Pertussis toxin blocks melatonin-induced pigment aggregation in *Xenopus* dermal melanophores

Beatrix H. White, Ronald D. Sekura*, and Mark D. Rollag

Department of Anatomy, Uniformed Services, University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, Maryland 20814-4799, USA

* Laboratory of Developmental and Molecular Immunity, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20205, USA

Accepted November 1, 1986

Summary. The molecular mechanism of action for the pineal hormone melatonin was explored by testing melatonin interaction with the components of the hormone-sensitive adenylate cyclase complex in a *Xenopus* dermal melanophore bioassay. Forskolin was employed to stimulate melanosome dispersion. The ability of melatonin to reverse forskolin-stimulated pigment dispersion was assessed, as was the effect of pertussis toxin on the ability of melatonin to aggregate dispersed pigment.

Forskolin elicited dispersal of melanosomes in a dose dependent manner (EC$_{50}$ = 12 nM) in meninges from stage 52–56 tadpoles of *Xenopus laevis*. Maximal pigment dispersion was obtained with 100 nM forskolin. Melatonin reversed this effect of forskolin (EC$_{50}$ = 1.5 nM), causing pigment aggregation. Pertussis toxin blocked the melatonin-induced aggregation (EC$_{50}$ = 358 ng/ml). Prior treatment of the melanophore containing meningeal explants with pertussis toxin results in blockade of melatonin induced pigment aggregation. A 41 kDa pertussis toxin substrate is found in explant homogenates treated with $^{32}$P-NAD and pertussis toxin. The availability of this substrate is reduced by prior treatment of intact explants with pertussis toxin and depletion of melatonin responsiveness corresponds to depletion of the 41 kDa substrate. Together, these data suggest that melatonin action upon amphibian dermal melanosomes is mediated by a system requiring a protein similar to the regulatory protein Ni used by mammalian cells to mediate the action of hormones which inhibit adenylate cyclase through a cell surface receptor.

Abbreviations: MI melanophore index; MSH melanocyte stimulating hormone; FCS fetal calf serum

Introduction

Melatonin, an indole produced by the pineal gland from serotonin, serves to coordinate information about daylength with the timing of reproduction in seasonally breeding mammals (Tamarkin et al. 1985) and of locomotor and thermoregulatory behavioral rhythms in birds and reptiles (Binkley 1974; Gwinner and Benzinger 1978; Underwood 1981). Despite numerous demonstrations of an important role for melatonin in regulation of physiological rhythms, its target site and mechanism of action remain to be elucidated.

The following experiments were designed to define the mechanism of action of the pineal hormone melatonin. The dermal melanophore of *Xenopus laevis* was selected as a model target cell because this specific cell type is well-characterized in its response to melatonin (McCord and Allen 1917; Bagnara 1963; Ralph and Lynch 1970). Melatonin acts to aggregate melanosomes in dermal melanophores; consequently, melatonin induces a visible change in pigment distribution which can be used to assess the efficacy of stimulatory or inhibitory agents upon melatonin action.

Pigment dispersion in amphibian melanophores has been associated with increased levels of cAMP. Abe et al. (1969a, b) demonstrated that MSH (melanocyte stimulating hormone), disperses pigment in dorsal skin of *Rana pipiens* and increases cAMP within this tissue. Similar experiments by van de Veerdonk and Konijn (1970) demonstrated that dorsal skin from dark-adapted *Xenopus laevis* contains 2 to 4-fold the amount of cAMP as does blanched dorsal skin from white background-adapted toads. MSH-induced pigment dispersion can be mimicked by cAMP, dB-
cAMP, or forskolin (de Graan et al. 1984). Melatonin inhibits the MSH-induced increase in cAMP in dorsal thigh skin of *Rana pipiens* and causes pigment aggregation (Abe et al. 1969b). The specific interaction of melatonin with components of the adenylate cyclase regulatory system has not been described previous to this report.

Hormone-sensitive adenylate cyclase consists of three primary components: 1) hormone receptors, 2) guanine-nucleotide binding proteins, Ns and Ni, and 3) a catalytic protein (Rodbell 1980; Gilman 1984). The guanine nucleotide-binding regulatory proteins modulate the activity of the catalytic component, with Ns mediating the action of stimulatory hormones, and Ni mediating the action of hormones which inhibit adenylate cyclase (Cooper 1982). The receptor specifically binds to either a stimulatory or inhibitory hormone reversibly.

In order to establish whether melatonin acts on adenylate cyclase in meningeal dermal melanophores, pertussis toxin and forskolin were used. Forskolin, a plant diterpine obtained from the roots of *Coleus forskohlii*, directly activates the catalytic component of adenylate cyclase (Seamon et al. 1981). Pertussis toxin blocks the ability of inhibitory hormones to lower adenylate cyclase activity (Katada and Ui 1979, 1981; Hazeki and Ui 1981) by catalyzing the NAD-dependent ADP-ribosylation of the 41 kDa inhibitory guanine nucleotide-binding protein, Ni (Bokoch et al. 1983). ADP-ribosylation prevents Ni from coupling with the inhibitory receptor (Cote et al. 1984); thus, pertussis toxin prevents inhibitory hormones from reversing the action of substances which stimulate adenylate cyclase. Reported here are findings that pertussis toxin prevents melatonin from reversing forskolin-induced pigment dispersion in melanophores of meningeal explants from *Xenopus laevis* tadpoles. These findings provide a basis for understanding melatonin's mechanism of action.

