

## Signal Transduction Pathway Involved in $\beta_3$ -Adrenoceptor-Mediated Relaxation in Guinea Pig Taenia Caecum

Katsuo Koike, Takahiro Horinouchi and Issei Takayanagi\*

Department of Chemical Pharmacology, Toho University School of Pharmaceutical Sciences, 2-2-1, Miyama, Funabashi, Chiba 274, Japan

Received November 24, 1994 Accepted January 31, 1995

**ABSTRACT**—Experiments were carried out to examine the components of the intracellular second messenger system that is involved in  $\beta_3$ -adrenoceptor (atypical  $\beta$ -adrenoceptors)-mediated relaxation in the guinea pig taenia caecum. Propranolol and butoxamine caused competitive antagonism of the relaxant response to isoprenaline. However, propranolol or butoxamine did not significantly affect the relaxant responses to CGP 12177 (4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazol-2-one), a  $\beta_3$ -adrenoceptor agonist. The concentration-response curves of the isoprenaline-induced increase in adenosine 3',5'-cyclic monophosphate (cyclic AMP) levels were shifted to the right in a parallel manner by propranolol and butoxamine. However, propranolol or butoxamine did not significantly affect the concentration-response curve for the CGP 12177-induced increase in cyclic AMP levels. MDL 12330 (*cis-N*-(2-phenylcyclopentyl)-azacyclotridec-1-en-2-amine) inhibited the isoprenaline- or CGP 12177-induced increase in cyclic AMP levels. These results suggest that the production of cyclic AMP contributes to the  $\beta_3$ -adrenoceptor (or atypical  $\beta$ -adrenoceptor)-mediated relaxation of the guinea pig taenia caecum.

**Keywords:** Taenia caecum (guinea pig),  $\beta$ -Adrenoceptor,  $\beta_3$ -Adrenoceptor, CGP 12177, Cyclic AMP

$\beta$ -Adrenoceptors are integral membrane proteins mediating a wide variety of the physiological actions of catecholamines, through coupling to guanine nucleotide-binding regulatory proteins (G proteins) and activation of adenylate cyclase. The existence of two subtypes of the  $\beta$ -adrenoceptor is generally accepted (1). However,  $\beta$ -adrenoceptors have now been reclassified by molecular biological studies into three subtypes:  $\beta_1$ ,  $\beta_2$  and  $\beta_3$  (2, 3). Therefore, it is timely to re-evaluate the pharmacological characterization of many  $\beta$ -adrenoceptor-mediated effects and to establish the role of  $\beta_3$ -adrenoceptors in comparison with those of  $\beta_1$ - and  $\beta_2$ -adrenoceptors (4–7).

We have reported that predominantly  $\beta_2$ - and partly  $\beta_3$ -adrenoceptors (atypical  $\beta$ -adrenoceptor) are involved in the  $\beta$ -adrenoceptor-mediated relaxation of the guinea pig taenia caecum and that CGP 12177 (a  $\beta_1/\beta_2$ -adrenoceptor antagonist and a  $\beta_3$ -adrenoceptor agonist)-induced relaxation of the guinea pig taenia caecum is solely mediated through  $\beta_3$ -adrenoceptors (8).

In general, it is accepted that all three  $\beta$ -adrenoceptor subtypes ( $\beta_1$ -,  $\beta_2$ - and  $\beta_3$ -subtypes) are coupled to the acti-

vation of adenylate cyclase and acted through the same effector pathway-cAMP as a primary mechanism for signal transduction (9). However, the signal transduction pathway that is activated by the occupation of  $\beta_3$ -adrenoceptors on the guinea pig taenia caecum is not known. The aim of the present study is, therefore, to examine elements of the possible signal transduction pathway that is involved in  $\beta_3$ -adrenoceptor (or atypical  $\beta$ -adrenoceptors)-mediated relaxation in the guinea pig taenia caecum.

