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Effects of high energy phosphates and L-arginine on the electrical parameters of ischemic-reperfused rat skeletal muscle fibers

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Abstract

In skeletal muscle, 4 h of ischemia followed by 30 min of reperfusion depolarizes the fibers, markedly increases the Cl^- and glibenclamide-sensitive K^+ conductances and reduces the excitability of the fibers. The ischemia-reperfusion also significantly decreases the ATP content of the muscles. In the present work, the electrical parameters of reperfused extensor digitorum longus muscle of rats were measured in vitro at 30°C, by a computerized two-intracellular microelectrode technique, before and after in vivo pretreatment with equimolar doses of phosphocreatine disodium salt tetrahydrate, phosphocreatine di-L-arginine salt and L-arginine hydrochloride. In the same experimental situations the ATP content of the muscles was also measured. Both phosphocreatine salts prevented the increase of membrane ion conductance due to muscle reperfusion by preloading the muscle fibers with extra ATP. Phosphocreatine disodium salt also prevented the depolarization and restored the normal excitability of the reperfused fibers. In contrast, phosphocreatine di-L-arginine salt did not restore the resting potential nor the excitability of the fibers, but it decreased the amplitude of the action potential by reducing the overshoot. The pretreatment with L-arginine also failed to protect the electrical parameters of the fibers from the ischemic-reperfusion insult. Furthermore, the L-amino acid produced a more pronounced reduction of the excitability of the fibers by increasing the threshold current needed to elicit an action potential and reducing its overshoot. The in vitro application of L-arginine to the muscle also reduced the overshoot of the action potential, suggesting a direct interaction of the L-amino acid with Na^+ channels.

Keywords: Ischemia; Reperfusion; Skeletal muscle; Phosphocreatine disodium salt; Phosphocreatine di-L-arginine salt; L-Arginine; Current clamp; Electrophysiology

1. Introduction

Skeletal muscle, which is generally considered to have great tolerance to sustained ischemia, may instead develop important abnormalities after a brief post-ischemic reflow period (Smith et al., 1989; Yokota et al., 1989; Walker, 1991). Several authors have shown that short-term reperfusion may cause alterations of the metabolic state of the cells such as a decrease of phosphocreatine and ATP content, lowering of intracellular pH as well as morphological and functional derangement of the muscle fibers (Walker, 1991; Eckert and Schnackerz, 1991; Mars and Gregory, 1991). In agreement with these observations, we have recently found that, in rat extensor digitorum longus muscle, 4 h of ischemia followed by 30 min of reperfusion lowered the ATP content of the muscle, depolarized the fibers, markedly increased the resting chloride and glibenclamide-sensitive potassium conductance, and reduced the fiber excitability (Tricarico and Conte Camerino, 1994a). On the basis of these findings we have hypothesized that the energy depletion of the cell occurring during reperfusion may cause a loss of Na^+/K^+ ATPase activity and the opening of ATP-sensitive K^+ channels (K_{ATP}), whose activity is strictly dependent on the intracellular ATP content (Tricarico and Conte Camerino, 1994a). Additional support for the role of ATP in the modulation of the activity of K_{ATP} channels and Na^+/K^+ ATPase pump of the sarcolemma is that these membrane transporters seem to be more sensitive to a decrease in ATP concentra-

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tion than other cytosolic enzymes (Jones, 1986; Niki et al., 1989). One of the hypotheses to explain this phenomenon involves the existence of an ATP gradient into the cells that provides a lower level of ATP at the inner side of the sarcolemma in the vicinity of ion channel and transporter proteins than in the rest of the cytosol (Jones, 1986; Niki et al., 1989). Given the importance of the role of ATP in controlling the ion channel function of the sarcolemma, we tested the protective effects of a high-energy phosphate compound, such as phosphocreatine (as phosphocreatine disodium salt), in preventing the alteration of ion membrane conductance and excitability characteristics of skeletal muscle fibers due to reperfusion. Indeed, it is well established that the reaction mediated by creatine kinase plays an important role in muscle energy and metabolism, buffering the changes of ATP concentration and providing inorganic phosphate at the onset of muscle contraction, which are required for activation of muscle glycogenolysis and glycolysis (Meyer et al., 1986; Meyer, 1989; Godt and Nosek, 1989). In addition, phosphocreatine and creatine are important in the diffusive transport of high-energy phosphate from the mitochondria to the contractile proteins (Meyer et al., 1984).

