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# Antispasmodic Action of 1-Diethylaminoethyl-3-(*p*-methoxybenzyl)-2-quinoxalone (P 201-1) and Its Inhibitory Effect on Cyclic 3′,5′-Nucleotide Phosphodiesterase (PDE) Activity

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The action on various smooth muscle of 1-diethylaminoethyl-3-(*p*-methoxybenzyl)-2-quinoxalone (P 201-1) and its inhibitory effect on the phosphodiesterase (PDE) activity in the supernate prepared from the rat ileum, cerebral cortex, and liver were investigated by comparison with those of papaverine.

P 201-1 had more potent antispasmodic action on the isolated guinea pig ileum, gastric smooth muscle and uterus of the rat, rabbit ileum and so on than papaverine, and showed little influence upon the autonomic nervous system.

P 201-1 inhibited the PDE activity in the supernate prepared from the rat ileum, cerebral cortex, and liver. The *Ki* values of P 201-1 for the supernate of the ileum, cerebral cortex, and liver were 1370, 700, and 650 μM, respectively, and the values were 5—10 times those of papaverine. The inhilitory effect of P 201-1 on PDE from the rat ileum or cerebral cortex was competitive and that from the liver noncompetitive, and the type of inhibition of P 201-1 was different from that of papaverine in each tissue. When the influence of $Mg^{2+}$ concentration on the inhibitory effect of P 201-1 was examined, the liver PDE inhibition of P 201-1 occurred in a different way from the ileum or cerebral cortex PDE inhibition, and completely disappeared at high concentration of $Mg^{2+}$ (10 mM).

The mechanism of smooth muscle relaxation induced by so-called "papaverine-like" drugs has not completely been clarified yet. It was reported that the smooth muscle relaxing effect of epinephrine in the guinea pig taenia coli was mediated by cyclic 3′,5′-adenosine monophosphate (3′,5′-AMP) levels.[2] Moore, *et al.*[3] reported that the isoprenaline-induced relaxation of the isolated guinea pig trachea was stimulated by dibutyryl cyclic AMP. Recently, Pöch, *et al.*[4] reported that smooth muscle relaxants such as papaverine were potent inhibitors of phosphodiesterase (PDE) and showed the relaxing effect especially on the coronary artery. Moreover, based on the above-described fact, Pöch, *et al.*[4b] suggested that the smooth muscle relaxing effect of papaverine-like drugs was attributable to their inhibitory action on PDE and was mediated by accumulation of cyclic 3′,5′-AMP. Furthermore, it was reported by Triner, *et al.*[5] that papaverine inhibited PDE activity also in the homogenates of the uterus, vascular and striated muscle tissues *in vitro*.

On the other hand, Hornkiewiez, *et al.*[6] studied on quinoxaline derivatives, found 1-diethylaminoethyl-3-(*p*-methoxybenzyl)-2-quinoxalone (P 201-1) which showed the most potent antispasmodic action, and reported that the antispasmodic action of P 201-1 in the isolated guinea pig ileum was about twenty times as potent as that of papaverine and that

1) Location: *a*) *3–2, 1-Chome, Minamidai, Kawagoe-shi*; *b*) *Aobayama, Sendai-shi.*
2) E. Bueding and E. Bülbring, "Pharmacology of Smooth Muscle," Pergamon Press, Oxford, 1964, p. 37; R.W. Butcher, I. Hawkins, A.R. Timms, and E.W. Sutherland, *Biochim. Biophys. Acta*, **115**, 173 (1966).
3) P.F. Moore, L.C. Iorio, and J.M. McManus, *J. Pharm. Pharmacol.*, **20**, 368 (1968).
4) *a*) G. Pöch, H. Juan, and W.R. Kukovetz, *Naunyn-Schmiedebergs Arch. Pharmak.*, **264**, 293 (1969); *b*) W.R. Kukovetz and G. Pöch, *ibid.*, **267**, 189 (1970); *c*) G. Pöch and W.R. Kukovetz, *Life Sci.*, **10**, 133 (1971).
5) L. Triner, Y. Vulliemoz, I. Schwartz, and G.G. Nahas, *Biochem. Biophys. Res. Commun.*, **40**, 64 (1970).
6) A.L. Hornkiewiez, G. Hitzenberger, and W.H. Zellner, *Wiener Klin. Wochenshrift*, **75**, 189 (1963).

