Biochimica et Biophysica Acta, 976 (1989) 42–52
Elsevier

BBABIO 43047

Effects of altering carbohydrate metabolism on energy status and contractile function of vascular smooth muscle

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(Received 22 November 1988)
(Revised manuscript received 17 April 1989)

Key words: Glycolysis; High energy phosphate; Muscle contraction; (Pig)

Substrate-dependent changes in vascular smooth muscle energy metabolism and contractile function were investigated in isolated porcine carotid arteries. In media containing glucose glycogen catabolism accounted for all the estimated high-energy phosphate turnover that occurred in conjunction with contraction induced by 80 mM KCl. However, in glucose-free media glycogen catabolism accounted for only a portion of the estimated ATP utilization in resting and contracting arteries, even though glycogen stores were not depleted. The glycogenolysis and lactate production that ordinarily accompanies contraction was completely inhibited by 5 mM 2-deoxyglucose (2-DG). However, there was no decrease in the high-energy phosphate levels when compared to control resting arteries similarly treated with 2-DG. The results suggest that an endogenous non-carbohydrate source may be an important substrate for energy metabolism. Treatment of arteries with 50 μM iodoacetate (IA) in media containing glucose resulted in a marked reduction of high energy phosphate levels and an accumulation of phosphorylated glycolytic intermediates, as demonstrated by ^{31}P-NMR spectroscopy. In glucose-free media, 50 μM IA had only a slight effect on high-energy phosphate levels, while glycogenolysis proceeded unhindered. With 1 mM IA in glucose-free media, the oxidative metabolism of glycogen was inhibited as evidenced by the depletion of high-energy phosphates and the appearance of sugar phosphates in the ^{31}P-NMR spectra. Thus, the titration of enzyme systems with IA reveals a structural partitioning of carbohydrate metabolism, as suggested by previous studies.

Introduction

Carbohydrate metabolism is an important process involved in the physiologic properties exhibited by vascular smooth muscle [1]. Indeed, early studies have suggested that the primary substrate utilized by this tissue is carbohydrate [2,3]. Subsequent investigations have suggested that different carbohydrate sources are utilized by smooth muscle to support various physiologic processes. For instance the metabolism of extracellular glucose has been linked to ion transport mechanisms [4], while glycogen has been implicated as the substrate utilized preferentially during contraction [4,5]. Other studies, however, have provided experimental evidence that is contrary to the premise that carbohydrate is the primary substrate. Intracellular lipid has been implicated as the primary fuel for quiescent, non-contracting vascular smooth muscle. These latter studies indicate that carbohydrate utilization is of minor importance in this functional state under normal conditions [6,7].

Since the extent to which carbohydrate utilization satisfies the energy requirements of vascular smooth muscle remains uncertain, the present studies were undertaken to investigate carbohydrate metabolism in relation to energy production in vascular smooth muscle. In this paper, we investigated the effects of glucose depletion and inhibitors of glycolysis on high-energy phosphates and metabolite levels in resting and contracting porcine carotid artery. With this approach, we were able to provide more information on the relative roles of glucose, glycogen and endogenous non-carbohydrate substrates in energy metabolism and physiological function.

Abbreviations: 2-DG, 2-deoxyglucose; IA, iodoacetate; PSS, physiological salt solution; PCr, phosphocreatine; P_i, inorganic orthophosphate; PME, phosphomonoester.

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Methods

Porcine carotid arteries were obtained from an abattoir soon after killing, and transported to the laboratory in media at ambient temperature. Segments of approx. 4.5 cm in length and 0.5 cm in diameter were dissected – free of fat and loose connective tissue. Helical strips were prepared and mounted in jacketed chambers and attached to Grass FT03 force transducers connected to an Electronics-for-Medicine VR12 recorder for monitoring of tension. Sufficient resting tension was applied to simulate 100 mmHg mean arterial pressure, as described in detail previously [8]. The strips were equilibrated at 37°C for 1 h in a physiological salt solution containing glucose (glucose-PSS) consisting of 118 mM NaCl, 20 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.6 mM $CaCl_2$ and 5.6 mM glucose. This electrolyte solution was aerated with a gas mixture of 95% O_2/5% CO_2. The pH of the medium remained constant at pH 7.3–7.4. Phosphate buffer was omitted because of interference of the inorganic phosphate with determination of phosphate metabolites by ^{31}P-NMR spectroscopy (see below).

After pre-equilibration with glucose PSS for 1 h, tension was re-adjusted and strips were incubated for an additional $1\frac{1}{2}$ h in either glucose PSS, or glucose-free PSS with or without iodoacetate (IA), or 2-deoxyglucose (2-DG). Carotid strips were then contracted maximally for 30 min by challenge with 80 mM KCl. Resting, control strips with passive tension applied were left in another muscle chamber for the same incubation period, so that total experimental incubation time (2 h) was the same for both resting and contracting strips. At the end of the experimental incubation period, resting or contracting strips, were immersed immediately in Dewar flasks containing liquid nitrogen while still mounted in the muscle chambers. In those experiments in which tissue glycogen levels were determined, carotid strips were immersed in liquid N_2 after the 1 h pre-equilibration period in glucose PSS. All tissues subsequently were stored in liquid N_2 until the analyses described below were performed.

Preparation of perchloric acid tissue extracts. Perchloric acid extracts were prepared as described previously [9,10]. Briefly, two frozen carotid strips were weighed and then pulverized to a fine powder in a stainless steel mortar and pestle maintained in a liquid N_2 bath. The tissue powder was weighed and then added to a liquid N_2 chilled polyallomer centrifuge tube containing a frozen aliquot (0.2 vol/wt.) of 60% perchloric acid. The frozen powder and perchloric acid were then stirred slowly while gradually warming until a paste consistency was achieved. In this manner, the powdered tissue particles were coated with ultracold perchloric acid. The resultant tissue paste was centrifuged for 15 min at $45\,000 \times g$ at 4°C. The supernatant was immediately transferred using a cold Pasteur pipette to a 10 ml beaker containing an equivalent volume of 10 M KOH and an aliquot of potassium-form Chelex-100 resin. The resultant suspension was pH-adjusted to 10.0 and centrifuged for 15 min at 4°C at $45\,000 \times g$ to pellet the $KClO_4$ precipitate. The final supernatant volume was measured and the supernatant was passed through a potassium-form Chelex-100 column to remove divalent cations, and lyophilized. The lyophilized extract was then dissolved in 1 ml of 20% (vol/vol) ^2H_2O, pH-adjusted to 10.0, filtered through glass wool into a 12 mm NMR microcell assembly, and analyzed by ^{31}P-NMR spectroscopy (see below).

