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TENSION RESPONSES OF SHEEP AORTA TO SIMULTANEOUS DECREASES IN PHOSPHOCREATINE, INORGANIC PHOSPHATE AND ATP

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SUMMARY

1. Tension responses of sheep aortae were investigated when different substrates were included in the superfusing medium. The magnitude of tension development was similar whether or not 5 mM glucose was present in the medium. However, the rate of tension development was greater in the absence of glucose.

2. When 5 mM 2-deoxyglucose (2DG) was present in the medium, the magnitude of tension generation was 1·6 times that in the absence of exogenous substrate. A second sequential contraction with 2DG generated tension 1·25 times that in the absence of exogenous substrate. The rate of tension development during the first contraction in the presence of 2DG was similar to that in the absence of substrate. However, the second contraction in the presence of 2DG had a substantially increased rate of tension development.

3. ^{31}P nuclear magnetic resonance (NMR) spectroscopy revealed that, at resting tone, in the presence of 2DG, inorganic phosphate (P_i) and phosphocreatine (PCr) simultaneously decrease while 2-deoxyglucose-6-phosphate accumulates. During contraction–relaxation cycles, in the presence of 2DG, P_i and PCr become undetectable while ATP declines to $\sim 50\%$ of control values as determined by NMR. During the second contraction in the presence of 2DG, the area of the ADP resonance was similar to that of the α-ATP resonance.

4. The increase in the magnitude of tension generation, during 2DG administration, correlated with a decrease in P_i levels. The rate of relaxation from a contraction, in the presence of 2DG, was slower than in the presence of glucose or in the absence of exogenous substrate. These results are consistent with the role of P_i in the release of the proposed 'latch-bridge' state of maintained contraction at low energy demand.

5. The increase in isometric tension generation during contraction in the presence of 2DG appears to be related to the decreased levels of P_i. In the presence of 2DG, the reduction of PCr and of ATP occur to a similar extent to that during hypoxia, yet no inhibition of force takes place. The low levels of ATP and PCr reported with 2DG administration in these studies do not energetically limit the contractile apparatus.

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INTRODUCTION

In many smooth muscles, hypoxia is associated with a decrease in force generation. During hypoxia, ATP production by the mitochondria is inhibited, often resulting in decreased levels of phosphocreatine (PCr) and ATP, and increased levels of inorganic phosphate (P_i). Controversy surrounds the mechanisms proposed to account for the observation that in many smooth muscles force generation decreases during hypoxia. Proposed mechanisms for the hypoxia-induced relaxation range from energy supply limitations to direct effect of P_i on the contractile elements. Ishida & Paul (1990) have reported that in taenia coli tension development correlated with oxygen consumption under aerobic and hypoxic conditions, and concluded that decreases in tension during hypoxia were due to inhibition of energy metabolism. Furthermore, Lovgren & Hellstrand (1985) reported a correlation between the concentration of PCr and force generation in rat portal vein under hypoxic conditions. However, the levels of ATP during hypoxia in taenia coli are substantially higher than those required to maintain contraction in a demembranated preparation of this tissue (Iino, 1981; Arner & Hellstrand, 1985). Furthermore, in rabbit aortae, Scott & Coburn (1989) showed that when tissues were loaded with creatine, either hypoxia or cyanide treatment resulted in decreased force despite levels of PCr that were higher than those in control tissues.

Part of the difficulty in determining the mechanism of vascular relaxation during hypoxia is that decreases in PCr and ATP are concomitant with increases in P_i. To determine whether the decreased force during hypoxia is due to decreased levels of high energy phosphates or to increased P_i levels, a method of decreasing high energy phosphates without increasing the level of P_i is needed. By administration of 2-deoxyglucose (2DG) to vascular smooth muscle, the resulting accumulation of 2-deoxyglucose-6-phosphate acts as a phosphate sink and reduces the concentrations of all exchangeable phosphates (PCr, ATP and P_i). However, during 2DG administration, oxidative metabolism is not impaired (Hardin, Wiseman & Kushmerick, 1992*b*). In our study, we directly examined the effects of decreased PCr and ATP on contraction independently of changes in the ability to resynthesize ATP and without the usual concomitant increase in P_i. With 2-deoxyglucose administration, we were able to decrease P_i at the same time as PCr and ATP decreased. With PCr levels undetectable and ATP levels reduced to 50% of control, isometric force generation increased. Therefore at these low concentrations of PCr and ATP, the contractile elements are not energy limited since force did not decrease. The increase in force may be the result of decreased levels of P_i. We conclude that hypoxia-induced relaxation of many smooth muscles does not result from energy limitation but from an increased concentration of P_i.

