and 1.04 mM (see Methods). After loading under different [ADP] conditions, the fibre was washed in $Wash_1$ solution and the SR Ca^{2+} was always released in Release solution to facilitate quantification of results. The results are shown in Fig. 2 and can be accurately described by exponential expressions of the type:

$$a(1 - e^{-bt}), \quad (2)$$

where a and b are related to the two major processes that occur at the SR level during SR Ca^{2+} loading. First, Ca^{2+} is being loaded into the SR by the SR Ca^{2+} pump, and second, Ca^{2+} is leaking out of the SR. Thus the rate of SR Ca^{2+} change, $(d[Ca^{2+}]/dt)_{SR}$, can be assumed to be:

$$(d[Ca^{2+}]/dt)_{SR} = \text{pump rate} - \text{SR } Ca^{2+} \text{ leak rate}. \quad (3)$$

The pump rate is supposed to be constant in the first approximation for the loading duration and the SR Ca^{2+} leak rate is assumed to be proportional to the overall SR permeability to Ca^{2+} and to the ionised Ca^{2+} in the SR, which in turn is proportional to the total SR Ca^{2+}, $[Ca^{2+}]_{SR}$, considering that in all our experiments the SR was submaximally loaded with Ca^{2+} (i.e. calsequestrin was far from being saturated with Ca^{2+}, see Methods). Therefore:

$$(d[Ca^{2+}]/dt)_{SR} = \text{pump rate} - \beta[Ca^{2+}]_{SR}, \quad (4)$$

where β is a rate constant proportional to the overall SR permeability to Ca^{2+}. Integration of differential eqn (4) leads to the following expression:

$$[Ca^{2+}]_{SR}(t) = \text{pump rate}/\beta(1 - e^{-\beta t}), \quad (5)$$

where t is the loading time and $[Ca^{2+}]_{SR}(t)$ is the Ca^{2+} content of the SR at time t. From eqns (2) and (5) it follows that $\beta = b$ and pump rate $= ab$. Therefore, in principle one should be able to estimate the pump rate and the relative Ca^{2+} permeability of the SR, β, for each condition. The r^2 value for all fits to the data points was 0.96 or greater, indicating a high correlation and goodness of fit of all curves. From eqns (4) or (5) it is also possible to calculate the SR Ca^{2+} loading capacity for $t = \infty$(pump rate/β) when the Ca^{2+} uptake equals the Ca^{2+} leak and no further accumulation of Ca^{2+} into the SR occurs.

To increase accuracy of the data, experiments were performed using six preparations loaded with Ca^{2+} at pCa 6.7 and different ADP concentrations (< 0.10 μM, ~40 μM, 0.14 mM, 0.24 mM and 1.04 mM; see Methods) and the results are shown in Fig. 2*A* and *B*. As can be clearly seen in Fig. 2*A* and *B*, the SR was able to load and subsequently release substantially less Ca^{2+} when the

loading took place in the presence of elevated ADP. Figure 2*A* shows the time course of SR Ca^{2+} loading at pCa 6.7 in the presence of CP when the [ADP] was estimated to be < 0.10 μM (see Methods) and in the absence of CP in the solution where the [ADP] was estimated to be about 40 μM (see Methods). As one can see, the SR Ca^{2+} loading capacity was greatly reduced when CP was omitted from the loading solution. Further experiments were performed to determine whether the effects shown in Fig. 2*A* were due to the removal of CP from solutions or due to the presence of ADP in the preparation when no CP was present. Assuming that about 40 μM ADP would be present in fibres during SR Ca^{2+} loading in the absence of CP (see Methods), we have chosen to buffer [ADP] to close to 40 μM with 12 mM creatine in the presence of 8 mM ATP, 10 mM CP and endogenous creatine phosphokinase (ADP + CP $\rightleftharpoons$ ATP + creatine, $K = 260$), for conditions similar to those used in this study (Chase & Kushmerick, 1995). The results from these experiments (Fig. 2*C*) show that the curve used to fit the loading points in the absence of CP passed close to the data for loading in the presence of 40 μM ADP buffered with creatine. This demonstrates clearly that (i) the reduction in loading ability of the SR following the removal of CP is due to a build-up of ADP rather than the removal of CP *per se* and (ii) the average [ADP] in preparations bathed in solutions without CP is indeed about 40 μM as estimated in Methods. It was also of interest to determine the time course of SR Ca^{2+} loading at a buffered ADP level (10 μM) close to that encountered in the muscle fibre at rest (Dawson *et al.* 1978). For this we added 3 mM creatine to the solutions containing 10 mM CP and 8 mM ATP, to buffer ADP to 10 μM in the fibre and the results are shown in Fig. 2*A*. As expected, the curve for 10 μM ADP lies between the curves for < 0.10 μM and ~40 μM ADP.

