Does the sarcoplasmic reticulum achieve chemiosmotic equilibrium in relaxed muscle?

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Measurements of $^{45}Ca^{2+}$ efflux from bovine skeletal muscle fibres preloaded with this isotope indicate that the sarcoplasmic free Ca^{2+} concentration rises and falls under anaerobic (or uncoupled) and aerobic conditions, respectively. Dantrolene has only a small effect on these responses which are larger in predominantly slow-twitch fibre preparations. Dantrolene slightly reduces the resting free Ca^{2+}; this is much more pronounced in rectus abdominis (mainly fast-twitch) than in sternomandibularis (mainly slow-twitch). It is proposed that the sarcoplasmic reticulum in the resting muscle fibre achieves a state close to chemiosmotic equilibrium and that aerobic metabolism generates a higher phosphorylation potential, permitting a larger Ca^{2+} electrochemical potential difference to develop across the sarcoplasmic reticulum membrane under aerobic conditions.

Sarcoplasmic reticulum *Bovine muscle fibre* *Phosphorylation potential* *Dantrolene* *Chemiosmotic equilibrium*

1. INTRODUCTION

The calcium pump of the sarcoplasmic reticulum catalyses, with high efficiency, the transformation of chemical energy into osmotic energy [1]. Experimental evidence [2–4] indicates that the overall chemiosmotic [5–7] reaction catalysed by the Ca^{2+}-translocating ATPase in isolated sarcoplasmic reticulum vesicles achieves a state of near-equilibrium when net Ca^{2+} flux ceases in the steady state. The same near-equilibrium may exist in a relaxed muscle cell, since most of the free energy available from the hydrolysis of sarcoplasmic ATP (the sarcoplasmic 'phosphorylation potential') is apparently conserved in the electrochemical potential difference of Ca^{2+} across the sarcoplasmic reticulum membrane [8]. Such a near-equilibrium state implies that, if Ca^{2+} fluxes across the plasma membrane may be disregarded, then the sarcoplasmic free calcium concentration should rise or fall when the sarcoplasmic phosphorylation potential decreases or increases, respectively.

The phosphorylation potential in the cytosol has been measured for a number of mammalian tissues including (aerobic) skeletal muscle [9] and is apparently close to 14 kcal/mol (similar to the value generated by isolated mitochondria in state 4) (review [10]). In the case of muscle fibres, it seems likely that the phosphorylation potential maintained aerobically by oxidative phosphorylation is considerably higher (particularly in mitochondria-rich oxidative fibres [10–14]) than that which may be sustained by anaerobic glycolysis.

It is noteworthy in this context that in human skeletal muscle studied by ^{31}P NMR, the net resynthesis of phosphocreatine after a bout of contractile work is rapid under aerobic conditions but that under anaerobic (ischaemic) conditions the rate of recovery is exceedingly slow [15]. Even in frog skeletal muscle which relies very heavily upon glycolysis for recovery metabolism, rephosphorylation of creatine after a contraction may be only partial in the anaerobic state [16,17]. If the phosphorylation potential is higher aerobically than anaerobically and if the sarcoplasmic reticulum comes close to chemiosmotic equilibrium, then the

Abbreviations: Dantrolene, 1-(5-*p*-nitrophenyl) furfurilidene amino hydantoin sodium hydrate; SR, sarcoplasmic reticulum

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sarcoplasmic free calcium concentration would be expected to rise and fall under anaerobic and aerobic conditions, respectively. Here, the consequences of preventing oxidative phosphorylation during ^{45}Ca efflux from a muscle fibre preparation are examined. The results show that this is the case and that oxidative phosphorylation lowers the resting free Ca^{2+} concentration in mammalian skeletal muscle fibres.

2. MATERIALS AND METHODS

Loading of muscle fibre preparations with $^{45}Ca^{2+}$, $^{45}Ca^{2+}$ efflux measurements and mechanical observations were made as in [18]. Fibre preparations from 3 different beef muscles (sternomandibularis, longissimus dorsi and rectus abdominis) were employed. Increases and decreases in the rate constant for $^{45}Ca^{2+}$ efflux in the later (first order) phase of tracer efflux [18] were used as an indication of increases and decreases, respectively, in the Ca^{2+} concentration free in the sarcoplasm. The fibre preparation (~0.3 mm diam. and with the cut fibre ends sealed with vaseline), was perfused at a flow rate of 0.8 ml/min with a saline solution containing 130 mM NaCl, 5 mM KCl, 1.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 3 mM Na-phosphate (pH 7.1), with 20 μg/ml of D-tubocurarine chloride at 14°C.

