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THE EFFECT OF pH ON THE RATE OF RELAXATION OF ISOLATED BARNACLE MYOFIBRILLAR BUNDLES

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CO_2-induced acidosis in barnacle muscle fibres prolongs the relaxation phase of the electrically stimulated contraction (Ashley, C.C., Franciolini, F., Lea, T.J. and Lignon, J. (1979) J. Physiol. 296, 71P). In order to test if this effect is due to a direct action of H^+ on the relaxation kinetics of the myofilaments, isolated myofibrillar bundles were contracted and relaxed in Ca^{2+} buffer solutions at pH 6.0 and 7.1, in the presence of 20 mM caffeine to inactivate the sarcoplasmic reticulum. At pH 7.1, the relaxation half-time was reduced from 1.5 to 0.3 s as the EGTA concentration in the relaxing solution was progressively increased from 0.3 to 50 mM. The resulting curve was shifted in the direction of increasing EGTA concentration by lowering the pH to 6.0. This effect could be explained by the reduction in affinity of Ca^{2+} for EGTA at pH 6.0, since relaxation half-times for a given relaxing p*Ca* (calculated from the contaminating Ca^{2+} concentrations in the relaxing solutions) were shorter (by about 40%) at pH 6.0 compared with 7.1. However, similar experiments using the new Ca^{2+}-chelating agent 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid (BAPTA), which is much less pH sensitive than EGTA, indicated that there was no significant difference between relaxation half-times at pH 6.0 and 7.1 for a given relaxing p*Ca*. It is concluded that because no prolongation of relaxation of the myofibrils was observed on lowering the pH from 7.1 to 6.0, the effect of CO_2 on the relaxation of intact muscle fibres is probably due to a modification of sarcoplasmic reticulum activity.

Introduction

Mechanical responses of skeletal muscle are fairly insensitive to changes in extracellular pH (pH_o) [1], unless changes in intracellular pH (pH_i) also occur. Under conditions where pH_i is thought to change, such as through the introduction of extracellular CO_2, NH_4^+ or phthalate [2], the amplitude of the twitch tension falls with decreasing pH_i [3–5]. Each of the steps in the development of twitch tension is sensitive to pH_i to some degree. Thus, the surface membrane permeability of barnacle muscle fibres to K^+ and Cl^- is affected by both pH_o and pH_i, with the most marked effects being observed below pH 5 [6]. Other reported membrane effects include a slowing of inactivation gating in the Na^+ channels of frog skeletal muscle by reduced pH_i [7] and the blocking of the voltage-dependent, outward K^+ current of crayfish slow muscle fibres by reduced pH_i, thus causing an all-or-nothing Ca^{2+} action potential [8]. E-C * coupling is probably sensitive to pH, since Ca^{2+}-induced release of Ca^{2+} from the sarcoplasmic reticulum of skinned cardiac muscle fibres is reduced by lowering solution pH [9]. Other experiments using skinned muscle fibres have demonstrated that, at the myofilaments themselves, Ca^{2+} is less effective in generating

Abbreviations: BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid; HDTA, hexamethylenediamine-*N*,*N*,*N'*,*N'*-tetraacetate; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N'*-tetraacetic acid.

* E-C, excitation-contraction.

tension when the pH of the surrounding medium is reduced [9–11]. Finally, the sequestration of Ca^{2+} by the sarcoplasmic reticulum is pH dependent: loading of the sarcoplasmic reticulum of skinned muscle fibres has a pH optimum of 6.2–7.4 depending on the p*Ca* of the solution [9], while the activity of isolated Ca^{2+}-ATPase from barnacle fragmented sarcoplasmic reticulum has an optimum pH of 7.4 [12].

In muscle fibres of the barnacle, CO_2- and phthalate-induced acidosis not only reduces the amplitude of the contraction (and free Ca^{2+} transient) but also prolongs the relaxation phase [5]. Both effects have also been observed with cardiac muscle [13,14]. The aim of the experiments described in this paper was to test whether the effect on relaxation could be attributed to a direct effect of pH on the relaxation kinetics of the myofilaments. This was done by activating isolated myofibrillar bundles in Ca^{2+} solutions and relaxing them in Ca^{2+} buffer solutions in the presence of caffeine to inactivate the sarcoplasmic reticulum. Solution pH was decreased from 7.1 to 6.0 to simulate changes in pH_i of crustacean muscle fibres exposed to 100% CO_2 [15]. It is concluded that such a reduction in pH_i in the intact muscle would not significantly affect the relaxation kinetics per se of the myofilaments, suggesting that the prolongation of relaxation arises from changes in the properties of the sarcoplasmic reticulum.