METHODS

Tissue handling

Sheep (typically Suffolk) were held under general endotracheal anaesthesia which was induced with thiamylal sodium (18 mg/kg) and maintained with halothane in 100% O_2. After a lethal overdose of sodium pentobarbitone (90 mg/kg I.V.), segments of the thoracic aorta were dissected out and rinsed in isotonic NaCl for 10 to 30 min before being placed in a physiological saline solution (PSS) (pH 7·4) at room temperature. The composition of the PSS was (mM): 4·6 KCl,

116 NaCl, 26 3-(*N*-morpholino)propanesulphonic acid, 2·5 $CaCl_2$, 1·16 $MgCl_2$, 5 glucose plus 10 mg/l gentamicin; the solution was pre-equilibrated with a gas mixture of 95% O_2 and 5% CO_2. Fat, connective tissue and adventitia were removed and segments of ~ 5 cm in length and ~ 17 mm in diameter were placed unmounted in a 20 mm nuclear magnetic resonance (NMR) tube and superfused with gas-equilibrated PSS at room temperature. Segments ~ 4 mm in length were placed into separate water-jacketed glass chambers filled with PSS.

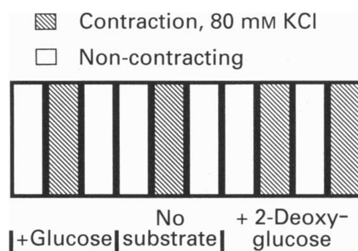

Fig. 1. Diagrammatic representation of the experimental protocol. After equilibration of aortae for 1 h, tissues underwent relaxation–contraction–relaxation cycles under three different metabolic conditions; in the presence of glucose, 2-deoxyglucose, or in the absence of these substrates. Relaxation (open bars) is defined as the metabolic and mechanical state when tissues are superfused with physiological saline solution (PSS) (see Methods). Contraction (hatched bars) is defined as the metabolic and mechanical state when aortae are superfused with PSS containing 80 mM KCl concentration (in molar replacement of NaCl). Each relaxation or contraction lasted 1 h. Glucose or 2-deoxyglucose (2DG), when provided, was included at a concentration of 5 mM. At the end of the relaxation–contraction–relaxation cycle in the presence of 2DG, aortae were contracted in the presence of 2DG again for 1 h and then tissues were rapidly frozen for further chemical analysis.

Isometric force measurements

The aortic rings were held isometrically at a length 1·4 times the excised resting length. Isometric force was measured with transducers from Harvard Apparatus on tissues in a water-jacketed glass chamber equipped with a magnetic stirrer and oxygen electrodes with temperature maintained at 22 °C. Resting tone was defined as the steady-state tone maintained by aortae while superfused in PSS at the set length of 1·4 times the excised resting length. Contractions were elicited by changing the solution to PSS containing a high concentration of KCl (final concentration of 80 mM KCl in molar replacement of NaCl). Relaxation was induced by replacing the bathing solution with PSS.

^{31}P NMR spectroscopy

^{31}P NMR spectroscopy measurements were performed in parallel with force measurements. Spectra were acquired using a General Electric GN300 Omega spectrometer operating at 121 MHz. Data were acquired using a 90 deg pulse (32 μs), a predelay of 3·0 s, a sweep width of 6000 Hz and a 2K block size. Serial spectra, consisting of 200 acquisitions (10 min of data averaging), were obtained throughout the experimental protocol. Data were zero filled to 4K points and filtered using 15 Hz exponential line broadening prior to Fourier transformation. In some experiments, a 5 mm NMR tube filled with 2 mM methylene diphosphonate (chemical shift of 17 p.p.m. relative to H_3PO_4) was included within the 20 mm sample NMR tube to serve as an external standard.