Moreover a beneficial role of L-arginine has been recently proposed in preventing reperfusion injury of the heart (Masini et al., 1991). In particular, it has been shown that the perfusion of isolated ischemic-reperfused guinea pig heart with L-arginine and nitric oxide (NO) donors significantly reduces the abnormal release of histamine and the Ca^{2+} overload due to ischemia and reperfusion (Masini et al., 1991). It is also known that the inhibition of NO production in different tissues is strictly related to various microvascular dysfunctions and to reperfusion injury of various organs (Kubes and Granger, 1992; Schulz and Triggle, 1994). Indeed, numerous evidence indicates that inhibition of the release of NO from endothelial cells favors the adherence of leukocytes to postcapillary venules, which in turn leads to various pathophysiological effects such as production of reactive oxygen metabolites, increase of protein flux out of the vasculature, and edema formation (Kubes and Granger, 1992; Schulz and Triggle, 1994). Similar abnormalities have been found in ischemic-reperfused skeletal muscle, in which it has been proposed that the leukocytes are the major source of free radical generation through NADPH oxidase enzyme (Smith et al., 1989; Yokota et al., 1989). On the basis of these considerations, we performed experiments to evaluate the protective effects of phosphocreatine di-L-arginine salt and L-arginine on the ion membrane conductances and excitability characteristics of reperfused rat skeletal muscle. We have also monitored the changes in the ATP content of reperfused muscles dissected from rats pretreated or not pretreated with the phosphocreatine salts.

2. Materials and methods

2.1. Surgical preparations and experimental conditions

The animals were operated in accordance with the 'Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Sciences. Adult male Wistar rats (260–300 g) were anesthetized with an intraperitoneal injection of pentobarbital sodium salt (50 mg/kg). The animals were under deep anesthesia during the ischemia and reperfusion period. After muscle dissection, they were rapidly killed by an overdose of pentobarbital. Before the ischemia, 400 units of heparin were injected intravenously. A model of severe muscle ischemia was produced by placing four temporary ligatures of the right-hand common artery and its branches feeding the extensor digitorum longus muscle (Tricarico and Conte Camerino, 1994a). During and after surgery, the animals were kept at a constant temperature of 28–30°C and were allowed to breath room air.

After 4 h of ischemia, reflow of the right-hand extensor digitorum longus muscle was obtained by declamping the temporary ligatures for 30 min (Tricarico and Conte Camerino, 1994a).

The experimental animals were divided into five groups. The first group of rats (group I) underwent 4 h of ischemia followed by 30 min of reperfusion. The other rats were pretreated with phosphocreatine disodium salt (group II), phosphocreatine di-L-arginine salt (group III) and L-arginine (group IV). Group V consisted of control rats that did not undergo any surgical intervention.

2.2. Electrophysiological recordings

The reperfused right-hand muscles from untreated (group I) and treated rats (groups II, III and IV) and their left-hand contralateral limbs (contralateral controls) as well as the corresponding muscles from unoperated animals (controls, group V) were dissected from the bones, mounted in a bath at 30°C, and perfused with physiological solution gassed with 95% O_2 and 5% CO_2. The contralateral control muscles from treated and untreated rats were dissected from the bones about 2 h after the surgical intervention. The passive cable properties of the fibers were measured by using a standard two-intracellular microelectrode current clamp technique. In brief, a constant hyperpolarizing current pulse was injected into a muscle fiber and the resulting voltage deflection was observed by a second microelectrode inserted at two different dis-

tances (50 μm and about 2 mm) from the current electrode (Bryant, 1969). The current pulse generation and the acquisition of the voltage records were done under computer control. The voltage records were filtered with an 8-pole Bessel filter (Frequency Devices, model 902LPF) set to a cutoff frequency (−3 dB) one-half of the sampling frequency. The acquisition rate was set as a function of the time constant of the fibers, being 11 kHz in normal physiological solution (approx. 2 ms time constant). 2048 data points were acquired per voltage record. Other detailed information about the software and the data acquisition system has been reported elsewhere (Bryant and Conte Camerino, 1991).

The basic cable model parameters we measure are the space constant (λ), the fiber input resistance (Rin), and the time constant (τ). These constants are experimentally determined as follows: λ is the logarithmic decay of the electrotonic potential, with distance from the current electrode; Rin is the steady state electrotonic potential at the current electrode divided by the current intensity; and τ is taken to be the 84% rise time of the electrotonic potential which is theoretically equal to the membrane time constant. From these quantities are calculated the membrane resistance and the membrane capacitance. The fiber diameter is calculated from the measured value of λ and Rin, assuming a constant value for the myoplasmic resistivity (R_i) of 125 · Ω cm (Boyd and Martin, 1959; Bryant, 1969). The value of R_i has previously been determined in experiments where diameter was accurately measured with histological methods. R_i is then adjusted for temperature using Q_{10} of 1.34. The R_i of the single fiber may change following reperfusion of the muscle. However, because the diameter is related to the square root of R_i (Boyd and Martin, 1959; Bryant, 1969), considerable changes in R_i will not significantly affect the diameter and therefore the membrane resistance. The reciprocal of the membrane resistance was assumed to be the total membrane conductance (G_m).