Materials and Methods

Myofibrillar bundles were prepared from muscle fibres of the barnacle *Balanus nubilus*, then tension was detected using an RCA transducer as described previously [10,11], and the output displayed on a Devices M2 pen recorder. The response time of the pen recorder was less than 10 ms for 95% full-scale deflection.

The composition of the bathing solutions is given in Table I. The values of the apparent affinity constants of Ca^{2+} and Mg^{2+} to the ligands are given in Table II; the free Ca^{2+} and Mg^{2+} concentrations were calculated using methods previously described [11,16]. Relaxing solutions containing EGTA concentrations between 0.3 (low relaxing, LR) and 50 mM (high relaxing, HR)

TABLE I

COMPOSITION OF SOLUTIONS

Contaminant $[Ca]_t$ was measured by atomic spectrometry to be approx. 10 μM in both pH 7.1 and 6.0 solutions; activating solutions had $CaCl_2$ added. Subscript t refers to total concentration. VLA, very low activating; VLR, very low relaxing; HR, high relaxing. The tonicity of BAPTA solutions was maintained by 140 mM sucrose. All concentrations are expressed as mM. In addition all solutions contained: creatine phosphate (10 mM), creatine kinase (20 units/ml) and caffeine (20 mM)

Solution No.	pH	$[K^+]$	$[Na^+]$	$[Cl^-]$	$[Tes]_t$	$[ATP]_t$	[MgATP]
(1) VLA	7.1	131	30	12	18	5.0	4.3
(2) VLR	7.1	133	30	12	18	5.0	4.3
(3) HR	7.1	116	30	16	18	5.0	4.4
(4) HR	7.1	133	30	12	18	5.0	4.4
(5) VLA	6.0	131	30	10	160	5.0	3.5
(6) VLR	6.0	129	30	11	160	5.0	3.5
(7) HR	6.0	128	30	10	160	5.0	3.5
(8) VLA	6.0	242	30	11	160	5.0	3.6
(9) VLR	6.0	242	30	11	160	5.0	3.6
(10) HR	6.0	244	30	10	160	5.0	3.3
(11) HR(BAPTA)	7.1	137	30	12	18	5.0	4.3
(12) HR(BAPTA)	6.0	156	30	9	160	5.0	3.2

were prepared by mixing solutions (very low relaxing, VLR; and HR) in the appropriate proportions (e.g., at pH 7.1, Nos. 2 and 3, Table I). To maintain ionic strength at the low EGTA concentrations, HDTA was used [17,18].

A limited number of experiments were performed using the newly described Ca^{2+} buffer, BAPTA [19,20]. Although this chelator shares with EGTA a high selectivity (greater than 10^5) for Ca^{2+} over Mg^{2+}, it is much less sensitive to pH than EGTA. Table II shows that the apparent affinity constant of Ca^{2+} to BAPTA is reduced by a factor of only 3.3 on lowering the pH from 7.1 to 6.0, compared with a factor of 154 for EGTA under the same conditions. Relaxing solutions (Nos. 11 and 12, Table I) were prepared containing 5.0 mM BAPTA at pH 7.1 and 16.8 mM BAPTA at pH 6.0 so that the p*Ca* values of these two solutions were identical (assuming an equal Ca^{2+} contamination from the other solution constituents). As for the EGTA solutions, ionic strength was maintained constant using HDTA. In addition, an attempt was made to balance the BAPTA-containing solutions with respect to tonicity by adding 140 mM sucrose to all the solutions at pH 7.1 (i.e., very low activating, VLA; VLR and HR) (not shown in Table I). Experiments were performed at room temperature (18–23°C), unless otherwise stated.

