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European Journal of Pharmacology 415 (2001) 257–263

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β_1- and β_3-adrenoceptor mediated smooth muscle relaxation in hypothyroid rat ileum

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Received in revised form 19 January 2001; accepted 26 January 2001

Abstract

The effect of hypothyroidism on gastrointestinal β_1- and β_3-adrenoceptor function and expression was examined in rat ileal smooth muscle preparations. (−)-Isoprenaline and the selective β_3 agonist disodium (*R*,*R*)-5-[2-[[2-3-chlorophenyl)-2-hydroxyethyl]-amino]propyl]-1,3-benzodioxole-2,2-dicarboxylate (CL 316234) relaxed both control and hypothyroid tissues in a dose-dependent manner. Responses to isoprenaline were reduced in tissues from hypothyroid rats, as was the shift produced with the β_3-adrenoceptor antagonist, 3-(2-ethylphenoxy)-1-[(1*S*)-1,2,3,4-tetrahydronaphth-1-ylamino]-(2*S*)-2-propanol oxalate (SR 59230A). No change was seen in responses to CL 316243. Experiments with a selective β_1-adrenoceptor antagonist produced results suggesting that isoprenaline did not act at this receptor. Messenger RNA levels for both β_1- and β_2-adrenoceptors were not affected by hypothyroidism. These results show that, unlike in adipose tissues, ileal β_1- and β_3-adrenoceptors are not directly regulated by thyroid hormone and that β_3-adrenoceptor coupling to the relaxation response is reduced in a rat model of hypothyroidism.

Keywords: β-Adrenoceptors; Smooth muscle, ileal; Hypothyroidism; Messenger RNA; relaxation

1. Introduction

Both β_1- and β_3-adrenoceptors are expressed together in many tissues where they share many of the same signal transduction pathways. In brown adipose tissue, agonist stimulation of β_1- and β_3-adrenoceptors causes thermogenic responses by activation of adenylyl cyclase and cAMP accumulation. Differentiated rat and mouse adipocytes express both receptors at high levels but functional responses to agonist stimulation switches during differentiation from a wholly β_1- to a predominantly β_3-adrenoceptor mediated effect (Bronnikov et al., 1999). In β_3-adrenoceptor knockout mice β_1-adrenoceptor up-regulation functionally compensates for the lack of β_3-adrenoceptors in brown and white adipose tissues and in ileum (Susulic et al., 1995; Hutchinson et al., 2001).

In both brown and white adipose tissue, thyroid hormone regulates the function and expression of β_1- and β_3-adrenoceptors. In white fat, hypothyroidism reduces (Rubio et al., 1995b) and hyperthyroidism increases β_3-adrenoceptor-binding, mRNA, responsiveness and sensitivity to β_3-adrenoceptor agonists (Fain et al., 1997; Wahrenberg et al., 1994) without affecting expression or function of β_1-adrenoceptors (Germack et al., 2000). Conversely, in brown fat, hypothyroidism increases β_3-adrenoceptor expression (Rubio et al., 1995b) and reduces cAMP (Carvalho et al., 1996; Chaudhry and Granneman, 1997) and thermogenic responses to β_3-adrenoceptor agonists (Ilyes and Stock, 1990), while simultaneously reducing the expression of the β_1-adrenoceptor mRNA (Rubio et al., 1995a). In the same tissue, hyperthyroidism down-regulates β_3-adrenoceptor expression and increases thermogenesis and expression of β_1-adrenoceptors (Adli et al., 1999; Ilyes and Stock, 1990).

β_3-Adrenoceptors occur throughout the gastrointestinal tract in many species, including man. Selective β_3-adrenoceptor agonists including CL 316243, CGP 12177, SR 58611A and BRL 37344 relax the smooth muscle of the oesophagus, small and large intestine (for review see (Manara et al., 1995). Relaxation responses to β_1-adrenoceptor agonists in ileum are small (Roberts et al., 1999).