### MATERIALS AND METHODS

#### *Mechanical responses*

Male guinea pigs weighing 300 to 500 g were killed by a blow on the head. A 2- to 3-cm piece of the taenia caecum was isolated and suspended in a 20-ml organ bath filled with a Ringer-Locke solution (154 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl<sub>2</sub>, 2.1 mM MgCl<sub>2</sub>, 5.9 mM NaHCO<sub>3</sub>, and 2.8 mM glucose) kept at 32°C and bubbled with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The mechanical responses of the smooth muscle preparations were recorded isotonically under a tension of 0.7 g. The experiments

\*To whom correspondence should be addressed.

were started after the preparations had been allowed to develop their spontaneous tone for 2 hr. The concentration-response curves of the test drugs were obtained cumulatively, and the relaxation induced by these drugs was expressed as a percentage of the maximal relaxation produced by isoprenaline. To test the antagonism, one of the antagonists was added to the bath 30 min before the addition of the agonist. The concentration-response curves to isoprenaline were then obtained in the presence of an antagonist. The time interval between two consecutive curves was usually set at 60 min. When cumulative concentration-response curves for isoprenaline were successively obtained two or three times with the 60-min interval, the maximum response and sensitivity did not change markedly during the procedure (data not shown). The agonistic potency was expressed as the  $pD_2$  value (10). The competitive antagonistic potency was expressed as the  $pA_2$  value. It was calculated according to the method of Tallarida et al. (11), which was originally reported by Arunlakshana and Schild (12).

#### *Estimation of cyclic AMP levels*

The tissue content of cyclic AMP was estimated by the method of Steiner et al. (13). Two pieces of taenia were removed from one caecum and suspended in two similar 20-ml baths filled with a Ringer-Locke solution kept at 32°C and gassed with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub>. They were frozen in liquid nitrogen immediately after the test drugs were added to the baths for 2 min (14). One piece was used for measuring the control level of cyclic AMP and the other for estimating any change of cyclic AMP concentration after treatment with the test drugs. The tissues were homogenized with a glass homogenizer in 2 ml of cold trichloroacetic acid (6% w/v) that contained 0.5 pmol of [<sup>3</sup>H]cyclic GMP to estimate the recovery of cyclic AMP (15). The homogenate was centrifuged at 3000 rpm at 0°C for 15 min, the supernatant was then acidified with 1 N HCl, and the trichloroacetic acid was extracted with ether. The cyclic AMP samples were lyophilized. The lyophilized samples were dissolved with sodium acetate buffer, pH 6.2, and they were used for the estimation of cyclic AMP and for calculating the recovery (15). The quantity of cyclic AMP was determined by a radioimmunoassay, using a commercial kit (Yamasa, Chiba).

The concentration-response curves of the increase in cyclic AMP levels induced by isoprenaline and CGP 12177 were obtained. Five different concentrations, ranging from 10<sup>-8</sup> to 10<sup>-6</sup> M, were used for isoprenaline, while four different concentrations, ranging from 10<sup>-8</sup> to 10<sup>-5</sup> M, were used for CGP 12177. Each series of concentrations represents one experiment. Cyclic AMP levels were determined as the percentage of the maximum isoprenaline response for each experiment separately. To

test the antagonism, one of antagonists was added to the bath 30 min before the addition of the agonists. The agonistic potency was expressed as the  $pD_2$  value (10). The competitive antagonistic potency was expressed as the  $pA_2$  value, which was estimated as the negative logarithm of the dissociation constant from the antagonism experiments. It was calculated according to the method of Tallarida et al. (11), which was originally reported by Arunlakshana and Schild (12).

#### *Protein assay*

Protein concentrations were determined by the method of Lowry et al. (16) with bovine serum albumin as a standard.

#### *Data analyses*

Numerical results are expressed as means  $\pm$  S.E., and statistical analyses were performed by Student's *t*-test and Duncan's new multiple range test as appropriate. A *P* value of less than 0.05 was considered to indicate a significant difference.