Direct electrical stimulation of the preparation was achieved via platinum bars embedded in the walls of the 0.05 ml experimental chamber. The preparation was suspended between a semiconductor isometric force transducer (Kulite) mounted on one micromanipulator and a second micromanipulator which was used to alter the length of the preparation. Sarcomere length was measured by optical diffraction at 633 nm using a 2 mW helium–neon laser (Scientifica and Cook type SL-H2) and was set at 2.5 μm.

$^{45}Ca^{2+}$ efflux is expressed as a first-order rate constant and the early portion of the time course of washout of the isotope, which does not exhibit first-order kinetics [18], was not employed in these experiments.

3. RESULTS AND DISCUSSION

The time course of changes in the rate constant for $^{45}Ca^{2+}$ efflux from a fibre preparation from beef sternomandibularis, a predominantly slow-twitch muscle [19,20] is indicated in fig.1 for two preparations of this type. The replacement of normal saline by cyanide-containing saline resulted in an increase in the rate constant for $^{45}Ca^{2+}$ efflux. This was reversed following the restoration of normal saline. Anaerobic (N_2-saturated) saline had an effect similar to that of cyanide. The efflux rate constant rose ~60–70% during both treatments. Dantrolene, which was present in both experiments and which inhibits Ca^{2+} release from the

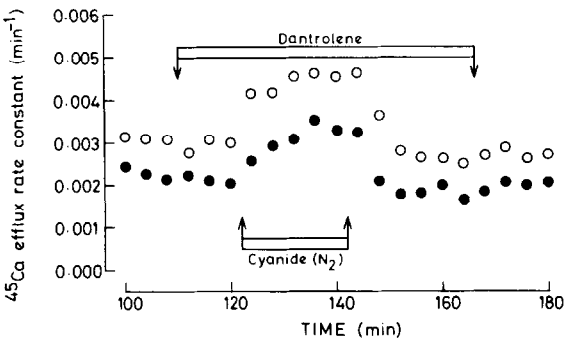

Fig.1. Effect of dantrolene, cyanide and anaerobic conditions upon $^{45}Ca^{2+}$ efflux from a beef sternomandibularis muscle preparation. For the duration of the upper bar, dantrolene (30 μM) was present in the superfusing saline and, for the duration of the lower bar, cyanide (1 mM NaCN) (•) was present or the medium was rendered anaerobic by bubbling with nitrogen (○). The time axis indicates the time since isotope washout had started.

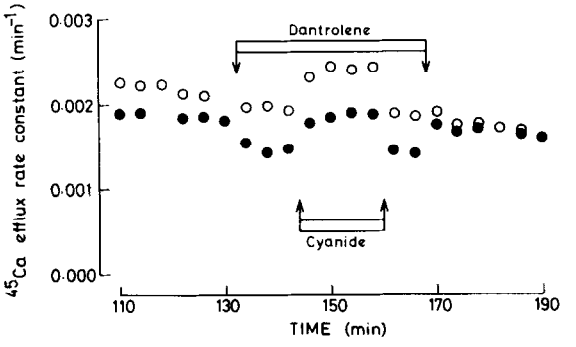

Fig.2. Effect of dantrolene, cyanide and anaerobic conditions upon $^{45}Ca^{2+}$ efflux from a beef longissimus dorsi muscle preparation (○) or a preparation from beef rectus abdominis (•). The experiment was conducted as for fig.1 except that for the duration of the lower bar, cyanide (1 mM NaCN) was present in both cases.

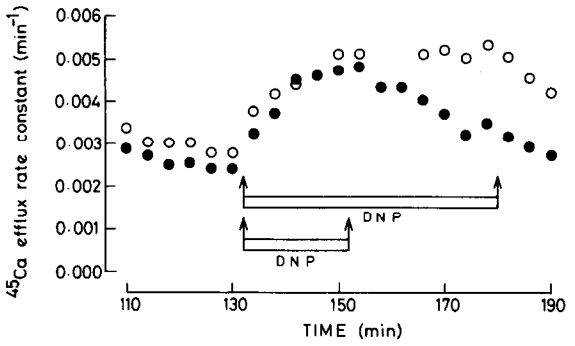

Fig.3. Effect of 2,4-dinitrophenol upon $^{45}Ca^{2+}$ efflux from a beef sternomandibularis muscle preparation. For the duration of the upper bar (○) or the lower bar (●), 2,4-dinitrophenol (100 μM) was present in the superfusing saline.

SR [21–24] had little effect on efflux although in its presence, the twitch tension produced by electrical stimulation of the fibres (15 V, 0.7 ms) was substantially reduced (not shown). Similar experiments conducted in the absence of dantrolene also showed a 50–70% increase in efflux rate constant under cyanide or nitrogen (not shown). No increase in steady tension was noted under cyanide or nitrogen and, for comparison, a 2 min tetanic contraction (0.7 ms pulses at 25 Hz) transiently increased the $^{45}Ca^{2+}$ efflux rate constant by 6.8 times in the cyanide-treated preparation of fig.1 when elicited 40 min after the preparation had been returned to normal saline.

