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Cyclic AMP-independent relaxation mediated by β_3-adrenoceptors on guinea pig gastrointestine

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Abstract

In this study, we investigated the signal transduction pathway involved in β_3-adrenoceptor-mediated relaxations of guinea pig gastric fundus and duodenum. In the presence of β_1- and β_2-adrenoceptor blockade, the potency (pD_2 value) of catecholamines ((−)-isoprenaline, (−)-noradrenaline and (−)-adrenaline) and β_3-adrenoceptor agonists ((*R**, *R**)-(±)-4-[2-[(2-(3-chlorophenyl)-2-hydroxyethyl)amino]propyl]phenoxyacetic acid sodium (BRL37344) and (±)-[4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2*H*-benzimidazol-2-one] hydrochloride ((±)-CGP12177A)) to induce relaxation was not affected by the adenylate cyclase inhibitor, 9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine (SQ-22,536, 100 μM). Catecholamines induced an elevation of cyclic AMP and SQ-22,536 significantly abolished the responses of gastric fundus. However, cyclic AMP levels were unaltered by the β_3-adrenoceptor agonists in gastric fundus and by the five agonists in duodenum. Furthermore, the relaxant responses to catecholamines and to β_3-adrenoceptor agonists were unaffected by the cyclic AMP-dependent protein kinase inhibitor, *N*-(2-[*p*-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide (H-89, 10 μM) in gastric fundus. These results suggest that β_3-adrenoceptor-induced relaxation is mediated through both cyclic AMP-dependent and cyclic AMP-independent pathways in gastric fundus and through a cyclic AMP-independent pathway in duodenum. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: β-Adrenoceptor, atypical; β_3-Adrenoceptor; Gastric fundus, guinea pig; Duodenum, guinea pig; SQ-22,536; cAMP

1. Introduction

Atypical β-adrenoceptors including β_3-adrenoceptors, which are different from classical β_1- and β_2-adrenoceptors (Arch and Kaumann, 1993), are the predominant β-adrenoceptors mediating relaxation of gastrointestinal smooth muscles in various species (Manara et al., 1995). In the guinea pig gastric fundus and duodenum, β_3-adrenoceptor mediated relaxant responses to catecholamines ((−)-isoprenaline, (−)-noradrenaline and (−)-adrenaline), to the selective β_3-adrenoceptor agonist, (*R**, *R**)-(±)-4-[2-[(2-(3-chlorophenyl)-2-hydroxyethyl)amino]propyl]phenoxyacetic acid sodium (BRL37344), and to the non-conventional partial β_3-adrenoceptor agonist, (±)-[4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2*H*-benzimidazol-2-one] hydrochloride ((±)-CGP12177A) are resistant to blockade by classical β-adrenoceptor antagonists but are antagonized by (±)-bupranolol, a non-selective β_1-, β_2- and β_3-adrenoceptor antagonist (Horinouchi and Koike, 1999a,b).

Stimulation of classical β-adrenoceptors and β_3-adrenoceptors increases adenosine 3′:5′-cyclic monophosphate (cyclic AMP) production via activation of G_s protein (Rasmussen, 1986; Guan et al., 1995; Deng et al., 1997). The relaxation of smooth muscle induced by cyclic AMP is mediated through the activation of cyclic AMP-dependent protein kinase and subsequent phosphorylation of specific proteins (Hardman, 1984; Bennet et al., 1989; Emorine et al., 1991). However, there is little information on the signal transduction pathway involved in β_3-adrenoceptor-mediated relaxation of gastrointestinal smooth muscle. Furthermore, in some cell types, although β_3-adrenoceptors are predominant, increases in cyclic AMP occur primarily through β_1- and β_2-adrenoceptors (Jockers et al., 1998). This suggests that β_3-adrenoceptor-induced responses may be mediated through other signal transduction pathways.

The purpose of the present study was to investigate the effects of the adenylate cyclase inhibitor, 9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine (SQ-22,536; Haslam et al.,

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1978), on the relaxation and cyclic AMP levels elicited by catecholamines ((−)-isoprenaline, (−)-noradrenaline and (−)-adrenaline) and β_3-adrenoceptor agonists (BRL37344 and (±)-CGP12177A) in the guinea pig gastric fundus and duodenum. In addition, the effects of the cyclic AMP-dependent protein kinase inhibitor, *N*-(2-[*p*-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide (H-89; Chijiwa et al., 1990; Engh et al., 1996), were examined to clarify whether the β_3-adrenoceptor-mediated relaxation in response to these agonists is mediated through a cyclic AMP/cyclic AMP-dependent protein kinase pathway.

2. Materials and methods

2.1. Animals and tissue preparation

Male Hartley guinea pigs weighing 300–500 g (Saitama Experimental Animals, Saitama, Japan) were used in accordance with the Guide for the Care and Use of Laboratory Animals of Toho University School of Pharmaceutical Sciences (which is accredited by the Ministry of Education, Culture, Sports, Science and Technology, Japan), and the protocol of the present study was approved by the Institutional Animal Care and Use Committee. Guinea pigs were housed under laboratory standard conditions on a 12-h light/dark cycle (lights on 8:00 AM; lights off 8:00 PM) in temperature- (20–22 °C) and relative air humidity-(50 ± 5%) controlled room. Food and water were available ad libitum.

Guinea pigs were killed by cervical dislocation and the gastric fundus and duodenum were isolated. The stomach and luminal contents were removed immediately and the connective tissue was dissected away. After the mucosa was removed, strips of approximately 15–20 mm in length and 4–6 mm in width were cut in the direction of the longitudinal smooth muscle, with a maximum of four strips from one guinea pig gastric fundus. The outer layer of duodenum (approximately 10–15 mm in length) containing longitudinal smooth muscle was carefully removed with a cotton swab. Strips were mounted vertically under an initial tension of 0.5 × *g* in a 20-ml organ bath containing Ringer–Locke solution (NaCl, 154; KCl, 5.6; $CaCl_2$, 2.2; $MgCl_2$, 2.1; $NaHCO_3$, 5.9 and glucose, 2.8 mM), maintained at 32 °C and bubbled continuously with a mixture of 95% O_2 and 5% CO_2 (pH 7.4). Imipramine (1 μM, a neuronal uptake inhibitor), normetanephrine (10 μM, an extraneuronal uptake inhibitor), phentolamine (10 μM, an α-adrenoceptor antagonist) and L-ascorbic acid (10 μM, to prevent oxidation of catecholamines) were present in the medium throughout all experiments.

