THE INHIBITORY EFFECT OF PAPAVERINE ON RESPIRATION-DEPENDENT CONTRACTURE OF GUINEA PIG TAENIA COLI IN HIGH-K MEDIUM. III. THE DIFFERENTIAL EFFECT OF PAPAVERINE AND ROTENONE ON DT DIAPHORASE

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Abstract—The differential effects of papaverine (Pap) and rotenone (Rot) were studied on the highly respiration-dependent contracture of guinea pig taenia coli in 40 mM potassium (40-K) medium, on isolated DT diaphorase activity and on mitochondrial respiration. The inhibition of guinea pig taenia coli to the 40-K induced tension by Rot (5 × 10⁻⁷ M) was fully reversed by the addition of a water soluble vitamin K₃ (VK₃) derivative or menadione sodium bisulfite (MSB). A low concentration (10⁻⁷—10⁻⁶ M) of Pap which had no effect on the 40-K induced tension inhibited the VK₃ restored tension from the Rot suppression, corresponding to a Pap inhibition of the isolated DT diaphorase. Inhibition of the effective concentration of Pap to the 40-K induced tension development was never reversed by addition of VK₃ or MSB. In taenia coli, both MSB and VK₃ established a bypass of the Rot sensitive site on the mitochondrial respiratory chain by means of the DT diaphorase system. The difference in washout-efficacy between Pap and Rot on the inhibition of 40-K induced tension was ascribed to a difference in their mitochondrial binding properties.

Our previous paper (1) confirmed the early finding that the tonic response of guinea pig taenia coli in 40 mM potassium (40-K) medium is highly respiration-dependent (2, 3). Furthermore, it was suggested that Pap enters the cell, inhibits the respiration and the 40-K induced tonic tension development (1). Tsuda et al. reported that NADH-linked respiration plays an important role in smooth muscle respiration (4). In the preceding paper it was demonstrated that Pap inhibited the NADH-linked respiration of guinea pig taenia coli mitochondria to about the same degree as it inhibited the increased O₂ consumption of the muscle strip in 40-K medium, and further suggested that Pap inhibits the mitochondrial respiration in the vicinity of NADH dehydrogenase acting on the same site as amytal and Rot, i.e., on the O₂ side of the NADH dehydrogenase (5). Rot at low concentrations is a highly specific inhibitor of NADH oxidation (6). In the present work, we confirmed the previous proposal (1, 5) that Pap inhibits the 40-K induced tonic tension by a direct inhibition of mitochondrial respiration, by using a comparison of Rot and its effects on tension, on the

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isolated DT diaphorase and on the respiration of mitochondria pretreated by the drugs.

MATERIALS AND METHODS

Most of the materials and methods were as described in our preceding papers (1, 5). Rot and VK₃ were dissolved in acetone and ethanol respectively and the concentrations of these solvents never exceeded 0.1% or 1% in the Tyrode solution or in the suspension media of mitochondria, respectively. Protein determination in Fig. 3 was done by using absorption at 280 nm. Vitamin K₃ was from Nakarai Chemicals Co., and dicoumarol was from Tokyo Kasei Co.

Purification of DT diaphorase: DT diaphorase was purified from the soluble fraction of rat liver homogenates. Rat liver was homogenized in 2 volumes of 0.25 M sucrose solution and centrifuged at 105,000×g for 60 min at 1°C. The 9 ml supernatant was purified by G-200 Sephadex gel filtration. A Sephadex G-200 column (2.6×100 cm) was used at 4°C after equilibration with 10 mM sodium phosphate buffer (pH 6.4) and fractions of 10 ml each were collected at a flow rate of about 30 ml per hr. The 34th fraction was used as DT diaphorase. The assay system for DT diaphorase was that of Ernster (7).

Pretreatment of mitochondria with Rot or Pap and subsequent washing: Pretreatment of the mitochondria with Rot or Pap was made by adding 5×10⁻⁷ M Rot or 10⁻⁴ M Pap (final concentration) to suspensions of mitochondria in 0.25 M sucrose (the suspension contained mitochondria from 100 mg of liver, wet weight, per ml). The samples were allowed to stand for 10 min at 0°C, and then centrifuged at 8,000×g for 15 min. The mitochondria were resuspended in 0.25 M sucrose and recentrifuged.

