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ACIDOTIC DEPRESSION OF CYCLIC AMP ACCUMULATION AND PHOSPHORYLASE *b* TO *a* TRANSFORMATION IN SKELETAL MUSCLE OF MAN

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SUMMARY

1. Intravenous infusion of adrenaline was performed in three healthy subjects on two occasions. In one case subjects performed a maximal isometric contraction before infusion. Biopsies were taken from the quadriceps femoris muscle before and after infusion for 0·5 and 2 min, and analysed for muscle pH, cyclic AMP, metabolites and activities of glycogen phosphorylase and synthetase.

2. Isometric contraction resulted in a decrease of muscle pH to 6·60 (normal value at rest 7·0–7·1). By this experimental procedure the effect of adrenaline infusion could be studied on a muscle with normal pH and one with low pH.

3. Cyclic AMP increased from 3 to about 9·5 μmol per kg dry weight after 0·5 min of adrenaline infusion. When isometric contraction preceded the infusion, cyclic AMP increased more slowly and was about 5·5 μmol per kg dry weight after the same time of infusion.

4. Phosphorylase *a* constituted about 22 % of total phosphorylase in resting muscle but increased rapidly to 80 % after 0·5 min infusion. When exercise preceded infusion phosphorylase *a* decreased and was still lower after 2 min infusion.

5. The results can be explained by inhibition of adenylcyclase and phosphorylase *b* kinase at low muscle pH.

INTRODUCTION

During intense exercise and in connexion with oxygen deficiency part of the energy is provided through lactic acid formation. However, uncontrolled lactic acid formation is a potential danger to the muscle tissue and the whole organism, since acid-labile components or structures could be damaged as a result. It is well recognized that hydrogen ions can regulate the glycolytic flux through feed-back inhibition of phosphofructokinase, which is a key regulatory enzyme in glycolysis. The activity of phosphofructokinase is highly sensitive to changes in pH and is almost completely inhibited at a cellular pH of 6·4 (Trivedi & Danforth, 1966) which is close to the value in fatigued muscle (Sahlin, 1978). However, the inhibition of phosphofructokinase can

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be overcome by increased levels of the substrate fructose 6-phosphate (Trivedi & Danforth, 1966) and a control point higher up in glycolysis is required. Phosphorylase is the flux-generating step for glycolysis from glycogen and exists in two interconvertible forms (*b* and *a*). The activity of phosphorylase *b* is dependent upon the activator AMP and the inhibitors glucose 6-phosphate and ATP and is generally considered to be less active than the *a* form. Inhibition of phosphofructokinase by

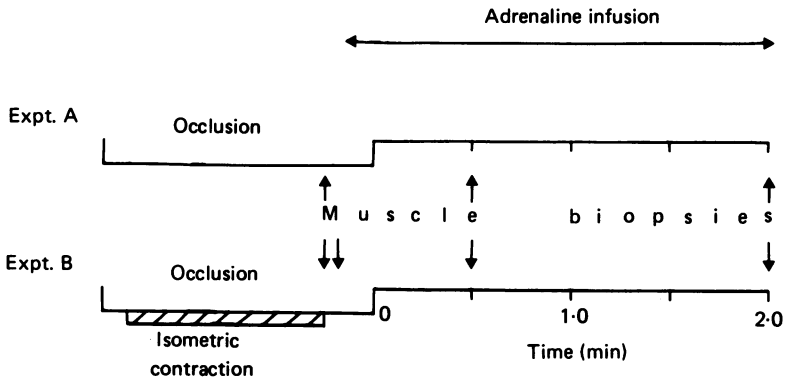

Fig. 1. Design of the experimental procedures. Time zero denotes the time at which the circulation was restored.

protons could cause inhibition of phosphorylase due to accumulation of glucose 6-phosphate. It was reported by Danforth 1965*a* that transformation of phosphorylase *b* to *a* in isolated frog muscle in response to electrical stimulation was slower during acidosis (induced by CO_2) than in control muscles.

