Na\textsuperscript{+}-K\textsuperscript{+} pumps in the transverse-tubular system of skeletal muscle fibers preferentially use ATP from glycolysis

T. L. Dutka and G. D. Lamb

Department of Zoology, La Trobe University, Melbourne, Victoria, Australia

Corresponding author:

Dr. T. L. Dutka
Department of Zoology, La Trobe University, Melbourne 3086, Victoria, Australia
Telephone: +61-3-9479 2983
Facsimile: +61-3-9479 1551
E-mail: t.dutka@latrobe.edu.au

Running head: Preferential ATP usage by T-system Na\textsuperscript{+}-K\textsuperscript{+} pumps
Abstract

The Na\(^+\)-K\(^+\) pumps in the transverse tubular (T-) system of a muscle fiber play a vital role keeping [K\(^+\)] in the T-system sufficiently low during activity so as to prevent chronic depolarization and consequent loss of excitability. These Na\(^+\)-K\(^+\) pumps are located in the triad junction, the key transduction zone controlling excitation-contraction (EC-) coupling, a region rich in glycolytic enzymes and likely having high localised ATP usage and limited substrate diffusion. This study examined whether Na\(^+\)-K\(^+\) pump function was dependent on ATP derived via the glycolytic pathway locally within the triad region. Single fibers from rat fast-twitch muscle were mechanically skinned, sealing off the T-system but retaining normal EC-coupling. Intracellular composition was set by the bathing solution, and action potentials (APs) triggered in the T-system, eliciting intracellular Ca\(^{2+}\) release and twitch and tetanic force responses. Conditions were selected such that increased Na\(^+\)-K\(^+\) pump function could be detected from the consequent increase in T-system polarization and resultant faster rate of AP repriming. Na\(^+\)-K\(^+\) pump function was not adequately supported by maintaining cytoplasmic [ATP] at its normal resting level (~8 mM), even with 10 mM or 40 mM creatine phosphate (CP) present. Addition of as little as 1 mM of phospho(enol)pyruvate (PEP) resulted in a marked increase in Na\(^+\)-K\(^+\) pump function, supported by endogenous pyruvate kinase (PK) bound within the triad. These results demonstrate that the triad junction is a highly restricted micro-environment, where glycolytic re-synthesis of ATP is critical to meet high demand of the Na\(^+\)-K\(^+\) pump and maintain muscle excitability.

Key words: Muscle fatigue, Na\(^+\)-K\(^+\)-ATPase, Excitation-contraction coupling, T-system, Excitability
**Introduction**

$\text{Na}^+\text{-K}^+$ pumps play a crucial role in skeletal muscle by maintaining the normal electrochemical gradients for $\text{K}^+$ and $\text{Na}^+$, which keeps the membrane adequately polarized and permits continued muscle excitability and contraction (3). Approximately 50% of all the $\text{Na}^+\text{-K}^+$ pumps are located in the transverse-tubular (T-) system (36), the extensive network of invaginations of the surface membrane by which action potentials (APs) penetrate throughout a muscle fiber, triggering $\text{Ca}^{2+}$ release from the adjacent sarcoplasmic reticulum (SR) and subsequent contraction (6, 16, 33). The T-tubules have a very high surface to volume ratio, constituting ~80% of the total fiber surface area but only ~1% of the total fiber volume. As a result of the efflux of intracellular $\text{K}^+$ with each AP the [$\text{K}^+$] in the T-tubule lumen may increase substantially above its resting level of ~4 mM (3, 45), which could depolarize the membrane and interfere with AP propagation and normal $\text{Ca}^{2+}$ release. Thus, proper function of the $\text{Na}^+\text{-K}^+$ pumps in the T-system is particularly important for normal activity.

So it is very telling to note that the T-system $\text{Na}^+\text{-K}^+$ pumps, which require ATP to function, are situated in a rather extraordinary local environment. Firstly, there is likely to be restricted diffusional access, because the T-system is abutted on both sides for >90% of its length by the terminal cisternae of the SR (7), forming what is known as the triad junction. Secondly, during muscle activity there is likely high local consumption of ATP in this region, owing to the presence of many $\text{Ca}^{2+}$ pumps on the SR terminal cisternae just outside the junction (17, 44) and the high density of $\text{Na}^+\text{-K}^+$ pumps and other ATPases on the T-system membrane itself (22, 36). Thirdly, there is a high density of glycolytic enzymes in the triad junction, and in isolated triads it has been shown that glycolytic substrates have ready access to the triadic space but the ATP produced there is not in dynamic equilibrium with the ATP outside the junction (21). Fourthly, mitochondria are present in high density in the vicinity of the triad junction, some typically
situated immediately adjacent (2). These observations suggest that the triad junction is likely to be a special micro-environment in respect to production, diffusion and utilisation of ATP, and this is likely to have a major bearing on Na\(^{+}\)-K\(^{+}\) pump function in the T-system.

Using oxygen removal and various inhibitors, it has been shown previously in resting skeletal muscles from both frog (12) and rat (38) that glycolysis can be an important source of ATP for Na\(^{+}\)-K\(^{+}\) pumping and the maintenance of Na\(^{+}\) and K\(^{+}\) homeostasis in whole muscles over a time period of an hour. It was nevertheless evident that cytoplasmic ATP had some direct access to the pumps and that cellular creatine phosphate (CP) could also be used to some extent to re-synthesize ATP for Na\(^{+}\)-K\(^{+}\) pumping (12). Furthermore, when glycolytic ATP production was inhibited with iodoacetate the total amounts of both CP and ATP decreased to ~50% or less of their resting levels (12, 38), leaving it unclear whether Na\(^{+}\)-K\(^{+}\) pump function normally might be adequately supported if cytoplasmic [ATP] could be maintained close to its resting level. It was also not evident whether the findings were primarily reflecting the properties of the sarcolemmal or the T-system Na\(^{+}\)-K\(^{+}\) pumps or both. In perfused cardiac muscle cells, Na\(^{+}\)-K\(^{+}\) pump function was found to be preferentially fuelled by glycolytic synthesis of ATP (19), though in those experiments there was no CP present to buffer cytoplasmic [ATP]. Very recently using whole soleus muscles stimulated at high frequency in-vitro, it was found that limiting both glycolysis and aerobic metabolism caused faster loss of tetanic force and sarcolemmal excitability than did limiting aerobic metabolism alone (34). It is likely that the force decreased faster because the greater constraints on the ATP supply meant that the Na\(^{+}\)-K\(^{+}\) pumps were unable to maintain an adequate K\(^{+}\) electrochemical gradient (34). It was unclear however whether the T-system Na\(^{+}\)-K\(^{+}\) pumps were coping adequately in these circumstances or were the prime cause of the problem, and also whether the effects were due to local ATP micro-environments or to changes in bulk [ATP] and CP buffering of ATP.
The aims of the present study were to investigate i) whether the triad junction does indeed function in-situ as a specialised ATP micro-environment, ii) whether the T-system Na\(^+\)-K\(^+\) pumps require ATP from glycolysis to function adequately during periods of high demand, and if so, iii) whether this is purely because the cytoplasmic [ATP] is not being properly maintained at close to its normal level. These were investigated using a skinned muscle fiber preparation, where it is possible by means of the bathing solution to control and vary the cytoplasmic constituents as desired, but importantly where the structure of the triad junction is largely unperturbed and EC-coupling is fully functional (40, 43). The T-system seals off when the fiber is mechanically skinned (27), and electric field stimulation can be used to trigger action potentials (APs) in the T-system and initiate SR Ca\(^{2+}\) release and twitch and tetanic force responses. In this preparation the T-system potential is critically dependent on the maintained function of the T-system Na\(^+\)-K\(^+\) pumps (28, 37, 39), and changes in potential can be monitored by the alteration it causes in the AP refractory period, the recovery time needed after one AP before another can be propagated (11, 37, 50). In this way it was possible to examine whether the T-system Na\(^+\)-K\(^+\) pumps functioned adequately when cytoplasmic [ATP] was kept at the normal rest level (~8 mM), buffered by CP at levels occurring at rest or in fatigue, but where there was no glycolytic production of ATP. Then various concentrations of phospho(enol)pyruvate (PEP) could be added, with or with pyruvate kinase, to investigate whether this substrate for the final ATP production step in the glycolytic pathway was utilised locally within the triad junction to support Na\(^+\)-K\(^+\) pump function.
Methods

