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Pharmacological characterization of the 5-HT_{1A}, 5-HT_2 and 5-HT_3 receptors in the bovine ciliary muscle

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Abstract

We aimed to investigate the effects of serotonin (5-hydroxytryptamine, 5-HT) on the bovine ciliary muscle and subsequently to characterize and identify the subtypes of 5-HT receptors involved in the serotonin-evoked contractility muscle. The binding of [^{3}H]ketanserin, [^{3}H]granisetron and [^{3}H]8-hydroxy-2-(di-*n*-propylamino)tetralin ([^{3}H]8-OH-DPAT) was analyzed. All labelled compounds bound with high affinity to a single site in the membrane preparations studied. The affinity (K_d) of the binding site was 7.5 ± 1.2 nM for [^{3}H]ketanserin, 6.9 ± 0.8 nM for [^{3}H]granisetron and 4.4 ± 0.31 nM for [^{3}H]8-OH-DPAT. The density of receptors (B_{max}) was 1062 ± 43.0 fmol/mg protein for [^{3}H]ketanserin, 566 ± 2.32 fmol/mg protein for [^{3}H]granisetron and 205 ± 4.63 fmol/mg protein for [^{3}H]8-OH-DPAT. The serotonin-induced contraction appeared to be competitively antagonized by ketanserin (0.1, 1 and 10 μM) and ondansetron (0.1, 10 and 100 μM) which produced a pA_2 value of 8.5 ± 0.12 and 8.0 ± 0.19, respectively. 8-OH-DPAT and 5-carboxamidotryptamine (5-CT) proved to be completely ineffective. We conclude that serotonin induces bovine ciliary muscle contraction via 5-HT_2 and 5-HT_3 receptors while the 5-HT_{1A} receptors, although present, do not mediate the contractile response.
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Keywords: 5-HT receptor; Binding assay; 5-HT (5-hydroxytryptamine, serotonin) neurotrasmission; Ciliary muscle

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is known to be a neurotransmitter having different functions in both central and peripheral nervous systems. Its actions are mediated through interactions with specific receptors (Boess and Martin, 1994; Hoyer et al., 1994; Barness and Sharp, 1999). Up to now, seven classes of 5-HT receptors have been recognized and most of them are coupled to the production of second messengers via interactions with G-protein. The 5-HT_1 family of receptors, including six different subtypes, is negatively linked to adenylate cyclase; particularly, it has been mostly investigated the 5-HT_{1A} receptor subtype, which shows high affinity to 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT). The 5-HT_2 class of receptors is coupled to phospholipase C (Conn and Sanders-Bush, 1986) and shows high affinity for antagonists like ketanserin. The 5-HT_4, 5-HT_5, 5-HT_6 and 5-HT_7 receptors are positively coupled to adenylate cyclase provoking increase of cAMP intracellular concentration. On the contrary, 5-HT_3 receptors show properties of a typical gated cation channel (Derkach et al., 1989; Maricq et al., 1991; Boess et al., 1995).

In the last 20 years, evidence has been progressively accumulated suggesting that serotonin plays an important physiological role in the eye especially acting as an effective intraocular pressure modulating agent. A number of reports show that serotonin and some serotonergic agents can influence the intraocular pressure. Topical application of 5-HT seems to reduce (Krootila et al., 1987) the intraocular pressure in rabbits and increase (Meyer Bothling et al., 1993) intraocular pressure in mice and dogs, whereas intracameral injection of 5-HT in rabbits increases it (Krootila et al., 1987), and intravenous injection of serotonin into dogs and rabbits lowers intraocular pressure (Chiang, 1974). Different studies revealed the presence of serotoninergic neurons in the iris–ciliary body complex of rabbit (Tobin et al., 1988) and human (Martin et al., 1992). Furthermore, radioligand binding

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(Mallorga and Sugrue, 1987; Chidlow et al., 1995) and second messenger studies (Barnett and Osborne, 1993; Tobin et al., 1988; Tobin and Osborne, 1989) have clearly proved the existence of at least two types of serotoninergic neurons in the ciliary body: the 5-HT_1 subtype, negatively linked to the metabolism of cAMP, and the 5-HT_2 subtype, linked to a stimulation of inositol phosphates.