The object of the present study was to investigate whether decreased muscle pH exerts an inhibitory effect on the transformation of phosphorylase *b* to *a* in human skeletal muscle.

METHODS

Material. Three healthy male subjects, aged between 21 and 29 years, participated in the present study. The mean body weight was 80 kg (range 78–82). The mean height was 190 cm (range 185–197). Their training status was not investigated in any detail, but none of the subjects could be classified as an athlete. Every subject was informed of the purpose and the nature of the experiments before his voluntary consent was obtained. The experiments are part of a project which has been approved by the Ethical Committee of the Karolinska Institute, Stockholm, Sweden.

Experimental. Each subject participated in two experimental sessions explained in Fig. 1. In both experiments blood circulation to the legs was occluded by means of a tourniquet placed around the upper thigh and inflated to 250 mmHg.

Experiment A. After occlusion of the leg for 56–60 s a muscle biopsy was taken and infusion of adrenaline was started. The circulation was restored 10 s later. In Fig. 1 the time at which the circulation was restored is set as zero min. Muscle biopsies were taken after infusion for 0·5 and 2 min with intact circulation.

Experiment B. After the onset of occlusion subjects performed sustained isometric contraction at 66% of their maximum voluntary contraction force to fatigue (56–60 s) in a chair of the type described by Tornvall (1963). Thereafter two muscle biopsies were taken and adrenaline infusion started. The procedure was then the same as in Experiment A. Adrenaline (adrenalin 'Aco' solution, 1 mg ml^{-1}) was given by continuous intravenous infusion with an infusion pump (IVAC 630 volume pump, U.S.A.). The stock solution of adrenaline was diluted with isotonic NaCl to a concentration of 5 μg ml^{-1}. The infusion rate was 2 ml min^{-1}, corresponding to about 0·15 μg ml^{-1}

adrenaline kg^{-1} body weight min^{-1} and the infusion was performed until the last muscle biopsy was taken.

All muscle samples were taken from the lateral portion of quadriceps femoris muscle by the needle biopsy technique (Bergström, 1962). Immediately after withdrawal, the biopsy needle was immersed into liquid Freon (dichlorodifluoromethane) maintained at its melting point (−150 °C) with liquid N_2. The time delay between insertion of the needle in the muscle and freezing was about 2–3 s. Muscle samples were stored in liquid N_2 until further treatment.

Analytical methods. Muscle pH was determined by homogenization of the sample at 0 °C with a solution (5 μl/mg muscle) containing 145 mM-KCl, 10 mM-NaCl and 5 mM-Na iodoacetate. The pH of the muscle homogenate was measured at 38 °C by micro-electrode equipment (IL-213 Radiometer, Copenhagen). (For details see Sahlin, Harris & Hultman, 1975.)

Muscle samples intended for determination of metabolites, cyclic AMP or enzymatic activity were freeze-dried and dissected free from blood, fat and all visible connective tissue.

Cyclic AMP was determined by extraction of muscle samples (2–3 mg) with 500 μl trichloroacetic acid (TCA) solution (6% w/v) for about 15 min and centrifugation for 15 min at 4000 rev/min at 4 °C; 400 μl extract was washed three times with 4·0 ml water-saturated diethylether warmed to 60 °C, lyophilized and dissolved in 0·05 M-sodium acetate buffer, pH 6·2. The amount of cyclic AMP was determined by the procedure developed by Steiner, Kipnis, Utiger & Parrer (1969) with a radioimmunoassay kit (Becton Dickinson). All assays were performed in duplicate on aliquots of 50–100 μl.