2.2. Experimental protocols

After the preparations were allowed to equilibrate for 30 min in the absence of β-adrenoceptor antagonist, they were contracted with prostaglandin $F_{2\alpha}$ (3 μM; gastric fundus) or histamine (10 μM; duodenum). The β-adrenoceptor-mediated relaxation caused by test drugs was determined by measuring the inhibition of the contractile drug-induced contraction. First, concentration–response curves for (−)-isoprenaline (up to 3 μM) were generated as controls (100%). Prostaglandin $F_{2\alpha}$ (3 μM; gastric fundus) or histamine (10 μM; duodenum) was added to the bath 30 min after wash out of the drug, and then test drugs were added cumulatively until a maximal relaxant response was observed. The relaxation induced by these drugs is expressed as a percentage of the maximal relaxation produced by the reference drug, (−)-isoprenaline (3 μM), in the absence of β-adrenoceptor antagonist.

In order to assess the β_3-adrenoceptor-mediated relaxation, a combination of the selective β_1-adrenoceptor antagonist, (±)-atenolol (100 μM), and the selective β_2-adrenoceptor antagonist, (±)-butoxamine (100 μM) (gastric fundus; Horinouchi and Koike, 1999a), or the non-selective β_1- and β_2-adrenoceptor antagonist, (±)-propranolol (1 μM) (duodenum; Horinouchi and Koike, 1999b), was added to the bath 60 min before the cumulative addition of the test drug. (±)-Atenolol (100 μM), (±)-butoxamine (100 μM) and (±)-propranolol (1 μM) themselves did not inhibit the drug-induced contraction (data not shown). Concentration–response curves for catecholamines ((−)-isoprenaline, (−)-noradrenaline and (−)-adrenaline) and for selective β_3-adrenoceptor agonists (BRL37344 and (±)-CGP12177A) were obtained in the absence or presence of the adenylate cyclase inhibitor, SQ-22,536 (100 μM), or the cyclic AMP-dependent protein kinase inhibitor, H-89 (10 μM). To test the effect of SQ-22,536, one of the agonists was added to the bath after a maximal relaxant response to SQ-22,536 (100 μM) was established. H-89 was applied 60 min before the addition of the agonists and remained throughout. In preliminary experiments, the tissue sensitivity and the maximal response to BRL37344 and (±)-CGP12177A decreased when two consecutive concentration–response curves for these drugs were recorded with the same segment (data not shown); therefore, a single cumulative concentration–response curve for each test drug was made for each strip.

2.3. Cyclic AMP assay

At the end of the protocol, the tissues were quickly removed from the organ bath and immediately frozen in liquid N_2. The frozen tissues were then homogenized in ice-cold 6%wv^{-1} trichloroacetic acid containing 100 μM 3-isobutyl-1-methylxanthine (IBMX) with a Teflon pestle homogenizer. Homogenates were stored at 4 °C for 60 min, and then centrifuged (3000 rpm) for 30 min at 4 °C. The pH of the supernatant was acidified by the addition of 1 N HCl. Trichloroacetic acid was extracted by washing supernatants four times with water-saturated diethyl ether (five volumes of ether to one volume of supernatant). Aqueous phases were

lyophilized to dryness and the residue was resuspended in 0.05 M sodium acetate buffer (pH 6.2, 4 °C). The cyclic AMP content of samples was measured using a radioimmunoassay kit (Yamasa, Chiba, Japan) following the instructions of the manufacturer. Tissue pellets were dissolved in 1 N NaOH and protein content was determined by the method of Lowry et al. (1951) with bovine serum albumin used as standard. The cyclic AMP content is expressed as picomoles per milligram of sample protein. In most other studies, tissues were often exposed to phosphodiesterase inhibitors, because cyclic AMP is broken down by phosphodiesterase (mainly phosphodiesterase type 3) (Beavo et al., 1994). However, phosphodiesterase inhibitors inhibit prostaglandin $F_{2\alpha}$- and histamine-induced contractions and cause relaxation by themselves in the guinea pig gastric fundus and duodenum (data not shown). Moreover, phosphodiesterase inhibitors elevate cyclic AMP to unphysiologically high levels, which complicates the interpretation of data (Turcato and Clapp, 1999). In this study, therefore, tissues were not exposed to phosphodiesterase inhibitors in order to compare directly tension and cyclic AMP.

2.4. Data analysis

The results are expressed as means ± S.E.M. of the number (n) of experiments. Agonist potency is expressed as the pD_2 value (Van Rossum, 1963). The intrinsic activity of each drug was calculated as the ratio of the maximal relaxation induced by each agonist to the maximal relaxation induced by (−)-isoprenaline (3 μM), the full agonist, in the absence of β-adrenoceptor antagonist. Statistical significance between two data sets was tested by Student's t-test. A P value of less than 0.05 was considered statistically significant.

