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EFFECT OF ISOPRENALINE AND PHENYLEPHRINE ON THE ADENOSINE 3′,5′-MONOPHOSPHATE CONTENT AND MECHANICAL ACTIVITY OF COLD-STORED AND FRESH TAENIA CAECUM FROM THE GUINEA-PIG

F. HONDA & S. KATSUKI
Research Laboratories, Fujisawa Pharmaceutical Company Ltd.

J.T. MIYAHARA & S. SHIBATA
Department of Pharmacology, School of Medicine,
University of Hawaii, Honolulu, Hawaii 96822, U.S.A.

1 Cold storage treatment of the guinea-pig taenia caecum had a greater inhibitory effect on the isoprenaline-induced relaxation than that induced by phenylephrine. Prolonged cold storage (12–14 days) almost abolished the effect of isoprenaline but only reduced the phenylephrine effect. The ED_{50} of cyclic adenosine 3′,5′-monophosphate (cyclic AMP) that elicited muscle relaxation was not altered by the prolonged cold storage.

2 After cold storage treatment, tissue cyclic AMP content was decreased; however, isoprenaline still caused a dose-dependent increase in the cyclic AMP level. The threshold dose of isoprenaline for cyclic AMP accumulation was the same in fresh and cold-stored preparations.

3 In the fresh preparation, the onset of the isoprenaline (10^{-6} M)-induced relaxation preceded the increase in tissue cyclic AMP.

4 Isoprenaline, phenylephrine, adrenaline and noradrenaline at doses (ED_{50}) sufficient to induce muscle relaxation did not always increase the cyclic AMP level.

5 Similarly, the responses to papaverine and nitroglycerine were not accompanied by an increase in cyclic AMP.

6 The adenylate cyclase and phosphodiesterase (low and high *Km*) activities of taenia caecum were not attenuated by the prolonged cold storage.

7 Propranolol inhibited both the isoprenaline-induced relaxation and cyclic AMP accumulation; however, the pA_2 values were significantly different for the two events.

8 Based on these results, both the relaxation and cyclic AMP accumulation caused by isoprenaline are mediated by activation of β-adrenoceptors but are independent phenomena.

Introduction

Evidence has been presented that in various smooth muscles stimulation of β-adrenoceptors by catecholamines is accompanied by an increase in intracellular cyclic adenosine 3′,5′-monophosphate (cyclic AMP) (Butcher, Ho, Meng & Sutherland, 1965; Bueding, Butcher, Hawkins, Timms & Sutherland, 1966; Robison, Butcher & Sutherland, 1971; Andersson, Lundholm, Mohme-Lundholm & Nilsson, 1972; Bar, 1974). It has also been reported that the relaxation of intestinal smooth muscle induced by β-adrenoceptor stimulation is associated with an increase in cyclic AMP concentration (Andersson & Mohme-Lundholm, 1970; Andersson, 1972; Takayanagi, Uchida, Inatomi, Tomiyama & Takagi, 1972; Inatomi, Takayanagi, Uchida & Takagi, 1974). However, the correlation between cyclic AMP production and intestinal smooth muscle relaxation as effected by β-adrenoceptor agonists has not been fully established and there is still some question as to whether these effects reflect a parallel process or represent a cause and effect.

Our previous data showed that cold treatment of the guinea-pig taenia caecum for 7 days does not interfere with the inhibitory action of the catecholamines but does affect that of adenosine triphosphate and cyclic AMP. This suggested that the underlying mechanisms mediating the catecholamine relaxation may not involve an adenine nucleotide or

related compound (Shibata, Hattori & Timmerman, 1970). Moreover, the electrochemical activity following α-adrenoceptor stimulation was more resistant to cold storage than was the β-adrenoceptor response (Fukuda & Shibata, 1972). Therefore, in the experiments described here, the relationship between cyclic AMP production and relaxation was investigated.

