THE REGULATION OF RESPIRATION OF GUINEA PIG TAENIA COLI IN HIGH-K MEDIUM; THE ROLE OF NICOTINAMIDE-ADENINE DINUCLEOTIDE, ADENOSINE DIPHOSPHATE AND Ca**

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Abstract—In an attempt to elucidate the regulation mechanism of respiration in the smooth muscle cell, we investigated the roles of nicotinamide-adenine dinucleotide (NAD), adenosine diphosphate (ADP) and Ca** in the muscle respiration using the tissues and subcellular fractions from guinea pig taenia coli. The tension in the strips of taenia coli increased with a concomitant increase in O2 consumption in high-K medium (40 mM K) containing 2.5 mM Ca. 10^-3 M amytal and 10^-6 M ouabain decreased the high-K induced tension and O2 consumption of the muscle. 10^-4 M 2,4-dinitrophenol (DNP) relieved the decreased respiration induced by ouabain, but not that with amytal. From these data it is suggested that NADH-linked respiration plays an important role in the respiration of the muscle. Ca++ in concentrations ranging from 0.5 to 2.5 mM in the high-K medium resulted in an increase in tension and in O2 consumption progressively. In spectrophotometric observations of subcellular fractions of the taenia coli, ADP increased in absorbance change at 340 nm. Such occurred in mitochondrial fractions and was initiated by the addition of NADH. Therefore it is deduced that the increase in ADP level of the cytoplasm is primarily due to a contraction triggered by Ca** thus stimulating respiration. On the other hand, at 0.1 mM of Ca** concentration, the muscle strip increased O2 consumption without tension development in high-K medium. In the spectrophotometric observations, Ca** and Sr** increased the absorbance change in the homogenate and in the mitochondrial fraction. Hence, it seems that one part of the Ca** entering into the smooth muscle treated with the high-K increased O2 consumption in mitochondria independent of an increase in muscle tension. From these results it is concluded that NADH-linked respiration plays an important role in the smooth muscle respiration in high-K medium and that ADP and Ca** also play a role in regulating respiration.

It has been reported that the smooth muscle of guinea pig taenia coli requires the aerobic breakdown of carbohydrate to maintain a continuous tension in a high-K (40 mM) medium (1) and that during the tonic contraction, Ca++ enters across the cell membrane into the muscle (2) and also that both the changes in tension and Ca++ movement induced by the high-K are abolished by an application of 10^-4 M DNP or anoxia (3). In preliminary work we have found that the inhibitors of NADH oxidation in mitochondrial respiratory
chain which have no effect on succinoxidase activity, such as amytal, piericidine A, and rotenone, also abolished the high-K induced tonic tension in the taenia coli. However, the relationship between NADH-linked respiration and smooth muscle respiration in a stimulated state has not been reported.

Saito et al. (4) found that an application of the high-K caused an increase in O₂ consumption accompanied by an increase in tension change in the taenia coli, and also that addition of 10⁻⁴ M DNP to the high-K medium further increased or maintained the O₂ consumption although it abolished that developed tension, and that the change in O₂ consumption was not modified by the Ca⁺⁺ removal from the medium (5). In light of these results, the possibility of the essential role of ADP in stimulating respiration of the smooth muscle during the tonic tension maintenance and the active Ca⁺⁺ movement warrants further investigation.

According to Urakawa et al. (6) the increase in O₂ consumption and in tension change of the taenia coli in the high-K medium was abolished by the depletion of Ca⁺⁺ from the high-K medium, and that Sr⁺⁺ added to Ca⁺⁺ depleted high-K medium showed an increase in O₂ consumption without notable increase in tension. These authors suggested that Ca⁺⁺ entering into the smooth muscle treated with the high-K probably plays an essential role in O₂ consumption, independent of increase in the muscle tension.

In the present work, further studies on the regulatory role of NADH, ADP and Ca⁺⁺ in the respiration of guinea pig taenia coli were carried out.

MATERIALS AND METHODS

Strips of taenia coli were isolated from guinea pigs weighing from 400-500 g.