Experimental protocol

Aortae in the tissue chambers and in the spectrometer were subjected to relaxation–contraction–relaxation cycles (all three stages lasting 1 h) in PSS with (*a*) 5 mM glucose as substrate, (*b*) no exogenous substrate, and (*c*) with 5 mM 2-deoxyglucose (2DG) as the only

exogenous substrate (see Fig. 1). A second contraction in the presence of 2DG immediately followed the relaxation–contraction–relaxation cycle in the presence of 2DG (see Fig. 1). At the end of the experimental protocol, samples were rapidly frozen by submersion in liquid N_2 and stored at −80 °C for high-performance liquid chromatography (HPLC) analysis of ATP and PCr.

Analytical techniques

Neutralized perchloric acid extracts of frozen tissues were analysed for PCr and ATP by HPLC on a strong anion exchange column (Vydac model 303 NT305) using a phosphate gradient from 25 mM (pH 4·5) to 500 mM (pH 2·7) phosphate buffer. Absorbance was measured at 210 nm and quantified by comparison with standards previously calibrated spectrophotometrically using standard enzyme-linked metabolite assays (Lowry & Passoneau, 1972).

RESULTS

Isometric contractions with glucose and with no substrate

As shown previously (Hardin *et al.* 1992*b*), sheep aorta reaches peak isometric force in ~ 1 h of KCl stimulation. The magnitude of isometric force generation by aortae, during contractions in the presence of and in the absence of glucose, and with 5 mM 2DG as the sole exogenous substrate, is shown in Fig. 2. Each point represents the average isometric force of four aortic segments measured every 3 min and normalized to the peak isometric force obtained at the end of 60 min of contraction with no exogenous substrate. When no glucose was provided as substrate, the rate of contraction was slightly greater than when 5 mM glucose was present. The time to half-maximal contraction was estimated by linear interpolation of normalized force at the two points just prior to and just following attainment of half-maximum force. Estimates of the time to half-maximal force development are shown in Table 1. It is possible that the contraction in the presence of 5 mM glucose had not reached maximal isometric force during the 60 min of measurement; however, the time to half-maximal contraction would have been increased even further if this had been the case. The apparently similar maximum isometric force development in the presence and absence of glucose may be expected since the measured P_i, ATP and PCr do not change during sequential contraction–relaxation cycles with or without glucose as substrate (Hardin *et al.* 1992*b*).

Isometric contractions with 2-deoxyglucose as substrate

When 2DG was the sole exogenous substrate, the maximum isometric force generation increased by 60% compared to that obtained with no exogenous substrate (Fig. 2). This occurred despite the fact that 2DG administration in PSS, prior to contraction, did not alter the resting tone (data not shown). This increase in isometric force development in the presence of 2DG was not likely to be due to inhibition of glycolysis since no significant glycolytic flux was expected in the absence of exogenous substrate (Fig. 2). The time to half-maximum contraction was estimated to be 16 min, equivalent to that measured for contraction when no exogenous substrate was provided (Table 1).

The second sequential contraction with 2DG as substrate also exhibited a greater isometric force generation than when 2DG was not provided in PSS although the increase was less than that of the first contraction (Fig. 2). The level of isometric force development in the second contraction in the presence of 2DG was 1·25 times that

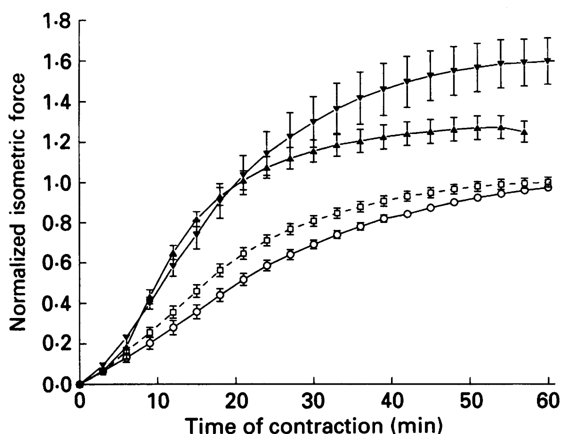

Fig. 2. Isometric force generation by sheep aortae normalized to the level of isometric force generated at 60 min of contraction with no substrate present. Each point represents the mean force measured from four aortae as sampled every 3 min. Error bars represent one standard deviation from the mean. Aortae were contracted under three different metabolic conditions: in the presence of 5 mM glucose only (○), with no exogenous substrate provided (□) and in the presence of 2DG (▼ for the first contraction, ▲ for the second sequential contraction).