By fitting eqn (5) to the curves in Fig. 2 the following parameters were calculated: SR Ca^{2+} loading capacity, SR Ca^{2+} pump rate and SR Ca^{2+} leak rate constant (β); the results obtained for these parameters are shown in Fig. 3. Under conditions of 10 mM CP (< 0.10 μM ADP), the SR Ca^{2+} pump was found to be pumping at a rate of 80.0 ± 2.0% endogenous SR Ca^{2+} min^{-1}, where 100% corresponds to the physiological endogenous Ca^{2+} level in the SR. Thus, in the presence of 10 mM CP, the SR Ca^{2+} pump was able to fill the SR to its endogenous level within about 70 s at pCa 6.7. The calculated Ca^{2+} leak parameter from the SR (β) was 0.15 ± 0.04 min^{-1}. The SR Ca^{2+} loading capacity at pCa 6.7 when ADP < 0.10 μM was 6.23 times the endogenous physiological level of Ca^{2+} in the SR.

By plotting these parameters against $\log_{10}$[ADP] (Fig. 3), it is possible to determine the apparent K_d^{ADP} for each parameter. The K_d^{ADP} values for the SR Ca^{2+} pump rate, SR Ca^{2+} leak rate constant and SR Ca^{2+} loading capacity were 0.22 ± 0.04 mM, 64 ± 27 μM and 2.7 ± 1.2 μM, respectively. Therefore, when the [ADP] is close to the physiological level in the muscle fibre at rest (10 μM), the

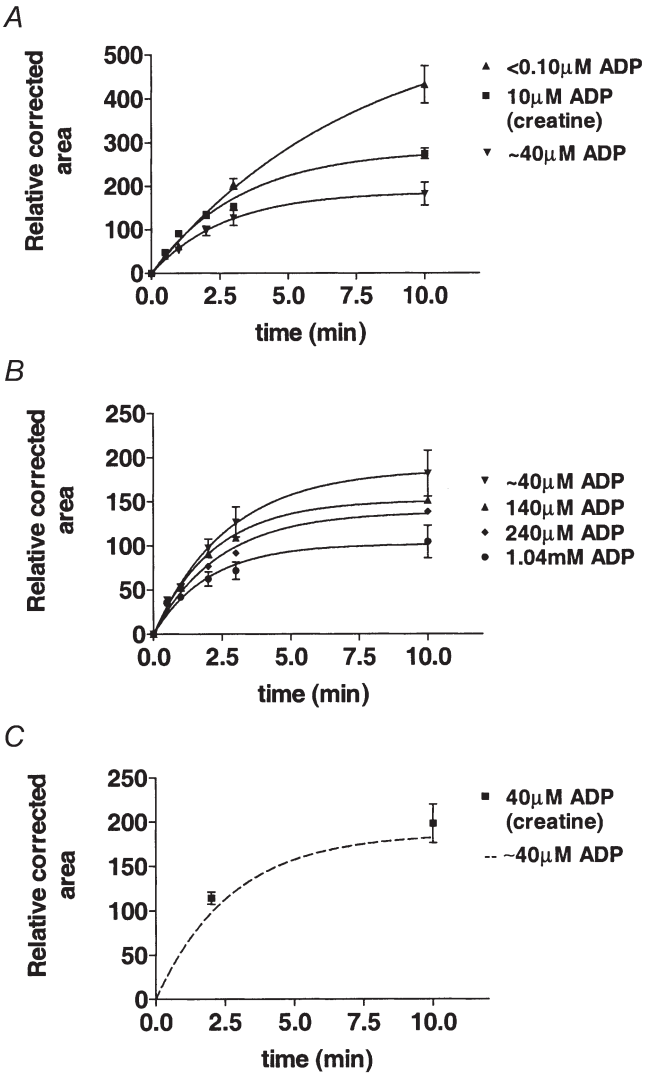

Figure 2. The effect of ADP on SR Ca^{2+} loading ability in mechanically skinned EDL fibres of the rat