The present study was undertaken to investigate the effect of different concentrations of serotonin on the bovine ciliary muscle and to characterize the serotonergic receptors involved in this effect. Knowing that ciliary muscles have an important role in the regulation of aqueous humor outflow, the presence of the 5-HT-receptor subtypes in these tissues could be an interesting target for intraocular pressure reduction.

2. Materials and methods

2.1. Preparation of ciliary muscles

Bovine eyes obtained from a slaughterhouse were enucleated within 5 min after death and immediately placed in an oxygenated modified Krebs solution at 4 °C, using them for the study within 30 min. The globe was equatorially opened and the vitreous and lens were removed. The ciliary muscle was quickly isolated under a binocular microscope (Nikon, Japan) and dissected from the scleral spur lens and choroid as previously described (Lograno and Reibaldi, 1986; Lograno et al., 1991), to be utilized for both functional experiments and binding assays. After the isolation, the tissues were immediately used or frozen on dry ice and stored at − 80 °C until assayed.

2.2. Biochemical experiments

2.2.1. Ciliary muscle membrane preparation

For the binding of 5-HT_2 receptors, ciliary muscles were homogenized with a polytron homogenizer in ice cold Tris–HCl buffer (50 mM, pH 7.4). The homogenate was centrifuged at 37,000 × *g* for 20 min and then washed with the same buffer three times. The final pellet was frozen and stored at − 80 °C until use.

For the binding of 5-HT_3 receptors, ciliary muscles were homogenized with a polytron homogenizer in ice cold HEPES buffer (50 mM, pH 7.4). The homogenate was centrifuged at 50,000 × *g* for 10 min and then washed with the same buffer three times. The final pellet was frozen and stored at − 80 °C until use.

For the binding of 5-HT_{1A} receptors, ciliary muscles were homogenized with a polytron homogenizer in ice cold Tris–HCl buffer (50 mM, pH 7.4). The homogenate was centrifuged at 30,000 × *g* for 15 min and then washed with the same buffer. The pellet was resuspended in 20 volumes of buffer and incubated at 37 °C for 10 min. After the incubation, the homogenate was centrifuged at 30,000 × *g* for 15 min. The final pellet was frozen and stored at − 80 °C until use.

Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as a standard. At the time of incubation, the membranes were resuspended in the respective incubation buffer.

2.2.2. Radioligand binding assays

Saturation binding assays were conducted with[^{3} H]ketanserin, [^{3}H]granisetron and [^{3}H]8-OH-DPAT. All experiments were performed in triplicate using a total assay volume of 1 ml.

[^{3}H]ketanserin binding studies were assayed in a reaction mixture containing 50 mM Tris–HCl buffer (pH 7.4), increasing concentration (1 nM–0.1 μM) of the tritiated compound and 10 μM of unlabelled ketanserin for the determination of non-specific binding. The incubation was initiated by the addition of the membrane suspension (60 μg membrane protein) and allowed to continue at 37 °C for 30 min. Incubation was terminated by rapid filtration under vacuum through glass fiber Whatman GF/B filters. Filters were washed three times with 4 ml of ice-cold buffer. Filter sections were transferred to vials and 8 ml of Formula 989 (New England Nuclear) scintillation cocktail was added to each vial. Vials were allowed to set overnight before counting on a Beckman LS6000 liquid scintillation counter. In competition studies, the ability of unlabelled analogs to displace 7 nM [^{3}H]ketanserin was assessed at concentrations ranging from 1 nM to 10 μM.

[^{3}H]granisetron binding studies were assayed in a reaction mixture containing 50 mM HEPES buffer (pH 7.4), increasing concentrations (0.1–40 nM) of the tritiated compound and 1 μM of unlabelled granisetron for the determination of non-specific binding. The incubation was initiated by the addition of the membrane suspension (50 μg membrane protein) and allowed to continue at 23 °C for 30 min. Incubation was terminated by rapid filtration under vacuum through glass fiber Whatman GF/B filters. The filters were pretreated in 0.1% polyethylenimine for 40 min to reduce radioactivity trapping on the filters and washed three times with 5 ml of ice-cold buffer. The radioactivity retained was determined as described above. In competition studies, the ability of unlabelled analogs to displace 7 nM [^{3}H]granisetron was assessed at concentrations ranging from 1 nM to 10 μM.