Methods

Mechanical activity

Adult guinea-pigs weighing 500 to 600 g were used in the experiments. The animals were stunned, exsanguinated and the taenia dissected from the caecum. To study the effects of drugs on the mechanical response, taenia strips of approximately 0.5 cm resting length, were suspended vertically in a 50 ml organ bath by silk ligatures, the lower end secured to a holder at the bottom of the chamber and the upper end connected to a Grass strain gauge, force transducer (FT.03) which led to a Grass model 7 polygraph. The bathing medium was Ringer solution of the following composition (mM): NaCl 154, KCl 5.4, $CaCl_2$ 2.4, $NaHCO_3$ 6, glucose 11, bubbled with a gas mixture (95% O_2, 5% CO_2) and maintained at 37°C and pH 7.4.

A 3 g tension was initially applied to the taenia strip and an equilibration period of 3 h was allowed before the tissue was exposed to any drug. The cumulative dose-response curves were obtained by a stepwise incremental addition of the agonist. Unless specified, the maximum relaxation evoked by phenylephrine in the fresh preparation served as the 100% control to determine the dose-response curves for the different agonists.

In some experiments, the tissues were treated with β-adrenoceptor blocking agents for 15 min before application of the agonists. The pA_2 value was calculated as the negative logarithm of that concentration of the antagonist propranolol, 10^{-6} M or 10^{-7} M, which requires twice as high a concentration of the agonists to elicit a given response (Rossum, 1963).

For cold storage treatment, the taenia caecum was placed in 250 ml Krebs solution and stored in a refrigerator (set at 2 ± 0.5°C) for 1–14 days. After cold treatment, the taenia strip was equilibrated in aerated Ringer at room temperature for 1 h and transferred into the warm medium (37°C) for an additional 2 h before the start of the experimental procedure. ED_{50} values were based on geometric means.

Biochemical assay

Attempts were made to perform all biochemical assays under conditions similar to those in the physiological experiments. The time course of cyclic AMP accumulation and relaxation for the various agonists was carried out simultaneously in different preparations but on tissues from the same animal. For measurements of cyclic AMP, the tissues were exposed to the agonists for 30 s or 1 min, which was sufficient time to cause a maximal relaxation.

Cyclic AMP

Immediately after the incubation or following exposure to the agonists, the taenia strip was pressed between two plates of dry ice. The weighed tissue sample (200–300 mg, 4–5 tissues together) was homogenized in cold 6% trichloroacetic acid and prepared for centrifugation (2000 *g*). The supernatant was extracted with ether (4 times) to remove the trichloroacetic acid before being subjected to the lyophilization procedure. The residue from the process was then dissolved in buffer, and a suitable aliquot was taken for cyclic AMP determination by the protein binding method. The binding protein was prepared from rat brain (Miyamoto, Kuo & Greengard, 1969) and the assay was carried out essentially as described by Gilman (1970).

Adenylate cyclase and phosphodiesterase

The taenia caecum was first homogenized in ice-cold Tris-maleate buffer (pH 7.4) containing 2 mM 1,2, bis, 2 aminoethoxyethane-NNN'N'-tetra-acetic acid (EGTA) and then filtered through a gauze. A portion of the filtrate was taken for the phosphodiesterase (PDE) assay; the remainder was centrifuged at 4°C for 10 min at 2500 rev/min and the precipitate was resuspended in 5 mM Tris-maleate-2 mM EGTA buffer and used for the determination of adenylate cyclase activity.

The adenylate cyclase assay was a modified method of Kebabian, Petzold & Greengard (1972). The reaction mixture (0.6 ml) consisted of 80 mM Tris-maleate (pH 7.4), 2 mM $MgSO_4$, 10 mM theophylline, 0.2 mM EGTA, 10 mM creatine phosphate, 6 i.u. of creatine phosphokinase, 0.5 mM ATP and the enzyme preparation (ca. 2 mg as protein). The reaction was started by the addition of ATP in the presence of 10 mM NaF. After incubation at 30°C for 2.5 min, the enzyme mixture was immersed for 2 min in a boiling water bath to stop the reaction and then centrifuged. The resultant supernatant was taken and the amount of cyclic AMP produced was determined as described above. The enzyme activity is expressed as picomoles of cyclic AMP formed during 2.5 min/mg of protein. Protein content was determined by a Biuret method described by Gornall, Bardawill & David (1949).