P 201-1 was a musculotropic antispasmodic agent with no atropine-like parasympatholytic action.

In the present paper, the antispasmodic effect of P 201-1 on various smooth muscle by comparison with that of papaverine and the inhibitory effect of P 201-1 on PDE are studied.

## Experimental

**Preparation of the Drug**——P 201-1 is a yellowish white, odorless crystalline powder and has the melting point of 69—71°. It is freely soluble in ether and in alcohol and sparingly soluble in water. P 201-1 as the hydrochloride salt is dissolved in water up to the concentration of 2%. In this experiment, P 201-1 as the hydrochloride salt was employed and papaverine hydrochloride as control. The chemical structure of P 201-1 is shown in Fig. 1.

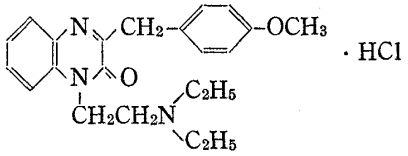

Fig. 1. Chemical Structure of P 201-1

**Effect on the Isolated Guinea Pig Ileum**——The inhibitory effect of P 201-1 on the contraction of the ileum induced by barium chloride, histamine and acetylcholine (Ach) was examined. A strip of the isolated guinea pig ileum was mounted in a magnus bath containing oxygenated Tyrode's solution at 22° (anti-barium action) or 28° (anti-histaminic and anti-Ach actions). One liter of the Tyrode's solution contained 8.0 g of NaCl, 0.2 g of KCl, 0.2 g of $CaCl_2$, 0.1 g of $MgCl_2$, 0.05 g of $Na_2HPO_4$, 1.0 g of $NaHCO_3$, and 1.0 g of glucose.

**Effect on the Isolated Rat Gastric Smooth Muscle**——Male rats of Sprague-Dawley JCL strain were sacrificed and the stomach with the vagus was isolated by cutting it off at the part of the lower portion of the esophagus. The esophagus was ligated at the upper part of the cardia and the vagus was separated from the esophagus. A cannula was then inserted into the stomach through the pyrolus. The isolated stomach was mounted in a 50 ml organ bath containing oxygenated Tyrode's solution at 37° with the mixed gas of 95% $O_2$ and 5% $CO_2$. The endogastric pressure was recorded on a polygraph with a low pressure transducer. The endogastric pressure was adjusted to 20—30 mmHg prior to recording.

**Effect on the Isolated Guinea Pig Tracheas**——The isolated tracheal chain preparation was mounted in a magnus bath containing Tyrode's solution at 37°. The inhibitory effect of P 201-1 on the contraction induced by Ach or histamine was recorded on a kymograph.

**Effect on the Motility of the Isolated Rabbit Ileum**——In order to examine the effect of P 201-1 on the intestinal motility, the isolated ileum from the 24 hr-fasted rabbit was mounted in a magnus bath containing Tyrode's solution at 37°. The amplitude was used as an index for the action of P 201-1.

**Effect on the Motility of the Isolated Rat Uterus**——Virgin female rats of Sprague-Dawley JCL strain weighing 150—180 g were used. In order to exclude variation in response of the uterus to P 201-1, the ovary was removed under pentobarbital anesthesia (30 mg/kg, *i.p.*). After one week, the rats were killed by exsanguination and the uterine horn of each side was isolated. The isolated uterine horn was mounted in a magnus bath containing a modified Locke-Ringer's solution with low concentration of $Ca^{2+}$ (9.0 g of NaCl, 0.42 g of KCl, 0.1 g of $CaCl_2$, 0.5 g of $NaHCO_3$, and 1 g of glucose per liter) at 27°. Then, the effect of P 201-1 on the uterine motility was examined.

