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A 5-HT_{1A}-like receptor is involved in the regulation of the embryonic rotation of *Lymnaea stagnalis* L.

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ABSTRACT

Cilia driven rotation of the pond snail *Lymnaea stagnalis* embryos is regulated by serotonin (5-HT). In the present study, physiological and biochemical assays were used to identify the 5-HT receptor type involved in rotation. The 5-HTergic agonists applied stimulated the rotation by 180–400% and their rank order potency was as follows: LSD>5-HT>8-OH-DPAT>WB4101≫5-CT. The applied antagonists, spiperone, propranalol and mianserin inhibited the 5-HT or 8-OH-DPAT stimulated rotation of the embryos by 50–70%. ^{3}H-5-HT was bound specifically to the washed pellet of the embryo homogenates. The specific binding of ^{3}H-5-HT was saturable and showed a single, high affinity binding site with K_d 7.36 nM and B_{max} 221 fmol/mg pellet values. This is the first report demonstrating the high affinity binding of ^{3}H-5-HT to the native receptor in molluscs. All of the pharmacons that stimulated the rotation or inhibited the 5-HT or 8-OH-DPAT evoked stimulation displaced effectively the binding of ^{3}H-5-HT. 5-HT resulted in the inhibition of forskolin stimulated cAMP accumulation, showing that 5-HT is negatively coupled to adenylate cyclase. Our results suggest that in the 5-HTergic regulation of the embryonic rotation in *L. stagnalis* a 5-HT_{1A}-like receptor of the vertebrate type is involved.

1. Introduction

5-HT is present in significant concentration in different tissues of gastropods, including the pond snail *Lymnaea stagnalis* L. (Kemenes et al., 1989, Hetherington et al., 1994, Croll et al., 1999), and plays a regulatory role in feeding (Kemenes et al., 1990, Yeoman et al., 1996, Elliott and Vehovszky 2000, Hernadi et al., 2004, Patel et al., 2005), locomotion (Syed and Winlow, 1991), axonal regeneration (Koert et al., 2001) and reproduction (de Lange et al. 1998). The regulatory role of 5-HT in the embryonic development and behavior was also demonstrated both in the planorbid *Helisoma trivolis* (Diefenbach, et al., 1991, 1995, Goldberg et al., 1994,) and in the lymnaeid *L. stagnalis* (Voronezhskaya et al., 2004, Filla et al., 2009). However, in most cases the types of the 5-HT receptors that might be involved in these funtions are not known. In the central nervous system (CNS) of snails, native 5-HT receptors identical with any of those identified in vertebrates have not yet been demonstrated. In the adult CNS of *Helix pomatia* (Drummond et al., 1980), *Aplysia californica* (Kadan and Hartig, 1988) and *L. stagnalis* (Hiripi and Elekes, unpublished results) the native 5-HT receptor was shown to bind 5-HT with low affinity and the receptor was positively coupled to the second messenger system. The cloned 5-HT receptor in the CNS of *L. stagnalis*, 5-HT_{lym}, bound 5-HT also with low affinity but displayed some pharmacological properties that were attributed to a 5-HT_1-like receptor (Sugamori et al., 1993). 5-HT_{ap1} receptor was cloned from the kidney and CNS of *A. californica* (Angers et al., 1998), and the receptor stucture and its biochemical properties described were also closely related to those of the vertebrate 5-HT_1 receptor. On the basis of the studies on both the ligand binding and the 5-HT stimulated adenylyl cyclase, the native receptor in different peripheral tissues of adult *L. stagnalis* (Balogh, Hiripi, Filla, Voronezhskaya, Elekes, unpublished results) and *H. pomatia* (Hiripi, Pirger, Kiss, Elekes, unpublished results) proved to be identical with the vertebrate 5-HT_6 type receptor.

Cilia driven rotation of the early embryos of pond snails (reviewed by Goldberg et al., 2008, and Byrne et al., 2009) and other molluscan species (Beiras and Widdows, 1995, Braubach et al., 2006) is a crucial locomotory behavior. In both *H. trivolis* (Diefenbach et al., 1991; Goldberg et al., 1994) and *L. stagnalis* (Voronezhskaya et al., 2004; Filla et al., 2009) embryos 5-HT was shown to participate in the regulation of the cilia driven behavior. In *H. trivolis*, a consistent ciliary expression of a 5-HT_1-like, receptor (5-HT_{1Hel}) was demonstrated (Doran et al., 2004). Our preliminary pharmacological experiments also suggested that a 5-HT_1-like receptor is present in *L. stagnalis* embryos (Filla et al., 2009).