Preparations. With approval of the La Trobe University Animal Ethics Committee, male Long-Evans hooded rats (~6 months old) were anesthetized with Fluothane (2 % vol/vol) in a glass chamber and killed by asphyxiation. Both extensor digitorum longus (EDL) muscles were rapidly excised and immediately pinned at resting length under paraffin oil in a Petri dish lined with Sylgard 184 (Dow Corning, Midland, MI) and kept cool (~10°C) on an ice pack. Individual fibers were mechanically-skinned by rolling back the sarcolemma by microdissection with fine forceps. A segment of the skinned fiber was then connected to a force transducer (AME801, resonance frequency >2 kHz; SensoNor, Horten, Norway) and stretched to 120 % of resting length. The mounted skinned fiber segment (~2 mm long, diameter 30-50 µm) was transferred to a small Perspex well containing 2 ml of the standard K+-hexamethylene-diamine-tetraacetate (K-HDTA) solution (see below) for 2 min to replace all of the in vivo diffusible myoplasmic constituents with the experimental bathing solution. All experiments were conducted at ~25°C.

Solutions. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless specified otherwise. The standard K-HDTA solution (control solution, 126 mM K+) contained (in mM): K+, 126; Na+, 36; HDTA2- (Fluka, Buchs, Switzerland), 50; total ATP, 8; creatine phosphate (CP), 10; total Mg2+, 8.5; total EGTA, 0.075; 4-(2-hydroxyethyl)-1-piperazine-ethane-sulphonic acid (HEPES), 90; pH 7.1 and pCa (= -log10 [Ca2+]) 6.9, except where stated. A matching ‘partial depolarising’ solution with 66 mM K+ was made with additional Na+ replacing 60 mM of the K+. A similar 66 mM K+ solution was also made with the [CP] increased from 10 mM to 40 mM by equimolar reduction of HDTA and with 8.74 mM total Mg2+. All these solutions had an osmolality of 295 ± 5 mOsmol/L and a free [Mg2+] of 1 mM based on the assumed apparent Mg2+ affinity constants of 6.9 x 10³ M⁻¹ for ATP, 8 M⁻¹ for HDTA, and 15 M⁻¹ for CrP (14, 48). The
pCa of solutions (for pCa < 7.2) was measured with a Ca²⁺-sensitive electrode (Orion Research, Cambridge, Mass., USA). Solutions containing glybenclamide (50 µM) were made by addition of 1 part in 1000 from stock solutions in DMSO, with the same amount of DMSO (0.1% final concentration) added to all comparison solutions.

Solutions with phospho(enol)pyruvate (PEP) were made by adding the trivalent Na⁺ salt at 1 mM or 5 mM, with and without pyruvate kinase (PK, 25 units per ml) as indicated. When using 5 mM PEP, the comparison solutions without PEP contained an additional 7.5 mM Na₂HDTA so as to have the same [Na⁺] and to largely compensate for the increase in ionic strength and osmolality, with the minor remaining differences likely to cause only a small decrease in maximum force and Ca²⁺-sensitivity (29). No adjustment was made for the slight decrease in free [Mg²⁺] (to ~0.9 mM) occurring in solutions with 5 mM PEP, as this would have had very little effect on either contractile properties or on AP repriming time (see definition below), the latter not detectably changing when free [Mg²⁺] is varied in the range 0.5 to 3 mM (results not shown). Creatine kinase was added to solutions where indicated.

Solutions used to examine the properties of the contractile apparatus were as follows. i) To ascertain maximum Ca²⁺-activated force at the end of an experiment measuring AP-induced responses, the fiber was exposed to the maximum activating solution (‘max’), which matched the control K-HDTA solution but with the [Ca²⁺] heavily-buffered at pCa 4.5 by replacing all 50 mM HDTA with Ca-EGTA (see (48)). The fiber was subsequently relaxed in a matching solution with 50 mM free EGTA (pCa > 10) (‘relaxing solution’). ii) For measuring whether the force-[Ca²⁺] relationship differed in the different base solutions (e.g. 66 mM K⁺ solution versus
126 mM K\textsuperscript{+} solution), sets of the appropriate solutions were made with 5 mM CaEGTA-EGTA replacing 5 mM of the HDTA, with other constituents unaltered, with the pCa of the solutions in each set ranging between 6.5 and 4.5.

