

5-HT₂ Receptor-Mediated Phosphoinositide Hydrolysis in Bovine Ciliary Epithelium

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ABSTRACT

The serotonin 2 (5-HT₂) receptor antagonists, MCI-9042 (Anplag[®]) and ketanserin, have been shown to lower intraocular pressure in rabbits (1) and humans (2). The mechanism of action of these drugs has not been determined, but it is hypothesized that 5-HT₂ receptors, and possibly α -adrenergic receptors, (3) may regulate in part aqueous humor production via an intracellular signal transduction pathway in the ciliary body. We therefore examined whether 5-HT₂ receptors were coupled to phosphoinositide hydrolysis in an organ culture system of isolated bovine ciliary epithelium. 5-HT stimulated [³H]inositol phosphates ([³H]InsPs) accumulation in a dose-dependent manner with a maximum increase approximately twice over the basal level. The mean EC₅₀ value was 1.1 μ M, which was calculated from four dose-response curves. The 5-HT stimulated accumulation of [³H]InsPs was inhibited by spiperone (5-HT_{2A/1A} and dopamine 2 (D₂) antagonists), M-1 (a major metabolite of MCI-9042), ketanserin (5-HT_{2A} antagonist), SB-206553 (5-HT_{2B/2C} antagonist), and mesulergine (5-HT_{2C} antagonist and D₂ agonist). It was not inhibited by chlorpromazine, which is a D₂ receptor antagonist. Accordingly, our study demonstrates that 5-HT₂ receptors are coupled to phospholipase C in bovine ciliary epithelium.

INTRODUCTION

In the central and peripheral nervous system, serotonin (5-hydroxytryptamine or 5-HT) is a neurotransmitter/autocoid with diverse regulatory roles in various physiological functions. The diversity of this neurotransmitter is imparted by the large family of 5-HT receptors, which are classified by pharmacological and molecular biological properties into 14 subtypes: 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT₃, 5-HT₄, 5-HT_{5A}, 5-HT_{5B}, 5-HT₆, and 5-HT₇ (4). With the exception of 5-HT₃, which is a ligand-gated ion channel, all other 5-HT receptors belong to the family of G protein-coupled receptors. The transmembrane signaling of the 5-HT receptors has

been extensively studied. The 5-HT₁ receptor family is negatively coupled to adenylate cyclase via Gi/o, and receptor activation lowers intracellular cAMP (5). The 5-HT₂ receptor family is coupled to phospholipase C via Gq/11, and receptor activation produces inositol 1,4,5-trisphosphate and diacylglycerol, which leads to an increase of intracellular Ca²⁺ and protein kinase C activation (6).

Within the anterior segment of the eye, 5-HT has been detected in human aqueous humor (7,8) and the 5-HT enzymatic pathways assayed in human ciliary body (9). 5-HT receptors are present in rabbit iris-ciliary bodies (10), and have been characterized as the 5-HT_{1A} receptor subtype (11). It appears that both 5-HT_{1A} receptor agonists, e.g., 8-hydroxy-2-(di-N-propylamino)tetralin hydrobromide (12) and 5-HT_{2A} receptor antagonists, e.g., MCI-9042 (1,2), ketanserin (13–15) and ketanserin analogs (16), lower intraocular pressure (IOP). Given the growing evidence for the role for 5-HT in modulating aqueous humor dynamics, we sought to determine whether 5-HT₂ receptors were coupled to phosphoinositide hydrolysis in isolated bovine ciliary epithelium.

MATERIALS AND METHODS

Materials

M-1 (Fig. 1) was provided by Mitsubishi Pharma Corporation (Osaka, Japan). *Myo*-[³H]Inositol (92.0 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). AG 1-X8 formate resin (200–400 mesh) and scintillation cocktail were purchased from Bio-Rad (Hercules, CA) and Fisher Scientific (Chicago, IL), respectively. Other reagents were purchased from Sigma (St. Louis, MO). The AcuPunch™ was obtained from Acuderm Inc. (Ft. Lauderdale, FL).