Parallel experiments were performed for measurement of tissue lactate and total extractable tissue phosphate. For the lactate determinations, tissue extracts were prepared as described above, except that the centrifuged tissue pellets were saved and weighed before and after drying in an oven and the supernatant was adjusted to pH 8.0. The precipitate of the neutralized supernatant was also saved and weighed before and after drying. For total extractable tissue phosphate, the tissue pellets were similarly weighed before and after drying, but the supernatant was not neutralized.

Analytical determination of lactate and total phosphate. Tissue lactate was assayed spectrophotometrically with the use of lactate dehydrogenase as described by Lowry and Passonneau [11], and referenced to the total wet weight of the tissue. Acid soluble phosphates in perchloric acid extracts were digested to inorganic orthophosphate as previously described and the total inorganic phosphate assayed [12].

Tissue glycogen determination. Parallel experiments were undertaken for determination of tissue glycogen. Carotid strips were immersed in liquid N_2 and pulverized, and weighed as before. The tissue powder was solubilized in 5 vol of 1.5 M NaOH at 70°C for 2 h. This mixture was cooled, neutralized to pH 7.0, and centrifuged. Aliquots were assayed analytically for glycogen according to the method of Lowry and Passonneau [11]. Tissue glycogen was referenced to the wet weight of the strips.

^{31}P-nuclear magnetic resonance spectroscopy (^{31}P-NMR) of tissue extracts. The ^{31}P-NMR analyses of porcine carotid artery perchloric acid extracts were performed according to procedures described and detailed elsewhere for other tissues [9,10]. Briefly, tissue extract samples were analyzed under constant temperature (24°C) conditions with proton decoupling. The polyvalent-cation-purged extract samples prepared as described above were found, based on repeated analyses of test extracts, to be chemically stable for at least 24 h at 24°C. The NMR spectrometer used in this study was a Nicolet NT-200 system interfaced to a 4.7 T, 89 mm bore Oxford magnet, operating at 80.99 MHz for ^{31}P. The spectrometer signal acquisition conditions were as

follows: pulse sequence, 1 pulse; pulse width, 8 μs (45° flip angle); acquisition delay, 200 μs; number of data points per free-induction decay, 16384; number of acquisitions, 20000; acquisition time, 1.64 s; sweep width, ±2500 Hz; and a free-induction decay multiplication factor was used which introduced 0.6 Hz artificial line-broadening. Chemical shift values corresponding to the different phosphate resonances were determined relative to the conventional standard of 85% orthophosphoric acid. The chemical identification of the tissue extract resonance signals was based on multiple physicochemical criteria. These criteria included the standardized chemcial shift position of the peak, the proton-coupled peak characteristics, the J-coupling values of the peak multiplets, peak super-positioning with an added compound of known identity, pH-dependent shift curve characteristics, and the demonstration of co-migration with a known compound. Whenever possible, biochemical enzymatic procedures were employed to provide evidence confirming the presence and the relative content of specific metabolites in the extract.

Data reduction, resonance peak chemical shift determinations, and quantification of spectral resonance peak distributions based on peak area integrations were performed by computer analysis. Relative resonance signal areas were corrected for small differential spectroscopic saturation and nuclear Overhauser enhancement, as necessary. Metabolite levels are reported either as % of total phosphorus detected by spectral analysis, or as concentrations based on the product of the mol% of the metabolite detected and the total phosphorus concentration extracted from the tissue sample.

Previous studies have shown that smooth muscle contains, in addition to ATP, appreciable quantities of the nucleotide triphosphates, GTP and UTP [13,14]. The α-, β- and γ-phosphate groups of GTP and UTP co-resonate with those of ATP and are poorly resolved at this field strength. Therefore, in this paper the total nucleotide triphosphate pool is designated as 'ATP'.

Results

Comparison of arterial strips incubated in glucose-PSS or glucose-free PSS revealed that there was no difference in resting tone between the two groups. The time course of isometric tension development in response to K^+ depolarization (80 mM KCl for 30 min) is shown in Fig. 1 for the two groups. The rate of tension development and maximum isometric tension attained was greater is muscles incubated in glucose-free PSS. There was no diminution of tension throughout exposure to KCl. These results agree with previous studies showing no inhibitory effect of prolonged glucose depletion on contractile responses to KCl in rabbit aorta [15]. On the contrary, glucose depletion appeared to enhance

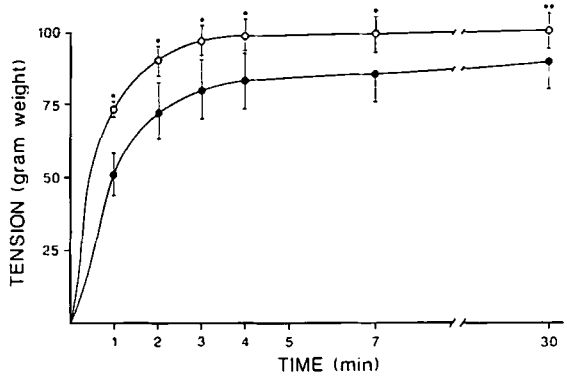

Fig. 1. Time-course of isometric tension rise in response to KCl depolarization. Closed circles, contractile response in normal glucose PSS; $n = 12$ carotid strips from different animals; open circles, contractile response in glucose-free PSS, $n = 10$ carotid strips from different animals. * $P < 0.001$; ** , $P < 0.02$.

both the rate and magnitude of the force generated by the contractile process.