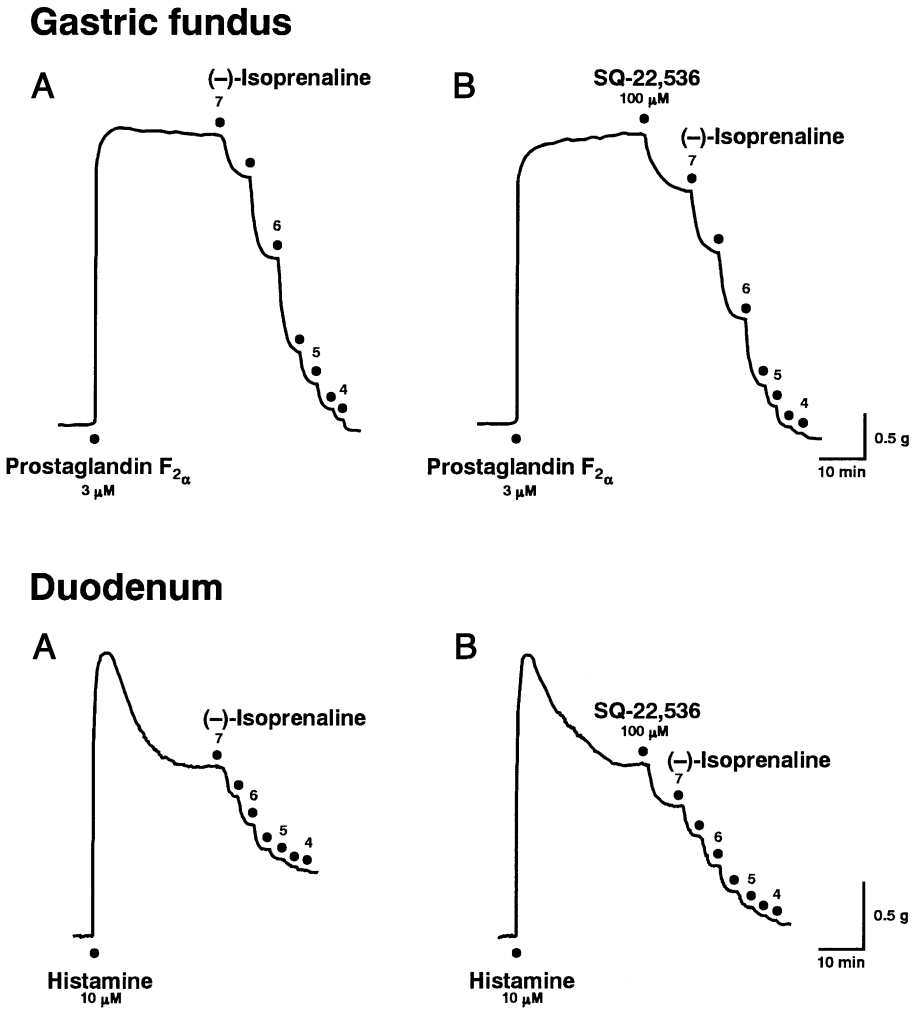

Fig. 1. Representative traces of the relaxant responses to (−)-isoprenaline in the absence (A) and presence (B) of SQ-22,536 (100 μM) in the guinea pig gastric fundus and duodenum. Gastric fundus; results were obtained in the presence of (±)-atenolol (100 μM) plus (±)-butoxamine (100 μM). Duodenum; results were obtained in the presence of (±)-propranolol (1 μM). ● indicates administration of drugs. Half log unit increments in (−)-isoprenaline concentration were added.

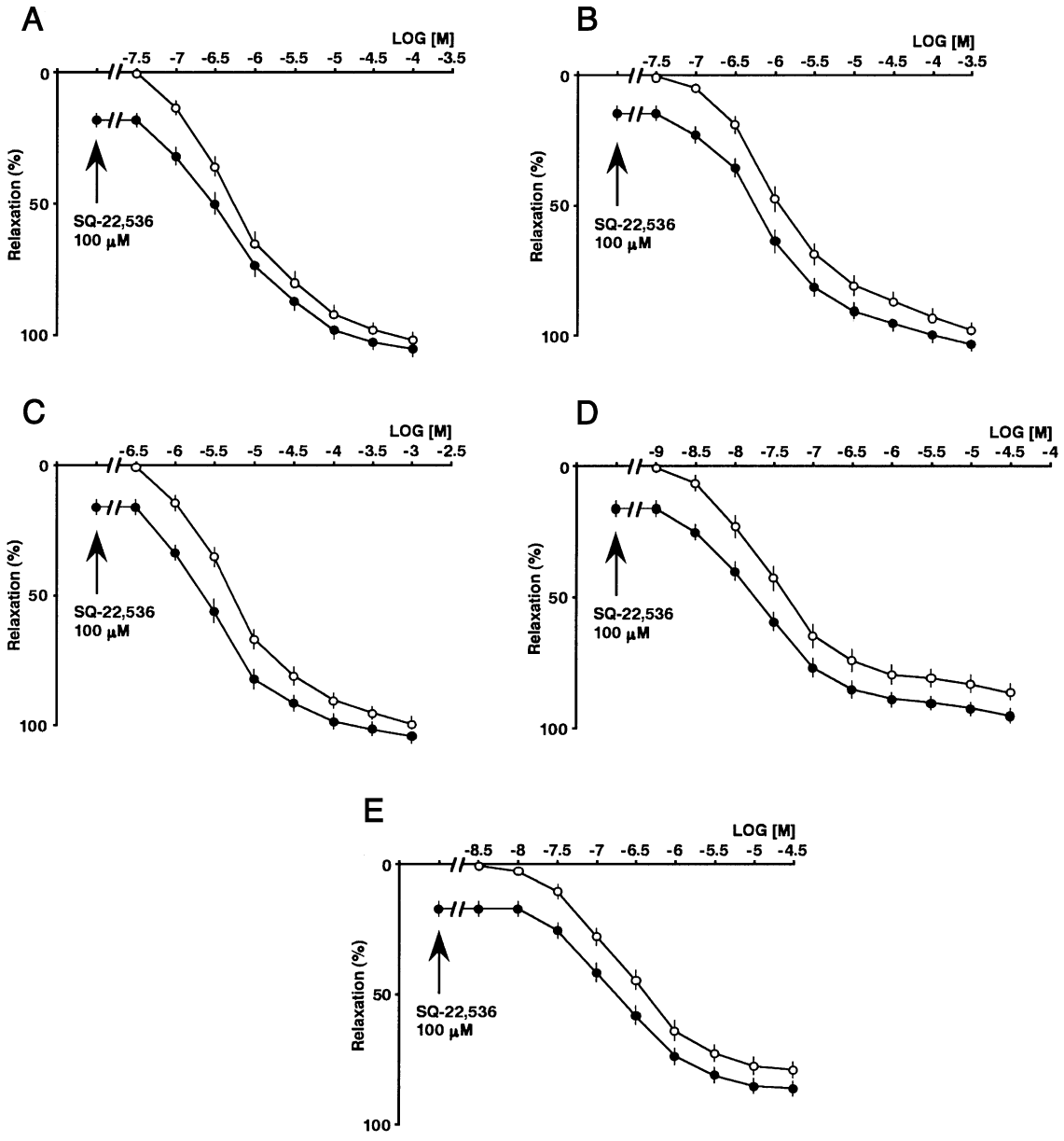

Fig. 2. Effects of SQ-22,536 (100 μM) on concentration–response curves for (−)-isoprenaline (A), (−)-noradrenaline (B), (−)-adrenaline (C), BRL37344 (D) and (±)-CGP12177A (E) in the presence of (±)-atenolol (100 μM) plus (±)-butoxamine (100 μM) in the guinea pig gastric fundus. Ordinate: relaxation (%), expressed as a percentage of the maximum relaxation (in the absence of β-adrenoceptor antagonists) induced by (−)-isoprenaline (3 μM), abscissa: concentration (M) of the test drugs. Each point represents the mean ± S.E.M. of *n* experiments. The *n* values and the results are shown in Table 1.

