EFFECTS OF NONSPECIFIC SMOOTH MUSCLE RELAXANTS ON CALCIUM-UPTAKE BY MICROSOMAL FRACTION AND THEIR INHIBITORY ACTION IN RABBIT TAENIA COLI

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Effects of nonspecific smooth muscle relaxants, papaverine and Aspaminol (1,1-diphenyl-3-piperidinobutanol hydrochloride) on Ca-uptake by a microsomal fraction and mechanical activity in the rabbit taenia coli were studied. Papaverine which inhibits cyclic AMP phosphodiesterase increased Ca-uptake by the microsomal fraction in a concentration of 10^{-5} M but not in a concentration of 10^{-4} M. Potentiation of Ca-uptake by papaverine is considered to be due to the increase of cyclic AMP as the results of the inhibition of phosphodiesterase. Aspaminol which is found to have little inhibitory action on phosphodiesterase activity inhibited Ca-uptake by the microsomal fraction. This inhibition by Aspaminol was abolished by the increase of Ca-concentration in the reaction medium. Non-competitive antihistaminic activity of Aspaminol was considerably reduced by an increase of external Ca-concentration, while the increase of external Ca-concentration slightly decreased antihistaminic activity of papaverine. Furthermore, contraction of KCl-depolarized taenia coli induced by CaCl_2 was inhibited competitively by Aspaminol and noncompetitively by papaverine. These results suggest that Aspaminol competes with Ca ions at a surface site of muscle membrane concerned with Ca-uptake process, thus decreasing the supply of Ca ions to the contractile elements and that papaverine may have inhibitory actions on Ca-uptake by the microsomal fraction in addition to stimulatory action which seems to be mediated through cyclic AMP. These actions on Ca-uptake may induce smooth muscle relaxation.

Keywords—Ca-uptake; microsomal fraction; nonspecific smooth muscle relaxant; papaverine; rabbit taenia coli

INTRODUCTION

The mechanisms of smooth muscle relaxation by papaverine-like antispasmodics, nonspecific smooth muscle relaxants are obscure. Evidence has been presented that papaverine, in concentrations which have a relaxing action, increases the cyclic AMP level in some smooth muscles (see Ferrari,¹⁾ for references). Some authors^{1,2-4)} have indicated that papaverine-like action of some antispasmodics is not mediated through an increase in cyclic AMP level. For example, 1,1-diphenyl-3-piperidinobutanol hydrochloride (Aspaminol), a nonspecific relaxant,⁵⁾ whose potency relative to papaverine is about 0.5 has

been found to have little inhibitory action on cyclic AMP phosphodiesterase activity²⁾ and to have no effect upon the cyclic AMP level even in concentrations that have a maximum antispasmodic action.⁶⁾ It has been reported that binding of Ca in microsomal membranes derived from the rabbit aorta and colon and rat uterus was increased by cyclic AMP and dibutyryl cyclic AMP.⁷⁻⁹⁾ The effects of Ca-antagonist on Ca-accumulation in microsomes from rat uterus and the effects of chlorpromazine on Ca-movements, using microsomes prepared from the longitudinal muscle of guinea pig have been reported.^{10,11)} It has also been suggested by Mas-Oliva *et al.*¹²⁾ that

ATP increases Ca-binding to isolated cardiac sarcolemma. In order to study a difference in mechanisms of smooth muscle relaxation induced by papaverine and Aspaminol, we obtained a microsomal fraction from the rabbit taenia coli, which was considered to contain the plasma membrane^{13,14)} and examined the effects of papaverine and Aspaminol on Ca-uptake by the microsomal fraction in the presence of ATP. The effects of Ca-concentration in reaction medium on actions of papaverine and Aspaminol were also studied, by comparing the effects of external Ca-concentration on the actions of both drugs in the mechanical and electrical activities of smooth muscle.