In order to further characterize and so identify the 5-HT receptor, we studied the effect of 5-HT agonists and antagonists on the rotation of *L. stagnalis* embryos, the kinetic and pharmacological properties of [^{3}H]-5-HT binding to the embryonic membrane pellet, and the effect of 5-HT on the forskolin stimulated adenylyl cyclase in the embryonic membrane pellet.

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2. Materials and methods

2.1. Animals

Egg masses were collected from our laboratory population of *L. stagnalis* maintained at 25 °C with a 12:12 h ligh/dark cycle in aquaria supplied with Lake Balaton water and fed on lettuce. The embryonic development was staged according to Mescheriakow (1990) and Raven (1996). The experiments were carried out on the isolated eggs containing individual embryos during June, July and August.

2.2. Compounds

Serotonin, 5-hydroxytryptamine creatinine sulphate complex (5-HT), 5-carboxamidotryptamine maleate (5-CT), (±)-8-hydroxy-2-(di-N-propyl-amino) tetralin hydrobromide (8-OH-DPAT), D-lysergic acid diethylamide tartarate (LSD), WB-4101 hydrochloride (WB-4101), clozapine, spiperone–HCl, mianserin–HCl, propranalol–HCl, forskolin, adenosine 3′,5′-cyclic monophosphate (cAMP), adenosine triphosphate (ATP), 3-isobutylmethylxantine, guanosine 5′-triphosphate (GTP), ditiothreitol, bovine serum albumin, ethylenediaminetetraacetic acid (EDTA), ethyleneglycol-bis(2-aminoethylether)N,N,N′,N′-tetraacetic acid (EGTA), creatine phosphate, and creatine phosphokinase, were obtained from Sigma-Aldrich. 5-Hydroxy[^{3}H]-tryptamine trifluoroacetate, ([^{3}H]-5-HT, 4.44 TBq/mmol and cyclic AMP assay kits were purchased from GE Healthcare UK Limited.

2.3. Pharmacological manipulation of embryonic rotation

Embryonic rotation was monitored under a stereomicroscope at E30–40% stage. The counting of the rotation was carried out in three parallels and the experiments were repeated three times ($N=9$). Isolated egg capsules containing single embryos were separated from egg masses and maintained in filtered Balaton water (BW) at 25 °C. Individual embryos were placed each into a small chamber containing 200 µL BW for 10 min at 25 °C temperature, then the rotation of the embryos was counted for 2 min (control). Only embryos which rotated three times during the 2 min period were used for further experiments. BW was then changed for 200 µL solution containing an agonist in 10^{-9}–10^{-3} M concentration diluted in BW. The incubation was continued for an additional 10 min, then the rotation of the embryos was counted for 2 min again. Agonists applied were the following: 5-HT, 8-OH-DPAT, LSD, WB4101, and 5-CT. Since the basal rotation rate was low, the inhibitory effect of the antagonists, clozapine, spiperone, mianserin, and propranalol was investigated on a 5-HT or 8-OH-DPAT stimulated rotation in a concentration of 10^{-4} M. The embryos were incubated for 20 min in 200 µL of 100 µM solution of an antagonist, then 100 µM 5-HT or 100 µM 8-OH-DPAT was added to the antagonist containing solution and the incubation was continued for an additional 10 min, and by the end of the incubation the rotation was counted for 2 min. In the control experiments of the antagonistic effects, the incubation mixture contained only 100 µM 5-HT or 100 µM 8-OH-DPAT. The experiments were carried out in three parallels and repeated three times.

2.4. Measurement of adenylyl cyclase activity

Thousand embryos of E30–40% development were prepared and homogenized in a glas–Teflon homogenizer containing 20 vol. of 50 mM Tris–HCl buffer (pH 7.4) and 0.5 mM EDTA and then centrifuged 15000 *g* for 20 min in order to obtain pellets. Pellets were thereafter washed three times by resuspension and centrifugation in the same volume of buffer. The final membrane pellet wet weight was measured and than resuspended in 5 vol. of the buffer and stored at −80 °C.