#### *Drugs and chemicals*

The drugs used were obtained from the following sources: (–)-isoprenaline hydrochloride, butoxamine hydrochloride, (±)-propranolol hydrochloride (Sigma Chemical Co., St. Louis, MO, USA); (±)-CGP 12177 (4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazol-2-one) hydrochloride, MDL-12330 (*cis-N*-(2-phenylcyclopentyl)-azacyclotridec-1-en-2-amine) hydrochloride (Research Biochemicals Inc., Natick, MA, USA); and [<sup>3</sup>H]cyclic GMP (specific activity, 36.4 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear, Boston, MA, USA). All the drugs were used as a solution in distilled water. The other chemicals used were of analytical grade.

## RESULTS

#### *Mechanical responses*

Isoprenaline and CGP 12177 caused graded relaxation of the guinea pig taenia caecum where tone had been raised spontaneously. The  $pD_2$  values were  $8.32 \pm 0.05$  (mean  $\pm$  S.E., *N* = 6) for isoprenaline and  $7.00 \pm 0.17$  (mean  $\pm$  S.E., *N* = 6) for CGP 12177. Propranolol (10<sup>-8</sup>–10<sup>-6</sup> M), a non-selective  $\beta_1$ - and  $\beta_2$ -adrenoceptor antagonist, competitively antagonized the relaxation responses to isoprenaline. The Schild plot of the data revealed the  $pA_2$  value of  $8.39 \pm 0.02$ , the slope of the regression line ( $1.01 \pm 0.03$ ) not being significantly different from unity (Table 1). However, propranolol ( $\sim 10^{-6}$  M) did not significantly affect the relaxant responses to CGP 12177 (Table 1).

**Table 1.** The  $pA_2$  values for propranolol and butoxamine

Antagonists	$pA_2$ value			
	Cyclic AMP levels		Mechanical responses	
	(agonist) Isoprenaline	(agonist) CGP 12177	(agonist) Isoprenaline	(agonist) CGP 12177
Propranolol	$8.49 \pm 0.06$	No effect <sup>*1</sup>	$8.39 \pm 0.02$	No effect <sup>*1</sup>
Butoxamine	$6.32 \pm 0.02$	No effect <sup>*2</sup>	$6.46 \pm 0.06$	No effect <sup>*2</sup>

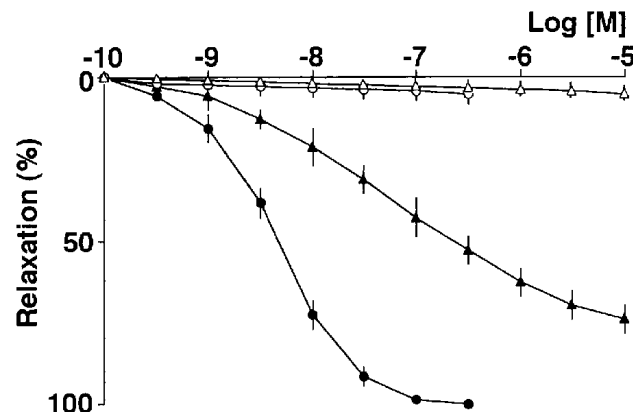
Each value is presented as a mean  $\pm$  S.E. of 6 experiments. <sup>\*1</sup>No effect up to  $10^{-6}$  M. <sup>\*2</sup>No effect up to  $10^{-4}$  M.

Butoxamine ( $10^{-6}$ – $10^{-5}$  M), a selective  $\beta_2$ -adrenoceptor antagonist, caused competitive antagonism of the relaxant responses. The Schild plot of the data revealed the  $pA_2$  value of  $6.46 \pm 0.06$ , which was the same as the previously reported one (8). Butoxamine did not significantly affect the relaxant responses to CGP 12177, as previously reported (8).

