Melatonin-induced suppression of PC12 cell growth is mediated by its Gi coupled transmembrane receptors

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Abstract

The effects of pertussis toxin, an uncoupler of Gi protein from adenylate cyclase, and luzindole, a competitive inhibitor of melatonin receptor binding, were examined for their ability to inhibit melatonin-induced suppression of PC12 cell growth. Both agents inhibited the melatonin response suggesting that melatonin may be acting through one of its Gi coupled cell surface receptors. This is confirmed by Western blots demonstrating the presence of MT1 receptors in PC12 cells. Coupling of the Gi protein to these receptors is demonstrated by failure of melatonin to suppress cell growth in PKA deficient A126-1B2-1 mutant PC12 cells. Similarly, melatonin failed to prevent cell proliferation when cells were incubated in the presence of the PKA inhibitor, Rp-cAMP. Retinoic acid and dexamethasone, agents known to effect PC12 cell growth and/or differentiation, displayed differential effects on the actions of melatonin. In the presence of melatonin and low concentrations of retinoic acid (100 nM), PC12 cell proliferation was additive with that of melatonin whereas, 1,25-dihydroxyvitamin D$_3$ (IC$_{50}$=10 nM), which by itself had no effect on PC12 cell growth, was found to inhibit the melatonin response. This study demonstrates that inhibition of PC12 cell growth, at physiological concentrations of melatonin, is mediated by cAMP-dependent cell surface receptors and this response is altered by other growth factors known to effect PC12 cell proliferation and differentiation. © 2001 Elsevier Science B.V. All rights reserved.

Theme: Development and regeneration

Topic: Biological effects of trophic factors: neurotrophins

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1. Introduction

Melatonin, the primary endocrine product of the pineal gland, mediates the photic control of the circadian rhythms [52] in vivo. This hormone is responsible for regulation of the timing of the reproductive cycle that occurs in response to changes in duration of daylight in seasonally breeding animals [36,37] and can affect seasonal rhythms for fasting, hibernation and thermoregulation in animals [52]. In addition, pharmacological levels of melatonin have been shown to prevent cell death and protect against cell damage caused by free radicals both in vitro and in vivo [33,34]. In contrast, endogenous levels of melatonin have been reported to have an antiproliferative effect in a number of cell culture systems prompting the suggestion that this hormone has the potential to be used as an oncostatic agent [13,30].

Although the biochemical pathways responsible for the biological actions of melatonin have not been clearly defined, it is generally believed that the cell surface transmembrane receptors are primarily responsible for mediating its actions in vivo. Three high affinity mammalian cell surface receptors termed MT1 and MT2 (previously labeled Mel 1a and 1b, respectively) have been identified for melatonin [14,37,49]. Both, melatonin transmembrane receptors are coupled to a pertussis toxin sensitive guanine nucleotide-binding G$_i$ protein and their main response is presumed to be mediated via inhibition of cAMP formation by adenylate cyclase [22,53]. The MT1 and MT2 receptor isoforms share 60% homology and both

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contain seven hydrophobic transmembrane spanning domains [15]. Interestingly, the melatonin receptors do not share significant sequence homology with previously characterized G protein-coupled receptors [14,37].

The antiproliferative effects of melatonin have been demonstrated in a number of cell culture systems including human breast cancer MCF-7 cells [47,21,11], chick skeletal muscle cells [24], ME-180 human cervical cancer cells [6], rat hepatoma AH 130 [7] and 7288CTC [3] cells, rat [45] and human [38] melanoma cells, human benign [17–19] and malignant [28] prostate cells and ovarian carcinoma cells [31]. Studies in our laboratory have revealed that physiological concentrations of melatonin were also capable of suppressing rat pheochromocytoma (PC12) cell growth [41]. The mechanism for the antiproliferative effects of melatonin are not well understood although several investigators [1,3] have suggested that the transmembrane receptor may be mediating this response. Prior studies [41] from our laboratory were inconsistent with this hypothesis in that melatonin failed to inhibit forskolin-induced cAMP formation suggesting that these receptors may not be involved in suppression of PC12 cell growth. However, this latter observation failed to consider that melatonin-induced inhibition of forskolin-stimulated cAMP production is dependent on the expression of the cell surface receptor; i.e. when expression is low the Gi coupled response is not detected and when overexpressed, suppression of the response is observed [9]. In light of this, we decided to re-examine whether the antiproliferative actions of melatonin in PC12 cells are, in fact, mediated by the transmembrane receptors.