**Force-[Ca\textsuperscript{2+}] relationship in different solution conditions.** The direct effects of the 66 mM K\textsuperscript{+} solution (with or without 5 mM PEP-PK) on the force-[Ca\textsuperscript{2+}] relationship of the contractile apparatus were examined by activating a skinned fiber under each condition in a set of solutions with the free [Ca\textsuperscript{2+}] buffered at progressively higher levels in the range pCa 6.3 to 4.5. This procedure has been extensively discussed elsewhere (see (9, 41)). Briefly, the skinned fiber segment was exposed to the Ca\textsuperscript{2+}-buffered solutions only after being treated first in Triton X-100 (1 % vol/vol) in relaxing solution for 5 min to completely lyse all membranous compartments, which might otherwise affect the free Ca\textsuperscript{2+} level within the fiber, with all traces of the Triton X-100 removed by two 1 min washes in relaxing solution. The fiber was then exposed to solutions of progressively higher [Ca\textsuperscript{2+}] until maximum force was achieved, with the sequence repeated twice for each circumstance to verify reproducibility. Control conditions (126 mM K\textsuperscript{+}) were examined first, and then either the ‘66 mM K\textsuperscript{+} solution’ or the ‘66 mM K\textsuperscript{+}+5 mM PEP-PK’ test condition. Control conditions were examined after each test condition. The isometric force responses elicited at each different pCa under a given condition were expressed as a percentage of the corresponding maximum Ca\textsuperscript{2+}-activated force, plotted against the pCa values and a Hill curve fit using GraphPad Prism 3 (GraphPad Software, San Diego, Ca., USA). The pCa required to elicit 50 % of the maximum Ca\textsuperscript{2+}-activated force (pCa\textsubscript{50}) under the test conditions was compared to the average of the control (126 mM K\textsuperscript{+}) values obtained before and after treatment to eliminate any change in force associated with repeated activation.

<<Figure 1 near here>>
The Ca$^{2+}$-sensitivity of the contractile apparatus in the 66 mM K$^+$ solution was reduced relative to that in the 126 mM K$^+$ solution (see fiber example in Fig 1). Very similar effects were found in all four fibers examined, with the pCa$_{50}$ decreasing by 0.15 ±0.01 (P<0.05, from a mean of ~6.01) and the Hill coefficient (h) increasing slightly (from ~4.1 to ~4.6, change 0.5 ±0.1), without significant change in maximum Ca$^{2+}$-activated force (99.0 ±0.8% in 66 mM K$^+$ relative to 126 mM K$^+$). Virtually identical changes in contractile apparatus properties were also seen when 5 mM PEP (with 25 units of PK) was present in the 66 mM K$^+$ solution (changes in pCa$_{50}$ and h value, and maximum force relative to bracketing 126 mM K$^+$ case: 0.15 ±0.01, ~0.3 ±0.2, and 99.6 ±0.9%, respectively). The lower Ca$^{2+}$-sensitivity in the 66 mM K$^+$ solutions compared to the 126 mM K$^+$ solutions is due to the fact that the [K$^+$] was lowered by replacing it with additional Na$^+$ (to maintain constant osmolality and ionic strength) and this change reduces contractile sensitivity (14).

_T-system AP generation by transverse electric field stimulation._ The skinned fiber segment mounted on the force transducer was positioned parallel to, and midway between, two platinum electrodes in a stimulating chamber containing 130 µl of the control K-HDTA solution. Electric field pulses (duration, 1 ms; 75 V cm$^{-1}$) were applied by an in-house stimulator in order to generate APs in the sealed T-system (see (8, 43, 50)). The electric field pulse strength was ~2.5-fold greater than required to elicit maximum twitch force (i.e. supramaximal). Twitch and tetanic force responses were elicited with single pulse and 50 Hz stimulation (400 ms, 20 pulses), respectively. Peak force was reached well before the end of the tetanic stimulation. Whenever a fiber was transferred from one solution condition to another, the fiber was first equilibrated in the new conditions for 20 to 30 s before stimulation. Fibers were kept in depolarizing solution for no more than 3 min at a time and returned to fully-polarizing control conditions between each
episode (these are not shown in some Figures). The fiber properties remained approximately constant over such a period in the depolarizing conditions.

To quantify the ability of the T-system to generate a second closely-spaced AP, pairs of identical pulses (duration, 1 ms; 75 V cm⁻¹) were applied, with various time spacings (1-20 ms) between the end of the first pulse and the start of the second pulse (‘inter-pulse spacing’). If the second pulse of the pair was unable to generate a second AP, the twitch response would be no different to when stimulating with a single pulse alone. If the second pulse did elicit an AP then the submaximal twitch response, a sensitive indicator of changes in the amount of Ca²⁺ released, should increase substantially in peak (see (8, 43, 50)). In this study, the repriming period was defined as being the time, in nearest whole ms, taken for >50 % incremental increase in the twitch size elicited by a pair of pulses compared to the twitch size elicited by a single pulse alone under the same conditions. In practice, the demarcation line of a 50% incremental increase was not critical for responses in control conditions, because twitch force was observed typically to increase by >70% of the maximum increment over a very narrow range of inter-pulse spacings (e.g. open circles in Figures 2B, 3B & 4A).

Statistics. Data are expressed as mean ± standard error of the mean (SE), with the number of fibers studied denoted as ‘n’. In Tables 1 and 2, Student’s paired t-test was used to determine statistical significance in each separate subset of fibers (significance accepted at a probability value (P) <0.05). The significance level was not adjusted by Bonferroni’s correction because this was not appropriate for this particular presentation format. In this design, the main treatments (e.g. effect of 66 mM K⁺, or addition of PEP plus PK) were independently examined in two or more separate fiber subsets, with the same statistical outcome, and the effect of a single further
treatment (e.g. addition of pyruvate to 66 mM K\(^+\), Table 1 subset 4) was examined by paired comparison in one of these fiber subsets
Results

**AP-repriming and twitch and tetanic responses in well-polarised fibers.**

As described previously, electric field stimulation can be used to elicit APs in the sealed T-system of mechanically-skinned muscle fibers, and thus trigger twitch and tetanic force responses (8, 11, 40, 43). When the T-system was maintained at a well-polarised level by bathing the skinned fiber in the high [K⁺] (126 mM K⁺) ‘control’ solution, single AP stimulation elicited a highly reproducible twitch response, which in different fibers ranged in size from ~25 to 50% of the maximum Ca²⁺-activated force level, with tetanic stimulation at 50 Hz eliciting maximum force (e.g. Fig. 2A). The extent and characteristics of AP-induced Ca²⁺ release in the skinned fibers are very similar to those in comparable intact muscle fibers (1, 42), though the peak twitch force is slightly larger, and fusion frequency for tetanic stimulation lower, in skinned fibers compared to intact fibers, most likely because parvalbumin, an endogenous cytoplasmic Ca²⁺-buffer, is not present in the solution perfusing the skinned fibers.

