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A Novel Phototransduction Pathway in the Pineal Gland and Retina

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Light is a major environmental signal for entrainment of the circadian clock, but little is known about the phototransduction pathway triggered by light-activation of photoreceptive molecule(s) responsible for the phase shift of the clock in vertebrates. The chicken pineal gland and retina contain the autonomous circadian oscillators together with the photic entrainment pathway, and hence they provide useful experimental model for the clock system. We previously demonstrated the expression and light-dependent activation of rod-type transducin α-subunit ($Gt_1\alpha$) in the chicken pineal gland. It is unlikely, however, that the pineal $Gt_1\alpha$ plays a major role in the photic entrainment, because the light-induced phase shift is unaffected by bloking the signaling function of $Gt_1\alpha$. Here, we show the expression of G11α, an α-subunit of another heterotrimeric G-protein, in the chicken pineal gland and retina by cDNA cloning, Northern blot and Western blot analyses. G11α-immunoreactivity was colocalized with pinopsin in the chicken pineal cells and it was found predominantly at the outer segments of photoreceptor cells in the retinal sections, suggesting functional coupling of G11α with opsins in the both the tissues. By coimmunoprecipitation experiments using the retina, we showed the light- and GTP-dependent interaction between rhodopsin and G11α. Upon ectopic expression of a Gq/11-coupled receptor in cultured pineal cells, pharmacological (non-photic) activation of endogenous G11 induced phase-dependent phase shifts of the melatonin rhythm in a manner very similar to the effect of light. These results suggested opsin-G11 pathway contributing to the photic entrainment of the circadian clock.

Key words: pineal gland, retina, circadian clock, G-protein, phototransduction, circadian photoentrainment

INTRODUCTION

Daily rhythms in biochemistry, physiology, and behavior of living organisms are driven by endogenous oscillators called circadian clocks. The circadian clock oscillates with a period of approximately 24 hours under constant conditions, and it is entrained to the 24-hour cycle of a change in the ambient conditions, for example, the solar light-dark cycle. The light-dependent phase control represents one of the most important properties of the biological clock, but little is known about the phototransduction mechanism responsible for photic entrainment in vertebrates. Among vertebrate clock-containing cells, light-sensitive chicken pineal cell is a prominent model for studying the photic entrainment mechanism, since the photo-entrainable clock machineries reside in individual cells and well maintained in dispersed cell culture [1]. In this system, the endogenous photoreceptive molecule pinopsin [2] has been postulated to mediate the phase-shifting effect of light via a G-protein signaling pathway. We previously demonstrated that rod-type transducin α-subunit ($Gt_1\alpha$) is expressed in the chicken pineal gland and that it is activated by light [3]. It is unlikely, however, that pineal $Gt_1\alpha$ plays a major role in photic entrainment, because the light-induced phase shift is unaffected by treatment of the pineal cells with pertussis toxin (PTX) [4], which blocks signaling of $Gt_1\alpha$ by ADP-ribosylation. Here we describe a novel phototransduction pathway mediated by a PTX-insensitive G-protein, G11, and discuss the biological significance of this signal transduction pathway.

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MATERIALS AND METHODS

Pineal cell culture and drug administration for transfection assay.

Pineal glands were isolated from one-day-old chicks. The dispersed cells were cultured in 24-well cloning plates (~6×10^6 cells/well; Greiner Labortechnik) at 39.5 + 0.2 °C under 95% air/5% CO_2 in the light-dark cycle (12 hour:12 hour) using Medium 199 (Invitrogen)

supplemented with 10 mM HEPES-NaOH (pH 7.4), 2.2 mg/ml $NaHCO_3$, 100 U/ml penicillin, 100 μg/ml streptomycin, 250 μg/ml Fungizone, 2.5 μM arabinosylcytosine, and 10% fetal bovine serum. Cultured cells were transfected with pREP9 expression vector (Invitrogen) harboring porcine cDNA for m1 or m2 mAChR (muscarinic acetylcholine receptors) or with a control (empty) vector. The transfected cells were selected in medium containing 200 μg/ml G418 for 4 days (day 5-8) and then cultured in medium containing 50 μg/ml G418 and 20 μM atropine until the end of the experiment. On day 10, the cells were transferred to constant darkness and treated with atropine-free medium containing an agonist of mAChRs, carbamylcholine (CCh; 100 μM) for 4 hours at the time period when light induces the phase-advance or phase-delay. The medium was collected and replaced at 4-hour intervals and the amount of secreted melatonin was measured using HPLC.

RESULTS AND DISCUSSION

To explore the possibility of a pinopsin-mediated pathway for photic entrainment, we first investigated whether specific G-protein(s) colocalize with pinopsin. We found that pinopsin-positive membrane structures of the pineal cells were immunolabeled with both anti-$Gt_1\alpha$ antibody [3] and anti-Gq/11α antibody that recognizes mammalian Gqα and G11α [5, 6]. In Western blot analysis of the chicken pineal and retinal homogenates, the anti-Gq/11α antibody detected a single band (42 kDa) with mobility identical to that of a band recognized by G11α-specific antibody [6]. The entire coding sequence of pineal G11α was determined by 5'- and 3'-RACE [6]. According to the deduced amino acid sequence, the Cys residue susceptible to ADP-ribosylatation by PTX is absent, indicating that the protein is PTX-insensitive [6]. Altogether, these observations suggest a role for G11α in light-signaling processes, possibly as a mediator for the phase-shifting effect of light in the pineal gland and retina.

