Fluorometric studies of recovery metabolism of rat fast- and slow-twitch muscles

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MAMMALIAN SKELETAL MUSCLES may be classified as either fast or slow twitch, depending on the speed of their contractile response, and in recent years it has been demonstrated that there are also marked differences in the histochemical characteristics of these muscles. Indeed, three histochemically distinct types of fiber have been identified in mammalian skeletal muscle. (See ref. 7 for review.) The overall characteristics of a muscle are reflected in its fiber composition, and although most muscles are heterogeneous with regard to their fiber type, it may be generalized that histochemically, slow-twitch muscles appear to have high activities of oxidative enzymes and rather low activities of glycolytic enzymes; whereas fast-twitch muscles display higher activities of glycolytic enzymes and lower oxidative enzyme activities.

These different enzymatic profiles imply differences in the energy metabolism of fast- and slow-twitch muscles. Recent experiments in this laboratory (21) have revealed differences in the recovery heat production of fast- and slow-twitch muscles, particularly in the susceptibility of the recovery heat to anaerobiosis. In the present series of experiments, the recovery metabolism of fast- and slow-twitch muscles has been further investigated using the fluorometric technique developed by Chance and Jobsis (3) for measuring the reduction level of nicotinamide adenine dinucleotide (NAD) in intact tissue. These measurements are based on the fact that light of the 366-nm region elicits fluorescence from the reduced molecule, NADH, but not from the oxidized form, NAD⁺.

In this paper we describe the fluorescence variations which follow contractile activity in fast- and slow-twitch muscles. In an attempt to elucidate the relative importance of oxidative and glycolytic pathways in the recovery metabolism of these muscles, the effects of anaerobiosis and iodoacetic acid (IAA) on the fluorescence changes were also investigated.

METHODS

The experiments were performed on extensor digitorum longus (EDL) and soleus (SOL) muscles obtained from the hindlimbs of 4- to 5-wk-old hooded Wistar rats of either sex. These muscles are respectively representative of mammalian fast-twitch (EDL) and slow-twitch (SOL) muscles. The choice of the above age group ensured that the postnatal differentiation of fast- and slow-twitch muscles was complete (6), yet provided muscles which were still small enough to maintain in good condition in the isolated state (8, 13, 20). The dissection of the muscle preparations was carried out as described in the previous paper (21).

For fluorometric observations, the muscle was suspended vertically in a clear acrylic plastic bath containing 35 ml of Krebs-Henseleit solution. This solution had the following composition, in millimoles per liter: NaCl, 118.00; KCl, 4.75; CaCl₂, 2.54; KH₂PO₄, 1.18; MgSO₄, 1.18; NaHCO₃, 24.80; glucose, 10.00; it was continuously bubbled with 95% O₂-5% CO₂, had a pH of 7.4, and also contained insulin (0.01 U/ml). The bath was fitted with a quartz window to allow efficient illumination and observation of tissue fluorescence. All experiments were conducted at room temperature which was maintained at 22°C.

Mechanical measurements. One tendon of the muscle was firmly clamped near the bottom of the bath, and the other tendon was attached, via a light stainless steel tube, to a Sanborn FTA-100 tension transducer. All contractions were isometric.

Muscle length. At the beginning of each experiment, the length of the muscle was set to be optimal for tetanic tension development.
Stimulation. Square pulses (1.0 ms, 10-15 V) from a Grass S8 stimulator passed through an isolation unit and were delivered directly to the muscle via two large silver electrodes, one on either side of the muscle. Tetanic contractions were produced by stimulation at 80 Hz for EDL and 40 Hz for SOL. The tetanic duration was generally 1 s.

Fluorometric measurements. The fluorometric measurements were made using a microfluorometer with feedback regulation basically similar to that introduced by Jobsis and Stainsby (17) and described in detail by Jobsis et al. (16). Light (366 nm) was focused onto the surface of the muscle by means of an epi-illumination microscope attachment. Light from a 2-mm diam field of observation was collected by the objective lens and split after the ocular lens system with a 95-5% beam splitter. The 95% fraction was passed through a Wratten 5% filter to select the fluorescent light, which was measured with an end-window photomultiplier tube. The 5% fraction passed directly to a second photomultiplier tube, which gave a measure of the reflected excitation light. Since the light returning to the objective lens consisted almost entirely of reflected light, with only a small amount of fluorescent light, filtering of the 5% fraction was not necessary to obtain a reliable measure of the reflected excitation light. The AC outputs of the phototubes were demodulated, and the resulting DC signals of fluorescence and reflectance were amplified and recorded on an oscillograph. The optical difference signal (fluorescence minus reflectance) and the isometric tension were also recorded oscillographically.