After an AP has been elicited, the T-system membrane is refractory for several milliseconds and unable to support the generation and propagation of a second closely-spaced AP (24). Consequently, application of a second field pulse during this period fails to elicit any further Ca²⁺ release, and the size of the twitch response to two closely-spaced pulses is no different than to a single pulse alone (see 0 to 3 ms spacings in Fig. 2A). If the T-system is sufficiently well polarised, the Na⁺ channels recover rapidly from fast inactivation and the K⁺ and Cl⁻ conductance changes subside (13, 47). In this situation, the T-system can again support a propagating AP within a few milliseconds, which results in a second discrete phase of SR Ca²⁺ release and a larger force response (1, 42). Note that the development of force is relatively slow (~30 ms 10-90% rise time for twitch response to a single AP, (10)) and only a single force response is seen to the two AP-induced Ca²⁺ release events. In the skinned EDL fibers examined
here when the T-system was well-polarized in the 126 mM K\textsuperscript{+} ‘control’ solution, the force response to a pair of pulses was highly reproducible, with the twitch response showing a marked quantal increase when the two pulses were applied 4 ms or more apart (Fig. 2), indicating a ‘repriming period’ (RP) of 4 ms (see definition in Methods).

<< Figure 2 near here >>

In these skinned fibers, the maintenance of the T-system potential is highly dependant on the continuous function of the Na\textsuperscript{+}-K\textsuperscript{+} pump (see Introduction). Pump function was supported in the control solution by the presence of 8 mM ATP, with 10 mM CP also present to facilitate resynthesis of ATP via the creatine kinase (CK) reaction. In these well-polarized conditions, addition of 5 mM PEP (with pyruvate kinase, PK, 25 units ml\textsuperscript{-1}) had no significant effect on the repriming time or on the twitch size (e.g. Fig. 2 & Table 1 subset 1). (There was a slight reduction in peak tetanic tension, likely caused by direct effects on the contractile apparatus of a small remaining difference in ionic strength of the solution – see Methods).

<< Table 1 near here >>

**Effect of PEP and PK on AP repriming when the T-system is partially depolarised.** In order to examine whether ATP-resynthesis via the glycolytic pathway can aid the function of the Na\textsuperscript{+}-K\textsuperscript{+} pumps in the T-system, we devised circumstances in which any increase in Na\textsuperscript{+}-K\textsuperscript{+} pump function would be readily detectable because it would make the T-system potential more negative and result in a noticeable increase in the rate of AP repriming. To achieve this, it was first necessary to partially depolarize the T-system so that AP repriming was slowed (11) and any improvement in AP repriming rate could be readily detected. The skinned fiber was transferred to a solution with the [K\textsuperscript{+}] reduced to 66 mM, which resulted in the T-system becoming partially
depolarized (from ~-80 mV to ~-60 mV, (11, 40). This slowed the AP repriming from ~4.1 ms to
~7.8 ms (Figure 3 and Table 1 top rows). The change in constituents of the bathing solution,
replacing 60 mM of the K+ with additional Na+, also caused a substantial decrease in the Ca2+-
sensitivity of the contractile apparatus (see Methods and Fig. 1), and this was a major cause of the
decline in twitch size in the 66 mM K+ solution, with the T-system depolarization itself being
directly responsible for only some of the decline in twitch size (see considerations in (9, 11)).

Under these conditions, addition of 5 mM PEP and exogenous PK (25 units ml⁻¹) resulted
in a marked improvement in the rate of AP repriming, decreasing the RP from a mean value of
7.9 ±0.2 ms to 5.8 ±0.3 ms in the 11 fibers examined (Table 1 subset 2). This can be seen in the
example in Figure 3, where it is also apparent that with PEP-PK there was an increase in the size
of the twitch response to single AP stimulation, which must have been due to improved T-system
polarization and increased Ca2+ release because the properties of the contractile apparatus were
unchanged by PEP-PK (Fig. 1 and Methods). On average the presence of 5 mM PEP and PK in
the 66 mM K+ solution resulted in the twitch peak increasing from 71.8 ±5.9% to 82.6 ±5.1% of
its control level and the peak tetanic force to 50 Hz stimulation increasing from 56.6 ±7.3% to
70.6 ±8.0% of its control level.

The improvement in AP repriming rate with PEP and PK occurred to a similar extent
when the [K+] was lowered to 66 mM by replacement with NH4⁺ rather than Na⁺, so as to
maintain [Na⁺] constant (RP: (i) 3.7 ±0.2 ms for control, (ii) 6.3 ±0.3 ms for 66 mM K⁺, and (iii)
5.0 ±0.0 ms for 66 mM K⁺ with 5 mM PEP-PK, n=3, all cases significantly different, P<0.05).
Substituting NH4⁺ for K⁺ also has a comparatively smaller effect on the Ca²⁺-sensitivity of the
contractile apparatus (11). These data further confirm that the effects on AP repriming are due to
the changes in T-system polarization and not to the particular ions used.
Endogenous PK activity and PEP requirement. Addition of PEP was found to increase both the AP repriming rate and the twitch and tetanic responses in the 66 mM K\(^+\) conditions irrespective of whether or not exogenous PK was also added (Figure 4; Table 1 subsets 2 and 3). This indicates that some of the normal endogenous PK was retained in the skinned fibers and was bound near the T-system Na\(^+\)-K\(^+\) pumps, and that this was adequate for the increased pump function. When exogenous PK was added without any PEP there was no significant change in any of the parameters (Table 1 subset 3), consistent with the PK requiring PEP as a substrate. (Note that in the comparisons made in Table 1 subset 3, the case of PEP alone was always examined before the case of exogenous PK addition, so as to avoid any possibility of the former being affected by residual exogenous PK; in all other experiments, matched cases were examined in pseudo-random order or by bracketing as appropriate). The effects of PEP were not due to the pyruvate produced during ATP resynthesis by the PK reaction; addition of pyruvate (1 mM) alone had no significant effect on the rate of repriming or the force responses (Table 1 subset 4).

Effects on tetanic responses. Figure 5 shows examples of the effects of PEP or PK alone on the tetanic responses in one fiber, presented on two different time scales. Depolarization in the 66 mM K\(^+\) conditions cause a greater proportional decrease in tetanic force than in twitch force, because single APs, or the first AP in a train, may be little affected whereas the slowed repriming greatly interferes with the generation and propagation of the second and subsequent APs in a tetanic train (11). As expected from this, addition of PEP appeared to cause proportionately greater improvement in tetanic force than in twitch force (Table 1, subsets 2 and 3). The comparatively early decay of tetanic force occurring when the T-system is depolarized in 66 mM
K⁺ conditions in some fibers (e.g. Figure 5B) is indicative of inadequate Ca²⁺ release at later times during the 400 ms stimulus train. It seems that net uptake and diffusional loss of Ca²⁺ exceeds the rate of SR Ca²⁺ release, leading to a fall in cytoplasmic free [Ca²⁺] and consequent force relaxation. This reduced Ca²⁺ release is again simply a reflection of the relatively poor AP repriming occurring in the depolarized conditions (Fig. 3 and see also (11)). In general, the tetanic force reached a peak slightly later when PEP was added to aid Na⁺-K⁺ pump function and AP repriming.