All fibres were loaded under similar conditions for [Ca^{2+}] (pCa 6.7), pH, ionic strength, cationic composition and anionic composition except for ADP, which was substituted with HDTA, and the SR Ca^{2+} was always released in the same Release solution after the preparation was washed in $Wash_1$ solution to ensure an accurate comparison between results. The data points for individual fibres were normalised to the corrected area obtained under the standard loading condition (2 min) for that fibre. The points were fitted by eqn (5). Data points are means ± S.E.M. *A*, effect of ADP ⩽ 40 μM on SR Ca^{2+} loading ($n = 6$ for all points except for 10 μM ADP where $n = 5$); *B*, effect of 0.04–1.04 mM ADP on SR Ca^{2+} loading ($n = 6$ for all points); *C*, comparison of results when [ADP] was buffered to 40 μM using 12 mM creatine, 10 mM CP and 8 mM ATP ($n = 6$) with the time course of SR Ca^{2+} loading when the SR was loaded with Ca^{2+} in a solution without CP which was estimated to contain 40 μM ADP (from *A*). The solutions with 10 μM ADP were buffered using 3 mM creatine, 10 mM CP and 8 mM ATP.

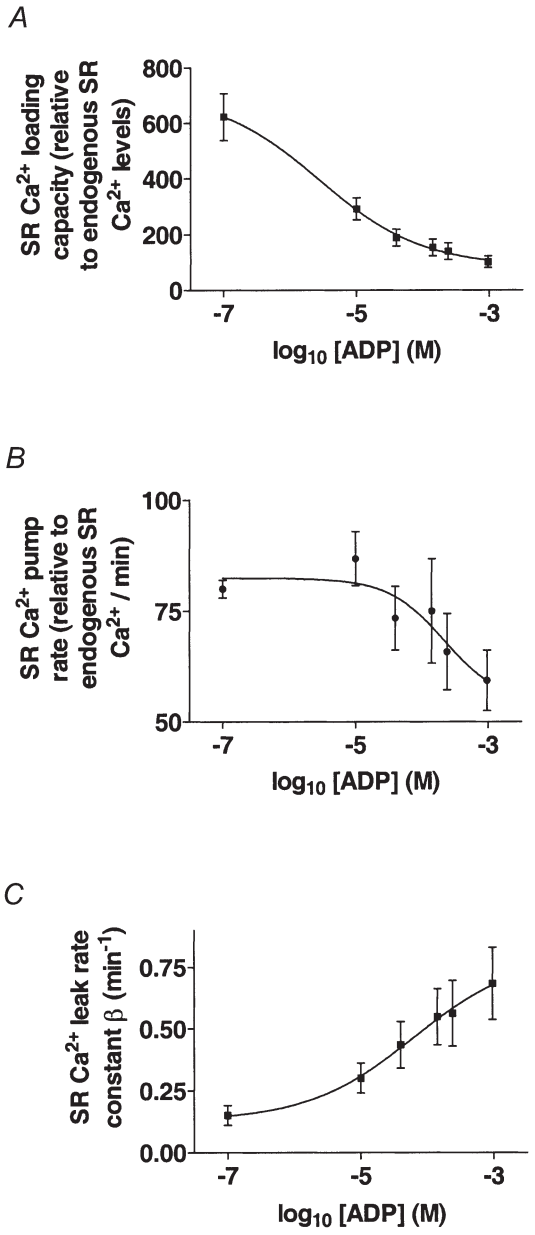

Figure 3. ADP effects on key functional parameters of the SR from mechanically skinned EDL fibres of the rat

A, SR Ca^{2+} loading capacity; *B*, SR Ca^{2+} pump rate; *C*, SR Ca^{2+} leak rate constant. The data points obtained from eqn (5) for each of the 6 fibres were fitted by Hill equations with variable Hill coefficient (n_H) for *A* and *B*:

$$y = \min + (\max - \min)/(1 + ([ADP]/K_d)^{n_H}),$$

and for *C*:

$$y = \min + (\max - \min)/(1 + (K_d/[ADP])^{n_H}).$$

The values for min, max and n_H were for *A*: 85, 708 and 0.56; for *B*: 54, 82 and 1.02; and for *C*: 0.13, 0.80 and 0.55, respectively.