In mice, β_3-adrenoceptors are differentially regulated in gastrointestinal and adipose tissues (Evans et al., 1998;

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0014-2999/01/$ - see front matter
PII: S0014-2999(01)00795-6

Hutchinson et al., 2000). Glucocorticoids, forskolin and CL 316243 down-regulate β_3-adrenoceptor expression in brown and white adipose tissues but do not affect receptor expression in ileum, even after 24 h when levels in adipose tissues return to normal. The β_3-adrenoceptor antagonist SR 59230A up-regulates β_3-adrenoceptor mRNA in ileum and brown fat but not in white fat, and obesity is associated with down-regulation in adipose tissues but not in ileum.

The experiments described here examine for the first time the effect of hypothyroidism on β_1- and β_3-adrenoceptor mediated responses and mRNA levels in rat ileum. We found that in contrast to effects in adipose tissues, thyroid hormone does not affect receptor regulation in ileal smooth muscle but does modulate coupling of the β_3-adrenoceptor.

2. Materials and methods

2.1. Tissue preparation

This study was approved by the Monash University Animal Ethics Committee in accordance with the laws and regulations of Australia. Hypothyroidism was induced in male Sprague–Dawley rats 8–12 weeks old, (250–400 g) by the administration of 500 mg/l methimazole in drinking water for 14 days while control rats received normal drinking water. This regime causes hypothyroidism based on established plasma methimazole and thyroid hormone clearance rates (Cooper et al., 1984). Rats were anaesthetised with 80% CO_2/20% O_2, decapitated and the ileum removed. Intestinal contents were flushed out with Krebs–Henseleit buffer (118.4 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4.7H_2O$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 11 mM glucose, 2.5 mM $CaCl_2$) containing 0.1 mM ascorbic acid and 0.04 mM EDTA, the ileum cut into segments and muscle preparations obtained by opening each segment along the mesenteric line, pinning it flat in a Petri dish and gently dissecting off the smooth muscle layer with forceps and a scalpel blade. The muscle layer was cut to provide four to six strips that were either snap-frozen in liquid nitrogen for molecular analysis or directly used for organ bath studies.

2.2. Organ bath studies

Muscle strips were mounted under 0.5 g resting tension in organ baths containing Krebs–Henseleit buffer at 37°C bubbled continuously with 95% O_2/5% CO_2. Changes in the length of the tissue were measured with Ugo Basile isotonic transducers connected to a Maclab system with an Apple Macintosh IIci computer. Preliminary experiments carried out in the presence of hydrocortisone (30 μM) to block extraneuronal uptake of (−)-isoprenaline, showed that this did not affect the concentration–response relationship and so was subsequently omitted. Tissues were preincubated for 30–50 min with phentolamine (10 μM) to block α-adrenoceptors, in the presence or absence of the selective β_1 antagonist 2-hydroxy-5(2-((hydroxy-3-(4-((1-methyl-4-trifluoromethyl)1*H*-imidazole-2-yl)-phenoxy) propyl)amino)ethoxy)-benzamide monomethane sulfonate (CGP 20712A) or the selective β_3 antagonist 3-(2-ethylphenoxy)-1-[(1*S*)-1,2,3,4-tetrahydronaphth-1-ylamino] (2*S*)-2-propanol oxalate (SR 59230A; 30, 100 and 300 nM). Tissues were precontracted with a submaximal dose of carbachol (3 μM) and allowed to equilibrate for 20 min. Relaxation curves were generated by the cumulative addition of (−)-isoprenaline (1 nM–30 μM) or disodium (*R*,*R*)-5-[2-[[2-3-chlorophenyl)-2-hydroxyethyl]-amino]propyl]-1,3-benzodioxole-2,2-dicarboxylate (CL 316243; β_3-adrenoceptor agonist; 0.1 nM–1 μM) in half-log increments. Time to equilibration between agonist doses ranged from 3–6 min for (−)-isoprenaline to 20–30 min for CL 316243. After completion of the concentration–response curves, maximal relaxation was induced by the addition of papaverine (0.1 mM). All responses are expressed as a percentage (%) of the maximum papaverine relaxation.