MATERIALS AND METHODS

The taenia coli isolated from male rabbits weighing 2.0 to 3.0 kg, was suspended in a 30-ml organ bath filled with a physiological saline solution kept at 32° and gassed with a mixture of 95% O₂ and 5% CO₂. The pH of physiological saline solution under these conditions was about 7.6. Responses to drugs were recorded isotonicly.

Experiments to the effects of Aspaminol and papaverine on membrane activities were conducted on strips of the taenia caecum from the male guinea pig (300 to 400 g). A strip of isolated taenia coli was mounted in the sucrose gap apparatus according to the method of Bülbiring and Burnstock.¹⁵⁾ In this apparatus, an arrangement was made to record isometric contractions of smooth muscle with electrical charges.

The physiological saline solution used had the following composition (mM); NaCl 154, KCl 5.6, CaCl₂ 2.2, MgCl₂ 2.1, NaHCO₃ 5.9 and glucose 2.8. To test the effects of the increase of external Ca ions on noncompetitive antagonistic activities of Aspaminol and papaverine, the concentration of CaCl₂ in the physiological saline solution was increased 10 times (termed as a high Ca-solution in this paper). The high Ca-solution contained 100 mM NaCl.

In some experiments the taenia coli of rabbit was suspended in the 30 ml organ bath filled with Ca-free KCl-physiological saline solution (KCl

159.6, MgCl₂ 2.1, NaHCO₃ 5.9 and glucose 2.8 mM), bubbled with a mixture of 95% O₂ and 5% CO₂ and kept at 32°. After an initial incubation (lasting about 60 min), CaCl₂ was added cumulatively and the contraction induced by CaCl₂ was recorded isotonicly. Aspaminol or papaverine was added to the bath 3 min before the addition of CaCl₂.

Estimation of Ca-uptake by the microsomal fraction was done by the method of Carsten¹⁶⁾ and as modified by Takayanagi *et al.*¹⁷⁾ Rabbits, weighing 2.0 to 3.0 kg, were killed by a blow on the neck and taenia coli was immediately removed and washed with ice-cold 0.25 M sucrose solution. The isolated tissue was minced with scissors and homogenized in 10 volumes of ice-cold 0.25 M sucrose containing 0.01 M Tris-HCl buffer (pH 7.4) in a Polytron (P.T.-10, Brinkman Instruments) with the rheostat setting 8 for 5 seconds. The homogenate was centrifuged at 2500× *g* for 15 min. The supernatant was again centrifuged at 15000× *g* for 20 min. Centrifugation of the supernatant at 40000× *g* for 90 min resulted in a pellet which was used as the microsomal fraction in this study. Ca-uptake was studied at 32° in the incubation mixture which contained 100 mM KCl, 5 mM MgCl₂, 3 mM ATP, 5 mM oxalic acid, 5 mM sodium azide, 30 mM histidine buffer (pH 7.4), 20 μM Ca plus ⁴⁵Ca (0.02 μCi/ml) in a total volume of 5 ml. To test the effect of the increase of Ca-concentration on drug actions, a mixture of 200 μM Ca plus ⁴⁵Ca (0.08 μCi/ml) in a total volume of 5 ml (high Ca-reaction medium) was used in some experiments. The reaction was stopped by filtration of the mixture through a Millipore filter (HA 0.45 μm). The radioactive Ca²⁺ retained was estimated by a liquid scintillation spectrometer, after the filters had been dissolved in a toluene scintillator. The values were corrected for the ⁴⁵Ca remaining on the filters in the absence of the microsomal fraction. Ca-uptake was estimated from the radioactivity of the filter. Protein was assayed by the method of Lowry *et al.*,¹⁸⁾ using bovine serum albumin as the standard. The amount of protein contained in the incubation mixture was 50 to

100 $\mu\text{g/ml}$. Drugs used: Aspaminol (1,1-diphenyl-3-piperidinobutanol hydrochloride; Kowa Co., Japan), papaverine hydrochloride (Wako-junyaku Co., Japan), histamine dihydrochloride (Wako-junyaku Co., Japan), Tris (Sigma chemical Co.), ATP-2Na (Sigma Chemical Co.) and $^{45}\text{CaCl}_2$ (specific activity: 16 mCi/mg ^{45}Ca ; New England Nuclear). Other chemicals used were analytical grade. Drugs were dissolved in double-distilled and deionized water.