PDE assay was carried out in Tris-HCl buffer (40 mM, pH 7.5), containing 1 mM $MgCl_2$. The high *Km*-PDE activity was determined as described by

Butcher & Sutherland (1962) with 2 mM cyclic AMP substrate. Inorganic phosphate determination was by the method of Post & Sen (1967). The low *Km*-PDE was assayed according to the method of Thompson & Appleman (1971) with [^{3}H]-cyclic AMP at a concentration of 1.5×10^{-7} M. The high *Km*-PDE activity was expressed as nanomoles of cyclic AMP hydrolyzed during 10 min/mg of protein and the low *Km*-PDE activity was expressed as picomoles of cyclic AMP hydrolyzed during 10 min/mg of protein.

The following agents were used: (±)-isoprenaline sulphate, (−)-phenylephrine hydrochloride, (−)-noradrenaline bitartrate, (−)-adrenaline bitartrate, papaverine hydrochloride, nitroglycerine (2% ethanol solution) and adenosine 3′,5′-cyclic monophosphoric acid (cyclic AMP) (Sigma). Propranolol hydrochloride (Ayerst Lab) was used as a β-adrenoreceptor blocking agent.

Results

Relaxant effect of isoprenaline, phenylephrine and cyclic AMP

Figure 1 illustrates the dose-related relaxant effect of isoprenaline and phenylephrine on the guinea-pig taenia strips before and after cold storage treatment. Cold storage of the taenia strip for 9 days caused the dose-response curve of isoprenaline to shift to the right along with a slight attenuation of the maximum relaxant effect, whereas such treatment had little effect on the dose-response curve of phenylephrine and the maximum efficacy of phenylephrine was not appreciably affected. Even after prolonged cold storage (12–14 days), the maximum relaxant effect evoked by phenylephrine was diminished by only 25%, whereas the response to isoprenaline was barely detectable.

In Table 1 are shown the ED_{50} for isoprenaline, phenylephrine and cyclic AMP in the fresh and cold-stored taenia. The ED_{50} for isoprenaline in the 9 day cold-stored taenia strips was approximately 100 times that in the fresh preparations. In the 12–14 day-treated strips, the relaxant effect of isoprenaline was so severely depressed that the ED_{50} was not measurable. On the other hand, the reactivity of the taenia strip to phenylephrine after 9 days of cold storage was no different from that of the fresh preparation. Moreover the ED_{50} for phenylephrine in the 12–14 day-treated taenia was only increased twofold over the untreated control. In contrast, the ED_{50} for cyclic AMP was not significantly altered by the cold storage treatment, although the maximal efficacy of cyclic AMP progressively decreased to approximately 52% ($n=7$).

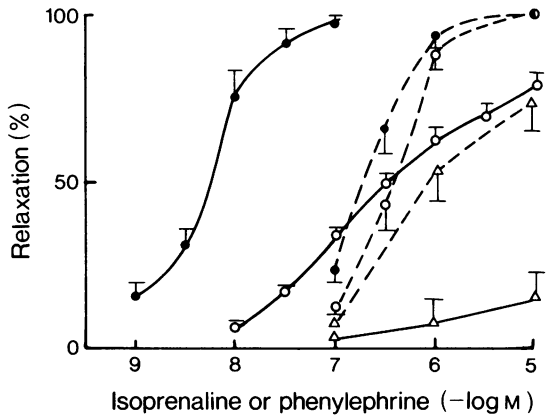

Figure 1 Relaxant effect of isoprenaline and phenylephrine on the fresh and cold-stored guinea-pig taenia caecum. Fresh preparations: (●——●) isoprenaline; (●----●) phenylephrine. Cold-stored strips: (○——○) isoprenaline 9 days; (△——△) isoprenaline 12–14 days; (○----○) phenylephrine 9 days; (△---△) phenylephrine 12–14 days. Each plot is the mean of 14 strips. Vertical lines show s.e. mean. Different animals were used in each of the experiments.