The excitability was determined by observing the intracellular potential response, recorded by a 'voltage' microelectrode, to a square constant current pulse (100 ms duration) delivered by a 'current' microelectrode inserted within 50 μm of the voltage electrode. In each fiber, the membrane potential was set by a steady holding current to −80 mV before the depolarizing pulse was passed (Tricarico and Conte Camerino, 1994a).

2.3. Physiological solution and drug tested

The composition of the normal physiological solution used was (mM): NaCl 148; KCl 4.5; $CaCl_2$ 2; $NaHCO_3$ 12, NaH_2PO_4 0.44, glucose 5.55; pH 7.2. The phosphocreatine salt solutions were freshly prepared by dissolving the chemicals in NaCl solution (0.9 p/v) and phosphate buffer (pH = 7.2) to obtain stock equimolar solutions of 30 mg/ml, 51 mg/ml and 19.5 mg/ml for phosphocreatine disodium tetrahydrate (from Fluka), phosphocreatine di-L-arginine salt (from Nuovo Consorzio Sanitario Nazionale, Rome; Italian Patent No. 1232152, 25.01.1992) and L-arginine hydrochloride (from Fluka), respectively. The drug solutions were injected intramuscularly into the gluteus superficialis muscle of the right-hand contralateral limb to the operated one, in equimolar doses of 40 mg/kg, 68 mg/kg and 26 mg/kg for phosphocreatine disodium salt, phosphocreatine di-L-arginine salt and L-arginine hydrochloride, respectively. The concentration of phosphocreatine disodium salt was selected according to the dose of the drug used as cardioprotective agent. The doses of the other drugs tested, phosphocreatine di-L-arginine salt and L-arginine, were then adjusted to the phosphocreatine disodium salt concentration. These doses were injected about 1 h before ischemia to preload the muscle with extra creatine. It is known that phosphocreatine is rapidly converted into creatine by plasma creatine kinase enzymes and then transported into the extensor digitorum longus muscle of rat against a concentration gradient by energy-dependent transport (Fitch and Shields, 1966; Shields et al., 1975; Chevli and Fitch, 1979; Shoubridge and Radda, 1984; Meyer et al., 1986).

2.4. Determinations of tissue ATP

The reperfused and the contralateral extensor digitorum longus muscles from untreated (group I) and treated animals (groups II, III and IV) and the control muscles from unoperated animals (group V) were exposed and freeze-clamped in situ by aluminum tongs precooled in liquid N_2 (Ruff and Weissman, 1991). The frozen muscles were then dissected from the bones, wrapped in aluminum foil precooled at −80°C and stored in liquid N_2 until chemical analysis. The muscles were pulverized in a mortar filled with liquid N_2 and extracted in cold $HClO_4$ 0.4 N (10 μl of $HClO_4$/mg of tissue) and centrifuged at 2000 × g at 0°C for 10 min. Supernatants were removed and neutralized with 0.4 N NaOH, and then analyzed for ATP content. The ATP content was measured using a spectrophotometric method (Lamprech and Trautschold, 1974). Other detailed information about the ATP assay method has been reported elsewhere (Tricarico and Conte Camerino, 1994a).

2.5. Statistical analysis

The values were expressed as means ± S.E. The effects between preparations: ischemia-reperfusion, contralateral controls, and left-hand (sx) and right-hand

(dx) controls from unoperated rats, and the difference in response between rats for each experimental group, were evaluated by 2-way analysis of variance (ANOVA) (Ludbrook, 1994). Significant differences between individual pairs of means were determined by Student's t-test.