2.3. Data analysis

Concentration–response curves were plotted as mean ± S.E.M. of *n* individual experiments and a pEC_{50} value obtained using PRISM™ (Graphpad software). In some experiments, the antagonist did not produce measurable shifts so a single pK_B value was calculated for each treatment from the mean pEC_{50} values in the presence and absence of the antagonist. Unpaired *t*-test or one-way analysis of variance (ANOVA) with Tukey's test was performed where appropriate, to determine the statistical significance of results.

2.4. Reverse transcription-polymerase chain rection (PCR)

RNA extraction was carried out as previously described (Hutchinson et al., 2000). One microgram of each total RNA sample was used for reverse transcription (Evans et al., 1996) with oligo(dT)15 as the primer. Twenty microliters of 1 mM EDTA was added after the reaction to maintain the integrity of the cDNA after storage at −70°C.

PCR amplification was done on the equivalent of 0.1 μg of starting RNA using primers specific for rat β actin, β_1- and β_3-adrenoceptors (Table 1). For each reaction, one primer was end-labelled in a 25 μl reaction mix containing 20 ng μl^{-1} oligonucleotide, 1× One-Phor-All-plus buffer (Pharmacia), 50 μCi [γ-^{33}P]ATP (Geneworks) and 1.5 μl T4 polynucleotide kinase (Pharmacia) and incubated at 37°C for 30 min then 95°C for 5 min. PCR mixes for β_3-adrenoceptor and β actin mRNAs were as previously described (Roberts et al., 1999), while the mix for β_1-adrenoceptor PCR contained 1× Amplification buffer (Life Technologies), 130 μM dNTPs, 1.5 mM $MgSO_4$,

Table 1
Oligonucleotide primers and parameters used in PCR experiments

cDNA	Cycles	Temperature (°C)	Direction	5′ to 3′ sequence
β-Actin	16	64	Forward	ATCCTGCGTCTGGACCTGGCTG
			Reverse	CCTGCTTGCTGATCCACATCTGCTG
β_1-Adrenoceptor	30	60	Forward	CCGCTGCTACAACACCCCAAG
			Reverse	ACGCAGTTGAAGAAGACGAAGAGGCG
β_3-Adrenoceptor	30	64	Forward	TGCCAACTCTGCCTTCAACCCGCTC
			Reverse	CGCTCACCTTCATAGCCATCAACC

1× enhancer solution (Life Technologies), 1 μl of labelled primer, 1 ng μl^{-1} of unlabelled primer, 0.5 U of Platinum Taq polymerase (Life Technologies) and cDNA in a volume of 20 μl. PCR products were electrophoresed on 1.3% agarose gels and transferred onto Hybond N+ membrane by Southern blotting in 0.4 M NaOH/1 M NaCl. Membranes were rinsed in 0.5 M Tris–HCl (pH 7.5)/1 M NaCl, then 2× salt sodium citrate (SSC; 0.3 M NaCl/30 mM sodium citrate) and radioactivity quantitated using a Molecular Dynamics (SI) phosphorimager after 24 h exposure to imaging plates. Receptor mRNA levels were normalised against actin and expressed as a percentage relative to control tissues. Unpaired *t*-tests were performed using PRISM software.

3. Results

3.1. Effect of hypothyroidism on responses to (−)-isoprenaline

(−)-Isoprenaline relaxed carbachol (3 μM) precontracted ileal smooth muscle and ileal segments from control and hypothyroid rats in a concentration-dependent manner. Hypothyroidism significantly reduced the effectiveness of (−)-isoprenaline in relaxing the tissues by three-fold compared to control (Fig. 1). The pEC_{50} value for (−)-isoprenaline in controls was 7.13 ± 0.14 ($n = 9$) compared to 6.66 ± 0.08 ($n = 7$) in hypothyroid rats ($P < 0.05$). Although a tendency toward an increase in the maximum response was observed in tissues taken from hypothyroid rats this did not reach significance ($P = 0.082$). Sensitivity to carbachol was not affected by hypothyroidism (data not shown).