Phosphorylase and synthetase were determined in muscle homogenates prepared in a Potter–Elvehjem-type homogenizer at −33 °C in an aqueous solution of 60% (v/v) glycerol, containing 30 mM-Na glycerophosphate, 50 mM-NaF, 5 mM-EDTA, 0·5 g bovine serum albumin l^{-1}, and 20 mM-dithiothreitol. The pH was adjusted to 7·0. The same muscle homogenate was used for assay of glycogen phosphorylase and synthetase. Glycogen phosphorylase was assayed at 35 °C, pH 7·0, in the direction of glycogen breakdown using a method adapted from Holmes & Mansour (1968), as previously described (Chasiotis, Sahlin & Hultman, 1982*a*). The reaction was started by addition of AMP-free glycogen and formation of hexose phosphates was measured enzymatically according to Harris, Hultman & Nordesjö, (1974). Values are reported as mmol glucosyl units kg^{-1} dry muscle min^{-1}. Glycogen synthetase was assayed at 35 °C and pH 7·0 in the direction of glycogen synthesis using a method adapted from Kornfeld & Brown (1962), as previously described by Chasiotis *et al.* (1982*a*). The reaction was started by addition of UDP-glucose and formation of UDP was measured enzymatically according to Danforth (1965*b*). Values are reported as mmol glucosyl units kg^{-1} dry muscle min^{-1}.

Glucose 6-P, glucose 1-P, fructose 6-P, and lactate were analysed in perchloric acid extracts of freeze-dried muscle by enzymatic methods as previously described (Harris *et al.* 1974).

RESULTS

Infusion of adrenaline resulted in a slight increase in muscle content of hexose monophosphate (hexose-P) in all subjects, but almost unchanged lactate content (Table 1), which is indicative of an enhanced rate of glycogenolysis. Isometric contraction resulted in highly increased contents of lactate and hexose-P in muscle which were of similar magnitude as previously reported (Bergström, Harris, Hultman & Nordesjö, 1971; Harris, Hultman & Sahlin, 1981). Hexose-P content was unchanged and maintained at a high level when isometric contraction was followed by adrenaline infusion. This contrasts to recovery without adrenaline infusion where hexose-P decreases at a rate of approximately 4 mmol kg^{-1} dry muscle min^{-1} (Harris *et al.* 1981). The difference might be due to the fact that the activity of synthetase I which decreases during exercise was maintained at a low level during adrenaline infusion (see below), thus preventing resynthesis of glycogen from accumulated hexose-P. Muscle pH was found to decrease to 6·60 after isometric contraction, a finding similar to that previously observed (Sahlin *et al.* 1975). In order to keep the total number

of biopsies at a minimum no samples intended for assay of muscle pH were taken at rest or during adrenaline infusion. Infusion of adrenaline has been found not to influence intracellular pH of resting muscle (K. Sahlin, J. Henrikson & A. Juhlin-Dannfeldt, unpublished work) and muscle pH could therefore be calculated from the lactate content by utilizing the previously observed relationship between muscle pH

TABLE 1. Muscle pH and metabolites in human skeletal muscle during infusion of adrenaline. The subjects ($n = 3$) participated in two sessions. In one case adrenaline infusion was preceded by a maximal isometric contraction and in the other case not. Metabolites are expressed as mmol/kg dry muscle

		Adrenaline infusion	
	Rest	0·5 min	2 min
Muscle pH	7·0*	7·0*	7·0*
Hexose monophosphates	2·1 (1·0–2·7)	2·9 (2·6–3·2)	4·7 (3·1–6·4)
Lactate	5·8 (4·0–8·3)	6·1 (3·1–9·2)	6·20 (4·7–7·5)
		Adrenaline infusion	
	Isometric contraction	0·5 min	2 min
Muscle pH	6·60 (6·56–6·60)	6·6*	6·7*
Hexose monophosphates	19·1 (17·4–21·2)	19·5 (18·6–20·4)	18·4 (17·2–19·6)
Lactate	99·3 (92·4–103·7)	95·2 (92·3–98·0)	61·4 (50·5–72·3)

* Calculated from the lactate contents (Sahlin *et al.* 1975).

and lactate (Table 1). Muscle lactate content following isometric contraction decreased at a similar rate as during recovery without adrenaline infusion (Harris *et al.* 1981).