**PDE Preparation from Rat Ileum, Cerebral Cortex and Liver**——Sprague-Dawley JCL male rats weighing 250—300 g were decapitated, and the brain, ileum, and liver were immediately isolated and chilled in icecold solution containing 0.32 M sucrose and 50 μM Tris-HCl buffer at a final pH of 7.4. Continuously, tissues of each isolated organ were homogenized in the same icecold isotonic solution nine times the volume of the tissues using a teflon homogenizer. The homogenate was centrifuged for 10 min at 1000 ***g*** at 0° and the nuclei fraction was discarded. The supernate was centrifuged at 10000 ***g*** for 20 min at 0° and the mitochondrial fraction was discarded. The supernate was centrifuged at 105000 ***g*** for 60 min at 0° and the supernate thus obtained was used as an enzyme preparation.

**Enzymic Assay**——All PDE assays were performed by the method of Pöch.[7] with a cyclic 3′,5′-AMP concentration of $3.6 \times 10^{-4}$ M as the substrate unless otherwise described. Enzyme activity w as measured as the rate of hydrolysing cyclic 3′,5′-AMP in a standard reaction medium (final volume 500 μl) containing $^3$H-cyclic 3′,5′-AMP (0.05 μCi), 3 mM Mg-acetate, 2 mM 5′-AMP, 100 mM Tris-HCl buffer (pH 7.4) and 100 μl of enzyme preparation containing 0.4—3 mg of protein per ml.

Incubations were run at 37° for 15 min (cerebral cortex) or for 40 min (other tissues) and the reaction was stopped by adding 200 μl of 0.17 M $ZnSO_4$ and 200 μl of 0.15 M $Ba(OH)_2$. After centrifugation (2500 rpm, 10 min), 300 μl of the supernate was transferred into a counting vial with 10 ml of scintillation medium (4 g of PPO, 100 mg of dimethyl POPOP, 1000 ml of toluene, 400 ml of 99.5% ethanol and 100 ml of dioxane). The radioactivity was then determined by a liquid scintillation spectrometer (Model 3380, Paccard Co.).

7) G. Pöch, *Naunyn-Schmiedebergs Arch. Pharmak.*, **268**, 299 (1971).

The results were corrected for quenching by the use of internal standards. PDE activity was determined after incubation with $^3$H-cyclic 3′,5′-AMP as substrate by the decrease in radioactivity after removal of the reaction product ($^3$H-5′-AMP) by $ZnSO_4$ and $Ba(OH)_2$-precipitation.

**Protein Determination**——Protein contents were determined by the method of Lowry, *et al.*[8] using serum albumin as standard.

## Results

### Effect on the Isolated Guinea Pig Ileum

Atropine, diphenhydramine, and papaverine were served as antagonists against Ach, histamine and barium chloride, respectively. Each effective dose 50% ($ED_{50}$) of P 201-1 of anti-Ach, anti-histaminic and anti-barium actions was compared with the $ED_{50}$ of these antagonists. As shown in Table I, the anti-Ach, anti-histaminic and anti-barium actions of P 201-1 were approximately 1/100, 1/25 and 20 times as potent as those of atropine, diphenhydramine and papaverine, respectively, and the $ED_{50}$ values of P 201-1 of anti-Ach, anti-histaminic and anti-barium actions were approximately similar, *i.e.*, $5.8\times10^{-7}$, $2.3\times10^{-7}$, and $1.4\times10^{-7}$ g/ml, respectively. That is, P 201-1 was proved to have a direct antispasmodic action on the smooth muscle which was more potent than that of papaverine. The dose-response curves of P 201-1 and papaverine for anti-barium action are shown in Fig. 2.

Table I. Effect on the Isolated Guinea Pig Ileum

| Compound (g/ml) | $ED_{50}$ | | |
|---|---|---|---|
| | Barium chloride ($5\times10^{-4}$) | Ach ($1\times10^{-7}$) | Histamine ($5\times10^{-7}$) |
| P 201-1 | $1.4\times10^{-7}$ ($8.3\times10^{-8}$—$5.8\times10^{-7}$) | $5.8\times10^{-7}$ ($6.9\times10^{-8}$—$4.9\times10^{-6}$) | $2.3\times10^{-7}$ ($3.3\times10^{-8}$—$1.0\times10^{-7}$) |
| Papaverine | $3.1\times10^{-6}$ ($1.6\times10^{-6}$—$1.7\times10^{-4}$) | — | — |
| Diphenhydramine | — | — | $9.0\times10^{-9}$ ($3.0\times10^{-9}$—$2.7\times10^{-8}$) |
| Atropine | — | $6.0\times10^{-9}$ ($1.7\times10^{-9}$—$2.1\times10^{-8}$) | — |

Figures in parentheses indicate confidence limit ($p=0.05$).