We then addressed the question of whether or not G11 signaling inputs to the circadian oscillator in cultured chicken pineal cells (Fig. 1). In order to activate G11 without stimulating the other (redundant) photic pathway(s), we adopted transient expression of a Gq/11-coupled receptor, m1 muscarinic acetylcholine receptor (m1 mAChR), the activity of which can be controlled pharmacologically in the dark. A primary

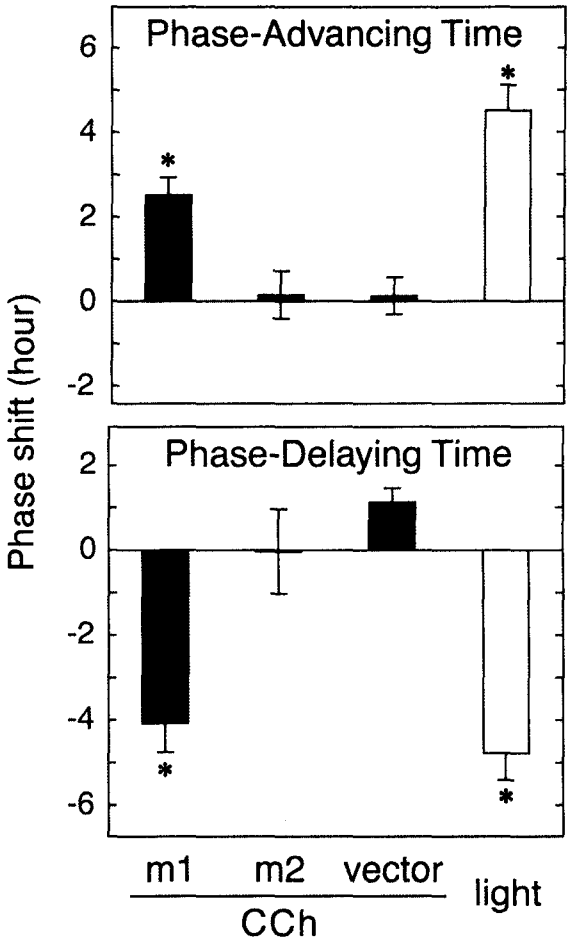

Fig. 1 Carbamycholine or light induces phase-shifts of the melatonin rhythm of chicken pineal cells transfected with m1 or m2 mAChR. A primary culture of pineal cells was transfected with pREP9 vector harboring cDNA for m1 or m2 mAChR or with a control vector, and the transfected cells were treated in the dark with carbamylcholine (CCh; 100 μM) for 4 hours at the time period when light induces the phase-advance (upper panel) or phase-delay (lower panel). The phase shifts induced by the CCh-treatment or by light were calculated and plotted (± SEM) as negative and positive values, respectively. * A significant phase shift relative to that observed in the cells transfected with a control vector ($p < 0.025$; one-tailed Student's t-test). A similar result was obtained in the other experiment using two independent wells of the cell culture.

culture of pineal cells was transfected with an expression plasmid containing cDNA for m1 mAChR or for m2 mAChR (as a control for the Gi/Go-coupled receptor), and transfected cells were selected by incubation in the presence of G418. After entrainment of the transfected cells to the light-dark cycle, cells were transferred to constant darkness and treated for 4 hours with 100 μM CCh. In the m1 mAChR-expressing cells, the transient CCh-treatment induced a clear phase shift of the melatonin rhythm. The direction of the phase shift, either delay or advance, was dependent on the phase of the CCh-treatment, and, importantly, the CCh-induced phase-dependent phase shift (Fig. 1, m1) was very similar to that elicited by 4-hour exposure to light (Fig. 1, light). In sharp contrast, the CCh-treatment had little or no effect on the melatonin rhythm of m2 mAChR-expressing cells (Fig. 1, m2) and of control vector-transfected cells (Fig. 1, vector). We conclude that the phase-shifting effect of light on the pineal circadian oscillator was mimicked by selective activation of Gq/11-coupled receptor, most probably via endogenous G11, but not by activation of Gi/Go-coupled receptors.

Since the establishment of the central role of cGMP as an intracellular messenger in the vertebrate visual transduction pathway, no definitive role has been assigned to light-induced retinal PI turnover that is thought to involve G11α and PLC-β4, both of which were found in bovine retinal photoreceptor cells [7]. Based on the present results, together with structural and functional similarities between the photosensitive retinal and pineal cells, we speculate that photic entrainment of the circadian clock in the two cells is triggered by an opsin-G11 pathway accompanying PI turnover.

The novel opsin-G11 pathway in vertebrate photoreceptive cells is analogous to the early steps in fruitfly visual transduction, i.e., rhodopsin-DGQα (Gq/11α homolog)-NorpA (PLC-β4 homolog). In this species, the rhodopsin-DGQα-NorpA phototransduction pathway also plays a role in entrainment in a redundant manner together with pathways present in the extra-retinal tissues. The redundancy in the vertebrate photic entrainment system has also been suggested by several loss-of-function studies. Now it would be waited to reevaluate negative results in loss-of-function studies on the photic entrainment system such as those observed with inhibitors and mutant mice.

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