Effect of catecholamines on cyclic AMP production

In Figure 2 are presented the net (a) and percentage (b) increases of tissue cyclic AMP following the

Table 1 Relaxant effect (ED_{50}) of isoprenaline, phenylephrine and cyclic AMP on fresh and cold stored guinea-pig taenia caecum

	Fresh	*Cold stored*	
		9 days	12–14 days
Isoprenaline (M)	$5.5 \pm 1.0 \times 10^{-9}$	$6.0 \pm 1.2 \times 10^{-7}$*	——**
Phenylephrine (M)	$3.0 \pm 0.8 \times 10^{-7}$	$4.0 \pm 0.6 \times 10^{-7}$	$6.2 \pm 0.6 \times 10^{-7}$*
Cyclic AMP (M)	$4.9 \pm 1.1 \times 10^{-4}$	$5.1 \pm 0.6 \times 10^{-4}$	$6.7 \pm 0.8 \times 10^{-4}$

*Significantly different from value of fresh tissue (Student t-test, $P<0.05$)
**Unmeasurable response.
Each value is mean ± s.e. mean of 7 experiments.

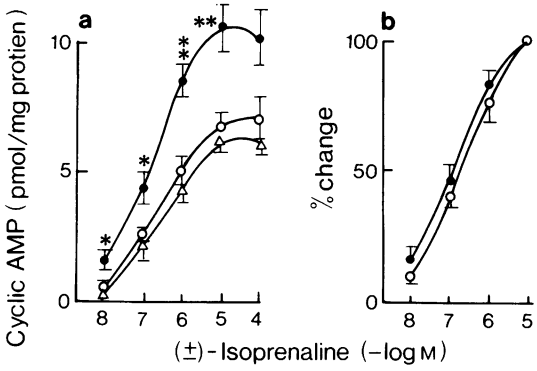

Figure 2 Effect of isoprenaline on the cyclic AMP content of fresh and cold-stored guinea-pig taenia caecum. (a) Net increase in cyclic AMP content; the cyclic AMP content before exposure to isoprenaline was subtracted from each individual value (see text). (b) Percentage change of net increase in cyclic AMP level. (●) Fresh strips; (○) cold stored strips (9 days); (△) cold stored strips (12–14 days). Each value is the mean of 11 experiments. Vertical lines show s.e. mean.

* $P<0.05$ (Student's t-test); ** $P<0.01$ between fresh and cold-stored strips.

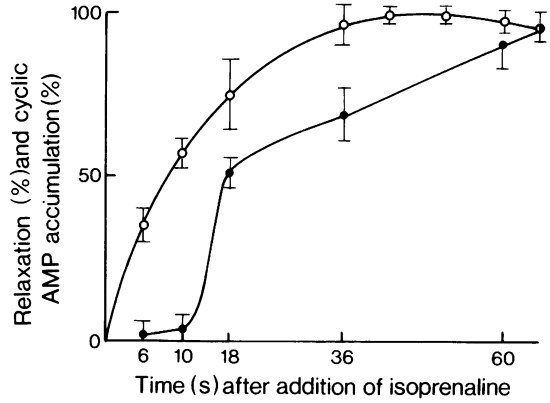

Figure 3 Time course of isoprenaline (2×10^{-7} M) induced relaxation and cyclic AMP production (pmol/mg protein) in the guinea-pig taenia caecum. (○) Relaxation; (●) cyclic AMP. Each value is the mean of 7 experiments. Vertical lines show s.e. mean. Cyclic AMP content of control tissue before exposure to isoprenaline was 9.48 ± 0.68 pmol/mg protein.