2.5. Drugs

The following drugs were used: (−)-isoprenaline hydrochloride, (−)-noradrenaline (+)-bitartrate, (−)-adrenaline (+)-bitartrate, (±)-propranolol hydrochloride, imipramine hydrochloride, normetanephrine hydrochloride, histamine dihydrochloride, (±)-butoxamine hydrochloride, L-ascorbic acid, reactive blue 2, tetraethylammonium chloride, glibenclamide, N^G-methyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (L-NNA), N^G-nitro-L-arginine methyl ester (L-NAME) (Sigma-Aldrich, St. Louis, MO, USA); phentolamine mesylate (Novartis, Basel, Switzerland); (*R**, *R**)-(±)-4-[2-[(2-

Table 1
Effects of SQ-22,536 (100 μM) on relaxant responses to catecholamines and β_3-adrenoceptor agonists at β_3-adrenoceptors in the guinea pig gastric fundus

Agonists	*n*	pD_2 value	pD_2 value
		Absence of SQ-22,536	Presence of SQ-22,536
(−)-Isoprenaline	6	6.27 ± 0.05	6.30 ± 0.08
(−)-Noradrenaline	5	5.93 ± 0.09	6.06 ± 0.10
(−)-Adrenaline	6	5.28 ± 0.03	5.44 ± 0.05
BRL37344	5	7.55 ± 0.07	7.61 ± 0.06
(±)-CGP12177A	5	6.66 ± 0.05	6.70 ± 0.07

Values are means ± S.E.M. of *n* experiments. Results were obtained in the presence of (±)-atenolol (100 μM) plus (±)-butoxamine (100 μM).

(3-chlorophenyl)-2-hydroxyethyl)amino]propyl]phenoxyacetic acid sodium (BRL37344), 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (Nacalaitesque, Kyoto, Japan); (±)-atenolol, (±)-[4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2*H*-benzimidazol-2-one] hydrochloride ((±)-CGP12177A), *N*-(2-[*p*-bromocinnamy-

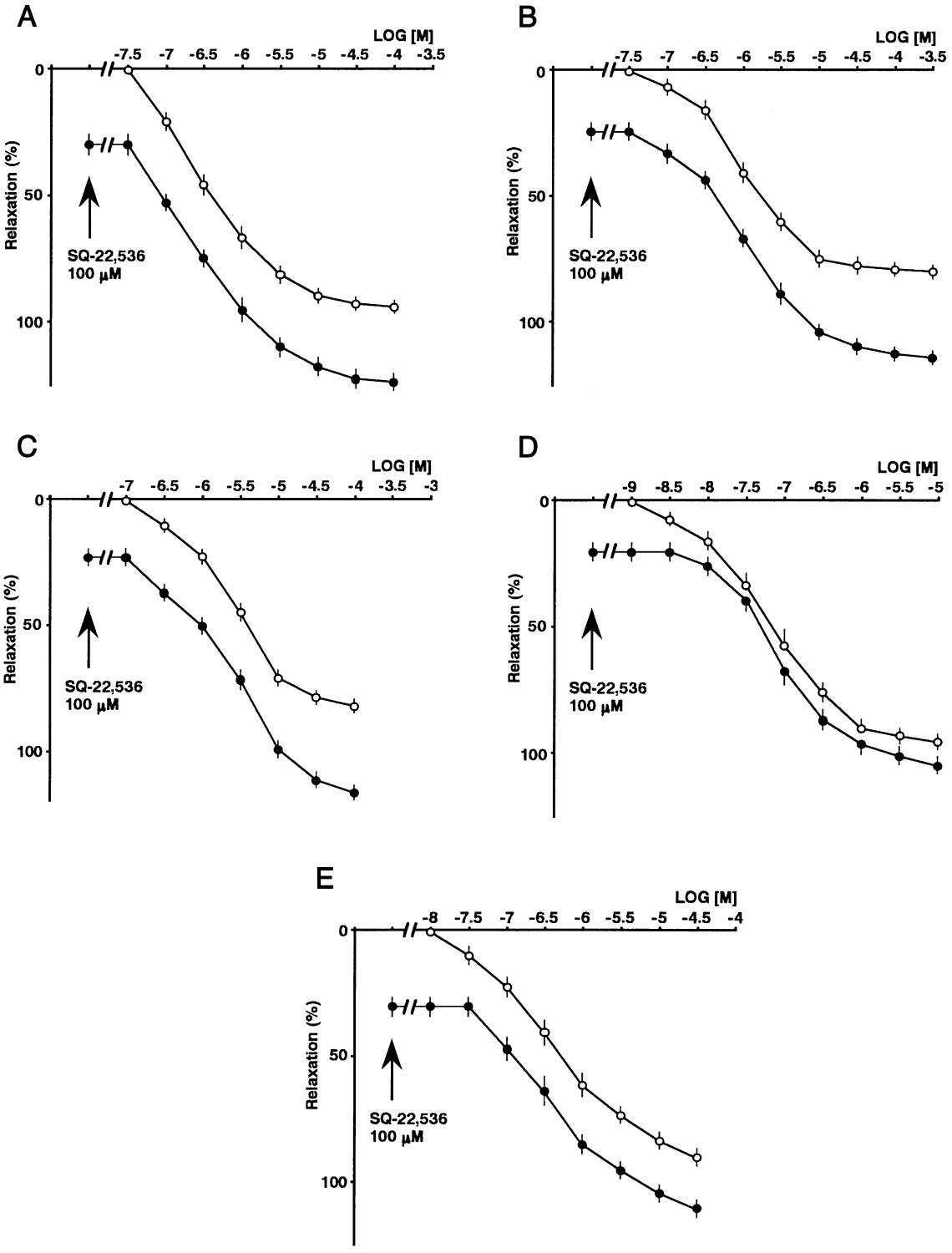

Fig. 3. Effects of SQ-22,536 on concentration–response curves for (−)-isoprenaline (A), (−)-noradrenaline (B), (−)-adrenaline (C), BRL37344 (D) and (±)-CGP12177A (E) in the presence of (±)-propranolol (1 μM) on the guinea pig duodenum. Ordinate: relaxation (%), expressed as a percentage of the maximum relaxation (in the absence of (±)-propranolol (1 μM)) induced by (−)-isoprenaline (3 μM), abscissa: concentration (M) of the test drugs. Each point represents the mean ± S.E.M. of *n* experiments. The *n* values and the results are shown in Table 2.