<< Figure 5 near here >>

Relative efficacy of CP and PEP in supporting Na⁺-K⁺ pump function. Direct comparisons made in 3 fibers showed that addition of 1 mM PEP was just as effective as 5 mM PEP in increasing the AP repriming rate and the force responses (Table 1 subset 5, PK present in both cases). Increasing the [CP] from 10 mM to 40 mM (with [ATP] maintained at 8 mM), caused a small increase in the AP repriming rate (Figure 6; Table 2 subset 1). The twitch and tetanic force responses were seemingly smaller in the 40 mM CP solution, and this was likely due simply to the known effects of high [CP] in directly reducing maximum force production and the Ca²⁺-sensitivity of the contractile apparatus (18). Importantly, even when there was 40 mM CP present, addition of 5 mM PEP (with PK) resulted in a marked further increase in the AP repriming rate (Figure 6; Table 2 subset 2).

<< Figure 6 and Table 2 near here >>

Effects of glibenclamide or presence of Cl⁻. Finally, we investigated the influence of other T-system ion channel conductances on the properties observed here, in particular Kₐₕₜ channels, which might be activated by local [ATP] and elevated [ADP], and also Cl⁻ channels. Blocking
$K_{ATP}$ channels with glybenclamide (50 $\mu$M) (46) in the depolarizing conditions (66 mM K$^+$) had no effect either on the rate of AP repriming or on the twitch and tetanic responses (Table 1, subset 6), indicating that such channels were not substantially activated by the conditions.

Cl$^-$ was absent from the solutions used throughout this study so that the T-system potential and properties were not dominated by the high Cl$^-$ permeability of the T-system (4, 5), but instead influenced primarily by K$^+$ movements alone, thus allowing changes in Na$^+$-K$^+$ pump function to be rapidly apparent. When T-system potential was instead clamped by having Cl$^-$ present, and [Cl$^-$] was raised reciprocally when decreasing [K$^+$] such that the [K$^+$][Cl$^-$] product remained constant (see (23)), addition of PEP caused no noticeable improvement in AP repriming (RP: 4.0 ±0.1 ms for control 126 mM K$^+$ with 3 mM Cl$^-$; 8.3 ±0.9 ms for 66 mM K$^+$ with 6 mM Cl$^-$; 8.3 ± 0.9 ms for latter conditions with 5 mM PEP and PK, n=3; contrast this with improvement occurring with PEP in absence of Cl$^-$ in Table 1 subset 2).
Discussion

This study has provided specific evidence that the Na\(^+\)-K\(^+\) pumps in the T-system of skeletal muscle fibers are situated in a specialised micro-environment and require localised ATP re-synthesis via the glycolytic pathway within the triad junction for maximal pump function. This pump dependence on local glycolytic ATP production is likely to be important in the maintenance of adequate T-system polarization and excitability during vigorous activity in muscle fibers in-situ.

Examining Na\(^+\)-K\(^+\) pump activity in the highly confined and isolated environment of the T-system is a difficult matter, especially when considering dynamic changes rather than total ion movements over a long period. Here, pump activity was not assayed directly but instead via its effect on T-system excitability, which in fact is the key matter in considerations of the properties and importance of the T-system Na\(^+\)-K\(^+\) pumps. The skinned fiber preparation used gave information specifically about the T-system pumps, without any confounding effects arising from the presence of pumps in the sarcolemma. Importantly too the cytoplasmic concentrations of the key substrates and ions could be accurately controlled and manipulated on a short time-scale. One drawback of this method is that such solution changes can affect the measured force responses by direct effects on the properties of the contractile apparatus (e.g. see Figure 1), but this can be readily quantified and separated from any effects on T-system excitability and AP-induced Ca\(^{2+}\) release (see (9, 11)).

Diffusional access to T-system Na\(^+\)-K\(^+\) pumps. It was found that cytoplasmic ATP does have at least some diffusional access to the Na\(^+\)-K\(^+\) pumps in the T-system in the skinned fibers here, a preparation where the normal in-situ structural arrangement and coupling at the triad are preserved (27, 40). This is evident because excitability was retained when the fibers were kept in
the 126 mM K⁺ polarizing solution with 8 mM ATP but with no means of glycolytic ATP production (e.g. control case in Figure 2). We have previously shown that these skinned fibers lose excitability if the T-system Na⁺-K⁺ pump function is stopped, such as by pre-addition of ouabain into the T-system before skinning the fiber (28) or by removing all cytoplasmic Na⁺, which causes complete or near complete loss of excitability within ~60 s (37, 39). This high dependency on Na⁺-K⁺ pump function arises because the T-system has a comparatively small volume and is no longer open to the extracellular solution as in an intact fiber. Consequently, the Na⁺-K⁺ pumps are needed to keep the T-system membrane polarised and they do this by maintaining the [K+] within the T-system at a low level and also likely by the electrogenic action of the pump, which can increase membrane polarization by up to a further 8 mV (3). Thus, there must have been sufficient ATP reaching the T-system Na⁺-K⁺ pumps to support some ongoing level of pump activity. It was apparent that the findings were not due simply to CP, rather than ATP, having diffusional access to the pumps, because excitability was also maintained when there was no CP present at all (results not shown, and see (31)).

It was evident, nevertheless, that diffusional access of ATP into the triad junction was quite limited, because it was not adequate to support higher levels of Na⁺-K⁺ pumping, despite the [ATP] in the bulk cytoplasmic solution being held at 8 mM by an effectively unlimited supply of ATP from the bathing solution (2 ml volume compared to <10 nL fiber volume), and additionally being locally buffered by CP. This was shown by depolarizing the T-system in 66 mM K⁺ solution, likely to ~-60 mV (40), where the rate of AP repriming was steeply dependent on the membrane potential (11, 13), and where AP propagation was hampered by the accompanying increase in K⁺ leakage current and Na⁺ channel inactivation (47) and an increase in intracellular [Na⁺] (37). In these conditions, the AP repriming time increased from ~4 ms to ~7.7 ms, and it was apparent that the Na⁺-K⁺ pump was not functioning at its maximal rate
because the AP repriming rate could be considerably accelerated in the same conditions simply by providing PEP to support glycolytic ATP supply to the pumps in the triad junction (RP repriming time ~5.8 ms, Figure 3 and Table 1).