Tissue Preparation

Ciliary bodies were dissected from bovine eyes, which were put on ice within 3 hours of death from Wolverine Packing Company (Detroit, MI). The ciliary epithelial dissections were performed as described in Moroi et al (17). In brief, ciliary bodies were incubated in Ca²⁺- and Mg²⁺-free Hanks' Balanced Salt Solution (HBSS) at 37°C and 5% CO₂ for 30 minutes. The culture medium was exchanged for Ca²⁺- and Mg²⁺-free HBSS containing 0.25 mg/ml trypsin and 0.1 mg/ml EDTA, and the ciliary bodies gently shaken at 4°C overnight. The following day, the epithelium was gently pulled off the ciliary body, and incubated at 37°C and 5% CO₂ in Dulbecco's Modified Eagle's Medium (DMEM) containing high glucose (4.5 g/L), 10% fetal bovine serum, gentamicin (10 μg/ml), penicillin (10 U/ml) and streptomycin (10 μg/ml).

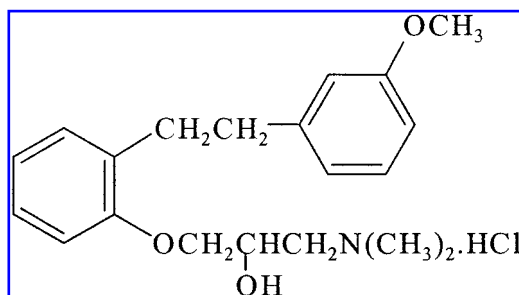


FIGURE 1. Structure of M-1, the Major Metabolite of Anplag® (sarpogrelate HCl, MCI-9042).

Phosphoinositide Assay

Six millimeter diameter epithelial explants were prepared using the AcuPunch™. A single epithelial explant was placed into a 96 well plate, and the bovine ciliary epithelial explants were cultured overnight at 37°C and 5% CO₂ in HBSS with Ca²⁺ and Mg²⁺ containing 2 μCi *myo*-[³H]inositol and antibiotics. The culture medium was exchanged for serum-free DMEM (1.0 g/L glucose content) containing 10 mM lithium chloride (LiCl). After a 15 minute incubation at 37°C and 5% CO₂, 5-HT was added at various concentrations with 10 μM ascorbic acid and 10 μM pargyline and the epithelial explants were stimulated for 30 minutes. When examining the effect of antagonists, 10 μM of each antagonist was added 10 minutes prior to the addition of 5-HT. After a 30 min stimulation period, the reaction was stopped with 0.4 M of perchloric acid. The samples containing epithelial explants and culture media were neutralized with 0.72 M KOH-0.6 M KHCO₃, transferred to polypropylene tubes, and homogenized (Tissumizer; Tekmar, Cincinnati, OH). The supernatant of the homogenate was applied to polypropylene columns filled with anion exchange resin, and washed with 5 mM *myo*-inositol and 5 mM Na₂B₄O₇/60 mM NaO₂CH. The total [³H]InsPs were eluted with 1.0 M NH₄COOH, and an aliquot was used counted using a liquid scintillation counter.

Data Analysis

The 5-HT dose-response curves were analyzed using GraphPad Prism (San Diego, CA). Two-tailed Student's *t* test was used to compare [³H]InsPs accumulation between basal and 5-HT. The effects of the various antagonists on [³H]InsPs accumulation were evaluated by one-way ANOVA.

RESULTS

We have previously demonstrated the suitability of these bovine ciliary epithelial explants by histology, viability assessed by trypan blue staining, and total cellular protein content (17). The effect of 30 minute stimulation of 5-HT on [³H]InsPs accumulation was compared in the ciliary epithelial explants at 24 versus 48 hours recovery following the trypsin dissection. The epithelial explants that had a 48 hour recovery had a 2.2-fold increase in [³H]InsPs over basal levels, which was greater than the 1.4-fold increase in those explants that had a 24 hour recovery (Fig. 2). Stability of the 5-HT agonist was maximized by fresh preparation prior to each experiment, and the presence of pargyline, a monoamine oxidase inhibitor, and of the antioxidant ascorbic acid. Neither pargyline nor ascorbic acid had any effect on [³H]InsPs accumulation (17). All subsequent experiments were performed at 48 hours after the trypsin-mediated dissection of the ciliary epithelial explants.

5-HT stimulated accumulation of [³H]InsPs in a dose-dependent manner, and a representative dose-response curve is shown in Figure 3. The mean EC₅₀ value calculated from four independent curves was 1.1 μM.