MDL 12330 ( $3 \times 10^{-4}$  M), an adenylate cyclase inhibitor, inhibited the relaxation responses to isoprenaline and CGP 12177 (Fig. 1). Moreover, MDL 12330 ( $3 \times 10^{-4}$  M) increased the spontaneous tone of the taenia caecum in some degree.

#### Cyclic AMP levels

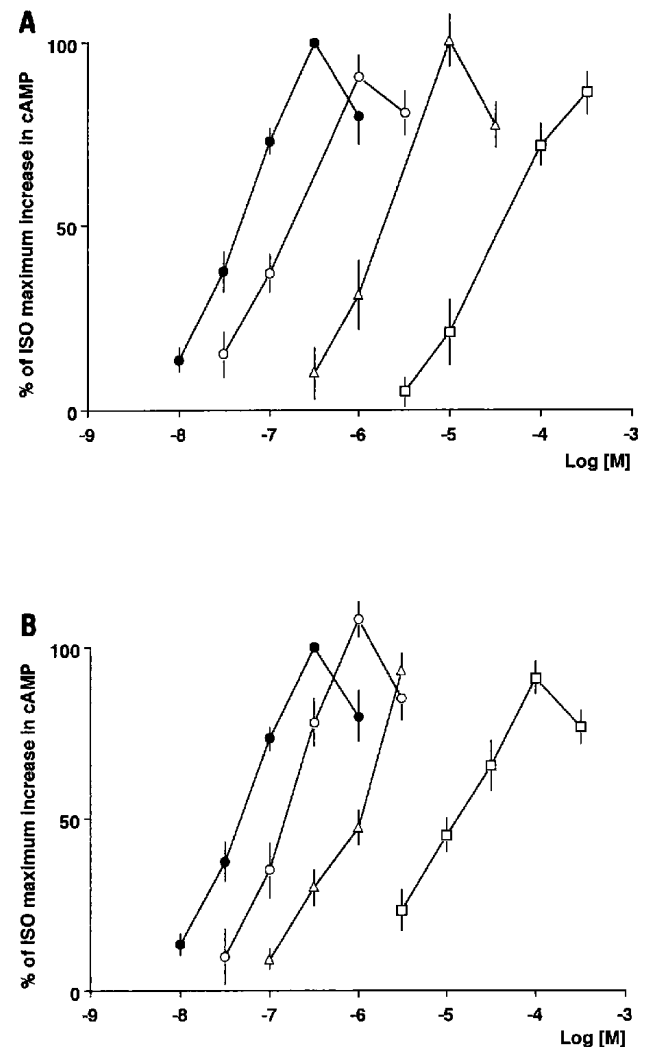
Isoprenaline and CGP 12177 increased cyclic AMP levels in the guinea pig taenia caecum. The  $pD_2$  values were  $7.33 \pm 0.07$  (mean  $\pm$  S.E.,  $N=6$ ) for isoprenaline and  $7.03 \pm 0.11$  (mean  $\pm$  S.E.,  $N=6$ ) for CGP 12177. The concentration-response curves from isoprenaline-induced increase in cyclic AMP levels were shifted to the right in