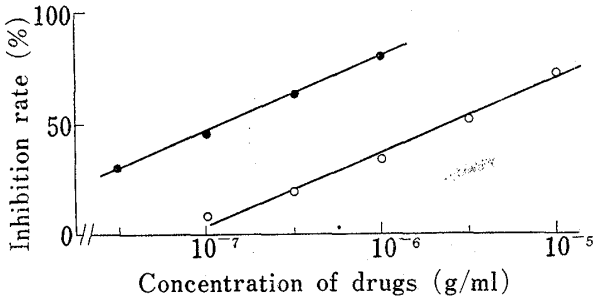


Fig. 2. Dose-response Curves of P 201-1 and Papaverine for Anti-barium Action in the Isolated Guinea Pig Ileum

—●— : P 201-1
—○— : papaverine

### Effect on the Isolated Rat Gastric Smooth Muscle

As shown in Table II, the anti-barium action of P 201-1 was approximately 10 times as potent as that of papaverine.

### Effect on the Isolated Guinea Pig Tracheal Preparation

The inhibitory effect of P 201-1 and papaverine on the contraction of the trachea induced by Ach or histamine is shown in Table III. The inhibitory action of P 201-1 was weak as compared with that of papaverine, *i.e.*, approximately half as potent as that of papaverine.

8) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

Table II. Effects of P201-1 and Papaverine on the Smooth Muscle

| Compound (g/ml) | $ED_{50}$ | |
|---|---|---|
| | P 201-1 | Papaverine |
| Anti-barium action in the isolated rat gastric smooth muscle | $2.9\times10^{-7}$ (10.3) | $3.0\times10^{-6}$ (1) |
| Inhibitory effect on the motility of the isolated rabbit ileum | $9.2\times10^{-7}$ (2.9) | $2.7\times10^{-6}$ (1) |
| Anti-Ach action in the isolated rat uterus | $7.0\times10^{-7}$ (18.6) | $1.3\times10^{-5}$ (1) |
| Anti-oxytocin action in the isolate-ratd uterus | $1.5\times10^{-7}$ (20.0) | $3.0\times10^{-6}$ (1) |

Figures in parentheses indicate the relative potencies between P 201-1 and papaverine.

Table III. Effects of P 201-1 and Papaverine on the Isolated Guinea Pig Trachea

| Compoud (g/ml) | Inhibition (%) | | | |
|---|---|---|---|---|
| | p 201-1 | | Papaverine | |
| | $10^{-5}$ | $10^{-4}$ | $10^{-5}$ | $10^{-4}$ |
| Ach ($1\times10^{-5}$) | 10.2 | 26.9 | 27.2 | 55.2 |
| Histamine ($5\times10^{-5}$) | 41.0 | 65.5 | 100 | 100 |

## Effect on the Motility of the Isolated Rabbit Ileum

The inhibitory effects of P 201-1 and papaverine on the ileum motility are presented in Table II. As the results shown, the inhibitory effect of P 201-1 on the rabbit ileum motility was approximately 3 times as potent as that of papaverine.

## Effect on the Isolated Rat Uterus

The anti-Ach and anti-oxytocin actions of P 201-1 in the isolated rat uterus were compared with those of papaverine. As shown in Table II, the effect of P 201-1 was approximately 20 times as potent as that of papaverine.

## Effect of P 201-1 on PDE Activity

As seen from the results of the experiment in which the supernate of the rat ileum, cerebral cortex and liver were used, it was proved that P 201-1 had a marked and dose-dependent inhibitory effect on PDE activity. Fig. 3 presents the inhibitory effect of P 201-1 on PDE activity in the ileum, and the 50% inhibition of the enzyme activity of P 201-1 was 1300 μM. According to Lineweaver-Burk plots, the *K*m values for the enzyme preparations isolated from the ileum, cerebral cortex, and liver were 390, 270 and 500 μM, respectively, as seen in Fig. 4 and 5. The *K*i values of P 201-1 for the enzyme preparations from the ileum and cerebral cortex were 1370 and 700 μM, respectively, which were 10 times those of papaverine, whereas the *K*i value in the case of the enzyme preparation from the liver was 650 μM which was 5 times that of papaverine.