2. Materials and methods

2.1. Materials

Dulbecco’s modified Eagle’s medium (DMEM), penicillin and streptomycin were obtained from Gibco, Grand Island, NY, USA. Normal and charcoal-stripped fetal bovine serum was purchased from Hyclone Laboratories, Logan, UT, USA and heat inactivated horse serum from bovine serum was purchased from Hyclone Laboratories, 0.5% NP-40, 10 mM Tris, 0.5 M NaCl, containing 10% fetal bovine serum, 5% horse serum and 100 U/ml of penicillin, and 100 μg/ml of streptomycin as described previously [41]. Both wild type and A126 mutant cells were maintained in 95% air/5% CO2 at 36.5°C and passaged at 70–80% confluency [40]. In some experiments charcoal-stripped serum was used to deplete the growth media of vitamin D3. To determine the effects of luzindole, pertussis toxin, all-trans-retinoic acid, dexamethasone and the adenosine Rp-isomer of 3’,5’-cyclic monophosphothioate on melatonin-induced suppression of PC12 cell growth, cells were plated onto 35-mm culture dishes in media described above and reagents were added at time of plating. All reagent solutions were prepared in DMSO except for the Rp-isomer of adenine 3’,5’-cyclic monophosphothioate which was suspended in phosphate-buffered saline, pH 7.4. In each experiment, the appropriate amount of DMSO was added to control cultures, which never exceeded 1 μl/ml of media. Prior studies have demonstrated this concentration of DMSO is not toxic to the cells.

2.3. Assay for PC12 cell growth

PC12 cell nuclei were counted according to the method as described by Lin et al. [26]. In brief, media was removed from the culture dish and the attached cells were washed with 1 ml of phosphate-buffered saline, pH 7.4. Cell membranes were disrupted and nuclei were stained using a lysing solution containing 13.2 mM cetyltrimethylammonium bromide, 0.28% glacial acetic acid, 2.8 mM NaCl, 0.5% Triton X-100 and 0.25 mg/ml methylene blue in 10% PBS, pH 7.4. Cells were gently scraped from the culture dishes using a rubber policeman and the stained nuclei were counted on a hemocytometer.

2.4. Immuno blot

PC12, HEK293 and MCF7 cells were grown on 100-mm dishes, rinsed in 10 ml PBS and scraped in 200 μl cold extraction buffer (20 mM Tris pH 7.6, 120 mM NaCl, 0.5% NP-40, 10 μg/ml aprotinin, 2 mM benzamidine, 10 μg/ml leupeptin, 100 mM NaF, 200 μM Na2VO4, 4 mM PMSF). Lysates were incubated for 30 min at 4°C, clarified by centrifugation for 10 min, and boiled in Laemmli sample buffer for 10 min. Protein concentrations were determined using the BCA protein assay (bicinchoninic acid; Pierce, Rockford, IL, USA). Fifty micrograms of protein from each sample were subjected to SDS–PAGE on a 12% acrylamide gel. Proteins were transferred to an immobilon-P membrane and blocked for 1 h at 25°C with 5% nonfat dry milk in TTBS (20 mM Tris, 0.5 M NaCl, 0.05% Tween-20). Membranes were incubated with primary MT1 antibody (CIDTech Research Inc., Cambridge, ON, Canada) for 1 h at 25°C, rinsed with TTBS, and incubated with horseradish peroxidase conjugated secondary antibody for 1 h at 25°C. The membranes
were rinsed again with TTBS and treated with Pierce SuperSignal chemiluminescent substrate to detect the transferred proteins.

2.4.1. Statistical analysis

To determine significant differences between treatment groups and control group, data were analyzed by one-way analysis of variance using a Duncan’s multiple range test or unpaired T-test with a P-value less than 0.05 as being statistically significant.

3. Results

To determine whether the cell surface receptors for melatonin are mediating melatonin-induced suppression of PC12 cell growth, the effect of pertussis toxin [4,39], an uncoupler of Gt protein from adenylate cyclase, and luzindole, a competitive inhibitor of melatonin binding to its receptor, on cell proliferation [4] was assessed. The data reported in Fig. 1 demonstrate that 10 nM melatonin produced approximately 13% inhibition of cell growth 2 days after initiating treatment. This is consistent with results published previously from our laboratory [41]. Addition of either 10 μM luzindole or 40 ng/ml pertussis toxin to the culture media resulted in essentially total inhibition of the melatonin response suggesting that melatonin may be acting through one of its cell surface receptors in PC12 cells.

