Retinal melatonin is metabolized within the eye of Xenopus laevis
(eyecup/aryl acylamidase/monoamine oxidase/methoxyindoles/circadian rhythm)

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ABSTRACT Retinal synthesis of melatonin, a potent modulator of rhythmic retinal processes, is elevated at night as a result of regulation by a circadian clock. Despite high nocturnal synthetic capacity, both melatonin content and release are low in the retina of the frog Xenopus laevis. We report here that cultured eyecups from Xenopus have the capacity for rapid metabolic breakdown of melatonin. Pharmacological analysis indicates that the initial step in this degradation pathway is deacetylation of melatonin by the enzyme ary1 acylamidase (aryl-acylamide amidohydrolase, EC 3.5.1.13). This produces 5-methoxytryptamine, which is then deaminated by monoamine oxidase [amine:oxygen oxidoreductase (deaminating) (flavin-containing), EC 1.4.3.4], producing 5-methoxyindoleacetic acid and 5-methoxytryptophol. Inhibition of ary1 acylamidase with eserine dramatically increases the release of endogenous melatonin by eyecups cultured at night, indicating that this pathway is the normal fate of retinal melatonin. Metabolism within the eye suggests a local neuromodulatory role for retinal melatonin, in contrast to the hormonal role of pineal melatonin.

Melatonin (N-acetyl-5-methoxytryptamine) appears to play a major role in circadian regulation of retinal physiology (1). It mimics darkness in its effects on rod photoreceptor disc shedding (2), core myoid length (3), movement of pigment granules in pigment epithelium (4-6), and retinal dopamine release (7). Melatonin synthesis in the retina, as in the pineal gland, is elevated at night as a result of regulation by light and a circadian oscillator (1, 8-13). Melatonin may therefore be a circadian signal for light in the retina.

Pineal melatonin content and release into the circulation appear to reflect directly its synthesis (8, 9). However, evidence from several species suggests that other unidentified mechanism(s) may be involved in the regulation of retinal melatonin (14, 15). For example, in chicken retina, the activity of serotonin N-acetyltransferase, the penultimate enzyme in the synthesis of melatonin, and melatonin content are both high at night, but very little retinal melatonin enters the bloodstream (14). Despite high nocturnal serotonin N-acetyltransferase activity in the retina of Xenopus (12, 13), we found in preliminary experiments that retinal melatonin content and release of melatonin by cultured eyecups were both surprisingly low (<10 pg per retina and <10 pg/hr, respectively). One possible explanation for these results is that retinal melatonin is degraded within the eye. We have used a cultured Xenopus eyecup preparation to examine this possibility.

MATERIALS AND METHODS

Eyecup Culture. Postmetamorphic juvenile Xenopus laevis were maintained in a cycle of 12 hr light/12 hr dark for at least 4 weeks before use in experiments. Eyecups, including the retina, pigment epithelium, choroid, and sclera, were prepared and cultured individually in 1 ml of defined culture medium as described (16). For experiments in which the metabolism of exogenous melatonin was measured, eyecups were prepared in the light just before the time of dark onset and cultured for 3 hr in the dark in medium that included either 8 μCi of [methoxy-3H]melatonin (DuPont/NEN) per ml (1 Ci = 37 GBq) (100 nM) or 1 μM melatonin (Sigma). For studies of endogenous melatonin release, eyecups were prepared and cultured for 4 hr at different times of day in the light or darkness. Dissections during the dark period were performed under infrared light with the aid of infrared viewers (FJW Industries, Mount Prospect, IL).

High-Performance Liquid Chromatography (HPLC). Tissue extracts were made by sonicating the combined retina, pigment epithelium, and choroid from each eyecup in 150 μl of methanol. A sample of this suspension was reserved for determination of protein content by the method of Lowry et al. (17). After centrifugation, 100 μl of supernatant was added to 200 μl of 0.05 M ammonium acetate (pH 4.25). Samples (100 μl) of culture medium and tissue extracts were analyzed by reverse-phase HPLC. A Rainin Microsorb 5-μm C18 column (150 × 4.6 mm) was used. The standard mobile phase was 33% methanol in 0.05 M ammonium acetate (pH 4.25) with a flow rate of 1 ml/min. To identify unknown peaks, standard compounds were added to the samples and chromatographic conditions were altered by varying the methanol concentration in the range of 20–40%, and by substitution of 0.1 M sodium acetate for ammonium acetate. Fluorescence was measured with a Laboratory Data Control Milton Roy (Riviera Beach, FL) Fluoromonitor III with a Hg lamp, 245-nm excitation filter, and 300- to 400-nm emission filter. For radiotracer experiments, fractions of 0.3 ml were collected and 3H activity was determined by liquid scintillation spectrometry. Counts per minute (cpm) are not corrected for counting efficiency, which was essentially constant.