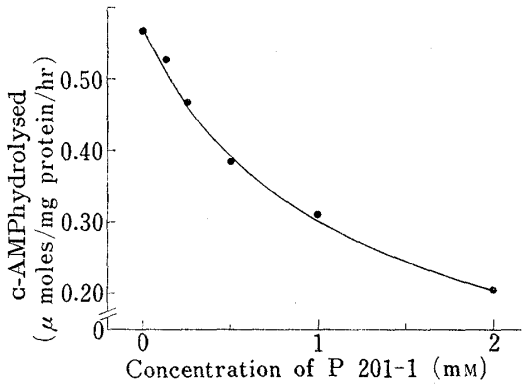


Fig. 3. Inhibitory Effect of P201-1 on Cyclic 3′,5′-Nucleotide Phosphodiesterase from the Rat Ileum

Assays were performed as described in Experimental. Incubations were run at 37° for 40 min at 256 μg protein levels.

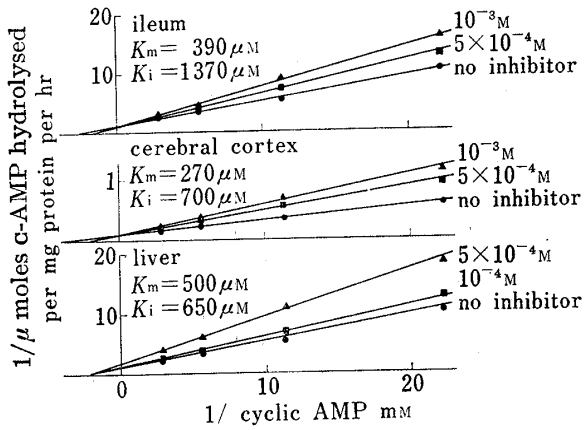


Fig. 4. Inhibitory Effect of P201-1 on PDE from the Rat Ileum, Cerebral Cortex and Liver

Assays were performed as described in Experimental. The concentration of cyclic AMP as substrate ranged from 45 μM to 360 μM.
The concentration of protein from the rat ileum, cerebral cortex, and liver in the assay medium were 256, 40, and 298 μg, respectively.

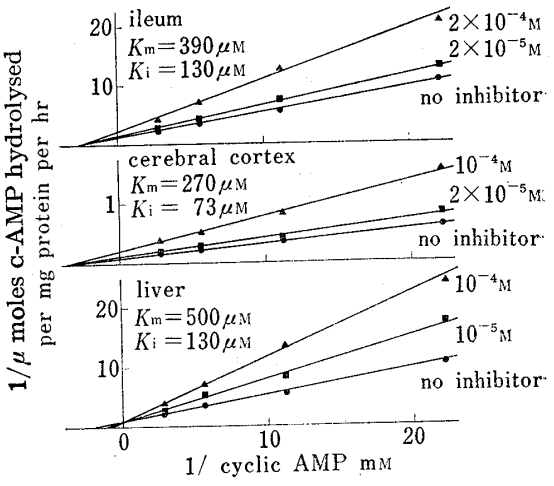


Fig. 5. Inhibitory Effect of Papaverine on PDE from the Rat Ileum, Cerebral Cortex and Liver

Assays were performed as described in Experimental. The concentration of cyclic AMP as substrate ranged from 45 μM to 360 μM. The concentration of protein from the rat ileum, cerebral cortex, and liver in the assay medium were 256, 40, and 289 μg, respectively.