3. Results

3.1. Effects of in vivo pretreatment with phosphocreatine disodium salt, phosphocreatine di-L-arginine salt and L-arginine on ischemic-reperfused extensor digitorum longus muscle fibers

Cable parameters and ion conductance

As previously reported (Tricarico and Conte Camerino, 1994a), a postischemic reflow period of 30 min significantly increased the total membrane ion conductance of the fibers as compared to that of the contralateral control muscles (Fig. 1). The variance ratio, F, between preparations was 113.14 ($df = 1/4$, $P < 0.001$), while it was 0.48 between rats ($df = 4/4$, n.s.), suggesting that the main source of variation in the group I was due to ischemia-reperfusion. The short-term reperfusion did not change the diameter and the capacitance of the fibers, these being 30 ± 1 μm and 3.9 ± 1 $\mu F/cm^2$ ($n = 46$), respectively, in contralateral control muscles, and 32 ± 2 μm and 3.8 ± 3 $\mu F/cm^2$ ($n = 63$) after ischemia-reperfusion. The pretreatment with phosphocreatine disodium salt prevented the abnormal increase of membrane ion conductance (Fig. 1). This observation was statistically supported by the 2-way ANOVA test (Fig. 1). No significant alterations of the diameter and capacitance of the fibers were observed, these being 29 ± 2 μm and 3.7 ± 1 $\mu F/cm^2$ ($n = 20$), respectively, in contralateral control muscles, and 33 ± 2 μm and 3.6 ± 4 $\mu F/cm^2$ ($n = 21$) in ischemic-reperfused muscle after pretreatment of the rats with the phosphocreatine disodium salt.

As well as pretreatment with its disodium salt homolog, pretreatment with phosphocreatine di-L-arginine salt fully restored the total membrane ionic conductance of the fibers (Fig. 1). No changes were observed in the diameter and capacitance of the fibers, these being 31 ± 3 μm and 3.9 ± 3 $\mu F/cm^2$ ($n = 13$), respectively, in contralateral control muscles, and 33 ± 2 μm and 3.6 ± 4 $\mu F/cm^2$ ($n = 25$) in ischemic-reperfused muscle after pretreatment with phosphocreatine di-L-arginine salt. In contrast, the pretreatment of the rats with L-arginine did not show any beneficial effect on the electrical parameters of ischemic-reperfused muscle fibers, leaving unaltered the membrane ionic conductance (Fig. 1). The diameter and capacitance of

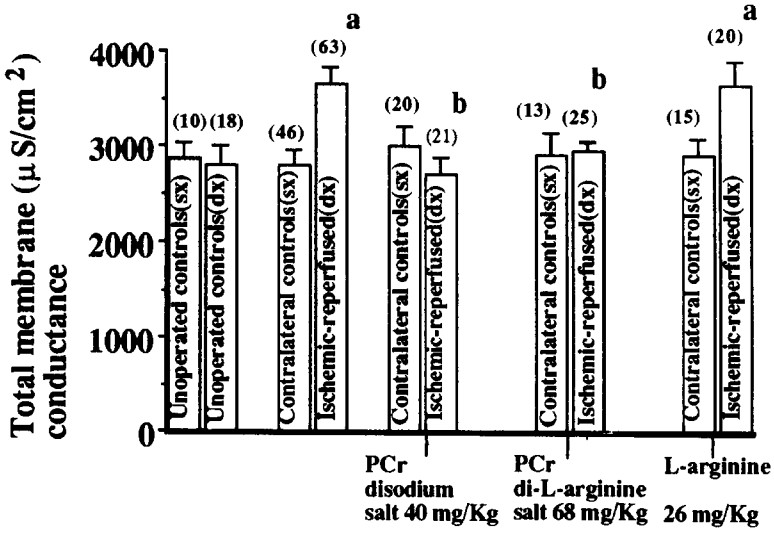

Fig. 1. Each group of bars shows the total membrane conductance (G_m) values from different muscle preparations: the right-hand (dx) extensor digitorum longus muscle fibers that underwent 4 h of ischemia followed by 30 min of reperfusion (ischemic-reperfused), and the left-hand (sx) muscles contralateral to the operated one (contralateral controls), with no treatment or after pretreatment with drugs indicated below the bars. G_m values from sx and dx muscle fibers of unoperated rats (controls) are also shown. The values are expressed as means ± S.E. The numbers in brackets are the numbers of the fibers sampled. The variance ratio (F) was calculated for each experimental condition by 2-way ANOVA. The F between ischemic-reperfused and contralateral control fibers from untreated rats was 113.14 ($df = 1/4$, $P < 0.001$), and between rats 0.48 ($df = 4/4$, n.s.). After in vivo pretreatment with phosphocreatine disodium salt, phosphocreatine di-L-arginine salt, and L-arginine the F values between preparations were 3.52 ($df = 1/3$, n.s.), 9.78 ($df = 1/3$, n.s.), and 17.83 ($df = 1/3$, $P < 0.025$); between rats 0.493 ($df = 3/3$, n.s.), 4.36 ($df = 3/3$, n.s.), and 0.74 ($df = 3/3$, n.s.), respectively. A comparison of the G_m values between control dx and control sx muscles from unoperated rats shows F values of 0.8 ($df = 1/2$, n.s.) between muscle preparations, and 0.3 ($df = 1/2$, n.s.) between rats. The comparison between individual pairs of means was made with Student's t-test. Means significantly different from unoperated controls are indicated by [a] ($P < 0.005$ or less) and values significantly different from untreated ischemic-reperfused muscles are indicated by [b] ($P < 0.005$ or less).