application of isoprenaline (10^{-8} M–10^{-4} M) in the fresh and cold-stored taenia strips. Both fresh and cold-stored preparations showed a dose-dependent increase in cyclic AMP content in response to isoprenaline. Although the tissue cyclic AMP content in fresh preparations was always greater than that in the cold-treated taenia in the whole range of isoprenaline concentration examined, the drug effect was not different in the cold-stored preparations. Even at the maximally effective concentrations of isoprenaline the cyclic AMP level was the same in the 9 day and 12–14 day cold-treated taenia strips. A measurable increase in cyclic AMP was detected at isoprenaline 10^{-8} M and the maximum effect occurred at a concentration of 10^{-5} M in both fresh and cold-treated strips. Other catecholamines such as adrenaline, noradrenaline and phenylephrine at ED_{50} for the relaxation failed to increase significantly the cyclic AMP content of the taenia. Even high concentrations of phenylephrine (10^{-5} M) did not increase the cyclic AMP content. The dose-response curves for isoprenaline in the fresh and cold-treated taenia became almost identical when the drug effect was expressed as a percentage increase in cyclic AMP (Figure 2b). The ED_{50} was $2.6\pm0.6\times10^{-7}$ M ($n=11$) in the fresh taenia and increased slightly to $3.1\pm0.6\times10^{-7}$M ($n=11$) with cold storage. Apparently cyclic AMP biosynthesis was not adversely affected by cold storage, as the adenylate cyclase activity and the low and high *Km*-PDE values in the cold-stored strips were not significantly different from those of the fresh preparations. Adenylate cyclase activity in the fresh tissue was 219 ± 18 pmol cyclic AMP formed and in the cold-treated strip 225 ± 36. The PDE values for low and high *Km* were 252 ± 15 pmol/mg^{-1} protein 10 min^{-1} and 129 ± 15 nmol/mg^{-1} protein 10 min^{-1} for the fresh and 278 ± 32 and 121 ± 7 for the cold-treated taenia, respectively. The cyclic AMP content meanwhile progressively declined from 9.73 ± 0.57 pmol/mg protein ($n=18$) in the fresh preparations to 6.22 ± 0.47 ($n=18$) in the 9 day and 6.08 ± 0.32 in the 12–14 day cold-stored preparations.

Time course of relaxation and cyclic AMP accumulation

Figure 3 illustrates the time course of relaxation and the changes in the tissue cyclic AMP following the administration of isoprenaline (2×10^{-7} M). In all cases, the relaxation occurred rapidly and preceded the increase in cyclic AMP. Within 6 to 10 s after the isoprenaline application, the relaxation was already approximately 35% and 57% of maximum, while the cyclic AMP content was not yet significantly increased. The inhibitory response reached a maximum after about 36 s and remained at this level for approximately 2 min when the cyclic AMP content was still increasing.

Antagonistic effect of propranolol on the isoprenaline-induced relaxation and cyclic AMP accumulation

Pretreatment with propranolol (10^{-7} and 10^{-6} M) for 15 min competitively inhibited the relaxant effect of

Table 2 The antagonistic effect of propranolol on the isoprenaline-induced relaxation of the taenia caecum

	Relaxation (%)		
		Treatment with propranolol	
Isoprenaline (M)	*Control*	1×10^{-7} M	1×10^{-6} M
10^{-9}	5.6 ± 1.1	3.1 ± 1.6	2.2 ± 0.7
3×10^{-9}	16.1 ± 2.2	6.3 ± 2.0	5.3 ± 1.6
10^{-8}	34.0 ± 4.8	10.8 ± 2.4	8.8 ± 1.6
3×10^{-8}	53.2 ± 5.6	19.0 ± 3.1	13.6 ± 1.6
10^{-7}	78.6 ± 2.7	34.2 ± 4.4	20.0 ± 2.2
3×10^{-7}	91.9 ± 2.7	56.7 ± 6.2	32.6 ± 2.9
10^{-6}	100	79.9 ± 4.2	58.4 ± 4.2
3×10^{-6}		93.7 ± 1.7	72.4 ± 3.2
10^{-5}		100	90.5 ± 2.5
3×10^{-5}			100

The maximum relaxation in response to isoprenaline served as 100% control.
Each value expresses mean ± s.e. mean of 16 and 12 experiments in control and propranolol treatment, respectively.

