The inhibitory effect of papaverine on respiration-dependent contracture of guinea pig taenia coli in high-K medium. II. Inhibition of mitochondrial respiration

Shuji Tsuda, Norimoto URAKAWA and Jun-ich FUKAMI

Department of Veterinary Pharmacology, Faculty of Agriculture, University of Tokyo, Bunkyo-ku, Tokyo 113,

and *Laboratory of Insect Toxicology, The Institute of Physical and Chemical Research, Wako-shi, Saitama 351, Japan

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Abstract—To elucidate the inhibitory action of papaverine (Pap) on the highly respiration-dependent contracture of guinea pig taenia coli in 40 mM potassium (40-K) medium, the effects of Pap and rotenone (Rot) on the respiration of mitochondria (Mt) were examined using guinea pig taenia coli, rat liver and pigeon heart. The addition of Pap to taenia coli Mt at a concentration which inhibits the muscle contracture rapidly inhibited the aerobic oxidation of glutamate but not succinate. A peak of cytochrome (cyt) b1 was not observed in the Mt which were treated with Pap and contained glutamate. These results were confirmed and extended to rat liver Mt. Both the taenia coli Mt and the heart Mt oxidized NADH when added as a substrate. Pap (10^-5 - 10^-4 M) inhibited the oxidation of NADH by both Mt to almost the same degree as it inhibited the respiration of the muscle strip in 40-K medium. Raising the NADH concentration did not antagonize inhibition by Pap. NADH-ferricyanide reaction in both Mt occurred essentially as reported in the electron transport particle of heart Mt. The reduction of ferricyanide was scarcely inhibited by Pap. The effects of Pap and Rot were identical throughout the experiment. It is suggested that Pap inhibits the electron transport of the mitochondrial respiratory chain between NADH dehydrogenase and coenzyme Q10 (CoQ) on the O2 side of the dehydrogenase, thereby inhibiting the contracture.

We reported (1) that the inhibitory effects of papaverine (Pap) on the 40-K induced tonic tension of the guinea pig taenia coli, which is highly dependent on respiration, were attributed to the inhibition of muscle respiration.

Discussing the effect of Pap on the respiration of Mt, there is only a brief report by Santi et al. in 1963 (2). They showed that Pap inhibited the oxidation of glutamate and β-hydroxybutyrate, but not succinate, in rat liver Mt without affecting the P/O ratio, and that the inhibitory effects of Pap were not overcome by the addition of 2,4-dinitrophenol (DNP). From these results they assumed that Pap inhibited the electron transfer reactions in the mitochondrial respiratory chain between NAD and flavoprotein. Singer et al. reported in 1971 (3) that rhein was the only known inhibitor of this site, while amyntal, Rot and piericidin A act on the O2 side of the flavoprotein. Kean et al. (4, 5) reported that rhein inhibited

† Present Address: Laboratory of Pharmacology, Toxicology Division, The Institute of Environmental Toxicology, Kodaira-shi, Tokyo 187, Japan
the oxidation of NADH-linked substrates in rat liver Mt, but did not inhibit respiration in beef liver Mt. Therefore, the effects of Pap may differ between smooth muscle Mt and rat liver Mt.

However, data on the Mt of smooth muscle have been rarely reported and there is no evidence that Pap inhibits the respiration of smooth muscle Mt.

To clarify this situation, the mode and the site of action of Pap on mitochondrial respiration were investigated in taenia coli Mt, rat liver Mt or pigeon heart Mt.

MATERIALS AND METHODS

Strips of taenia coli were isolated from Hartley strain guinea pigs weighing 400 to 500 g. Mitochondrial fractions were obtained from the taenia coli, pigeon heart and rat liver.

Preparation of Mt: Rat liver Mt were prepared according to the method of Nakazawa (6). Taenia coli and pigeon heart Mt were prepared according to the method of Chance and Hagihara (7), with slight modifications. Such is termed the "protease method" and the procedure was as follows: 4 g of muscle strips isolated from about 20 guinea pigs or from one pigeon were cut into small pieces with scissors and immersed (in 40 ml of a solution containing 0.25 M sucrose, 0.2 mM EDTA, 12.5 mM Tris-phosphate buffer and 20 mg alkali protease of B. subtilis (Sigma Co.) for 20 min at 0°C, pH 7.6. Next, they were homogenized lightly with a loose glass-Teflon homogenizer, allowed to stand for 20 min and diluted in an equivalent volume of solution without protease then homogenized completely with a glass homogenizer. The taenia coli homogenate was centrifuged for 10 min at 600×g and the resulting supernatant was centrifuged at 12,000×g for 20 min to produce the pellet which was used as the mitochondrial fraction. Pigeon heart homogenate was centrifuged for 5 min at 600×g to produce a supernatant which was centrifuged for 20 min at 12,000×g to yield the resultant pellet that was centrifuged for 15 min at 5,500×g. This pellet was used as the pigeon heart mitochondrial fraction. A taenia coli homogenate and Mt were also prepared using the Ultra-Turrax as reported previously (UT method) (8). All procedures were performed at low temperatures (0°C–4°C).