RESULTS

Effect of Pap and Rot on the 40-K induced tonic tension development

As reported previously (1), a wide concentration range of Rot, 10⁻⁷-10⁻⁵ M, selectively inhibited the tonic tension development of guinea pig taenia coli in 40-K medium without affecting the phasic response. The inhibition of Rot to the tonic tension development was never restored by repeated washing of Rot for 3 hr or longer. The Rot was dissolved in acetone at a concentration of less than 0.1% (v/v). Acetone at a concentration of 0.1% had no effect on either the developed tension or the tissue calcium content of the muscle strip in 40-K medium, the latter was measured using an atomic absorption spectrophotometer. Pap, in concentrations of 2×10⁻⁶ M-2×10⁻⁵ M selectively inhibited the tonic tension development, whereas the inhibitory effect of Pap was readily restored by reducing the concentration in the external medium (1).

The inhibition of Rot on the 40-K induced tonic tension development was restored by addition of VK₃ or its water-soluble derivative, MSB. In particular, the inhibition of Rot (5×10⁻⁷ M) was fully overcome by the addition of 5×10⁻⁸ M MSB. The inhibition of Pap at each effective concentration was never restored by the addition of VK₃ or MSB, even at concentration ranges of 5×10⁻⁶ to 10⁻³ M. Moreover, a low concentration of Pap (10⁻⁷-10⁻⁶ M), which had no effect on the 40-K induced tonic tension, inhibited the VK₃
Effects of Rot, VK₃, and Pap on the tonic tension development of guinea pig taenia coli induced by 40-K. The inhibition (%) of Rot was calculated from \( \frac{b}{a} \times 100 \), the restoration (%) from the Rot inhibition by VK₃ was from \( \frac{c}{b} \times 100 \) and the inhibition (%) of Pap to the restored tension was from \( \frac{d}{c} \times 100 \).

**Fig. 1.** Effects of Rot, VK₃ and Pap on the tonic tension development of guinea pig taenia coli induced by 40-K. Ordinate: Residual (%) of the 40-K induced tonic tension development at 30 min after the Rot application (open circles), and the restoration (%) of the tension from the Rot inhibition by 10⁻⁴ M MSB (filled triangles), or by 5 10⁻⁶ M VK₃ (filled circles). The sample number of the point was 4-6 when S.E.M. was indicated by a vertical bar, and 2-3 without a vertical bar. These values were calculated using the same formula as in Fig. 1.

Restored tension from the Rot inhibition. A typical result is shown in Fig. 1, and the dose-inhibition curve of Pap to the VK₃ restored tension from the Rot inhibition is shown in Fig. 6 (open circles). The relationships between the Rot concentration and the residual tension from Rot inhibition, and between its concentration and the restoration rates by VK₃ or MSB from the Rot inhibition are shown in Fig. 2. From the results in Fig. 2 it is evident that the residue of the tension from the Rot inhibition reaches almost zero at a concentration of 10⁻⁶ M, while the restoration by VK₃ or MSB from the inhibition of Rot at a concentration higher than 10⁻⁸ M decreases according to the increase in Rot concentration.

**Effect of Pap and Rot on isolated DT diaphorase**

Ernster et al. (6) demonstrated that the inhibition of respiration in rat liver mitochondria by Rot (4 x 10⁻⁷ M) can be overcome by the addition of a catalytic amount of VK₃, which
induces a bypass of the Rot-sensitive site on the respiratory chain by utilizing a dicoumarol sensitive enzyme, DT diaphorase, and that VK₃-mediated respiration was inhibited by antimycin A (AnA).

Chromatography of the rat liver soluble fraction of Sephadex G-200 with sodium phosphate buffer eluted DT diaphorase, primarily in fraction 34, corresponding to a molecular weight of 48,000 based on the chromatographic distributions of bovine serum albumin, ovalbumin and myoglobin (Fig. 3). The DT diaphorase of 1 mg oxidized 171 nM NADH/min in the same assay system as in Fig. 3 and the Km for NADH was $7.7 \times 10^{-5}$ M. This enzyme reacted with MSB, ferricyanide and VK₃, but its reaction with MSB was weak (Table 1).

![Figure 3](image)

**Table 1.** Activity of DT diaphorase with different electron acceptors

| Electron acceptor | Concentration (M) | Relative activity |
|-------------------|-------------------|------------------|
| VK₃               | $10^{-7}$         | 1.7              |
|                   | $10^{-6}$         | 3.1              |
|                   | $10^{-5}$         | 4.1              |
|                   | $5 \times 10^{-5}$| 100              |
|                   | $10^{-4}$         | 86               |
|                   | $2 \times 10^{-4}$| 48               |
| MSB               | $10^{-7}$         | 1.7              |
|                   | $10^{-6}$         | 1.7              |
|                   | $10^{-5}$         | 1.7              |
|                   | $10^{-4}$         | 4.0              |
| Ferricyanide      | $10^{-3}$         | 10.3             |