SR Ca^{2+} pump rate does not change, but the SR Ca^{2+} leak rate constant (β) significantly increases and the SR Ca^{2+} loading capacity is dramatically reduced. The SR Ca^{2+} pump rate became significantly reduced only at [ADP] in the millimolar range. The marked reduction in the ability of the SR to load Ca^{2+} when ADP rose from less than 0.10 μM to 1.04 mM results mainly from an increase in the leak rate constant of Ca^{2+} from the SR rather than from a decrease in the SR Ca^{2+} pumping rate.

SR Ca^{2+} leak at low [Ca^{2+}] (pCa > 8)

As the rate constant of SR Ca^{2+} loss at pCa 6.7 in the solution appeared to increase 4-fold over the range of [ADP] investigated, it was important to look specifically at the SR Ca^{2+} leak at pCa > 8 in order to determine whether the rate constant of SR Ca^{2+} loss is Ca^{2+} dependent. The Leak solutions were very low in [Ca^{2+}] (pCa > 8), as they contained 1.0 mM EGTA, which was able to chelate Ca^{2+} leaking out of the SR. Therefore for all functional purposes the SR Ca^{2+} pump was not actively pumping Ca^{2+} into the SR under these conditions, thus reducing the likelihood of any Ca^{2+} being recycled back into the SR by the SR Ca^{2+} pump while the preparation was kept in the Leak solution. Therefore, for these conditions, eqn (4) becomes:

$$(d[Ca^{2+}]/dt)_{SR} = -\beta[Ca^{2+}]_{SR}, \quad (6)$$

and integration of eqn (6) results in the following expression:

$$[Ca^{2+}]_{SR}(t) = [Ca^{2+}]^0_{SR} e^{-\beta t}, \quad (7)$$

where $[Ca^{2+}]^0_{SR}$ is the total Ca^{2+} content of the SR at $t = 0$. Since the preparations were always loaded to the same level for the leak tests (2 min in $Load_3$ solution), and then the SR Ca^{2+} was leaked for 60 s for each test in a particular solution with ADP, it was possible to measure directly the effect of ADP on the SR Ca^{2+} leak rate constant at pCa > 8.

The first response depicted in Fig. 4 is a control response, corresponding to the SR Ca^{2+} content prior to being subjected to the $Leak_1$ solution. The second response was elicited in Release solution after a 60 s exposure of the preparation to the $Leak_1$ solution containing < 0.10 μM ADP, and response 3 is a control response similar to the first response. The difference in size between these two types of responses gives a measure of the SR Ca^{2+} leak that had occurred in the fibre during exposure to $Leak_1$ solution. In six fibres, the corrected area of the force responses (see Methods) elicited after exposure to low ADP (< 0.10 μM) $Leak_1$ solution was markedly smaller than controls (56.4 ± 3.4 % of the control response), indicating that significant Ca^{2+} had been lost from the SR during the leak period with a rate constant of the order of 0.2 min^{-1}.

