Journal of Physiology (1989), **412**, *pp.* 155–180
With 8 text-figures
Printed in Great Britain

CHANGES OF INTRACELLULAR MILIEU WITH FATIGUE OR HYPOXIA DEPRESS CONTRACTION OF SKINNED RABBIT SKELETAL AND CARDIAC MUSCLE

BY ROBERT E. GODT AND THOMAS M. NOSEK
From the Department of Physiology and Endocrinology, Medical College of Georgia, Augusta, GA 30912-3000, USA

(*Received 2 March 1988*)

SUMMARY

1. Maximal calcium-activated force (F_{max}) and calcium sensitivity were markedly decreased in detergent-skinned fibres from skeletal and cardiac muscle by solutions that mimicked the total milieu changes associated with fatigue and hypoxia. Further experiments determined the relative contribution of each of the individual changes in milieu.

2. Both Ca^{2+} sensitivity and F_{max} of skeletal and cardiac fibres were decreased with increased [H^+] or inorganic phosphate (P_i). These effects were greater in cardiac muscle.

3. Decreasing MgATP over the range observed with fatigue and hypoxia (6·18–4·7 mM) had no effect on F_{max} or Ca^{2+} sensitivity of either muscle type.

4. Decreasing phosphocreatine (PCr: 15–1 mM) increased F_{max} but had little effect on Ca^{2+} sensitivity in both muscle types. In cardiac fibres, the effect on F_{max} could be mimicked by inhibition of endogenous creatine kinase.

5. ADP (0·7 mM) increased F_{max} and Ca^{2+} sensitivity, while AMP (0·06 mM) slightly increased F_{max} but had no effect on Ca^{2+} sensitivity of either skeletal or cardiac fibres.

6. Creatine (25 mM) had no significant effect on either Ca^{2+} sensitivity or F_{max} of skeletal and cardiac muscle fibres. At higher levels (50 mM), however, creatine depressed F_{max} and slightly altered Ca^{2+} sensitivity.

7. Thiophosphorylation of myosin P light chains (phosphorylatable light chains of myosin) in rabbit psoas fibres had no effect on Ca^{2+} sensitivity, yet slightly but significantly increased F_{max} under fatigue conditions.

8. Reducing the affinity for ATP hydrolysis (by adding ADP, AMP and creatine) over the range calculated for fatigue/hypoxia (60–45 kJ/mol) produced the enhancement in F_{max} expected from added ADP and AMP in cardiac but not skeletal muscle, indicating that changes in affinity influence F_{max} of skeletal muscle. Reducing affinity produced little change in Ca^{2+} sensitivity of skeletal muscle. In contrast, the change produced in cardiac muscle was greater than that expected from addition of ADP and AMP; i.e. decreasing affinity increases calcium sensitivity of the heart.

9. Simple summation of all significant changes expected from each constituent

altered by fatigue/hypoxia adequately predicted the observed changes in F_{max} and Ca^{2+} sensitivity in both cardiac and skeletal muscle fibres with but one exception (the change in Ca^{2+} sensitivity of skeletal muscle at pH 7 was slightly overestimated).

INTRODUCTION

In their classic paper on fatigue, Eberstein & Sandow (1963) proposed that the decline in force of isolated skeletal muscle with prolonged, low-frequency stimulation is due to either a reduction of 'the intensity of the E–C (excitation–contraction) link or [to a decrease in] the sensitivity of the contractile system to activation by the link'. They concluded that, under their conditions, the predominant mechanism responsible for fatigue was disruption of excitation–contraction coupling (ECC).

On the other hand, changes at the level of the contractile apparatus seem likely as well. Considerable alterations in intracellular milieu are observed in fatigued muscle (e.g. Dawson, Gadian & Wilkie, 1980) and certain of these are known to directly affect the contractile apparatus (e.g. the decline in pH and marked rise in inorganic phosphate: Fabiato & Fabiato, 1978; Brandt, Cox, Kawai & Robinson, 1982).

In cardiac muscle, contractile force declines dramatically with hypoxia. Studies on fatigued skeletal muscle (Dawson *et al.* 1980) and hypoxic cardiac muscle (Kammermeier, Schmidt & Jüngling, 1982; Allen, Morris, Orchard & Pirolo, 1985) demonstrate that the changes in intracellular milieu under these conditions are remarkably similar. While there is controversy as to whether ECC in the heart is affected in hypoxia (Allen & Orchard, 1983; MacKinnon, Gwathmey & Morgan, 1987), there is agreement that direct depressant effects on the contractile apparatus also play a significant role in hypoxia.

The purpose of this study was to mimic the changes in the intracellular milieu known to occur with fatigue and hypoxia (i.e. changes in the concentrations of adenine nucleotides, inorganic phosphate (P_i), H^+, phosphocreatine and creatine, and the affinity ('free energy') for ATP hydrolysis) and to determine if there is a direct effect of these changes, both alone and in combination, on the properties of the contractile apparatus of skeletal and cardiac muscle. To this end, we measured calcium-activated isometric force of chemically 'skinned' rabbit psoas and papillary muscles. Our results indicate that the changes in the intracellular milieu associated with fatigue and hypoxia have a net depressant effect on the contractile apparatus and that this effect is primarily due to the intracellular increase in P_i and to any decrease in pH that may accompany these conditions. Therefore, in Eberstein and Sandow's terms, the sensitivity of the contractile system to 'activation by the E–C link' is decreased under conditions mimicking fatigue and hypoxia. Preliminary reports of this work have already appeared (Godt, Fender, Shirley & Nosek, 1985*a*; Godt & Nosek, 1985).

METHODS

Small bundles of fibres were dissected from psoas muscles of rabbits killed by pentabarbitone overdose (*ca* 160 mg/kg) delivered via an ear vein. Bundles were stored at −20 °C in 50% glycerol–50% CTP (cytidine-5′-triphosphate) relaxing solution (see Table 1) which also contained 100 μM-leupeptin and purified Triton X-100, a non-ionic detergent. Storage in CTP

TABLE 1. Standard bathing solutions

	CTP relax	Standard bathing solution	Fatigue/hypoxia			
			Control	Test No. 1	Test No. 2	Test No. 3
Mg^{2+}	1	1	1	1	1	1
MgATP	0	1	6·18	4·7	4·7	4·7
MgCTP	2	0	0	0	0	0
ADP total	0	0	0·05	0·7	0·7	0·00155*
AMP total	0	0	0·0002	0·062	0·062	0
P_i total	0	0	0·88	17·38	17·38	17·38
Phosphocreatine	0	15	14·16	1·42	1·42	1·42
Creatine	0	0	11·46	21·14	47·30	0·045*
EGTA	5	5	5	5	5	5
Imidazole	20	20	20	20	20	20
K (acetate or Cl)	109	110–120	81–91	92–103	93–104	95–106
$CaCl_2$	0	Varied	Varied	Varied	Varied	Varied
pH	7	7	7	7	6·65	7
Creatine kinase (mg/ml)	0	1	1	1	1	1
Ionic strength (M)	150	200	200	200	200	200
Leupeptin	0·1	0	0	0	0	0
Affinity (kJ/mol)	†	†	60	45	45	60

Concentrations are in mM unless otherwise indicated. EGTA concentration in the stock solution was determined using the pH titration method of Moisescu & Pusch (1975). Affinity (A) for ATP hydrolysis (Dawson *et al.* 1978) was calculated from:

$$A = -\Delta G_{obs} + RT \ln [ATP]/[ADP][P_i],$$

where the standard free energy (ΔG_{obs}) for these conditions was taken as −30·5 and −30·1 kJ/mol for pH 7 and 6·65 respectively (Alberty, 1972). [ATP], [ADP] and [P_i] refer to total concentrations of these moieties, and R and T have their usual significance (Dawson *et al.* 1980).

* No ADP or creatine was added to test solution No. 3. However, as detailed in Methods, ATP used in this solution (Pharmacia) contained about 1% ADP. Therefore, the creatine kinase equilibrium produces the ADP and creatine concentrations shown.

† High, since no P_i or ADP are added to solution. Within the fibre, however, some P_i and ADP result from ATP hydrolysis.

and leupeptin was undertaken to prevent phosphorylation of the myosin light chains because leupeptin inhibits the proteolysis of myosin light chain kinase (MLCK) to a calcium-insensitive form and CTP is not a substrate for MLCK (Pires & Perry, 1977; Srivastava & Hartshorne, 1983). Single fibres selected from these bundles were attached between an opto-electronic force transducer and a movable arm using the method of D. Martyn & A. M. Gordon (personal communication). The

TABLE 2. Additional stability constants (K, in M^{-1})

Species	log K
HADP/H*ADP	6·40
H_2ADP/H*HADP	3·96
MgADP/Mg*ADP	3·17
MgHADP/Mg*HADP	1·68
CaADP/Ca*ADP	2·81
CaHADP/Ca*HADP	1·57
KADP/K*ADP	0·7
HAMP/H*AMP	6·19
H_2AMP/H*HAMP	3·80
MgAMP/Mg*AMP	1·97
CaAMP/Ca*AMP	1·80
KAMP/K*AMP	0·2
HPO_4/H*PO_4	11·74
H_2PO_4/H*HPO_4	6·72
$MgHPO_4$/Mg*HPO_4	1·7
MgH_2PO_4/Mg*H_2PO_4	0·7
$CaHPO_4$/Ca*HPO_4	1·5
CaH_2PO_4/Ca*H_2PO_4	0·6
$KHPO_4$/K*HPO_4	0·49
H acetate/H* acetate	4·56
Ca acetate/Ca* acetate	0·53
Mg acetate/Mg* acetate	0·51
HBES/H*BES	7·12
HTES/H*TES	7·46
HMES/H*MES	6·13
MgMES/Mg*MES	0·8
CaMES/Ca*MES	0·7

Constants for ADP and AMP were obtained from Smith & Martell (1975), for inorganic phosphate from Smith & Martell (1976), for acetate from Martell & Smith (1977), and for MES, BES and TES at 22 °C from Good, Winget, Winter, Connolly, Izawa & Singh (1966).

transducer and arm were terminated by small hooks made from thin (*ca.* 150 μm diameter) stainless-steel wire which had been roughened by gentle sandblasting. For attachment, the fibre was wrapped several times around the hook and immersed in a Ca^{2+} activating solution whereupon the fibre wrapped itself tightly to the hooks. At the end of the experiment the fibre was removed by immersing the hooks in common bleach. The attached fibre was then stretched to a striation spacing of 2·6 μm, as monitored with a He–Ne laser. The fibre was transferred between experimental solutions contained in small troughs on a movable, spring-mounted holder. We added Triton X-100 (*ca* 0·5% w/v final concentration) to each bathing trough. All experiments were performed at room temperature (*ca* 22 °C).