Test system: 0.2 mM NADH, 10 mM Tris-phosphate buffer of pH 7.8, 0.18 mg/ml DT diaphorase protein, final volume 3 ml.
Fig. 4. Effects of dicoumarol, Pap and Rot on DT diaphorase activity. Ordinate, inhibition (%) of NADH oxidation. Abscissa, molar concentration of drugs. Test system: 10 mM of Tris-phosphate buffer, pH 7.8, 0.2 mM NADH, 0.1 mM VK₃, DT diaphorase 0.18 mg protein/ml. Final volume, 3 ml. Open circles, filled circles and filled triangles represent data from dicoumarol, Pap and Rot, respectively.

Fig. 5. Competitive inhibition of DT diaphorase by Pap at a concentration of 10⁻⁶ M. Ordinate, reciprocal optical density change per min at 340 nm. Abscissa, reciprocal NADH concentration represented in mM. Filled circles indicate control and open circles, the presence of Pap. Test system was the same as in Fig. 4.

Fig. 6. Inhibitory effect of Pap on isolated DT diaphorase and on muscle tension development. Abscissa: concentration of Pap. Ordinate: inhibition (%) of Pap on isolated DT diaphorase, same result in Fig. 4 (filled circles, mean value of 2 experiments); inhibition (%) of Pap to the restored tension of the muscle by 5 x 10⁻⁶ M VK₃ from the inhibition of 10⁻⁶ M Rot in 40-K medium, which was calculated by the same procedure of d/c × 100 in Fig. 1 (open circles, mean value of 2-5 experiments).

Fig. 7. Effects of Rot, MSB and Pap on the oxygen consumption of rat liver mitochondria (Mt) during the oxidation of glutamate. The pH 7.4 medium contained 250 mM sucrose, 10 mM KH₂PO₄, 10 mM Tris-Cl, 10 mM KCl, 2 mM MgCl₂, 0.2 mM EDTA and mitochondrial protein of about 3 mg/ml in a final volume of 2 ml.
The DT diaphorase activity was almost completely inhibited by $10^{-7}$ M dicoumarol. Low concentrations of Pap (ID50, $2.8 \times 10^{-7}$ M) or high concentrations of Rot (ID50, $6.5 \times 10^{-6}$ M) inhibited the DT diaphorase activity according to their concentrations and their inhibitions were irrespective of electron acceptors. These results are shown in Fig. 4. As shown in Fig. 5, the inhibition of Pap at a concentration of $10^{-6}$ M was competitive to NADH concentrations. As shown in Fig. 6, there is a progressive relationship between the Pap inhibition of the restored tension from Rot inhibition and the inhibition of the isolated DT diaphorase.

Relation of DT diaphorase to the respiratory chain of mitochondria

Respiration of rat liver mitochondria (Fig. 7), in state 3 or 4 was inhibited by Rot and the inhibition was fully overcome by the addition of MSB. Moreover, the restoration by MSB from the Rot inhibition in the mitochondrial respiration of state 4 was enhanced by the addition of ADP. These results were identical with that of VK₃ in rat liver mitochondria reported elsewhere (6, 8).

As reported in a preceding paper (5), the addition of MSB or VK₃ slightly restored the inhibition of Rot in the mitochondrial preparation of guinea pig taenia coli, but did not do so in the pigeon heart mitochondria lacking DT diaphorase (7). Addition of the isolated DT diaphorase to taenia coli mitochondria with MSB added (respiration had been inhibited by Rot), produced a situation where the inhibition of NADH oxidation was fully restored, and the NADH oxidizing activity of the DT diaphorase coupled with the Rot-treated mitochondria was about 2 fold higher than without the mitochondria. The restored NADH oxidizing activity produced by the addition of the DT diaphorase was again inhibited by application of Pap to the medium. These results are shown in Fig. 8. Furthermore as shown in Fig. 9, in a homogenate of the taenia coli, the addition of MSB overcame the inhibition of NADH oxidation produced by Rot, and this restored NADH oxidation was again inhibited by addition of AnA. These findings are identical to those reported in the

Fig. 8. Effects of Rot, MSB, isolated DT diaphorase and Pap on the NADH oxidation by taenia coli mitochondria (M). The medium contained NaCl, 20 mM; KCl, 25 mM; MgCl₂, 5 mM; 70 mM K₂HPO₄ and 140 mM KH₂PO₄ mixture of pH 7.2; NADH, 0.1 mM; ADP, 0.5 mM; cyt c, 2 $10^{-6}$ M, mitochondrial protein, 0.34 mg/ml; DT diaphorase protein, 0.18 mg/ml in a final volume of 3 ml. At $t'$, the height of initial point of the following curve was adjusted artificially to the end point of the preceding one.
respiration of rat liver mitochondria with VK₃ (6, 8). The addition of MSB to the homogenate did not restore the inhibition induced by Pap.