Various antagonists were used to determine whether the 5-HT₂ receptor subtype stimulated [³H]InsPs accumulation (Fig. 4). In the 5-HT stimulated control group, [³H]InsPs accumulation increased 191.4 ± 10.1% over basal. The 5-HT₂ receptor antagonists spiperone (5-HT_{2A/1A} and D₂ antagonist), M-1 (5-HT_{2A}), ketanserin (5-HT_{2A}), SB-206553 (5-HT_{2B/2C}) and mesulergine (5-HT_{2C} antagonist and D₂ agonist) decreased the 5-HT stimulation with only 118.7 ± 12.5%, 104.9 ± 6.5%, 125.8 ± 18.2%, 105.0 ± 7.4%, and 104.1 ± 15.3% over basal, respectively. The D₂ receptor selective antagonist, chlorpromazine, did not lower the 5-HT stimulation. In addition, WAY-100635, a selective 5-HT_{1A} antagonist, did not affect the 5-HT stimulation (data not shown). Hence, these antagonists indicate that the stimulatory effect of 5-HT on [³H]InsPs accumulation is mediated by the 5-HT₂ receptor.

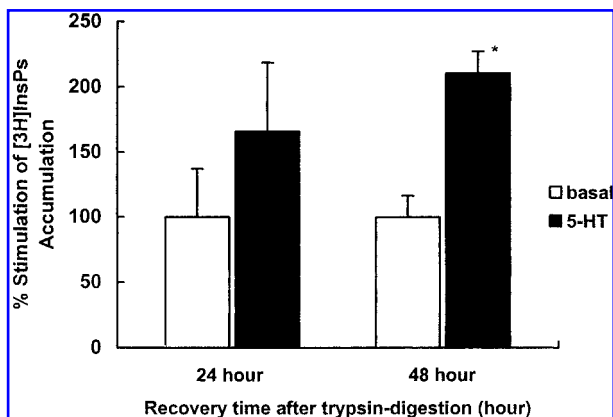


FIGURE 2. 5-HT-mediated Phosphoinositide Hydrolysis in Bovine Ciliary Epithelial Explants. After trypsin-mediated dissection, the epithelium recovered for 24 or 48 hours. The epithelial explants (6 mm punches) were labeled with *myo*-[³H]inositol, and stimulated with 500 μ M 5-HT. The data are shown as percent basal (mean \pm SE, n = 3–5). *Significant stimulation (Student's *t*-test, *p* < 0.01).

DISCUSSION

Given the recent results of the Ocular Hypertension Treatment Study (18), it is clear that lowering IOP is important in lowering the risk for developing glaucomatous optic neuropathy. Numerous pharmacological approaches have been developed to decrease the aqueous humor inflow and to enhance the trabecular and uveoscleral outflow pathways. Two commonly prescribed classes of drugs are the β -adrenergic receptors (β -ARs) and α_2 -ARs, both of which lower IOP by decreasing aqueous humor flow (19–21). Given the profound effect of these medications on aqueous flow, the presumed mechanism of action of these drugs is at the level of the ciliary epithelium.

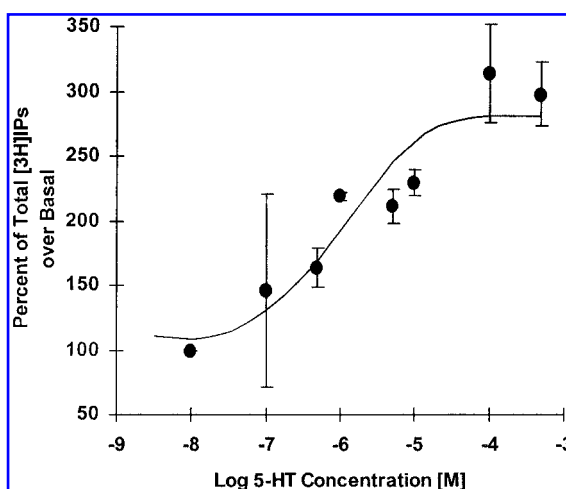


FIGURE 3. 5-HT Dose Response Curve for [³H]InsPs Accumulation in Bovine Ciliary Epithelial Explants. After a 48 hour recovery following trypsin-mediated digestion, the explants were radiolabeled with *myo*-[³H]inositol. Various concentrations of 5-HT were added for a 30 min stimulation period. Each point represents the mean of duplicate determinations \pm SE. The average EC₅₀ value was 1.1 μ M based on four independent 5-HT concentration-response curves.