Simultaneous measurements of O₂ consumption and tension change of the strip: This method and apparatus have been described in detail in a previous paper (4). In the perfusion chamber, PO₂ in the perfused medium was measured polarographically using the platinum electrode and tension change was recorded isometrically using a mechano-electro transducer. Tyrode solution used was as follows (mM): NaCl, 136.8; KCl, 5.4; CaCl₂, 2.5; MgCl₂, 1.0; NaH₂PO₄, 0.4; NaHCO₃, 11.9 and glucose, 5.5. The solution was equilibrated with gas mixture (95 % O₂, 5 % CO₂) at 37°C. High-K medium (40 mM KCl) was made by subtracting 40 mM NaCl from normal Tyrode solution and adding 40 mM KCl.

Spectrophotometric measurements of NADH oxidation in subcellular fractions: Pieces of the muscle strips approximately 1 mm square were homogenized using Ultra turrax (TP 18/2) for 10 sec in 9 volumes of artificial intracellular physiological solution (ICPS). The homogenate was centrifuged in a refrigerated centrifuge (Hitachi 24P) for 10 min at 600 g. The supernatant was re-centrifuged at 12,000 g for 20 min. This yielded pellet served as the mitochondrial fraction (Mt fraction). The residual supernatant was again centrifuged using an automatic preparative ultracentrifuge (Hitachi 65P) at 105,000 g for 60 min. The resultant supernatant was used as a soluble fraction. Absorbancy changes at 340 μm ensuing upon addition of NADH to the subcellular fractions were recorded using a multipurpose spectrophotometer (Shimazu MPS-50L) at 25°C. Preparations of
the Mt and soluble fractions were made at the low temperature of (0-4°C) and these fractions were suspended or diluted in ICPS of 25°C. The prepared homogenate, the Mt and soluble fractions were promptly used for measurements of absorbance changes. ICPS was made based on the intracellular ion concentrations of guinea pig taenia coli for Na⁺, K⁺, Cl⁻ (7) and Mg²⁺ (8). This ICPS contained: NaCl, 20 mM; KCl, 25 mM; MgCl₂, 5 mM; 70 mM K₂HPO₄ and 140 mM KH₂PO₄ mixture of pH 7.2. In subcellular fractions, 0.1 mM Ca²⁺ which developed almost maximal tension in glycerol extracted muscle (9) and the same concentration of Sr²⁺ were mainly used. When glycolether-diaminetetraacetic acid (EGTA) was used, Ca²⁺ and Sr²⁺ concentrations were calculated by the following formula, proposed by Danzuka and Ueno (10):

\[
[M] = \frac{1}{2} \left( \frac{[Z]t - [M]t}{[kM/Z]H} \right) - \sqrt{\frac{1}{4} \left( \frac{[Z]t - [M]t}{[kM/Z]H} \right)^2 + \frac{1}{[K/M/Z]H} - \frac{1}{[M]t} \left( \frac{1}{[K/M/Z]H} \right)^2}
\]

[M] : free metal concentration

[M]t : total metal concentration

[Z]t : total EGTA concentration

\[kM/Z]H = \frac{M}{k_M [Z]t}, \quad k_M \frac{M}{[Z]t} = \frac{[M]}{[Z]}, \quad a_H = 10^{0.43} \text{ (at pH 7.2)}
\]

NADH was diluted in 0.5 M Tris-phosphate buffer at pH 7.6 and promptly used. Protein was assayed by the method of Lowry et al. (11).

RESULTS

Simultaneous measurements of O₂ consumption and tension change of the muscle strips

Amytal is one of the electron transport inhibitors in the NADH-cytochrome b segment of the mitochondrial respiratory chain (12). Fig. 1 shows that 10⁻³ M amytyl abolished the high-K induced tension development and the increment of the respiration, 10⁻⁴ M DNP neither relieved amytyl inhibition of the respiration, nor the tension. On the other hand, although 10⁻⁵ M ouabain which does not inhibit a respiration of mitochondria abolished the developed tension and then decreased the increased O₂ consumption in the high-K medium, 10⁻⁴ M DNP reincreased O₂ consumption without accompanying tension development in the presence of ouabain.