In rat liver mitochondria, using the same protease digestion isolation procedure as used for taenia coli mitochondria did not alter the effect of VK₃ or MSB on the restoration of respiration. Moreover, in the mitochondrial preparation of taenia coli prepared with Ultra-Turrax without protease, MSB showed an effect similar to the protease-treated taenia coli mitochondria.

Effect of washing out Rot or Pap from the mitochondria on its respiration

The inhibition of Pap on the 40-K induced contracture was readily restored by washing, while that of Rot was not.

The pretreatment of rat liver mitochondria (Table 2), with Rot and subsequent washing (for details of pretreatment, see Materials and Methods) resulted in a persisting inhibition of the oxidation of glutamate (6), whereas the Pap-pretreated and subsequently washed mitochondria, oxidized glutamate at a rate almost equal to that of the washed control.

| Treatment                        | Ratio (mean value of 2 experiments) |
|----------------------------------|-------------------------------------|
| Control, rewashed                | 0.24                                |
| Treated with 10⁻⁴ M Pap, rewashed| 0.21                                |
| Treated with 5 × 10⁻⁷ M Rot, rewashed | 0.00                                |

Ratio represents the ratio of oxidation of 3 mM glutamate to that of glutamate and subsequently added 3 mM succinate in the same mitochondrial preparation. The medium of pH 7.4 contained 250 mM sucrose, 10 mM KH₂PO₄, 10 mM Tris-Cl, 10 mM KCl, 2 mM MgCl₂, 0.2 mM EDTA, 0.5 mM NAD, about 1 mg/ml of mitochondrial protein. The final volume was 2 ml.
DISCUSSION

The inhibition of Rot at the concentration of $5 \times 10^{-7}$M on the 40-K induced tonic tension development of taenia coli was reversed by the addition of a water soluble VK$_3$ derivative, MSB. VK$_3$ and MSB restored the inhibition produced by a high concentration (more than $10^{-6}$ M) of Rot, but the restoration rate decreased proportionally to the increased Rot concentration. The decreasing tendency of VK$_3$ or MSB to restore the tension produced by Rot inhibition coincided with the increasing trend of Rot to inhibit isolated DT diaphorase. It is postulated that the addition of MSB or VK$_3$ to taenia coli establishes a bypass of the Rot-sensitive site in the respiratory chain of mitochondria via DT diaphorase which is contained in the cytoplasm in the immediate vicinity of the mitochondria. Therefore, it is suggested that Rot inhibits the 40-K induced tonic tension development by the inhibition of mitochondrial respiration. The low concentration of Pap, which had no effect on the 40-K induced tension development, inhibited the tension restored by VK$_3$, from the Rot inhibition. The same concentration of Pap also inhibited isolated DT diaphorase. There was a progressive relationship between the inhibition of the restored tension by Pap and the inhibition of the isolated DT diaphorase. Consequently, it was deduced that the low concentration of Pap enters to the taenia coli cells across the cell membrane in the immediate vicinity of the mitochondria and inhibits the DT diaphorase activity, which is linked to the mitochondrial respiratory chain by way of VK$_3$, thus inhibiting the VK$_3$ restored tension from the Rot inhibition in 40-K medium. In our previous papers (1, 5) we stated that effective concentrations of Pap in guinea pig taenia coli inhibited to about the same degree both the mitochondrial respiration acting on the same site as that of Rot and the 40-K induced O$_2$ consumption of the muscle strip. Hence, the lack of restoration by VK$_3$ or MSB, from the inhibition of Pap at the effective concentration in the 40-K induced tonic tension, can be ascribed to its potent inhibitory effect on DT diaphorase activity.

Furthermore, the difference of efficacy in washing out the Pap or Rot from the medium can be ascribed to the differences in their ability to bind to the mitochondria. No other difference between Rot and Pap in their effect on the 40-K induced tonic tension development has been reported. These results support the previous suggestion (1, 5) that inhibitory concentrations of Pap enter the cell and inhibit the mitochondrial respiration, acting on the same site as that of Rot, thereby inhibiting the highly respiration-dependent contracture of guinea pig taenia coli in 40-K medium. The site of action of Pap is shown in scheme 1.

![Scheme 1](image-url)
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