The reflected light excited a significant amount of background fluorescence in the microscope optics and Wratten filter. The amount of such fluorescence was estimated by using a stainless steel "nonfluorescent" spatula to reflect 366 nm light at intensities ranging from 0 to 200% of the levels reflected by the muscles. Graphs of fluorescence versus reflectance were plotted to allow for fluorescence of the spatula itself (assumed constant for small variations of reflecting angle). The graphs were linear and gave values of about 20% fluorescence for 100% reflectance. Thus it must be concluded that 20% of the "tissue fluorescence" as recorded in the present work is an inert artifact due to fluorescence of the optical system. All fluorescence changes reported in the present work are referred to the 100% resting level which includes the 20% inert artifact. Hence, the changes in physiologically labile fluorescence are at least 1.25 times as large as indicated.

RESULTS

Fluorometric standardization. The muscles used in these experiments occupied a variable portion of the field of observation depending on their size and, therefore, the actual area of muscle surface from which fluorescence was detected varied from preparation to preparation. For this reason, no absolute comparison of the fluorescence of different muscles was attempted. Instead, for each muscle the 100% level of fluorescence was defined as that obtained for the muscle resting at its L length in oxygenated Krebs-Henseleit solution. Subsequent changes in fluorescence were the expressed as percentage changes of this resting oxygenated level. Both EDL and SOL have tendon covering a substantial portion of their surfaces, and particular care was always taken to avoid having tendon-covered regions in the field of observation.

Optical artifacts. In the subsequent figures, it can be seen that the optical traces contain movement artifacts associated with the mechanical events. These appeared in either, or both, the reflectance and fluorescence but were extremely variable, differing in both magnitude and direction. Presumably the artifacts arise from changes in the geometry of the muscle surface (reflectance) and light-scattering properties of the tissue (fluorescence), and perhaps also from changes in the mass of muscle in the optical field. To have confidence in the fluorescence records, it is essential that the reflectance returns to its control value following any artifact associated with the contractile event. No attempts were made to eliminate these artifacts from the records, but the optical traces were filtered with a time constant of 300 ms to reduce the noise inherent in these recordings. In all figures upward deflections of the optical traces correspond to increased in fluorescence, reflectance, and the fluorescence minus reflectance difference, respectively.

Twitch contractions. When the muscles performed 10 twitches at a frequency of 0.25/s both the EDL and SOL muscles responded with decreases in fluorescence followed by a return to the resting level when the contractile activity ceased. These responses are illustrated on the left sides of Figs. 1A and 2A for EDG and SOL, respectively. Although the nature of the response was the same for the two muscles, there was clearly a difference between the time courses of the fluorescence changes. In EDL fluorescence decrease had a rapid onset, beginning with the first twitch, reached its nadir after about five to six twitches, and began to return to baseline immediately after contractile activity ceased. With SOL fluorescence did not begin to decrease appreciably until after three to four twitches had been completed. It then decreased with subsequent twitches and, following the last twitch, continued to decrease further before finally reaching a minimum level and subsequently returning to the base line.

The rapid type of fluorometric response was observed in 14 out of 17 EDL muscles, and the slower response occurred in 9 out of 12 SOL muscles. There was some overlap in that the three remaining EDL muscles showed the slower type of response, and three out of the 12 SOL muscles showed the rapid type of response. An additional complexity occurred in one SOL and two EDL muscles. When the fluorescence returned toward the base line in these muscles, it overshoot into a phase of increased fluorescence before finally returning to the resting level (cf. ref. 5, Fig. 5).

The right-hand panels of Figs. 1A and 2A show the response of the same muscles to 10 twitches (0.25/s) 45 min after the 5% CO2 gas mixture bubbling the bathing solution had been replaced with 5% NaHCO3 gas mixture. It can be seen that the fluorescence changes accompanying the contractions have been virtually abolished. (A small decrease in fluorescence can still be
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CONTROL A ANOXIA