Table 3 The antagonistic effect of propranolol on the isoprenaline-enhanced cyclic AMP content of the taenia caecum

	Cyclic AMP (pmol/mg of protein	
Isoprenaline (M)	*No treatment*	*Propranolol* (10^{-7} M)
Control A	5.69 ± 0.53	———
Control B	———	5.08 ± 0.34
10^{-8}	6.70 ± 0.52	5.68 ± 0.34
10^{-7}	6.36 ± 0.41	5.84 ± 0.57
10^{-6}	10.50 ± 0.73	6.79 ± 0.50
10^{-5}	10.81 ± 0.90	7.28 ± 0.91
10^{-4}	10.93 ± 0.11	10.50 ± 1.1

Control A was not exposed to any agent; Control B was exposed to propranolol only.
Tissues were exposed to isoprenaline 15 min after treatment with propranolol. The tissues were exposed to isoprenaline for 1 minute. The full scale relaxation was observed within 1 min after application of isoprenaline. For each experiment, the taeniae from five different animals were used. Medium contained 2 mM theophylline. Each value of cyclic AMP is the mean ± s.e. mean of 7 experiments.

various concentrations (10^{-9} M to 3×10^{-5} M) of isoprenaline in the fresh taenia (Table 2). Similar treatment also inhibited the increase in cyclic AMP accumulation (Table 3).

The corresponding pA_2 values for the propranolol-isoprenaline interaction were 7.92 ± 0.10 for relaxation and 8.57 ± 0.14 for cyclic AMP accumulation.

Effect of papaverine and nitroglycerine on mechanical activity and cyclic AMP content

Papaverine (5×10^{-7} M – 5×10^{-5} M) and nitroglycerine (3×10^{-8} M – 3×10^{-6} M) also caused a similar dose-dependent relaxation of the taenia caecum; however, these agents did not increase the tissue cyclic AMP content (Table 4). For both drugs, the cyclic AMP level even at concentrations that elicited maximal relaxation was not significantly greater than the control.

Discussion

Early studies on the effects of catecholamines in smooth muscle demonstrated that adrenaline at doses sufficient to cause muscle relaxation induced a slight but significant increase in tissue cyclic AMP content (Butcher *et al.*, 1965; Bueding *et al.*, 1966). More

Table 4 Effect of papaverine and nitroglycerine on the mechanical activity and cyclic AMP level of the guinea-pig taenia caecum

Treatment (M)	*Relaxation* (%)	*Cyclic AMP* (pmol/mg protein)
Control	0	10.9 ± 1.0
Papaverine		
5×10^{-7}	10	10.5 ± 0.6
5×10^{-6}	50	11.0 ± 1.2
5×10^{-5}	100	10.6 ± 0.9
5×10^{-5} (3 min)	100	12.1 ± 1.3
Nitroglycerine		
3×10^{-8}	2	11.1 ± 0.4
3×10^{-7}	45	10.4 ± 1.3
3×10^{-6}	100	11.0 ± 1.6

Controls were exposed to neither agent. All experiments were carried out in the presence of theophylline (2 mM). The tissues were exposed to agents for 1 min or as specified in parentheses. The full scale relaxation was observed within 1 min after application of papaverine or nitroglycerine. Each value of cyclic AMP is the mean s.e. mean of 7–10 experiments. For each experiment, the taeniae from five different animals were used.

recently, several investigators have reported that the relaxation induced by a β-adrenoceptor stimulation is accompanied by an increase in tissue cyclic AMP in rabbit colon and guinea-pig taenia caecum (Andersson & Mohme-Lundholm, 1969; 1970; Andersson, 1972; Takayanagi *et al.*, 1972; Inatomi *et al.*, 1974). These results suggest that cyclic AMP may be the intracellular mediator of the intestinal smooth muscle relaxation produced by β-adrenoceptor activation. However, the relationship between cyclic AMP accumulation and intestinal muscular relaxation has not been carefully examined and the question still arises as to whether they are independent effects or causally interrelated.