TABLE 1. Mechanical responses

	Maximum force (relative)	Time to half-maximum contraction (min)	Time to half-relaxation (min)
+5 mM glucose	0·97	19·8	10·9
No substrate	1·00	16·2	9·9
+5 mM 2DG (first contraction)	1·60	16·1	14·7
+5 mM 2DG (second contraction)	1·25	11·6	

Mean maximum isometric force generation, time to half-maximum contraction, and time to half-relaxation in sheep aortae under two different substrate conditions and in the absence of substrate. Mean isometric force levels are taken from the data in Fig. 2. Time to half-peak isometric contraction and half-relaxation are determined from a linear interpolation of the data in Fig. 2.

obtained with no exogenous substrate present. However, the time to half-maximal contraction decreased to 11·6 min (Table 1).

^{31}P NMR during contractions with 2DG

The ^{31}P NMR spectra revealed substantial changes during the administration of 2DG. Figure 3 is a representative stack-plot of serial ^{31}P NMR spectra of an unmounted sheep aorta in the spectrometer. Each spectrum shown was taken after 30 min of each treatment (fourth of six acquisitions). The bottom of the plot shows the spectrum acquired while the aorta was at resting tone with no exogenous

substrate provided; on the right are the periods of addition of 2DG and of KCl-induced contracture. As previously reported (Hardin *et al.* 1992*b*), the ^{31}P NMR spectra do not change during contraction in the absence of exogenous substrate compared to those obtained in the presence of glucose. Therefore, the slower rate of

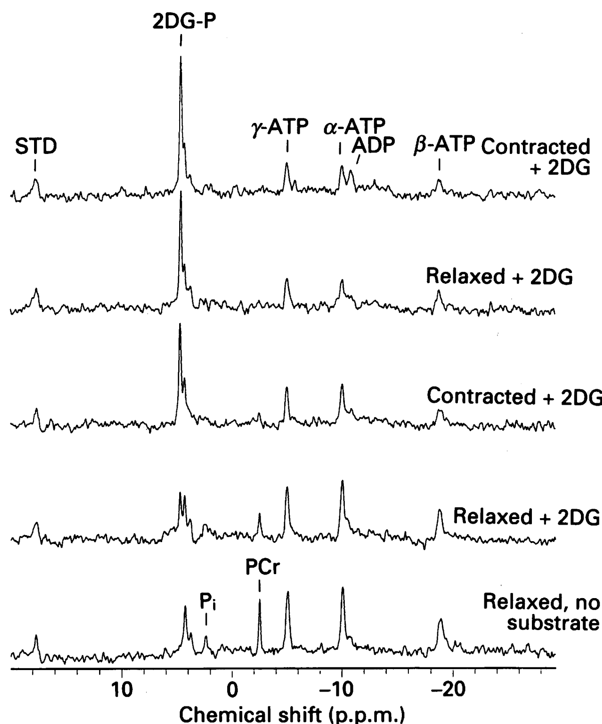

Fig. 3. Representative ^{31}P NMR spectra taken during the final relaxation prior to 2DG administration and from throughout the 2DG administration period. Spectra shown were selected from the middle of the 1 h treatment periods and represent the time period from 30 to 40 min of a 1 h treatment (fourth of six sequential 10 min acquisition periods). Resonances are identified as follows: STD designates the external standard methylene diphosphonate; 2DG-P designates the phosphomonoester region which includes the accumulating 2-deoxyglucose-6-phosphate; P_i, PCr, ADP and ATP are as designated in the text. Each of the five plots are on the same scale (same noise).