Small bundles of papillary muscle cells (*ca* 125–200 μm diameter) were prepared from rabbits killed as above (or, in a few cases, from guinea-pigs killed by cervical dislocation). Fine human hairs were tied to each end with a single overhand knot. The bundle was attached between an opto-electronic transducer and a movable arm by wedging the hair into roughened, stainless-steel hooks bent into a tight V-shape. Dissection and tying were performed under relaxing solution (pCa $>$ 8·5). Bundles were skinned by exposure to relaxing solution with 0·5% w/v purified Triton X-100 for at least 60 min. After mounting, the bundle was extended until passive force was just perceptible.

Bundles were either used on the day they were prepared or, as with skeletal muscle, were stored up to 2 weeks at −20 °C in 50% glycerol–50% CTP relaxing solution. No significant difference in performance between fresh and stored preparations was observed.

The force produced by skinned fibres typically declines somewhat with repeated activation. To control for any deterioration, each test contraction was bracketed by control contractions and compared with the mean of these contractions.

TABLE 3. Phosphorylation test solutions

	Control	Test
Mg^{2+}	1	1
MgATP	3·74	2·9
ADP total	0·03	0·33
P_i total	4·4	28·6
Phosphocreatine	24·2	1·1
Creatine	8·8	33
EGTA	5	5
Imidazole	20	20
KCl	59–69	94–105
$CaCl_2$	Varied	Varied
pH	7·0	6·65
Creatine kinase (mg/ml)	1	1
Ionic strength	200	200
AP_5A	0·1	0·1
Affinity (kJ/mol)	56	44

Unless otherwise noted, all concentrations are in mM.

Bathing solution composition is shown in Table 1. In most cases ionic strength was kept constant by adjustment of the concentration of potassium acetate, although some experiments were performed in solutions using KCl for this purpose. Sodium acetate was used only in experiments on effect of pH on maximal force; see below. Calculation of the concentration of constituents required to mix these solutions was accomplished using a microcomputer program written in Turbo Pascal (Borland International, Scotts Valley, CA, USA). The stability constants used in these calculations are those commonly used in our laboratory (Godt & Lindley, 1982) with the addition of those shown in Table 2.

In the fatigue/hypoxia series (see Table 1), the control solution mimics the intracellular composition of resting muscle, while the test solutions mimic changes accompanying fatigue or hypoxia. The values for ATP, ADP, P_i, PCr and creatine are for rat hearts before (control) and after 2 min of hypoxia (test solution No. 1) assuming intracellular pH is 7 in control and early hypoxia (Kammermeier *et al.* 1982). Test solution No. 2 (used to test for an effect of acidosis) is the same as test solution No. 1 except that the pH is lower and creatine is higher. The value for pH in test solution No. 2 (pH 6·65) was estimated from Dawson *et al.* (1980), their Fig. 4*a*, for fatigued frog skeletal muscle. At this lower pH creatine must be elevated to keep ATP, ADP and PCr constant because protons are reactants in the Lohmann reaction:

$$H^+ + ADP + PCr \Leftrightarrow ATP + Cr. \qquad (1)$$

The appropriate concentration of creatine was calculated using an equilibrium constant ([ATP][Cr]/[ADP][PCr][H]) of $10^9\,M^{-1}$ (Kammermeier *et al.* 1982). Endogenous adenylate kinase (myokinase) has been observed in glycerinated rabbit skeletal muscle (Abbott & Leech, 1973), so we included AMP to satisfy the adenylate kinase reaction:

$$2ADP \Leftrightarrow ATP + AMP, \qquad (2)$$

at the levels of ATP and ADP desired. Concentrations of AMP were calculated using an equilibrium constant ($[ATP][AMP]/[ADP]^2$) of 0·6 derived from high-performance liquid chromatography (HPLC) measurements (D. J. E. Luney, K. Y. Fender & R. E. Godt, unpublished observations).

Test solution No. 3 (used to test for an effect of affinity for ATP hydrolysis) was basically the same as test solution No. 1 except that no extra ADP, AMP or creatine were added. Commercially available ATP, however, is contaminated with some ADP which we could estimate using HPLC. Of the ATP available to us, that used in these experiments (P.L.-Pharmacia) contained the lowest concentration of contaminating ADP (approximately 1%). The level of ADP in the final solution was decreased by the Lohmann reaction so that the affinity of test solution No. 3 was 60 kJ/mol, the same as that for the control solution. Most of our experiments, however, were conducted using ATP from Sigma Chemical Co., which contained approximately 4% ADP.

Experiments on the effects of pH (6·2–7·4) on maximal force required different pH buffers to achieve adequate buffering over this wide range. We used MES (2-(*N*-morpholino)ethanesulphonic acid) at pH 6·2, BES (*N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid) at pH 6·6 and 7·0, and TES (*N*-tris(hydroxymethyl)methyl-2-aminosulphonic acid) at pH 7·4. Buffer concentration was 50 mM in all cases; ionic strength was adjusted with sodium acetate. Exchange of buffer *per se* had no effect on our estimation of any pH effects because maximum force was not significantly different in solutions (at pH 6·6) buffered with either MES, BES or TES.

For the phosphorylation experiments, the myosin P light chains of skinned psoas fibres were irreversibly thiophosphorylated following the procedure of Cooke, Franks & Stull (1982). Single fibres were exposed for 10–20 min to a thiophosphorylating solution containing (in mM); 50 KCl; 50 Tris; 1 EGTA; 7 $MgCl_2$; 2 ATP-γ-S; 0·98 $CaCl_2$ so that pCa was 5·5; 0·1 dithiothreitol; 50 nM-myosin light chain kinase (MLCK) and 2 μM-calmodulin, at pH 7·5. Calmodulin and MLCK were kindly provided by R. John Solaro. Thiophosphorylation of P light chains was verified in parallel experiments using alkaline urea gel electrophoresis of small bundles of fibres (Godt, Fender, Pan & Solaro, 1985*b*). Force–Ca^{2+} relations before and after thiophosphorylation were determined in solutions given in Table 3.

To determine calcium sensitivity, fibres were transferred sequentially through a series of solutions with increasing [Ca^{2+}]. Force–Ca^{2+} data were fitted (using a non-linear least-squares technique) to a Hill equation of the form:

$$\%\ \text{maximum force} = 100[Ca^{2+}]^N/((Ca_{50})^N + [Ca^{2+}]^N), \quad (3)$$

where N is a constant related to the steepness of the relationship and Ca_{50} is the calcium concentration required for half-maximal activation. In most cases, N and Ca_{50} were determined for each fibre under each condition. These values were then used to calculate the average N and Ca_{50} for each condition. When this was not possible (i.e. when the entire force–Ca^{2+} relation was not generated in each fibre), N and Ca_{50} were computed by fitting the Hill equation to the average force at each [Ca^{2+}]. Under all experimental conditions, Ca_{50} computed using both methods was similar, whereas N computed from the average of individual N values was always somewhat larger than that generated by fitting average force–Ca^{2+} data. In any event, the computed N values are very sensitive to force at low and high calcium and its precise determination requires measurements of force for Ca^{2+} concentrations more narrowly spaced than we chose to use. Thus we report N values for each condition but have carried out no statistics on them.

Statistical significance was determined by Student's t test with $P < 0.05$ as criterion. Statistical comparisons were only possible in those fibres where average N and Ca_{50} values could be determined. In most cases, control and test values were determined in the same fibre, allowing application of a paired test. In those cases where data were normalized or summed, the standard error of the resultant value was calculated using the relation for probable error of a function (Margenau & Murphy, 1956).

Chemicals were obtained from: Aldrich Chemical Co., potassium acetate; Baker Chemical Co., $MgCl_2$, KCl, EGTA, acetic acid; Sigma Chemical Co., ATP, ADP, AMP, PCr, creatine, creatine kinase, leupeptin; P.L.-Pharmacia, ATP, CTP; Fisher Chemical Co., NaH_2PO_4, imidazole; Calbiochem, imidazole; BDH Chemicals Ltd, $CaCl_2$; Boehringer Mannheim, creatine kinase, purified Triton X-100, diadenosine-5′-pentaphosphate (AP_5A), ATP-γ-S; Orion, $CaCl_2$.

RESULTS

Fatigue/hypoxia conditions

Do the changes in intracellular milieu associated with fatigue and hypoxia have any effect on the contractile apparatus? We mimicked the intracellular conditions

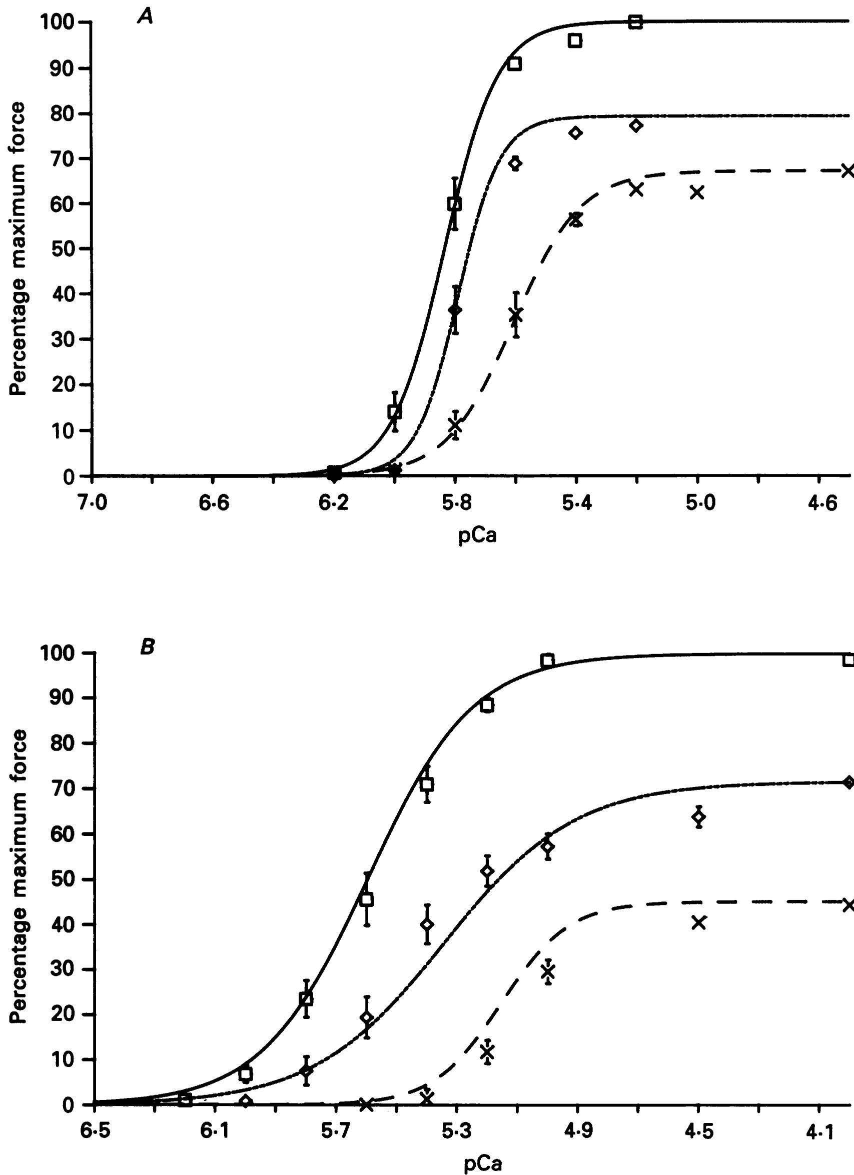

Fig. 1. Effects of solutions mimicking fatigue/hypoxic conditions on the force–pCa relationship of skinned skeletal (rabbit psoas, *A*) and cardiac (rabbit ventricle, *B*) muscle fibres. Control conditions, continuous line and □; early hypoxia (pH 7, test solution No. 1), double dot–dashed line and ◇; hypoxia with acidosis (pH 6·65, test solution No. 2), dashed line and ×. See Table 1 for complete solution composition. Solution ionic strength was adjusted with potassium acetate. In this and all subsequent figures, points show mean values from at least six fibres (except that $n = 5$ for control and hypoxia, pH 7 in Fig. 1*B*); bars are standard error of the mean. Curves represent non-linear least-squares fits to the data of a modified Hill equation (see text) using N and Ca_{50} values given in Table 4. Curves scaled relative to the maximum force in control conditions (100%). In this and all other figures, maximum force of skeletal fibres was obtained at pCa 4. In cardiac muscle, however, the maximum force was often achieved at a free calcium below pCa 4 and, unlike skeletal muscle, higher calcium led to significantly lower force. Thus, data for cardiac muscle are normalized to the actual maximum force attained in each fibre.