both the contralateral controls and ischemic-reperfused muscle fibers were unaltered by the pretreatment with the L-amino acid, these being 29 ± 4 μm and 3.8 ± 3 $\mu F/cm^2$ ($n = 15$), respectively, in contralateral control muscles, and 36 ± 5 μm and 3.8 ± 2 $\mu F/cm^2$ ($n = 20$) in ischemic-reperfused muscle after pretreatment of the rats with L-arginine.

Action potential and excitability characteristics

In agreement with earlier results (Tricarico and Conte Camerino, 1994a), reperfusion dramatically depolarized the fibers and increased the threshold current needed to elicit an action potential (Table 1). These effects always led to a reduction of the fiber excitability. The pretreatment of the ischemic-reperfused muscle fibers with phosphocreatine disodium salt prevented both the membrane depolarization and the increase of threshold current, thus restoring the excitability of the fibers (Table 1). However, the duration of the action potential of the contralateral controls and ischemic-reperfused muscle fibers of the rats treated with phosphocreatine disodium salt was abnormally prolonged as compared to that of the unoperated control group (Table 1).

In contrast, the pretreatment of the rats with phosphocreatine di-L-arginine salt did not restore the resting potential nor the threshold current of the ischemic-reperfused fibers. However, as with its disodium salt homolog, phosphocreatine di-L-arginine prolonged the duration of the action potential (Table 1). Furthermore, phosphocreatine di-L-arginine salt produced a significant decrease of the amplitude of the action potential, reducing the overshoot of both contralateral controls and ischemic-reperfused fibers (Table 1).

Also the pretreatment of the rats with L-arginine did not produce any beneficial effects on the resting potential or on the threshold and amplitude of the action potential of the ischemic-reperfused fibers but rather it caused a more pronounced alteration of these parameters (Table 1). Indeed, the pretreatment of the rats with the L-amino acid increased the threshold current

Table 1
Effects of pretreatment with phosphocreatine disodium salt, phosphocreatine di-L-arginine salt and L-arginine on the resting potential and excitability characteristics of ischemic-reperfused extensor digitorum longus muscle of rats

Experimental condition	No. of muscles/ No. of fibers	RP (mV)	I_{Th} (nA/cm^2)	Dur (ms)	Os (mV)	Repetitive activity
Unoperated controls (sx)	3/11	-71 ± 3	70 ± 9	6.9 ± 0.7	9 ± 2	4 ± 0.6
Unoperated controls (dx)	3/13	-67 ± 4	68 ± 7	6.4 ± 0.8	10 ± 2	5 ± 0.7
Contralateral controls (sx)	4/19	-68 ± 0.9	65 ± 3	6.3 ± 0.4	12 ± 1	4 ± 0.3
Ischemic-reperfused (dx)	4/18	-53 ± 1 [a]	100 ± 11 [a]	7.0 ± 1	13 ± 1	5 ± 0.3
Phosphocreatine disodium salt 40 mg / kg						
Controlateral controls (sx)	3/20	-69 ± 1	68 ± 7	10.0 ± 2 [a]	9 ± 0.5	4 ± 0.3
Ischemic-reperfused (dx)	3/24	-65 ± 1 [b]	77 ± 4 [b]	11.0 ± 0.8 [a,b]	10 ± 1	6 ± 0.4
Phosphocreatine di-L-arginine salt 68 mg / kg						
Contralateral controls (sx)	5/10	-67 ± 2	85 ± 8	8.0 ± 0.7	-7 ± 2 [a]	4 ± 0.3
Ischemic-reperfused (dx)	5/24	-57 ± 1 [a]	106 ± 5 [a]	9.0 ± 0.6	3 ± 1 [a,b]	7 ± 0.3 [a]
L-Arginine 26 mg / kg						
Contralateral controls (sx)	3/10	-64 ± 4	78 ± 9	7.0 ± 0.8	-2 ± 0.8 [a]	5 ± 0.9
Ischemic-reperfused (dx)	3/14	-51 ± 1 [a]	178 ± 24 [a,b]	6.0 ± 0.7	2 ± 1 [a,b]	4 ± 0.4