[^{3}H]8-OH-DPAT binding studies were assayed in a reaction mixture containing 50 mM Tris–HCl buffer (pH 7.4), increasing concentrations (0.2–35 nM) of the tritiated compound and 10 μM of unlabelled 8-OH-DPAT for the determination of non-specific binding. The incubation was initiated by the addition of the membrane suspension (200 μg membrane protein) and allowed to continue at 37 °C for 10 min. Incubation was terminated by filtration under vacuum through glass fiber Whatman GF/B filters. The filters were washed three times with 5 ml of ice-cold buffer. The radioactivity retained was determined as described above. In

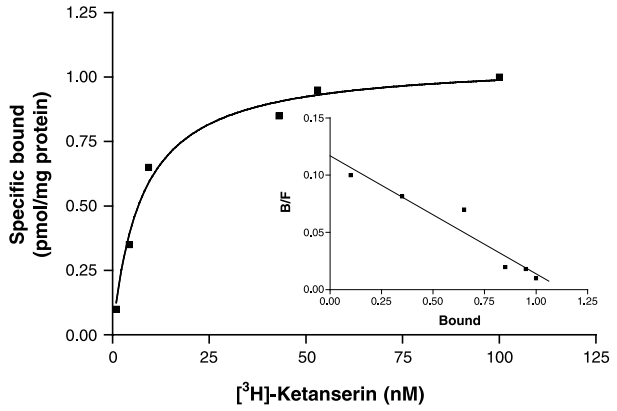

Fig. 1. Representative saturation analysis and Scatchard plot (inset) of [^{3}H]ketanserin to binding to bovine ciliary muscle membranes. K_d = 7.5 ± 1.2 nM, B_{max} = 1062 ± 43.0 fmol/mg of protein. Data were analyzed with GraphPad Prism program and a one-site analysis gave the best fit. Assays were performed as described in Materials and methods. Values represent a mean ± S.E.M. of triplicate determinations.

competition studies, the ability of unlabelled analogs to displace 4 nM [^{3}H]8-OH-DPAT was assessed at concentrations ranging from 1 nM to 10 μM.

Scatchard analysis of the data was performed with GraphPad Prism (GraphPad Software, San Diego, CA). The K_d and B_{max} values were obtained and averaged ($n \geq 3$) and then provided with the standard error of the mean (S.E.M). The apparent inhibition constant (K_i) for each drug was calculated by means of the Cheng–Prusoff equation: $K_i = IC_{50}/1+([C]/K_d)$, where [C] = concentration of the radioligand and K_d = affinity value. The curves were analyzed according to a one-site model.

2.3. Functional experiments

To investigate mechanical properties, ciliary muscle strips were set up under a tension of 200 mg in an organ bath and superfused by means of the laminar flow superfusion technique (Ferreira and De Souza Costa, 1976). The optimal passive tension at which the maximal concentration to carbachol (100 μM) was obtained averaged 450 ± 50 mg (n = 5). The isolated tissues were immersed in mineral oil. Krebs solution was superfused at rates of 0.1–0.2 ml/min by means of an infusion pump; the oil was maintained at 37 °C by water circulating through a jacket. The preparations were superfused by dropping the Krebs solution and oxygenated with 95% O_2 and 5% CO_2, on the thread which connects the tissue to the transducer. Contractile responses were isometrically measured by means of a force–displacement transducer (DYO, U. Basile, Italy) and recorded on a linear recorder coupled to a Grass polygraph. The tissues were allowed to equilibrate for 80 min during which they relaxed and reached a final length of about 4–6 mm, before any drug addition. Spontaneous activity was absent. Ketanserin, ondansetron and propranolol were added to the superfusing buffer to obtain the appropriate concentrations expressed as molar concentration. Agonists (serotonin, 8-OH-DPAT and 5-CT) were injected in 50 μl into the drops of the superfusing Krebs solution. Contractile responses were expressed as percentages of the maximal effect produced by serotonin before drug treatment.

Differences were examined by Student's t-test for paired observations and were considered to be significant for $P < 0.05$.

pA_2 values were calculated from Arunlakshana and Schild (1959) plot; concentration ratios were calculated at the EC_{50} level.

The composition of the Krebs solution was as follows (mM): NaCl 136.8, KCl 5.4, $CaCl_2$ 2.7, $MgSO_4 \cdot 7H_2O$ 0.8, Glucose 5 and HEPES [4-(2-hydroxyethyl) 1-piperazine-ethane sulfonic acid] 6 (pH 7.4).