FIG. 1. A: original records of (top to bottom) fluorescence minus reflectance, reflectance, fluorescence, and isometric tension taken from an EDL muscle performing 10 twitches (0.25/s) in presence first of oxygen (control) and then nitrogen (anoxia). Two successive responses in nitrogen are shown. B: original optical and mechanical records of same EDL muscle performing a 1-s tetanus in oxygen and 2 successive 1-s tetani in nitrogen. In this and all subsequent figures, the fluorescence calibration bars apply also to reflectance and difference records. Note difference in calibration between aerobic and anaerobic records. Fluorescence base line at commencement of anoxic records was increased relative to aerobic base line by 53% (A) and 57% (B). Dots mark 60 s of lapsed time following tetanic contractions.

detected in the case of EDL.) A marked deterioration in mechanical performance also occurred in both muscles (average 41% for EDL, 30% for SOL). However, these mechanical deteriorations were insufficient to account for the large quantitative changes that occurred in the anoxic fluorescence wave forms. (See calibration changes between aerobic and anoxic traces.) These and all subsequent effects of anoxia described in this paper were consistent within the six muscles of each type subjected to this treatment.

Tetanic contractions. The fluorescence cycle following a tetanus showed marked differences between the fast- and slow-twitch muscles. In EDL fluorescence decreased following a 1-s tetanus but rapidly increased, crossing the base line within several seconds and continuing to rise, reaching a maximum within 35-45 s after the tetanus. Following this there was a gradual decrease in fluorescence intensity back to the resting level. A typical response of EDL to a 1-s tetanus is shown in the left-hand panel of Fig. 1B. In complete contrast to this was the response of SOL which is illustrated in the left-hand panel of Fig. 2B. Immediately after a 1-s tetanus, there was a transient increase in the fluorescence of SOL which reached a maximum in about 5 s. This was quickly reversed and the fluorescence decreased, crossing the base line and reaching a minimum about 35-45 s after the tetanus was completed. From this minimum level, the fluorescence then slowly increased, returning finally to the resting level.

The fluorometric transients shown on the left of Figs. 1B and 2B appeared to represent the typical responses evoked by tetanic contractions of these fast- and slow-twitch muscles, respectively. The fluorescence cycle illustrated for EDL was observed in 15 out of the 17 EDL muscles studied, whereas 10 out of 12 SOL muscles showed the type of responses illustrated for SOL in Fig. 2B. The remaining two EDL and two SOL muscles exhibited responses which consisted of predominant phases of increased fluorescence but with substantial oscillations in the fluorescence intensity. It should be noted that the two EDL and two SOL muscles that showed atypical tetanic responses corresponded to two of the three muscles of each type showing the atypical twitch responses. Variation of the tetanic duration between 0.5 and 2.0 s for EDL and 0.5 and 4.0 s for SOL did not produce any alterations in the nature of the respective fluorescence changes.

The right-hand panels of Figs. 1B and 2B show the responses of the same muscles after the solution had been bubbled continuously for 45 min with 95% N₂ - 5% CO₂. In the case of EDL, fluorescence appeared to be transiently increased immediately following a 1-s tetanus in nitrogen, but the predominant response was a rapid decrease in fluorescence, followed by a slower increase back to the base line. With SOL, a 1-s tetanus in nitrogen evoked only a small, rather fast, initial increase in fluorescence followed by a slower return to the base line. Note the change in optical calibrations between the aerobic and anaerobic records.

There was always a large increase in the resting level of fluorescence intensity of both EDL and SOL following the transition from 95% O₂ - 5% CO₂ to 95% N₂ - 5% CO₂ bubbling. After 45-min exposure to nitrogen, the resting level of fluorescence was increased by an aver-
age 62% in EDL and 37% in SOL. As a result of this, the fluorescence traces obtained in nitrogen often occurred on a rising base line. This is evident in Fig. 1, and the fluorescence records should not be interpreted as overshooting the base line during the late recovery phase. The detrimental effect of exposure to nitrogen on the tetanic tension development of these muscles is also evident from the records presented in Figs. 1B and 2H. However, comparison of the calibrations for the aerobic and anoxic traces shows that the mechanical deterioration in anoxia was insufficient to account for the large qualitative and quantitative changes in the fluorescence wave forms.

Figure 3 shows records of 1-s tetani taken from an EDL and a SOL muscle before and after these muscles had been poisoned with IAA. The IAA was added to the bath to a concentration of 0.75 mM, and continuous bubbling with 95% O₂–5% CO₂ was maintained. Following addition of IAA, the muscles were left for 30 min during which there was always a decrease in the resting level of fluorescence intensity. This decrease averaged 14% in EDL and 28% in SOL. In the presence of IAA, the large phase of increased fluorescence which usually follows a tetanus of EDL was markedly inhibited. In the case of SOL, IAA supressed the brief, initial phase of increased fluorescence which was observed under normal conditions while leaving the subsequent phase of decreased fluorescence unimpaired. The respective effects of IAA described above were consistent within the five EDL and four SOL muscles subjected to this treatment.