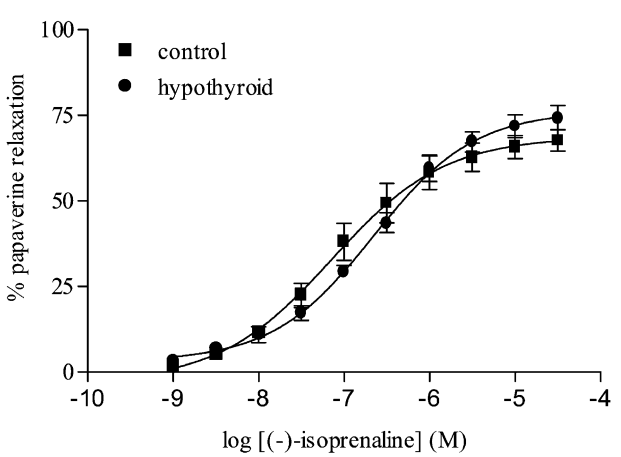

Fig. 1. Effect of hypothyroidism on relaxation responses to the non-selective β adrenoceptor agonist (−)-isoprenaline. Points show mean ± S.E.M. and are expressed as a percentage (%) of maximum relaxation by 100 μM papaverine. pEC_{50} values for these curves are given in Table 2.

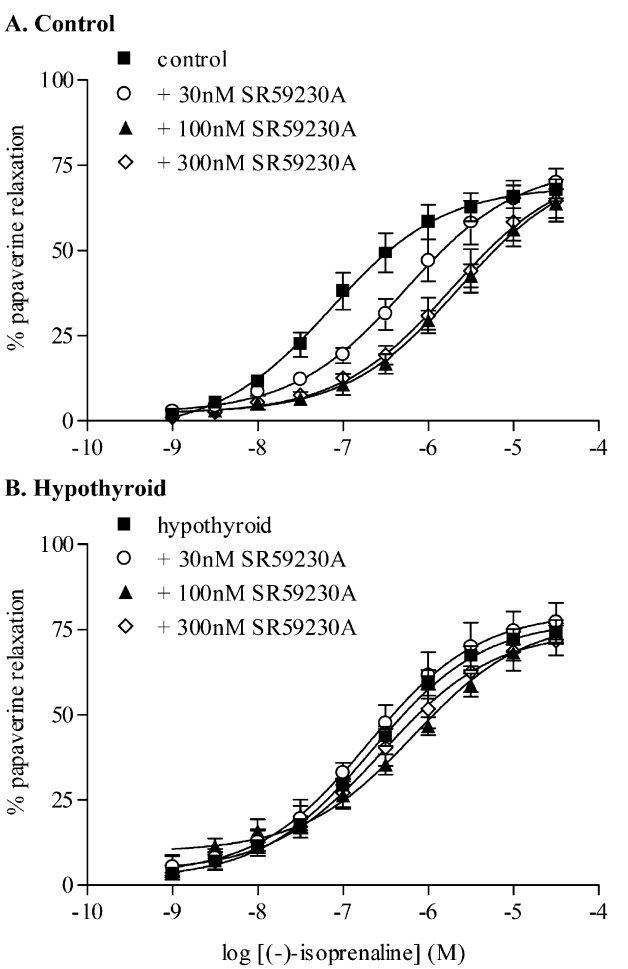

Fig. 2. Relaxation responses to (−)-isoprenaline alone and in the presence of SR 59230A in (A) control ($n = 8–9$) and (B) hypothyroid rat ileal smooth muscle ($n = 7–8$). pK_B values were calculated for each concentration of antagonist from mean pEC_{50} values obtained for each curve (Table 2).