Results

Myofibrillar bundles of diameters 80–163 μm and about 5 mm in length were used to study the effect of EGTA concentration and hence the free Ca^{2+} concentration in the relaxing solutions upon the rate of relaxation. Caffeine (20 mM) was present in all the solutions to ensure the depletion of the sarcoplasmic reticulum Ca^{2+} and the effective inactivation of the sarcoplasmic reticulum pump [11]. A typical contraction-relaxation cycle of a bundle is shown in Fig. 1; after equilibration in a relaxing solution (VLR, No. 2, Table I) without EGTA, the bundle contracted in an activating solution (VLA, No. 1, Table I) which contained added Ca^{2+} weakly buffered by ATP, so that the estimated free Ca^{2+} concentration at pH 7.1 was 13 μM. After equilibration in this solution, the bundle was relaxed in a solution in which the EGTA concentration was varied between 0.3 (LR) and 50 mM (HR) (No. 3, Table I). The effects of EGTA concentration in the relaxing solution upon the relaxation rate were examined at pH 7.1 (Fig. 2) and 6.0 (Fig. 3), using separate bundles. These relaxing solutions caused force to decline in an approximately exponential way; the half-times for relaxation are plotted against EGTA concentration in Fig. 4 and against values of the p*Ca* in the relaxing solution in Fig. 5.

$[Mg]_t$	$[Mg^{2+}]$	$[HDTA]_t$	$[EGTA]_t$	$[BAPTA]_t$	$[Ca]_t$	p*Ca*	Ionic strength (mM)
6.0	1.1	50	0	0	0.05	4.9	220
6.0	1.1	50	0	0	0.01	5.6	220
7.8	1.0	0	50	0	0.01	10.4	220
5.8	1.0	0	50	0	42.76	6.0	220
5.2	1.2	50	0	0	0.04	5.0	220
					0.24	4.2	220
5.2	1.2	50	0	0	0.01	5.6	220
4.9	1.1	0	50	0	0.01	8.3	220
5.6	1.5	100	0	0	0.04	5.0	370
					0.24	4.2	370
5.6	1.5	100	0	0	0.01	5.6	370
5.1	1.1	0	100	0	0.01	8.6	370
6.0	1.0	45	0	5	0.01	9.6	220
4.6	1.0	33	0	16.8	0.01	9.6	220

TABLE II

APPARENT AFFINITY CONSTANTS (K^{app}) OF Ca^{2+} AND Mg^{2+} TO THE LIGANDS USED IN THE BATHING SOLUTIONS (At 22°C)

Ligand	Cation	pH	K^{app} (M^{-1})	Conditions	Reference
EGTA	Ca^{2+}	7.1	$6.0 \cdot 10^6$		11
		6.0	$3.9 \cdot 10^4$	50–100 mM K^+	11 [b]
	Mg^{2+}	7.1	46	20–50 mM Na^+	11
		6.0	3		11 [b]
BAPTA	Ca^{2+}	7.1	$7.9 \cdot 10^6$		19
		6.0	$2.4 \cdot 10^6$		19
	Mg^{2+}	7.1	50	100 mM KCl	19
		6.0	15		19
ATP	Ca^{2+}	7.1	3324	130 mM K^+, 30 mM Na^+	11 [a]
		6.0	1050	130–240 mM K^+, 30 mM Na^+	11 [a,b]
	Mg^{2+}	7.1	6224	130 mM K^+, 30 mM Na^+	11 [a]
		6.0	1825	130–240 mM K^+, 30 mM Na^+	11 [a,b]
HDTA	Ca^{2+}	7.1	6.5		18
		6.0	0.5		18 [b]
	Mg^{2+}	7.1	8	100–200 mM K^+, 35–40 mM Na^+	18
		6.0	0.6		18 [b]
Creatine phosphate	Ca^{2+}	6.0 and 7.1	20	30 mM Na^+, 90–170 mM K^+	11
	Mg^{2+}	6.0 and 7.1	12		11
Tes	Ca^{2+}	6.0 and 7.1	1	90–170 mM K^+, 30 mM Na^+	11
	Mg^{2+}	6.0 and 7.1	1		11

[a] With corrections for different K^+ concentrations.
[b] Values extrapolated from pH 7.1 using procedure of Portzehl et al. [16].

At pH 7.1, the relaxation half-time approached a plateau minimum value as the EGTA concentration was increased. At 50 mM EGTA, the rate-limiting step is less likely to be the reduction in the free Ca^{2+} concentration in the myofibrillar space and more likely to reflect the kinetic properties of the contractile apparatus. The procedure of using high EGTA concentrations to relax bundles previously equilibrated in Ca-EGTA or unbuffered activating solutions of 20 μM free Ca^{2+} has been employed before [17,21] in order to minimize Ca^{2+} diffusional problems.