As illustrated in Fig. 2, raising Ca concentration from 0 to 2.5 mM in high-K medium increased O₂ consumption and tension progressively and 0.1 mM Ca increased O₂ consumption without tension development.

Spectrophotometric measurements of NADH oxidation in subcellular fractions

NADH oxidation was measured based on the absorbance change at 340 mμ initiated by addition of NADH. When NADH was used as a substrate, the respiratory control ratio in Mt was low, and the same result was obtained when glutamate was used instead of NADH. Both reactions were sensitive to rotenone or antimycin A. However, rotenone did not show an inhibitory effect when succinate was used as a substrate. From these
Fig. 1. Effects of $10^{-3}$ M amyntal (upper) and $10^{-6}$ M ouabain (lower) on $P_{O_2}$ in the medium and the muscle tension of taenia coli induced by the application of 40 mM K in Tyrode solution. Upper curve shows $P_{O_2}$ level (mm Hg), lower one shows muscle tension (g). The dotted line indicates the $P_{O_2}$ level in the medium without the muscles. Time in minutes represents the time lapsed after suspending the muscle in the chamber. The wet weights of taenia coli were 28.3 mg in the case of amyntal and 39.6 mg in the case of ouabain.

At A & A': normal medium changed to the high-K medium
At B: high-K medium to high-K medium containing $10^{-3}$ M amyntal
At B': high-K medium to high-K medium containing $10^{-6}$ M ouabain
At C: high-K medium containing $10^{-3}$ M amyntal to high-K medium containing $10^{-3}$ M amyntal and $10^{-4}$ M DNP.
At C': high-K medium containing $10^{-3}$ M ouabain to high-K medium containing $10^{-3}$ M ouabain and $10^{-4}$ M DNP.

observations, the Mt may have been somewhat damaged, but was still feasible for use in this experiment.

Fig. 3 shows that 0.1 mM Ca$^{++}$ increased NADH oxidation by about 50% and 0.1 mM Sr$^{++}$ also increased NADH oxidation by about 160% in the homogenate in the presence of 3 mM EGTA. As illustrated in Fig. 4, addition of 0.3 mM ADP increased NADH oxidation of Mt by about 150%. Fig. 5 shows that the addition of 0.1 mM CaCl$_2$ increased NADH oxidation by about 100% in Mt fraction in the absence of ADP. The addition of cytochrome c increased the NADH oxidation of the Mt as also reported in rat liver Mt (13, 14). However the NADH oxidation increase by cytochrome c was inhibited by rotenone (unpublished), so the effects of divalent ions were measured in the presence of 8 μM cytochrome c. In this case, as indicated in Fig. 6, 0.1 mM Ca$^{++}$ also increased NADH oxidation of the Mt fraction by about 40% in the absence of ADP and in the presence of 3 mM EGTA and the same concentration of Sr$^{++}$ duplicated the effect of Ca$^{++}$ in the same conditions.
NADH was oxidized in the soluble fraction, and the oxidation was not affected by mitochondrial electron transport inhibitors, such as $10^{-6} - 5 \times 10^{-5}$ M rotenone and $10^{-3}$ M NaN$_3$, and by enzyme inhibitors in glycolysis, such as $10^{-3}$ M iodoacetic acid and $10^{-3}$ M NaF. Among the metabolites of glycolysis and citric acid cycle tested (alpha-glycerophosphate, lactate, pyruvate, glutamate and succinate) only pyruvate was effective, i.e. pyruvate stimulated the NADH oxidation drastically. For this reason, it seems that the NADH oxidation
FIG. 6. Effects of Ca++ (A) and Sr++ (B) on the absorbance changes at 340 mμ induced by NADH in Mt fractions. Mt fractions were prepared with ICPS containing 3 mM EGTA. Taenia coli Mt, 0.5 mg(A) and 0.5 mg(B) of protein per ml were suspended in ICPS containing 3 mM EGTA. pH 7.2; temperature, 25°C. Refer to the note in Fig. 3.