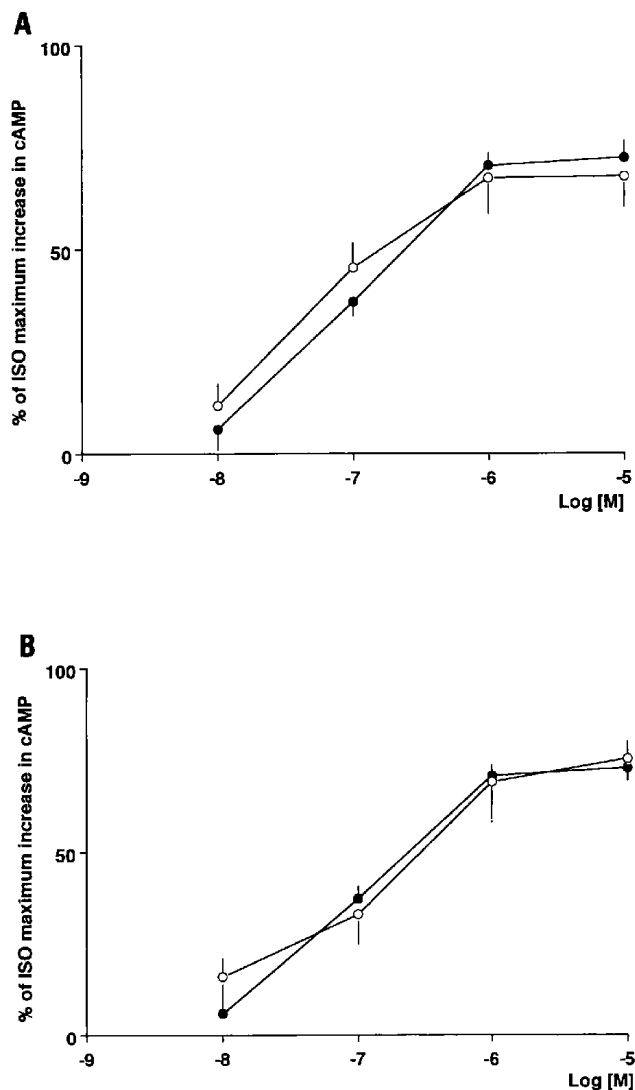
**Fig. 1.** Concentration-response curves for isoprenaline and CGP 12177 in the presence or absence of MDL 12330. Isoprenaline (●); MDL 12330 at  $3 \times 10^{-4}$  M (○). CGP 12177 (▲); MDL 12330 at  $3 \times 10^{-4}$  M (△). Ordinate: relaxation (%), expressed as a percentage of the maximal relaxation induced by isoprenaline. Abscissa: Log concentration (M) of isoprenaline or CGP 12177. Each point is presented as a mean  $\pm$  S.E. of 6 experiments.

a parallel manner by propranolol or butoxamine (Fig. 2, A and B). The  $pA_2$  values of propranolol and butoxamine against isoprenaline, which were calculated from the shift of each curve, are summarized in Table 1. However, propranolol or butoxamine did not significantly affect the concentration-response curve for the CGP 12177-induced increase in cyclic AMP levels (Fig. 3, A and B).

Moreover, MDL 12330 significantly inhibited the isoprenaline- or CGP 12177-induced increase in cyclic AMP levels (Fig. 4). As shown in Fig. 4, MDL 12330 ( $3 \times 10^{-4}$  M) also inhibited the basal cyclic AMP level.



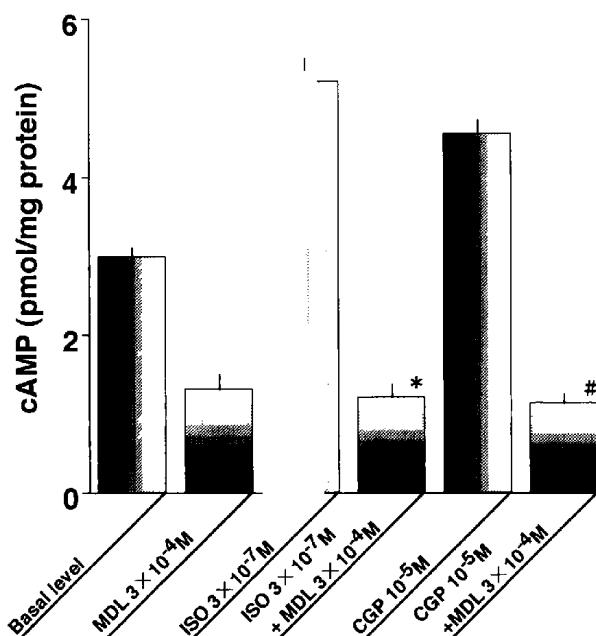
**Fig. 2.** Concentration-response curve for isoprenaline in the presence or absence of propranolol or butoxamine in increasing cyclic AMP levels. A: Control (●); propranolol at  $10^{-8}$  M (○),  $10^{-7}$  M (△) or  $10^{-6}$  M (□). B: Control (●); butoxamine at  $10^{-6}$  M (○),  $10^{-5}$  M (△) or  $10^{-4}$  M (□). Ordinate: percent of the maximal increase in cyclic AMP levels induced by isoprenaline. Abscissa: Log concentration (M) of isoprenaline. Each point is presented as a mean  $\pm$  S.E. of 6 experiments.