Spectrophotometric measurements: Spectra and absorbancy changes at 340 nm (NADH oxidation) and 420 nm (ferricyanide reduction) were recorded with a multipurpose spectrophotometer (Shimadzu MPS-50L), using cuvettes with a 1 cm light path and a final volume of 3 ml. The measurements were performed at 25°C. When ferricyanide was used as the oxidant, NADH was added just prior to the addition of Mt and the rate of the nonenzymatic reduction of ferricyanide was recorded for a brief period. The Mt were then added to the same sample, the ensuing reaction was recorded and the "blank" rate was subtracted from that of the enzymatic reaction.

Measurements of oxygen consumption in Mt: The oxygen consumption in Mt was measured using a conventional Warburg manometer at 37°C or polarographically with a platinum oxygen electrode using an Oxygen Consumption Recorder Model PO-100 (Yanagimoto Co., Ltd.) at 25°C.

Suspension media: The taenia coli Mt were suspended in the intracellular physiological
solution (ICPS), except for polarographical measurements. The composition of the ICPS was based on the intracellular ion concentrations of guinea pig taenia coli as mentioned previously (8) and contained: NaCl, 20 mM; KCl, 25 mM; MgCl₂, 5 mM; 70 mM K₂HPO₄ and 140 mM KH₂PO₄ mixture of pH 7.2. In polarographical measurements of the taenia coli Mt, and in all experiments utilizing pigeon heart and rat liver Mt, the suspension medium contained 250 mM sucrose, 10 mM KH₂PO₄, 10 mM Tris-Cl, 10 mM KCl, 2 mM MgCl₂ and 0.2 mM EDTA, at pH 7.4. Rot and antimycin A (AnA) were dissolved in acetone and the acetone concentration in the suspension media never exceeded 1 %.

Protein assay: Assay was carried out by the method of Lowry et al. (9).

Drugs: Drugs used were papaverine hydrochloride (Tokyo Kasei Co.), rotenone (prepared by J. Fukami), antimycin A (Kyowa Hakko Co.), 2,4-dinitrophenol (Wako Pure Chemical Co.), flavine mononucleotide (Boehringer Mannheim GmbH), coenzyme Q₁₀ (gift from Dr. H. Fukawa, Nissin Flour Milling, Central Research Laboratory), cyclic 3',5'-adenosine monophosphate (Kyowa Hakko Co.), D-600 (Knoll A. G.), menadione sodium bisulfite (Kongo Kagaku Co.), and sodium azide (Wako Pure Chemical Co.).

RESULTS

Property of the subcellular fractions

Based on the difference spectra of oxidized and reduced pigments, no appreciable difference between the UT method and the protease method was observed in the homogenates and the Mt of taenia coli. The spectra of the homogenate and the Mt were essentially the same as those reported in rat liver (10, 11). The electron micrograph of the Mt prepared using the protease method showed that the mitochondrial fraction contained almost pure Mt about 0.2-0.4 × 0.3-0.5 μm in size. However, the matrix density of much of the Mt was low. The Mt may have been partially digested by the protease.

Effects of Pap on difference spectra of taenia coli Mt

As shown in Fig. 1, a typical cyt b₁₁ peak of the taenia coli Mt was obtained by the addition of AnA and a substrate of 1-glutamate or succinate in the aerobic state. Using 1-glutamate as the substrate, the cyt by peak did not appear in the Pap treated Mt. This is true of Rot tested in other Mt (12). AnA did not produce a peak in the presence of Pap or Rot, however the subsequent addition of succinate as a substrate did produce a cyt by peak.