Table 2
pEC_{50} values for (−)-isoprenaline relaxation of normal and hypothyroid ileum in the presence of SR 59230A. pK_B values were calculated from the mean pEC_{50} values

[SR59230A]	Control Tissues		Hypothyroid tissues	
	$pEC_{50} \pm$S.E.M. (n)	pK_B	$pEC_{50} \pm$S.E.M. (n)	pK_B
(Alone)	7.13 ± 0.14 (9)	–	6.66 ± 0.08 (7)[a]	–
30 nM	6.27 ± 0.15 (8)[b c]	8.34	6.71 ± 0.16 (8)	n/c
100 nM	5.64 ± 0.12 (9)[d]	8.49	6.18 ± 0.13 (7)	7.31
300 nM	5.77 ± 0.18 (8)[d]	7.92	6.54 ± 0.16 (8)	6.03

[a] $P < 0.05$ compared to (−)-isoprenaline alone in control tissues.
[b] $P < 0.01$ compared to (−)-isoprenaline alone in control tissues.
[c] $P < 0.05$ compared to 100 nM concentration.
[d] $P < 0.001$ compared to (−)-isoprenaline alone in control tissues.

3.2. Effect of a selective β_3-adrenoceptor antagonist on responses to (−)-isoprenaline

The selective β_3-adrenoceptor antagonist SR 59230A was used to determine the β_3-adrenoceptor-mediated component of the response. SR 59230A produced significant rightward shifts in the (−)-isoprenaline dose–response curve in control tissues at all concentrations of antagonist tested. Shift was produced by SR 59230A at 30 and 100 nM, but no further shift was produced by 300 nM SR 59230A (Fig. 2A). Schild analysis produced a regression line with a slope significantly < 1. No significant shift was seen in tissues from hypothyroid animals at any concentration of SR 59230A (Fig. 2B). A single pK_B value was calculated for each concentration from the mean pEC_{50} values in the presence and absence of the antagonist. pK_B values for SR 59230A against (−)-isoprenaline relaxation for control and hypothyroid tissues are given in Table 2.

3.3. Effect of hypothyroidism on responses to a selective β_3-adrenoceptor agonist

The β_3-adrenoceptor agonist CL 316234 caused a dose-dependent relaxation of tissues from both groups of rats. pEC_{50} values were not significantly different between control and hypothyroid groups of rats (8.13 ± 0.16 and 8.02 ± 0.20, respectively).

Both curves were shifted to the right by 100 nM SR 59230A (Fig. 3). pK_B values were only slightly reduced

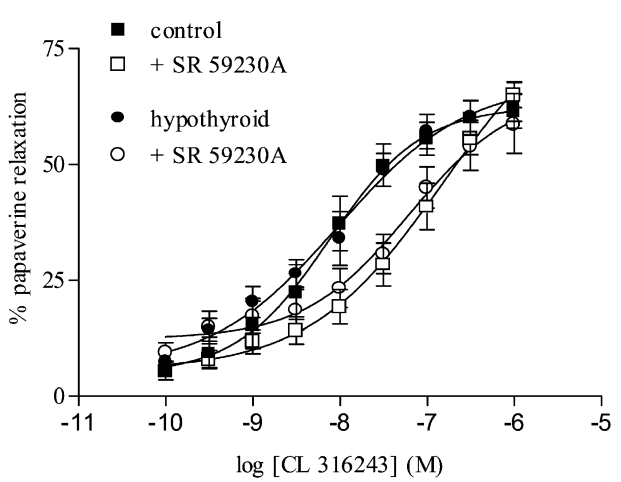

Fig. 3. Relaxation responses to CL 316243 alone and in the presence of 100 nM SR 59230A in control ($n = 11$) and hypothyroid ($n = 6–7$) rat ileal smooth muscle.