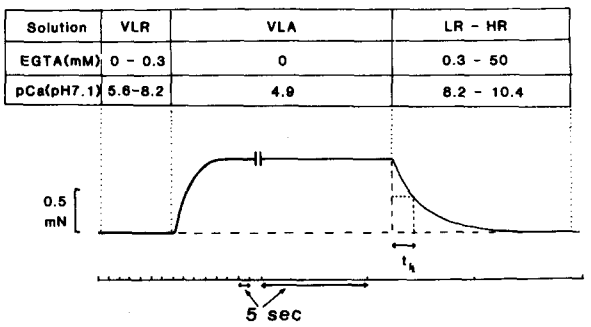

Fig. 1. A typical contraction-relaxation cycle of a myofibrillar bundle. VLR, very low relaxing; VLA, very low activating; LR, low relaxing; HR, high relaxing. Notice change in chart speed during the contraction.

For a given EGTA concentration the rate of relaxation was longer at pH 6.0 than at pH 7.1 (Fig. 4). This can at least be partly explained by the fact that the apparent affinity constant of Ca^{2+} to EGTA is reduced by a factor of about 154 on reducing pH from 7.1 to 6.0 (Table II), so that there is a change in buffering power for Ca^{2+}. If this fact is taken into account, a plot of half-time against the p*Ca* of the relaxing solution shows that at pH 6.0 there is a shift in the curve to the left, i.e., to higher p*Ca* values, suggesting that relaxation at a given p*Ca* is accelerated at a lower pH (Fig. 5). This was also confirmed in single myofibrillar bundles, relaxed at both pH values and a single relaxing p*Ca* (8.3). It is likely that at

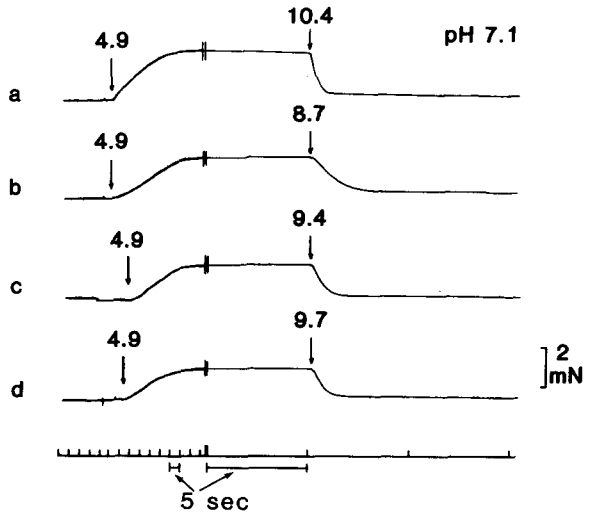

Fig. 2. Successive contraction cycles from a myofibrillar bundle of diameter 90 μm, showing the effect of different EGTA concentrations in the relaxing solution at pH 7.1. In each cycle the bundle was equilibrated in VLR solution for 3 min (p*Ca* = 5.6) before activation in VLA solution and relaxation in solutions containing the following total EGTA concentration (mM): (a) 50, (b) 1.0, (c) 5 and (d) 10. Arrows show time of solution change and p*Ca* values are given above them. Relaxation half-times (s) are (a) 0.35, (b) 1.17, (c) 0.55, (d) 0.52. Note the 10-fold increase in chart speed during the plateau phase of the contraction. Contraction amplitude decreased as the series progressed, but this did not affect the relaxation rate, since a fifth cycle in which the relaxing solution p*Ca* was 10.4 had the same relaxation half-time as in a.

