Stimulation of cAMP accumulation by the cloned *Xenopus* melatonin receptor through G_i and G_z proteins

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Abstract **The *Xenopus* melatonin receptor was expressed in human embryonic kidney 293 cells and assayed for cAMP accumulation. In transfected 293 cells expressing the melatonin receptor, melatonin dose-dependently inhibited the endogenous adenylyl cyclases. In contrast, melatonin stimulated the accumulation of cAMP in cells co-expressing the type II adenylyl cyclase. Both the inhibitory and stimulatory responses to melatonin were mediated via G_i-like proteins as they were blocked by pertussis toxin. Upon co-transfection with the α subunit of G_z, the ability of melatonin to regulate both type II and the endogenous adenylyl cyclases became refractory to pertussis toxin, indicating that the melatonin receptor can also couple to G_z. However, other pertussis toxin-insensitive G proteins such as G_q, G_{12} and G_{13} were unable to interact with the melatonin receptor.**

Key words: Melatonin receptor; Adenylyl cyclase; G protein; Signal transduction

1. Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine) plays an important role in the regulation of circadian rhythms as well as many physiological functions in mammals [1]. The recent cloning of the melatonin receptor from *Xenopus* [2] and rat [3] has confirmed the long-suspected notion that this receptor belongs to the superfamily of G protein-coupled receptors. Numerous reports suggest that melatonin can inhibit adenylyl cyclase via pertussis toxin (PTX) sensitive G_i-like proteins [4,5]. Expression of the cloned melatonin receptors in NIH-3T3 and CHO cells has revealed that this receptor can indeed inhibit adenylyl cyclase in a PTX-sensitive manner [2,3].

The complexity of G protein-mediated signal transduction networks has increased tremendously with the isolation of large numbers of signaling molecules exhibiting very similar functions. Multiple G protein subunits [6] and many isoforms of adenylyl cyclase [7] have now been identified. In order to fully appreciate the physiological importance of melatonin, it is necessary to examine the functional capability of the melatonin receptor to regulate diverse signaling pathways. An increasing number of G_i-coupled receptors have been shown to share the ability to stimulate type II adenylyl cyclase (AC II) as well as to interact with the PTX-insensitive G protein, G_z. Thus far, this group of G_i-coupled receptors include the α_2-adrenergic, dopamine-D_2, adenosine-A_1, formyl peptide, complement C5a, and all three types of opioid receptors [8–12]. Although there is no indication of melatonin-mediated stimulation of adenylyl cyclase nor of possible coupling to PTX-insensitive G proteins, it is conceivable that the melatonin receptor may behave like the other G_i-coupled receptors. In this study, we report that the *Xenopus* melatonin receptor is capable of stimulating AC II in a PTX-senitive manner. Moreover, we present evidence that the melatonin receptor can interact with the PTX-insensitive G protein, G_z, but not with other PTX-insensitive G proteins such as G_q, G_{12}, and G_{13}.

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Abbreviations: AC II, type II adenylyl cyclase; G protein, guanine nucleotide-binding regulatory protein; α_z, α subunit of the G protein G_z; PTX, pertussis toxin; hCG, human choriogonadotropin; LHR, luteinizing hormone receptor.

2. Experimental

2.1. Materials

The cDNA encoding *Xenopus* melatonin receptor [2], a generous gift from Dr. Steve Reppert (Massachusetts General Hospital, Boston), was subcloned into pcDNAI (Invitrogen) at *Eco*RI sites. cDNAs encoding the α subunit of G_{12} and G_{13} in pCIS were kindly provided by Dr. Melvin Simon (California Institute of Technology, Pasadena). The cDNA encoding AC II in pcDNAI was from Dr. Randall Reed (Johns Hopkins University, MD). Other cDNAs were constructed or obtained as previously described [13]. PTX was purchased from List Biological Laboratories (Campbell, CA). Human choriogonadotropin (hCG) was supplied by the National Pituitary Agency (Bethesda, MD). [2-^{3}H]Adenine was purchased from Amersham Corp. Plasmid purification columns were obtained from Qiagen Inc. Cell culture reagents were obtained from Gibco and all other chemicals were purchased from Sigma.

2.2. Transient transfection of 293 cells

The human embryonic kidney 293 cells (ATCC CRL-1573) were kept in Eagle's minimum essential medium (MEM) with 10% fetal calf serum (FCS), 50 units/ml penicillin and 50 μg/ml streptomycin, and in 5% CO_2. Cells were seeded in 12-well plates at 2×10^5 cells per well and were transfected 24 h later with various cDNAs by the DEAE-Dextran method as described previously [14].