lamino]ethyl)-5-isoquinolinesulfonamide (H-89), 9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine (SQ-22,536) (Research Biochemicals International, Natick, MA, USA); and prostaglandin $F_{2\alpha}$ (Ono Pharmaceutical, Osaka, Japan). All other chemicals used were of analytical grade. H-89, glibenclamide and ODQ were dissolved in dimethylsulfoxide at a stock solution of 20 mM. Final dimethylsulfoxide concentrations in the bath solution did not affect the relaxant responses (data not shown). All other drugs were dissolved in distilled water.

3. Results

3.1. Effects of the adenylate cyclase inhibitor SQ-22,536 on relaxant responses

Fig. 1 shows the relaxant responses to (−)-isoprenaline in the absence (Fig. 1A) or presence (Fig. 1B) of the adenylate cyclase inhibitor, SQ-22,536 (100 μM), in the guinea pig gastric fundus and duodenum. In the presence of the combination of the selective β_1-adrenoceptor antagonist, (±)-atenolol (100 μM), and the selective β_2-adrenoceptor antagonist, (±)-butoxamine (100 μM) (gastric fundus), or in the presence of the non-selective β_1- and β_2-adrenoceptor antagonist, (±)-propranolol (1 μM) (duodenum), SQ-22,536 itself induced relaxation (the maximal relaxation: 15 ± 3%, gastric fundus; 27 ± 3%, duodenum) (Fig. 1B). SQ-22,536 increased the maximal relaxant response to catecholamines ((−)-isoprenaline, (−)-noradrenaline and (−)-adrenaline) and to β_3-adrenoceptor agonists (BRL37344 and (±)-CGP12177A) slightly, but caused no change in pD_2 values in the gastric fundus ($P > 0.05$) (Fig. 2, Table 1). In the duodenum, the maximal relaxant responses to these agonists were significantly ($P < 0.05$) increased by SQ-22,536 while pD_2 values were unaffected ($P > 0.05$) (Fig. 3, Table 2).

3.2. Effects of SQ-22,536 on cyclic AMP levels

In the presence of the combination of (±)-atenolol (100 μM) and (±)-butoxamine (100 μM), cyclic AMP levels were clearly elevated by catecholamines ((−)-isoprenaline, (−)-noradrenaline and (−)-adrenaline) ($P < 0.05$) while β_3-adrenoceptor agonists (BRL37344 and (±)-CGP12177A) had no effect on cyclic AMP levels ($P > 0.05$) in the guinea pig gastric fundus (Table 3). These increases in cyclic AMP in response to catecholamines were significantly inhibited by SQ-22,536 (100 μM) ($P < 0.05$) (Table 3).

In the presence of (±)-propranolol (1 μM), SQ-22,536 (100 μM) produced no statistically significant decrease of the basal cyclic AMP level in the guinea pig duodenum

Table 2
Effects of SQ-22,536 (100 μM) on relaxant responses to catecholamines and β_3-adrenoceptor agonists at β_3-adrenoceptors in the guinea pig duodenum

Agonists	*n*	pD_2 value	pD_2 value
		Absence of SQ-22,536	Presence of SQ-22,536
(−)-Isoprenaline	7	6.49 ± 0.05	6.48 ± 0.08
(−)-Noradrenaline	8	6.01 ± 0.04	5.95 ± 0.03
(−)-Adrenaline	8	5.64 ± 0.04	5.63 ± 0.03
BRL37344	8	7.20 ± 0.03	7.10 ± 0.02
(±)-CGP12177A	7	6.41 ± 0.03	6.37 ± 0.03

Values are means ± S.E.M. of *n* experiments. Results were obtained in the presence of (±)-propranolol (1 μM).

Table 3
Intracellular cyclic AMP levels measured in the guinea pig gastric fundus under different experimental conditions

Experimental conditions	Cyclic AMP (pmol/mg protein)	*n*
Basal level	3.42 ± 0.21	11
$PGF_{2\alpha}$ 3 μM	3.79 ± 0.21	10
$PGF_{2\alpha}$ 3 μM + SQ 100 μM	3.31 ± 0.24	11
$PGF_{2\alpha}$ 3 μM+(−)-ISO 100 μM	5.39 ± 0.52[a]	6
$PGF_{2\alpha}$ 3 μM + SQ 100 μM+(−)-ISO 100 μM	3.40 ± 0.31[a]	6
$PGF_{2\alpha}$ 3 μM+(−)-NA 300 μM	5.40 ± 0.38[b]	6
$PGF_{2\alpha}$ 3 μM + SQ 100 μM+(−)-NA 300 μM	3.87 ± 0.51[b]	6
$PGF_{2\alpha}$ 3 μM+(−)-AD 1 mM	6.62 ± 0.38[c]	9
$PGF_{2\alpha}$ 3 μM + SQ 100 μM+(−)-AD 1 mM	4.94 ± 0.20[c]	7
$PGF_{2\alpha}$ 3 μM + BRL 30 μM	4.05 ± 0.41[d,f]	7
$PGF_{2\alpha}$ 3 μM + SQ 100 μM + BRL 30 μM	3.95 ± 0.44[d,g]	7
$PGF_{2\alpha}$ 3 μM+(±)-CGP 30 μM	3.93 ± 0.48[e,f]	7
$PGF_{2\alpha}$ 3 μM + SQ 100 μM+(±)-CGP 30 μM	4.07 ± 0.40[e,g]	7

Values are means ± S.E.M. of *n* experiments. Results were obtained in the presence of (±)-atenolol (100 μM) plus (±)-butoxamine (100 μM). After mechanical responses were recorded, tissues were frozen in order to measure cyclic AMP levels. $PGF_{2\alpha}$ = prostaglandin $F_{2\alpha}$; SQ = SQ-22,536; (−)-ISO=(−)-isoprenaline; (−)-NA=(−)-noradrenaline; (−)-AD=(−)-adrenaline; BRL = BRL37344; (±)-CGP=(±)-CGP12177A. [a,b,c] $P < 0.05$, [d,e] $P > 0.05$, compared with its control (the absence of SQ). [f] $P > 0.05$, compared with $PGF_{2\alpha}$ 3 μM. [g] $P > 0.05$, compared with $PGF_{2\alpha}$ 3 μM + SQ 100 μM.