RESULTS

Concentration action curve of histamine in contraction of the rabbit taenia coli shifted toward its higher concentrations by the increase of external Ca ions. This may be due to stabilization of membrane. The noncompetitive antihistamine activity of Aspaminol was greatly decreased by the increase of external Ca ions, while that of papaverine was decreased slightly (Fig. 1). Similar results were observed in 10 strips from 10 animals.

Aspaminol (10^{-5} to 10^{-4} M) clearly shifted the

concentration action curve of CaCl_2 towards higher concentrations, indicating a competitive inhibitory action (Fig. 2). Antagonism of papaverine (10^{-5} to 10^{-4} M) against CaCl_2 was noncompetitive (Fig. 2). these results were similar to our previous ones tested on the ileum from the guinea pig.¹⁷⁾ It is known that papaverine parallelly shifted the concentration action curve of CaCl_2 in the depolarized aorta of rabbit and taenia caecum of guinea pig.¹⁹⁾ The mode of action of papaverine observed in this work differed from that on the depolarized rabbit aorta and guinea pig taenia caecum.

To test the effects of external Ca ions on the noncompetitive antihistaminic action of Aspaminol and papaverine, high concentration (10^{-5} M) of histamine was applied to the taenia caecum of guinea pig. After administration of histamine (10^{-5} M) to the taenia, the membrane was depolarized, the spikes becoming more frequent and smaller. These phenomena were accompanied by a rise in tension. Aspaminol (3×10^{-4} M) hyperpolarized the membrane and

FIG. 1. Effects of External Ca-concentration on the Noncompetitive Antihistamine Activities of Aspaminol and Papaverine tested on the Rabbit Taenia Coli

CaCl_2 in the normal physiological saline solution; 2.2 mM, CaCl_2 in the high Ca-solution: 22 mM, concentrations of drugs: M.

abolished the spontaneous spike. The phenomena were accompanied by a fall in tension. Histamine (10^{-5} M) still depolarized the membrane in the presence of Aspaminol (3×10^{-4} M) but spike was not observed. Tension was not developed with these phenomena. However, in high Ca-solution, histamine (10^{-5} M) also depolarized the membrane and spike was observed even in the presence of Aspaminol (3×10^{-4} M). Tension of the smooth muscle developed accordingly. These are demonstrated in Fig. 3. As reported previously^{20,21} the inhibitory effects of papaverine on the mechanical and electrical activities were decreased by the increase of external Ca-concentrations (data not shown). However, the

effects of increasing Ca-concentration on the actions of Aspaminol were much more prominent than those on the actions of papaverine.

The microsomal fraction used in this study contained high concentrations of markers of plasma membrane, such as true cholinesterase, 5'-nucleotidase and Na, K-dependent ATPase as reported previously.^{13,14} Electron microscopic observation indicated that there were no intact mitochondria in this fraction used. Amounts of Ca-uptake by the microsomal fraction were expressed as n mol/mg protein. Ca-uptake increased in the presence of ATP (3 mM). Aspaminol (3×10^{-5} and 3×10^{-4} M) inhibited Ca-uptake in the presence of ATP (3 mM) at all times of incubation. Inhibition of Ca-uptake by 3×10^{-4} M of Aspaminol was greater than that by 3×10^{-5} M. These are summarized in Table I. This inhibitory action of Aspaminol (3×10^{-4} M) was abolished by 10 fold increase of Ca-concentration (Table I).

However, Ca-uptake in the presence of ATP (3 mM) was potentiated by papaverine in the concentration of 10^{-5} M at incubation times of 6 and 10 min but not in the concentration of 10^{-4} M (Table I). Papaverine (10^{-4} M) did not influence Ca-uptake even in high Ca-reaction medium (Table I).