2.4. Materials

[^{3}H]ketanserin (60 Ci/mmol), [^{3}H]granisetron (90 Ci/mmol) and [^{3}H]8-OH-DPAT (143.8 Ci/mmol) were purchased from New England Nuclear (Milan, Italy). All drugs and reagents were purchased from Sigma (St. Louis, MO, USA) except 5-carboxamidotryptamine (5-CT), spiperone and ketanserin TocrisCookson (Bristol BS11 8TA UK); 8-OH-DPAT, granisetron and ritanserin RBI (Natick, MA, USA).

3. Results

3.1. Binding to membrane preparations of ciliary muscle

Time course studies of [^{3}H]ketanserin, [^{3}H]granisetron and [^{3}H]8-OH-DPAT binding in bovine ciliary muscle membrane were initially performed. Non-specific binding

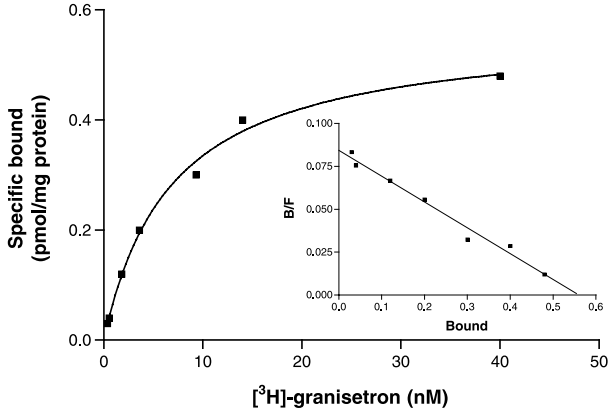

Fig. 2. Representative saturation analysis and Scatchard plot (inset) of [^{3}H]granisetron to binding to bovine ciliary muscle membranes. K_d = 6.9 ± 0.8 nM, B_{max} = 565 ± 32 fmol/mg of protein. Data were analyzed with GraphPad Prism program and a one-site analysis gave the best fit. Assays were performed as described in Materials and methods. Values represent a mean ± S.E.M. of triplicate determinations.

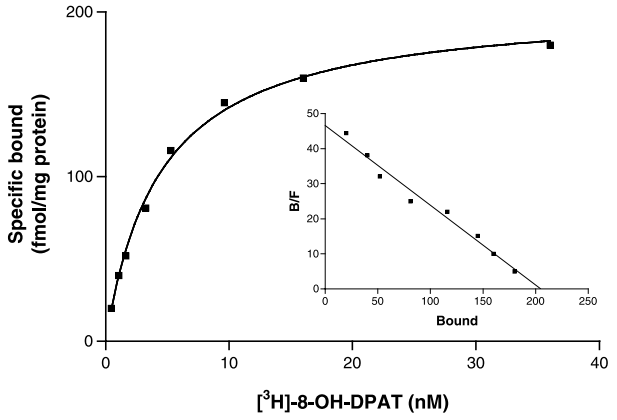

Fig. 3. Representative saturation analysis and Scatchard plot (inset) of [^{3}H]8-OH-DPAT to binding to bovine ciliary muscle membranes. $K_d = 4.4 \pm 0.31$ nM, $B_{max} = 205 \pm 4.63$ fmol/mg of protein. Data were analyzed with GraphPad Prism program and a one-site analysis gave the best fit. Assays were performed as described in Materials and methods. Values represent a mean ± S.E.M. of triplicate determinations.

for [^{3}H]ketanserin, [^{3}H]granisetron and [^{3}H]8-OH-DPAT represented less than 10% of the total binding.

Specific binding of [^{3}H]ketanserin increased linearly with membrane protein concentration over a range of 10–100 μg/ml. Scatchard analysis of the saturation data produced a linear plot indicating the likelihood of one population of labelled [^{3}H]ketanserin binding sites. K_d and B_{max} values of 7.5 ± 1.2 nM and 1062 ± 43.0 fmol/mg protein were respectively obtained (Fig. 1).

Specific binding of [^{3}H]granisetron increased linearly with membrane protein concentration over a range of 10–75 μg/ml. Scatchard transformations of the saturation data produced a linear plot indicating the likelihood of one population of labelled [^{3}H]granisetron binding sites. K_d and B_{max} values of 6.9 ± 0.8 nM and 566 ± 2.32 fmol/mg protein were respectively obtained (Fig. 2).