**Importance of glycolytic production of ATP.** Addition of PEP caused an increase in T-system Na⁺-K⁺ pumping when there was 10 mM CP or even 40 mM CP present in the cytoplasm with the 8 mM ATP (Tables 1 and 2). The latter case (40 mM CP and 8 mM ATP) reflects the approximate levels present in the cytoplasm in a fully rested fast-twitch fiber. In exercise, the cytoplasmic [ATP] is initially maintained at this level though the [CP] may drop to 10 mM or lower, and with vigorous exercise the [ATP] can also decline considerably (15, 25). Thus, the findings here show that even when the cytoplasmic [ATP] and [CP] are at or above their maximal in-vivo levels, diffusion into the triadic region of ATP alone, or even of ATP and CP together, is not able to provide sufficient ATP for maximal Na⁺-K⁺ pumping. Furthermore, it can also be concluded that aerobic metabolism alone would not be sufficient in-vivo to support maximal Na⁺-K⁺ pumping because even though the mitochondria may be located quite close to the triad junction, some being positioned on the opposite side of SR terminal cisternae from the junction itself (2), any ATP and CP reaching the triad from the mitochondria must do so via the cytoplasmic space, and such supply would not be expected to exceed that prevailing in the present experiments. Moreover, it should be noted that in the experiments here local production of ATP from CP by the CK reaction was greatly favoured because there was no creatine present in the bathing solution, and this effect was sufficient to keep the local [ADP] outside the SR at <10 μM (32), well below the cytoplasmic levels present even in a rested fiber.

The fact that the experiments here were performed at ~25 ºC rather than at normal body temperature does not prevent us reaching the above conclusion about the inadequacy of aerobic
metabolism in-vivo, because the limitation to the supply process is the diffusion into the triad junction, and the rate of diffusion in solution is affected comparatively little by such a temperature difference, whereas the maximal rate of consumption of ATP by the Na\(^+\)-K\(^+\) pumps is likely to be considerably increased at body temperature.

**Pump rate and substrate concentrations within the triad junction.** It is not known whether the addition of PEP in the experiments here was sufficient to allow the Na\(^+\)-K\(^+\) pumps to operate at their maximal rate, which is 20 fold or more higher than the resting rate (3). The repriming time in the depolarizing conditions with PEP added (~5.8 ms) was longer than that in the fully polarized conditions (~4.0 ms), but this just means that the increased pump function with PEP was not sufficient to fully polarize the T-system, and it does not indicate that the pumps were necessarily operating sub-maximally. What is known, however, is that the effect of PEP had saturated over the range examined, as 5 mM PEP had no more stimulatory effect than 1 mM PEP (Table 1). Here we note that whilst a concentration of 1 mM PEP may seem a comparatively high value, it cannot be directly compared to values that might be presumed to be present in-vivo, because the latter cannot be readily estimated and the local concentration of PEP near its source of production within the highly confined volume of triad may well be very high. Furthermore, it is quite likely that there is limited diffusional access of PEP into the triadic space from the cytoplasm, and the concentration established there may be much lower than the 1 mM or 5 mM present in the bathing solution. We emphasise that this type of argument concerning the restricted space and unknown local concentrations within the triad junction does not bear on the arguments presented above concerning ATP and CP, because it was the bulk cytoplasmic concentrations of these substrates, not the triadic concentrations, that were pertinent, and these were set in the present experiments at appropriate known physiological values.
There also was no problem with other localised regions of high ATP usage interfering with the control of ATP and CP in the cytoplasm outside the triad junction, in particular ATP usage by the SR Ca\(^{2+}\) pumps and by the myosin heads, because there would have been virtually no such ATP usage until the moment that each AP was triggered and Ca\(^{2+}\) was released from the SR, and hence the cytoplasmic conditions should have been tightly set by the bathing solution. In contrast, within the triad junction itself there would have been a considerable ongoing usage of ATP by the Na\(^{+}\)-K\(^{+}\) pumps, and perhaps by other ATPases (22), and the steady-state level of ATP there was very likely much lower than in the cytoplasm. The maximal rate of ATP usage by the Na\(^{+}\)-K\(^{+}\) pumps is likely to be only ~10% of the total maximal ATP usage in a contracting fiber (49), but again as this occurs within a confined region, it could result in a considerable decrease in the [ATP] within the triadic junction. Nevertheless, given that ATP evidently has some diffusional access into the triad junction from the cytoplasm, and that the cytoplasmic [ATP] was set at a much higher level than that required for ATP hydrolysis by the pumps (3), we presume that the primary cause of the deficient pump function in the absence of PEP was not that there was insufficient ATP but rather that the [ADP] near the pumps was comparatively high and this interfered with pump function by decreasing the free energy for ATP hydrolysis.

*Glycolysis occurs within the triad junction.* As the stimulatory effect of PEP on T-system Na\(^{+}\)-K\(^{+}\) pump function was similar irrespective of whether or not exogenous PK was added (Table 1), it can be concluded that the ATP re-synthesis by PEP was catalysed by endogenously present PK that remained bound in the fibers following the mechanical skinning procedure. This is not surprising, because it has been observed previously that many glycolytic enzymes remain associated with the triad even after biochemical fractionation procedures required to obtain isolated triads (21). Also, we have shown previously that much of the endogenous CK also remains bound in the muscle fibers after mechanical skinning, and is lost only comparatively
slowly to the open bathing solution (10). Importantly, it is further apparent that some of this endogenous PK must have been present in the triad junction itself in the present experiments, because the results cannot be explained by PK catalysing ATP re-synthesis outside the triadic region, as the [ATP] there was well already maintained. Thus, the results here demonstrate that PEP was being used within the triad junction to better maintain the local [ATP] and [ADP] there.

The effect of PEP on increasing the rate of AP repriming was evidently not due to it reducing or preventing the opening of $K_{\text{ATP}}$ channels in T-system membrane (46), which if it occurred could hinder AP generation and propagation (20), because addition of 50 $\mu$M glybenclamide had no effect at all (Table 1), and this concentration of glybenclamide has been shown to be sufficient to fully block all $K_{\text{ATP}}$ channels in skeletal muscle fibers when added from either the cytoplasmic or T-system luminal side (46). Thus the present findings demonstrate that the T-system Na$^+$.K$^+$ pump do indeed require local glycolytic production of ATP to support high levels of pump activity.