Table 4
Intracellular cyclic AMP levels measured in the guinea pig duodenum under different experimental conditions

Experimental conditions	Cyclic AMP (pmol/mg protein)	*n*
Basal level	3.58 ± 0.40	13
His 10 μM	3.83 ± 0.44	14
His 10 μM + SQ 100 μM	2.91 ± 0.17[a]	15
His 10 μM+(−)-ISO 100 μM	3.67 ± 0.31[a]	6
His 10 μM + SQ 100 μM+(−)-ISO 100 μM	3.15 ± 0.24	8
His 10 μM+(−)-NA 100 μM	3.66 ± 0.30[a]	7
His 10 μM + SQ 100 μM+(−)-NA 100 μM	3.19 ± 0.30	8
His 10 μM+(−)-AD 1 mM	3.39 ± 0.25[a]	6
His 10 μM + SQ 100 μM+(−)-AD 1 mM	2.76 ± 0.12	8
His 10 μM + BRL 10 μM	3.64 ± 0.29[a]	5
His 10 μM + SQ 100 μM + BRL 10 μM	3.36 ± 0.21	9
His 10 μM+(±)-CGP 30 μM	3.13 ± 0.27[a]	8
His 10 μM + SQ 100 μM+(±)-CGP 30 μM	3.03 ± 0.21	9

Values are means ± S.E.M. of *n* experiments. Results were obtained in the presence of (±)-propranolol (1 μM). After mechanical responses were recorded, tissues were frozen in order to measure cyclic AMP levels. His = histamine; SQ = SQ-22,536; (−)-ISO=(−)-isoprenaline; (−)-NA=(−)-noradrenaline; (−)-AD=(−)-adrenaline; BRL = BRL37344; (±)-CGP=(±)-CGP12177A.

[a] $P > 0.05$, compared with His 10 μM.

($P>0.05$) (Table 4). Cyclic AMP levels were unaltered by catecholamines ((−)-isoprenaline, (−)-noradrenaline and (−)-adrenaline) and β_3-adrenoceptor agonists (BRL37344 and (±)-CGP12177A) ($P>0.05$) (Table 4).

3.3. Effects of the cyclic AMP-dependent protein kinase inhibitor H-89 on relaxant responses in the gastric fundus

Table 3 shows that the catecholamines induced a significant increase in cyclic AMP levels compared with the basal level in the guinea pig gastric fundus. The relaxation of smooth muscle induced by cyclic AMP is believed to involve the cyclic AMP/cyclic AMP-dependent protein kinase pathway through phosphorylation by cyclic AMP-dependent protein kinase (Hardman, 1984; Bennet et al., 1989; Emorine et al., 1991). Therefore, to evaluate whether cyclic AMP-dependent protein kinase is involved in the signal transduction pathway activated by β_3-adrenoceptors, we examined the effects of the cyclic AMP-dependent protein kinase inhibitor, H-89 (10 μM), on the relaxant responses induced by catecholamines and β_3-adrenoceptor agonists in the guinea pig gastric fundus. However, pretreatment with H-89 (10 μM) did not significantly ($P>0.05$) change pD_2 values and the maximal relaxant responses of these agonists (Table 5).

3.4. Characterization of β_3-adrenoceptors mediating relaxant responses in the gastric fundus

Since relaxant responses to catecholamines and to β_3-adrenoceptor agonists were not affected by either the adenylate cyclase inhibitor, SQ-22,536, or the cyclic AMP-dependent protein kinase inhibitor, H-89, in the guinea pig gastric fundus, we investigated whether the relaxant responses to these agonists are mediated through other receptors and signal transduction pathways. In the presence of a combination of (±)-atenolol (100 μM) and (±)-butoxamine (100 μM), the relaxant responses to catecholamines and to β_3-adrenoceptor agonists were not affected by either tetraethylammonium (1 mM; a Ca^{2+}-dependent K^+ channel inhibitor), glibenclamide (10 μM; an ATP-sensitive K^+ channel inhibitor), ODQ (10 μM; a soluble guanylate cyclase inhibitor), L-NAME (300 μM; a nitric oxide (NO) synthase inhibitor), L-NNA (300 μM; a NO synthase inhibitor), L-NMMA (30 μM; a NO synthase inhibitor) or reactive blue 2 (100 μM; a selective P_{2y} receptor antagonist) (data not shown).

Table 5
Effects of H-89 (10 μM) on relaxant responses to catecholamines and β_3-adrenoceptor agonists at β_3-adrenoceptors in the guinea pig gastric fundus

Agonists	n	pD_2 value	pD_2 value
		Absence of H-89	Presence of H-89
(−)-Isoprenaline	9	6.27 ± 0.09	5.99 ± 0.10
(−)-Noradrenaline	4	6.06 ± 0.11	6.15 ± 0.13
(−)-Adrenaline	6	5.35 ± 0.06	5.40 ± 0.07
BRL37344	5	7.34 ± 0.05	7.35 ± 0.09
(±)-CGP12177A	5	6.64 ± 0.07	6.56 ± 0.08

Values are means ± S.E.M. of n experiments. Results were obtained in the presence of (±)-atenolol (100 μM) plus (±)-butoxamine (100 μM).

