ANTIPHOSPHODIESTERASE ACTIVITY AND NONSPECIFIC
SMOOTH MUSCLE RELAXATION TESTED ON
INTESTINAL SMOOTH MUSCLES

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Abstract—Mitochondrial, microsomal and soluble fractions separated from the guinea pig taenia and from the dog longitudinal smooth muscle were used as phosphodiesterase preparations. Each preparation had low and high Km values, indicating the existence of at least two kinds of phosphodiesterase. Papaverine and Aspaminol (1, 1-diphenyl-3-piperidinobutanol hydrochloride), hydralazine, caffeine Na benzoate and aminophylline were used as test drugs. Aspaminol had little inhibitory effect on phosphodiesterase. Ki value of papaverine almost equalled the concentration (M) which was necessary to produce 50% relaxation. Relaxation of the guinea pig taenia by papaverine was preceded by an increase of intracellular cyclic AMP. Therefore, the action of papaverine is likely to be mediated by an increase in cyclic AMP, which is caused by inhibition of the phosphodiesterase-catalyzed breakdown of cyclic AMP. There was little correlation between relaxing activities of the drugs used and their antiphosphodiesterase activities. Relaxation of the smooth muscle induced by the smooth muscle relaxants excepting papaverine is not due to inhibition of phosphodiesterase.

Papaverine, a nonspecific smooth muscle relaxant, is well known to be a potent phosphodiesterase inhibitor (1, 2). Evidence has been also presented that papaverine increases the intracellular cyclic AMP level in some smooth muscles (3-7). On the other hand, Aspaminol (1, 1-diphenyl-3-piperidinobutanol hydrochloride, Kowa Co. Ltd., Japan) and benactyzine hydrochloride, synthetic antispasmodics, have a papaverine-like nonspecific relaxing action in high concentrations. These drugs have, however, been found to have little inhibitory effects on phosphodiesterase activity in concentrations which have a maximum antispasmodic action (7, 8). Furthermore, Aspaminol does not increase the intracellular cyclic AMP in concentrations which have a relaxing action (6, 9). These results suggest the possibility that the nonspecific relaxing action or papaverine-like action of antispasmodics is not due to an increase in the intracellular cyclic AMP, which is caused by inhibition of the phosphodiesterase-catalyzed breakdown of cyclic AMP. The present paper describes modes of action of papaverine and some nonspecific smooth muscle relaxants or so-called papaverine-like drugs on phosphodiesterase. The tests have been also carried out to determine whether or not relaxation of the intestinal smooth muscle produced by all the nonspecific smooth muscle relaxants or so-called papaverine-like drugs is mediated by the increase in the intracellular cyclic AMP, which is caused by inhibition of phosphodiesterase.
Some of the results were at the Second International Conference on Cyclic AMP (10).

MATERIALS AND METHODS

Estimation of phosphodiesterase activity.

Enzyme preparations used were obtained from the male guinea pig taenia and from the male dog intestine.

Dog intestine: A dog was anesthetized with sodium pentobarbital 30 mg/kg (i.v.) and after blood depletion by cutting the carotid arteries, the small intestine was isolated. The isolated small intestine was immediately immersed in oxygenated Locke Ringer solution. After blood vessels and the adipose tissue were removed, the mucous layer was peeled off from the intestine and then the circular muscle was removed.

Guinea-pig taenia: After sacrificing a male guinea pig by a blow on the neck, the taenia was isolated from the caecum.