## Type of PDE Inhibition by P 201-1

The kinetics of the hydrolysis of cyclic 3′,5′-AMP by PDE in the rat ileum, cerebral cortex and liver and the inhibition of this hydrolysis by P 201-1 and papaverine were investigated by double-reciprocal plots and are presented in Fig. 4 and 5. As can be seen, the PDE inhibition of P 201-1 was competitive in the case of the ileum or cerebral cortex whereas it was non-competitive in the case of the liver. The inhibitory effect papaverine in the rat ileum and cerebral cortex was non-competitive whereas the type of inhibition was competitive in the case of the rat liver. These results on papaverine coincide with studies of Pöch, *et al.*[4c]

## Requirement for $Mg^{2+}$

By addition of $Mg^{2+}$ of varying concentrations, influence of $Mg^{2+}$ concentration on PDE in the rat ileum, cerebral cortex, and liver and on the PDE inhibitory effect of P 201-1 were examined and are presented in Fig. 6. The PDE activity was increased up to 140—200%.

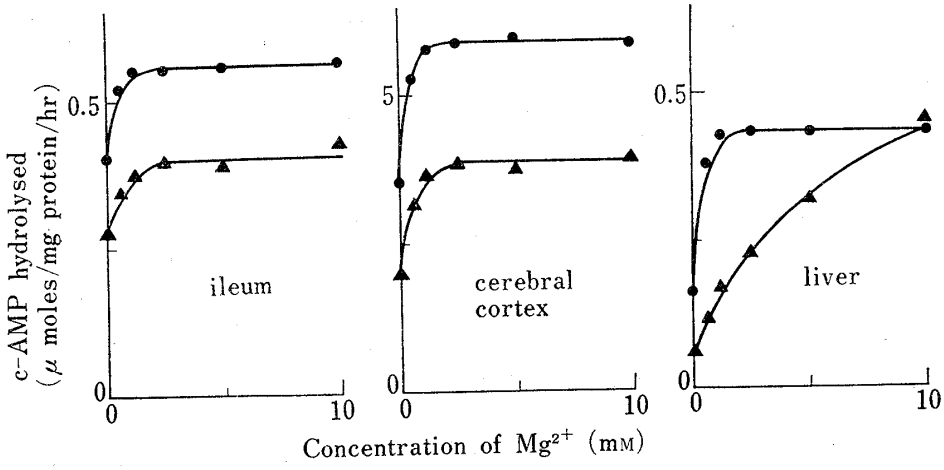


Fig. 6. Influence of $Mg^{2+}$ Concentration on PDE from the Rat Ileum, Cerebral Cortex, and Liver

Conditions were as described in Experimental. $Mg(CH_3COO)_2$ of varying concentrations was used.

—●— : no inhibitor
—▲— : P 201-1 at $5\times10^{-4}$ M

of the basal activity in all cases by addition of $Mg^{2+}$, and reached a plateau at a $Mg^{2+}$ concentration of 1.25 mM. When P 201-1 at $5\times10^{-4}$M was added to the incubation medium, the PDE activity in the rat ileum or cerebral cortex was decreased. The PDE activity in the rat liver, however, showed a decrease different from that observed in the ileum or cerebral cortex and the inhibitory effect of P 201-1 completely disappeared by addition of $Mg^{2+}$ in a concentration of 10 mM.

## Discussion

The antispasmodic action of P 201-1 on smooth muscle was examined and it was noted that P 201-1 exerted a potent papaverine-like action upon the isolated ileum, uterus and gastric smooth muscle. P 201-1 showed a potent anti-barium action on the isolated ileum which was approximately 20 times as potent as that of papaverine, but the anti-Ach and anti-histaminic actions of P 201-1 were very weak. Moreover, the anti-barium action of P 201-1 on the gastric smooth muscle was 10 times as potent as that of papaverine. And P 201-1 showed a musculotropic antispasmodic action on the rabbit ileum which was approximately 3 times as potent as that of papaverine, and thus the difference in potency of the antispasmodic action between P 201-1 and papaverine was not so clear in the rabbit ileum as was seen in the guinea pig ileum. Furthermore, it was previously reported in our pharmacological investigation of P 201-1 that the adverse effects of P 201-1 upon blood pressure were weak and that P 201-1 was a potent musculotropic antispasmodic agent having little effect on the autonomic nervous system.[9]

