β_3-Adrenergic relaxation of bovine iris sphincter

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Abstract Bovine iris sphincter in vitro responded to β-adrenergic stimulation with pronounced relaxation (EC_{50} of isoproterenol = 0.3 nM), which was potentiated by the cAMP phosphodiesterase inhibitor, isobutylmethylxanthine, and mimicked by the adenylyl cyclase activator, forskolin. The β_1/β_2 antagonist, propranolol, exhibited low potency with calculated K_i of 200 nM. The β_3-selective antagonist, bupranolol, exhibited a biphasic inhibition profile, with calculated K_is of approximately 20–50 and 200–300 nM. The β_3-selective agonist, BRL 37344, elicited 70% of maximal relaxation (EC_{50} = 30 nM). When relaxation was induced by BRL 37344, bupranolol exhibited much higher potency (calculated K_i = 1 nM). Our data suggest that the β-adrenergic relaxation response in bovine iris sphincter is mediated by a mixed population of β-adrenergic receptors, with a predominant contribution of atypical, most likely β_3 subtype, receptors.

Key words: Bovine iris sphincter; Relaxation; β_3-Adrenergic receptor; Bupranolol; BRL 37344

1. Introduction

Atypical β-adrenergic receptors have been described quite often in the pharmacological literature. Recently, some of the phenomenology ascribed to atypical β-adrenergic responses has been identified with a novel receptor subtype, β_3-adrenoceptor. First found in brown fat (see [1] for review), it has been subsequently cloned [2–5] and characterized pharmacologically [3,6]. Currently, β_3-adrenoceptors have been identified in human brown fat cells [7], colon and gall bladder [8]. A mutation of the β_3-adrenoceptor in Pima Indians, Europeans and Japanese has been linked to their genetic propensity for obesity and high incidence of diabetes [9].

We investigated the pharmacology of β-adrenergically evoked relaxation of bovine iris sphincter. It is generally accepted that β-adrenergic effects are mediated by an increase in cellular cAMP. However, in view of the atypical β-adrenergic characteristics of the bovine sphincter response (see Section 3) and considering the reports that some of the β-adrenergic effects may be mediated by NO (e.g. [10–12]), we investigated the role of cAMP in the β-adrenergic relaxation of the bovine iris sphincter. Our results conform with the hypothesis that the relaxation induced by β-adrenergic is most probably mediated to a significant extent by the β_3-adrenoceptor subtype and proceeds via an increase in cellular cAMP concentrations. This finding extends the distribution of β_3-adrenoceptors to the ocular smooth muscle.

2. Materials and methods

Bovine eyes were freshly enucleated after the slaughter of the animal and kept on ice. Eyes were used within 72 h after enucleation. After removal of the cornea, the iris sphincter was excised. The muscle was tied at opposing sides and mounted on an isometric tension apparatus in an organ bath (10 ml) in Krebs-bicarbonate solution. The assays were conducted essentially as described by Patil [13]. Briefly, the tension was adjusted to 1 g and the muscle was allowed to initially relax, then to spontaneously regain its tonus (usually to more than 1 g tension) over 1–3 h. The bath was continuously aerated with an O_2:CO_2 mixture (95:5%) and kept at a constant temperature of 32°C. Drugs were added directly to the bath in a 20–100 μl volume.

To control for the possible occlusion of the β-blockers by the melanin-rich iris sphincter, we performed a bio-assay of propranolol (PPL) pre-exposed for 30 min to excised sphincter and then tested on rat right atria for its inhibition of the positive chronotropic effect of isoproterenol (ISO). Briefly, spontaneously contracting right atria were mounted on the isometric tension apparatus and a dose-response to ISO was performed. In the absence of PPL, the half-maximal stimulation of atrial contraction rate was observed at [ISO] = 0.6 nM. 10 nM PPL shifted the EC_{50} to 0.9 nM and the same value was obtained with PPL pre-incubated with the bovine sphincter tissue. Addition of 1 μM PPL shifted the EC_{50} to 0.4 μM, and for PPL pre-incubated with a bovine sphincter to 0.1 μM. Hence, although melanin could potentially bind β-adrenergic antagonists, this was found to be practically negligible. Similar conclusions were reached also by Patil and Weber [14].