Table 1

Effect upon the $^{45}Ca^{2+}$ efflux rate constant of dantrolene, cyanide, anaerobic conditions and 2,4-dinitrophenol

Treatment	Muscle	Change in $^{45}Ca^{2+}$ efflux rate constant (%)	
Dantrolene	st.m.	− 3 ± 4	(6)
for 10 min	l.d.	−10 ± 6	(5)
	r.a.	−17 ± 5	(4)
Cyanide	st.m.	+67 ± 7	(9)
for 18 min	l.d.	+35 ± 10	(5)
	r.a.	+22 ± 6	(4)
Anaerobic	st.m.	+65 ± 6	(3)
for 18 min	l.d.	+28	(1)
	r.a.	+30	(1)
2,4-Dinitrophenol	st.m.	+107 ± 14	(5)
for 18 min	l.d.	+45	(1)
	r.a.	+44	(2)

Abbreviations: st.m., sternomandibularis; l.d., longissimus dorsi; r.a., rectus abdominis

Results compiled from experiments like those of fig.1–3 are quoted as means ± SEM with the number of observations in parentheses

When fibre preparations from longissimus dorsi (a muscle containing predominantly fast-twitch fibres [20]) were employed in an experiment like that of fig.1 (cyanide treatment), the result was similar, although the amplitude of the enhancement of efflux was smaller (fig.2). In the case of rectus abdominis preparations, however (fig.2), dantrolene treatment caused a decrease in efflux rate constant which was almost as large as the subsequent increase caused by cyanide. Beef rectus abdominis is predominantly a fast-twitch muscle [19,20].

The classical uncoupling agent 2,4-dinitrophenol had an effect in these experiments similar to that produced by inhibition of mitochondrial electron transport and this too could be reversed by perfusion with saline free of uncoupling agent (see fig.3). Reversal, in this case, took a considerable time, perhaps reflecting the kinetics of washout of the lipid-soluble uncoupling agent from the preparation. The stimulation of efflux (~2-fold) was greater than that produced by respiratory inhibition, and a slight increase in tension (to 0.08 times that produced by tetanic stimulation) was recorded during the application of uncoupling agent.

Uncoupling oxidative phosphorylation in this way might be expected to produce an effect somewhat different from the inhibition of electron transport since, in addition to eliminating oxidative phosphorylation, the dinitrophenol-stimulated mitochondrial ATPase might be expected to augment cellular ATP turnover sufficiently to cause a further decline in the phosphorylation potential which could be maintained by anaerobic glycolysis and the creatine phosphate buffering system. Classical uncoupling agents and other proton-transporting ionophores also cause (transient) Ca^{2+} release from the sarcoplasmic reticulum of skinned rabbit psoas fibres [25].

The results of various experiments similar to those of fig.1–3 are summarised in table 1. It is evident that treatment with dantrolene causes a decrease in the efflux rate constant in all 3 types of

preparation; this is, however, greatest in those from the rectus abdominis. This difference probably indicates that upon treatment with dantrolene, a larger decrease in resting free Ca^{2+} concentration occurs in the rectus abdominis preparations than in the other two. The effect of the other 3 treatments is qualitatively similar (see table 1). In all 3 preparations, these treatments, each of which prevents oxidative phosphorylation, produce an increase in $^{45}Ca^{2+}$ efflux, and, by inference, an increase in sarcoplasmic free Ca^{2+} concentration. The increase is largest with the uncoupling agent and in this case only, was a small rise in tension recorded. This latter observation is consistent with the conclusion drawn from the efflux data, that the uncoupling agent produces a larger increase in cytoplasmic free Ca^{2+} than do treatments which merely inhibit electron transport.

The simplest interpretation of the data is that dantrolene decreases the resting intracellular free calcium level, whilst when oxidative phosphorylation is prevented in three different ways this level rises. It therefore seems likely that a decreased capability for ATP resynthesis underlies the similar response to these 3 different experimental treatments.

It might be argued that the observed effect of all 3 treatments could be the outcome of Ca^{2+} release from mitochondria.

However, recent work indicates that, in a resting muscle cell, very little Ca^{2+} is located within the mitochondrial matrix. Ca^{2+} does not appear to come to electrochemical equilibrium across the cristae membrane of respiring mitochondria which are in or near state 4 [26], and the free Ca^{2+} concentration within the mitochondrial matrix space is $< 10^{-5}$ M under these conditions [27,28], and indeed, as in the cytosol, variation in the free Ca^{2+} concentration within the mitochondrial matrix space probably plays a part in metabolic regulation [27,28]. Similarly, measurements indicate that the affinity of mitochondria for Ca^{2+} is very much lower than that of the sarcoplasmic reticulum [29–31]. Electron probe microanalysis also indicates that very little Ca^{2+} is located within the mitochondrial matrix in resting muscle cells [31–33]. The notion, therefore, that when respiration is inhibited, significant mitochondrial Ca^{2+} release occurs, appears to be an unlikely explanation of these results.