Based on the report by Pöch, *et al.*[4b] that the smooth muscle relaxation of papaverine-like drugs was due to their inhibitory action on PDE, the effect of P 201-1 on PDE activity in the rat ileum, cerebral cortex, and liver was investigated. As summarized in Table IV, the results discerned by double-reciprocal plots of Lineweaver-Burk show that the *K*i value for the ileum PDE was the highest (1370 μM) which was twice that for the cerebral cortex PDE or for the liver PDE and that the cerebral cortex and liver showed the same *K*i values. The *K*i values of P 201-1 for the ileum and cerebral cortex PDE were approximately 10 times as high as those of papaverine, while the *K*i value of P 201-1 for the liver PDE was approximately 5 times as high as that of papaverine. The antispasmodic action of P 201-1 on various smooth muscle was more potent than that of papaverine, but the inhibitory effect of P 201-1 on PDE was weaker than that of papaverine. It may be considered that the antispasmodic action was not always in parallel with the PDE inhibitory effect.

The type of PDE inhibition of P 201-1 was competitive in the ileum and cerebral cortex and non-competitive in the liver, but the type of the inhibition of papaverine was conversely non-competitive in the ileum and cerebral cortex and competitive in the liver. This difference in the type of PDE inhibition between P 201-1 and papaverine may be explained by the diversity of PDE as a multi-enzyme that the type of inhibition was varied with compounds or with tissue sources, as has been reported by many investigators.[10] One of the explanations for the diversity of PDE as a multi-enzyme may be suggested from the result of this experiment that the liver PDE showed a $Mg^{2+}$-dependent reaction curve different from that in the case of the ileum or cerebral cortex PDE when influence of $Mg^{2+}$ dependency on the PDE inhibitory effect of P 201-1 was examined, as shown in Fig. 6.

Despite the fact that papaverine-like drugs are potent PDE inhibitors as compared with theophylline, there is no evidence that papaverine markedly stimulates cardiac contraction,

9) H. Ozawa and A. Tobe, *Pharmacometrics*, **4**, 233 (1970).
10) J. Vernikos-Danellis and C.G. Harris, *Proc. Soc. Exptl. Biol. Med.*, **128**, 1016 (1968); J.B. Smith and D.C.B. Mills, *Biochem. J.*, **120**, 20 p. (1970); M.S. Amer and G.R. Mckinney, *Pharmacologist*, **12**, 291 (1970); M. Chsin, D.N. Harris, M.B. Phillips, and S.M. Hess, *Biochem. Pharmacol.*, **21**, 2433 (1972); S. Hayashi and H. Ozawa, *Chem. Pharm. Bull.* (Tokyo), **22**, 587 (1974).

heart rate, glycogenolysis in the liver, and lipolysis in the adipose tissue, and the actions of papaverine are mainly confined to the smooth muscle. The most likely explanation at present would be that theophylline is able to penetrate the cellular membranes of many tissues more easily than papaverine. From the well-known fact that cyclic 3′,5′-AMP produced by certain compounds or hormones not only mediates various metabolic effects as has been already clarified[11] but also controls the mechanical stimulation of the heart[12] or relaxation of the smooth muscle,[4a,4b] it may be speculated that the mode of action of papaverine-like drugs would become apparent if a strict proof that cyclic 3′,5′-AMP levels do not increase in the tissues which show no response to papaverine could be obtained.

TABLE IV. Inhibitory Effect of P201-1 and Papaverine on PDE Activity

| Compound | | PDE from rat tissues | | |
|---|---|---|---|---|
| | | Ileum | Cerebral cortex | Liver |
| *k*m values (μM) | | 390 | 270 | 500 |
| *K*i values (μM) | P201-1 | 1370 | 700 | 650 |
| | papaverine | 130 | 73 | 130 |
| Type of inhibition | P201-1 | A* | A* | B* |
| | papaverine | B* | B* | A* |

A*: competitive inhibition
B*: non-competitive inhibition

11) G.A. Robinson, R.W. Butcher, and E.W. Sutherland, *Ann. N. Y. Acad. Sci.*, **139**, 703 (1967).
12) W.R. Kukovetz and G. Pöch, *Naunyn-Schemiedebergs Arch. Pharmak.*, **266**, 236 (1970).