The composition of the Krebs-bicarbonate solution was (in mM): NaCl 118; KCl 4.7; $MgCl_2$ 1.2; $CaCl_2$ 2.5; $NaHCO_3$ 25; KH_2PO_4 1.2; glucose 11; pH 7.4. ISO, isobutylmethylxanthine (IBMX), salbutamol (SAL), PPL and forskolin were purchased from Sigma (Israel). BRL 37344 was purchased from Research Biochemicals, Natick, MA, USA. Bupranolol was a gift of Schwarz Pharma, Monheim, Germany. SR 59230A was a gift of Dr. Manara of Sanofi Midy Research Center, Milan, Italy. All other chemicals were of analytical grade.

Grass isometric force transducer type FTO3C and Grass Polygraph amplifier and recorder were used. All experiments were performed several times on sphincters obtained from different animals. Results are presented as means ± S.E.M. Student's *t*-test was used to assess the significance of the results at $P < 0.05$.

3. Results

3.1. The effect of β-adrenergic stimulation on basal tension is mediated by cAMP accumulation

β-Adrenergic stimulation alone caused a significant relaxation of basal tension in iris sphincter muscle, mechanically adjusted to 1 g tension. Addition of 10 nM SAL or 1 nM ISO (not shown) caused a rapid decrease in basal tension by 0.87 ± 0.21 g (Fig. 1A, $n = 10$, $P < 0.001$).

To confirm that the β-adrenergic effect was indeed mediated by an increase in cAMP, we employed forskolin, a diterpene that stimulates adenylyl cyclase. 10 μM forskolin alone also caused a major decrease in basal tension (by 0.65 ± 0.15 g, Fig. 1B, $n = 9$, $P < 0.001$). Subsequent addition of 10 nM SAL did not further decrease basal tension (Fig. 1B). Conversely, the addition of forskolin after SAL also did not produce further relaxation (Fig. 1A). Hence, elevation of cAMP

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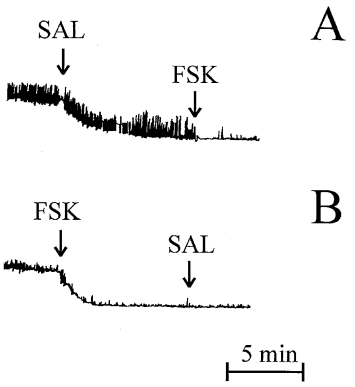

Fig. 1. Non-additivity of SAL and forskolin. Isolated iris sphincter was pre-equilibrated in the tissue bath, as described in Section 2. 10 nM SAL was added (A, first downward arrow). When the relaxation caused by SAL reached a plateau, the tension was re-adjusted and 10 μM forskolin (FSK) was added (A, second downward arrow). An identical experiment, but with the drugs added in the reverse order, is shown in B. Tracings from two representative experiments are shown.

alone was sufficient to produce maximal relaxation, be it via stimulation of the β-adrenergic receptors or by direct stimulation of adenylyl cyclase.

In nine out of the 15 experiments performed, the cAMP phosphodiesterase inhibitor, IBMX (3 μM), potentiated the relaxing effect of 1 nM ISO (not shown). In six experiments, there was no effect of IBMX, suggesting that in these experiments ISO alone caused sufficient elevation of cAMP to produce maximal relaxation. Hence, the β-adrenergic relaxation of bovine iris sphincter appeared to be mediated by cAMP.

3.2. *Pharmacological characterization of the response to β-adrenergic drugs*

In order to characterize further the β-agonist-induced relaxation of the sphincter, we performed cumulative dose-response experiments to β-adrenergic stimulation, either alone or in the presence of antagonists.

ISO alone caused a dose-dependent relaxation of resting sphincter, with a half-maximal relaxation (EC_{50}) obtained at approximately 0.3 nM and maximal effect at 10–30 nM (Fig. 2). The potent $\beta_{1,2}$-adrenergic antagonist, PPL, caused competitive inhibition of the ISO-induced relaxation. The apparent EC_{50} for ISO shifted to 10–20 nM at 10 μM PPL, yielding an approximate K_i value of 0.2 μM. The dose response to ISO in the presence of PPL spread to over three orders of magnitude of the concentration of the agonist, suggesting more than a single receptor population. The low potency of PPL was not due to its binding to the iris pigment, as determined by bio-assay on the positive chronotropic effect of ISO in rat right atria (see Section 2). The very low potency of PPL and the shape of the dose-response curve (see above) suggested an atypical β-adrenergic response. We therefore tested the relatively selective β_3-adrenoceptor antagonist, bupranolol. Bupranolol competitively inhibited ISO-induced relaxation, with apparent potency equal to or better than PPL. The apparent EC_{50} was shifted to 3 and 10 nM at 1 and 10 μM bupranolol, respectively (Fig. 3). This indicated that the apparent K_i for bupranolol was approximately 0.2 μM. However, the dose responses to ISO in the presence of bupranolol were distinctly biphasic, suggesting more than a single population of β-adrenoceptors and yielding two different K_i values for the antagonist (see Section 4).