The columns from left to right indicate: the experimental conditions, compounds tested and dose intramuscularly administered; number of muscles on number of fibers sampled; RP, resting potential of the fibers; I_{Th}, minimal depolarizing current necessary to evoke a single action potential (rheobase current); Dur, duration of action potential; Os, overshoot of action potential; maximum number of spikes elicited by a depolarizing stimulus of 100 ms of duration. The data shown were recorded from different muscle preparations: the right-hand (dx) extensor digitorum longus muscle fibers that underwent 4 h of ischemia followed by 30 min of reperfusion (ischemic-reperfused), and from the left-hand (sx) muscles contralateral to the operated one (Contralateral controls), with no treatment or after in vivo pretreatment with drugs. The excitability parameters from sx and dx muscle fibers of unoperated rats (Controls) are also shown. The values are expressed as means $\pm$ S.E. The variance ratio (F) was calculated for each experimental group by 2-way ANOVA. The F values for RP between ischemic-reperfused and contralateral control muscle preparations from untreated rats were 132.1 ($df = 1/3$, $P < 0.001$) and between rats 2.3 ($df = 3/3$, n.s.). After in vivo pretreatment with phosphocreatine di-sodium salt, phosphocreatine di-L-arginine salt and L-arginine the F values between preparations were 1.2 ($df = 1/2$, n.s.), 120.2 ($df = 1/4$, $P < 0.001$) and 198.3 ($df = 1/2$, $P < 0.005$); between rats 0.9 (2/2, n.s.), 12 ($df = 4/4$, $P < 0.025$), and 0.8 ($df = 1/2$, n.s.), respectively. A comparison of the RP values between unoperated control dx and unoperated control sx muscles yielded F values of 0.3 ($df = 1/3$, n.s) between muscle preparations, and 0.2 ($df = 3/3$, n.s.) between rats. The F values for I_{Th} between ischemic-reperfused and contralateral control muscles were 242.1 ($df = 1/3$, $P < 0.001$), and between rats 5.1 ($df = 3/3$, n.s.). After in vivo pretreatment with phosphocreatine di-sodium salt, phosphocreatine di-L-arginine, and L-arginine the F values between preparations were 7.1 ($df = 1/2$, n.s.), 131.1 ($df = 1/4$, $P < 0.001$), and 212.1 ($df = 1/2$, $P < 0.001$); between rats 3.1 ($df = 2/2$, n.s.), 9.1 ($df = 4/4$, n.s.), and 2.1 ($df = 1/2$, n.s.). A comparison of the I_{Th} between unoperated control dx and unoperated control sx muscles from unoperated rats shows F values of 0.22 ($df = 1/3$, n.s.) between muscle preparations, and 0.4 ($df = 3/3$, n.s.) between rats. The comparison between individual pairs of means was made with Student's t-test. Means significantly different from unoperated controls are indicated by [a] ($P < 0.05$ or less), from ischemic-reperfused of untreated rats are indicated by [b] ($P < 0.05$ or less).

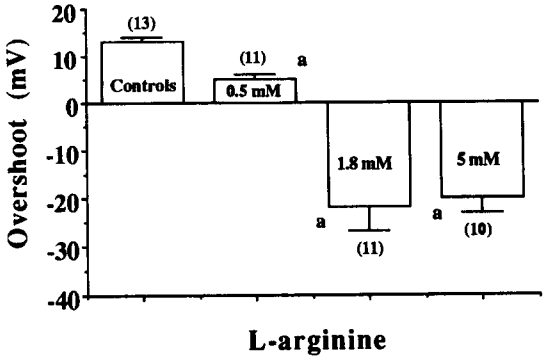

Fig. 2. Effect of in vitro application of increasing concentrations of L-arginine on the action potential overshoot of rat extensor digitorum longus muscle fibers. The values are expressed as means ± S.E. The numbers in brackets are the numbers of the fibers sampled. The comparison between individual pairs of means was done with Student's *t*-test. Means significantly different from control are indicated by [a] ($P < 0.005$ or less).

but decreased the amplitude of the action potential of the contralateral controls and ischemic-reperfused muscle fibers. As it has been already observed for phosphocreatine di-L-arginine salt, the overall decrease of the amplitude of the action potential was mainly due to a reduction of the overshoot (Table 1).