As shown in Fig. 4, the caffeine-induced response was reduced even further when the estimated [ADP] was

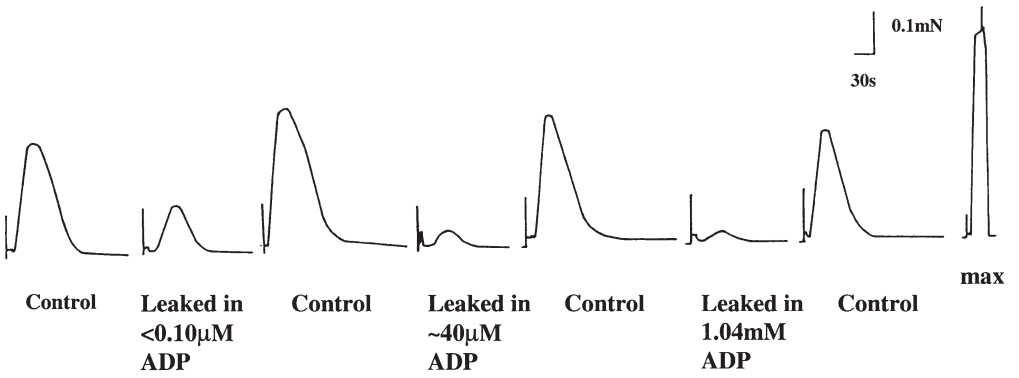

Figure 4. Representative traces showing the effects of ADP on SR Ca^{2+} leak

Responses 1, 3, 5 and 7 are 30 mM caffeine-induced force responses in Release solution after the preparation was loaded under standard conditions (2 min in $Load_3$ solution, pCa 6.7) and then briefly washed (30 s) in $Wash_1$ solution. Responses 2, 4 and 6 are 30 mM caffeine-induced force responses after the preparation was loaded under standard conditions and leaked for 60 s in $Leak_1$ (response 2) or $Leak_2$ solution (responses 4 and 6) with different ADP concentrations followed by 30 s wash in $Wash_1$ solution. The last response represents the maximum force produced in the preparation.

raised from $< 0.10\ \mu$M to about 40 μM in the Leak solutions. This suggests that the SR was leaking more Ca^{2+} when [ADP] was increased in the leak solutions. In six fibres, the area of the caffeine-induced force response was reduced to 37.4 ± 1.1% of control responses when [ADP] was about 40 μM, and this was significantly lower ($P < 0.001$, t test), than that obtained in the presence of 10 mM CP and ADP $< 0.10\ \mu$M (56.4 ± 3.4% of control responses).

The increase in [ADP] to 0.14 and 0.24 mM in $Leak_2$ solution did not significantly reduce the subsequent caffeine-induced force response further. However, the presence of 1.04 mM ADP in the $Leak_2$ solution led to a significantly larger ($P < 0.05$, t test) leak of Ca^{2+} from the SR. In a total of five fibres there was only 33.9 ± 0.8% Ca^{2+} left in the SR after 60 s leak in the presence of 1.04 mM ADP. This elevation of ADP in the millimolar range increases the passive SR Ca^{2+} leak and agrees with the results calculated from the SR Ca^{2+} loading curves.

The results for the leak rate constant at pCa > 8 at different [ADP] are summarised in Fig. 5 together with the leak rate constant results at pCa 6.7. The rate constant for the leak was greater at lower [Ca^{2+}] in solutions by a similar value at all ADP concentrations. This result suggests that the Ca^{2+} leak may not take place through the ryanodine receptor Ca^{2+} release channel because then it would have been expected that the leak would have increased rather than decreased when the [Ca^{2+}] in solution was raised from < 10 nM to 200 nM (Meissner, 1984).

Effect of blocking the SR Ca^{2+} pump on SR Ca^{2+} leak

In order to find out whether there is a SR Ca^{2+} leak pathway via the pump, particularly at low [Ca^{2+}] in solutions, 20 μM TBQ was used to inhibit the SR Ca^{2+} pump (Nakamura *et al.* 1992); it has also been shown to inhibit SR Ca^{2+} loading in rat EDL fibres (Bakker *et al.* 1996).