Melatonin RIA. The melatonin content of unextracted samples (50–200 μl) of culture medium was determined by the RIA method of Rolfag and Nisswender (18) as modified by Takahashi et al. (19). The 125I-labeled melatonin analogue for the assay was obtained from Hazleton Biotechnologies (Vienna, VA). Melatonin concentrations >5 pg/ml were quantifiable in this assay.

Validation of the RIA. Inhibition curves for various quantities of melatonin and pooled medium from nighttime eserine-treated eyecups were fitted to a 4-parameter logistic equation by a nonlinear least-squares curve fitting routine (20). The curves were parallel, with slopes of −0.914 ± 0.028 and −0.927 ± 0.067, respectively. Melatonin (2.5, 10, 25, 50, and 100 pg) added to samples of culture medium from eserine-treated day and night and control night eyecups was quantitatively recovered in the assay. The slopes of the recovery curves were 1.03, 1.07, and 0.92, respectively.

RESULTS

To determine whether ocular tissue has the capacity for metabolism of melatonin, we cultured Xenopus eyecups in...
defined medium containing 100 nM [methoxy-3H]melatonin. After a 3-hr incubation, samples of the culture medium and extracts of the combined retina, pigment epithelium, and choroid were separated by reverse-phase HPLC. Analysis of fractions revealed the production of three radioactive metabolites from the added melatonin (Fig. 1 A–C). In this experiment, the amount of [3H]melatonin in the medium was reduced 18%, from 687,000 cpm per 100 μl in control medium to 560,000 ± 47,500 cpm per 100 μl (mean ± SD; n = 4) during a 3-hr incubation. This reduction was entirely accounted for by the production of the three metabolites. The tissue concentration of [3H]melatonin at the end of this experiment was 0.24 ± 0.084 pmol per mg of protein, and metabolites were produced at an average rate of 16.9 ± 4.9 pmol per hr per mg of protein (means ± SD). Thus, under these conditions, the entire tissue content of [3H]melatonin was turned over in <1 min.

The HPLC retention times of melatonin and its metabolites are reduced slightly by the tritiated methoxy group, preventing direct identification of the radiolabeled metabolites by comparison with unlabeled standards. Therefore, eyecups were incubated in medium containing 1 μM unlabeled melatonin, and the culture medium was analyzed by HPLC with fluorescence detection. Three fluorescent compounds, similar to the radiolabeled metabolites in relative amounts and chromatographic behavior, were released by the eyecups into the culture medium (Fig. 1D). These compounds, with retention times of 8.2, 10.9, and 12.1 min under standard chromatographic conditions, were identified as 5-methoxytryptamine, 5-methoxyindoleacetic acid, and 5-methoxytryptophol, respectively. Under several different chromatographic conditions, these compounds were indistinguishable from standards. When eyecups were incubated without added melatonin, 5-methoxyindoleacetic acid was detectable at low levels by HPLC with fluorescence detection in culture medium; endogenous melatonin and the other metabolites were detectable only after extraction and concentration (data not shown).

A pathway that produces these metabolites from melatonin has previously been described as a minor route of melatonin metabolism in the liver (21). This pathway (Fig. 2) involves deacetylation of melatonin by aryl acylamidase (arylacylame amidohydrolase, EC 3.5.1.13) to produce 5-methoxytryptamine. Deamination of 5-methoxytryptamine by monoamine oxidase [amine:oxygen oxidoreductase (deaminating) (flavin-containing), EC 1.4.3.4] produces 5-methoxyindoleacetaldehyde, which is further oxidized to 5-methoxyindoleacetic acid or reduced to 5-methoxytryptophol. We used inhibitors of monoamine oxidase and aryl acylamidase to test the hypothesis that this is the pathway for melatonin metabolism in the eye. When pargyline, a monoamine oxidase inhibitor, was added to the culture medium at a concentration of 100 μM, the production of 5-methoxyindoleacetic acid and 5-methoxytryptophol from [3H]melatonin was blocked, and 5-methoxytryptamine accumulated in the tissue (Fig. 3A). This indicates that the initial step in the breakdown of melatonin is deacetylation to 5-methoxytryptamine, which is then deaminated by monoamine oxidase.