DISCUSSION

Since ultraviolet light excites the fluorescence of NADH but not that of NAD⁺, the fluorescence variations described above are considered to reflect the oxidation-reduction state of the NAD-NADH couple and, consequently, they give some insight into the nature of the recovery metabolism occurring in these muscles. It is thought that, in skeletal muscle, mitochondrial NADH normally accounts for the greatest part of the fluorescent light emission (15, 17), although it was suggested by Godfraind-De Becker (11) that a significant contribution may arise from free cytoplasmic reduced coenzymes. During the present experiments, no attempts were made to elucidate the specific origin of the labile tissue fluorescence. The results suggest that a large part of the fluorescence originates from mitochondrial NADH, but glycolytic activity also appears to have a marked effect on the fluorescence changes. Whether this is directly due to the fluorescence of cytoplasmic NADH or due to the mitochondrial NADH levels being altered by glycolysis cannot be resolved at present.

Groups of twitch contractions of both EDL and SOL always resulted in fluorescence decreases, indicating oxidation of NADH, which subsequently returned to the resting level when contractile activity ceased. These observations agree with similar experiments on isolated frog and toad sartorius muscles (3, 14, 15), circulated mammalian skeletal muscle (17), and isolated rabbit cardiac muscle (5). Also, with the exception of the occa-
tetanus proceeds differently in the fast- and slow-twitch muscles.

In terms of the oxidation-reduction level of NAD, a tetanus of SOL in the presence of adequate oxygen is followed by a rapid transition to a more reduced state and a subsequent oxidation of NADH which attains a maximum and then reverses, returning to the resting state. Qualitatively this cycle is identical to that described by Godfraind-De Becker (11) for toad sartorius muscles, and some insight into the mechanisms underlying this complex response was obtained. First, the initial step of reduction of the coenzymes is abolished by IAA, indicating that it is related to glycolytic activity, whereas the ensuing oxidation is unaffected. In the presence of nitrogen, however, the initial reduction persists, but the ensuing oxidation is prevented and the fluorescence returns toward the resting level. It appears, therefore, that the predominant phase of decreased fluorescence is related to mitochondrial events requiring the consumption of oxygen. These effects of IAA and nitrogen on the fluorescence cycle of SOL are identical to those which occur in toad sartorius muscles subjected to comparable treatment (11).

In complete contrast to this are the fluorescence changes following a tetanic contraction of EDL. Following the early transition to a more oxidized state of NAD, the predominant feature of the response is a long-lasting phase of increased fluorescence (i.e., reduction of the NAD+). The tentative interpretation of this large reduction of the coenzymes is that it results from glycolytic activity operating at a high level. This is supported by the effects of IAA which greatly inhibited the prolonged phase of increased fluorescence.

A puzzling feature of the results was the behavior of EDL muscles when contracting in nitrogen. In this situation, a tetanus was followed by a rapid decrease in fluorescence which subsequently returned to the resting level. The magnitude of the response was small compared with the control response in oxygen, but nonetheless it clearly indicated an oxidation of NADH.

In the preceding paper (21), it was reported that the anoxic tetanized EDL muscle apparently continued to produce substantial amounts of delayed heat considered to arise mainly from anaerobic glycolysis. Such glycolytic activity could be expected to produce, if anything, an increase in fluorescence intensity. The opposite effect was observed experimentally. Nitrogen produced a large increase in the resting level of fluorescence as the respiratory chain moved into a more reduced state when deprived of oxygen. In the case of EDL, this increase in the resting fluorescence level was of the order of 60%, well above what was ever achieved during a transient response following contraction. This greatly elevated level of reduction of the coenzymes in the resting state may complicate subsequent fluorescence transients recorded in response to contractile activity. Also it may be possible that an oxidation of NADH is produced during anaerobic glycolysis through lactate dehydrogenase and α-glycerophosphate dehydrogenase. Both lactate and α-glycerophosphate are indeed terminal metabolites in anaerobiosis (12). The possibility that a small amount of oxygen was still present during the "anoxia" experiments cannot be ruled out. Indeed, the continually rising base lines of Figs. 1 and 2 would tend to indicate this. However, small oxidations of NADH were also observed following 1-s tetani of EDL (but not SOL) muscles treated with 1 mM cyanide. Cyanide also increased the resting fluorescence level to an extent comparable with anoxia.