Figure 6 shows that after loading the SR with Ca^{2+} (2 min $Load_3$ solution) and then leaking SR Ca^{2+} for 120 s in the presence of 1.04 mM ADP ($Leak_2$ solution), only 18.0 ± 5.6% of control SR Ca^{2+} remained in the SR, where 100% was the amount of SR Ca^{2+} without the leak (control). The addition of 20 μM TBQ to the 1.04 mM ADP in $Leak_2$ solution increased the amount of SR Ca^{2+}

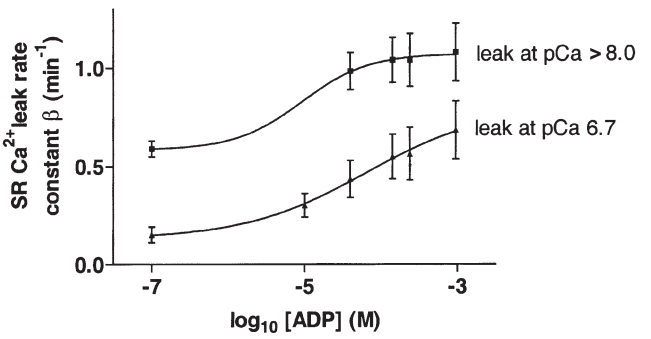

Figure 5. The effect of ADP on SR Ca^{2+} leak rate constant in rat mechanically skinned fibres

Lower curve, SR Ca^{2+} leak rate constant (β) at pCa 6.7 calculated from eqn (5) ($n = 6$); data fitted to a Hill curve:

$$y = \min + (\max - \min)/(1 + (K_d/[\mathrm{ADP}])^{n_H}),$$

where min = 0.130, max = 0.803 and $n_H = 0.546$. Upper curve, SR Ca^{2+} leak rate constant (β) at pCa > 8 ($n = 6$); data fitted to Hill curve:

$$y = \min + (\max - \min)/(1 + (K_d/[\mathrm{ADP}])^{n_H}),$$

where min = 0.340, max = 1.127 and $n_H = 0.381$.

retained in the SR to 33.7 ± 6.8 % control SR Ca^{2+}. Note that TBQ was removed from the preparation in paraffin oil before Ca^{2+} was released with caffeine (see Methods) and that TBQ did not significantly alter the sensitivity of the contractile apparatus to Ca^{2+} (Bakker *et al.* 1996). Therefore, the difference between the two results must be due to a reduction in the amount of Ca^{2+}, which leaked from the SR when the SR Ca^{2+} pump was partially blocked, suggesting that some of the Ca^{2+} leaking from the SR was in fact facilitated by the SR Ca^{2+} pump.

DISCUSSION

Effect of ADP on SR Ca^{2+} loading

The present results indicate that changes in [ADP] in the range expected to occur under resting physiological conditions and during fatigue have a significant effect on the ability of the SR to sequester Ca^{2+} in mammalian skeletal muscle. Increasing the [ADP] from < 0.10 μM to 10 μM and 1.04 mM decreases the ability of the SR to load Ca^{2+} by a factor of 2 and 4.4, respectively (Fig. 2), about 10–30 % of which is due to a decrease in the SR Ca^{2+} pump (Fig. 3*B*), and the rest is due to a marked increase in the leak of Ca^{2+} from the SR (Fig. 3*C*).

SR Ca^{2+} pump

It is important to be aware that increasing the [ADP] from < 0.10 μM to 10 μM had no effect on the SR Ca^{2+} pump rate and only when [ADP] was in the 100 μM range was a reduction in SR Ca^{2+} pump rate observed. Elevating the [ADP] to 1.04 mM only reduced the SR Ca^{2+} pump rate by 30 %. This is despite the [ADP] increasing 10 000-fold, which would have had a large effect on the ATP free energy under our two extreme conditions.

Mechanism of SR Ca^{2+} leak

The experiments using creatine to buffer the ADP levels in the presence of CP showed that the loading ability of the SR was affected by the increase in [ADP] rather than the reduction in [CP] *per se* (Fig. 2*C*). Furthermore, the reduction in SR Ca^{2+} handling ability at elevated [ADP] both in the presence and absence of an ATP regenerating system implies that the increase in the leak rate cannot be simply associated with local depletion of ATP.