Table 2
ATP content of extensor digitorum longus muscle of rat after pretreatment with phosphocreatine disodium and phosphocreatine di-L-arginine salt

Experimental condition	Homogenates sampled	ATP (μmol/g of tissue)
Unoperated control (sx)	5	5.96 ± 0.27
Unoperated controls (dx)	5	6.32 ± 0.4
Contralateral controls (sx)	7	5.83 ± 0.3
Ischemic-reperfused (dx)	7	2.59 ± 0.8 [a]
Phosphocreatine disodium salt 40 mg / kg		
Controlateral controls (sx)	4	8.20 ± 2
Ischemic-reperfused (dx)	4	7.10 ± 0.6
Phosphocreatine di-L-arginine salt 68 mg / kg		
Controlateral controls (sx)	4	8.41 ± 2
Ischemic-reperfused (dx)	4	5.30 ± 0.8

The ATP contents were measured of homogenates from different muscle preparations: the right-hand (dx) extensor digitorum longus muscle that underwent 4 h of ischemia followed by 30 min of reperfusion (ischemic-reperfused), and from the left-hand (sx) muscles contralateral to the operated one (Contralateral controls), with no treatment or after pretreatment with drugs. The values are expressed as means ± S.E. The variance ratio (F) was calculated for each experimental group by 2-way ANOVA. The F values between ischemic-reperfused and contralateral control preparations from untreated rats were 99.3 ($df = 1/6$, $P < 0.005$); between rats 4.1 ($df = 6/6$, n.s.). After in vivo pretreatment with phosphocreatine di-sodium salt and phosphocreatine di-L-arginine salt the F values between preparations were 4.2 ($df = 1/3$, n.s.) and 31.1 ($df = 1/3$, $P < 0.025$); between rats 2.1 (3/3, n.s.) and 12.2 ($df = 3/3$, $P < 0.05$). A comparison of the ATP contents between homogenates from unoperated control dx and unoperated control sx muscles yielded F values of 0.31 ($df = 1/4$, n.s.) between muscle preparations, and 0.41 ($df = 4/4$, n.s.) between rats. The comparison between individual pairs of means was made with Student's *t*-test. Means significantly different from unoperated controls are indicated by [a] ($P < 0.05$ or less).

3.2. Effects of in vitro applications of L-arginine on extensor digitorum longus muscle fibers

The in vitro application of increasing concentrations of L-arginine (0.5 mM–1.8 mM–5 mM) on extensor digitorum longus muscles of unoperated control rats did not produce significant effects on resting potential, cable parameters and membrane conductance, but it caused a significant dose-dependent decrease of the amplitude of the action potential, sustained by a reduction of the overshoot (Fig. 2). Moreover, the threshold current to elicit the action potential was increased up to 54–67% by L-arginine. These effects were fully reversible after about 15 min of washout.

3.3. Effects of in vivo pretreatment with phosphocreatine disodium salt and phosphocreatine di-L-arginine salt on ATP content of ischemic-reperfused extensor digitorum longus muscles

In agreement with preceding experiments (Tricarico and Conte Camerino, 1994a), the ATP content of ischemic-reperfused muscles was dramatically lowered in respect to contralateral control muscles (Table 2). Pretreatment with both phosphocreatine disodium salt and phosphocreatine di-L-arginine salt loaded the reperfused muscles with extra ATP. Both phosphocreatine salts showed almost the same efficacy in this effect (Table 2).

4. Discussion

4.1. Effects of phosphocreatine disodium salt on ischemic-reperfused rat skeletal muscle fibers

Earlier experiments have shown that reperfusion is responsible for most of the injuries occurring in rat skeletal muscle subjected to severe ischemia (Tricarico and Conte Camerino, 1994a). Indeed, 4 h of ischemia caused only a decrease of the total membrane ion conductance. In contrast, 4 h of ischemia followed by 30 min of reperfusion lowered the ATP content of the muscles, dramatically depolarized the fibers and increased the total membrane ion conductance, reducing their excitability. The increase of the total membrane ion conductance was mediated by an increase of both Cl^- and K^+ conductance. We also observed that the K^+ conductance was sensitive to glibenclamide, suggesting an involvement of K_{ATP} channels in the abnormal increase of macroscopic conductance due to reperfusion (Tricarico and Conte Camerino, 1994a). In the