2.3. cAMP accumulation

293 cells were labelled 16–20 h post-transfection with [2-^{3}H]adenine (1 μCi/ml) in MEM with 1% FCS. When needed, PTX (100 ng/ml) was added simultaneously. 18–24 h later, the cells were assayed in 20 mM HEPES-buffered MEM (pH 7.4) containing the appropriate drugs and 1 mM 1-methyl-3-isobutylxanthine for 30 min at 37°C. The reaction was stopped by adding 5% TCA containing 1 mM ATP. Intracellular [^{3}H]cAMP was determined by sequential chromatography [14]. Absolute values for cAMP accumulation varied between experiments, but variability within a given experiment was less than 10% in general.

3. Results and discussion

The cloned *Xenopus* melatonin receptor has been shown to inhibit adenylyl cyclase in transfected CHO cells [2]. We first begun by ensuring that the *Xenopus* melatonin receptor behaves similarly in transfected human embryonic kidney 293 cells. The 293 cells were co-transfected with cDNAs encoding the rat luteinizing hormone receptor (LHR) [15] and the *Xenopus* melatonin receptor. By co-expressing LHR with the melatonin

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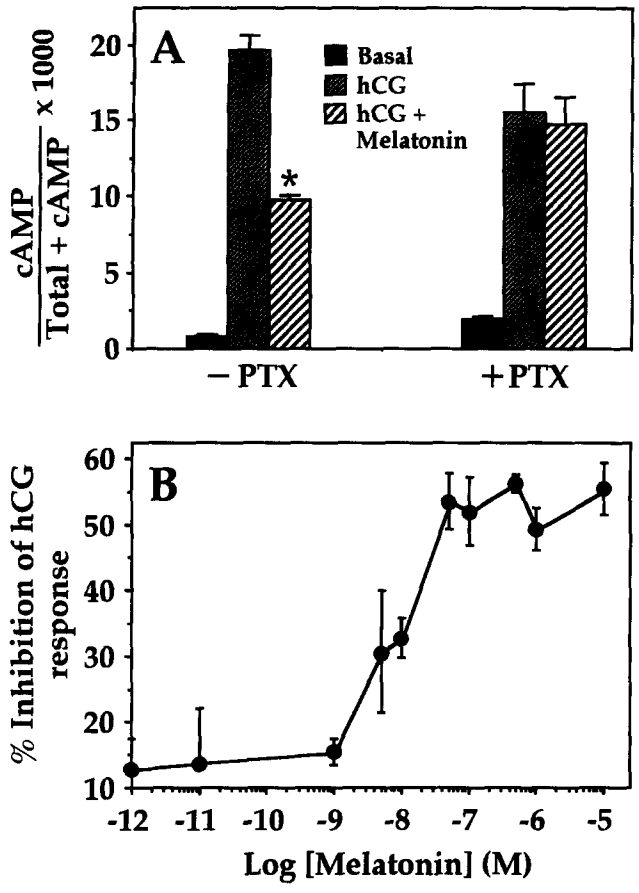

Fig. 1. Melatonin-mediated inhibition of cAMP accumulation in transfected 293 cells. 293 cells were co-transfected with cDNAs encoding the LHR (0.15 μg/ml) and melatonin receptor (0.25 μg/ml). (A) Transfected cells were labelled with [2-^{3}H]adenine (1 μCi/ml) in the absence or presence of PTX (100 ng/ml) and assayed for responses to hCG (5 ng/ml) with or without melatonin (100 nM). *Melatonin significantly reduced the hCG-stimulated activity; paired t-test, $P < 0.05$. (B) Transfected cells were assayed for cAMP accumulation after exposure to varying concentrations of melatonin (from 1 pM to 10 μM). Results are expressed as percent inhibition of the hCG-stimulated activity as compared with that measured in the absence of melatonin. Melatonin (100 nM) significantly inhibited the hCG-stimulated activity at concentrations >1 nM; paired t-test, $P < 0.05$. Data shown represent the mean ± S.D. of triplicate determinations in a single experiment; two additional experiments produced similar results.

receptor, we were able to selectively study the sub-population of cells that have taken up the cDNAs. As illustrated in Fig. 1A, addition of hCG (5 ng/ml) to transfected 293 cells resulted in a 20-fold increase in intracellular cAMP levels. In the presence of 100 nM of melatonin, the hCG-stimulated cAMP accumulation was reduced by about 50%. In contrast, 100 nM melatonin did not significantly inhibit the hCG-stimulated cAMP accumulation in 293 cells transfected with LHR alone ($P > 0.9$). Other indoleamines such as 2-iodomelatonin, 6-hydroxymelatonin, and *N*-acetylserotonin at 100 nM were able to inhibit the hCG response to similar magnitudes (unpublished results). The melatonin-induced inhibitory effect was dose dependent (Fig. 1B) and sensitive to PTX treatment (Fig. 1A), indicating the involvement of G_i proteins. Our results are in accordance with similar reports using NIH-3T3 or CHO cells [2,3].