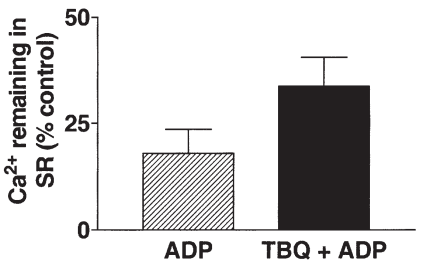

Figure 6. Effect of 20 μM TBQ on SR Ca^{2+} leak

Preparations were loaded under standard conditions and then leaked for 2 min in $Leak_2$ solution containing either 1.04 mM ADP or 1.04 mM ADP plus 20 μM TBQ (see Methods). Both responses are compared to Ca^{2+} remaining in SR after standard loading conditions (2 min $Load_3$ solution, pCa 6.7) and were found to be significantly different ($P < 0.05$, Student's paired *t* test, $n = 3$).

What is it then that causes the SR to become leakier when [ADP] is increased? Figure 5 shows that the leak did increase rather than decrease when the Ca^{2+} in the leak solution was decreased from 200 nM (pCa 6.7) to 10 nM (pCa 8), suggesting that the leak of Ca^{2+} due to elevated [ADP] did not occur through the ryanodine receptor. If the ryanodine receptor were responsible for the SR Ca^{2+} release from the SR, then increasing the myoplasmic [Ca^{2+}] would have caused an increase in the rate of SR Ca^{2+} leak (Meissner, 1984). Under conditions similar to those in this study, Launikonis & Stephenson (1997) showed that Ruthenium Red had no effect on the SR Ca^{2+} leak, which is also consistent with the Ca^{2+} leak being mainly via a different pathway from that involving the ryanodine receptor, which is blocked by Ruthenium Red. The application of the SR Ca^{2+} pump blocker TBQ reduced the leak of Ca^{2+} from the SR. Since TBQ reduces the activity of the SR Ca^{2+} pump by binding to the myoplasmic side of the pump like other types of SR Ca^{2+} pump blocking agents (Inesi & Sagara, 1994), the most likely explanation of results presented in this study is that the SR Ca^{2+} pump is at least partially responsible for the SR Ca^{2+} leak. Duke & Steele (1999) also observed a decrease in the Ca^{2+} loss from the SR in rat muscle, induced by the removal of CP from solution, when the Ca^{2+} pump was blocked with cyclopiazonic acid.

Slippage of the SR Ca^{2+} pump

Studies with isolated vesicles (de Meis, 1988) showed that in the presence of P_i, ADP and Mg^{2+}, and in the absence of ATP, the SR Ca^{2+} pump can be driven in the reverse direction. However, under our conditions of 8 mM ATP and 40 μM P_i (see Methods), even at 1.04 mM ADP it is not thermodynamically possible to drive the SR Ca^{2+} pump in the reverse direction with synthesis of ATP. Under our most extreme conditions of 1.04 mM ADP, the ATP free energy is -60 kJ mol^{-1} ($\Delta G^0_{ATP} = \Delta G^{0\prime}_{ATP} + RT\ln([ADP][P_i]/[ATP])$, where $\Delta G^{0\prime}_{ATP} = -30$ kJ mol^{-1}; Lehninger, 1970, p. 299). Even with a 10 mM [Ca^{2+}] in the SR it would thermodynamically not be possible to synthesise one ATP molecule following the transfer of two Ca^{2+} ions across the SR membrane during loading at pCa 6.7 (free energy associated with 2 mol of Ca^{2+} ions moving down the [Ca^{2+}] gradient $= 2 \times RT\ln([Ca^{2+}]$ in SR/[Ca^{2+}] in myoplasm) = $2 \times RT \times 2.303 \times 4.7 = 53.5$ kJ).

How is it then possible that the SR Ca^{2+} pump can mediate Ca^{2+} loss from the SR if not by pump reversal? As mentioned in the Introduction, a number of investigators have suggested that it is possible for the SR Ca^{2+} pump to act also as a Ca^{2+}–Ca^{2+} exchanger in the presence of ADP without being fully driven into reverse (Chiesi & Wen, 1983; Soler *et al.* 1990; Dalton *et al.* 1999).