Table I compares the steady-state levels of phosphate metabolites, including the high-energy phosphates, ATP and phosphocreatine (PCr), of resting strips and strips contracted for 30 min with KCl in the presence or absence of glucose. Glucose depletion did not affect the total acid soluble phosphate extracted from the tissue (without glucose: 9.74 ± 0.8 μmol phosphate per g vs. with glucose: 9.80 ± 1.1 μmol phosphate per g ($n = 4$ for both groups)). A representative ^{31}P-NMR spectrum of the phosphate metabolites demonstrable in carotid artery tissue extracts is presented in Fig. 2. As seen in Table I there was a small but significant decrease in PCr levels and a corresponding increase in inorganic phosphate (P_i) in contracting muscles as compared with resting muscles in glucose-PSS. These observations are consistent with reports showing a modest decrease in PCr in the steady-state condition during contractions of various smooth muscle preparations [1,15,16,17] but are at variance with others which did not detect such a change [9,13,18]. Contracted carotid strips were relaxed by wash-out of the KCl with rinses with glucose PSS, and then frozen after remaining in the resting state for 60 min. There was no significant difference between resting and contracting-relaxing levels of PCr (7.4 ± 0.2% vs. 6.8 ± 0.4%) and P_i (8.2 ± 0.3% vs. 8.9 ± 0.2%, $n = 4$), which demonstrates that the reported changes associated with contraction were reversible. Elimination of glucose from the incubation medium in resting carotid artery also resulted in a small but significant decrease in PCr content and a concomitant increase in P_i. When medium glucose was restored for an additional 60 min to resting, glucose-depleted muscles, there was return of PCr (7.4 ± 0.2 mol% vs. 7.3 ± 0.1 mol%, $n = 4$) and P_i (8.2 ± 0.3 mol% vs. 9.1 ± 0.4 mol%, $n = 4$) to normal levels. The results demonstrate the stability of the carotid artery preparation under the experimental

TABLE I

Steady-state levels of phosphate metabolites in porcine carotid artery at rest or at the end of 30 min contraction in the presence or absence of glucose

ATP + PCr is expressed in absolute content (μmol/g). Values for the remaining data represent percentages of total acid soluble phosphates (mean ± S.E.M.). Values for ATP represent the three phosphate groups. *n*, number of experiments; samples were prepared from two carotid arteries from different animals per experiment. PME, phosphates with resonances in the phosphomonoesterified region. Remaining phosphates includes glycerylphosphocholine, phosphocholine, glycerophosphoethanolamine, uridine diphosphosugars and dinucleotide phosphates.

Condition	*n*	ATP + PCr (μmol/g)	ATP (%)	PCr (%)	ADP (%)	P_i (%)	PME (%)	Remaining phosphates
Resting, glucose PSS	5	1.94 ± 0.02	37.5 ± 0.8	7.4 ± 0.2	11.1 ± 0.3	8.2 ± 0.3	20.6 ± 0.6	13.9
Contracting, glucose PSS	5	1.68 ± 0.05 *	36.7 ± 0.9	5.1 ± 0.4 *	11.9 ± 0.5	10.0 ± 0.3 *	23.8 ± 0.6 *	12.5
Resting, glucose-free PSS	4	1.75 ± 0.06 *	36.8 ± 0.7	5.7 ± 0.4 *	11.1 ± 0.5	11.7 ± 1.0 *	21.5 ± 0.7	13.2
Contracting, glucose-free PSS	4	1.67 ± 0.08 *	36.5 ± 1.2	5.0 ± 0.5 *	12.0 ± 0.3	11.5 ± 0.8 *	20.9 ± 1.3	14.0

* $P < 0.001$.

incubation conditions employed in this study, and further validate the ^{31}P-NMR methodology used to determine phosphorus balance. Upon KCl contraction, there was no change in PCr or total high-energy phosphate pools (ATP + PCr) when compared to resting strips in glucose-free PSS.

It was also observed that a compound at a chemical shift of 4.32 δ demonstrable in spectra from carotid artery strips incubated in glucose PSS was consistently absent from spectra from all strips equilibrated in glucose-free PSS, either at rest or contracted. This metabolite constituted 0.5 ± 0.04% of the total phosphate of resting muscles incubated in glucose PSS. Attempts to determine definitively the chemical identity of this compound have yielded inconclusive findings.

The compound with a chemical shift at 3.89 δ has been previously observed in intact rabbit taenia coli [16] and in extracts of rabbit aorta [9]. This compound was preliminarily identified as corresponding to a sugar phosphate. Subsequent studies, however, have identified this metabolite in rabbit bladder as phosphoethanolamine [14]. In the present study with porcine carotid artery extracts, standards of phosphoethanolamine and ribose 5-phosphate co-resonated with this peak at pH

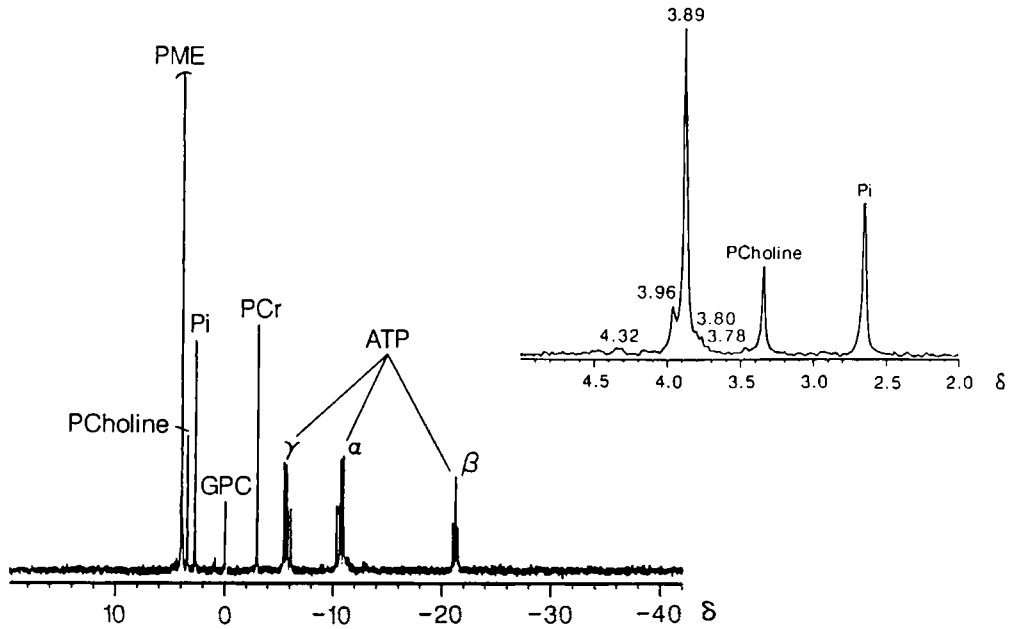

Fig. 2. ^{31}P-nuclear magnetic resonance spectrum of perchloric acid extract of resting porcine carotid artery equilibrated in glucose PSS. Major resonance signals from right to left: α, β, γ phosphates of ATP; PCr, phosphocreatine; GPC, glycerol 3-phosphorylcholine; P_i, inorganic orthophosphate; PCholine, phosphocholine; PME, compounds with resonance signals in the phosphomonoester region. Expanded phosphomonoester region is seen at the right. Resonance at 3.89 δ identified as phosphoethanolamine [17]. Remaining phosphates are unidentified. Resonance peaks are scaled to the 3.89 δ, phosphoethanolamine peak.