Muscle content of cyclic AMP was similar after isometric contraction as at rest (Fig. 2*A*) and increased during infusion of adrenaline in accordance with previous studies (Chasiotis, Sahlin & Hultman, 1982*b*). However, the increase was slower when infusion was preceded by isometric contraction. After 0·5 min adrenaline infusion the increase of cyclic AMP in the exercised leg was only 37 % of that in the non-exercised leg.

The total activity of phosphorylase ($a+b$) and of synthetase (I+D) was not changed during adrenaline infusion or by isometric contraction and was 80–103 and 8·0–9·9 mmol glucosyl units kg^{-1} dry muscle min^{-1} for the three subjects. Infusion of adrenaline resulted in a rapid increase of phosphorylase *a* in the non-exercised leg in accordance with previous results (Chasiotis *et al.* 1982*b*) whereas a slight decrease was observed when infusion was preceded by exercise (Fig. 2*B*). The activities of phosphorylase presented in Fig. 2*B* were obtained by using a concentration of 11 mM-inorganic phosphate (P_i) in the assay system. However, the K_m values of phosphorylase *a* and $a+b$ for P_i have previously been determined to be 26·6 and 7·3 mM respectively in resting muscle (Chasiotis *et al.* 1982*a*) and thus the activity of either form of the enzyme at saturating P_i concentrations will be substantially higher than that measured at 11 mM-P_i. In further studies it was found that the K_m values of phosphorylase *a* and $a+b$ for P_i were not affected by adrenaline infusion (Chasiotis *et al.* 1982*b*). From data on phosphorylase *a* activity at 11 mM-P_i and the values of K_m the ratio $V_{max,a}$: $V_{max,a+b}$ has been calculated (Table 2). This ratio may be used as an estimate of the proportion of phosphorylase in the *a* form (Chasiotis