TABLE 4. Collective changes in F_{max} and force–Ca^{2+} relation

	Skeletal			Cardiac		
	F_{max} (%) (S.E.M.)	N	Ca_{50} (μM) (S.E.M.)	F_{max} (%) (S.E.M.)	N	Ca_{50} (μM) (S.E.M.)
		Fatigue/hypoxia				
Control (A = 60 kJ/mol)	100	4·91 (0·34)	1·47 (0·07)	100	2·48 (0·09)	2·53 (0·18)
Test (A = 45, pH 7)	79·3* (2·6)	6·19 (0·73)	1·68* (0·07)	71·7* (4·6)	2·08 (0·16)	4·53* (0·73)
Test (A = 45, pH 6·65)	67·2*† (1·1)	3·90 (0·34)	2·48*† (0·16)	45·2*† (1·8)	3·88 (0·44)	7·02* (1·2)
		pH changes				
Control (pH 7)	100	7·71 (0·78)	1·45 (1·03)	100	4·34 (0·54)	2·32 (0·10)
Test (pH 6·65)	86·2* (3·9)	4·77 (0·59)	3·42* (0·23)	84·5* (2·2)	2·79 (0·18)	5·40* (0·22)
		Inorganic phosphate				
Control (zero added P_i)	100	7·71 (0·78)	1·45 (0·03)	100	4·34 (0·54)	2·32 (0·10)
15 mM-P_i	73·5* (3·9)	5·34 (0·22)	2·60* (0·12)	55·4* (1·5)	2·96 (0·25)	4·94* (0·51)
		MgATP				
Control (6·18 mM)	100	4·88 (0·70)	0·88 (0·06)	100 (1·5)	3·24 (0·07)	2·17 (0·14)
Test (4·7 mM)	99·7 (5·6)	—	—	101·7 (1·7)	—	—
Test (1 mM)	111·8* (2·1)	6·57 (0·66)	0·94 (0·02)	99·8 (1·5)	4·34 (0·54)	2·32 (0·10)
		Phosphocreatine				
Control (15 mM)	100	7·71 (0·78)	1·45 (0·03)	100	4·34 (0·54)	2·32 (0·10)
Test (1 mM)	110·2* (1·9)	6·49 (1·55)	1·24* (0·08)	112·0* (3·9)	2·67 (0·34)	2·39 (0·18)

			ADP			
Control (zero ADP with AP_5A)	100	7·45	0·32	100	2·67	1·37
		(NSP)	(NSP)		(0·32)	(0·22)
Test (0·7 mM with AP_5A)	106·6*	5·91	0·26	110·9*	2·31	0·62*
	(1·3)	(NSP)	(NSP)	(2·6)	(0·44)	(0·09)
			AMP			
Control (zero AMP with AP_5A)	100	6·31	0·79	100	3·19	2·24
		(1·00)	(0·06)		(0·33)	(0·16)
Test (0·06 mM with AP_5A)	105·7*	4·35	0·64	105·6*	2·50	2·45
	(0·8)	(0·16)	(0·03)	(1·3)	(0·09)	(0·23)
			Creatine			
Control (zero creatine)	100	4·88	0·88	100	3·24	2·17
		(0·70)	(0·06)		(0·07)	(0·14)
25 mM-creatine	97·2	3·91	0·82	104·9	2·47	2·65
	(2·1)	(0·33)	(0·05)	(2·9)	(0·39)	(0·56)
50 mM-creatine	89·2*	4·84	0·68*	90·2*	2·97	2·69*
	(3·2)	(1·03)	(0·05)	(2·0)	(0·27)	(0·17)
			Phosphorylation			
Control						
Before phosphorylation	100	4·03	1·17	—	—	—
		(NSP)	(NSP)			
After phosphorylation	99·3	14·26	0·96	—	—	—
	(2·2)	(NSP)	(NSP)			
Fatigue conditions:						
Before phosphorylation	100	3·66	1·98	—	—	—
		(NSP)	(NSP)			
After phosphorylation	105·2*	4·54	1·69	—	—	—
	(1·7)	(NSP)	(NSP)			
			Affinity for ATP hydrolysis			
Control ($A = 60$)	100	2·63	0·75	100	2·48	2·53
		(NSP)	(NSP)		(0·09)	(0·18)
Test solution No. 1 ($A = 45$)	64·8*	3·58	1·08	71·7*	4·28	2·63
	(2·4)	(NSP)	(NSP)	(4·6)	(0·44)	(0·09)
Test solution No. 3 ($A = 60$)	66·2*	6·79	1·02	63·5*†	2·59	4·41*†
	(2·6)	(1·27)	(0·07)	(4·9)	(0·18)	(0·35)

Parameters N and Ca_{50} are for modified Hill equation fitted to force–Ca^{2+} data (see Methods).

Standard error of the mean (S.E.M.) calculated for at least six determinations (except for cardiac fatigue/hypoxia control and test, affinity $(A) = 45$, where $n = 5$).

* Significantly different ($P < 0·05$) from control (note significant differences in N values not statistically evaluated, see text).

† Significantly different ($P < 0·05$) from other test condition.

Dashes, not measured.

NSP: no statistics possible since complete force–pCa curves were not determined in each fibre.

reported to exist in resting and fatigued/hypoxic muscle cells with the solutions given in Table 1. Figure 1 and Table 4 illustrate that in both skeletal and cardiac muscle fibres, calcium sensitivity as well as maximum calcium-activated force (F_{max}) is decreased, with a greater effect at the lower pH. Under identical ionic conditions,

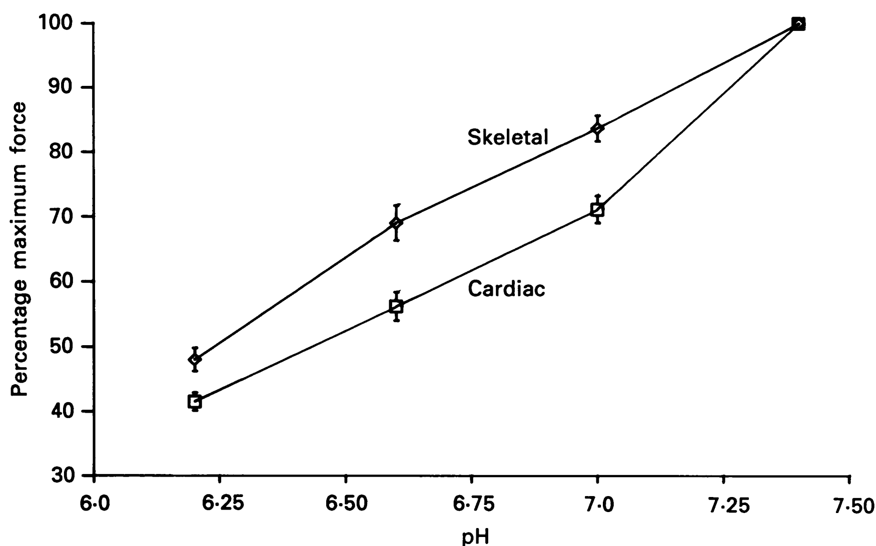

Fig. 2. Effect of pH on the maximal calcium-activated force in rabbit psoas (pCa 4, ◇) and cardiac (pCa 4–5, □; see legend of Fig. 1) muscle fibres. Data expressed relative to maximal force at pH 7·4. Ionic strength adjusted with sodium acetate. Solutions buffered with MES (pH 6·2), BES (pH 6·6 and 7) or TES (pH 7·4).

these effects are more profound in cardiac muscle. To examine which of the changes in milieu are responsible, we compared the individual influence of each on the force–pCa relationship of skeletal and cardiac fibres.

pH

Fatigue and long-term hypoxia of skeletal and cardiac muscle have been associated with a decline in intracellular pH (e.g. Dawson, Gadian & Wilkie, 1978; Allen *et al.* 1985). It is well established that a fall in pH has a depressant effect on the contractile machinery (e.g. Fabiato & Fabiato, 1978). In accord with these observations, we found a depressant effect of lowering pH on F_{max} (Fig. 2) and calcium sensitivity (Table 4) of skinned skeletal and cardiac muscle fibres under our experimental conditions.

Inorganic phosphate

Increases in intracellular inorganic phosphate (P_i) have been reported for fatigued skeletal and hypoxic heart muscle (Dawson *et al.* 1980; Kammermeier *et al.* 1982; Allen *et al.* 1985). The effects of P_i on active force developed by skinned skeletal and cardiac fibres were studied by including P_i in the standard bathing solution (see Table 1); all other constituents were kept constant with the exception of potassium

acetate which was decreased to maintain the ionic strength at 0·2 M. Figure 3 illustrates that P_i decreases F_{max} of skinned skeletal and cardiac fibres, with a greater effect in cardiac muscle. Increasing P_i also decreases the calcium sensitivity of the contractile apparatus (Table 4).