Many G_i-coupled receptors have the ability to stimulate the formation of cAMP by activating AC II [8–11]. As a G_i-coupled receptor, the melatonin receptor may also possess the ability to regulate AC II. We examined this hypothesis by co-transfecting 293 cells with cDNAs encoding AC II [16], α_s-Q227L, and melatonin receptor. The inclusion of α_s-Q227L (a constitutively active mutant of α_s harboring a point mutation at codon 227) is necessary because G_i-coupled receptors activate AC II by releasing $\beta\gamma$ subunits, and the stimulatory actions of $\beta\gamma$ subunits require the presence of GTP-bound α_s [8,17]. As shown in Fig. 2, activation of the melatonin receptor stimulated the cAMP accumulation by 80–120% over basal values. The melatonin-induced stimulatory response was apparently mediated by G_i-like proteins as the increase in cAMP accumulation was completely blocked by PTX treatment (Fig. 2). The significance of this finding is presently unclear. Thus far, no report on melatonin-induced stimulation of cAMP accumulation has been documented. Because the melatonin receptor can inhibit as well as stimulate different isoforms of adenylyl cyclase, the net change in intracellular cAMP will depend on the relative abundance of the various isozymes of adenylyl cyclase. In the absence of AC II, the endogenous type I, III, and VI adenylyl cyclases [18] were inhibited by the activated melatonin receptor (Fig. 1A). By over-expressing AC II in 293 cells, the inhibitory response of melatonin was converted to a stimulatory signal (Fig. 2). It should also be noted that the $\beta\gamma$-induced stimulation of AC II absolutely required the presence of GTP-bound a_s [8]. Studies using forskolin to stimulate adenylyl cyclases cannot provide the proper environment for G_i-coupled receptors to activate AC II. Interestingly, melatonin has been shown to potentiate the vasoactive intestinal peptide-stimulated cAMP accumulation in human lymphocytes [19]. Whether the melatonin response in lymphocytes is due to $\beta\gamma$-mediated stimulation of AC II remains to be determined.

Another property shared by many G_i-coupled receptors is their ability to functionally interact with the PTX-insensitive G_z protein [20] in the regulation of adenylyl cyclases [9–13]. Thus we sought to examine if similar coupling between G_z and the melatonin receptor exists. In 293 cells co-transfected with

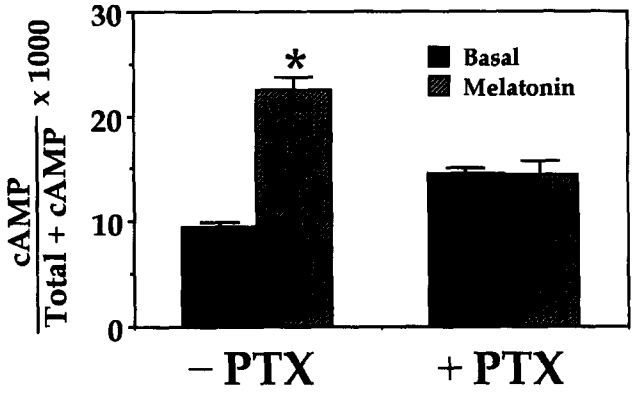

Fig. 2. Stimulation of AC II by the melatonin receptor. 293 cells were co-transfected with cDNAs encoding the AC II (0.25 μg/ml) and α_s-Q227L (0.025 μg/ml), with or without the melatonin receptor (0.25 μg/ml). Transfected cells were labelled with [2-^{3}H]adenine with or without PTX as in the legend to Fig. 1 and subsequently assayed for cAMP accumulation in the absence or presence of melatonin. *Melatonin significantly stimulated the AC II activity; paired t-test, $P < 0.05$. Data shown represent the mean ± S.E.M. of triplicate determinations from three independent experiments.