Adenylyl cyclase (EC: 4.6.1.1) activity was assayed by following the production of cyclic AMP. Assay medium (250 µL) contained 50 mM Tris–HCl, 50 µM forskolin, 10 µM GTP, 1.5 mM $MgCl_2$, 1 mM ditiothreitol, 0.5 mM EDTA, 0.2 mM EGTA, 0.5 mM 3-isobutyl-1-methylxantine, 1 mg/mL of bovine serum albumin, 0.5 mM ATP, 5 mM creatine phosphate, and 50 U/mL creatine phosphokinase. In a parallel experiment, the incubation mixture contained 100 µM 5-HT. Incubation was initiated by adding 50 µL of membrane suspension containing 2.5 mg of wet mass membrane pellet, and was continued for 20 min at 25 °C. Cyclic AMP formed during the incubation was measured by a competitive binding assay using an Amersham Kit. The experiments were carried out in parallels and repeated three times.

2.5. Measurement of [3H]-5-HT binding

Two-thousand embryos of E30–40% developmental stage were homogenized in 40 vol. of ice cold Tris–HCl buffer, 50 mM, pH 7.4 with Polytron PT 10. The homogenate was centrifuged four times at 30000 *g* for 30 min at 4 °C with intermediate resuspension. After the second centrifugation, the suspension was incubated at 25 °C for 20 min and, following the final centrifugation, the pellet wet weight was measured and then resuspended in cold 50 mM Tris buffer. For saturation experiments 2 mg membrane pellet was incubated with increasing concentrations of [^{3}H]-5-HT trifluoroacetate (0.25–10 nM) for 20 min at 25 °C. Competition binding assay was done in the presence of increasing concentrations (10^{-8}–10^{-4} M) of different competition agents and 5.0 nM [^{3}H]-5-HT trifluoroacetate. All assays were terminated by rapid filtration over Whatmann GF/C filters with three times rinse with 5 mL ice cold buffer. Non-specific binding was defined with 10 µM 5-HT creatinine sulphate. The filters were extracted overnight in 10 mL toluene based liquid scintillator and the radioactivity measured by scintillation spectrometry. Saturation and competition experiments were performed parallel and repeated four ($N=8$) and three ($N=6$) times, respectively. The results of kinetic and competition experiments were evaluated using the Grafit program (Leatherbarrow, 1992).

2.6. Statistical methods

Significance was determined using a one-way ANOVA followed by a Tukey HSD test. *p*-values less than 0.05 were considered statistically significant.

3. Results and discussion

The investigated agonists all stimulated the rotation of the embryos (Fig.1). The 5-HT and the 5-HT_{1A} receptor agonists 8-OH-DPAT and WB4101 caused 50% stimulation at a concentration of 3.1, 10 and 20 µM (EC_{50}), respectively. The maximal stimulation of 5-HT, 8-OH-DPAT and WB4101 were 200, 400 and 180 %, respectively, meanwhile the most effective agonist proved to be LSD ($EC_{50}=0.2$ µM) causing a maximum of 350% stimulation. In the CNS of *L. stagnalis* (Hiripi and Elekes, unpublished results) and *H. pomatia* (Drummond et al., 1980) LSD was shown to excert both agonistic and antagonistic effects on adenylyl cyclase activity. However, LSD had only an agonistic effect on the rotation of the *L. stagnalis* embryos. 5-CT produced the least potent agonistic effect ($EC_{50}=400$ µM). The effect of the antagonists was investigated on both 100 µM 5-HT and 100 µM 8-OH-DPAT evoked stimulation. Spiperon, propranalol, mianserin and clozapine inhibited significantly (18–80%) both the 5-HT and the 8-OH-DPAT evoked increase of rotation (Fig.2). Clozapine which is more specific to 5-HT_7 than 5-HT_1 receptor had a lower affinity (18–40% inhibition) to the embryonic 5-HT receptor than spiperone, propranalol or mianserin.

Comparing the effect of pharmacons applied on the rotation of *L. stagnalis* and *H. trivolis* (Goldberg et al., 1994) embryos, there are both similarities and differences. Ergot alkaloids (LSD, in case of *L. stagnalis*, methysergide, in the case of *H. trivolis*) acted in an agonistic way, similarly to 5-HT. However, the selective 5-HT_{1A} receptor agonist 8-