contraction in the presence of glucose compared to that in the absence of exogenous substrate cannot be explained in terms of changes detectable by ^{31}P NMR spectroscopy. With 2DG administration at resting tone, cellular P_i and PCr decreased and the P_i resonance was also reduced to an undetectable level. The decline in PCr continued during contraction in the presence of 2DG as substrate with PCr being almost undetectable by the end of the first contraction. No detectable changes in the intracellular pH occurred throughout the experimental protocol as inferred from the lack of change in the chemical shift of the phosphate resonance prior to 2DG administration, and of 2-deoxyglucose-6-phosphate during 2DG administration (since P_i was no longer detectable). In addition, the free Mg^{2+} concentration did not

change throughout the experiment as inferred from the lack of change of the chemical shift of the β-ATP resonance. Therefore, the primary change in the phosphorous spectrum during 2DG administration, prior to contraction, was a decrease in P_i and PCr. P_i levels remained undetectable from the first contraction in the presence of 2DG until the end of the experiment. However, with 2DG as substrate, the level of ATP began to decline during the first contraction and continued until the end of the experiment. Therefore, the key difference in the phosphorous spectrum between the first and the second contractions with 2DG as substrate was the continued decline of ATP levels.

The peak isometric force attained during a second contraction with 2DG as substrate was 1·25 times that attained when no exogenous substrate was provided (Fig. 2). Although P_i was still unmeasurable, PCr became almost undetectable and ATP was substantially decreased (Fig. 3). Therefore, if the decreased P_i was responsible for the increased force in the first contraction in the presence of 2DG, then the attenuation of the increase in force during the second contraction in the presence of 2DG may have been the result of a partial limitation of high energy phosphates. However, tension generation was still greater during both contractions in the presence of 2DG than during contractions in the presence of glucose. At the end of the experimental protocol, the levels of PCr and ATP measured by HPLC were 0·14 μmol/g blot wt ($\pm$0·04 S.E.M., $n = 4$) and 0·20 μmol/g blot wt ($\pm$0·04 S.E.M., $n = 4$) respectively. By peak height analysis, the β-ATP peak declined by 50% by the end of the second contraction in the presence of 2DG. In addition, a peak at -11 p.p.m. had formed by the end of the second contraction with 2DG, the likely result of free ADP accumulation. Although differences in the degree of peak saturation occur due to differences in spin relaxation (T_1), the changes in P_i levels during 2DG administration were well below 1 mM, probably in the 0·2 mM range, based on a comparison of the P_i peak area with that of ATP or PCr.

Relaxation of isometric force

If the increased force generation during contraction in the presence of 2DG was the result of decreased P_i levels, it would be predicted that the rate of relaxation would be slower in the presence of 2DG. This prediction is based on the work of Schneider, Sparrow & Ruegg (1981), Itoh, Kanmura & Kuriyama (1986) and Gagelmann & Guth (1987) who showed that increases in P_i are associated with increased rates of relaxation in smooth muscle. The isometric force during relaxation of sheep aortae following KCl-induced isometric contraction is shown in Fig. 4. In Fig. 4*A*, the levels of force are normalized to the levels of maximum isometric force shown in Fig. 2. The rate of relaxation of aortae in the presence or absence of glucose was similar, but was slower in presence of 2DG. For comparison, the level of isometric force just prior to each contraction is considered to be 1·0 in Fig. 4*B*. Table 1 shows the time to half-relaxation for all three relaxations. The decreased P_i levels correlated both with increased isometric force generation and decreased rate of relaxation.