10.0. The physicochemical properties of this phosphomonoester signal were characterized further through NMR spectroscopic techniques to elucidate its chemical identity. Although similar, detailed analysis of the pH-dependent resonance-shift curves obtained for this resonance peak and ribose 5-phosphate revealed a significant variance in the curves, indicating that ribose-5-phosphate is not the major metabolite contributing to the 3.89 δ resonance signal. Subsequent examination of the pH-resonance shift characteristics of phosphoethanolamine by ^{31}P NMR (4.7 T and 11.75 T) revealed that it completely superimposes and co-migrates throughout the pH range of 10.0–4.0 with the 3.89 δ peak, when added to a tissue extract. Moreover, both phosphoethanolamine and the 3.89 δ resonance have the same apparent pK_a value. Overall, these findings provide convincing evidence supporting the interpretation that the 3.89 δ resonance signal in the porcine carotid artery arises primarily from phosphoethanolamine. The concentration of this metabolite, as reflected in the peak area of this resonance peak in the ^{31}P NMR spectral profile, was not altered by any experimental intervention used in this study. This observation is at variance with a previous study involving rabbit aorta [9] and suggest that further studies are needed to identify the cause of this apparent disparity.

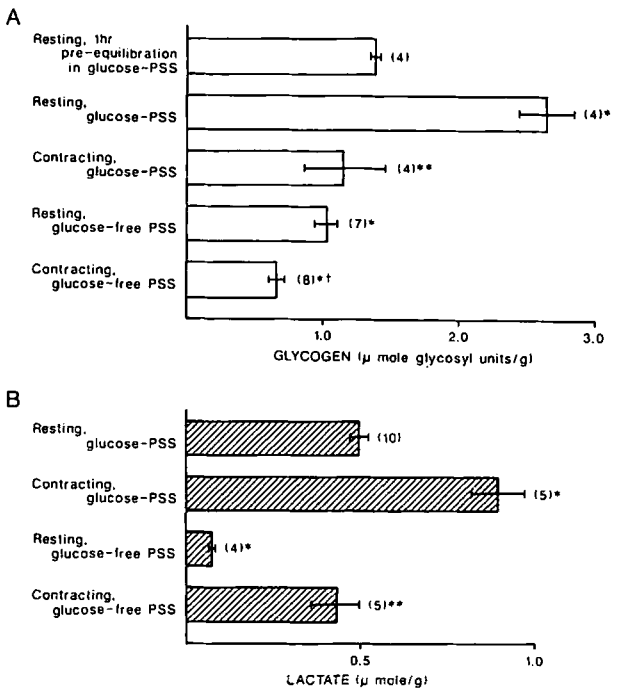

Fig. 3. Glycogen and lactate contents of porcine carotid artery in the presence and absence of glucose. (A) Glycogen content; number between parentheses is number of experiments; samples were prepared from single arteries from different animals per experiment. * $P < 0.001$ compared with "Resting, 1 h pre-equilibration in glucose PSS"; ** $P < 0.004$ compared with "Resting, glucose PSS"; *† $P < 0.05$ compared with "Resting, glucose-free PSS". (B) Lactate content; number between parentheses is number of experiments; samples were prepared from two arteries from different animals per experiment; * $P < 0.001$ compared with "Resting, glucose-PSS"; ** $P < 0.001$ compared with "Resting, glucose-free PSS".

The content of glycogen and lactate in carotid arteries in the presence or absence of medium glucose is depicted in Fig. 3. After 1 h of pre-equilibration with glucose PSS, the tissue glycogen content was 1.37 ± 0.05 μmol/g (mean ± S.E.M.). With 2 h additional incubation in glucose PSS smooth muscle continued to synthesize glycogen to a level of 2.84 ± 0.2 μmol/g. The lactate content was 0.49 ± 0.025 μmol/g. Subsequent contraction for 30 min produced a reduction in glycogen by 1.69 μmol/g (from 2.84 ± 0.2 to 1.15 ± 0.3 μmol/g) and a concomitant elevation of tissue lactate. Incubation of resting carotid strips for 2 h in glucose-free PSS after pre-equilibration for 1 h in glucose PSS resulted in a modest but significant decrease in glycogen to 1.04 ± 0.09 μmol/g, and a large decrease in tissue lactate content to 0.07 ± 0.01 μmol/g. This latter observation is consistent with previous reports showing that vascular smooth muscle in glucose-free media does not produce lactate [19]. By the end of 30 min contraction with KCl, the glycogen level decreased by 0.37 μmol/g to 0.67 ± 0.10 μmol/g, with no appreciable change in high-energy phosphate levels, when compared to resting muscles in glucose-free PSS (Table I). Lactate levels also rose markedly when compared to controls (Fig. 3B). Since media glucose was absent, the lactate must have been derived from an intracellular substrate source, possibly glycogen.

Effects of metabolic inhibitors

The effect of glycolytic pathway inhibitors (iodoacetate, 2-deoxyglucose) on metabolite levels of smooth muscle were investigated.