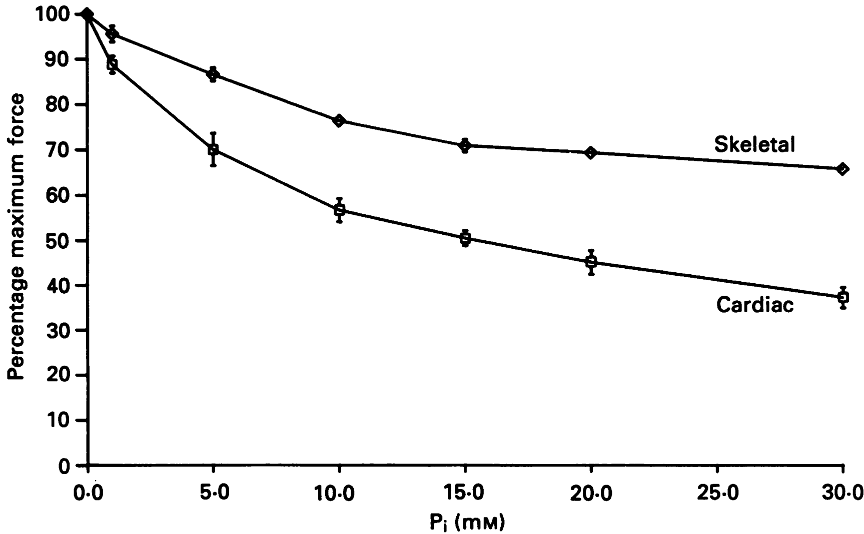

Fig. 3. Depression of maximum calcium-activated force of skinned rabbit psoas (pCa 4, ◇) and cardiac (pCa 4–5, □) muscle fibres by inorganic phosphate (P_i) at pH 7. Solution ionic strength adjusted with potassium acetate.

MgATP

With fatigue and hypoxia there is some decline in the intracellular level of ATP (cardiac, 6·18–4·7 mM, Kammermeier *et al.* 1982; approximately 20% decrease, no absolute concentrations given, Allen *et al.* 1985; skeletal, *ca* 3·5–2·3 mmol/kg [5·3–3·5 mol/l cytosol, see Godt & Maughan, 1988]. Dawson *et al.* 1978; *ca* 4–2 mmol/kg [6–3 mol/l cytosol], Dawson *et al.* 1980; 15% decline at most, Nassar-Gentina, Passonneau, Vergara & Rapoport, 1978). There was no significant effect on F_{max} of changing MgATP over the range 6·18–4·7 mM in either skeletal or cardiac fibres, although decreasing MgATP to 1 mM led to a slight increase in F_{max} of skeletal but not cardiac fibres. Moreover, in both tissues, calcium sensitivity was unaffected by changing MgATP from 6·18 to 1 mM (Table 4). Thus, the decrease in MgATP observed with fatigue/hypoxia would have little effect on the contractile apparatus of either skeletal or cardiac muscle.

Phosphocreatine

As has long been known, ATP levels within fatigued or hypoxic muscle cells are maintained relatively constant at the expense of phosphocreatine (PCr) (e.g. Nassar-Gentina *et al.* 1978). We found that lowering PCr from the control value, 15 mM, to 1 mM slightly increased calcium sensitivity in skeletal but not cardiac muscles (Table

4). Moreover, decreasing PCr increases maximal calcium-activated force (Fig. 4), an effect similar to that of decreasing MgATP (Best, Donaldson & Kerrick, 1977). Increasing PCr from 15 to 30 mM has no significant effect on maximal force. Similar effects of PCr on maximal force were seen in skinned guinea-pig cardiac bundles.

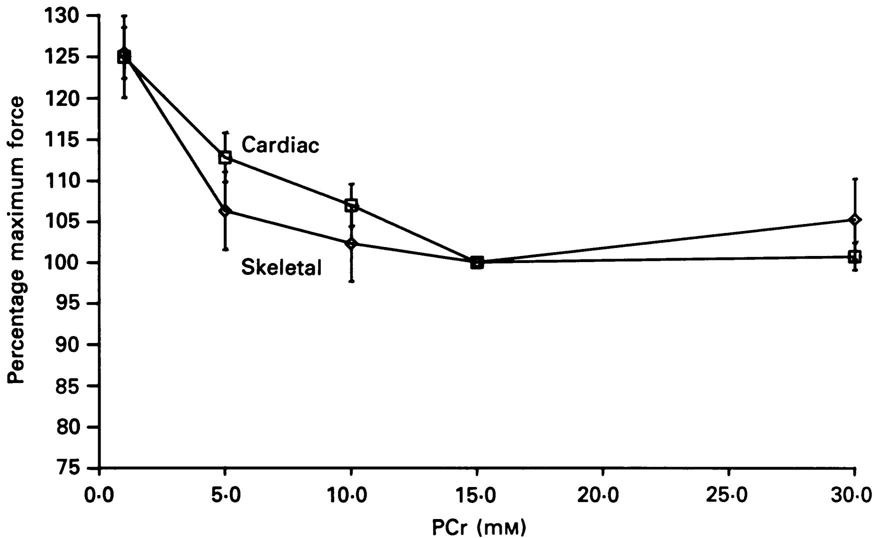

Fig. 4. Effect of phosphocreatine (PCr) on maximal calcium-activated force produced by skinned rabbit psoas (◇) and cardiac (□) muscle. Mean force shown relative to maximal force in 15 mM-PCr. Solution ionic strength adjusted with potassium acetate.

Since PCr is known to be involved only in the Lohmann (creatine kinase, CK) reaction, it seems unlikely on biochemical grounds that the effects of PCr shown above are due to a direct chemical influence on the contractile apparatus. More likely, the effect of drastically lowering PCr is indirect, resulting from poorer buffering of MgATP in the interior of the preparation by the CK reaction. We tested this possibility in the following fashion (see Fig. 5). Small bundles from guinea-pig papillary muscles were initially activated in a control solution, free of both PCr and added CK. Subsequently, each bundle was activated sequentially in two standard solutions (containing 15 mM-PCr, see Table 1), the first without (CK−) and the second with 1 mg/ml added CK (CK+). As expected from Fig. 4, force in 15 mM-PCr solutions, either with or without exogenous CK, was reduced relative to the 0 PCr control. Reduction of force in CK− suggests that there was endogenous CK in our preparations. The fibre was then bathed for 5 min in a relaxing solution containing 0·38 mM-fluorodinitrobenzene (FDNB), an irreversible CK inhibitor (Infante & Davies, 1965), to inhibit endogenous CK. After a brief rinse in FDNB-free relaxing solution, each fibre was again activated in the same CK− solution. Even though 15 mM-PCr was present, maximal force in CK− was not now significantly different from that in the 0 PCr control. Lastly, when the fibre was then activated in CK+, maximal force was reduced to the value obtained in this solution prior to FDNB

treatment, indicating that, under our conditions, FDNB had no deleterious effect on the contractile apparatus itself. Therefore, the effect of PCr on maximal force, shown in Fig. 4, is dependent upon the presence of active CK.

These findings indicate that the effect of PCr on maximal force is not a direct

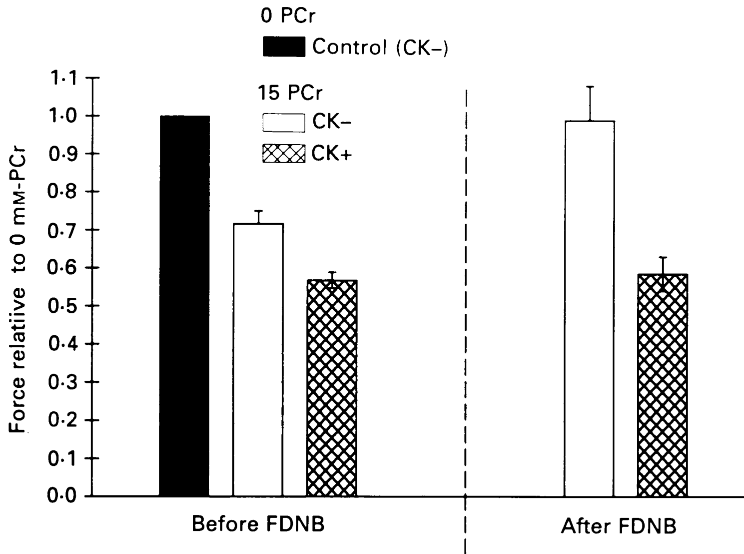

Fig. 5. Influence of PCr on maximum force in guinea-pig cardiac muscle in the presence (CK+) or absence (CK−) of active creatine kinase before and after FDNB treatment. All contractions are expressed relative to the maximal contraction in the control solution which contained no added PCr or CK. Activation sequence for each fibre is as shown along abscissa. Solution ionic strength adjusted with KCl.

chemical influence of the compound but is more likely related to its role in maintaining an adequate spatial and temporal buffering of ATP (and maintenance of low ADP; see below) within the preparation (Meyer, Sweeney & Kushmerick, 1984; Kentish, 1986).

ADP

Fatigue and hypoxia are also associated with an increase in intracellular ADP (Dawson *et al.* 1978, 1980; Kammermeier *et al.* 1982; Allen *et al.* 1985). To evaluate the direct effects of ADP, we modified standard bathing solutions (see Table 1) by eliminating PCr and CK to prevent the rephosphorylation of ADP by the CK reaction. In addition, all solutions contained 0·1 mM-diadenosine pentaphosphate (AP_5A), an inhibitor of adenylate kinase (Feldhaus, Froelich, Goody, Isakov & Schirmer, 1975), to prevent rephosphorylation of ADP by endogenous adenylate kinase (Abbott & Leech, 1973). Figure 6 illustrates that, in both skeletal and cardiac muscle fibres, ADP increases F_{max}. Addition of 0·7 mM-ADP, a concentration in the range likely for fatigued skeletal and hypoxic cardiac muscles (Dawson *et al.* 1980; Kammermeier *et al.* 1982), significantly increased the calcium sensitivity of cardiac skinned fibres (Table 4).

We observed that addition of 0·1 mM-AP_5A *per se* shifted the force–pCa relation of both skeletal and cardiac fibres to the left (decreasing Ca_{50} by 0·6 μM). This effect is not due to contaminating calcium accompanying the AP_5A since atomic absorption spectrophotometry (kindly performed by G. Whitford) revealed no more than 0·2 mol Ca^{2+} per mole AP_5A which is insufficient to account for the apparent shift in calcium sensitivity. Another possibility is that AP_5A binds sufficient

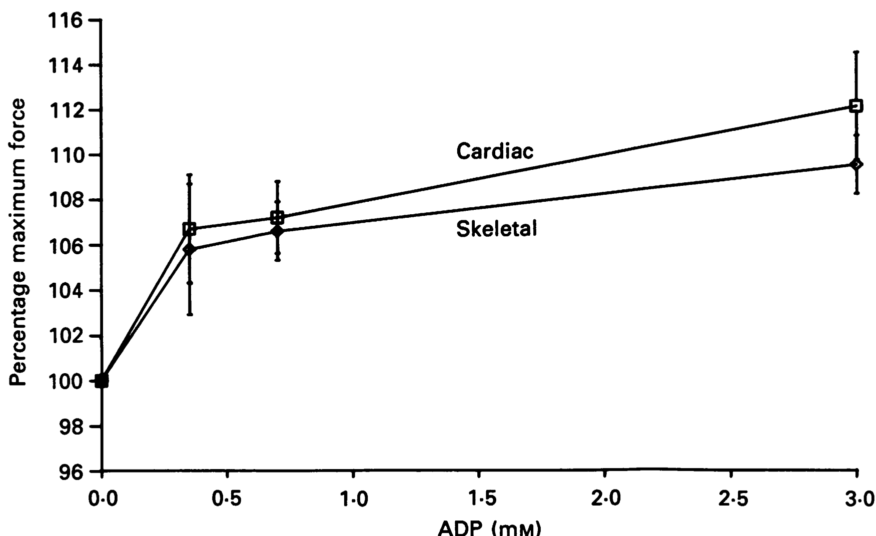

Fig. 6. Effect of ADP on maximum calcium-activated force of rabbit psoas (◇) and cardiac (□) muscle fibres. Force normalized to that at 0 ADP. KCl used to adjust solution ionic strength with skeletal fibres.

magnesium to appreciably lower free magnesium and thus increase calcium sensitivity (Donaldson & Kerrick, 1975; Godt & Lindley, 1982). Computations using association constants for the binding of 1 or 2 moles Mg per mole AP_5A (determined at very low ionic strength, Holler, Holmquist, Vallee, Taneja & Zamecnik, 1983) indicate that free Mg^{2+} would only be reduced about 0·1 mM in our solutions. Moreover, the association constants of Mg for AP_5A are likely to be decreased markedly at 200 mM ionic strength (Holler *et al.* 1983). Therefore, binding of Mg by AP_5A seems insufficient to explain the shift in Ca^{2+} sensitivity we observed with AP_5A. A third more likely possibility is that, in the absence of AP_5A, the endogenous adenylate kinase keeps the concentration of ADP within the fibre low. Thus the addition of AP_5A would increase calcium sensitivity by allowing intrafibre [ADP] to rise (cf. Cooke & Pate, 1985).