![Fig. 1. HPLC analysis of melatonin metabolites produced by cultured Xenopus eyecups. (A and B) When eyecups are incubated in medium containing [methoxy-3H]melatonin (Mel), three radiolabeled metabolites (arrowheads) are found in 100-μl samples of tissue extract (A) and culture medium (B). (C) Control medium incubated without eyecups. (D) When eyecups are incubated with 1 μM unlabeled melatonin, metabolites (arrowheads) are detectable in the culture medium by fluorescence. (E) Chromatogram of synthetic indole standards (1 ng). The metabolites cochromatograph with synthetic 5-methoxytryptamine (peak 4), 5-methoxyindoleacetic acid (peak 5), and 5-methoxytryptophol (peak 6). Other standard peaks shown are serotonin (peak 1), N-acetylserotonin (peak 2), 6-hydroxymelatonin (peak 3), and melatonin (peak 7).](image)

![Fig. 2. Proposed enzymatic pathway for ocular metabolism of melatonin. Aryl acylamidase and monoamine oxidase are inhibited by eserine and pargyline, respectively (21, 22).](image)
Aryl acylamidase, a deacetylase that appears to be related to acetylcholinesterase, can be inhibited by eserine (22). Eserine (100 μM) inhibited the production of all metabolites from [3H]melatonin and caused increased accumulation of melatonin in the tissue (Fig. 3B), consistent with a primary role for aryl acylamidase in the ocular metabolism of melatonin. Lower concentrations of eserine (1 and 10 μM) were ineffective.

To determine whether this metabolism is the normal fate of melatonin synthesized in the retina, we used eserine to block aryl acylamidase in eyecups cultured at different times of day and night in the absence of added melatonin. The endogenous melatonin released into the medium by individual eyecups was measured by radioimmunoassay (Fig. 4). At night, when serotonin N-acetyltransferase activity is high, eyecups treated with eserine released ≈7 times as much melatonin as controls. In all daytime groups, melatonin levels were near or below the level of accurate quantification by the RIA. Because eserine is expected to increase retinal acetylcholine levels by inhibiting acetylcholinesterase, other eyecups were cultured with carbachol, an agonist of acetylcholine receptors. Carbachol had no effect, indicating that the increased melatonin release was not the result of increased retinal acetylcholine levels.

**DISCUSSION**

We have shown that cultured *Xenopus* eyecups have the capacity for rapid metabolism of melatonin. The results of pharmacological experiments support the hypothesis that the
labor of cellular processes in photoreceptors (2, 3), the retinal pigment epithelium (4–6), and dopaminergic amacrine cells (7). An alternative function for this pathway may be in the synthesis of other biologically active methoxyindoles. 5-Methoxytryptophol has effects on disc shedding similar to those of melatonin (2), and 5-methoxytryptamine has been shown to affect serotonergic neurotransmission in the central nervous system (26). Synthesis of these compounds in the retina has been demonstrated previously, but it was assumed that they were made by methylation of serotonin (5-hydroxytryptamine) and deaminated serotonin metabolites through the action of hydroxindole-O-methyltransferase (27). Our results show that they can also be synthesized through the degradation of melatonin.

Previous attempts to demonstrate deacetylation of melatonin by aryl acylamidase in the brain and pineal gland have been unsuccessful (21, 23). In fact, no endogenous substrate has been found for the brain and pineal forms of this enzyme. The majority of circulating melatonin is cleared by conversion to 6-hydroxymelatonin in the liver; only 1–2% is deacetylated by liver aryl acylamidase (21, 28, 29). Thus, the eye may be unique in its use of this pathway as a major route of melatonin metabolism.

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