Effects of Pap on the aerobic oxidations of substrates in Krebs cycle by Mt

Santi et al. (2) reported that Pap inhibited the oxidation of glutamate and 3-hydroxybutyrate in rat liver Mt. The Krebs cycle plays an important role in energy metabolism, therefore, experiments were performed using various components of the Krebs cycle as substrates plus glutamate for rat liver Mt oxidations. The effect of Pap on the oxidation of 3 mM of the following substrates, malate plus pyruvate, α-ketoglutarate, 1-glutamate or succinate, were examined using polarographical measurement. Pap at concentrations sufficient to inhibit the contracture (2.5-5 × 10⁻⁵ M) inhibited the oxidation of all the NADH-
linked substrates tested by about 80% but did not inhibit that of succinate. Addition of 0.1 mM DNP, 1 mM NAD, 0.5-2 mM FMN, 1 mM CoQ and 0.02 mM cyt c did not antagonize the Pap inhibition. Bovine serum albumin prevented the inhibition of Pap and 5 \times 10^{-6} M Rot. The effect of Pap on the ADP/O ratio was examined at 2.5 \times 10^{-6} M in the presence of a glutamate substrate and at 10^{-4} M utilizing a succinate substrate. Both concentrations of Pap produced a slight inhibition of the ADP/O. Rat liver Mt did not utilize NADH as a substrate. However the addition of 2 \times 10^{-5} M cyt c increased the oxidation of NADH by the Mt. Pap (10^{-4} M) as well as AnA (2 \times 10^{-6} M) did not inhibit the NADH oxidation in the presence of cyt c. Cyclic AMP (10^{-4}-10^{-3} M) and D 600 (10^{-5}-10^{-4} M) had no effect on the Mt respiration in the presence of 1-glutamate and succinate as substrates. Addition of 1% acetone had no effect on oxidative phosphorylation in rat liver Mt.

In guinea pig taenia coli Mt, Pap at 2.5 \times 10^{-5} M inhibited the aerobic oxidation of 1-glutamate by about 70%, but had no effect on succinate as measured with a conventional Warburg manometer (Fig. 2). It can be seen in Fig. 3 that 5 \times 10^{-7} M Rot and 2.5 \times 10^{-5} M Pap inhibited state 3 respiration of taenia coli Mt produced by 1-glutamate as the substrate.
FIG. 2. Effects of Pap on oxidation of the L-glutamate and the succinate of the taenia coli Mt prepared with the protease method, measured using a Warburg manometer. The Mt, 3-4 mg protein/ml, were suspended in ICPS containing the final concentration of $10^{-5}$ M cyt c, $10^{-4}$ M NAD, $10^{-3}$ M ATP, $5 \times 10^{-5}$ M CaCl$_2$, $3 \times 10^{-2}$ M substrate. Total volume 3 ml; pH 7.2; 37 °C. Filled circles represent control and open circles, the presence of $2.5 \times 10^{-5}$ M Pap. Data represent the mean value of 2-4 experiments.

FIG. 3. Effects of Rot and Pap on L-glutamate oxidation of the taenia coli Mt as measured polarographically. The Mt, 2.9 mg/ml (upper) and 2.8 mg/ml (lower) of protein, were diluted with the suspension medium containing $10^{-5}$ M cyt c and 0.1 mM NAD; pH 7.4; 25 °C; total volume 2 ml. Final concentration of 3 mM L-glutamate, 1.25 mM ADP, $5 \times 10^{-7}$ M Rot, $2.5 \times 10^{-5}$ M Pap, 1 mM MSB and 3 mM succinate were added as indicated.

FIG. 4. Effect of raising the concentration of NADH on the Pap inhibition to NADH oxidation of guinea pig taenia coli Mt. The Mt, 0.25 or 0.5 mg/ml of protein, were suspended in ICPS containing $2 \times 10^{-5}$ M cyt c and 0.5 mM ADP in a final volume of 3 ml. NADH oxidation was measured by means of the optical density change at 340 nm. Filled circles indicate control and open circles, the presence of $2.5 \times 10^{-5}$ M Pap. Data represent the mean value of 2-7 experiments.
The inhibition of Pap reached a steady level within 1 min. The addition of 1 mM menadione sodium bisulfite (MSB) had little effect on the inhibition of Rot, and no effect on Pap. The addition of succinate reversed the inhibited respiration of the Mt. In this case (upper trace of Fig. 3) the taenia coli Mt produced a respiratory control ratio of about 2.6, which was smaller than 5–10 of rat liver Mt prepared by the same protease method.