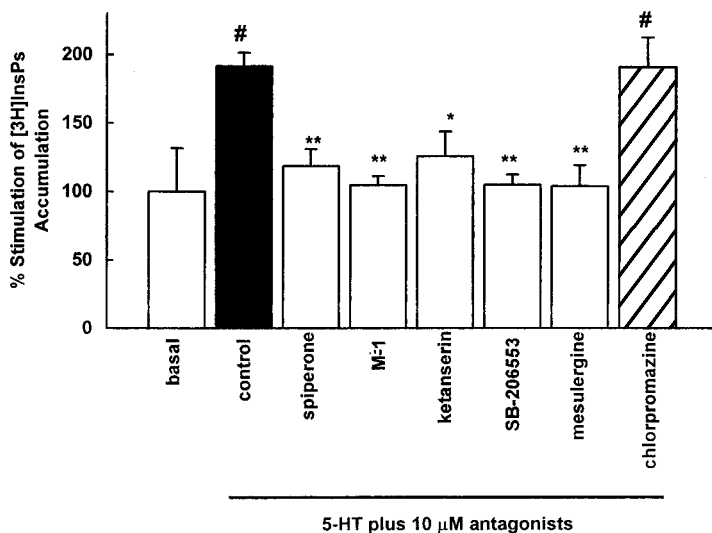


FIGURE 4. Effect of Serotonergic and Dopaminergic Antagonists on 5-HT Stimulated [³H]InsPs Accumulation. Data are shown as percentage of the basal (mean ± SE, n = 5). #Significant increase over basal (p < 0.05). *Significant decrease from 5-HT control (p < 0.05). **Significant decrease from 5-HT control (p < 0.01) by one-way ANOVA.

Other pharmacological classes have also been shown to affect aqueous humor dynamics, but are not used in the clinical management of patients with glaucoma. These are the α_1 -AR antagonists (22,23), 5-HT_{1A} receptor agonists (24,25), and 5-HT₂ receptor antagonists (13,26). It is not clear how the α_1 -ARs antagonists lower IOP since there is evidence for both decreased inflow (27) and increased outflow (28). The 5-HT_{1A} receptor agonists have been shown to lower IOP in rabbits (24) and to increase aqueous outflow, but not change IOP in monkeys (24). The 5-HT_{1A} receptors have been detected by radioligand binding (11), and by in situ hybridization and by reverse transcriptase polymerase chain reaction (rtPCR) in the rabbit eye (29). The biochemical and pharmacological evidence for the localization for the 5-HT₂ receptors is not as clear.

With the widespread use of drugs that affect the 5-HT receptors and transporters (4) for cardiac (30), psychiatric (31), and chemotherapeutic (32) indications, it is important to examine the role of indolamines in aqueous humor dynamics. Fluoxetine, an antidepressant which inhibits 5-HT uptake, increases IOP in rabbits, and the effect is attenuated by ketanserin, a selective 5-HT₂ receptor antagonist (33). Osborne et al have reviewed the evidence for the development of 5-HT_{1A} agonists to lower IOP in rabbits and humans, and to attenuate retinal damage in the ischemia-reperfusion of the rat eye (34). In order to determine the effect of 5-HT and related agonists and antagonists on aqueous humor flow, fluorophotometry would be required. The evidence to explain the effects of 5-HT on 5-HT₂ receptors on aqueous humor dynamics is controversial. Using [³H]5-HT, radioligand binding in iris-ciliary body membranes from rabbits showed the presence of 5-HT_{1A} binding sites and no 5-HT₂ binding sites, with the acknowledgement that low expression levels may account for this binding data (11). More sensitive detection methods, such as rtPCR, have not been performed in the various 5-HT₂ receptor subtypes on discrete ocular tissues.

In summary, we have demonstrated that 5-HT₂ receptors are coupled to phosphoinositide hydrolysis in bovine ciliary epithelial explants. Based upon the use of selective antagonists, it appears that the 5-HT_{2A/2C} receptor subtypes mediate the effect of 5-HT. Our findings also demonstrate the suitability of using isolated ciliary epithelial explants to study the biochemical mechanism of action of G protein-coupled receptors identified in the bovine ciliary epithelium (17,35). The regulation of

aqueous humor secretion at the level of the ciliary epithelium is complex, and our findings suggest that the 5-HT₂ receptors are involved in addition to the β -ARs and α_2 -ARs.

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