AMP

In the presence of active adenylate kinase, the intracellular level of AMP would increase during fatigue and hypoxia as ADP increases. Using our value for the equilibrium constant for this reaction, we calculate that AMP will be no greater than about 0·06 mM under fatigue or hypoxia conditions. We determined the direct effects of AMP on skinned fibres by adding AMP to our standard bathing solution (Table 1). All solutions also contained 0·1 mM-AP_5A to prevent phosphorylation of AMP by endogenous adenylate kinase. We found that AMP at this concentration has no effect on calcium sensitivity of either skeletal or cardiac fibres. However, this concentration of AMP produced a small but significant increase in the maximum force of both muscle types (see Table 4).

Creatine

During fatigue and hypoxia the intracellular level of creatine increases markedly, mirroring the decline in PCr (as expected from the creatine kinase reaction, Dawson *et al.* 1978, 1980). To determine the effects of creatine on contractile properties, we added 25 or 50 mM-creatine to a standard bathing solution (Table 1) in which MgATP was elevated to 6 mM. Inasmuch as the creatine kinase reaction is reversible, addition of creatine will lead to a reduction of ATP and an equimolar enhancement of PCr and ADP. Under these conditions, one can calculate (using an equilibrium constant of 10^9 M^{-1}) that with 25 or 50 mM added creatine, respectively, the solutions contained (in mM): 5·9 or 5·81 MgATP, 15·1 or 15·2 PCr, 24·9 or 49·8 creatine, and 0·097 or 0·19 ADP. As shown above, changes in MgATP, PCr and ADP of these magnitudes should have little or no significant effect on maximum force or calcium sensitivity in skeletal or cardiac fibres. The addition of 25 mM-creatine has no effect on the calcium sensitivity of either skeletal or cardiac fibres. It also has no significant effect on the Ca_{50} of these fibres. Increasing creatine to 50 mM, on the other hand, slightly increases the calcium sensitivity of skeletal fibres and decreases that of cardiac fibres. Moreover, this higher concentration of creatine slightly depresses F_{max} of both muscle types (Table 4).

Phosphorylation of myosin P light chains

With prolonged stimulation, the myosin P light chains (LC-2) of fast-twitch skeletal muscle become phosphorylated within a few seconds (Manning & Stull, 1979). Therefore, in a fatiguing muscle, P light chains are likely to be phosphorylated. Does this phosphorylation of myosin light chains have an effect on the contractile apparatus and does it alter the response to fatigue conditions? We examined this question in skeletal fibres using control and test solutions (Table 3) similar to the intracellular composition of resting and fatigued frog muscle fibres (Dawson *et al.* 1978).

For these experiments, the fibres were first activated in a series of pCa solutions. After irreversible thiophosphorylation as described in Methods, the fibres were retested in the same solutions. Thus, each fibre served as its own control since myosin P light chains in fibres prepared from resting rabbit psoas (see Methods) were not phosphorylated (Godt *et al.* 1985*b*). Under our conditions, thiophosphorylation had little or no effect on the calcium sensitivity in either control or test solutions or on maximal force in control conditions (Fig. 7 and Table 4). However, thiophosphorylation did lead to a slight but significant increase in F_{max} under fatigue conditions (Table 4).

Affinity for ATP hydrolysis

The increase in intracellular P_i and ADP and the small decrease in ATP that occur with fatigue and hypoxia decrease the calculated affinity for ATP hydrolysis from 55–60 to 40–45 kJ/mol (Dawson *et al.* 1978, 1980; Kammermeier *et al.* 1982). While the decline in tension with fatigue and hypoxia is *correlated* with the decline in affinity (Dawson *et al.* 1978; Kammermeier *et al.* 1982; Allen *et al.* 1985), a causal relationship is difficult to establish in intact cells because of the myriad other changes

in the intracellular milieu which take place as well. Even in a simplified system (e.g. skinned fibres) where one can control the milieu, determination of any affinity effects is confounded because affinity cannot be changed without altering the concentration of constituents which, as we have shown above, have effects in and of themselves on the contractile properties.

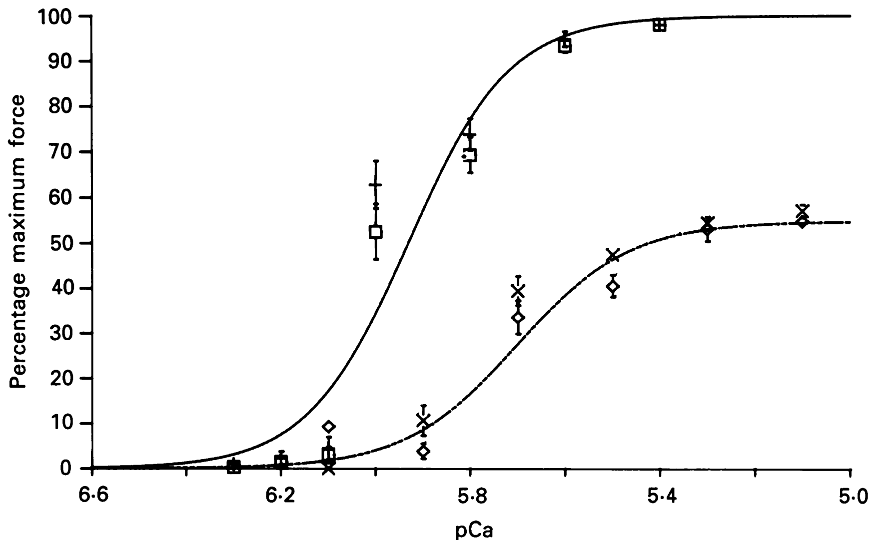

Fig. 7. Influence of thiophosphorylation of myosin P light chains on calcium activation of rabbit psoas skinned muscle fibres. The continuous curve (□) is the response to control solutions and the dashed curve (◇) to test (fatigue) conditions (see Table 3) before thiophosphorylation. After thiophosphorylation, response to control (+) and test (×) is shown. All data expressed relative to maximal force (pCa 4) in control solution before thiophosphorylation. Solution ionic strength adjusted with KCl.

Figure 1 illustrates that, in skinned skeletal and cardiac muscles, the milieu changes accompanying fatigue and hypoxia decrease both F_{max} and calcium sensitivity relative to control, even in the absence of a pH decline. The question is, how much of this shift of the force–pCa curve down and to the right is due to the effect of lowering affinity from 60 to 45 kJ/mol? The evidence reported above suggests that a shift in this direction would be produced by the large depressant effect of P_i (Fig. 3 and Table 4), counterbalanced to some extent by the lesser enhancing influences of increased ADP and AMP (Table 4) and of decreased PCr (Fig. 4).

We attempted to determine the contribution of the affinity change *per se* to the shift in the force–pCa curve by comparing the response of the fibres to test solution No. 1 with that of those in a solution (test solution No. 3 in Table 1) with the same [P_i] but with no added ADP, AMP or creatine, Due to the deletion of ADP, this solution has a calculated affinity equal to that of the control solution. If the change in affinity has no significant effect, the decrease in ADP and AMP in test solution No. 3 relative to that in No. 1 would cause the curve to shift downward and to the right

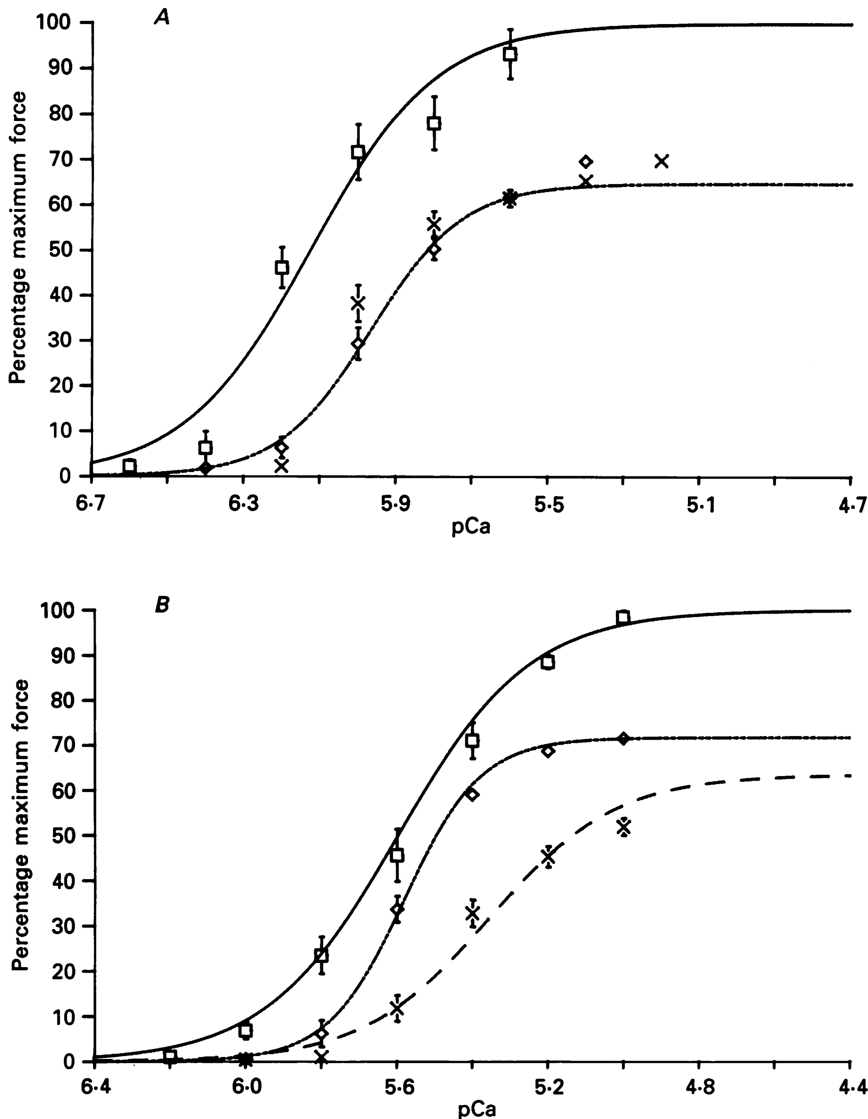

Fig. 8. Effect of affinity for ATP hydrolysis on calcium activation at pH 7 in rabbit psoas (*A*) and cardiac (*B*) skinned fibres. Continuous curve (□) represents control solution (low P_i and ADP; affinity, 60 kJ/mol); double dot–dash curve (◇) is for test solution No. 1 (high P_i and ADP; affinity, 45 kJ/mol). These data are identical to those shown in Fig. 1. Dashed curve represents (×) data for test solution No. 3 (high P_i, but low ADP; affinity, 60 kJ/mol). (For clarity, dashed lines deleted with skeletal muscle data in Fig. 8*A*.) See Table 1 for complete solution details. KCl used to adjust ionic strength with skeletal fibres.

relative to that of No. 1 (see Table 4). As discussed above, removal of creatine will have little or no effect. On the other hand, if affinity has a significant effect, the force–pCa relationship in test solution No. 3 would shift differently from that expected simply from removal of ADP and AMP.