Effects of Pap on the aerobic oxidation of added NADH and on the reduction of ferricyanide with NADH in the Mt

The Mt of taenia coli and pigeon heart both oxidized NADH when added as a substrate. Pap and Rot rapidly inhibited (within 1 min) the aerobic oxidation of added NADH by taenia coli Mt to almost the same degree as they inhibited the oxidation of the NADH-linked substrate, even in the presence of added cyt c. The inhibitory effect of Pap was not antagonized by raising NADH concentration (Fig. 4). Similar results were obtained in pigeon heart Mt. The oxidation of added NADH in the aerobic state by taenia coli Mt was increased more than 10 fold by the addition of 1 mM ferricyanide. As shown in Fig. 5, Pap (10^{-5}–10^{-4} M) inhibited the NADH oxidation by the taenia coli Mt in aerobic state in proportion to its concentration in the absence of ferricyanide. The inhibition values of Pap were much the same as those of the increased respiration of the muscle strip in 40-K medium (1). High

![Fig. 5. Inhibitory effects of Pap on the oxidation of NADH catalyzed by guinea pig taenia coli Mt in an aerobic state with (open circles) or without (filled circles) 1 mM K_3[Fe(CN)_6]. NADH oxidation was measured by means of optical density change at 340 nm. Mt, about 0.5 mg ml protein, were suspended in ICPS containing 0.1 mM NADH, 2 × 10^{-5} M cyt c and 0.5 mM ADP. Data represent the mean value of 2 experiments.]

![Fig. 6. Inhibitory effects of Pap and Rot on the aerobic oxidation of NADH and on the K_3[Fe(CN)_6] reduction with NADH catalyzed by pigeon heart Mt. NADH oxidation and K_3[Fe(CN)_6] reduction were measured according to optical density change at 340 nm and 420 nm, respectively. For NADH oxidation about 0.3 mg ml protein of Mt were suspended in the medium containing 0.1 mM NADH. For K_3[Fe(CN)_6] reduction about 0.05 mg ml protein of Mt were suspended in the medium containing 0.1–0.15 mM NADH and 1.5–2 mM K_3[Fe(CN)_6]. Filled circles and filled triangles represent the inhibition of NADH oxidation by Pap and Rot, respectively. Open circles and open triangles represent the inhibition of K_3[Fe(CN)_6] reduction by Pap and Rot, respectively.]

concentration of Pap \((10^{-4} - 5 \times 10^{-4} \text{ M})\) weakly inhibited the oxidation of NADH in the presence of ferricyanide. In pigeon heart Mt, the effective reduction of ferricyanide \((1.5 \text{ mM})\) by NADH \((0.15 \text{ mM})\) was obtained. The reduction was increased by 5% by 1 mM azide and inhibited 5-10% by \(10^{-6} \text{ M} \text{ AnA}\). Pap and Rot both inhibited the NADH oxidation by pigeon heart Mt in the absence of ferricyanide but neither drug inhibited the NADH reduction of ferricyanide by the Mt (Fig. 6). Furthermore, the pre-incubation of the Mt with \(10^{-4} \text{ M} \text{ Rot}\) for 5 min completely abolished the inhibition of \(10^{-4} \text{ M} \text{ Pap}\) to ferricyanide reduction.

**Effects of EGTA and cyclic AMP on the oxidation of NADH by taenia coli homogenate**

Pap \((10^{-4} \text{ M})\) rapidly inhibited the oxidation of NADH by homogenates (UT method) of taenia coli in ICPS with or without 1 mM EGTA. The inhibition was not changed by the presence of 1 mM EGTA and reached a steady level within 1 min. The addition of 1 mM cyclic AMP had no effect on the oxidation or on the inhibition of Pap.

**DISCUSSION**

In the preceding paper (1) it was suggested that the inhibition of Pap on the 40-K induced tonic tension resulted from respiration inhibition, and that a rapid inhibition of mitochondrial respiration by Pap was required.

The taenia coli Mt and pigeon heart Mt both utilized exogenous NADH as a substrate, as reported for freshly prepared pig heart Mt (13). Rat liver Mt did not utilize exogenous NADH, as was reported earlier (14). Rot and Pap inhibited the oxidation of added NADH by taenia coli Mt, even in the presence of added cyt c. Pap and AnA did not inhibit this oxidation by rat liver Mt in the presence of added cyt c as efficiently as did Rot (15). Taenia coli Mt may not have the same cyt b; pathway as rat liver Mt (15). As shown in Fig. 4 MSB had almost no effect on the Rot inhibition of respiration by taenia coli Mt, while MSB and vitamin K3 both reversed the Rot inhibition in rat liver Mt (16). Therefore, the taenia coli Mt prepared by the protease method may contain a small quantity of DT diaphorase.