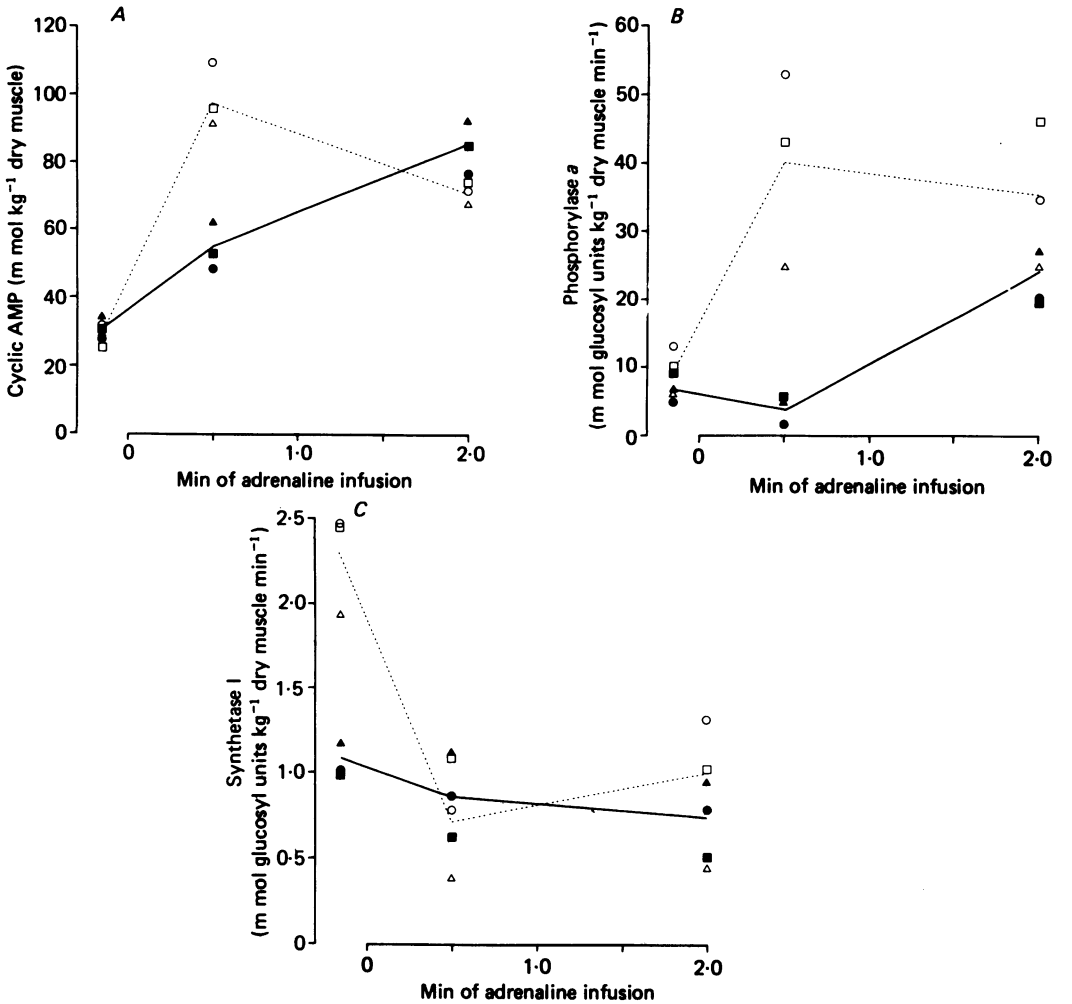

Fig. 2. Content of cyclic AMP (Fig. 2*A*) activity of phosphorylase *a* (Fig. 2*B*) and synthetase I (Fig. 2*C*) in biopsies taken from quadriceps femoris muscle of man during adrenaline infusion. In one experimental session infusion was preceded by an isometric contraction to fatigue (filled symbols). All subjects ($n = 3$) participated in both sessions and are represented by the same symbol. Total activity of phosphorylase and synthetase are represented in Table 2.

TABLE 2. Fraction of total phosphorylase present in the *a* form. Values have been calculated from phosphorylase activities measured at 11 mM P_i using a K_m of 26·6 for phosphorylase *a* and a K_m of 7·3 for phosphorylase *a*+*b* (Chasiotis *et al.* 1982 *a*)

	Adrenaline infusion		
	0	0·5 min	2 min
$V_{max,a}/V_{max,a+b}$, resting muscle	0·22 (0·19–0·25)	0·84 (0·67–0·99)	0·79 (0·65–0·90)
$V_{max,a}/V_{max,a+b}$, exercised muscle	0·14 (0·10–0·19)	0·10 (0·05–0·13)	0·62 (0·51–0·74)

et al. 1982*a*). The *a* form constituted 22% of total phosphorylase in resting muscle and increased to 84% after 0·5 min adrenaline infusion. Isometric contraction to fatigue resulted in lower values for phosphorylase *a* in all subjects, and after 0·5 min infusion a further decrease was observed (Table 2). After 2 min infusion the proportion of phosphorylase in the *a* form had increased but was still lower than for non-exercised muscle.