By noting the positions of the 60-s time lapse dots in Figs. 1 and 2 of this paper and in Fig. 2 of the preceding paper, it is apparent that the time courses of the fluorometric responses do not correlate in any simple way with the time courses of the heat evolution following tetanic contractions of EDL and SOL. More thorough examination of any such correlations will require simultaneous recording of heat and fluorescence change in single muscles.

Although EDL and SOL generally displayed distinct fluorometric responses which were apparently characteristic of these fast- and slow-twitch muscles, respectively, there were some instances of overlap where an EDL muscle would show the type of response more typical of SOL, and vice versa. The overall characteristics of a muscle are determined by its fiber composition, and neither EDL nor SOL is purely homogeneous with regard to its fiber type. SOL consists almost entirely of slow-twitch intermediate fibers but does have a small percentage of fast-twitch red fibers (18), and similarly EDL, although it is composed mainly of fast-twitch white fibers, contains a considerable proportion of fast-twitch red fibers (9, 10). Since, in these experiments, tissue fluorescence is sampled from only a limited area of the muscle surface, the possibility exists that fluorescence will be recorded occasionally from regions of EDL and SOL which have some similarity with regard to the fiber type. This may explain the occasional overlap between the type of response recorded from EDL and SOL. Alternatively, the anomalous responses seen with some muscles may be related to unusual resting oxidation reduction levels of the respiratory chain or particular metabolic peculiarities or disturbances in these muscles.

Jobsis and Duffield (15) have shown that in amphibian skeletal muscles the tissue fluorescence arising from cytoplasmic NADH is an insignificant fraction of that arising from mitochondrial NADH, and Jobsis and Stainsby (17) imply this is also true for mammalian skeletal muscles. In accord with this, the nature of the fluorometric transients associated with oxidative recovery metabolism are readily interpreted. Oxidative phosphorylation, stimulated by ADP liberated during contraction, causes a rapid oxidation of mitochondrial NADH as is represented, in greatly simplified form, by the reaction:

\[
\text{NADH} + 3 \text{ADP} + 3 \text{P}_i + \text{H}^+ + \frac{1}{2} \text{O}_2 \rightarrow \text{NAD}^+ + 3 \text{ATP} + \text{H}_2\text{O}
\]  

The fluorescence is then returned to the resting level by the inflow of reducing equivalents arising from the metabolism of endogenous lipid, carbohydrate, or exogenous substrate. These are the reactions of intermediary metabolism and may be summarized as:
NAD\(^+\) + substrate H\(_2\) ⇒ NADH + substrate + H\(^+\)

The effects of glycolysis on the fluorescence cycle are, however, not as readily interpretable. It is evident from the present results and those of others (1, 2, 11, 14, 15, 19, 22) that glycolytic reactions do influence the course of fluorescence changes and that this influence is manifested as a tendency toward increased fluorescence. This occurs because of an increased reduction of NAD, but uncertainty exists as to the subcellular location of the NAD responsible for the increased fluorescence. It could arise directly from the glycolytic reduction of cytoplasmic NAD at the glyceraldehyde-3-phosphate dehydrogenase step or indirectly through the mitochondrial NADH levels being altered by glycolytic activity. Such an indirect reduction of the mitochondrial couple could, for example, result from metabolism through the tricarboxylic acid cycle of some substrate (e.g., pyruvate) formed by glycolysis or through mitochondrial shuttles operating in association with the reoxidation of cytoplasmic NADH. However, the persistence of a rapid initial reduction phase seen in anaerobic SOL muscles is at least suggestive of a direct effect from cytoplasmic dehydrogenases. This would further be supported by the oxidation of NADH observed in anaerobic EDL muscles if it were in fact being produced by the dehydrogenases mentioned above.

It is apparent that the fluorescence cycle represents a complex pattern of metabolic processes, and even if it can be interpreted in terms of a single event (i.e., the reversible transformation of NADH into NAD), it is probable that pyridine nucleotides at more than one location are involved. Nonetheless, the present results reveal that there are marked differences between the recovery metabolism of the fast- and slow-twitch muscles. These differences appear to be consistent with the slow-twitch muscle having predominantly mitochondrial oxidative recovery metabolism and the fast-twitch muscle having a predominantly glycolytic recovery metabolism.

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