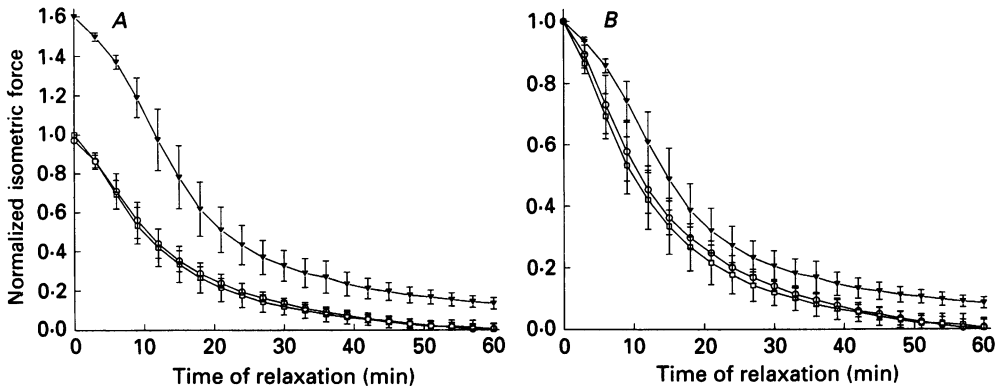

Fig. 4. Isometric force measured during relaxation immediately following contraction. *A*, force is normalized as in Fig. 3. Relaxations were performed under the same substrate conditions as contractions: in the presence of 5 mM glucose only (○), with no exogenous substrate provided (□) and in the presence of 2DG (▼). Aortae were not relaxed following the second contraction in the presence of 2DG. *B*, isometric force measurements during relaxations normalized to the peak isometric force level just prior to each relaxation (see text for details). Symbols as in *A*.

DISCUSSION

The most important conclusions from these experiments are that: (a) the levels of ATP and PCr decrease to levels similar to those observed during hypoxia in many smooth muscles yet isometric force increases, and (b) as the level of P_i decreases, the magnitude of isometric force generated during KCl-induced contraction increases. Since isometric force generation can be directly affected both by the levels of high energy phosphates and by P_i, and while P_i and high energy phosphates normally vary in opposite directions, it is useful to have a means of varying them both in the same direction to distinguish their effects on contractile function. In the current study we were able to decrease P_i, ATP and PCr simultaneously during contraction–relaxation cycles in vascular smooth muscle by administration of 2DG.

Contractions in the presence or absence of glucose

Since 2DG inhibits glycolysis, it was of interest to determine if the effects of 2DG administration resulted from inhibition of glycolysis or from the induced global changes in levels of PCr, ATP and P_i. We performed contraction–relaxation cycles with no glucose or other substrate present to serve as a control for inhibition of glycolysis. The rate of isometric force generation was lower when glucose was present in the superfusate, yet the level of isometric force generation was similar. This has been previously reported for hog carotid artery (Lynch, Kuettner & Paul, 1989) and is likely to be due to the role of glycolysis in supporting the calcium pump (Hardin, Raeymaekers & Paul, 1992*a*). The first contraction in the presence of 2DG occurred at the same rate as the contraction in the absence of any substrate; this would have been expected since glycolysis is inhibited in both cases. Therefore effects of 2DG

administration are due to global changes in PCr, ATP and P_i and not due to a simple inhibition of glycolysis.

Increased rate of tension development during the second contraction with 2DG

The rate of tension development during the second contraction in the presence of 2DG was substantially greater than during all previous contractions. This may be the result of increased free Ca^{2+} levels resulting from the decreased PCr and ATP levels which are entirely produced by oxidative metabolism. ATP produced by oxidative phosphorylation may preferentially support contractile activity (Paul, 1983; Paul, Krisanda & Lynch, 1984) while glycolysis may preferentially fuel the calcium pump (Paul, Hardin, Raeymaekers, Wuytack & Casteels, 1989). During glycolytic inhibition, as occurs with glucose removal or 2DG administration, oxidative metabolism can support membrane ion pump function. However, when global ATP levels are decreased, then ATP produced oxidatively may still support the contractile apparatus at the expense of fueling the calcium pump. Therefore the increased rate of the second contraction in the presence of 2DG may be the result of a further decrease in calcium pump function. The attenuation of tension between the first and second contractions in the presence of 2DG may indicate the beginning of a global ATP limitation. However, substantially greater (1·25 times) isometric force is generated during the second contraction in the presence of 2DG compared to contractions prior to 2DG administration suggesting that contractile activity may be supported better than calcium pump function at these low levels of phosphagens.