Specific binding of [^{3}H]8-OH-DPAT increased linearly with membrane protein concentration over a range of 50–300 μg/ml. Scatchard transformations of the saturation data produced a linear plot indicating the likelihood of one population of labelled [^{3}H]8-OH-DPAT binding sites. K_d and B_{max} values of 4.4 ± 0.31 nM and 205 ± 4.63 fmol/mg protein were respectively obtained (Fig. 3).

The data from competition experiment are shown in Table 1. These data demonstrate that selective agonists and antagonists of the 5-HT$_2$, 5-HT$_3$ and 5-HT$_{1A}$ receptors selectively displaced the labelled ketanserin, granisetron and 8-OH-DPAT binding respectively producing the K_i values in the low nanomolar range. Serotonin appears to have most selectivity for [^{3}H]8-OH-DPAT binding sites ($pK_i = 7.7$ M) compared to [^{3}H]ketanserin ($pK_i = 6.6$) and [^{3}H]granisetron ($pK_i = 6.4$) binding. Moreover, one can note that non-selective compounds like dopamine and noradrenaline did not produce any displacement of [^{3}H]8-OH-DPAT, [^{3}H]ketan-

Table 1
Potencies of 5-HT receptor agonists and antagonists in displacing radioligand binding in bovine ciliary muscle

Compound	pK_i (M)		
	[^{3}H]8-OH-DPAT	[^{3}H]ketanserin	[^{3}H]granisetron
Serotonin	7.7	6.6	6.4
8-OH-DPAT	8.1	>5	>5
5-CT	9.2	>5	>5
Granisetron	>5	>5	8.2
Ondansetron	>5	>5	7.9
Zacopride	>5	>5	7.1
Ketanserin	>5	8.4	>5
Ritanserin	>5	7.8	>5
Noradrenaline	>5	>5	>5
Dopamine	>5	>5	>5

Data represent mean ± S.E.M. of three to five experiments.
Radioligand binding studies were performed as described in Materials and methods. IC_{50} values were determined from inhibition curves using six triplicate concentrations of each inhibitor. Apparent inhibition constants, assuming competitive inhibition, were determined using the Cheng–Prusoff equation: $K_i = IC_{50}/1+([C]/K_d)$, where [C] = concentration of the radioligand and K_d = affinity value.

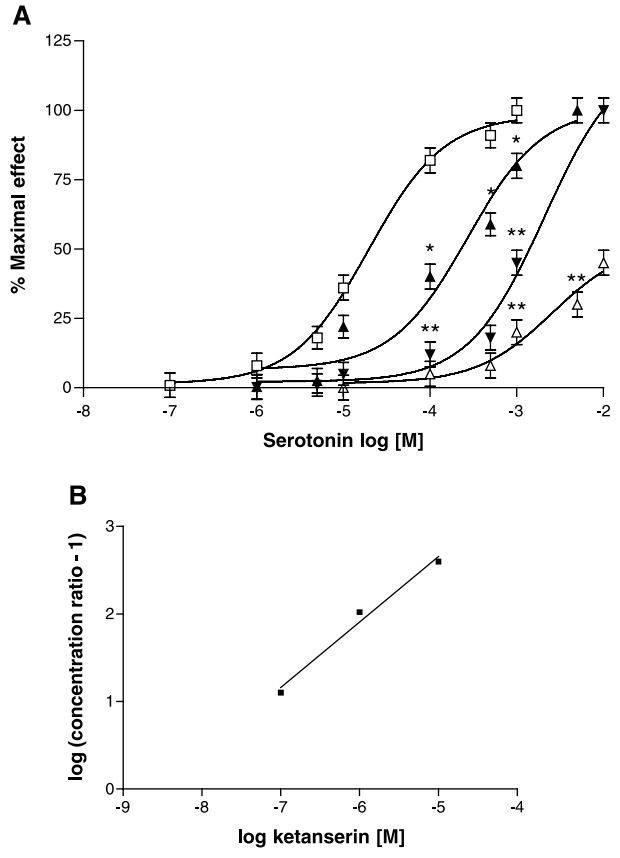

Fig. 4. Effects of 5-HT$_2$ receptor antagonist, ketanserin in the bovine ciliary muscle on contractions evoked by serotonin. (A) Concentration–effect curves in absence (□) and in presence of 0.1 μM (▲), 1 μM (▼) and 10 μM (△) ketanserin. Data are means ± S.E.M. of at least five separate experiments; *$P < 0.05$; **$P < 0.01$. (B) Schild plot for ketanserin with serotonin as agonist. Each point represents means ± S.E.M. of five experiments.

serin and [^{3}H]granisetron binding sites in the membrane preparations examined.