Effects of iodoacetate. In these experiments, following 1 h pre-equilibration with glucose-PSS, carotid strips were incubated in PSS containing 50 μM iodoacetate (IA) in the presence or absence of glucose. A comparison of spectra from the two treatment groups is seen in Fig. 4. Iodoacetate reacts with sulphydryl moieties of glyceraldehyde 3-phosphate dehydrogenase, thereby inactivating the enzyme and inhibiting glycolysis [20]. Consequently, phosphorylated glycolytic intermediates proximal to this step accumulate as extracellular glucose enters the cell and is phosphorylated by ATP [21], as seen in Fig. 4A. The resonance peaks at 3.96 δ and 4.06 δ demonstrable in these spectra, co-resonated with a fructose 1,6-diphosphate standard when it was added to the extract sample. Furthermore, analysis of these extract samples by enzymatic assay techniques [11] confirmed the presence of this intermediate in extracts of arteries treated with IA. This metabolite was undetectable in untreated arteries by the assay method used. The sugar phosphates so formed appeared to become a sink for the pool of intracellular free inorganic phosphate (P_i). (Note that phosphate buffer is omitted from the

PSS, and therefore intracellular P_i could not be replenished). Accordingly, tissue high-energy phosphate stores were markedly depleted as ATP was consumed by the transfer of the γ-phosphate to the sugar phosphate sink. Tissue ATP formation appeared to be restricted by the limited supply of free P_i (Table II). In contrast, when 50 μM IA was added in the absence of media glucose, there was only a slight accumulation of glycolytic intermediates in the ^{31}P-NMR spectrum (Fig. 4B) and the high-energy phosphate levels were diminished slightly by 16%, from 1.75 ± 0.06 μmol/g to 1.47 ± 0.08 μmol/g (Table II). Glycogenolysis continued to take place as evidenced by a decrease in glycogen from 1.37 ± 0.05 μmol/g at the beginning of incubation with IA, to 0.81 ± 0.12 μmol/g at the end of the 2 h incubation period. The tissue lactate content was 0.09 ± 0.01 μmol/g, which was not significantly different from the level detected in tissues incubated in glucose-free PSS in the absence of IA (0.07 ± 0.01 μmol/g). Thus glycogenolysis persisted, despite the apparent inhibition of the Embden-Meyerhof pathway. As was the case of glucose-free PSS, upon stimulation with 80 mM KCl muscles treated with 50 μM IA in the absence of glucose generated greater force (97 g ± 4 g, $n = 28$, vs. 91 g ± 4 g, $n = 40$, $P < 0.001$) and at a higher rate than muscles in glucose-PSS. Nevertheless, the high-energy phosphates decreased markedly and sugar phosphate intermediates accumulated, as detected in the ^{31}P spectrum. ADP was the major adenine nucleotide demonstrable in the spectrum (Fig. 4C). Recently, we demonstrated that contracted arterial strips treated with iodoacetate maintained full steady-state force, while the phosphorylation of the 20 kDa light chain of myosin decreased in concert with the ATP content [23], possibly reflecting the 'latch' state postulated by Murphy and coworkers [22]. In any case, the source of carbohydrate for formation of sugar phosphate intermediates was likely tissue glycogen, as evi-