As can be seen in Fig. 8*A*, the force–pCa relationship of skeletal muscle in test solution No. 3 was quite similar to that in test solution No. 1. In particular, we did not observe a significant change in F_{max}. This contrasts with the depression of F_{max} expected from removal of AMP and ADP (−12·3 %, calculated assuming that the

TABLE 5. Comparison of actual and predicted effects of milieu changes: changes in F_{max} and Ca_{50}

	Skeletal		Cardiac	
	F_{max} (%)* (S.E.M.)	Ca_{50} (μM)* (S.E.M.)	F_{max} (%) (S.E.M.)	Ca_{50} (μM) (S.E.M.)
Observed fatigue/hypoxia (pH 7)	−20·7 (2·6)	+0·21 (0·24)	−28·3 (4·2)	+2·00 (0·75)
Predicted value† (pH 7)	−16·3 (4·9)	+0·94‡ (0·15)	−16·1 (5·1)	+0·84 (0·72)
Observed fatigue/hypoxia (pH 6·65)	−32·8 (1·1)	+1·01 (0·17)	−54·8 (1·8)	+4·49 (1·21)
Predicted value (pH 6·65)	−40·9 (7·0)	+2·71 (1·06)	−41·4 (7·8)	+4·44 (0·79)

* A positive value indicates an increase in F_{max} or Ca_{50}.
† Calculated by addition of all significant changes due to individual constituents.
‡ Significantly different ($P < 0·05$) from actual experimental value.

ADP and AMP effects on F_{max} are additive). This difference between actual and expected results is statistically significant, thus indicating that decreasing affinity has an inhibitory effect on F_{max} of skinned skeletal fibres. The effect of affinity on Ca_{50} is negligible since no difference was expected or observed between Ca_{50} in solutions No. 1 or No. 3.

On the other hand, in cardiac muscle we saw a marked decrease in maximal force and Ca^{2+} sensitivity in test solution No. 3 relative to test solution No. 1 (see Fig. 8*B* and Table 4). The observed decrease in F_{max} (−11·5 %) is not significantly different from that expected from the additive effects of deletion of ADP and AMP (−16·5 %), which implies that affinity has no effect on F_{max} of cardiac muscle. In contrast, the change in Ca_{50} (1·78 μM) was significantly greater than that predicted from ADP and AMP removal (0·75 μM), i.e. decreasing affinity from 60 to 45 kJ/mol *increases* calcium sensitivity.

Does the sum of the individual effects equal the net effect of fatigue/hypoxic conditions?

Given the net effects of fatigue/hypoxic milieux on F_{max} and calcium sensitivity (Fig. 1 and Table 4), can these be explained simply by the additive effects of the individual solution changes? Table 5 compares the observed changes in F_{max} and Ca_{50} of skeletal and cardiac fibres with those predicted from simple summation of all significant changes due to each constituent determined individually (i.e. those due to pH, P_i, PCr, ADP, AMP, and affinity; see above). As can be seen, the observed effects of fatigue/hypoxia solutions on F_{max} are not significantly different from those

predicted in both skeletal and cardiac fibres. Moreover, with one exception, the observed changes in Ca_{50} are also as expected if the individual effects summate.

DISCUSSION

Locus of impairment with fatigue/hypoxia

With fatigue and hypoxia, the force generated by intact fast-twitch skeletal and cardiac muscles declines markedly and, under certain conditions, can approach zero (e.g. Eberstein & Sandow, 1963; Allen *et al.* 1985). Some have ascribed the decline in force primarily to a disruption of excitation–contraction coupling (ECC), with little or no effect on the contractile apparatus. Although we have not examined the role of ECC in fatigue, our data (Fig. 1) unequivocally demonstrate that the salient changes in the intracellular milieu associated with fatigue and hypoxia (as described by Dawson *et al.* 1978, 1980; Kammermeier *et al.* 1982) have direct depressant effects on both Ca^{2+} sensitivity and F_{max} of the contractile apparatus in skeletal and cardiac muscle. Note that the shift of the force–pCa relation under these conditions is reminiscent of the shift in the relation between peak K^+-contracture force and depolarization in fatigued frog fibres (Grabowski, Lobsiger & Lüttgau, 1972; their Fig. 6) which puts into question the conclusion that the impairment in fatigue lies *exclusively* in ECC. However, because F_{max} of skinned fibres does not decline to zero under ionic conditions mimicking fatigue/hypoxia, some impairment of ECC is likely to occur *in addition to* direct depression of the contractile apparatus. This notion is bolstered by recent evidence from aequorin-injected frog skeletal muscle which indicates that calcium transients are decreased during fatigue (Allen, Lee & Westerblad, 1988).

Experiments in intact cardiac muscle injected with aequorin support our contention that depression of the contractile apparatus is a significant cause of force decline under fatigue/hypoxic conditions. Allen *et al.* (1985) used ^{31}P-NMR to measure the intracellular milieu changes in hypoxic ferret hearts. In hypoxic hearts with glycolysis intact, they found that developed pressure declines by approximately 60% as the typical changes in intracellular constituents develop. However, in their hands, these changes were not accompanied by a measurable effect on ECC, insofar as calcium transients estimated from aequorin luminescence were essentially unchanged (Allen & Orchard, 1983). On the other hand, MacKinnon *et al.* (1987) found that hypoxia causes a significant decrease in the calcium transient of ferret papillary muscles. However, because the effects of hypoxia on force were greater than the changes in Ca^{2+}, they infer that the contractile apparatus is affected as well.

Effects of individual changes in milieu

Having established that the collective changes in intracellular milieu associated with fatigue and hypoxia have direct effects on the contractile apparatus, we determined the effects of changes in each constituent individually.

pH

We found that decreasing pH decreases F_{max} and calcium sensitivity of both skeletal and cardiac fibres. Our results are in accord with data from other laboratories (e.g. Fabiato & Fabiato, 1978; Donaldson & Hermansen, 1978; Metzger

& Moss, 1987; Chase & Kushmerick, 1988). The effect on Ca^{2+} sensitivity is thought to arise from competition between H^+ and Ca^{2+} at the thin filament with H^+ acting either directly at troponin C (Robertson & Kerrick, 1979) or indirectly by altering the net charge on the thin filament (Godt, 1981). The mechanism for the effect of acidification on maximum force has yet to be established.

It has become clear that there is no necessary relationship between the fall of force accompanying fatigue or hypoxia and cellular acidification. Some NMR studies of skeletal muscle report that fatigue is associated with a decrease in intracellular pH (pH_i) (Dawson *et al.* 1978) while others do not (Kushmerick & Meyer, 1985). These differences may be due to the specific conditions under which fatigue was induced (Edwards, 1981; Lännergren & Westerblad, 1988). In any event, there is general agreement that the cellular acidification accompanying fatigue is insufficient to fully explain the corresponding decline in force (Renaud, Allard & Mainwood, 1986; Westerblad & Lännergren, 1988).

In cardiac muscle, a number of studies have demonstrated that the decline in contractile force in early hypoxia (first several minutes) is not correlated with a decline in pH_i (Matthews, Radda & Taylor, 1981; Allen *et al.* 1985; Marban & Kusuoka, 1987). Our data demonstrate (Fig. 1) that, even in the absence of a pH decrease, the influence of the other changes in intracellular milieu cause significant decreases in calcium sensitivity and maximum force in both cardiac and skeletal muscle, and that any decline in pH_i would have an additional depressant effect on these contractile properties.

Inorganic phosphate

A number of laboratories have reported an increase in the intracellular concentration of P_i under fatigue and hypoxic conditions and have correlated this change in milieu with the accompanying decrease in contractile function (Dawson *et al.* 1978, 1980; Kusuoka, Weisfeldt, Zweier, Jacobus & Marban, 1986; Marban & Kusuoka, 1987). In an intact cell, this increase in P_i could affect cellular metabolism by stimulating the glycolytic flux (Neely & Morgan, 1974) or by precipitating calcium within certain regions (Kubler & Katz, 1977). Neither of these effects would influence the contractile properties of skinned fibres where the composition of the bathing medium is under rigid control. In skinned fibre preparations, any effects of P_i on calcium-activated force would have to be directly on the contractile apparatus. Our results indicate that P_i is very effective in decreasing the maximum force developed by skinned fibres as well as their sensitivity to calcium, with a greater effect in cardiac than in skeletal muscle. Our data are similar to those of others from both skinned skeletal (Rüegg, Schaedler, Steiger & Muller, 1971; Brandt *et al.* 1982; Cooke & Pate, 1985; Brozovich, Yates & Gordon, 1988) and cardiac fibres (Herzig, Peterson, Rüegg & Solaro, 1981; Kentish, 1986; Mekhfi & Ventura-Clapier, 1988). This evidence agrees with the conclusion of Marban & Kusuoka (1987) from intact heart that P_i depresses maximal force but differs from their conjecture that P_i does not influence Ca^{2+} sensitivity. In intact, hypoxic hearts, however, such conclusions can only be tentative in the face of the many uncontrolled changes in intracellular milieu under these conditions.

The effect of P_i on maximum force can be explained by the finding of Hibberd,

Dantzig, Trentham & Goldman (1985) that P_i appears to reverse the putative force-producing step of the cross-bridge cycle (P_i release) by shifting the distribution of cross-bridges towards those states with a full complement of bound products (ADP and P_i) and can lead to an actual decrease in the instantaneous number of attached bridges. If so, this could explain the effect of P_i on calcium sensitivity of the contractile apparatus inasmuch as attached cross-bridges are thought to increase the Ca^{2+} sensitivity of troponin on the thin filament through co-operative interactions (Bremel & Weber, 1972; Brozovich *et al.* 1988). However, an alternative explanation based on the effects of P_i on the relative rates of cross-bridge attachment to the thin filament and Ca^{2+} binding to troponin has been advanced by Brandt *et al.* (1982).