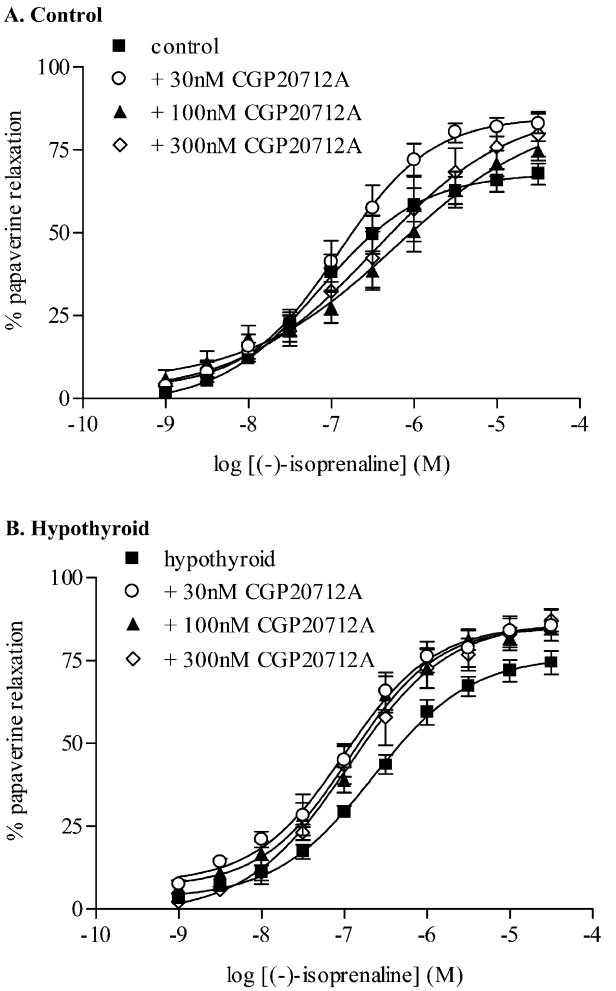

Fig. 4. Relaxation responses to (−)-isoprenaline in the presence of CGP 20712A in (A) control ($n = 6–9$) and (B) hypothyroid rat ileal smooth muscle ($n = 5–7$). pK_B and pEC_{50} values obtained for each curve are presented in Table 3.

Table 3
pEC_{50} values for (−)-isoprenaline relaxation of normal and hypothyroid ileum in the presence CGP 20712A. pK_B values were calculated from the mean pEC_{50} values

[CGP20712A]	Control Tissues		Hypothyroid tissues	
	$pEC_{50} \pm$S.E.M. (n)	pK_B	$pEC_{50} \pm$S.E.M. (n)	pK_B
(Alone)	7.13 ± 0.14 (9)	–	6.66 ± 0.08 (7)	–
30 nM	6.91 ± 0.11 (6)	7.34	7.00 ± 0.11 (5)	n/c
100 nM	6.23 ± 0.30 (9)[a]	7.84	6.93 ± 0.11 (6)	n/c
300 nM	6.48 ± 0.28 (6)	7.06	6.96 ± 0.12 (5)	n/c

[a] $P < 0.05$ compared to (−)-isoprenaline alone.

by hypothyroidism being 8.29 in controls and 7.74 in the hypothyroid group.

3.4. Effect of a selective β_1-adrenoceptor antagonist on responses to (−)-isoprenaline

The β_1-adrenoceptor antagonist CGP 20712A was used to examine whether there was a β_1-adrenoceptor mediated component of the (−)-isoprenaline response curve. In control tissues a small but significant shift occurred at 100 nM CGP 20712A but not at 30 or 300 nM (Fig. 4A). No significant shift was produced by any concentration of antagonist in the hypothyroid tissues although the presence of the antagonist appeared to slightly sensitise these tissues to the relaxant effects of (−)-isoprenaline (Fig. 4B). In the presence of CGP 20712A the maximal relaxations to (−)-isoprenaline appeared to be greater in both sets of tissues but this did not reach significance. Where possible, a single pK_B value was calculated for each antagonist concentration from the mean pEC_{50} values in the presence and absence of CGP 20712A. These values are given in Table 3.