Studies (using ^{31}P NMR) of fatigued contracting frog muscles [14] indicate that the decrease in the rate of relaxation which is characteristic of fatigue, occurs to an extent which may be predicted from the degree to which fatigue has reduced the cellular phosphorylation potential. Studies of the 'calcium transient' in aequorin-injected frog muscle fibres indicate that the decrease in the rate of relaxation during fatigue is associated with a decrease in the rate of sequestration of Ca^{2+} [34]. Taken together these observations indicate that the rate of net Ca^{2+} translocation by the SR is a function of the available phosphorylation potential. It seems likely that this behaviour of the SR when displaced rather far from equilibrium (during relaxation in a 'calcium transient') is closely related to the tight coupling between ATP hydrolysis and ion translocation which is implied by the experimental observation that under other conditions (low net Ca^{2+} flux in SR vesicle suspensions in vitro) near-equilibrium relations obtain.

It seems reasonable to deduce from the available experimental data that most of the free energy which is available from ATP hydrolysis in the resting muscle cell in vivo is conserved in the electrochemical potential difference of Ca^{2+} established across the SR membrane [8]. Thus the variations in intracellular free Ca^{2+} inferred from the present results may well directly reflect variations in the magnitude of the phosphorylation potential to which the Ca^{2+} pump of the SR has access, rather than kinetic factors modifying the rate at which the pump may operate.

When in the relaxed resting state, net calcium flux across the SR membrane has ceased and influx balances efflux, one of two states may obtain. The condition may be one of thermodynamic equilibrium; alternatively, a steady state may exist, with net Ca^{2+} influx driven by the Ca^{2+}-translocating ATPase at the expense of ATP hydrolysis, balanced by a significant net efflux of Ca^{2+} down its electrochemical potential gradient. Ca^{2+} release from the SR during muscle contraction involves a very great enhancement of this Ca^{2+} efflux pathway and it is not clear that the calcium permeability of the SR membrane may be sufficiently reduced in the relaxed state to permit an approach to equilibrium on the part of the Ca^{2+} pump. Dantrolene [22] apparently inhibits rather specifically the Ca^{2+} release process from the SR

[21–24] without affecting the rate of Ca^{2+} translocation by the SR Ca^{2+} pump or the process of Ca^{2+} permeation through the plasma membrane. The absence of an appreciable inhibition by dantrolene of the Ca^{2+} efflux in experiments with sternomandibularis muscle and the presence of such an effect in preparations from longissimus dorsi and rectus abdominis probably indicates that in the former case, a significant leak of Ca^{2+} from the SR does not occur in the relaxed muscle fibre and in the latter cases that it does. Thus, in these sternomandibularis preparations at 14°C the SR is probably closer to a chemiosmotic equilibrium state than in the other preparations used in these experiments. In all cases examined, however, effectively anaerobic conditions cause an enhanced Ca^{2+} efflux which appears to imply that inhibition of oxidative phosphorylation causes an increase in free calcium concentration in the cytosol. Although various 'kinetic' explanations of this phenomenon are possible in terms of altered rates of Ca^{2+} pumping, it seems probable that such an inhibition causes a drop in the phosphorylation potential to which the SR Ca^{2+}-translocating ATPase has access and that the associated rise in free Ca^{2+} in the cytosol may be simply explained if a state close to chemiosmotic equilibrium is achieved by the Ca^{2+} pump in resting mammalian skeletal muscle.

It seems likely that the inferences drawn from these experiments concerning mammalian skeletal muscle have a greater generality since, in a wide variety of non-muscle cells (e.g., [35–38]) Ca^{2+}-dependent phenomena are elicited or cellular free Ca^{2+} is observed to rise upon the application of uncouplers and inhibitors of oxidative phosphorylation or after the inhibition of respiration. It has been widely assumed that the increase in cytosol free calcium concentration inferred from or measured in these responses reflects the loss of Ca^{2+} from the mitochondrial matrix. This interpretation seems improbable in view of the low levels of Ca^{2+}, both free and bound within mitochondria in vivo. Such responses may well reflect the drop in phosphorylation potential which these treatments almost certainly cause and the consequent impaired ability of the ATP-dependent Ca^{2+}-translocating systems to maintain a large Ca^{2+} electochemical potential difference across the endoplasmic or sarcoplasmic reticulum membrane.

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