As previously demonstrated [4,22,39,53], the transmembrane receptors for melatonin are coupled to the Gt protein resulting in the suppression of cAMP formation. The fact that melatonin suppression of PC12 cell growth is pertussis toxin sensitive, implies that the mechanism for this process is likely mediated, at least in part, by PKA. In order to further establish the involvement of PKA in the melatonin response, we examined its effect on a mutant PC12 cell line (A126-1B2-1 cells) deficient in PKA [51]. The data shown in Table 1 reveal that melatonin, at the three concentrations examined, failed to suppress A126 mutant PC12 cell proliferation, further supporting the premise that melatonin suppression of cell growth is PKA dependent and is likely mediated via the Gi-coupled cell surface receptors.

| Table 1: Effect of melatonin on the cell growth in PKA deficient PC12 cells |
|-----------------|-----------------|
| Melatonin conc. (nM) | % Cell growth |
| 0.1             | 100±1.2        |
| 10              | 99.1±0.8       |
| 100             | 101±1.8        |

Cells were treated with melatonin and nuclei were counted 2 days later, as described in Materials and methods. The data above are the mean±S.E. of three separate experiments, each performed in duplicate. The percentage cell growth is a ratio of treatment/control×100.

Fig. 1. Effect of luzindole (Luzin) and pertussis toxin (PT) on melatonin (Mel)-induced suppression of PC12 cell growth. Cells were treated with 10 nM melatonin in the presence or absence of 10 μM luzindole or 40 ng/ml pertussis toxin and cell nuclei were counted 2 days later, as described in Materials and methods. The data presented are the mean±S.E. of three separate experiments, each performed in duplicate. The percentage cell growth is a ratio of treatment/control×100. *Significant differences between treatment groups and control groups (P<0.05).

Fig. 2. Western blots of the Mel 1a receptor from PC12 cells, HEK293 and MCF-7 cells. Western blots were performed on 50 μg protein isolated from each of the three cell lines as described in Materials and methods.
melatonin-induced suppression of PC12 cell growth. For these experiments, PC12 cells were cultured with 10 nM melatonin in the presence or absence of 100 nM and 100 μM retinoic acid, 100 nM 1,25-dihydroxyvitamin D₃, (vitamin D₃) or 100 nM dexamethasone. Cells were also cultured in the presence of 2 μM Rp-cAMP, a known antagonist of PKA. Initial studies focused on dexamethasone since dexamethasone, like melatonin, was also found to suppress PC12 cell growth. The data illustrated in Fig. 3 reveal that after 2 days of treatment with 10 nM melatonin or 100 nM dexamethasone, cell growth was suppressed approximately 12 and 20%, respectively. When dexamethasone was co-incubated with melatonin, cell growth was reduced approximately equal to the sum of the two agents added separately (34%). Also shown in Fig. 3 are results of experiments performed with the PKA inhibitor, Rp-cAMP. In this case, 2 μM Rp-cAMP had no effect on its own on PC12 cell proliferation but was capable of totally suppressing the melatonin response.

Like dexamethasone, retinoic acid was also found to suppress PC12 cell growth. The data illustrated in Fig. 4 reveal that retinoic acid at both 100 nM and 100 μM suppresses cell growth approximately 20% but, in contrast to the results with dexamethasone, no additional inhibition was observed when it was co-incubated along with melatonin. Surprisingly, cell survival actually increased upon the addition of melatonin to cells grown in the presence of 100 nM retinoic acid. This implies that the two agents mutually suppress the response by which the other promotes its inhibitory actions. However, when the concentration of retinoic acid was increased to 100 μM, melatonin was unable to overcome the inhibition produced by the retinoid on cell proliferation.

Experiments were also performed to determine the effects of vitamin D₃ on melatonin-induced suppression of PC12 cell growth. As illustrated in Fig. 5 and in direct contrast to both retinoic acid and dexamethasone, vitamin D₃ had little effect of its own on PC12 cell growth. However, as the data demonstrate, vitamin D₃ was capable of inhibiting the suppressive actions of melatonin on cell proliferation. The ability of vitamin D₃ to inhibit the melatonin response in PC12 cells is somewhat surprising since this vitamin has never been reported to effect other PC12 cell related activities. Thus, it was of interest to determine the dose–response curve for vitamin D₃, which is responsible for suppressing the actions of melatonin on cell proliferation. As illustrated in Fig. 6, total inhibition of the melatonin response was achieved at approximately 100 nM of vitamin D₃ with an IC₅₀ value of approximately 10 nM. This latter value is in a concentration range known for the binding of vitamin D₃ to its nuclear receptor, VDR. Since serum contains vitamin D₃ as well as other steroid and polycyclic aromatic compounds that might influence the response observed with vitamin D₃, we performed additional experiments with charcoal stripped FBS and horse serum, which removes polycyclic aromatic agents from the media. As indicated by the data in Table 2, the
response observed with vitamin D₃ in charcoal-stripped media was essentially identical to that seen with normal serum. Under these conditions, vitamin D₃ again failed to elicit any response of its own but still totally inhibited the melatonin-induced suppression of PC12 cell growth.

Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (nM)</th>
<th>% Cell growth</th>
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<tbody>
<tr>
<td>Vitamin D</td>
<td>100</td>
<td>97.9 ± 1.0</td>
</tr>
<tr>
<td>Melatonin</td>
<td>10</td>
<td>85.8 ± 1.0*</td>
</tr>
<tr>
<td>Vit. D+Melatonin</td>
<td>100+10</td>
<td>99.5 ± 1.8</td>
</tr>
</tbody>
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Cells were treated with 100 nM 1.25(OH)₂ Vit. D₃ and/or 10 nM Melatonin and cell nuclei were counted 2 days later, as described in Materials and methods. The data above are averages ± S.E. of three separate experiments, each performed in duplicate. The percentage cell growth is a ratio of treatment/control × 100. *Significant differences between treatment groups and control groups (P<0.05).

4. Discussion

As noted in the Introduction, melatonin has been reported to suppress cell growth in a number of cell culture systems including PC12 cells [41]. The mechanism by which this hormone suppresses cell growth is not known but it has been reported to increase in the length of the cell cycle in human breast cancer MCF-7 cells [10] presumably by arresting progression from the G₁ to the S-phase of the cell cycle [10]. The accumulated data in the literature suggests that melatonin-induced inhibition of cell proliferation may be mediated by its transmembrane receptors [1,3]. Accordingly, we attempted to establish whether these receptors are responsible for inhibition of PC12 cell growth by using the selective competitive inhibitor of the cell surface receptors, luzindole, and the G₁ uncoupler, pertussis toxin and Western blot analysis.

Two high affinity cell surface receptors for melatonin have been identified in mammals, MT1 and MT2 [14,37,49]. These transmembrane receptors are linked to the guanine nucleotide G₁ and G₂ proteins [35,53] and thus, can suppress adenylate cyclase activity [22]. Although melatonin suppression of endogenous levels of cAMP has not been observed, it is capable of inhibiting forskolin-stimulated cAMP formation. A prior publication from our laboratory revealed that melatonin did not inhibit forskolin production of cAMP in PC12 cells [41]. Our initial conclusion from these studies was that these cells, most likely, do not possess functionally active cell surface receptors for melatonin or these transmembrane receptors are not involved in the actions of melatonin. However, in light of the findings presented in this paper we now know that the original assumption was incorrect. This discrepancy is best explained by the prior observation [9] demonstrating that observable inhibition of the forskolin response by melatonin directly relates to the quantity of receptor on the cell surface. These investigators demonstrated that melatonin failed to interfere with forskolin-stimulated production of cAMP in human embryonic kidney cell line (HEK293) when receptor levels were in low abundance. However, when the MT1 receptor was overexpressed by transfection into these cells, melatonin-induced suppression of the forskolin response was observed. Based on
these latter findings we can conclude that the levels of the melatonin receptors are probably in low abundance in PC12 cells, thus accounting for the ineffectiveness of melatonin to inhibit forskolin stimulated cAMP production.

The MW of approximately 60 kDa obtained for the MT1 receptor in this paper corresponds to that obtained by Brydon et al. [5] in MT1 transfected COS cells. The latter investigators noted the larger than expected MW is characteristic of other highly hydrophobic and glycosylated seven transmembrane-spanning receptor proteins. However, the MW we obtained in MCF-7 cells is almost twice that reported by Ram et al. [32] for this receptor. Whether this reflects dimerization of the MT1 receptor or a glycosylated species is not known.