FIG. 2. Effects of Aspaminol (Left) and Papaverine (Right) on Concentration Action Curve of CaCl_2 tested on the Depolarized Rabbit *Taenia Coli*

DISCUSSION

It has been reported that the microsomal fractions obtained from several smooth muscles are

FIG. 3. Records of Electrical and Mechanical Changes in the *Taenia Caecum* from the Guinea Pig. Upper record: electrical activity, lower record: mechanical activity. A and B: in the normal physiological saline solution ($\text{CaCl}_2 = 2.2$ mM), C-1 and C-2: in the high Ca-solution ($\text{CaCl}_2 = 22$ mM), C-2: 3 min after application of Aspaminol. Aspaminol = 3×10^{-4} M, Histamine = 10^{-5} M.

able to take up Ca ions in the presence of ATP.⁷⁻⁹⁾ It has been recently indicated that Ca-uptake by microsomes isolated from rabbit uterus was potentiated by oxalate as well as by inorganic phosphate and evidence has been also provided that two anions increase Ca-accumulation by different mechanisms.²²⁾

The microsomal fraction used in this study was considered to form microsacks. Therefore, we used it as a smooth muscle model. The fact that Aspaminol inhibits Ca-uptake by KCl-depolarized intestinal smooth muscle has been reported by Takayanagi *et al.*²³⁾ Aspaminol inhibited Ca-uptake by the microsomal fraction dose-dependently and this inhibition by Aspaminol was also antagonized by the increase of Ca-concentration in the reaction medium, suggesting that the antagonism was competitive. Furthermore, Aspaminol shifted parallelly the concentration action curve of CaCl₂ in the KCl-depolarized preparations. Antihistaminic actions of Aspaminol in the mechanical and electrical activities were considerably decreased by the increase of external Ca-concentration. These results suggest that Aspaminol competes with Ca

ions at a surface site of muscle membrane concerned with Ca-uptake process, thus decreasing the supply of Ca ions to contractile elements. The inhibition of Ca-uptake by Aspaminol may induce smooth muscle relaxation, though details of the mechanisms involved in this process are still unknown.

Ca-uptake by the microsomal membranes from the rabbit aorta, colon and rat uterus was enhanced by cyclic AMP and dibutyryl cyclic AMP.⁷⁻⁹⁾ These results suggest that papaverine which inhibits cyclic AMP breakdown increases Ca-uptake by microsomal fraction. In the present work, potentiation of Ca-uptake by papaverine in the concentration of 10⁻⁵ M is considered to be due to the increase of cyclic AMP as the results of the inhibition of cyclic AMP phosphodiesterase. Papaverine in the concentration of 10⁻⁴ M did not potentiate Ca-uptake, notwithstanding that the concentration of papaverine used was higher. Papaverine was also considered to have an inhibitory action on Ca-uptake by the microsomal fraction. This inhibitory action of papaverine was little influenced by Ca-concentration in the reaction medium in this study. The noncompetitive

TABLE I. *Effects of Aspaminol and Papaverine on Ca-uptake by the Microsomal Fraction from the Rabbit Taenia Coli in the Presence of 20 μ M Ca(A) and 200 μ M Ca(B) in the Reaction Medium*