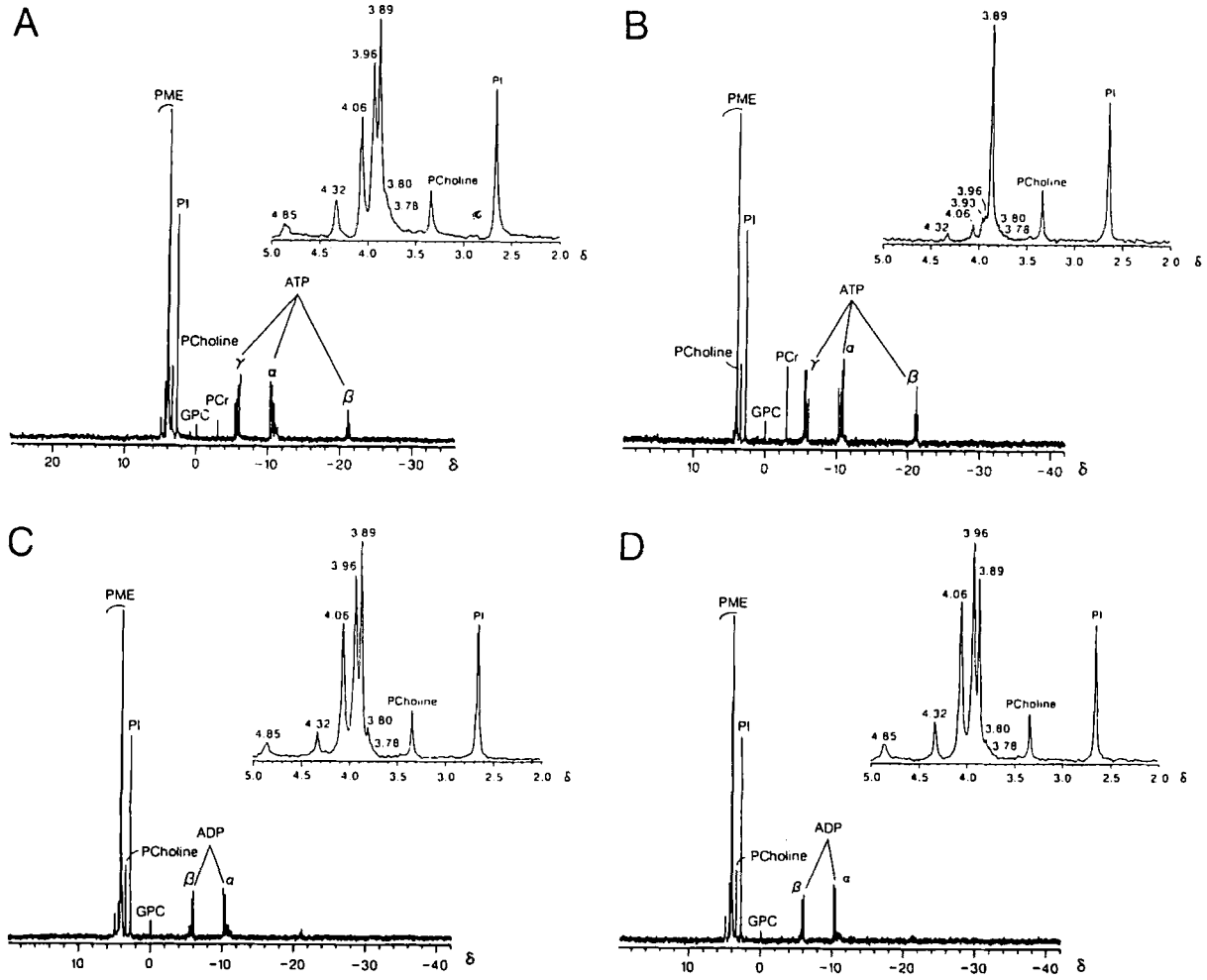

Fig. 4. Effect of iodoacetate on phosphate metabolites in porcine carotid artery. (A) Resting carotid strips incubated in glucose-PSS and 50 μM IA. Abbreviations for phosphate compounds same as those in Fig. 2. Expanded monoesterified phosphate region (PME) is seen at the right. Resonance at 4.06 δ and 3.96 δ definitively identified as fructose 1,6-diphosphate. Resonance at 3.89 δ corresponds to phosphoethanolamine. Remaining resonances unidentiifed. (B) Resting carotid strips incubated in glucose-free PSS with 50 μM IA. (C) Carotid strips incubated in glucose-free PSS with 50 μM IA and contracted with 80 mM KCl. (D) Resting carotid strips incubated in glucose-free PSS and 1 mM IA. All resonance peaks are scaled to the constant intensity of the phosphoethanolamine peak at 3.89 δ.

TABLE II

Metabolite contents of porcine carotid artery treated with iodoacetate (IA)

Values are means ± S.E.M. Phosphomonoesters include phosphorylated sugar intermediates of glycolysis. *n*, number of experiments using two arteries from different animals per experiment. *n* +, number of experiments using single arteries from different animals.

Condition	ATP + PCr (μmol/g)	Phosphomonoesters (% total phosphorus)	*n*	Glycogen (μmol glycosyl units/g)	*n* +	Lactate (μmol/g)	*n*
Resting 1 h pre-equilibration in glucose PSS				1.37 ± 0.05	4		
Resting, glucose-free PSS	1.75 ± 0.06	21.5 ± 0.7	5	1.04 ± 0.09 *	7	0.07 ± 0.01	4
Resting, glucose-free PSS + 50 μM IA	1.47 ± 0.08 +	26.9 ± 2.1 +	4	0.81 ± 0.12 *	6	0.09 ± 0.01	4
Resting, glucose-free PSS + 1 mM IA	0.07 ± 0.02 **	57.6 ± 3.1 **	3	0.71 ± 0.14 *	4	0.29 ± 0.03 **	4
Contracting, glucose-free PSS + 50 μM IA	0.12 ± 0.02 **	57.7 ± 1.9 **	5	0.40 ± 0.08 *	6	0.31 ± 0.01 **	6
Resting, glucose PSS, + 50 μM IA	0.62 ± 0.11 **	50.9 ± 3.3 **	4	2.18 ± 0.14	4	0.29 ± 0.02 **	8
Resting, glucose PSS + 1 mM IA	0.17 ± 0.11 **	56.8 ± 8.1 **	4	1.40 ± 0.07	4	0.36 ± 0.04 **	4

* $P < 0.001$ compared with Resting, 1 h pre-equilibration in glucose-PSS.
** $P < 0.001$.
\+ $P < 0.004$, compared with Resting, glucose-free PSS.

denced by the decrease in tissue glycogen stores to 0.40 ± 0.08 μmol/g. It is noteworthy that whereas the glycogen breakdown of approx. 0.41 μmol/g in contracting smooth muscle was accompanied by accumulation of sugar phosphates (Fig. 4C and Table II), the 0.56 μmol/g of glycogen degradation in resting muscle was not accompanied by appreciable accumulation of intermediates.

Although IA at a concentration of 50 μM had little effect on glycogen metabolism in resting smooth muscle in glucose-free PSS, a concentration of 1 mM IA produced a large decrease in tissue ATP and PCr levels, and the accumulation of phosphorylated glycolytic intermediates (Fig. 4D). As before, the source of the sugar phosphate intermediates was glycogen, as indicated by the decrease in glycogen to 0.71 ± 14 μmol/g (Table II).

The extent of the inhibition of aerobic glycolysis by IA was examined by incubating resting strips in glucose PSS containing either 50 μM or 1 mM IA. Although lactate content was reduced with 50 μM IA, appreciable lactate production was still evident (Table II). Contracting carotid strips in glucose-free PSS treated with 50 μM IA also displayed increased lactate levels, as compared to resting, unstimulated arteries. Treatment of resting strips in glucose PSS with a greater concentration of IA (1 mM) did not, however, result in any further diminution in lactate content, suggesting that a cellular compartment involved in lactate production may exist, which was impervious to IA at either 50 μM or 1 mM. Additional evidence for such a compartment was provided by the presence of tissue lactate in contracted carotid strips incubated in glucose-free PSS and 50 μM IA, and in resting strips incubated in glucose-free PSS containing 1 mM IA. Under these conditions, some glycosyl units from glycogen apparently entered a compartment in which glycolysis was inhibited by IA and accumulated as sugar phosphates (Fig. 4C and D), while other glycosyl units entered a compartment unaffected by IA and were subsequently metabolized to lactate.

Effects of 2-deoxyglucose (2-DG). After 1 h pre-equilibration with glucose PSS carotid strips were incubated in glucose-free PSS with 5 mM 2-DG for $1\frac{1}{2}$ h and then contracted with 80 mM KCl, while resting muscles were left in muscle chambers for the same period. 2-Deoxyglucose enters the cell and is phosphorylated by ATP to form 2-deoxyglucose 6-phosphate, which is not metabolized further. This compoud is a competitive inhibitor of glucose 6-phosphate for the reaction with phosphoglucose isomerase, thereby inhibiting glycolysis [24]. A representative ^{31}P-NMR spectrum of tissue extracts of 2-DG treated smooth muscle is presented in Fig. 5. The resonance peak seen at 4.62 δ co-resonated with a glucose 6-phosphate standard and enzymatic assay of glucose 6-phosphate [11] confirmed the presence of this metabolite in extracts of 2-DG-treated arteries. As with the case of IA, phosphorylated sugar intermediates proximal to the point of inhibition accumulated, and high-energy phosphate levels decreased as ATP was consumed. The phosphate metabolites so formed acted as a sink for the limited supply of inorganic phosphate and ATP stores were not maintained.