MgATP

In the presence of an adequate ATP regenerating system (PCr/CK), we find that changes in MgATP over the extent likely with fatigue or hypoxia have no effect on either maximal force or Ca^{2+} sensitivity of skinned skeletal and cardiac fibres. These results are in accord with those reported by others (see below).

Phosphocreatine

We found that decreasing bath [PCr] from 15 to 1 mM increases maximal force exerted by the contractile apparatus. Such effects have been seen by others (Kentish, 1986; Mehkfi & Ventura-Clapier, 1988). Our experiments with the CK inhibitor FDNB suggest that this response is not a direct effect of PCr. Nor is it due to a significant contamination of PCr with P_i (Kentish, 1986). More likely, this effect is related to the role PCr plays in buffering [MgATP] and maintaining low [ADP] within the skinned fibre preparation (Kentish, 1986). This interpretation is in qualitative agreement with the calculations of Cooke & Pate (1985), which demonstrate that, in the absence of an active PCr/CK system, fibre ATPase activity can lead to marked depletion of ATP and elevation of ADP within the preparation. It is well known that marked decreases in MgATP increase maximal force of skinned fibres from both skeletal and cardiac muscle (e.g. Best *et al.* 1977; Ferenczi, Goldman & Simmons, 1984). These results are consistent with the dissociating effect of MgATP on actomyosin (Ferenczi *et al.* 1984). Moreover, elevated ADP also increases F_{max} (see below).

In our hands, increasing PCr from 15 to 30 mM (values nearer to those in intact skeletal muscle, Godt & Maughan, 1988) has no effect on maximal force. This is expected if PCr acts solely as a buffer of MgATP. Kentish (1986), using skinned rat cardiac fibres, found a shallow monotonic decline of maximal force in the 10–30 mM range, which may be due to the difference in species or in bathing solution composition.

ADP

In the range likely with fatigue or hypoxia (i.e. sub-millimolar), ADP slightly increased F_{max} in both skeletal and cardiac fibres and increased calcium sensitivity of cardiac (but no skeletal) fibres. Similar effects on maximum force of skinned skeletal and cardiac fibres have been reported by others (Cooke & Pate, 1985; Ventura-Clapier, Mekhfi & Vassort, 1987). Ventura-Clapier *et al.* (1987), however, did

not observe any effect of ADP (10 mM) on calcium sensitivity of skinned rat cardiac fibres. These effects, albeit slight, would tend to oppose the inhibitory effects of P_i and pH during fatigue or hypoxia. ADP is thought to inhibit dissociation of the actomyosin complex by ATP during the cross-bridge cycle (Siemankowski, Wiseman & White, 1985; Cooke & Pate, 1985). If ADP thus increases the average number of attached cross-bridges, maximal force should increase. Again, if attached cross-bridges increase the Ca^{2+} affinity of troponin, this could explain an increase in calcium sensitivity.

Affinity for ATP hydrolysis

Dawson *et al.* (1978) noted that the fall in force with fatigue was correlated with a decline in affinity ('free energy'), although they considered that the steepness of the relation argued against a causal connection. More recently, Kammermeier *et al.* (1982) and Allen *et al.* (1985) have also correlated the fall in force that occurs during experimentally induced hypoxia of the heart with a decline in affinity. Obviously, if affinity is lowered below some critical point, ATP-dependent processes must be compromised. Has this critical affinity level been reached under fatigue or hypoxic conditions? Our results for the contractile apparatus suggest it has. We found that reducing affinity from a control value of 60 to 45 kJ/mol, the value achieved during early hypoxia or fatigue, has a small but significant depressant effect on F_{max} of skeletal (but not cardiac) muscle fibres. A similar conclusion for cardiac muscle has been reached by Kentish (1986) and Kentish & Allen (1986). In addition, this reduction in affinity significantly increased the calcium sensitivity of cardiac (but not skeletal) muscle fibres. The mechanism for this is unclear.

AMP and creatine

The adenylate kinase and creatine kinase enzyme systems produce AMP and creatine respectively as they regenerate ATP from ADP. These metabolites build up during fatigue and hypoxia and could have a direct effect on the contractile apparatus. We found that at levels likely with early hypoxia (Kammermeier *et al.* 1982; our Table 1, test solution No. 1), AMP increased F_{max} but not calcium sensitivity in both muscle types, and tended to oppose the depressant effects of P_i and pH. We have no explanation for this effect. On the other hand, creatine at hypoxic levels (25 mM) had no effect on either F_{max} or Ca_{50}. These data on creatine agree with those from Kentish (1986) in cardiac skinned fibres. However, elevation of creatine to higher levels (50 mM) decreased maximal force and had opposite effects on Ca^{2+} sensitivity in the two tissues. The effect of high creatine concentration on F_{max} may be due to the accompanying increase in solution osmolarity (Ashley & Moisescu, 1977; Chase & Kushmerick, 1988) operating through an as yet unknown mechanism.

Phosphorylation

Assuming that irreversible thiophosphorylation of myosin P light chains mimics the reversible phosphorylation which occurs with prolonged stimulation, our results indicate that, under control conditions, such phosphorylation of skinned skeletal muscle has no effect on F_{max} and little if any influence on Ca^{2+} sensitivity. Similar findings with F_{max} have been reported by others, although significant increases in

sensitivity at sub-maximal Ca^{2+} are reported with reversible P light chain phosphorylation (Persechini, Stull & Cooke, 1985). The present results, however, indicate that F_{max} under fatigue conditions is significantly elevated after thio-phosphorylation. This would indicate that the phosphorylation of myosin P light chains likely to be present in fatigued skeletal muscle fibres may play a small, ameliorative role in the response of the contractile machinery to fatigue.

Can the individual changes explain the net effect?

One of the unique aspects of this study was the opportunity to compare the observed effects of solutions mimicking fatigue/hypoxia with those predicted by simple summation of the effects of each individual change in milieu studied separately. Our data indicate that, with one exception, the net effect on F_{max} and Ca_{50} can be explained from the additive effects of the individual solution changes. This being the case, this implies that there is no significant interaction among the effects of the various solution constituents. This seems at variance with our previous results (Nosek, Fender & Godt, 1987) which demonstrated an interaction between the effects of pH and P_i. However, these interactive effects are only disclosed at high P_i, when pH is varied over a wide range (6–7·25 in our case). Such interaction between pH and P_i is not likely to be significant over the pH range investigated in the present study (7–6·65; see Fig. 1 in Nosek *et al.* 1987).

We wish to thank K. Y. Fender, K. Cheeks, D. J. E. Luney, L. Bigler and G. Shirley for technical assistance. Additionally, we would also like to thank Dr Howard E. Morgan for stimulating our interest in this area. Rabbit muscle was kindly supplied by Dr Keith Green, Departments of Opthalmology and Physiology, Medical College of Georgia. This work was supported by NIH grants AR 31636 (R.E.G.) and HL/AR 37022 (T.M.N.) and a grant from the American Heart Association, Georgia Affiliate (T.M.N.). R.E.G. was an Established Investigator of the American Heart Association.

REFERENCES

ABBOTT, R. H. & LEECH, A. R. (1973). Persistence of adenylate kinase and other enzymes in glycerol extracted muscle. *Pflügers Archiv* **344**, 233–243.

ALBERTY, R. A. (1972). Calculation of the standard Gibbs free energy, enthalpy, and entropy changes for the hydrolysis of ATP at 0°, 25°, 37° and 75°. In *Horizons of Bioenergetics*, ed. SAN PIETRO, A. & GEST, H., pp. 135–147. New York: Academic Press.

ALLEN, D. G., LEE, J. A. & WESTERBLAD, H. (1988). Intracellular calcium during fatigue in single fibres from *Xenopus* toe muscles. *Journal of Physiology* **407**, 75*P*.

ALLEN, D. G., MORRIS, P. G., ORCHARD, C. H. & PIROLO, J. S. (1985). A nuclear magnetic resonance study of metabolism in the ferret heart during hypoxia and inhibition of glycolysis. *Journal of Physiology* **361**, 185–204.

ALLEN, D. G. & ORCHARD, C. H. (1983). The effect of hypoxia and metabolic inhibition on intracellular calcium in mammalian heart muscle. *Journal of Physiology* **339**, 107–122.

ASHLEY, C. C. & MOISESCU, D. G. (1977). Effect of changing the composition of the bathing solutions upon the isometric tension–pCa relationship in bundles of crustacean myofibrils. *Journal of Physiology* **270**, 627–652.

BEST, P. M., DONALDSON, S. K. B. & KERRICK, W. G. L. (1977). Tension in mechanically disrupted mammalian cardiac cells: effects of magnesium adenosine triphosphate. *Journal of Physiology* **265**, 1–17.

BRANDT, P. W., COX, R. N., KAWAI, M. & ROBINSON, T. (1982). Regulation of tension in skinned muscle fibers. Effect of cross-bridge kinetics on apparent Ca^{2+} sensitivity. *Journal of General Physiology* **79**, 997–1016.

BREMEL, R. D. & WEBER, A. (1972). Cooperation within actin filament in vertebrate skeletal muscle. *Nature* **238**, 97–101.

BROZOVICH, F. V., YATES, L. D. & GORDON, A. M. (1988). Muscle force and stiffness during activation and relaxation. Implications for the actomyosin ATPase. *Journal of General Physiology* **91**, 399–420.

CHASE, P. B. & KUSHMERICK, M. J. (1988). Effects of pH on contraction of rabbit fast and slow skeletal muscle fibers. *Biophysical Journal* **53**, 935–946.

COOKE, R., FRANKS, K. & STULL, J. T. (1982). Myosin phosphorylation regulates the ATPase activity of permeable skeletal muscle fibers. *FEBS Letters* **144**, 33–37.

COOKE, R. & PATE, E. (1985). The effects of ADP and phosphate on the contraction of muscle fibers. *Biophysical Journal* **48**, 789–798.

DAWSON, M. J., GADIAN, D. G. & WILKIE, D. R. (1978). Muscular fatigue investigated by phosphorus nuclear magnetic resonance. *Nature* **274**, 861–866.

DAWSON, M. J., GADIAN, D. G. & WILKIE, D. R. (1980). Mechanical relaxation rate and metabolism studied in fatiguing muscle by phosphorous nuclear magnetic resonance. *Journal of Physiology* **299**, 465–484.

DONALDSON, S. K. B. & HERMANSEN, L. (1978). Differential, direct effects of H^+ on Ca^{2+}-activated force of skinned fibers from the soleus, cardiac and adductor magnus muscles of rabbits. *Pflügers Archiv* **376**, 55–65.