3.2. Functional assay

In quiescent strips, serotonin (0.1 μM–1 mM) produced a significant concentration-dependent contraction with EC_{50} value of 0.02 mM; the mechanical thresholds in terms of serotonin concentration were about 1 μM, whereas the maximal contractions were obtained with 1 mM serotonin. The 5-HT_{1A} receptor agonists 8-OH-DPAT and 5-CT had no effect on the ciliary muscle at the concentration 0.1 μM–1 mM. Furthermore, one analyzed the effect of 5-HT_{1A}/β-adrenoceptor antagonist propranolol which did not alter the contractile tone of the serotonin even at high concentration (1 mM). The 5-HT_2 receptor antagonist ketanserin (0.1, 1 and 10 μM) significantly antagonized in a competitive manner the contractile responses to serotonin (0.1 nM–1 mM) producing EC_{50} values of 0.27, 2.08 and 2.58 mM, respectively (Fig. 4A); the concentration ratios of serotonin, when plotted as a function of ketanserin concentration, resulted in a line with a slope of 0.75, from which a pA_2 value of 8.5 was calculated (Fig. 4B). The effect of ondansetron (0.1, 10 and 100 μM), a selective 5-HT_3 receptor antagonist, on the contractile responses induced by serotonin (0.1 nM–1 mM), was investigated. The ondansetron shifted to the right the concentration–response curves to serotonin (Fig. 5A) in a parallel and surmountable manner producing EC_{50} values of 0.083, 0.9 and 4.3 mM, respectively; the concentration ratios of serotonin, when plotted as a function of ondansetron concentration, resulted in a line with a slope of 0.58, from which a pA_2 value of 8.0 was calculated (Fig. 5B).

The high concentration of drugs used (10 μM kentanserin and 100 μM ondansetron) alter the slope factors which differed from unity; probably, these concentrations modified the affinity of binding versus 5-HT_2 and 5-HT_3 receptors, respectively, so that the interaction is not strictly competitive, as one can observe from curves (Figs. 4A and 5A); indeed, at lower concentrations of drugs the slope factors were near unity (0.93).

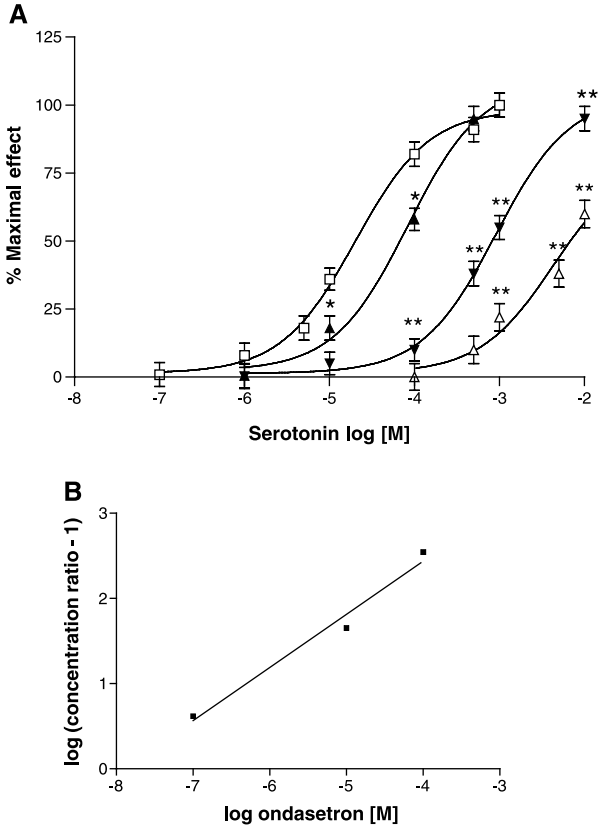

Fig. 5. Effects of 5-HT_3 receptor antagonist, ondansetron in the bovine ciliary muscle on contractions evoked by serotonin. (A) Concentration–effect curves in absence (□) and in presence of 0.1 μM (▲), 10 μM (▼), and 100 μM (△) ondansetron. Data are means ± S.E.M. of at least five separate experiments; *$P<0.05$; **$P<0.01$. (B) Schild plot for ondansetron with serotonin as agonist. Each point represents means ± S.E.M. of five experiments.