**Fig. 3.** Concentration-response curve for CGP 12177 in the presence or absence of propranolol or butoxamine in increasing cyclic AMP levels. **A:** Control (●); propranolol at  $10^{-6}$  M (○). **B:** Control (●); butoxamine at  $10^{-4}$  M (○). Ordinate: percent of the maximal increase in cyclic AMP levels induced by isoprenaline. Abscissa: Log concentration (M) of CGP 12177. Each point is presented as a mean  $\pm$  S.E. of 6 experiments.

## DISCUSSION

A previous study suggested that isoprenaline-induced relaxation of the guinea pig taenia caecum predominantly involves  $\beta_2$ -adrenoceptors, whereas CGP 12177-induced relaxation is solely mediated through  $\beta_3$ -adrenoceptors (8). In the present study, the action of isoprenaline was blocked by propranolol, whereas the response to CGP 12177 was completely insensitive to blockade with propranolol. One of the predominant pharmacological characteristics of the  $\beta_3$ -adrenoceptor is its relatively low affinity towards some  $\beta$ -adrenoceptor antagonists; i.e.,



**Fig. 4.** Effects of MDL 12330 on the isoprenaline- and CGP 12177-induced increase in cyclic AMP levels. Basal level: control; MDL: MDL 12330, ISO: isoprenaline, CGP: CGP 12177. Each column is presented as a mean  $\pm$  S.E. of 6 experiments. The asterisk denotes a significant difference from isoprenaline alone ( $P < 0.05$ ). The sharp denotes a significant difference from CGP 12177 alone ( $P < 0.05$ ).

propranolol and its many analogs (2, 3, 5, 17). The results confirmed the previous observation that the CGP 12177-induced relaxation of the guinea pig taenia caecum was solely mediated by  $\beta_3$ -adrenoceptors.

Tate et al. (18) have shown that three subtypes of  $\beta$ -adrenoceptors are expressed in an eukaryotic cell type in which the receptors may be coupled with a stimulatory guanosine-nucleotide-binding protein for activation of adenylate cyclase. From the results reported here, it appears that CGP 12177 stimulates cyclic AMP production in the guinea pig taenia caecum. However, this production is not abolished by propranolol, a non-selective  $\beta_1$ - and  $\beta_2$ -adrenoceptor antagonist or butoxamine, a selective  $\beta_2$ -adrenoceptor antagonist, suggesting that CGP 12177-induced increase in cyclic AMP levels is mediated by  $\beta_3$ -adrenoceptor (atypical  $\beta$ -adrenoceptors). On the other hand, the isoprenaline-induced increase in cyclic AMP levels (mediating  $\beta_2$ -adrenoceptors) was inhibited by propranolol or butoxamine, and the  $pA_2$  values of those antagonists obtained from the increase in cyclic AMP levels were in good agreement with their  $pA_2$  values obtained from the mechanical responses (Table 1). These results suggest that the production of cyclic AMP contributes to the  $\beta_3$ -adrenoceptor-mediated relaxation of the guinea pig taenia caecum, like  $\beta_2$ -adrenoceptors. Moreover, our results suggest that if  $\beta_2$ - and  $\beta_3$ -adrenocep-

tors coexist and both have an important role in catecholamine-mediated relaxation of the guinea pig taenia caecum, it is necessary to stimulate both receptors to induce relaxation.