As described previously (Fukuda & Shibata, 1972; Hattori, Kurahashi, Mori & Shibata, 1972) cold-storage treatment of the taenia caecum caused a differential effect on the isoprenaline- and phenylephrine-induced relaxation. The cold treatment reduced the efficacy as well as potency of isoprenaline, whereas it had much less effect on the response to phenylephrine, suggesting that the muscle relaxation through α- and β-adrenoceptors involves separate mechanisms. Cold storage decreased the basal cyclic AMP content of the taenia caecum; however, even in the 12–14 day cold-stored tissues in which muscle relaxation was barely measurable, isoprenaline still caused a prominent dose-related increase in cyclic AMP. In fact the ED_{50} of isoprenaline for tissue cyclic AMP increase was the same for the fresh and cold-stored preparations (Figure 2). These results would suggest a dissociation of the isoprenaline-induced events: muscle relaxation and cycle AMP accumulation,

In the fresh taenia strips the isoprenaline dose-relaxation curve was not correlated with that for cyclic AMP accumulation. At a concentration corresponding to the ED_{50} for muscle relaxation, isoprenaline did not cause an observable increase in cyclic AMP and the concentration for maximum relaxation was approximately 100 times less than that required for maximum cyclic AMP increase. Similarly, other catecholamines such as adrenaline, noradrenaline and phenylephrine at muscle relaxing concentrations failed to increase the cyclic AMP level. Therefore, it would appear that even in the fresh tissue relaxation and cyclic AMP accumulation can be separate events.

In the rabbit colon an increase in cyclic AMP preceded the relaxation induced by isoprenaline (Andersson & Mohme-Lundholm, 1969; Andersson, 1972). Conversely, in the present experiments, the relaxation started before any increase in cyclic AMP. The onset of the relaxant effect was not in phase with cyclic AMP production.

The antagonism of the isoprenaline-induced relaxation and cyclic AMP accumulation by propranolol would indicate that both phenomena are mediated through activation of β-receptors. However, the pA_2 value for cyclic AMP accumulation was much greater than for the relaxation effect. Presumably, the β-adrenoceptors that stimulate the relaxing mechanism have different properties from the receptors that activate cyclic AMP production. This would explain the lack of inhibitory effect of exogenously administered dibutyryl cyclic AMP on intestinal smooth muscle (Levine, 1968; Andersson & Mohme-Lundholm, 1970; Fukuda & Shibata, 1972).

Takayanagi *et al.* (1972) and Inatomi *et al.* (1974) showed that papaverine increases the intracellular

cyclic AMP level in guinea-pig taenia caecum and Andersson (1973) reported that the relaxation of the rabbit colon by papaverine and nitroglycerine is preceded by an increase in the nucleotide. However, in the present investigation the relaxation by papaverine and nitroglycerine occurred without any accompanying change in tissue cyclic AMP, adenylate cyclase or PDE activity. These contrasting results may be explained by the different drug concentrations employed. In these experiments the concentrations of papaverine and nitroglycerine were approximately 5 to 200 times less than employed by Takayanagi *et al.* (1972), Inatomi *et al.* (1974) and Andersson (1973). In this regard, Polacek, Bolan & Daniel (1971) observed little or no elevation of tissue cyclic AMP level in the rat uterus with low but relaxation-producing concentrations of papaverine. Therefore, the present data also provide evidence that the relaxation induced by papaverine and nitroglycerine is not mediated by an increase in tissue cyclic AMP.

The cold-storage treatment did not alter the adenylate cyclase or phosphodiesterase (low *Km* and high *Km*) activities in the taenia. Kuchii, Miyahara & Shibata (1973) previously demonstrated that cold storage had little effect on the biotransformation of adenosine to adenosine triphosphate in the taenia caecum. Therefore, the low level of cyclic AMP in the cold-stored tissue compared to the fresh preparation probably stems from a decrease in endogenous ATP.

The present study was concerned with measurement of total tissue cyclic AMP and does not eliminate the possibility that cyclic AMP changes in subcellular compartments may be related to the relaxant effect.

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