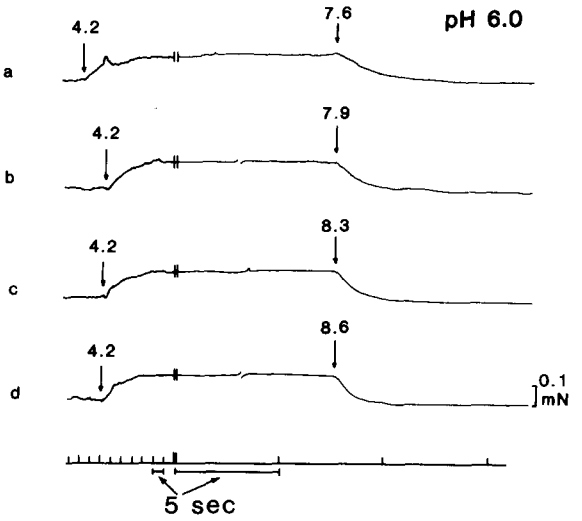

Fig. 3. The effect of EGTA concentration on relaxation rate at pH 6.0 and 370 mM ionic strength. Successive contraction cycles from a single bundle of diameter 90 μm. Bundle was equilibrated in VLR solution for 3 min before each contraction. Solution p*Ca* values are shown above each arrow. Total EGTA concentration (mM) in the relaxing solutions: (a) 10, (b) 20, (c) 50, (d) 100. Half-times (s) of relaxation are (a) 1.7, (b) 1.0, (c) 0.8, (d) 0.7. Note change in chart speed during each contraction.

pH 6.0, however, the relaxing state was still determined by the buffering power and hence indirectly by the rate of EGTA diffusion into the bundle even at 100 mM EGTA, as further increases in the buffering power of the solution (at pH 7.1) substantially reduced the half-time (Fig. 5).

When the relaxation experiments were repeated using the BAPTA-containing solutions (Nos. 11 and 12, Table I) there was no significant difference between relaxation half-times at pH 6.0 and 7.1 (Table III). Because of the limited amount of BAPTA available, intermediate relaxing solutions were used containing 5.0 mM BAPTA at pH 7.1 and 16.8 mM BAPTA at pH 6.0 (p*Ca* of both, 9.6). Nevertheless, the acceleration of relaxation which could be predicted by extrapolating the curves in Fig. 5 to a p*Ca* of 9.65 was not observed. The absolute values for relaxation half-time were longer in the BAPTA experiments, probably on

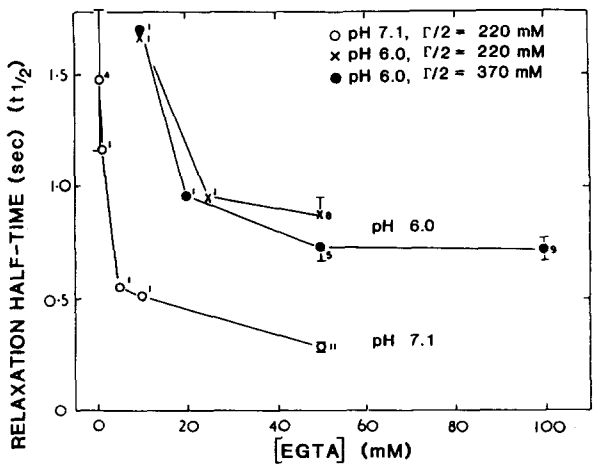

Fig. 4. Relaxation half-time ($t_{1/2}$) plotted against the EGTA concentration of the relaxing solution at pH 6.0 and 7.1. Points are mean values from several myofibrillar bundles (diameter 80–100 μm) with number of observations alongside the points and S.E. bars shown where appropriate. Relaxing solutions at pH 6.0 had an ionic strength ($\Gamma/2$) of either 220 (×) or 370 mM (●). Activating solutions at pH 6.0 (VLA) contained either 11 or 67 μM free Ca^{2+}.

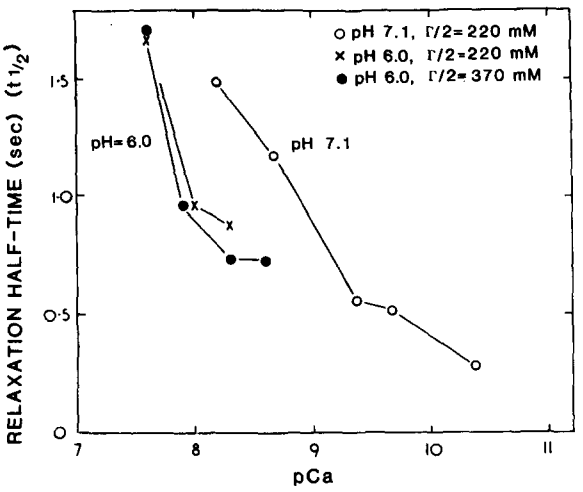

Fig. 5. The relaxation half-time data from Fig. 4, replotted against the p*Ca* values of the bulk relaxing solutions.