Increased isometric force generation despite low levels of PCr and ATP

The decline in the levels of ATP and PCr in this study did not appear to limit the energy available to the contractile apparatus. The mechanism for hypoxic relaxation in many smooth muscles may be related to the rate of ATP turnover rather than to the ATP concentration (Lovgren & Hellstrand, 1985; Scott & Coburn, 1989). In sheep aortae, with 2DG provided as substrate, we found that oxygen consumption was stimulated, indicating an unimpaired oxidative metabolism during 2DG administration (Hardin *et al.* 1992*b*). Since the ATP remaining during hypoxia should be sufficient to maintain contraction, the question remains as to how the decrease in the rate of ATP synthesis during hypoxia is translated into a decreased ATP demand by the contractile apparatus, while maintaining an ATP concentration adequate for the latter's demands. During hypoxia, a decline in PCr levels and an increase in P_i levels are typically associated with a limitation of the rate of ATP synthesis by the mitochondria. Possible mechanisms by which ATP demand by the contractile apparatus decreases during a decrease in aerobic ATP synthesis include: (1) a decrease in the phosphorylation potential or PCr/Cr ratio or in some other energetic term which limits turnover at the contractile apparatus and/or the plasmalemmal calcium pump; and (2) an increase in the P_i levels resulting in increased cross-bridge detachment. In the current study there is evidence that changes in P_i alter force generation in smooth muscle and it is likely that the PCr/Cr ratio substantially decreased with 2DG administration. The only way the PCr/Cr ratio would not have decreased would be if $> 90\%$ of creatine left the tissue. Yet despite the decreased PCr/Cr ratio, isometric force generation increased. This is in

contrast to the findings of Scott & Coburn (1989) where in rabbit aortae loaded with creatine, the isometric force varied with the PCr/Cr ratio regardless of the total concentration of creatine. The difference between our study and that of Scott & Coburn is that oxidative metabolism remains fully intact in our aortae. Therefore, some signal other than PCr/Cr may be the actual signal for relaxation in hypoxic aortae.

Possible mechanisms for increased force with 2DG administration

The global decreases in PCr, ATP and P_i during 2DG administration must act to increase isometric tension development by either increasing a signal for contraction (e.g. Ca^{2+} and myosin light chain phosphorylation) or decreasing an inhibitor of contraction (e.g. P_i or phosphatase effects on myosin light chain phosphorylation).

Decreased P_i levels – removal of inhibition of contraction

P_i levels become undetectable by the end of the 1 h exposure to 2DG prior to contraction. Therefore the increased magnitude of contraction during 2DG administration may be the result of removal of P_i-mediated inhibition of contraction. P_i inhibits contraction in chemically demembranated smooth muscle from taenia coli (Schneider *et al.* 1981; Gagelmann & Guth, 1987) and mesenteric artery (Itoh *et al.* 1986). However, at calcium concentrations which elicit maximal contraction ($\sim$ 10 μM), isometric force was decreased by only 12% by 10 mM P_i in fibres from taenia coli (Gagelmann & Guth, 1987) and to a similar extent by 6 mM P_i in fibres from mesenteric artery (Itoh *et al.* 1986). In both cases, increases in the concentration of P_i shifted the pCa–tension curve to the right. Therefore, at lower calcium concentrations the effect of P_i on force is greater. At pCa 6·17, in taenia coli fibres, 10 mM P_i inhibited isometric force generation by 70% (Gagelmann & Guth, 1987). In intact smooth muscle, the role of P_i in the inhibition of force generation is less clear. Ishida & Paul (1990) demonstrated an increase in P_i from 1 to 2·5 mM in taenia coli during hypoxia and a decrease in muscle tone. Under aerobic conditions in glucose-free solution, Nakayama, Seo, Tomita & Watari (1988) demonstrated an increase in P_i from 0·5 to 2 mM with an eventual decline in tension to about half that in the presence of glucose. The eventual decrease in tone occurred after a 30 min increase in tone immediately following removal of glucose and concomitant with the rise in P_i and with the decline in PCr. Clearly, the removal of glucose alone, under stimulated aerobic conditions, is likely to have affected force generation perhaps by affecting calcium extrusion (see below). The decrease in force occurred steadily over the 50 min following the onset of the raised P_i concentration. In our current study, the decrease in P_i occurred before the initiation of the contraction so we cannot determine if the P_i effect on force is delayed in sheep aortae as it appeared to be in aerobic taenia coli (Nakayama *et al.* 1988).