present experiments the pretreatment of the rats with phosphocreatine disodium salt loaded the extensor digitorum longus muscle with extra ATP, prevented the increase of the total membrane conductance as well as the fiber depolarization and restored the excitability of the fibers. These results suggest that most of the injuries due to reperfusion are related to the ATP decrement occurring in the muscle. It is known that the activity of ion channels responsible for the resting macroscopic conductance (mainly Cl^- and K^+ channels) depends also on the energy state of the cell or on the ATP molecule itself. The existence in various tissues of K_{ATP} channels that open in response to a low level of ATP is well documented (Spruce et al., 1985). The density of this type of channel in skeletal muscle is high (Tricarico and Conte Camerino, 1994b; Tricarico et al., 1994), therefore a lowering of ATP content due to reperfusion activates these channels and can increase the resting macroscopic K^+ conductance of the fibers. Opening of K_{ATP} channels can also account for the increase of threshold current needed to elicit an action potential and for the reduction of the duration of action potential occurring after reperfusion (Jonas et al., 1991; Tricarico and Conte Camerino, 1994b). In our experiments, the pretreatment with phosphocreatine disodium salt, loading the muscles with extra ATP, could have reduced the activity of K_{ATP} channels, thus preventing the abnormal increase of macroscopic K^+ conductance due to reperfusion and saving the internal K^+ content of the fibers. The closure of K_{ATP} channels could have also increased the duration of the action potential and decreased the threshold current needed to elicit the action potential. In regard to Cl^- conductance, at the moment the direct dependence of this parameter on ATP in skeletal muscle can only be speculated on (because of the difficulty of finding the channel on native skeletal muscle fibers by patch clamp method; the single channel properties are not yet known (Chua and Betz, 1991)). However, it has been recently reported that other types of chloride channels from epithelia cells need physiological levels of ATP to be fully activated (Quinton and Reddy, 1992).

4.2. Effects of phosphocreatine di-L-arginine salt and L-arginine on the ischemic-reperfused rat skeletal muscle fibers

Phosphocreatine di-L-arginine, in addition to restoring the energy state of the cell when the rate of ATP hydrolysis is high, should also protect the cell from reperfusion injury linked to free radical generation. Indeed, L-arginine, as precursor of NO, has been reported to reduce microvascular permeability, lowering the possibility of edema formation in reperfused tissues and reducing leukocyte mobility in the extravascular space (Kubes and Granger, 1992). It is widely accepted that most of these effects of NO are due to activation of cGMP-dependent protein kinase, which may interact with cytosolic proteins of the contractile apparatus or may increase openings of ion channels (Moncada et al., 1991; McCall and Vallace, 1992). Recent reports indicate that the molecular mechanisms underlying the effects of NO in tissues appear to be more complex, involving also a direct interaction of NO with ion channel proteins through a cGMP-independent pathway (Bolotina et al., 1994). However, in the present experiments, the phosphocreatine di-L-arginine salt as well as L-arginine did not show the expected beneficial effects on the electrical parameters of ischemic-reperfused muscle fibers. Although the phosphocreatine di-L-arginine salt restored the total membrane conductance and increased the ATP content of the reperfused muscle close to the physiological values found in the unoperated control muscles, the fibers were still depolarized and almost unexcitable. In addition, we found that the phosphocreatine di-L-arginine salt decreased the amplitude of the action potential, reducing the overshoot. Also the pretreatment with L-arginine failed to protect the muscle fibers from the reperfusion injuries. As with the phosphocreatine di-L-arginine salt, the L-amino acid reduced the amplitude of the action potential by reducing the overshoot. The inhibitory effect on action potential parameters shown by the in vitro application of L-arginine to the extensor digitorum longus muscle was rapid and reversible, supporting the hypothesis that the L-amino acid may directly interact with muscle Na^+ channels. Indeed, L-arginine shares a guanidine group with the tetrodotoxin molecule which is known to block Na^+ channels of various tissues by binding to receptor into the channel (Hille, 1984). The guanidine group will permeate through the pore, competing with the Na^+ ion for a common binding site located in the channel (Hille, 1984). The action of the guanidine group will lead to a reduction of the Na^+ channel current. The final effect will be an increase of the threshold current to elicit the action potential and a decrease of the overshoot and amplitude of the action potential, which reflect a decrease in the rate of depolarization of the fibers and therefore a decrease of the excitability. The lack of protective effects shown by phosphocreatine di-L-arginine salt and L-arginine can be also explained by considering that L-arginine is involved through the NO pathway in different toxic manifestations in various tissues. One of these involves the generation, during reperfusion, of new radical species such as peroxynitrite following the interaction of NO with superoxide anion (Matheis et al., 1992; Schulz and Triggle, 1994). Thus NO may also alter the single channel properties by interacting directly with reactive chemical groups located in the vicinity of the ion channel proteins such

as hydroxyl and thiol groups or disulfide bridge. It is known that an alteration of the redox state of these groups may modify the activity of ion channels and transporters including K^+ channels and Na^+ channels (Girard and Potier, 1993; Tricarico and Conte Camerino, 1994b).

In conclusion, the effects of the treatment with phosphocreatine disodium salt at a cellular level support the use of the chemical in reperfusion injury of skeletal muscle; however, we must exercise caution in suggesting the use of phosphocreatine di-L-arginine salt in the treatment of muscle disorders linked to ischemia/reperfusion.

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