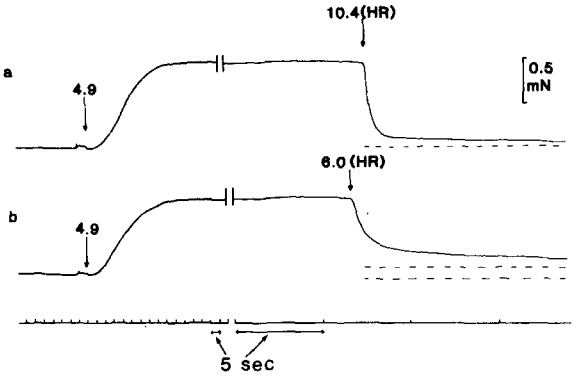

Fig. 6. Effect of an elevated free Ca^{2+} concentration on the relaxation rate at pH 7.1. The relaxing solutions are (a) HR with 50 mM total EGTA and no added Ca^{2+} (p*Ca* = 10.4) and (b) HR with 50 mM total EGTA and added Ca^{2+} (p*Ca* = 6.0). (b) Upper dashed line is steady tension to which bundle relaxed; lower dashed line is steady tension produced in subsequent exposure to HR solution (p*Ca* = 10.4). Relaxation half-times (s) are (a) 0.3, (b) 0.8. Bundle (diameter 100 μm) was first equilibrated in VLR solution (p*Ca* = 8.2) for 3 min and then activated in VLA solution. p*Ca* values shown above the arrows. Temperature 14°C.

account of the larger diameter bundles used, since with 5 mM BAPTA at pH 7.1 the rate of relaxation will be partly determined by the rate of inward diffusion of the Ca^{2+} buffer into the bundle.

Although the EGTA-containing solutions had higher tonicity values (386 and 549 mosmol/l for 220 and 370 mM ionic strength, respectively) at pH 6.0 than at pH 7.1 (249 mosmol/l), the relaxation rate in 50 mM EGTA was not significantly affected by the combined increases in ionic strength (from 230 to 370 mM) and in tonicity (from 386 to 549 mosmol/l) (Fig. 4). Unless these two factors have equal and opposite effects, it can be concluded that the increased tonicity of pH 6.0 solutions is unlikely to alter the relaxation rate. If anything it should prolong relaxation [22]. Tonicity was not tested specifically as a possible modifier of the relaxation rate. In the relaxation experiments using BAPTA, the solutions at pH 7.1 were iso-osmotic with those at pH 6.0.

The amplitude of contraction in response to VLA solution at pH 6.0 was smaller than that at pH 7.1 for the same p*Ca* value, a finding which has been previously observed in barnacle myofibrils [11]. Usually, at pH 6.0 it was necessary to raise the free Ca^{2+} concentration from 11 to 67 μM to get a maximal tension response. The half-

TABLE III

RELAXATION HALF-TIMES OBTAINED WITH BAPTA-CONTAINING SOLUTIONS

Two myofibrillar bundles were used; diameters = 138 and 163 μm. As in the experiments with EGTA-containing relaxing solutions, each bundle was first equilibrated in a VLR solution for 3 min and then contracted in a VLA solution which contained either 13 μM free Ca^{2+} (pH 7.1) or 75 μM free Ca^{2+} (pH 6.0).

Relaxing solution No. (see Table I)	Calcium chelator	p*Ca*	pH	Relaxation half-time (s) (±S.E.)	Number of determinations
2/3	5 mM EGTA	9.4	7.1	1.18±0.25	4
11	5 mM BAPTA	9.6	7.1	1.17±0.22	3
12	17 mM BAPTA	9.6	6.0	1.34±0.32	4

time for relaxation at this pH and EGTA concentration was detectably longer (by 34%) if the bundle was activated by the higher free Ca^{2+} solution. The nature of the equilibrating solution before contraction did not affect the subsequent relaxation rate, only the rate of tension development, as had previously been reported [21,23].

During exposure of intact muscle fibres to CO_2/saline the intracellular free Ca^{2+} concentration is elevated [24]. In order to test whether, at a given pH, an increase in resting free Ca^{2+} affects the rate of relaxation of myofibrillar bundles, a modified HR solution (No. 4, Table I) was used in which Ca^{2+} was added, as Ca-EGTA complex, to increase the free Ca^{2+} to 1 μM. This caused maximally activated bundles to relax to 13% of the initial contraction with a half-time of 0.8 s ($n = 2$) (Fig. 6), compared with 0.3 s ($n = 11$) for HR solutions of free Ca^{2+} less than 10^{-10} M. Thus, at a given pH, changes in free Ca^{2+} concentration in the relaxing solution apparently affected the rate of relaxation; however, the free EGTA concentration in the HR solution at p*Ca* 6 was only one-tenth of that in the HR solution at p*Ca* 10.4.