Since ATP is not produced under these conditions, the pump is not reversed as such but it rather slips. This slippage of the SR Ca^{2+} pump has been described previously (Inesi & de Meis, 1989; Dalton *et al.* 1999) and can be explained as follows. The SR Ca^{2+} pump has two distinct functional forms: the phosphorylated and the dephosphorylated form. The Ca^{2+} binding sites of the dephosphorylated form usually face the myoplasm and have an apparent dissociation constant for Ca^{2+} in the range 0.2–2 μM (high affinity), while the Ca^{2+} binding sites of the phosphorylated form normally face the SR lumen and have an apparent dissociation constant in the range 1–3 mM (low affinity) (de Meis, 1988). The phosphorylated form can exist in ADP-sensitive and ADP-insensitive states (de Meis, 1988). In the ADP-insensitive states, the Ca^{2+} binding sites face the luminal side of the SR, whilst in the ADP-sensitive states, the Ca^{2+} binding sites can face either the luminal or the myoplasmic sides. In the presence of ADP, ADP will bind to ADP-sensitive states of the phosphoenzyme, increasing the total concentration of these ADP-sensitive states that can act as Ca^{2+}–Ca^{2+} exchangers transferring luminal Ca^{2+} from a higher [Ca^{2+}] to the myoplasmic compartment with lower [Ca^{2+}]. Such a mechanism can fully explain all the results in this study. Indeed, increased [ADP] would cause an increased concentration of the ADP-sensitive phosphoenzyme and therefore increased Ca^{2+} loss from the SR by Ca^{2+}–Ca^{2+} exchange. One would expect that the ADP-sensitive fraction of pump sites is normally rather small and this explains why the SR Ca^{2+} pump rate decreases only relatively little when the SR Ca^{2+} leak rate increases severalfold. Furthermore, complete blockage of the SR Ca^{2+} pump with TBQ would be expected to only partially reduce the Ca^{2+} leak from the SR because TBQ, like other types of SR Ca^{2+} pump blocking agents (Inesi & Sagara, 1994), only binds to the dephosphorylated, Ca^{2+}-free, pump sites. After SR Ca^{2+} loading, the SR Ca^{2+} pump sites would be mainly phosphorylated and facing the luminal side of the SR where [Ca^{2+}] was high and the ADP-sensitive fraction of pump sites that would act as Ca^{2+}–Ca^{2+} exchangers would not be available for TBQ binding unless they became dephosphorylated.

Relevance to fatigue

One feature of muscle fatigue is the prolonged descending phase of the Ca^{2+} transient and the progressive increase in the resting [Ca^{2+}] at a time when the intracellular [Mg^{2+}] is only little changed (Westerblad & Allen, 1992). These features can be explained by reduced net Ca^{2+} uptake by the SR under conditions of fatigue. Westerblad *et al.* (1997) showed that impaired SR function was not due to acidosis and Westerblad & Allen (1996) also excluded an increase in P_i as the cause. Our results offer a direct explanation for these important features of muscle fatigue because it does not require complete CP depletion for [ADP] to rise above resting levels. For example, using the values from Fryer *et al* (1995), a 50 % depletion in CP would lead to a 2-fold increase in the [creatine], resulting in a 4-fold increase in [ADP]. This would lead to only a small drop in the SR Ca^{2+} pump rate but would cause a 3-fold increase in the SR Ca^{2+} leak rate, ultimately reducing the ability of SR to load Ca^{2+} by a factor of 3.5. Indeed the pCa in the myoplasm at [ADP] of 1.04 mM must be 6.7 in order for the SR to maintain the same SR Ca^{2+} capacity as in a fibre at rest. This compares with a pCa value of 7 (Williams *et al.* 1990) in a rested fibre and provides a direct explanation of why the resting [Ca^{2+}] increases. Thus ADP plays an important role in modulating the SR function in muscle over the entire range of physiological activities and, in turn, creatine plays an important role in buffering the [ADP].

In conclusion, we show here that under physiologically relevant conditions [ADP] elevation reduces the ability of the SR to re-sequester Ca^{2+}, by increasing the leak rate of Ca^{2+} from the SR and by decreasing the rate of the SR Ca^{2+} pump. This can be fully explained by the SR Ca^{2+} pump acting as a Ca^{2+}–Ca^{2+} exchanger under elevated [ADP]. Physiological levels of ADP occurring during fatigue play an important role in determining Ca^{2+} movements across the SR membrane and the myoplasmic [Ca^{2+}].

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