1 μM SR 59230A, a putative β_3-selective antagonist [15], had no effect on the response to 10 nM ISO (not shown). It is known, however, that this compound is fat-soluble and pigment-bound (Dr. Manara, personal communication). It is possible, therefore, that this lack of efficacy was related to its sequestration in the pigmented tissue.

To further verify the participation of β_3-adrenoceptors in the relaxation response, we used a selective β_3-agonist, BRL 37344 [6]. BRL 37344 alone caused a dose-dependent relaxation of the iris sphincter with EC_{50} of 30 nM (Fig. 4). The maximal effect of BRL 37344 was less than that of ISO (approximately 70% of maximal β-adrenergic relaxation). The effect of BRL 37344 was antagonized by 0.1 μM bupranolol. The apparent EC_{50} shifted to 3 μM (Fig. 4), yielding a K_i of approximately 1 nM. BRL 37344 did not, however, fully overcome the effect of 0.1 μM or 3 nM bupranolol, even at an

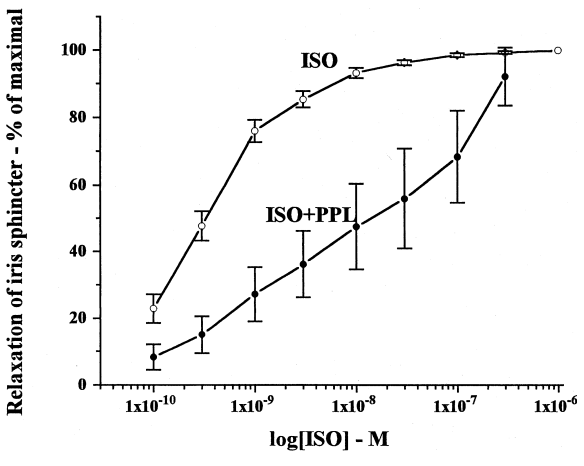

Fig. 2. Dose response to ISO. Isolated iris sphincters were mounted on an isometric tension apparatus and a cumulative dose response to ISO was performed in the presence of ISO alone (open circles) or in the presence of 10 μM PLL (closed circles, $n=5$). The data are presented as mean ± S.E.M. % of maximal relaxation (response to 1 μM ISO, 0.85 ± 0.05 g, $n=19$).

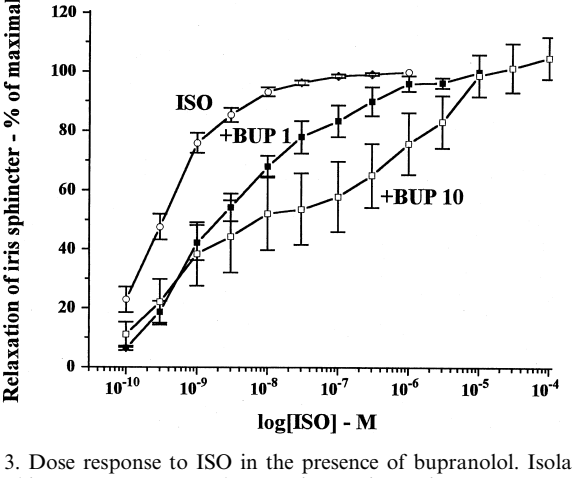

Fig. 3. Dose response to ISO in the presence of bupranolol. Isolated iris sphincters were mounted on an isometric tension apparatus and a cumulative dose response to ISO was performed in the presence of ISO alone (open circles), and in the presence of 1.0 (solid squares, $n=5$) or 10 μM (open squares) bupranolol (BUP, $n=8$). The data are presented as mean ± S.E.M. % of maximal relaxation (response to 1 μM ISO, 0.85 ± 0.05 g, $n=19$).