Discussion

The results presented here make it unlikely that the prolonged relaxation observed in intact muscle fibres exposed to CO_2 [5] can be attributed to a direct effect of acidification upon the relaxation kinetics of the contractile apparatus. A decrease in pH from 7.1 to 6.0 either shortened or had no effect on the relaxation time at a given p*Ca* in myofibrillar bundle preparations in which the sarcoplasmic reticulum was inactivated by 20 mM caffeine, depending on whether the Ca^{2+} chelator was EGTA or BAPTA. This decrease in pH was similar to the fall in intracellular pH (pH_i) of muscle fibres exposed to 100% CO_2 [15]. A more likely cause of the effect of CO_2 on intact fibres is an impairment of the sarcoplasmic reticulum Ca^{2+} pump as a result of the decrease in pH_i, although it is possible that an increase in intracellular CO_2 and HCO_3^- may be involved. One difference between the EGTA and the BAPTA experiments which could have been responsible for the discrepancies in the pH effect was that the EGTA relaxing solutions had higher tonicity values at pH 6.0 than 7.1. However, documented evidence from intact frog fibres [22] and myofibrillar bundles [25] suggests that if higher tonicity has any effect at all it should be to slow relaxation, which is the opposite of what was observed at pH 6.0. It may also be that the high pH sensitivity of EGTA means that the estimated p*Ca* values for the EGTA solutions at pH 6.0 are less reliable than those for the corresponding BAPTA solutions.

There is evidence from several sources that the activity of the sarcoplasmic reticulum Ca^{2+} pump could be impaired as a result of the intracellular acidification produced by CO_2. Garcia et al. [12] found that the Ca^{2+}-ATPase activity of sarcoplasmic reticulum vesicles from barnacle muscle was undetectable at pH values below 6.5. In frog skeletal muscle a reduction in pH_i of 0.7 units depresses Ca^{2+} transport by the sarcoplasmic reticulum [26]. This is supported by the findings of Fabiato and Fabiato [9], who showed that there is a pH optimum of 6.6–7.0 at p*Ca* 7.0 for the Ca^{2+} loading of the sarcoplasmic reticulum in skinned fibres from frog skeletal muscle, so that pH values of below 6.6 reduce Ca^{2+} loading.

Alternatively, the prolongation of the relaxation in fibres exposed to CO_2 may be a consequence of the increase in the intracellular concentrations of gaseous CO_2 and HCO_3^-, and not of the reduction in pH_i. Indeed, it has been shown that the increase in intracellular free Ca^{2+} which occurs in intact muscle fibres exposed to CO_2 [24] can be explained by a release of Ca^{2+} from an intracellular store, probably the sarcoplasmic reticulum, brought about by either gaseous CO_2 or HCO_3^- in the sarcoplasm [27–29]. Whether this Ca^{2+} release is due to an impairment of the sarcoplasmic reticulum Ca^{2+} pump or whether it represents an increase in the efflux from the sarcoplasmic reticulum by the enhancement of an existing release mechanism is not yet known. Obviously, an effect of these agents on the sarcoplasmic reticulum Ca^{2+} pump could explain the prolonged relaxation of intact fibres exposed to CO_2.

The observation that phthalate, an agent known to reduce pH_i readily, also prolongs the relaxation phase of intact muscle fibres when applied externally [5], supports the idea that a change in pH_i is responsible but does not exclude an additional effect of CO_2 or HCO_3^-.

In fatiguing muscle there is a decline in the rate of the relaxation phase, which is accompanied by a reduction in pH_i and changes in the intracellular concentrations of metabolites [30,31]. The reduction in pH_i does not seem to be the sole controlling influence on relaxation rate, since its prevention by iodoacetate did not prevent the decline in relaxation rate [30]. Evidence obtained from fatigued muscle using ^{31}P-NMR is consistent with a decrease in the affinity for ATP hydrolysis, as a result of the increases in H^+, ADP and P_i being responsible for the decreased relaxation rate during fatigue [31].

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