The observation that both luzindole and pertussis toxin prevent melatonin-induced suppression of cell growth strongly implies that the cell surface receptors via the Gi protein mediate the antiproliferative response. This suggests that the down-regulation of cAMP may be initiating the biochemical events promoting reduced cell turnover. This is supported by the findings that the PC12 cell A126-1B2-1 mutants, which are devoid of 80% of its PKA activity [51], are unresponsive to melatonin. However, it is somewhat difficult to reconcile these findings with those obtained with the Rp-isomer of cAMP. We anticipated that Rp-cAMP, being a potent inhibitor of PKA, should parallel the actions of melatonin and also suppress PC12 cell growth. However, Rp-cAMP had no effect of its own on PC12 cell proliferation, yet it was capable of inhibiting melatonin-induced suppression of PC12 cell growth. One plausible explanation for this discrepancy may relate to the actions of cAMP on expression of the melatonin cell surface receptors. In this regard, prior studies have reported that melatonin receptors in the ovine pars tuberalis (PT) are regulated by cAMP [2], i.e. increases in cAMP are associated with increased expression of the melatonin transmembrane receptors. Thus, it is feasible that inhibition of PKA activity resulted in the down regulation of these receptors. This may also account for the inability of melatonin to suppress cell growth in the PKA deficient mutants described above. Nuclear receptors for both dexamethasone and retinoic acid have been identified in PC12 cells [27,43] and as shown herein, both agents are capable of suppressing PC12 cell growth. Melatonin-induced inhibition of cell growth was additive with that of dexamethasone but not with that of retinoic acid. Although retinoic acid has been reported to induce apoptosis in PC12 cells [50], it is difficult to reconcile this action with its lack of additive with the response to melatonin. In the presence of retinoic acid, the remaining viable cells should still be sensitive to melatonin and one would have suspected that the effect of the two agents would have been, at least partially, additive. The lack of an additive response implies that retinoic acid and melatonin may mutually inhibit each other’s actions. In this regard, retinoic acid has been reported [23] to stimulate PKA dependent G proteins and both cAMP and retinoic acid act synergistically to augment the expression of the retinoic acid nuclear receptor, RAR-beta [48]. The promoter region of RAR-beta has been reported to contain a CRE-related motif indicating that its expression can be regulated by cAMP [48]. Thus, down regulation of cAMP by melatonin potentially can lead to suppression of RAR expression and simultaneously, RAR activation can lead to increased G protein activity which would counteract the response to melatonin. At suboptimal concentrations of both retinoic acid and melatonin, the net effect would be the mutual inhibition of the other’s response. Accordingly, it may be anticipated that high concentrations of retinoic acid may totally mask the melatonin’s response which is consistent with the data presented in Fig. 4 of this paper.

In contrast to retinoic acid, dexamethasone suppression of PC12 cell growth was additive with that of melatonin. These findings are consistent with prior studies by Lupowitz and Zisapel [28] demonstrating that melatonin-induced suppression of human prostate tumor cell growth was additive with the inhibitory actions of estradiol. This implies that the mechanism for the antiproliferative actions of steroids and melatonin may be different. Melatonin has been reported to down-regulate glucocorticoid receptors [42] but this would have resulted in suppression of the dexamethasone response and additivity of the two would not have been achieved. In contrast, the additive actions of the two agents may be accounted for by the fact that dexamethasone has been reported to decrease the G protein α-subunit, α1, in PC12 cells, which inhibits production of cAMP [25].

Vitamin D$_3$, in contrast to dexamethasone, displayed no effect of its own on PC12 cell growth, although it was capable of inhibiting the melatonin-induced suppression of PC12 cell growth. Our findings that vitamin D$_3$ had no effect on PC12 cell proliferation is consistent with the prior studies by Cosgaya et al. [12]. It is not known, however, whether PC12 cells actually possess the nuclear receptor, VDR, for this vitamin. Although the promoter regions of the transmembrane receptors for melatonin have not been characterized, it is conceivable they may contain hormone response elements specific for the nuclear receptors for both retinoic acid and/or vitamin D$_3$. If this is the case, then both retinoic acid and vitamin D$_3$ may be capable of down regulating transcription of the transmembrane receptors, thereby precluding the melatonin response in PC12 cells.

Vitamin D$_3$ inhibition of melatonin-induced suppression of PC12 cell growth was dose dependent with 100 nM providing complete inhibition. This effect does not appear to be dependent on the presence of either vitamin D$_3$ or other steroid and polycyclic aromatic compounds present in the growth media since cell growth in charcoal-treated serum resulted in the same outcome. Vitamin D$_3$ via activation of its nuclear receptor has been shown to
promote differentiation [29] and inhibit proliferation [8,16,44,46] of several normal and malignant cell types. In addition, there is evidence suggesting there may be cell surface receptors for vitamin D₃, but whether these receptors participate in the interaction with melatonin is not known [20]. In summary, this study shows that PC12 cell growth is inhibited by physiological concentrations (10 nM) of melatonin in a cell surface receptor dependent pathway probably mediated by cAMP. This response is inhibited by 1,25-dihydroxyvitamin D₃ and all-trans-retinoic acid but not by dexamethasone. The mechanisms for the suppression of the melatonin response in PC12 cells are not known, but may involve decreased expression of the melatonin receptors.

References