3.5. β_1- and β_3-adrenoceptor mRNA levels in normal and hypothyroid rats

Analysis of β actin, β_1- and β_3-adrenoceptor mRNA levels in ileal smooth muscle was performed by RT-PCR. Fig. 5 shows (A) β_1-adrenoceptor mRNA and (B) β_3-adrenoceptor mRNA expression in hypothyroid ileal muscle relative to the expression in controls. Expression of neither receptor in ileal smooth muscle was significantly altered by hypothyroidism. In hypothyroid tissues β_1-adrenoceptor mRNA levels were $92.7 \pm 24.3\%$ and β_3-adrenoceptor levels were $109.8 \pm 35.6\%$ relative to control ($n = 6$).

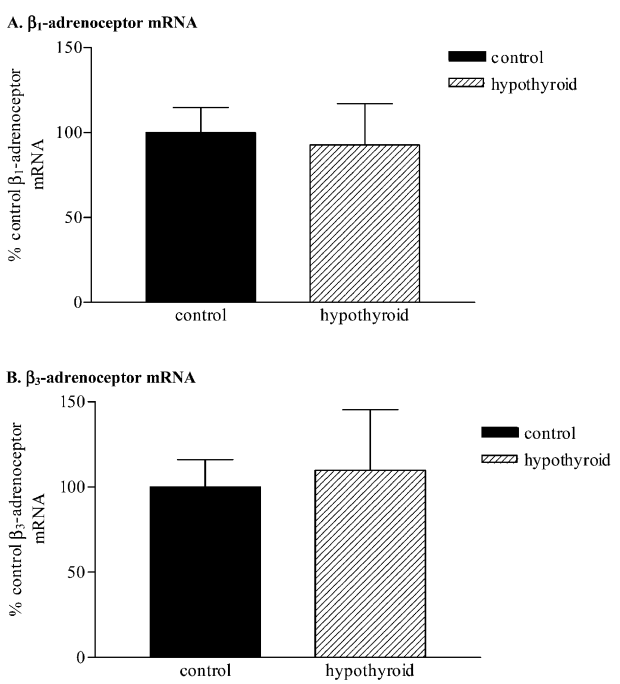

Fig. 5. Expression of (A) β_1- and (B) β_3-adrenoceptor mRNA in ileal smooth muscle from control and hypothyroid rats ($n = 6$). Values are expressed as a percentage (%) relative to the level of expression in control tissues.

4. Discussion

Regulation of β-adrenoceptors by thyroid hormone has been assessed by binding, functional and molecular approaches, and in heart, lung and several regions of the rat brain, hypothyroidism leads to down-regulation of β_1- and β_2- and up-regulation of α_1-adrenoceptor expression (Lazar-Wesley et al., 1991; Tejani-Butt and Yang, 1994). In liver, hypothyroidism increases β_2- and reduces α_1-mRNA, indicating that adrenoceptor regulation by thyroid hormone is both tissue and receptor specific (Lazar-Wesley et al., 1991). In brown adipose tissue hypothyroidism up-regulates β_3- and down-regulates β_1-adrenoceptor expression, and in white adipose tissue down-regulates β_3-adrenoceptors. Here we have examined the effect of hypothyroidism on β_1- and β_3-adrenoceptor function and expression in the smooth muscle of the rat small intestine.

Isoprenaline has been extensively used to characterise β-adrenoceptor responses in a number of tissues and stimulates all three known receptor subtypes. In rat gastrointestinal smooth muscle, isoprenaline mediates relaxation predominantly via β_3-adrenoceptors but also to a limited degree via β_1-adrenoceptors (MacDonald and Watt, 1999; Roberts et al., 1999). In the ileum of hypothyroid rats there was a reduction in responsiveness to (−)-isoprenaline and a reduction of the shift in response produced by SR59230A, suggesting that the β_3-adrenoceptor-mediated component of the (−)-isoprenaline relaxation response is reduced in hypothyroidism. However, it was also clear that there are functional β_3-adrenoceptors still present in tissue from hypothyroid rats since the selective β_3-adrenoceptor agonist, CL 316243, caused relaxation with pEC_{50} values that were not significantly different in normal and hypothyroid tissues.