DONALDSON, S. K. B. & KERRICK, W. G. L. (1975). Characterization of the effects of Mg^{2+} on Ca^{2+}- and Sr^{2+}-activated tension generation of skinned skeletal muscle fibers. *Journal of General Physiology* **66**, 427–444.

EBERSTEIN, A. & SANDOW, A. (1963). Fatigue mechanisms in muscle fibres. In *The Effect of Use and Disuse in Neuromuscular Function*, ed. GUTTMANN, E. & HNIK, P., pp. 515–526. Amsterdam: Elsevier.

EDWARDS, R. H. T. (1981). Human muscle function and fatigue. In *Human Muscle Fatigue: Physiological Mechanisms*, ed. PORTER, R. & WHELAN, J. pp. 1–18. London: Ciba Foundation Symposium 82, Pitman Medical Ltd.

FABIATO, A. & FABIATO, F. (1978). Effects of pH on the myofilaments and the sarcoplasmic reticulum of skinned cells from cardiac and skeletal muscles. *Journal of Physiology* **278**, 233–255.

FELDHAUS, P., FROEHLICH, T., GOODY, R. S., ISAKOV, M. & SCHIRMER, R. H. (1975). Synthetic inhibitors of adenylate kinases in the assays for ATPases and phosphokinases. *European Journal of Biochemistry* **57**, 197–204.

FERENZCI, M. A., GOLDMAN, Y. E. & SIMMONS, R. M. (1984). The dependence of force and shortening velocity on substrate concentration in skinned muscle fibres from *Rana temporaria*. *Journal of Physiology* **350**, 519–543.

GODT, R. E. (1981). A simple electrostatic model can explain the effect of pH upon the force–pCa relation of skinned muscle fibers. *Biophysical Journal* **35**, 385–392.

GODT, R. E., FENDER, K. J., SHIRLEY, G. C. & NOSEK, T. M. (1985*a*). Contractile failure with fatigue or hypoxia: Studies with skinned skeletal and cardiac muscle fibers. *Biophysical Journal* **47**, 293a.

GODT, R. E., FENDER, K. Y., PAN, B. S. & SOLARO, R. J. (1985*b*). Myosin light chain phosphorylation hastens relaxation of skinned skeletal muscle fibers. *Federation Proceedings* **44**, 1373.

GODT, R. E. & LINDLEY, B. D. (1982). Influence of temperature upon contractile activation and isometric force production in mechanically skinned muscle fibers of the frog. *Journal of General Physiology* **80**, 279–297.

GODT, R. E. & MAUGHAN, D. W. (1988). On the composition of the cytosol of relaxed skeletal muscle of the frog. *American Journal of Physiology* **254**, C591–604.

GODT, R. E. & NOSEK, T. M. (1985). The changes in intracellular milieu accompanying fatigue or hypoxia depress the contractile machinery of skeletal and cardiac muscle. *Journal of Physiology* **371**, 174*P*.

GOOD, N. E., WINGET, G. D., WINTER, W., CONNOLLY, T. N., IZAWA, S. & SINGH, R. M. M. (1966). Hydrogen ion buffers for biological research. *Biochemistry* **5**, 467–477.

GRABOWSKI, W., LOBSIGER, E. A. & LÜTTGAU, H. CH. (1972). The effect of repetitive stimulation at low frequencies upon the electrical and mechanical activity of single muscle fibres. *Pflügers Archiv* **334**, 222–239.

HERZIG, J. W., PETERSON, J. W., RÜEGG, J. C. & SOLARO, R. J. (1981). Vanadate and phosphate ions reduce tension and increase cross-bridge kinetics in chemically skinned heart muscle. *Biochimica et biophysica acta* **672**, 191–196.

HIBBERD, M. G., DANTZIG, J. A., TRENTHAM, D. R. & GOLDMAN, Y. E. (1985). Phosphate release and force generation in skeletal muscle fibers. *Science* **228**, 1317–1319.

HOLLER, E., HOLMQUIST, B., VALLEE, B. L., TANEJA, K. & ZAMECNIK, P. (1983). Circular dichroism and ordered structure of bisnucleotide oligophosphates and their Zn^{2+} and Mg^{2+} complexes. *Biochemistry* **22**, 4924–4933.

INFANTE, A. A. & DAVIES, R. E. (1965). The effect of 2,4-dinitrofluorobenzene on the activity of striated muscle. *Journal of Biological Chemistry* **240**, 3996–4001.

KAMMERMEIER, H., SCHMIDT, P. & JÜNGLING, E. (1982). Free energy change of ATP-hydrolysis: a causal factor of early hypoxic failure of the myocardium? *Journal of Molecular and Cellular Cardiology* **14**, 267–277.

KENTISH, J. C. (1986). The effects of inorganic phosphate and creatine phosphate on force production in skinned muscles from rat ventricle. *Journal of Physiology* **370**, 585–604.

KENTISH, J. C. & ALLEN, D. G. (1986). Is force production in the myocardium directly dependent upon the free energy change of ATP hydrolysis? *Journal of Molecular and Cellular Cardiology* **18**, 879–882.

KUBLER, W. & KATZ, A. (1977). Mechanism of early 'pump' failure of the ischemic heart: Possible role of adenosine triphosphate depletion and inorganic phosphate accumulation. *American Journal of Cardiology* **40**, 467–471.

KUSHMERICK, M. J. & MEYER, R. A. (1985). Chemical changes in rat leg muscle by phosphorus nuclear magnetic resonance. *American Journal of Physiology* **248**, C542–549.

KUSUOKA, H., WEISFELDT, M. L., ZWEIER, J., JACOBUS, W. E. & MARBAN, E. (1986). Mechanisms of early contractile failure during hypoxia in intact ferret heart: evidence for modulation of maximal Ca^{2+}-activated force by inorganic phosphate. *Circulation Research* **59**, 270–282.

LÄNNERGREN, J. & WESTERBLAD, H. (1988). The effect of temperature and stimulation scheme on fatigue and recovery in *Xenopus* muscle fibres. *Acta physiologica scandinavica* **133**, 73–82.

MACKINNON, R., GWATHMEY, J. K. & MORGAN, J. P. (1987). Differential effects of reoxygenation on intracellular calcium and isometric tension. *Pflügers Archiv* **409**, 448–453.

MANNING, D. R. & STULL, J. T. (1979). Myosin light chain phosphorylation and phosphorylase A activity in rat extensor digitorum longus muscle. *Biochemical and Biophysical Research Communications* **90**, 164–170.

MARBAN, E. & KUSUOKA, H. (1987). Maximal Ca^{2+}-activated force and myofilament sensitivity in intact mammalian hearts. *Journal of General Physiology* **90**, 609–623.

MARGENAU, H. & MURPHY, G. M. (1956). *The Mathematics of Physics and Chemistry*, p. 515. Princeton, NJ: Van Nostrand.

MARTELL, A. E. & SMITH, R. M. (1977). *Critical Stability Constants*, Vol. 3; *Other Organic Ligands*. New York: Plenum Press.

MATTHEWS, P. M., RADDA, G. K. & TAYLOR, D. J. (1981). A ^{31}P N.M.R. study of metabolism in the hypoxic perfused rat heart. *Biochemical Society Transactions* **9**, 236–237.

MEKHFI, H. & VENTURA-CLAPIER, R. (1988). Dependence upon high-energy phosphates of the effects of inorganic phosphate on contractile properties in chemically skinned rat cardiac fibres. *Pflügers Archiv* **411**, 378–385.

METZGER, J. M. & MOSS, R. L. (1987). Greater hydrogen ion-induced depression of tension and velocity in skinned single fibres of rat fast than slow muscles. *Journal of Physiology* **393**, 727–742.

MEYER, R. A., SWEENEY, H. L. & KUSHMERICK, M. J. (1984). A simple analysis of the 'phosphocreatine shuttle'. *American Journal of Physiology* **246**, C365–377.

MOISESCU, D. G. & PUSCH, H. (1975). A pH-metric method for the determination of the relative concentration of calcium to EGTA. *Pflügers Archiv* **355**, 243.

NASSAR-GENTINA, V., PASSONNEAU, J. V., VERGARA, J. L. & RAPOPORT, S. I. (1978). Metabolic correlates of fatigue and of recovery from fatigue in single frog muscle fibers. *Journal of General Physiology* **72**, 593–606.

NEELY, J. R. & MORGAN, H. E. (1974). Relationship between carbohydrate and lipid metabolism and the energy balance of heart muscle. *Annual Reviews of Physiology* **36**, 413–459.

NOSEK, T. M., FENDER, K. Y. & GODT, R. E. (1987). It is diprotonated inorganic phosphate that depresses force in skinned skeletal muscle fibers. *Science* **236**, 191–193.

PERSECHINI, A., STULL, J. T. & COOKE, R. (1985). The effect of myosin phosphorylation on the contractile properties of skinned rabbit skeletal muscle fibers. *Journal of Biological Chemistry* **260**, 7951–7954.

PIRES, E. M. V. & PERRY, S. V. (1977). Purification and properties of myosin light-chain kinase from skeletal muscle. *Biochemical Journal* **167**, 137–146.

RENAUD, J. M., ALLARD, Y. & MAINWOOD, G. W. (1986). Is the change in intracellular pH during fatigue large enough to be the main cause of fatigue? *Canadian Journal of Physiology and Pharmacology* **64**, 764–767.

ROBERTSON, S. P. & KERRICK, W. G. L. (1979). The effects of pH on Ca^{2+}-activated force in frog skeletal muscle fibers. *Pflügers Archiv* **380**, 41–45.

RÜEGG, J. C., SCHAEDLER, M., STEIGER, G. J. & MULLER, G. (1971). Effects of inorganic phosphate on the contractile mechanism. *Pflügers Archiv* **325**, 359–364.

SIEMANKOWSKI, R. F., WISEMAN, M. O. & WHITE, H. D. (1985). ADP dissociation from actomyosin subfragment 1 is sufficiently slow to limit the unloaded shortening velocity in vertebrate muscle. *Proceedings of the National Academy of Sciences of the USA* **82**, 658–662.

SMITH, R. M. & MARTELL, A. E. (1975). *Critical Stability Constants*, vol. 2; *Amines*. New York: Plenum Press.

SMITH, R. M. & MARTELL, A. E. (1976). *Critical Stability Constants*, vol. 4, *Inorganic Complexes*. New York: Plenum Press.

SRIVASTAVA, S. & HARTSHORNE, D. J. (1983). Conversion of a Ca^{2+}-dependent myosin light chain kinase from skeletal muscle to a Ca^{2+}-independent form. *Biochemical and Biophysical Research Communications* **110**, 701–708.

VENTURA-CLAPIER, R., MEKHFI, H. & VASSORT, G. (1987). Role of creatine kinase in force development in chemically skinned rat cardiac muscle. *Journal of General Physiology* **89**, 815–837.

WESTERBLAD, H. & LÄNNERGREN, J. (1988). The relation between force and intracellular pH in fatigued, single *Xenopus* muscle fibres. *Acta physiologica scandinavica* **133**, 83–89.