The stimulatory action of PEP found here was not due to production of pyruvate via the PK reaction, as pyruvate itself had no effect (Table 1). Furthermore, any beneficial effect of pyruvate would be expected to come from increased mitochondrial production of ATP, and as mentioned above, this would not be expected to influence pump function here given the already ample supply of cytoplasmic ATP. We did not attempt to investigate whether the entire glycolytic pathway for ATP production remained functional in the skinned fibers here. Such experiments would involve having to also supply and balance the diffusible substrates NADH and NAD$^+$, as well as glucose or other substrates, and is beyond the scope of this study, and indeed was not necessary to address the aims of this study.
**Triad junction functions as a key specialised ATP micro-environment.** It was established here that the triad junction does act as a specialised micro-environment in respect to the diffusion, synthesis and usage of ATP. Other specialised ATP micro-environments have been identified in skeletal muscle, in particular i) the vicinity of the SR Ca^{2+} pumps, where CK is bound and CP is preferentially utilised to re-synthesise ATP to meet the high local consumption (26), and ii) the myosin heads, which are responsible for force generation and utilise considerable ATP but are located in a region of restricted diffusion where CP may act as a shuttle to help maintain ATP supply (35, 51). The present study highlights unique aspects of the ATP micro-environment within the triad junction. It differs from the other cases in that the dynamic ATP supply is evidently dependent in large part on local glycolytic mechanisms rather than on diffusion of ATP and/or local buffering by CP. This of course is fully consistent with the high density of glycolytic enzymes located in the triad junction (21) and with glycogen deposits being located close to this region. Also, recent observations indicate that much of the glucose up-take by a muscle fiber occurs in the T-system (30), which means that this glucose actually enters the fiber within or close to the triad junction, where some can be readily utilised to support the high local ATP demands of the Na^+-K^+ pumps. Thus, the triad junction has a vital and quite unique role in skeletal muscle function and regulation, being the site where electrical activity is transduced to trigger Ca^{2+} release and contraction and where the energy status of the cell can be sensed and can influence the transduction process by modulating T-system excitability as well the opening of the Ca^{2+} release channels, which also are directly influenced by the local [ATP] in the triad junction (10).
Acknowledgments

We are grateful to Prof. D.G. Stephenson for helpful discussions during this study. We also wish to thank Mr Brian Taylor for his expertise in designing and building the in-house stimulator used in this study. This work was supported by the National Health and Medical Research Council of Australia (grant 280623).
References


Figure legends

Figure 1. Effect of 66 mM K⁺ solution, with and without 5 mM PEP-PK on Ca²⁺-sensitivity of the contractile apparatus in an EDL fiber. The force-pCa relationship in control (126 mM K⁺) solution (open circles ‘○’) was obtained before, between and after exposure to the 66 mM K⁺ solution (open squares ‘□’) and the 66 mM K⁺ solution with 5 mM PEP and PK (filled squares ‘■’). The force elicited at each pCa was normalized to the maximum Ca²⁺-activated force produced under the same condition. Mean changes in pCa₅₀ and h in four fibers are given in the Methods.

Figure 2. Twitch and tetanic force responses and AP repriming with a well-polarized T-system.
A. Representative examples of twitch response in a skinned EDL fiber to a single supramaximal electric field pulse or to a pair of identical pulses separated by 1-20 ms (inter-pulse spacing, shown above each response) when the T-system was well-polarised (control conditions, 126 mM K⁺ solution), with or without 5 mM PEP and PK (25 units ml⁻¹). ‘0 ms’ inter-pulse spacing indicates application of a single pulse alone. In control conditions there was a large increase in twitch force to a pulse pair if the inter-pulse spacing was ≥ 4 ms, indicating that the second pulse of the pair had also succeeded in eliciting an AP in the T-system, hence causing additional Ca²⁺ release and force. Tetanic stimulation (Tet 50 Hz) produced ~100 % of maximum Ca²⁺-activated force (‘max’). B: Same data plotted as relative twitch force versus inter-pulse spacing; with PEP-PK (●), or without PEP-PK both before and afterwards (○). All values normalised to average twitch size to a single pulse in control conditions; many of the values are averages from multiple measurements made for the indicated condition, including some not shown in panel A.

Figure 3. Effect of 66 mM K⁺ and 66 mM K⁺ + 5 mM PEP-PK on T-system AP repriming. A: Examples of twitch response to a single pulse (labelled ‘0’) or to a pair of pulses with indicated
inter-pulse spacing (0-20 ms) under various conditions. Responses also shown for tetanic stimulation (Tet 50 Hz). The T-system was repolarized in control conditions for 3 min between periods in 66 mM K⁺ (not shown). B: Twitch responses in A expressed as a percentage of the twitch size to a single pulse in control conditions. Open circles (‘○’) for control conditions (126 mM K⁺) both before and after 66 mM K⁺ (open squares ‘□’) and 66 mM K⁺ + 5 mM PEP with 25 units ml⁻¹ PK (solid squares ‘■’). AP repriming was greatly slowed when the T-system was depolarized in the 66 mM K⁺ solution, but improved in presence of 5 mM PEP-PK.

Figure 4. **PEP speeds AP repriming even in absence of exogenous PK.** A. Normalised twitch responses to single or paired pulses, as in Figure 3. B. Mean (±SE) values across ‘n’ fibers. Open circles (‘○’) for control conditions (126 mM K⁺) both before and after other conditions; 66 mM K⁺ (open squares ‘□’), n = 19; 66 mM K⁺ + 5 mM PEP with 25 units ml⁻¹ PK (solid squares ‘■’), n = 14; and 66 mM K⁺ + 5 mM PEP with no exogenous PK (gray squares ‘■’), n = 4. Note that averaging data from fibers with slightly different repriming times produces a mean response that tends to obscure the sudden increment in twitch size which is usually apparent in the data of an individual fiber when the inter-pulse spacing is close to the repriming time. The mean of the repriming time measured in individual fibers is shown in Table 1 for each condition.

Figure 5. **Effect of 66 mM K⁺ + 5 mM PEP and 66 mM K⁺ + PK on tetanic force responses.** A. Twitch and tetanic responses under indicated conditions and response to maximum Ca²⁺-activating solution (‘max’). B: Tetanic responses from panel A super-imposed on expanded time base.
Figure 6. *Effects of raising [CP] to 40 mM and adding 5 mM PEP on AP repriming.* Normalised twitch responses in a representative fiber in various conditions, each bracketed by measurements in control conditions (126 mM K⁺, open circles (‘○’), presented similarly to Figure 3B. 66 mM K⁺ (10 mM CP, open squares ‘□’); 66 mM K⁺ with 40 mM CP (open triangles ‘△’); 66 mM K⁺ with 40 mM CP and 5 mM PEP and 25 units ml⁻¹ PK (solid triangles ‘▲’). All solutions also contained 8 mM ATP and 30 units ml⁻¹ CK. Repriming time was measured individually for each fiber and condition and the means are shown in Table 2.
Table 1. Summarized data of twitch and tetanic responses and repriming period in various conditions. Values are means ± SE. Twitch and tetanic responses expressed as a percentage of bracketing measurements in control condition (126 mM K⁺); twitch and tetanic force in control conditions were 45 ± 2% and 96 ± 2% respectively of maximum Ca²⁺-activated force in same fiber. ‘*’ denotes significantly different from bracketing control level and ‘#’ denotes significant improvement relative to 66 mM K⁺ level (Student’s paired t-test, except for comparison of grand mean of RP across all fibers (two top rows) where an unpaired t-test was used). Repriming period (‘RP’) is the minimum spacing between a pair of pulses needed to elicit >50% of the maximal incremental increase in twitch size (see Methods). All solutions contained 10 mM CP and 8 mM ATP. 'Glyb' indicates glybenclamide (50 µM). PK at 25 units ml⁻¹ where indicated. In a given data subset, every fiber was examined under each indicated condition.