In tissues from normal rats SR 59230A shifted the (−)-isoprenaline response curve with pK_B values appropriate for an action at β_3-adrenoceptors. In contrast, in hypothyroid tissues there was almost no shift, indicating a reduction in the β_3-adrenoceptor mediated component of the response. This suggests functional compensation by another β-adrenoceptor subtype, and the close relationship between the β_1- and β_3-adrenoceptors marked the β_1-adrenoceptor as a likely candidate. This possibility was examined by performing studies with a selective β_1-adrenoceptor antagonist. (−)-Isoprenaline dose–response curves were challenged with the highly selective β_1-adrenoceptor antagonist CGP 20712A. In accordance with previous

findings (Roberts et al., 1999), CGP 20712A failed to produce a major shift in control tissues and pK_B values were inappropriate for an action at β_1-adrenoceptors. In hypothyroid rats CGP 20712A increased the maximal response and shifted the curve slightly to the left, apparently sensitising the tissue to (−)-isoprenaline. This occurred with all three concentrations of CGP 20712A although the pEC_{50} values were not significantly altered. Experiments were also conducted with the selective β_1-adrenoceptor agonist RO 363 (not shown) and produced weak relaxation and shallow concentration–response curves that were not shifted by CGP 20712A indicating that the effects were not mediated by β_1-adrenoceptors.

RT-PCR was conducted on β_1- and β_3-adrenoceptor mRNA to examine whether changes in receptor expression could be responsible for the differences in function. Hypothyroidism did not significantly affect mRNA expression for either receptor, despite the apparent changes in their relative contributions to relaxation and providing evidence that in ileal smooth muscle thyroid hormone is not necessary for the normal expression of these receptors in vivo. This suggests that thyroid hormone alters β_3-adrenoceptor coupling rather than receptor expression, in contrast to adipose tissues where function and receptor expression are linked.

Clinical observations on thyroid dysfunction have frequently identified changes in gastrointestinal transit time. Hyperthyroid subjects commonly suffer from malabsorption and diarrhoea associated with bowel hypermotility, whereas in hypothyroid subjects sluggish intestinal clearance leads to constipation and obstipation. In the present experiments, hypothyroidism reduced β_3-adrenoceptor coupling and relaxation responses to (−)-isoprenaline. This would be expected to increase motility and is in direct contrast to the delay in transit observed in hypothyroidism in humans.

Thyroid hormones do not appear to be required for the normal expression of rat ileal β_1- and β_3-adrenoceptors in vivo. However, sensitivity to (−)-isoprenaline is reduced in hypothyroid rat ileum as is the relative contribution of the β_3-adrenoceptor to the relaxation response, indicating that hypothyroidism interferes with β_3-adrenoceptor coupling to second messenger pathways mediating relaxation. β_1-adrenoceptors did not greatly contribute to (−)-isoprenaline relaxation, even when β_3-adrenoceptor-mediated responses were compromised, indicating that the functional compensation observed is not mediated by β_1-adrenoceptors.

In conclusion, in ileum thyroid hormones did not directly regulate β_1- and β_3-adrenoceptor expression but may affect ileal responsiveness to (−)-isoprenaline and the relative contribution of the β_3-adrenoceptor in producing these responses. This situation is dramatically different from the situation in adipose tissues where changes in function are directly related to changes in expression of the β_3-adrenoceptor.

Acknowledgements

The authors would like to thank Dr T. Nash (Wyeth, Australia), Dr. G. Anderson (Ciba-Geigy Australia) and Dr. L. Manara (Sanofi Recherche, Italy) for gifts of CL 316243, CGP 20712A and SR 59230A, respectively. This work was supported by a project grant from the National Health and Medical Research Council of Australia. KB is a Monash University Graduate Scholar.

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