DISCUSSION

Muscle content of cyclic AMP is determined by the balance between its formation through adenylcyclase and its breakdown catalysed by phosphodiesterase. It is well recognized that the increase of cyclic AMP during adrenaline infusion is due to activation of the membrane-bound adenylcyclase. In the present study a diminished rate of cyclic AMP accumulation was observed when muscle tissue was made acidotic by isometric contraction before infusion of adrenaline. This can be explained by a decreased rate of formation, which is supported by the observed depression of adenylcyclase activity in homogenates of human muscle tissue when pH decreases below 7·0 (Mawatari, Tagaki & Rowland, 1974). However, the results might be explained as well by increased rate of breakdown since an increased affinity of phosphodiesterase for cyclic AMP has been observed in adipose tissue when pH decreases (Fredholm & Hjemdahl, 1976). Thus, available data on enzyme kinetics suggest that the diminished rate of cyclic AMP accumulation *in vivo* observed in the present study is caused by the low muscle pH. Acidosis has also been found to decrease cyclic AMP accumulation and rate of lipolysis in isolated adipose tissue (Fredholm & Hjemdahl, 1976). Further, induced acidosis by NH_4Cl ingestion in human subjects has been found to decrease the concentration of glycerol and free fatty acids in blood during subsequent exercise, implying a reduced rate of lipolysis (Jones, Sutton, Taylor & Toews, 1977).

Transformation of phosphorylase *b* to *a* is catalysed by phosphorylase *b* kinase which exists in two enzymatically interconverting forms (inactive and active phosphorylase *b* kinase). The conversion of the inactive to the active form can be achieved by increased concentration of Ca^{2+} or cyclic AMP (through activation of protein kinase). Infusion of adrenaline results in transformation of phosphorylase *b* to *a* by a cascade mechanism initiated by increased levels of cyclic AMP. The lower cyclic AMP content found in muscle tissue when infusion was preceded by contraction partly explains the diminished rate of phosphorylase transformation. The total absence of transformation after 0·5 min infusion in spite of an increased cyclic AMP content does, however, suggest that a second step is inhibited by low pH. *In vitro* studies of phosphorylase *b* kinase have shown that both the activity of the active form as well as the rate of its transformation into the active form are decreased when pH decreases (Krebs, Love, Bratwold, Trayser, Meyer & Fischer, 1964). Results from the present study suggest that these *in vitro* findings are of physiological importance.

Available data suggest that decreased muscle pH results in diminished rates of cyclic AMP accumulation and transformation of phosphorylase *b* to *a*. However, other changes in muscle tissue following isometric contraction might influence the results. Exercise induces an enhanced blood flow in the working muscle and an increased

capillary bed will be available which would increase the local concentration of adrenaline. In spite of this 'advantage', a slower response compared to non-exercised tissue was observed. Isometric contraction to fatigue results in increased levels of AMP and inosine monophosphate (IMP) and a 20% decrease in ATP (Sahlin, Palmskog & Hultman, 1978). AMP and IMP are inhibitory to phosphorylase *a* phosphatase (Newsholme & Start, 1974) which catalysed transformation of phosphorylase *a* to *b*, and increased levels would thus tend to increase the *a* form of phosphorylase, which is contrary to the effect observed. Adenylcyclase has a high affinity for ATP (K_m = 0·29 mM; Mawatari *et al.* 1974) and will not be influenced by the relatively small change in ATP.

In common with animal studies, we have recently observed that phosphorylase *a* increases in human skeletal muscle during both isometric contraction and intensive bicycle exercise (Chasiotis *et al.* 1982*a*). However, when exercise was continued to fatigue phosphorylase *a* activity reversed to the value at rest or even below. This finding might be explained by an inhibitory effect of low pH on phosphorylase *b* kinase.

Glycogen and P_i are substrates for phosphorylase. However, only the dianion (HPO_4^{2-}) is believed to be the active substrate of phosphorylase (Kasvinski & Meyer, 1977) and a decrease of pH will convert the dianion into the inactive form. It can be calculated that a decrease of pH from 7·0 to 6·5 would convert about 50% of the active P_i into its inactive form. Acidosis will thus exert a double inhibitory function on glycogenolysis by affecting both the transformation of phosphorylase *b* to *a* and the active substrate concentration.

Conclusion

Accumulation of cyclic AMP and transformation of phosphorylase *b* to *a* is inhibited when the adrenaline infusion is preceded by isometric contraction. The findings can be explained by an effect of low muscle pH on the enzymes involved.

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