	Incubation time		
	2 min	6min	10 min
[A]			
ATP (—)	1.5 $\pm$ 0.8	2.4 $\pm$ 1.1	3.1 $\pm$ 1.0
ATP (3 mM) (control)	16.2 $\pm$ 0.9	21.6 $\pm$ 0.9	26.6 $\pm$ 1.1
ATP (3 mM) + Aspaminol (3 $\times$ 10 ⁻⁵ M)	11.6 $\pm$ 0.8*	16.8 $\pm$ 0.7*	20.8 $\pm$ 0.9*
ATP (3 mM) + Aspaminol (3 $\times$ 10 ⁻⁴ M)	6.4 $\pm$ 0.6**	9.8 $\pm$ 1.2**	12.8 $\pm$ 1.3**
ATP (—)	2.3 $\pm$ 0.4	2.8 $\pm$ 0.5	3.0 $\pm$ 0.5
ATP (3 mM) (control)	7.0 $\pm$ 0.3	11.8 $\pm$ 0.6	15.6 $\pm$ 0.7
ATP (3 mM) + papaverine (10 ⁻⁵ M)	7.8 $\pm$ 0.8	15.0 $\pm$ 0.4*	20.9 $\pm$ 0.6*
ATP (3 mM) + papaverine (10 ⁻⁴ M)	5.6 $\pm$ 0.7	12.6 $\pm$ 0.7	16.0 $\pm$ 0.5
[B]			
ATP (3 mM)	45.9 $\pm$ 4.2	51.3 $\pm$ 8.3	55.7 $\pm$ 6.1
ATP (3 mM) + Aspaminol (3 $\times$ 10 ⁻⁴ M)	50.3 $\pm$ 5.7	47.4 $\pm$ 6.7	58.8 $\pm$ 6.3
ATP (3 mM) + Aspaminol (3 $\times$ 10 ⁻⁴ M)	52.8 $\pm$ 7.2	48.9 $\pm$ 5.6	48.8 $\pm$ 7.6

Values of calcium binding are expressed in n mol Ca/mg protein and presented as mean $\pm$ S.E. of 10 experiments. *: significantly different from control at $p < 0.05$ and **: significantly different from control at $p < 0.01$. ATP(—): in the absence of ATP

antihistaminic activity of papaverine was slightly decreased by the increase in the external Ca-concentration, while this effect of external Ca-ions was clearly demonstrated in the experiments with Aspaminol which had little inhibitory action on phosphodiesterase activity. Papaverine noncompetitively inhibited contraction of KCl-depolarized smooth muscle induced by CaCl_2 . Therefore, inhibition of Ca-uptake by papaverine seems to be noncompetitive. The results mentioned above indicated that in the intestinal smooth muscle, papaverine have inhibitory action on Ca-uptake by the microsomal fraction in addition to stimulatory action which seems to be mediated through cyclic AMP. Relaxing action in the intestinal smooth muscle may be due to both actions thus decreasing the supply of Ca ions to the contractile elements.

Microsomal fraction used in this study is, however, considered to contain both muscle membrane and sarcoplasmic reticulum. We cannot rule out the possibility that the site(s) of inhibitory action of Aspaminol and papaverine is (are) also in the sarcoplasmic reticulum.