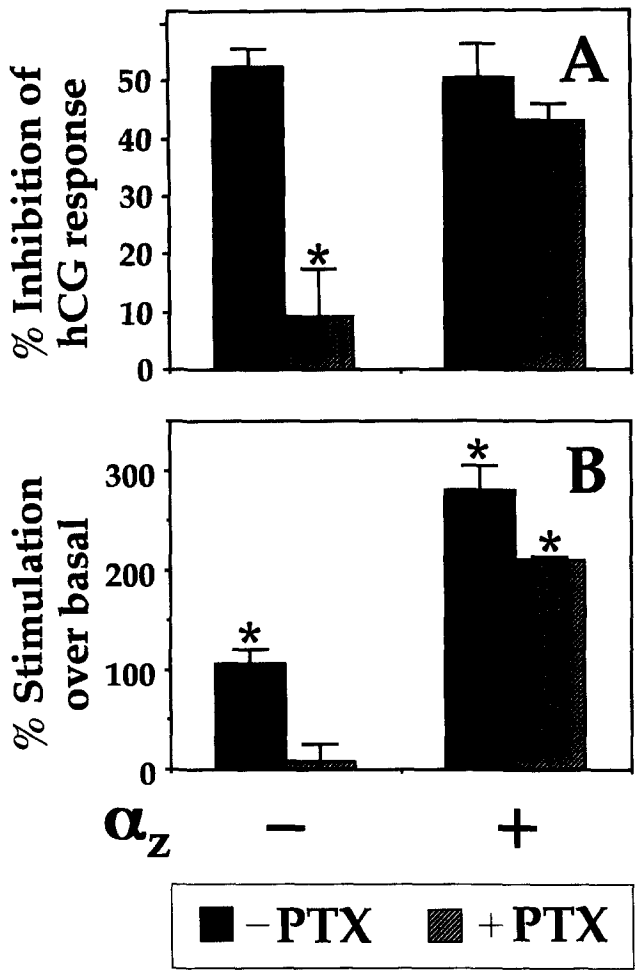

Fig. 3. Melatonin receptor coupling to the PTX-insensitive α_z. (A) 293 cells were co-transfected with cDNAs encoding the LHR (0.15 μg/ml) and the melatonin receptor (0.25 μg/ml) with or without α_z (0.125 μg/ml). Transfected cells were treated in the absence or presence of PTX (100 ng/ml) and assayed for cAMP accumulation in the presence of hCG (5 ng/ml) with or without 100 nM melatonin. Results are expressed as percent inhibition of the hCG-stimulated response. *PTX significantly attenuated the melatonin-induced inhibitory response; paired Bonferroni t-test, $P < 0.05$. (B) 293 cells were co-transfected with cDNAs encoding the AC II (0.25 μg/ml), α_s-Q227L (0.025 μg/ml), the melatonin receptor (0.25 μg/ml) and with or without α_z (0.125 μg/ml). Transfected cells were treated in the absence or presence of PTX (100 ng/ml) and assayed for cAMP accumulation in the presence of 100 nM melatonin. Results are expressed as percent stimulation over the basal activity by melatonin. *Melatonin significantly stimulated the AC II activity; paired t-test, $P < 0.05$. Data in each panel represent triplicate determinations in a single experiment; two independent experiments yielded similar results.

cDNAs encoding the LHR, melatonin receptor and the α subunit of G_z (α_z), the melatonin-mediated inhibition of hCG response became insensitive to PTX treatment (Fig. 3A). However, in the absence of α_z co-expression, PTX-mediated inactivation of endogenous α_i in the transfected 293 cells effectively abolished the response to melatonin (Fig. 3A). Likewise, to test whether the melatonin receptor can utilize G_z to stimulate AC II, we co-transfected 293 cells with cDNAs encoding the AC II, α_s-Q227L, and melatonin receptor in the absence or presence of α_z. In the absence of α_z, the melatonin-stimulated cAMP accumulation was almost abolished following PTX-mediated inactivation of endogenous α_i (Fig. 3B). However, in cells co-expressing α_z and the melatonin receptor, PTX only partially blocked the indoleamine-mediated stimulation of cAMP accumulation (Fig. 3B), presumably by inactivating the contribution by endogenous G_i proteins. These results suggest that α_z can indeed couple to the melatonin receptor, both for the inhibition of endogenous adenylyl cyclases and stimulation of AC II. There is no indication of any physiological significance of G_z coupling to the melatonin receptor, but it is interesting to note that both are present in the retina [1,20]. More importantly, PTX-insensitive G proteins appear to link melatonin receptors to inhibition of adenylyl cyclase in the ovine pars tuberalis [21].

