MOLECULAR AND FUNCTIONAL EVIDENCE FOR EXPRESSION OF ADENOSINE RECEPTORS IN ZEBRAFISH, *DANIO RERIO*

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Adenosine is a multifunctional nucleoside that in mammals acts as an endogenous antiarrhythmic (Olah, M.E. and Stiles, G.L., Ann. Rev. Pharmacol. Toxicol. 35:581-606, 1995) and cardioprotectant agent in the heart (Yang, B.C. and Mehta, J.L., J. Cardiovasc. Pharmacol. 24:779-785, 1994), as a neuroprotectant in the brain (Heurteaux et al., Proc. Nat. Acad. Sci. U.S.A., 92:4666-4670, 1995), and as an anti-inflammatory autocoid throughout the body (Cronstein, B.N., Invest. Med. 43:50-57, 1995). Most of the actions of adenosine result from binding to four different G-coupled membrane receptors: A1, A2a, A2b and A3 receptors. As compared to mammals, relatively little is known about the adenosine system in fish. In the Antarctic fish *Pagothenia borchgrevinki*, adenosine as well as the adenosine A1 receptor agonist N-6-cyclopentyladenosine (CPA) were found to cause a marked reduction in heart rate, with CPA being more potent than adenosine. The bradycardia was blocked by aminophylline or by the adenosine A1 receptor antagonist 8-cyclopentyltheophylline indicating that this response was mediated by adenosine A1 receptors (Sundin, L., J. Exp. Biol. 202:2259-2267, 1999). In the rectal gland of the dogfish shark *Squalus acanthias* adenosine acts as an endogenous feedback inhibitor of the stimulation of chloride secretion caused by secretagogues such as VIP (Forrest, J.N., Kidney Int. 49:1557-1562, 1996). In teleost fish adenosine has been found to influence photoreceptor metabolism and function in the retina by a receptor with an adenosine A2-like specificity (Rey, H.L., and Burnside, B., J. Neurochem. 72:2345-2355, 1999). The purpose of the present studies was: a) to identify an adenosine receptor-like gene in zebrafish *Danio rerio*; b) to study its expression in adult zebrafish; c) to assess a potential influence of endogenous adenosine on cardiac development of zebrafish embryos.

Searching the zebrafish EST database yielded a 529 bp cDNA clone that showed a 68% homology with the human adenosine A2a receptor. With extension and further sequencing we established a nucleotide sequence of about 1.2 kb with about half of the sequence representing the 3' segment of the coding sequence and the other half representing the complete 3'-UTR. A comparison of the entire obtained nucleotide sequence with human adenosine receptor subtypes revealed an identity for A1 of 49%, for A2a of 56%, for A2b of 52%, and for A3 of 47%. Comparing the obtained coding sequence with human adenosine receptor subtypes showed an identity for A1 of 58%, for A2a of 69%, for A2b of 59%, and for A3 of 55%. The reported sizes for human adenosine receptor subtypes are 326 amino acids (AA) for A1, 412 AA for A2a, 332 AA for A2b, and 318 AA for A3. Our partial coding sequence encodes a protein of 189 AA which at the protein level showed an identity and similarity with human adenosine receptor subtypes of 48 and 63% for A1, of 71 and 83% for A2a, of 53 and 68% for A2b, and of 41 and 60% for A3. Expression of this adenosine receptor-like gene in zebrafish was assessed by RT-PCR in total RNA from different zebrafish tissues. PCR product was confirmed by size and by restriction mapping. Gene expression levels were normalized for zebrafish ß-actin PCR product. The following primers were employed: sense 5'-TGTGAACTCAAGTCCCGCTC-3'; antisense 5'-GGAAAGTGTGTCTGAACTCTCG-3'. The RT-PCR results showed that the adenosine-

receptor-like gene was expressed to a similar extent in the liver, kidney, heart, brain, eye, muscle, and gill.

To evaluate cardiac development, fresh zebrafish eggs were harvested and dechorionated with protease (1 mg/ml). The eggs were divided into three groups and incubated in the respective media at 28°C. Group 1: vehicle (0.5% DMSO in methylene blue egg water; methylene blue egg water was made by adding 0.06 g Instant Ocean and 2 ml methylene blue stock (1 g/l) to 1 liter of distilled water); groups 2 and 3: 0.5% DMSO in methylene blue egg water plus the adenosine A1-receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) at a final concentration of 10^{-5} M and 10^{-4} M, respectively. Eggs were incubated in petri dishes that had been covered on the bottoms with 3% agarose to prevent adhesion of the eggs or embryos. Heart rates of the developing embryos were determined at 25, 32, 48, and 75 hr of incubation with the examiner being blinded for the respective groups. In untreated control eggs (n=12) as well as in the eggs treated with DPCPX at 10^{-4} M (n=14) or 10^{-5} M (n=8) the appearance of a heart beat occurred between 25 and 32 hrs. Initial heart rates were significantly lower in control than in treated embryos (84 ± 1 min^{-1} vs. 91 ± 3 and 92 ± 3 min^{-1} at 10^{-5} and 10^{-4} M DPCPX; $p < 0.05$). In all groups, the heart rate increased with time and reached a plateau between 48 and 72 hrs. At the 48 hour and 72 hour timepoints, heart rates were significantly higher in the embryos treated with either 10^{-5} or 10^{-4} M DPCPX than in the vehicle treated group (beats/min: 138 ± 2 or 140 ± 2 vs. 122 ± 1.9 at 48 hrs; 138 ± 1.8 or 140 ± 1.5 vs. 129 ± 1.5 at 75 hrs, all $p<0.05$). These findings indicate that endogenous adenosine decreases the heart rate in the developing zebrafish embryo.

We report the partial cloning of an adenosine receptor-like gene in zebrafish that has a high similarity to human adenosine A2a receptor. The gene was expressed ubiquitously in all tested tissues. Studies in the developing zebrafish embryo suggest that adenosine is generated endogenously and that it acts to slow the heart rate without affecting the time of onset of pacemaker activity. Since adenosine-mediated bradycardia is most consistent with an adenosine A1 receptor-mediated action we assume that this effect is not mediated by cardiac expression of the zebrafish adenosine receptor that was partially cloned in these studies. Thus, we speculate that teleosts, like mammals, possess at least two membrane receptors for adenosine. Adenosine A1 receptors do not appear to be involved in cardiac development or impulse generation.

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