<table>
<thead>
<tr>
<th>Condition</th>
<th>‘RP’ (ms)</th>
<th>Twitch Peak (%)</th>
<th>Tetanus Peak (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (126 mM K⁺) (40 fibers)</td>
<td>4.0 ± 0.1</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>66 mM K⁺ (28 fibers)</td>
<td>7.7 ± 0.1*</td>
<td>73.8 ± 4.1*</td>
<td>63.6 ± 4.1*</td>
</tr>
<tr>
<td>Subset 1 (4 fibers)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.3 ± 0.3</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>126 mM K⁺ + 5 mM PEP-PK</td>
<td>4.5 ± 0.3</td>
<td>102.7 ± 5.7</td>
<td>96.2 ± 2.1*</td>
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<td>Subset 2 (11 fibers)</td>
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<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>4.1 ± 0.2</td>
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<td>100</td>
</tr>
<tr>
<td>66 mM K⁺</td>
<td>7.9 ± 0.2*</td>
<td>71.8 ± 5.9*</td>
<td>56.6 ± 7.3*</td>
</tr>
<tr>
<td>66 mM K⁺ + 5 mM PEP-PK</td>
<td>5.8 ± 0.3*#</td>
<td>82.6 ± 5.1*#</td>
<td>70.6 ± 8.0*#</td>
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<tr>
<td>Subset 3 (4 fibers)</td>
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<td></td>
<td></td>
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<tr>
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<td>100</td>
<td>100</td>
</tr>
<tr>
<td>66 mM K⁺</td>
<td>8.5 ± 0.3*</td>
<td>68.5 ± 5.8*</td>
<td>56.7 ± 4.0*</td>
</tr>
<tr>
<td>66 mM K⁺ + 5 mM PEP</td>
<td>5.9 ± 0.3*#</td>
<td>79.1 ± 7.6*#</td>
<td>72.6 ± 5.9*#</td>
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<tr>
<td>66 mM K⁺ + PK</td>
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<td>59.5 ± 8.8*</td>
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<tr>
<td>Subset 4 (4 fibers)</td>
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<td>100</td>
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<tr>
<td>66 mM K⁺</td>
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<td>78.7 ± 3.5*</td>
<td>73.4 ± 4.0*</td>
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<td>66 mM K⁺ + 1 mM pyruvate</td>
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<td>81.2 ± 3.0*</td>
<td>71.2 ± 0.8*</td>
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<td>Subset 5 (3 fibers)</td>
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<tr>
<td>66 mM K⁺</td>
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<td>73.3 ± 6.3*</td>
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<td>66 mM K⁺ + 5 mM PEP-PK</td>
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<td>80.4 ± 5.9*</td>
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<tr>
<td>66 mM K⁺ + 1 mM PEP-PK</td>
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<td>86.3 ± 6.1*#</td>
<td>80.2 ± 8.0*</td>
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<td>Subset 6 (5 fibers)</td>
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<tr>
<td>Control</td>
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<tr>
<td>66 mM K⁺</td>
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<td>76.1 ± 6.3*</td>
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<td>66 mM K⁺ + 50 µM Glyb</td>
<td>7.4 ± 0.3*</td>
<td>72.0 ± 6.9*</td>
<td>70.0 ± 5.5*</td>
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Table 2. Comparative effects of raising CP concentration and adding PEP. Values are means ± SE, as in Table 1. In each data subset, every fiber was examined under each indicated condition. ‘*’ denotes twitch or tetanic force significantly smaller or repriming period (RP) significantly greater than in bracketing control cases; ‘#’ denotes significantly different from matching 66 mM K⁺ level in same fiber subset (Student’s paired t-test). All solutions contained 8 mM ATP, and also 10 mM CP unless indicated otherwise. All solutions contained 30 units activity ml⁻¹ of exogenous CK; 25 units ml⁻¹ of PK present with PEP.

<table>
<thead>
<tr>
<th>Condition</th>
<th>‘RP’ (ms)</th>
<th>Twitch Peak (%)</th>
<th>Tetanus Peak (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Subset 1 (3 fibers)</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control (126 mM K⁺)</td>
<td>3.2 ± 0.2</td>
<td>100</td>
<td>100</td>
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<tr>
<td>66 mM K⁺ (10 mM CP)</td>
<td>6.1 ± 0.4*</td>
<td>88.6 ± 5.3</td>
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<td>66 mM K⁺ (40 mM CP)</td>
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<td>82.8 ± 7.1*</td>
<td>71.8 ± 6.5*</td>
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<tr>
<td><strong>Subset 2 (5 fibers)</strong></td>
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<tr>
<td>Control (126 mM K⁺)</td>
<td>3.4 ± 0.2</td>
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<td>100</td>
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<tr>
<td>66 mM K⁺ (40 mM CP)</td>
<td>5.8 ± 0.3*</td>
<td>86.9 ± 6.5*</td>
<td>75.9 ± 5.9*</td>
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<td>66 mM K⁺ (40 mM CP) + 5mM-PEP-PK</td>
<td>4.3 ± 0.4*#</td>
<td>84.6 ± 4.3*</td>
<td>77.4 ± 5.1*</td>
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Figure 1. Effect of 66 mM K\(^+\) solution, with and without 5 mM PEP-PK on Ca\(^{2+}\)-sensitivity of the contractile apparatus in an EDL fiber.

60x41mm (600 x 600 DPI)
Figure 2. Twitch and tetanic force responses and AP repriming with a well-polarized T-system.

144x163mm (600 x 600 DPI)
Figure 3. Effect of 66 mM K\(^+\) and 66 mM K\(^+\)+ 5 mM PEP-PK on T-system AP repriming.
Figure 4. PEP speeds AP repriming even in absence of exogenous PK.

59x28mm (600 x 600 DPI)
Figure 5. Effect of 66 mM K⁺ + 5 mM PEP and 66 mM K⁺ + PK on tetanic force responses.
Figure 6. Effects of raising [CP] to 40 mM and adding 5 mM PEP on AP repriming.