The decrease in free P_i levels in our current study was much lower than the changes in P_i levels used in the skinned fibre experiments that demonstrated the role of P_i in smooth muscle relaxation ($\sim$ 5–10 mM) (Schneider *et al.* 1981; Itoh *et al.* 1986; Gagelmann & Guth, 1987). Based on relative resonance intensity, free P_i levels are well below 1 mM in resting aortae prior to 2DG administration unless the spin relaxation (T_1) of P_i is much greater than that for PCr. No such evidence exists for

a substantially longer T_1 for P_i compared to PCr in smooth muscle. However, if the T_1 for P_i is substantially longer than for PCr, P_i levels may be greater than our estimate thereby making P_i a more likely candidate as a modulator of force in smooth muscle. Therefore, either the effects of altered free P_i levels in sheep aortae are greater than in skinned smooth muscle preparations or the decrease in P_i coincidentally occurred when isometric force generation increased and the rate of relaxation was higher.

Increased myosin light chain phosphorylation

The decrease in P_i concentration associated with 2DG administration may have resulted in an increased myosin light chain phosphorylation resulting in higher levels of force generation and presumably a faster rate of contraction. However, in chemically demembranated taenia coli, increases of 10 mM P_i did not result in any changes in the level of phosphorylation of the 20000 Da regulatory myosin light chain (Gagelman & Guth, 1987). In addition, the time to half-maximal contraction of the first contraction following 2DG administration was the same as for the preceding contraction in the absence of any exogenous substrate (see Table 1). Therefore it is unlikely that the decreased P_i levels affected the level of myosin light chain phosphorylation in our experiments.

Increased steady-state cytosolic calcium concentration

If the 2DG administration resulted in increased cytosolic calcium levels, then an increase in force generation would have occurred because the original calcium concentration was insufficient to induce a maximal contraction. We have not verified whether or not 80 mM KCl elicits a maximal contraction in sheep aorta. However, if 2DG administration alone increases cytosolic calcium levels, then resting tone should have been altered during the first hour of 2DG administration. No such change occurred (data not shown). 2DG administration, it may be argued, increases cytosolic calcium levels during contraction by increasing calcium conductance or decreasing the rate of calcium extrusion. An increase in calcium conductance during contraction would result in an increased rate of tension development yet no such increase in the rate of contraction occurred during the first contraction in the presence of 2DG (see Table 1). 2DG administration may have decreased the rate of calcium extrusion by decreasing the rate of the plasmalemmal calcium pump. Glycolysis preferentially supplies ATP to the plasmalemmal calcium pump in smooth muscle (Paul *et al.* 1989; Hardin *et al.* 1992*a*) and 2DG administration in the absence of glucose should inhibit glycolysis. However, glycolysis should also have been inhibited during the contraction in the absence of glucose (Fig. 2) yet the force generation during 2DG administration was greater than when glucose was absent from the superfusate. Therefore an inhibition of glycolysis by 2DG administration does not appear to underlie the increased force generation.

In conclusion the decreased levels of ATP and PCr often attained during hypoxia do not appear to limit development of force generation implying that the decrease in ATP demand by the contractile apparatus during hypoxia, when the rate of oxidative ATP production is decreased, is not due to the concentration of ATP being limiting for contraction. Changes in the concentrations of P_i may modulate force

generation in smooth muscle and therefore may be one of several mediators of the decreased force during hypoxia. Alterations in the energy charge state (e.g. phosphorylation potential or PCr/Cr ratio) may directly affect the activity of the contractile apparatus or any of the intermediate steps of excitation–contraction coupling such as regulation by phosphorylation/dephosphorylation mechanisms. Since the concentration of ATP does not appear to limit function, the intermediate steps which couple the rates of ATP demand with ATP supply during hypoxia remain to be elucidated.

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