FIG. 7. Effects of ADP and Ca on the absorbance changes at 340 mμ initiated by addition of NADH in soluble fractions of taenia coli. These fractions contain 1.7 mg(A), 3.8 mg(B) and 1.7 mg(C) of protein per ml in ICPS. pH 7.2; temperature, 25°C. Refer to the note in Fig. 3.

of the soluble fraction may be mainly attributed to lactic dehydrogenase. As shown in Fig. 7, 0.5 mM ADP and 0.1, 1 and 2 mM CaCl₂ had no effect on the NADH oxidation.

DISCUSSION

It is widely accepted that NAD(H) plays an important role in energy metabolism, for example, as a cofactor of glyceraldehyde-3-phosphate dehydrogenase and lactic dehydrogenase in glycolysis, β-hydroxyacyl dehydrogenase in β-oxidation of fatty acids and glutamic dehydrogenase in amino acids’ metabolism. It also regulates the citric acid cycle itself, through NADH-linked isocitrate dehydrogenase (15), that is, the activity of this enzyme is inhibited by NADH or ATP and activated by ADP. As illustrated in Fig. 1, amytal which inhibits electron transport between NADH and cytochrome b and does not affect succinate oxidase activity abolished the increased respiration of the taenia coli in high-K medium. DNP one of the typical uncouplers of oxidative phosphorylation, which reversed the ouabain
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inhibition of respiration, did not relieve the amytal inhibition even though the succinate oxidase pathway was unaffected. It is thus suggested that NADH oxidation regulates the respiration of guinea pig taenia coli which has been stimulated in a high-K medium.

As shown in Fig. 2, the O₂ consumption always increased in a concomitant way with the tension development. The possibility of ATP breakdown to ADP during tension development has been reported even in guinea pig taenia coli (16). In liver Mt, the role of ADP regulating respiration is well established (17, 18). In the present experiment, as indicated in Fig. 4, ADP stimulated NADH oxidation in guinea pig taenia coli Mt. From these data, it is suggested that the ADP level in smooth muscle cells regulates respiration of the muscle and if so, this respiration stimulated by ADP may produce ATP, and the ATP may support the sustained tension development of this muscle in high-K medium.

On the other hand, in spectrophotometric measurements, 0.1 mM Ca²⁺ and Sr²⁺ stimulated NADH oxidation in the homogenate and in the Mt fraction, but Ca²⁺ had no effect on NADH oxidation in the soluble fraction. Therefore, the stimulation of NADH oxidation in the homogenate is most likely due to that of Mt. In this case, the stimulation of NADH oxidation by Ca⁺⁺ and Sr⁺⁺ in the Mt fraction occurred in the absence of ADP. In the case of rat liver Mt, Ca⁺⁺ and Sr⁺⁺ stimulation of respiration did not lead to production of ATP (19, 20). Hence, it can be presumed that in Mt of taenia coli, Ca⁺⁺ or Sr⁺⁺ stimulation of respiration does not lead to ATP production in the absence or presence of ADP. Saito et al. (21) suggested that the metabolic processes stimulated by Ca⁺⁺ supply energy for the muscle contraction and the related Ca⁺⁺ movement. Among these processes the possibility of ATP production due to direct stimulation of Ca in the smooth muscle mitochondria can be excluded. Furthermore as demonstrated in Fig. 2, 0.1 mM Ca increased O₂ consumption without an accompanying tension development. Urakawa et al. reported that during the tonic contraction of the muscle induced by 40 mM K, Ca⁺⁺ enters into the cell across the cell membrane (2, 22), and that Sr⁺⁺ also entered into the cell in 40 mM K medium accompanied by little tension and large O₂ consumption (6, 23). Although up to the present, there is little information about the affinity of Ca⁺⁺ to Mt and troponin in physiological environment, it is suggested that one part of the Ca⁺⁺ entering smooth muscle treated with high-K increases O₂ consumption independent of an increase in muscle tension.

Thus, it would appear that NADH-linked respiration plays an important role in the smooth muscle respiration, and that ADP and Ca⁺⁺ also play a role in regulating respiration.

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