Despite the differences referred to above, Pap and Rot both inhibited the NADH-linked respiration of taenia coli Mt, pigeon heart Mt and rat liver Mt almost equally. The magnitude of inhibition of Pap on the mitochondrial respiration was similar to the increased respiration of the taenia coli strip in 40-K medium (1).

In rat liver Mt, Pap inhibited the NADH-linked substrates in Krebs cycle. The results of these experiments on ADP/O ratio and the effect of DNP were compatible with the data of Santi et al. (2). Both Pap and Rot inhibited the oxidation of added NADH substrate in both taenia coli Mt and pigeon heart Mt. When glutamate was used as substrate, the cyt b; peak was not observed in the taenia coli Mt treated with Pap or Rot. Pap did not inhibit the oxidation of succinate by the Mt. These results suggest that Pap may inhibit the electron transport of mitochondrial respiratory chain between NADH and CoQ, without affecting the energy transfer system.

Investigations with chemicals which might interfere with Pap showed only bovine serum albumin to be effective. Keun (4) reported that added NAD reversed the rhein induced
inhibition of glutamate oxidation in rat liver Mt. This is a remarkable difference between Pap and rhein.

The identification of the sites of inhibition in the NADH dehydrogenase region of the respiratory chain was mainly obtained from the different effects of the inhibitors on the ferricyanide reduction of NADH, catalysed by the respiratory chain-linked NADH dehydrogenase (5, 13, 17–19). The electron transport particle (ETP) of Mt obtained by Crane et al. (20) contained NADH dehydrogenases, most of which were respiratory chain-linked dehydrogenases (13, 17). It was reported that pigeon heart Mt had no DT diaphorase (21), which catalyzes NADH-ferricyanide reaction. The similarity of the NADH-ferricyanide reaction to the drugs between the pigeon heart Mt of the present experiment and the reported ETP (13, 17) showed that the main part of the NADH-ferricyanide reaction of the heart Mt may be directly catalyzed by the respiratory chain-linked NADH dehydrogenase. From the similarity of Pap and Rot in the NADH-ferricyanide reaction and from the fact that NADH did not antagonize Pap inhibition, it is tentatively concluded that the site of action of Pap is on the O₂ side of NADH dehydrogenase as shown in scheme 1.

It was reported that the protease method is an excellent method to prepare the mitochondrial fraction, however, even with this method it is most difficult to obtain intact Mt from smooth muscle (22). Since the cell membrane of guinea pig taenia coli is strong and the quantity of the tissue is small, the tissue cannot be used for the homogenization with a glass-Teflon homogenizer and repeated washing of the mitochondrial fraction. Furthermore, the small sizes of the Mt make it difficult to separate the Mt from the microsomal fraction. The taenia coli Mt showed a low respiratory control ratio (Fig. 3), which may have resulted from damage during the isolation procedure. Rot, which has been considered to be a highly specific inhibitor of NADH oxidation by the respiratory chain (3, 15), however, inhibited the oxidation of added NADH and glutamate by the Mt.

Pap inhibited the NADH-linked respiration of the taenia coli Mt in the medium which imitated the intracellular ion composition of taenia coli tissue to almost the same degree as in the conventional medium. Furthermore, a similar effect of Pap was observed in the taenia coli homogenate independent of Ca⁺⁺ concentration. Cyclic AMP had no effect on the respiration of the Mt and on the homogenate. Hence, it is deduced that the inhibitory effect of Pap on the mitochondrial respiration functions in the intact smooth muscle cells of guinea pig taenia coli independent of its effect on Ca movement and on phosphodiesterase
activity. The essential role of NADH-linked respiration in the smooth muscle respiration in 40-K medium was reported previously (8). Moreover, the inhibition of Pap to the NADH-linked respiration reached a steady level within 1 min in both the homogenate and in the Mt of taenia coli. Therefore, it is suggested that Pap inhibits the electron transport of the mitochondrial respiratory chain between NADH and CoQ at the O2 side of the dehydrogenase, thus inhibiting the 40-K induced tonic tension development.

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