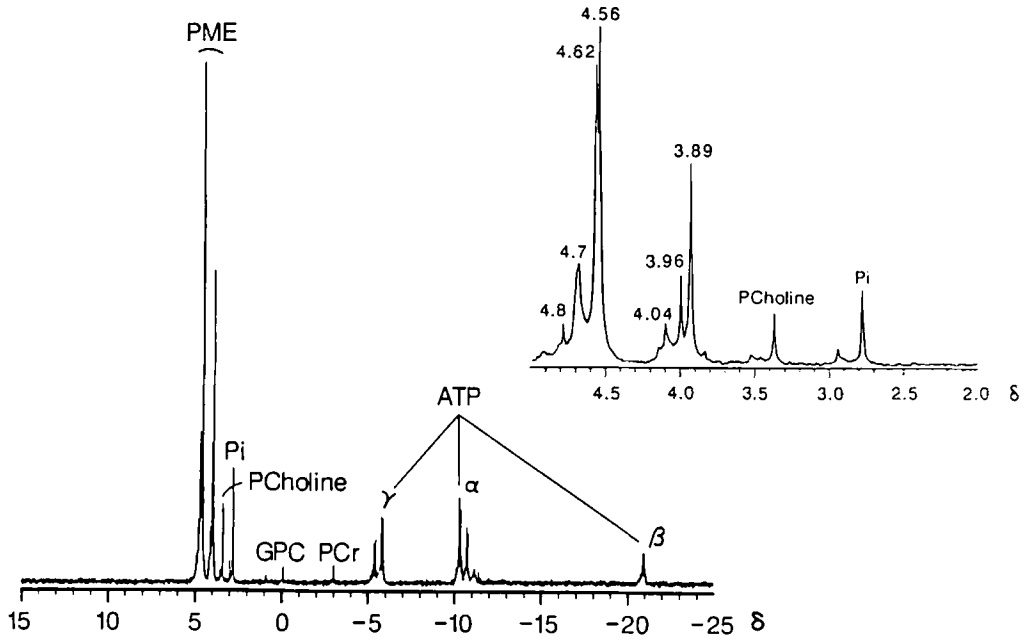

Fig. 5. ^{31}P-nuclear magnetic resonance spectrum of perchloric acid extract of porcine carotid artery treated with 2-deoxyglucose. Expanded phosphomonoester region (PME) is seen at the right. Abbreviations are given in legend of Fig. 2. Compounds with chemical shifts at 4.62 δ and 4.56 δ identified as glucose 6-phosphate and 2-deoxyglucose 6-phosphate, respectively. Resonance at 3.89 δ corresponds to phosphoethanolamine. Remaining resonances are unidentified. Resonance peaks are scaled to the 3.89 δ, phosphoethanolamine peak.

Since the carotid artery strips were incubated in glucose-free PSS, the most probable carbohydrate source for the sugar phosphates that accumulated was glycogen. Tissue glycogen levels in these arteries decreased from 1.37 ± 0.05 μmol/g in resting strips to 1.0 ± 0.10 μmol/g at the end of the 2 h incubation with 2-DG (Table III).

The maximal tension generated in response to 80 mM KCl by carotid artery strips treated with 2-DG was the same as that produced by strips incubated in normal glucose-PSS (89 ± 4 g vs. 90 ± 4 g, $n = 12$). The level of tension also remained constant throughout the 30 min exposure to KCl. There was no decrease in ATP and PCr levels in contracting strips when compared to resting strips, indicating that tissue high-energy phosphates were replenished at a rate comparable to the rate of ATP utilization during contraction. The endogenous substrate utilized by these strips to maintain cellular ATP stores could not have been glycogen, since tissue levels were comparable to those of resting strips (0.97 ± 0.11 μmol/g vs. 1.0 ± 0.10 μmol/g) and lactate levels were unchanged (Table III). These results strongly suggest that an endogenous, non-carbohydrate substrate supported the energy requirements of contraction under these experimental conditions.

Discussion

Disturbances in carbohydrate metabolism were induced experimentally in the present study to assess the resultant effects on the energy status and contractile activity of vascular smooth muscle. Specifically, glucose deprivation and inhibition of carbohydrate metabolism were utilized as experimental paradigms to assess the

TABLE III

Metabolite contents of porcine carotid treated with 2-deoxyglucose (2-DG)

Values are means ± S.E.M. Phosphomonoesters include phosphorylated sugar intermediates and 2-deoxyglucose 6-phosphate. n, number of experiments using two arteries from different animals per experiment; n^* = number of experiments using single arteries from different animals.

Condition	ATP + PCr (μmol/g)	Phosphomonoesters (% total phosphorus)	n	Glycogen (μmol glycosyl units/g)	n^*	Lactate (μmol/g)	n
Resting, glucose-free PSS +5 mM 2-DG	0.41 ± 0.3	58.4 ± 0.7	5	1.00 ± 0.10	4	0.08 ± 0.02	4
Contracting, glucose-free PSS +5 mM 2-DG	0.38 ± 0.02	57.6 ± 0.7	5	0.97 ± 0.11	4	0.09 ± 0.01	4

respective roles of extracellular glucose, endogenous glycogen and endogenous non-carbohydrate substrates in the energy metabolism and contractile function of vascular smooth muscle.