4. Discussion

The biological effects of β-adrenoceptor agonists on gastrointestinal smooth muscle are believed to be mediated by an elevation of cyclic AMP levels through the activation of adenylate cyclase. In the present study, we investigated the signal transduction pathway involved in β_3-adrenoceptor-mediated relaxation of the guinea pig gastric fundus and duodenum. To clarify whether cyclic AMP plays a major role in relaxant responses induced by catecholamines ((−)-isoprenaline, (−)-noradrenaline and (−)-adrenaline) and β_3-adrenoceptor agonists (BRL37344 and (±)-CGP12177A) in the guinea pig gastric fundus and duodenum, we measured the relaxant responses and cyclic AMP levels in the same tissue in the absence and presence of the adenylate cyclase inhibitor, SQ-22,536 (100 μM). Under conditions designed to assess only β_3-adrenoceptors, catecholamines and β_3-adrenoceptor agonists caused concentration-dependent relaxations of the guinea pig gastric fundus and duodenum in the absence and presence of SQ-22,536, and the potency (pD_2 value) of these agonists was not significantly ($P>0.05$) affected by SQ-22,536 when compared to control values. These results suggest that the relaxant responses to these agonists were not mediated through the adenylate cyclase pathway. However, SQ-22,536 increased the maximal relaxant responses induced by catecholamines and β_3-adrenoceptor agonists in the gastric fundus and duodenum. It appears that the maximal relaxation elicited by these agonists was potentiated by SQ-22,536, because the relaxation induced by SQ-22,536 was additive to the maximal relaxation elicited by these agonists.

Catecholamines ((−)-isoprenaline, (−)-noradrenaline and (−)-adrenaline) in the guinea pig gastric fundus significantly ($P<0.05$) increased cyclic AMP levels, whereas β_3-adrenoceptor agonists (BRL37344 and (±)-CGP12177A) in the guinea pig gastric fundus and the five agonists in the duodenum failed to significantly ($P>0.05$) increase cyclic AMP levels. Previously, we have shown that the non-selective β_1-, β_2- and β_3-adrenoceptor antagonist, (±)-bupranolol (100 μM), reduces the increase in cyclic AMP levels induced by catecholamines (data not shown). Furthermore, studies of human β_3-adrenoceptors based on computer modelling suggested that the hydroxyl groups on serine residues 209 and 212 probably form hydrogen bonds with the catechol hydroxyl groups at positions 3 and 4, respectively (Strosberg and Piétri-Rouxel, 1996). It is possible that the different increase in cyclic AMP levels between catecholamines ((−)-isoprenaline, (−)-noradrenaline and (−)-adrenaline) and noncatecholamines (BRL37344 and (±)-CGP12177A) in the guinea pig gastric fundus was

due to differences in the interaction of the five agonists with the serine residues. In addition, SQ-22,536 significantly inhibited the increase in cyclic AMP levels induced by catecholamines at concentrations producing maximal relaxation. These results are similar to those reported by Brawley et al. (2000) for atypical β-adrenoceptors of rat aorta and indicate that the cyclic AMP-dependent and cyclic AMP-independent pathways are involved in β_3-adrenoceptor-mediated relaxation. In the case of the gastric fundus, the effects of catecholamines on relaxant responses were mediated mainly by the cyclic AMP-independent pathway and in part by the cyclic AMP-dependent pathway, while the relaxations elicited by β_3-adrenoceptor agonists in the gastric fundus and by catecholamines and β_3-adrenoceptor agonists in the duodenum were mediated mainly by the cyclic AMP-independent pathway.

In isolated adipocyte cells, β_3-adrenoceptors are coupled to both the stimulatory (G_s) protein (increase in cyclic AMP accumulation) and the inhibitory (G_i) protein (decrease in cyclic AMP accumulation) (Chaudhry et al., 1994). For example, the stimulation of cyclic AMP by BRL37344 is limited by coupling of the β_3-adrenoceptors to G_i in these cells (Chaudhry et al., 1994). Furthermore, BRL37344 failed to increase basal cyclic AMP levels at 10 μM and it caused a slight, but not significant, reduction in the basal levels of cyclic AMP at a high concentration (10 μM) in rat soleus muscle (Roberts and Summers, 1998). In this study, β_3-adrenoceptor agonists (BRL37344 and (±)-CGP12177A) as well as catecholamines ((−)-isoprenaline, (−)-noradrenaline and (−)-adrenaline) did not reduce cyclic AMP levels, indicating that G_i protein was not involved in the β_3-adrenoceptor-mediated responses in the guinea pig gastric fundus and duodenum.

The adenylate cyclase inhibitor, SQ-22,536 (100 μM), produced relaxation of prostaglandin $F_{2\alpha}$ (3 μM; gastric fundus)- or histamine (10 μM; duodenum)-precontracted tissues. However, SQ-22,536 induced no statistically significant change in the basal level of cyclic AMP. Although MDL-12,330A and SQ-22,536 are adenylate cyclase inhibitors (Hunt and Evans, 1980; Lippe and Ardizzone, 1991), MDL-12,330A also inhibited cyclic AMP phosphodiesterase activity at a high concentration (Lippe and Ardizzone, 1991). For example, contractions induced by phenylephrine were inhibited by MDL-12,330A (30 μM) in rat thoracic aorta (Satake et al., 1996). In a preliminary experiment, we also confirmed that SQ-22,536 (100 μM) and MDL-12,330A (50 μM) inhibited prostaglandin $F_{2\alpha}$ (3 μM)-induced contractions in the guinea pig gastric fundus (data not shown). These facts suggest two possibilities, namely, that (i) SQ-22,536 acts as a potent adenylate cyclase inhibitor and as a weak phosphodiesterase inhibitor and that the relaxant responses to SQ-22,536 are due to the effect of the weak phosphodiesterase inhibitor, while changes in cyclic AMP levels could not be detected because of the opposite action between the adenylate cyclase inhibitory effect (decrease in cyclic AMP) and the phosphodiesterase inhibitory effect (increase in cyclic AMP); (ii) relaxations induced by SQ-22,536 are non-specific responses since a higher concentration of SQ-22,536 was used in this study.

In the present study, β_3-adrenoceptor-mediated relaxant responses induced by (−)-isoprenaline were not affected by SQ-22,536 (100 μM) in the guinea pig gastric fundus, while Dick et al. (2000) reported that SQ-22,536 (0.001–1 μM) inhibited relaxations induced by (−)-isoprenaline (100 μM) in a concentration-dependent manner in the absence of β-adrenoceptor antagonists in isolated smooth muscle cells of the guinea pig gastric fundus. These facts suggest that the (−)-isoprenaline-induced relaxation mediated through classical β-adrenoceptors involves the adenylate cyclase pathway, whereas the β_3-adrenoceptor-mediated relaxation elicited by (−)-isoprenaline is not mediated through the adenylate cyclase pathway.