Additionally, when differences between the  $pD_2$  values for isoprenaline and CGP 12177 obtained from the mechanical responses and from the increases in cyclic AMP levels were calculated, the difference for isoprenaline was larger than that for CGP 12177. These differences seem to be due to the existence of spare receptors as previously reported (14). These results suggest that there are spare receptors for isoprenaline, but few for CGP 12177, in the guinea pig taenia caecum.

It is generally accepted that the actions of a number of peptide hormones, catecholamines and prostaglandins are cAMP-mediated. Agents that specifically affect the activities of adenylate cyclase are useful tools for determining the importance of cyclic AMP in the regulation of cellular processes. For example, some adenylate cyclase inhibitors now available give additional support to the role of cyclic AMP, when they block the hormonal action. Recently, a cycloalkyl compound, MDL 12330, has been reported to inhibit adenylate cyclase activity in rat liver (19), kidney from rat, mouse and chick (20) and frog skin (21). Furthermore, it has been demonstrated that MDL 12330 at the concentration of  $3 \times 10^{-4}$  M, which was used in this study, decreases the basal cyclic AMP level by inhibiting the activities of adenylate cyclase (19, 20), and our result is in agreement with that observation. MDL 12330 at the concentration of  $3 \times 10^{-4}$  M seems to completely inhibit adenylate cyclase activity in the guinea pig taenia caecum. In the present study, the CGP 12177-induced increase in cyclic AMP levels was inhibited by MDL 12330. These results suggest that the CGP 12177-induced increase in cyclic AMP levels is directly mediated through the  $\beta_3$ -adrenoceptor-coupled adenylate cyclase system. Moreover, the isoprenaline-induced increase in cyclic AMP levels (mediated by  $\beta_2$ -adrenoceptors) was also inhibited by MDL 12330. Our results suggest that both  $\beta_2$ - and  $\beta_3$ -adrenoceptor-mediated relaxation of the guinea pig taenia caecum is related to activation of the same effector pathway-cAMP. However, since we did not examine whether  $\beta_2$ - and  $\beta_3$ -adrenoceptors are coupled to the same adenylate cyclase, much additional experimental support is necessary.

We conclude that CGP 12177-induced relaxation (mediating  $\beta_3$ -adrenoceptors) is strongly coupled to the production of cyclic AMP and that functional  $\beta_3$ -adrenoceptors coupled to adenylate cyclase are present in the guinea pig taenia caecum.