Partitioning of carbohydrate metabolism

Elimination of glucose from the supporting medium for 2 h prior to analysis resulted in a small but significant decrease in tissue glycogen content of resting carotid strips (Fig. 3). During treatment with IA in glucose-free PSS, glycogenolysis proceeded as before, and there was a slight decrease of the high-energy phosphates. ^{31}P-NMR spectra from carotid strips incubated in glucose free-PSS with 50 μMIA did not show the accumulation of phosphorylated glycolytic intermediates observed when extracellular glucose was included in the supporting medium (compare Fig. 4A with Fig. 4B). These results are consistent with previous results reported by Lynch and Paul [25,26], which have provided evidence suggesting that there are at least two functionally independent Embden-Meyerhof pathways within smooth muscle. One pathway subserves aerobic catabolism of extracellular glucose to lactate, while the other one purportedly is involved in the oxidative metabolism of glycosyl units derived from endogenous glycogen. The observation that glycogenolysis takes place without appreciable accumulation of sugar phosphates suggests that the glycosyl units so derived are inaccessible to the glycolytic enzymes inhibited by IA, and are metabolized instead by a separate set of glycolytic enzymes. The glyceraldehyde 3-phosphate dehydrogenase involved in glycogenolysis was apparently unaffected by IA at a concentration of 50 μM under the experimental conditions of the study. Our results also suggest that IA at a concentration of 50 μM inhibits at least a portion of the glyceraldehyde 3-phosphate dehydrogenase of the Embden-Myerhof pathway(s) that catabolize extracellular glucose to lactate. The presence of lactate in carotid artery strips in glucose PSS persisted, despite increasing IA to 1 mM, suggesting that a component of lactate production was not inhibitable by IA at the concentrations tested. ^{31}P-NMR spectral analysis of these vessels revealed a significant accumulation of phosphorylated sugar intermediates in extracts of strips treated with this IA concentration (Fig. 4D and Table II). The precursors of these intermediates could only have been derived from endogenous glycogen. The results of these series of experiments suggest that the glyceraldehyde-3-phosphate dehydrogenases of separate Embden-Meyerhof pathways may be titrated with increasing concentrations of IA. They also support the concept of a functional compartmentation of carbohydrate metabolism. That the division of the compartments of carbohydrate metabolism is not absolute is suggested by results of experiments in which carotid artery strips were incubated in glucose-free PSS and 50 μM IA and then contracted with KCl. Phosphorylated glycolytic intermediates appeared in the ^{31}P-NMR spectra from these vessels (Fig. 4C) and the lactate content increased (Table II), indicating that glycosyl units from glycogen 'crossed-over' to the compartment(s) containing the inactivated glyceraldehyde 3-phosphate dehydrogenases. Evidence for glycosyl unit cross-over between compartments in porcine carotid artery has been presented previously [26].

Partitioning of high-energy phosphate pools

The production of lactate from medium glucose has been linked to the activity of ion-transport pumps in vascular smooth muscle [1]. Ouabain, an inhibitor of Na,K-ATPase has been shown to stimulate isometric tension but inhibit lactate production [4]. Presumably, the activity of a matrix of glycolytic enzymes associated with the sarcolemma produces the ATP needed to drive the ion pumps [27]. The observation that the initial rate and magnitude of isometric tension development in strips contracted in glucose-free PSS was greater than that in normal glucose PSS is consistent with the hypothesis that with diminished activity of the Na,K-ATPase, Ca^{2+} availability to the contractile elements becomes enhanced, thereby increasing the rate of tension development. This interpretation of the results requires the existence of partitioned high-energy phosphate pools – one pool is derived from oxidative metabolism and another from aerobic glycolysis. Accordingly, this hypothesis would predict that there would be a fall in high-energy phosphate level upon withdrawal of medium glucose. Indeed, the present studies have detected a 10% decrease in the steady-state high-energy phosphate level in resting strips incubated in glucose-free PSS, as compared to strips in glucose PSS (Table I). Furthermore, whereas there was a decrease in high-energy phosphates upon contraction of strips in glucose PSS, there was no further decrease in ATP and PCr with contraction of strips in glucose-free PSS, when compared with resting strips in glucose-free PSS (Table I). Thus, we postulate that the decrement in high-energy phosphates in strips in glucose PSS during contraction is attributable to the consumption of ATP in the compartment subserving ion pumping, which is ordinarily fueled by the aerobic glycolysis of extracellular glucose. Although lactate production increased with contraction (Fig. 3), presumably from augmented metabolism of extracellular glucose in this compartment [26], the yield of high-energy phosphate from aerobic glycolysis in this compartment may be insufficient to keep pace with consumption. In contrast, in strips incubated with glucose there was no further change in tissue high-energy phosphate levels during contraction. These findings support the concept of partitioned high-energy phosphate pools.

Endogenous non-carbohydrate substrate as an energy source

The extent of glycogen breakdown measured in resting smooth muscle incubated in glucose-free media (Fig. 3A) appeared insufficient to account for estimated ATP turnover [28]. An endogenous substrate other than glycogen may have been metabolized to maintain tissue high-energy phosphate levels under these conditions. Evidence suggesting that vascular smooth muscle may utilize non-carbohydrate substrates is consistent with previous findings by Odessey and Chace [7].

Contraction of porcine carotid strips in glucose PSS was accompanied by a marked increase in lactate production and by glycogen breakdown (Fig. 3). The extent of glycogen breakdown that occurred during contraction in media containing glucose was more than sufficient to account for all of the estimated high-energy phosphate generated by oxidative metabolism [28] during the 30 min of K^+ depolarization, indicating that endogenous glycogen was a primary substrate supporting contraction. Under glucose-free conditions, however, this was not the case. Contraction of muscles in glucose-free PSS was characterized by greater isometric tenion but significantly less glycogenolysis than that which occurred in the presence of glucose. Thus, glycogen breakdown in contracting carotid artery in glucose-free conditions could not account for all of the estimated ATP turnover [28]. As with resting strips in glucose-free media, the results indicate that another endogenous substrate in addition to glycogen served as an energy source during contraction.

Additional evidence suggesting the involvement of an endogenous substrate in energy metabolism during contraction was obtained from the results of experiments iwth 2-deoxyglucose. Previous experiments by Shibata and Briggs [29] showed that 2-DG did not have an inhibitory effect on contractile responses of rabbit aorta to a variety of agonists. These results provided presumptive evidence suggesting that some other endogenous energy source supported contraction when glycolysis was inhibited. They did not, however, measure parameters of the energy state of the muscle. In our study with carotid artery, there was no difference in the maximal steady-state tension developed in response to KCl in strips treated with 2-DG when compared to artery strips incubated in glucose PSS or glucose-free PSS [23]. The inhibition of glycolysis under these conditions is suggested by the inhibition of lactate production. An endogenous non-carbohydrate energy source apparently supplied metabolic energy during contraction, since ATP and PCr were not decreased when compared to resting muscles. The energy source during contraction could not have been derived from glycogen, because there was no decrease in the glycogen level (Table III).

This study has demonstrated that the contractile process of vascular smooth muscle may utilize glycogen and another endogenous non-carbohydrate energy source. A decline in the high-energy phosphate pool was not demonstrable when the non-carbohydrate endogenous source was utilized together with glycogen, as was the case under glucose-free conditions during contraction. The regulatory mechanisms operating in smooth muscle that determine which endogenous fuel source and which metabolic pathway is engaged under various physiological and substrate conditions appear complex and await further elucidation.

Acknowledgement

This work was supported by a grant from the American Heart Association of Metropolitan Chicago.

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