**Materials and methods**

**Animals.** Larval *Xenopus laevis* frogs were raised from eggs (Nasco, Ft Atkinson, WI) in aerated tap water at 22–25 °C before their use at stages 52–56 (Normal Table of Nieuwkoop and Faber, 1956). They were fed “frog brittle” (Nasco, Ft Atkinson, WI) when water was changed biweekly.

**Dissection and culture of tissue.** Tadpoles were anesthetized by hypothermia (immersion in ice for 30 min). A tissue block consisting of the calvarium and underlying brain were excised using angled-side spring scissors and curved forceps. The brain and skin were removed, leaving the meningeal explants supported by a cartilaginous framework which was sufficiently flat to allow all the melanophores to be in one plane of focus when viewed through a dissecting microscope. The explants were maintained overnight in culture medium consisting of 47% L-15 without phenol red (Formula 78-5102, Gibco, Grand Island, NY), 40% distilled water, and 13% fetal calf serum (Gibco, Grand Island, NY) adjusted to 190 mOsm and pH 7.6. This culture medium is a modification of that described by Seldenrijk et al. (1979).

**Bioassay.** To test melanophore responses to drugs, explants were transferred to culture dishes (35 × 10 mm) containing L-15 medium lacking FCS (190 mOsm, pH 7.6) and allowed to incubate 1 h. Explants were subsequently transferred to media containing drugs as described below. Melatonin (Sigma, St Louis, MO) and forskolin (Calbiochem, San Diego, CA) stock solutions were dissolved in ethanol and control groups for these treatments received aliquots of the ethanol vehicle. The concentration of ethanol in the media (0.1% or less) did not affect pigment distribution or viability of the melanophores.

Meningeal melanophores were tested for their response to forskolin by transferring groups of 6 explants each to culture dishes with a given concentration of forskolin. The doses of forskolin tested were: 1 nM, 500 nM, 250 nM, 125 nM, 63 nM, 31 nM, 15 nM, 7.5 nM, 3.9 nM, 2 nM, 1 nM, 500 pM, 250 pM, and 0 (control with 0.1% ethanol). Photographs were taken of each explant before transferral to forskolin containing medium and after incubation in forskolin containing medium for 1 h. The degree of pigment expansion was determined from these photographs (see data analysis section).

The response to melatonin was assessed by first transferring explants to 3 ml FCS(−) culture medium containing 30 nM forskolin for 1 h. This was done to establish an initial dispersion of melanosomes so that melatonin's aggregating effect could be subsequently evaluated. An initial dilution of melatonin stock solution (1 ng/ml in 95% ethanol) was made with FCS(−) culture medium to obtain a concentration of 1 μg/ml. A 2-fold serial dilution was then made from this 1 μg/ml solution using FCS(−) culture medium as diluent. 30 μl aliquots from this dilution series were added after 1 h to dishes containing explants in 50 nM forskolin to establish the following final concentrations of melatonin: 10 ng/ml, 5 ng/ml, 2.5 ng/ml, 1.25 ng/ml, 630 pg/ml, 310 pg/ml, 160 pg/ml, 80 pg/ml, 40 pg/ml, 20 pg/ml, 10 pg/ml, 0 (control with 50 nM forskolin). Explants were exposed to these melatonin containing solutions for 30 min. Photographs for subsequent data analysis were obtained before forskolin pretreatment, after the one-hour forskolin pretreatment, and after the 30-min melatonin treatment.

The action of pertussis toxin was assessed by incubation of meningeal explants for 18 h in 500 μl FCS(−) culture medium containing the following concentrations of pertussis toxin: 5 μg/ml, 2.5 μg/ml, 1.25 μg/ml, 630 ng/ml, 315 ng/ml, 156 ng/ml, 78 ng/ml, 40 ng/ml, 20 ng/ml, 10 ng/ml, 5 ng/ml and 0 (control). Twenty-five μl of a 1 μM forskolin solution in FCS(−) medium was then added to each well and the tissue was exposed to this 50 nM forskolin solution in order to disperse pigment. After 1 h, 2.5 μl of a 1 μg/ml melatonin solution in FCS(−) culture medium was added to each well and 30 min later photographs were obtained so that the response to melatonin could be evaluated. Photographs were also obtained before and after the 1 h forskolin pretreatment.

**Data analysis.** Each melanophore within an individual explant was scored for degree of pigment dispersion from the photographic records. Melanophore expansion was rated visually, using the melanophore index (MI) of Hogben and Slome (1931). The investigator performing the scoring did not know the treatment to which a particular explant had been exposed. The overall melanophore index for the population of cells (explants usu-