## REFERENCES

- 1 Lands AM, Arnold A, McAuliff JP, Luduena FP and Brown G: Differentiation of receptor systems activated by sympathomimetic amines. *Nature* **214**, 597–598 (1967)
- 2 Emorine LJ, Marullo S, Briend-Sutren M-M, Patey G, Tate K, Delavier-Klutcho C and Strosberg AD: Molecular characterization of the human  $\beta_3$ -adrenergic receptor. *Science* **245**, 1118–1121 (1989)
- 3 Emorine LJ, Feve B, Pairault J, Briend-Sutren M-M, Marullo S, Delavier-Klutcho C and Strosberg AD: Structural basis for functional diversity of  $\beta_1$ -,  $\beta_2$ - and  $\beta_3$ -adrenergic receptors. *Biochem Pharmacol* **41**, 853–859 (1991)
- 4 Blin N and Strosberg AD: The human  $\beta_3$ -adrenoceptor mediates lipid metabolism; other physiological roles remain to be determined. *Trends Pharmacol Sci* **15**, 281–282 (1994)
- 5 Emorine LJ, Blin N and Strosberg AD: The human  $\beta_3$ -adrenoceptor: the search for a physiological function. *Trends Pharmacol Sci* **15**, 3–7 (1994)
- 6 Reverte M: Pharmacological effects of  $\beta_3$ -adrenoceptors: Additional physiological functions of the  $\beta_3$ -adrenoceptor. *Trends Pharmacol Sci* **15**, 281 (1994)
- 7 Silleuce MN and Matthews ML: Classical and atypical binding sites for  $\beta$ -adrenoceptor ligands and activation of adenylate cyclase in bovine skeletal muscle and adipose tissue membranes. *Br J Pharmacol* **111**, 866–872 (1994)
- 8 Koike K, Takayanagi I, Muramatsu M, Ohki S and Horinouchi T: Involvement of  $\beta_3$ -adrenoceptor in the relaxation response in guinea pig taenia caecum. *Jpn J Pharmacol* **66**, 213–220 (1994)
- 9 Zaagsma J and Hollenga Ch: Distribution and function of atypical  $\beta_3$ -adrenoceptors. In *Adrenoceptors: Structure, Mechanisms, Function*, Edited by Szabadi E and Bradshaw CM, pp 47–58, Birkhäuser Verlag, Basel (1991)
- 10 van Rossum JM: Cumulative dose-response curve. II. Techniques of making of dose-response curves in isolated organs and evaluation of drug parameters. *Arch Int Pharmacodyn Ther* **143**, 299–330 (1963)
- 11 Tallarida RJ, Cowan A and Adler MW:  $pA_2$  and receptor differentiation: a statistical analysis of competitive antagonism. *Life Sci* **25**, 637–654 (1979)
- 12 Arunlakshana O and Schild HO: Some quantitative uses of drug antagonists. *Br J Pharmacol Chemother* **14**, 48–58 (1959)
- 13 Steiner AL, Parker CW and Kipnis DM: Radioimmunoassay for cyclic nucleotides I. Preparation of antibodies and iodinated cyclic nucleotides. *J Biol Chem* **247**, 1106–1113 (1972)
- 14 Koike K and Takayanagi I: Relationship between intrinsic activity of  $\beta$ -adrenoceptor agonist and amount of spare receptors in guinea pig taenia caecum. *Jpn J Pharmacol* **33**, 327–333 (1983)
- 15 Ohkubo H, Takayanagi I and Takagi K: Relationship between the levels of intracellular cyclic nucleotides and mechanical responses induced by drugs. *Jpn J Pharmacol* **26**, 65–71 (1976)
- 16 Lowry OH, Rosebrough NJ, Farr AL and Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265–275 (1951)
- 17 McLaughlin DP and MacDonald A: Evidence for the existence of 'atypical'  $\beta$ -adrenoceptors ( $\beta_3$ -adrenoceptors) mediating relaxation in the rat distal colon in vitro. *Br J Pharmacol* **101**, 569–574 (1990)
- 18 Tate KM, Briend-Sutren M-M, Emorine LJ, Delavier-Klutcho C, Marullo S and Strosberg AD: Expression of three human  $\beta$ -

- adrenergic-receptor subtypes in transfected Chinese hamster ovary cells. *Eur J Biochem* **196**, 357–361 (1991)
- 19 Guellaen G, Mahu JL, Mavier P, Berthelot P and Hanoune J: RMI 12330 A, an inhibitor of adenylate cyclase in rat liver. *Biochim Biophys Acta* **484**, 465–475 (1977)
- 20 Hunt NH and Evans T: RMI 12330A, an inhibitor of cyclic nucleotide phosphodiesterase and adenylate cyclase in kidney preparations. *Biochim Biophys Acta* **613**, 499–506 (1980)
- 21 Lippe C and Ardizzone C: Actions of vasopressin and isoprenaline on the ionic transport across the isolated frog skin in the presence and the absence of adenylate cyclase inhibitors MDL 12330A and SQ 22536. *Comp Biochem Physiol* **99C**, 209–211 (1991)