REFERENCES

- 1) M.Ferrari; Effects of papaverine on smooth muscle and their mechanisms, *Pharmacol. Res. Commun.*, **6**, 97–115 (1974).
- 2) I.Takayanagi, M.Uchida, T.Abe, A.Tomiyama and K.Takagi; Relaxing action of synthetic antispasmodics on the intestinal smooth muscle of guinea pig, *Japan. J. Pharmacol.*, **23**, 737–739 (1973).
- 3) N.Inatomi, I.Takayanagi and K.Takagi; Anti-phosphodiesterase activity and nonspecific smooth muscle relaxation tested on intestinal smooth muscle, *Japan. J. Pharmacol.*, **25**, 63–69 (1975).
- 4) G.Pösch and W.Umfahrer; Differentiation of intestinal smooth muscle relaxation caused by drugs that inhibit phosphodiesterase, *Naunyn-Schied. Arch. Pharmacol.*, **293**, 257–268 (1976).
- 5) K.Takagi, Y.Kasuya, H.Fukuda and Y.Ohta; Pharmacology of 1,1-diphenyl-3-piperidinobutanol hydrochloride, *Japan. J. Pharm. Soc.*, **75**, 566–572 (1955).
- 6) N.Inatomi, I.Takayanagi, M.Uchida and K.Takagi; Intracellular cyclic AMP level and intestinal smooth muscle relaxation, *Europ. J. Pharmacol.*, **26**, 73–76 (1974).
- 7) R.Andersson, and K.Nilsson; Cyclic AMP and calcium in relaxation in intestinal smooth muscle, *Nature, New Biol.*, **238**, 119–120 (1972).
- 8) M.Baudouin-Legros and P.Meyer; Effects of angiotensin, catecholamines and cyclic AMP on calcium storage in aortic microsomes, *Brit. J. Pharmacol.*, **47**, 377–385 (1973).
- 9) K.Nishikori, T.Takanaka and A.Maeno; Stimulation of microsomal calcium uptake and protein phosphorylation by adenosine cyclic 3',5'-monophosphate in rat uterus, *Mol. Pharmacol.*, **13**, 671–678 (1977).
- 10) D.J.Crankshaw, R.A.Janis and E.E.Daniel; The effects of Ca^{2+} antagonist on Ca^{2+} accumulation by subcellular fractions of rat myometrium, *Can. J. Physiol. Pharmacol.*, **55**, 1028–1032 (1977).
- 11) A.K.Chaturvedi, E.J.Landon and B.V.Rama Sastry; Influence of chlorpromazine on calcium movements and contractile responses of guinea-pig ileum longitudinal smooth muscle to agonist, *Arch. int. Pharmacodyn.*, **236**, 109–129 (1978).
- 12) M.Mas-Oliva, A.J.Williams and W.G.Nayler; ATP-induced stimulation of calcium binding to cardiac sarcolemma, *Biochem. Biophys. Res. Commun.*, **87**, 441–447 (1979).
- 13) I.Takayanagi, T.Hongo and Y.Kasuya; Difference in the mechanisms by which acetylcholine and histamine interact with Ca^{2+} to contract the rabbit taenia coli, *J.Pharm. Pharmacol.*, **29**, 775–776 (1977).
- 14) I.Takayanagi, T.Hongo, T.Hisayama and Y.Kasuya; Effects of acetylcholine and histamine on mechanical activity of rabbit taenia coli, Ca-incorporation and Ca-release in its microsomal fraction, *J. Pharm. Dyn.*, **2**, 212–218 (1979).
- 15) E.Bülbring and G.Burnstock; Membrane potential changes associated with tachyphylaxis and potentiation of the response to stimulation drug in smooth muscle, *Brit. J. Pharmacol.*, **15**, 611–624 (1964).
- 16) M.E.Carsten; Role of calcium binding by sarcoplasmic reticulum in the contraction and relaxation of uterine smooth muscle, *J. Gen. Physiol.*, **53**, 414–426 (1969).
- 17) I.Takayanagi, H.Yamashita, T.Manda and K.Takagi; Calcium ions and relaxation of intestinal smooth muscle induced by papaverine and Aspaminol, *Japan. J. Pharmacol.*, **27**, 311–314 (1977).
- 18) O.H.Lowry, N.J.Rosebrough, A.L.Farr and R.U.Randall; Protein measurement with the Folin phenol reagent, *J. Biol. Chem.*, **193**, 265–275 (1951).
- 19) A.M.Simmonis, E.J.Ariens and J.J.Van den Broeke; Noncompetitive spasmolytics as antagonist of Ca-induced smooth muscle contraction, *J. Pharm. Pharmacol.*, **23**, 107–110 (1971).
- 20) K.Takagi, I.Takayanagi, K.Kubota and F.Taga; Effect of calcium ions and phospholipids on antiacetylcholine action of acetone and papaverine, *Japan. J. Pharmacol.*,

- 20, 398—412 (1970).
- 21) N.Tashiro and T.Tomita: The effects of papaverine on the electrical and mechanical activity of guinea pig taenia coli, *Brit. J. Pharmacol.*, **39**, 608—618 (1970).
- 22) S.Batra: Studies on calcium uptake by myometrial microsomes with particular reference to the dependence on inorganic phosphate and oxalate, *Acta Physiol. Scand.*, **104**, 68—73 (1978).
- 23) I.Takayanagi, A.Karasawa and Y.Kasuya: Relaxation of depolarized guinea pig taenia caecum induced by some antispasmodics, *Europ. J. Pharmacol.*, **50**, 137—143 (1978).