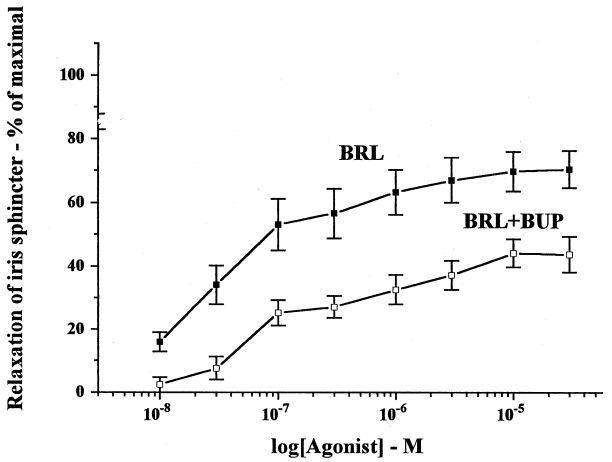

Fig. 4. Dose response to BRL 37344 ± bupranolol. Isolated iris sphincters were mounted on an isometric tension apparatus and a cumulative dose response to BRL 37344 was performed in the presence of the agonist alone (solid squares), or in the presence of 0.1 μM bupranolol (BUP, open squares, $n = 7$). The data are presented as mean ± S.E.M. % of maximal relaxation (response to 1 μM ISO, 0.85 ± 0.05 g, $n = 19$).

agonist concentration as high as 30 μM (Fig. 4), suggesting a non-competitive component in the action of the antagonist.

4. Discussion

β_3-Adrenergic receptor pharmacology has been investigated either in the tissues of origin (mostly fat cells from various species) or in CHO cells transfected with the receptor cDNA. In pure systems (i.e. transfectants), the potency of ISO (EC_{50}) was reported to be in a range of 4–700 nM [3,6]. The β_3-specific agonist, BRL 37344, yielded potency values of 0.4–80 nM. Hence, our values of 0.3 nM for ISO and 30 nM for BRL appear to be either more potent or within the same range. The very wide variations in the reported potencies suggest that the order of potency may be more indicative of receptor subtypes. However, this parameter also appears to vary considerably. BRL was reported to be 10-fold more potent than ISO in CHO cells transfected with the mouse receptors, but only 25% as potent in cells transfected with the human receptor [6]. Similarly, the potencies of PPL and bupranolol vary. In CHO cells transfected with human and mouse receptors, the K_i of bupranolol was 20 and 12 nM, respectively [6]. Our results yield K_i values of approximately 0.2 μM, comparable to the $\beta_{1,2}$-specific antagonist, propranolol.

Taken together, our results suggest a mixed receptor population with a predominant contribution of β_3-adrenoceptors. The main evidence for this hypothesis is: (1) the clearly biphasic dose response to ISO in the presence of bupranolol, yielding calculated approximate K_i values of 25–50 and 200–300 nM; (2) the ability of BRL 37344 to evoke only a partial response when compared to ISO. This was 70% of maximal relaxation, which matched the approximate proportion of the response highly sensitive to bupranolol (approximately 60–70%); (3) the very high potency of bupranolol to inhibit the response evoked by BRL 37344. Thus, when the response is evoked by a specific β_3-agonist, it is potently inhibited by a selective β_3-antagonist.

Our results resemble those described for a mixed receptor population rather than for cells expressing only the β_3-adrenoceptor. Galitzky et al. described a mixed population of β-adrenoceptors in dog fat cells [16]. Although ISO and BRL 37344 were equiefficacious when analyzed for their ability to induce lipolysis, BRL was only a 60% partial agonist for stimulation of adenylyl cyclase. Similarly, bupranolol was more potent than PPL in antagonizing the lipolytic effect stimulated by BRL 37344. Langin et al. [17] demonstrated that bupranolol exhibited more than 10-fold potency of PPL in inhibition of lipolysis induced by BRL 37344 in rat and hamster fat cells. They have suggested that mammalian fat cells express a mixed β-adrenoceptor population.

In conclusion, the β-adrenergic relaxation of the bovine iris sphincter appears to be mediated by a mixed adrenoceptor population, of which 60–70% may be attributed to atypical, most probably β_3-adrenoceptors.

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