Each smooth muscle was homogenized in 8 volumes of 0.25 M Tris-Cl (pH 7.5) with Polytrone (P.T. - 10 Brinkman Instruments) with rheostat setting on 8 for 5 seconds, three times. The homogenate was centrifuged at 1,000 x g for 15 min at 0-4°C. In most of the experiments, the 1,000 x g supernatant was used as an crude enzyme preparation. In some experiments, the 1,000 x g supernatant was further separated into mitochondrial, microsomal and soluble fractions. The 1,000 x g supernatant was centrifuged at 7,000 x g for 20 min. The resulting pellet was used as a mitochondrial fraction. The 7,000 x g supernatant was again centrifuged at 50,000 x g for 10 min. The 50,000 x g precipitates and supernatant were used as a microsomal fraction and a soluble fraction, respectively. These fractions were used as enzyme preparations. Phosphodiesterase activity was estimated with radioactively labeled cyclic AMP (adenosine 8-labeled ³H-cyclic AMP, Dai-ichi Pure Chemicals Co., Ltd. Japan) as a substrate. An appropriate dilution of enzyme was incubated in 0.4 ml of 40 mM Tris-Cl buffer solution (pH 7.5) containing 5 mM MgCl₂, 3 mM 2-mercaptoethanol and 10⁻⁷ M ³H-cyclic AMP. After 10 min incubation at 30°C the reaction was terminated by boiling for 3 min. In order to change 5'-AMP into adenosine, 5'-nucleotidase (1 unit/ml) was added into the reaction mixture at 37°C for 2 min. The reaction products were separated by paper chromatography (a solvent: a mixture of ethanol 70 ml and 1 M ammonium acetate 30 ml) and radioactivity of separated adenosine was counted in 10 ml of scintillation fluid. The rates of hydrolysis of substrates were adjusted to 20 to 60% in control samples. An antiphosphodiesterase activity of a test drug was shown as a dissociation constant (Ki-value).

Estimation of intracellular cyclic AMP concentration.

Two pieces of the taenia were isolated from one caecum of the guinea pig. One of them was used for measuring the control level of the cyclic AMP and the other for estimating any change of intracellular cyclic AMP content after drug treatment. The taenia was frozen in liquid nitrogen and used for measurement of cyclic AMP, immediately after the response to the drug was recorded. Measurement of the intracellular cyclic AMP was
carried out by the method of Gilman (11).

**Estimation of relaxing activities of drugs.**

A piece (4 to 5 cm) of the taenia isolated from the guinea pig caecum was suspended in a 30 ml organ bath filled with Locke Ringer solution, kept at 37°C and bubbled with air. Responses of the taenia to the drugs were recorded isotonically. Concentration action curves of the drugs were cumulatively obtained and relaxing activities of the drugs were estimated as negative logarithm of 50% effective concentration (M) (ED_{50}). Locke Ringer solution had the following composition (g/l): NaCl 9.0, KCl 0.4, CaCl₂ 0.2, NaHCO₃ 0.5 and glucose 0.5.

## RESULTS

**Antiphosphodiesterase activity and antispasmodic action**

Figure 1 shows a double reciprocal plot of cyclic AMP hydrolysis as a function of substrate concentration in the 1,000×g supernatant of the homogenate of the dog intestine. Extrapolation of the linear portion of these Lineweaver-Burk plots yields two apparent Michaelis constants \( (6.7 \times 10^{-8} \text{M} \) and \( 9.1 \times 10^{-3} \text{M} \) for cyclic AMP hydrolysis (Fig. 1). Quite similar results were obtained in all the other enzyme preparations from guinea pig taenia and from dog small intestine. Papaverine inhibited low Km phosphodiesterase

![Fig. 1. Kinetic analysis of cyclic AMP hydrolysis tested on the 1,000×g supernatant of the homogenate of the dog intestine. The unit of velocity is moles/μg of protein/min.](image-url)
from the dog intestine noncompetitively (Ki: 5.6 × 10⁻³ M) whereas it inhibited high Km enzyme competitively (Ki: 5.8 × 10⁻⁵ M). These results are shown in Figs. 2 and 3. Aspaminol, a synthetic antispasmodic drug, had little inhibitory action on either enzyme (Fig. 2).

**Fig. 2.** Effects of papaverine and Aspaminol (1,1-diphenyl-3-piperidinobutanol hydrochloride) on low Km phosphodiesterase in the 1,000 × g supernatant of the homogenate of the dog intestine. The unit of velocity is moles/μg of protein/min.

**Fig. 3.** Effects of papaverine on high Km phosphodiesterase in the 1,000 × g supernatant of the homogenate of the dog intestine. The unit of velocity is moles/μg of protein/min.
The following experiments were done to determine whether or not relaxing actions of some nonspecific smooth muscle relaxants or so-called papaverine-like drugs are concerned with inhibition of phosphodiesterase. The 1,000 xg supernatant of the homogenate of the guinea pig taenia was used as an enzyme preparation. Caffeine Na benzoate and hydralazine inhibited the low and high Km enzymes noncompetitively. Papaverine and aminophylline, however, inhibited the low Km enzyme noncompetitively and the high Km enzyme competitively. Antiphosphodiesterase activities of the test drugs were presented as negative logarithm of Ki values. As shown in Table 1, negative logarithm of ED50-value of papaverine almost equalled negative logarithm of its Ki value. Though other drugs used had almost the same relaxing activities (ED50-values), the antiphospho-