We have reported that functional β_2- and β_3-adrenoceptors are present in the guinea pig taenia caecum (Koike et al., 1994, 1995a,b, 1997a,b), and that the adenylate cyclase pathway is involved in the β_3-adrenoceptor-mediated relaxation of the guinea pig taenia caecum (Koike et al., 1995a,c, 2000). Our functional studies indicate that the predominant β-adrenoceptor subtypes are the β_2-adrenoceptor in the guinea pig taenia caecum (Koike et al., 1994, 1995a,b, 1997a) and the β_3-adrenoceptor in the guinea pig gastric fundus and duodenum, respectively (Horinouchi and Koike, 1999a,b). In human brown adipocytes, although β_3-adrenoceptors play a predominant role in the control of lipolysis and thermogenesis compared with β_1- and β_2-adrenoceptors (Zhao et al., 1994; Langin et al., 1995), β_3-adrenoceptors are poorly coupled to the adenylate cyclase pathway, contributing to only 10% of the (−)-isoprenaline-induced accumulation of cyclic AMP, whereas 20% and 70% of the signal depends on β_1- and β_2-adrenoceptors, respectively (Jockers et al., 1998). We therefore postulated that in the tissues where classical β-adrenoceptors (β_1- and β_2-adrenoceptors) and atypical (β_3) β-adrenoceptors coexist, atypical (β_3) β-adrenoceptors as well as classical β-adrenoceptors may couple to adenylate cyclase when classical β-adrenoceptors play a predominant role, but classical β-adrenoceptors only couple to adenylate cyclase when atypical (β_3) β-adrenoceptors play a predominant role.

As the increase in cyclic AMP in response to catecholamines was inhibited significantly by SQ-22,536 in the guinea pig gastric fundus, it can be hypothesized that the responses to catecholamines were mediated via the cyclic AMP-protein kinase A pathway. Therefore, we investigated the effects of the cyclic AMP-dependent protein kinase inhibitor, H-89 (Chijiwa et al., 1990; Engh et al., 1996), on the relaxation elicited by catecholamines ((−)-isoprenaline, (−)-noradrenaline and (−)-adrenaline) and β_3-adrenoceptor agonists (BRL37344 and (±)-CGP12177A) in the guinea pig gastric fundus. However, the relaxant responses to these agonists were not affected by H-89 (10 μM), indicating that cyclic AMP-dependent protein kinase was not involved in the β_3-adrenoceptor signaling pathway. The

β_3-adrenoceptor shares 40–50% amino acid sequence identity with β_1- and β_2-adrenoceptors (Granneman et al., 1993) and lacks phosphorylation sites for cyclic AMP-dependent protein kinase and β-adrenoceptor kinase implicated in the desensitization of β_2-adrenoceptors (Strosberg, 1993). Thus, β_3-adrenoceptors in the guinea pig gastric fundus and duodenum may lack the recognition sites for cyclic AMP-dependent protein kinase.

More recently, similar observations were reported for the guinea pig aorta, where SQ-22,536 completely abolished the elevation in cyclic AMP induced by iloprost, the stable prostacyclin analogue (Turcato and Clapp, 1999), but neither SQ-22,536 nor the cyclic AMP-dependent protein kinase inhibitor, H-89, had significant effects on the relaxation induced by iloprost (Turcato and Clapp, 1998, 1999). However, the relaxation in response to iloprost was markedly inhibited by blockers of Ca^{2+}-activated K^+ channels (Clapp et al., 1998), which would also support the idea that cyclic AMP-independent pathways are involved in mediating the relaxation in response to iloprost in these tissues.

β-Adrenoceptor agonists are known to induce cyclic AMP-dependent relaxation of various smooth muscle and also to hyperpolarize smooth muscle by increasing K^+ permeability (Kume et al., 1994; Torphy, 1994). For example, β-adrenoceptor agonists induce airway smooth muscle relaxation by a cyclic AMP-independent mechanism involving a direct coupling of β-adrenoceptors to Ca^{2+}-dependent K^+ channels (K_{Ca} channel) (Kume et al., 1994) and activate two types of K^+ channel, K_{Ca} channel and ATP-sensitive K^+ channel (K_{ATP} channel), via cyclic AMP-dependent phosphorylation in uterine smooth muscle (Hamada et al., 1994). Therefore, in the present study we further examined the involvement of K_{Ca} channels and K_{ATP} channels in the β_3-adrenoceptor-mediated relaxation of the guinea pig gastric fundus. However, our studies with tetraethylammonium (1 mM; a K_{Ca} channel inhibitor) and glibenclamide (10 μM; a K_{ATP} channel inhibitor) suggested that K^+ channels were not involved in the relaxation induced by catecholamines and β_3-adrenoceptor agonists (data not shown).

Several other mechanisms have been proposed to account for smooth muscle relaxation, including the activation of nitric oxide (NO)/guanylate cyclase pathway (Brookes, 1993) and P_{2y} purinoceptors (Fredholm et al., 1994). However, the relaxant responses of guinea pig gastric fundus to catecholamines and to β_3-adrenoceptor agonists were insensitive to ODQ (10 μM; a soluble guanylate cyclase inhibitor), L-NAME (300 μM; a NO synthase inhibitor), L-NNA (300 μM; a NO synthase inhibitor), L-NMMA (30 μM; a NO synthase inhibitor), reactive blue 2 (100 μM; a selective P_{2y} purinoceptor antagonist) (data not shown). These results suggest that the NO/guanylate cyclase pathway and P_{2y} purinoceptors could not modulate the β_3-adrenoceptor-mediated relaxation.

In summary, β_3-adrenoceptor-induced relaxations are mediated via cyclic AMP-dependent and cyclic AMP-independent mechanisms in the guinea pig gastric fundus and via a cyclic AMP-independent mechanism in the duodenum. The cyclic AMP-independent relaxation does not involve either K_{Ca} channels, K_{ATP} channels, NO/guanylate cyclase pathway or P_{2y} purinoceptor. The present study raises the question as to the signal transduction pathway of the β_3-adrenoceptor-mediated relaxation of the guinea pig gastric fundus and duodenum. It may be that the β_3-adrenoceptor-mediated relaxation of the guinea pig gastric fundus and duodenum is linked to novel transduction mechanisms.

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