Since the activation of AC II by G_i-coupled receptors is mediated by the $\beta\gamma$ subunits [8,17], one could detect receptor–G protein interactions in this system. For those G proteins that can functionally interact with the melatonin receptor, the released $\beta\gamma$ subunits would subsequently stimulate AC II. Taking advantage of this system, we examined the possible replacement of endogenous α_i polypeptides by other PTX-insensitive a subunits in transfected 293 cells. In particular, we wanted to test the capacity of the melatonin receptor to interact with G_q and G_{13} which regulate phospholipase C [22] and the Na^+/H^+ antiporter [23], respectively. Cells were co-transfected with cDNAs encoding the AC II, α_s-Q227L, melatonin receptor, and an a subunit from either G_z, G_q, G_{12}, or G_{13}. The transfected cells were then labelled with [2-^{3}H]adenine in the absence or presence of PTX prior to stimulation by melatonin. As shown in Fig. 4, neither α_q, α_{12}, nor α_{13} was able to replace the endogenous α_i in melatonin-induced activation of AC II, whereas the melatonin response was not suppressed by PTX in cells co-expressing α_z. Significant enhancement of the melatonin-induced stimulation of cAMP accumulation was observed in cells co-expressing α_z (Figs. 3B and 4). We have previously examined this phenomenon in other receptor systems and ascribed the enhancement effect to an increase in receptor-releasable pool of $\beta\gamma$ subunits which is due to the provision of

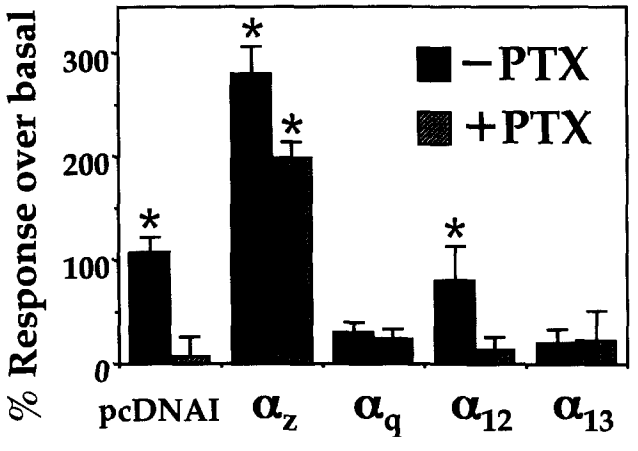

Fig. 4. Melatonin receptor coupling to other PTX-insensitive G proteins. 293 cells were co-transfected as in the legend to Fig. 3B, except where indicated, α_z was replaced by either α_q, α_{12}, α_{13}, or the control vector pcDNAI. Transfected cells were treated with or without PTX (100 ng/ml) and assayed for cAMP accumulation in the absence or presence of 100 nM melatonin. Results are expressed as percent stimulation over the basal activity by melatonin. *Melatonin significantly stimulated the AC II activity; paired t-test, $P < 0.05$. Data represent triplicate determinations in a single experiment; two independent experiments yielded similar results.

exogenous α_z subunit [9,11]. Potentiation of the melatonin response was specific and was not observed in cells co-expressing α_q, α_{12}, or α_{13} (Fig. 4). In fact, expression of α_q or a_{13} significantly ($P < 0.05$) inhibited the melatonin-mediated AC II response. Inhibitions seen with these a subunits are presumably due to their ability to scavenge free $\beta\gamma$ subunits that have been released by activated melatonin receptors, thus preventing the $\beta\gamma$ subunits from stimulating AC II. These results indicate a lack of coupling between the melatonin receptor and the a subunits of G_q, G_{12}, and G_{13}. Although we were unable to demonstrate coupling between the melatonin receptor and G_q, it does not necessarily rule out the possibility that the receptor can regulate phospholipase C. There is presidence that the complement C5a receptor selectively interacts with G_{16}, but not with G_q/G_{11}, to stimulate phospholipase C [24]. Moreover, reports of melatonin-mediated stimulation of phospholipase C are accumulating [25].

In summary, the present study is the first demonstration that the cloned *Xenopus* melatonin receptor can couple to a PTX-insensitive G protein, G_z, to either stimulate or inhibit different isoforms of adenylyl cyclases. Our findings indicate a lack of coupling to G_q, G_{12}, or G_{13} by the melatonin receptor.

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