4. Discussion

The present study clearly demonstrates the presence of specific serotonin receptor subtypes in the bovine ciliary muscle. Binding studies with [^{3}H]ketanserin, [^{3}H]granisetron and [^{3}H]8-OH-DPAT showed that these radioligands selectively, reversibly and respectively labelled a population of 5-HT_2, 5-HT_3 and 5-HT_{1A} receptors in the membrane preparations of the bovine ciliary muscle. Saturation data revealed that [^{3}H]ketanserin, [^{3}H]granisetron and [^{3}H]8-OH-DPAT bindings were saturable and of high affinity and occupying a single binding site with K_d value in the low nM range. Competition studies with [^{3}H]ketanserin, [^{3}H]granisetron and [^{3}H]8-OH-DPAT suggested that each radioligand could be displaced respectively by a variety of selective agonists and antagonists of 5-HT_2, 5-HT_3 and 5-HT_{1A} receptors and could define rank order potencies of competing substances for each radioligand. The rank order of pK_i values further supported the serotoninergic receptor specificity of [^{3}H]ketanserin, [^{3}H]granisetron and [^{3}H]8-OH-DPAT binding of membrane preparation. The relative potencies of these compounds in displacing [^{3}H]ketanserin and [^{3}H]granisetron binding are consistent with what has been found in functional assays.

The functional assays demonstrate that serotonin modulates the contractility of the bovine ciliary muscle in concentration-dependent fashion. The ability of ketanserin and ondansetron to antagonize competitively the serotonin-induced concentration–contraction curves suggests that 5-HT_2 and 5-HT_3 receptors were involved; however, ketanserin was more efficacious in inhibiting the serotonin-evoked contractile response. This was not unexpected, being in binding assay the density of 5-HT_2 receptor is higher when compared to 5-HT_3 and 5-HT_{1A}. Moreover, in secondary messenger study, Tobin et al. (1988) have

observed that in iris–ciliary body complex serotonin stimulates an increase in inositol phosphate accumulation in a dose-dependent manner.

Ondansetron, too, competitively antagonized the contraction to serotonin and it was not significantly different from ketanserin. Probably, the activation of 5-HT_3 receptors by serotonin involved a Ca^+ influx through the ligand-gated ion channels (Nichols and Mollard, 1996), which was responsible of the contraction of the bovine ciliary muscle. In contrast to the potent inhibition provoked by ketanserin and ondansetron versus the contractile response serotonin-induced, the 5-HT_{1A} receptor agonists 8-OH-DPAT and 5-CT were inactive even when using high concentration; furthermore, 5-HT_{1A}/β-adrenoceptor antagonist propranolol did not modify the tone serotonin-induced. The present evidence supports the hypothesis that 5-HT_{1A} receptors were not involved in the modulation of the contractility of the bovine ciliary muscle. Indeed, we have characterized binding sites of high affinity for 5-HT_{1A} receptor even if the receptor density is low (B_{max} = 204 fmol/mg protein) and any action on the contractility of the smooth muscle ciliary has been shown. These data confirmed the presence of 5-HT_{1A}, 5-HT_2 and 5-HT_3 receptors in the bovine ciliary muscle and the involvement of 5-HT_2 and 5-HT_3 receptors in the modulation of the contractility of the bovine ciliary muscle. It is known that the contractility of ciliary muscle leads to a decrease of the intraocular pressure for the extension of trabecular meshwork, thus promoting the outflow of aqueous humor.

The role of serotoninergic receptors in the bovine ciliary muscle should be examined in the context of the decrease of the intraocular pressure following contraction of smooth ciliary muscle. Hence, we have been hypothesized that the activation of 5-HT_2 and 5-HT_3 receptors probably promotes intracellular Ca^+ mobilization, and this could cause ciliary muscle contraction (Brown et al., 1998; Niemeyer and Lummis, 2001). Thus, the coexistence of 5-HT_2 and 5-HT_3 receptors in the ciliary muscle suggests that the regulation of the ciliary muscle tone by serotonin is based on a complex mechanism involving multiple receptor subtypes.

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