| Drug           | Negative Log of K_i on low Km | Negative Log of K_i on high Km | Negative Log of ED50 (mean ± S.E.) |
|----------------|-------------------------------|--------------------------------|-----------------------------------|
| Papaverine     | 4.3                           | 4.3                           | 4.7 ± 0.2                         |
| Aminophylline  | 3.1                           | 2.5                           | 3.8 ± 0.2                         |
| Caffeine       | 2.8                           | 2.3                           | 3.6 ± 0.2                         |
| Hydralazine    | 2.1                           | 2.0                           | 3.7 ± 0.3                         |
| Asaminol       | 0.7                           | 0.7                           | 4.3 ± 0.1                         |

PDE: phosphodiesterase

Fig. 4. Increase of the intracellular cyclic AMP and relaxation of the taenia caecum of the guinea pig produced by papaverine (10^-5 g/ml). Solid line: relaxation, solid bars: increased amount (%) of cyclic AMP, time: after applied papaverine (10^-5 g/ml). The mean (± S.E.) intracellular cyclic AMP content of the untreated taenia was 0.68 ± 0.07 moles/mg of wet tissue.
diesterase activities (Ki values) were considerably different (Table 1). Therefore it can be concluded that there is little correlation between the smooth muscle relaxing activities and the antiphosphodiesterase activities.

**Intracellular cyclic AMP and action of papaverine**

Papaverine (10⁻³ g/ml) relaxes the taenia from the guinea pig caecum as is already well known. This relaxation was preceded by an increase in the intracellular cyclic AMP level as shown in Fig. 4.

**DISCUSSION**

It is generally conceded there are at least two kinds of phosphodiesterase in mammalian tissues, since their apparent Km values are different. Thompson and Appleman (12) suggested that low Km enzyme is normally membrane bound and high Km enzyme is presumed to be of cytoplasmic origin in the brain. In the present study, two Km values were found in phosphodiesterase in the mitochondrial, microsomal and soluble fractions. Ki value of papaverine on phosphodiesterase was almost the same as its ED₅₀-value indicating the smooth muscle relaxing activity, and increase in the intracellular cyclic AMP preceded relaxation of the isolated taenia induced by papaverine. The action of papaverine at least in the concentrations used in this study is likely to be mediated by the increase in the intracellular cyclic AMP, which is caused by inhibition of phosphodiesterase.

Pöch and Kukovetz (13) reported that there was a definite correlation between relaxing activities of some drugs and their antiphosphodiesterase activities in the vascular smooth muscle. Since, in this study, there was no correlation between the nonspecific relaxing activities of the drugs and their antiphosphodiesterase activities in the intestinal smooth muscle, nonspecific relaxing action or so-called papaverine-like action of the drugs excepting papaverine is not mediated by the increase in cyclic AMP, which is caused by inhibition of phosphodiesterase.

Takagi et al. (14) have already reported that nonspecific smooth muscle relaxants or so-called papaverine-like antispasmodics are divided into two groups according to their mechanisms of action: strong basic substances such as Aspaminol and benactyzine belong to one group, and the other group includes weak bases such as papaverine and neutral substances such as isoamylesters, whose action is considered to be due to physico-chemical properties of their nonionic molecules. Papaverine may be able to penetrate into and through smooth muscle membrane to inhibit phosphodiesterase, because most molecules exist as a nonionized form in physiological hydrogen ion concentrations. Aspaminol and benactyzine, however, did not inhibit phosphodiesterase activity in the taenia from the guinea pig caecum (8). And it has been also reported in the previous papers (6, 9) that the action of Aspaminol is not mediated by the increase in intracellular cyclic AMP. Furthermore, it has been made clear in the present study that Aspaminol does not affect the high and low Km phosphodiesterases and that there is little correlation between relaxing activities of the drugs and their antiphosphodiesterase activities. It can be concluded from the observations mentioned above that there are at least two mechanisms for the nonspecific
smooth muscle relaxants or the so-called papaverine-like antispasmodics: one is due to
the increase of the intracellular cyclic AMP, which is caused by inhibition of phospho-
diesterase and the other is not concerned with the intracellular cyclic AMP level.

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