2005-08-31

Effect of Melatonin and Dopamine in Site Specific Phosphorylation of Phosducin in Intact Retina

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EFFECT OF MELATONIN AND DOPAMINE ON SITE SPECIFIC PHOSPHORYLATION OF PHOSDUCIN IN INTACT RETINA

by

Nkemdirim Okere Arinzechukwu

A thesis submitted to the faculty of Brigham Young University in partial fulfillment of the requirements for the degree of

Master of Science

Department of Chemistry and Biochemistry

Brigham young University

August 2005

Brigham Young University
BRIGHAM YOUNG UNIVERSITY

GRADUATE COMMITTEE APPROVAL

of a thesis submitted

Nkemdirim Okere Arinzechukwu

This thesis has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

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Date                      Steven W. Graves
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Date                      Craig Thulin
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Date                      Noel L. Owen
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As Chair of the candidate’s graduate committee, I have read the thesis of Nkemdirim Okere Arinzechukwu in its final form and have found that (1) its format, citations, and bibliographical style are consistent and acceptable and fulfill university and department style requirement; (2) its illustrative materials including figures, tables, and chart are in place; and (3) the final manuscript is satisfactory to the graduate committee and is ready for submission to the university library.

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Accepted for the Department

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Department Chair

Accepted for the College

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Associate Dean,
College of physical and Mathematical Sciences
ABSTRACT

EFFECT OF MELATONIN AND DOPAMINE IN SITE SPECIFIC PHOSPHORYLATION OF PHOSDUCIN IN INTACT RETINA

Nkemdirim Okere Arinzechukwu

Department of Chemistry and Biochemistry

Master of Science

Phosducin (Pdc) is a 28 kDa binding partner for the G protein βγ subunit dimer (Gβγ) found abundantly in the photoreceptor cells of the retina and pineal gland. In the retina, light-dependent changes in cAMP and Ca^{2+} control the phosphorylation of Pdc at serine 73 and 54, respectively, which in turn controls the binding of Pdc to Gβγ. Gβγ binding has been proposed to facilitate light-driven transport of Gβγ from the site of phototransduction in the outer segment of the photoreceptor cell to the inner segment, thereby decreasing light sensitivity and contributing to the process of light adaptation.

Dopamine and melatonin are neuromodulators whose concentrations in the retina vary reciprocally during the circadian cycle, with dopamine high during the day and
melatonin high during the night. Together, they control numerous aspects of light and
dark adaptation in the retina. In this study, we have investigated the possible roles of
dopamine and melatonin in regulating Pdc phosphorylation. Using phosphorylation-site
specific antibodies to serines 54 and 73, we show that dopamine decreases the
phosphorylation of both sites. This decrease is blocked by D4 receptor antagonists and
pertussis toxin, indicating that dopamine causes a decrease in photoreceptor cell cAMP
and Ca\textsuperscript{2+} concentration via the D4 receptor coupled to the G\textsubscript{i} protein. Conversely,
melatonin increases the phosphorylation of both S54 and S73, most likely via the
inhibition of dopamine synthesis. These results demonstrate that dopamine and
melatonin control the phosphorylation state of phosducin by changing the concentration
of cAMP and Ca\textsuperscript{2+} in photoreceptor cells, and they suggest that dopamine and melatonin
may contribute to the light-induced movement of the photoreceptor G protein by
regulating Pdc phosphorylation.
ACKNOWLEDGEMENTS

I would like to express my gratitude to my adviser, Barry M. Willardson for his relentless and selfless effort with my research, experiments and writing. I would like to thank all the members of Willardson Lab, for their help with my research. I would express my appreciation to Dr. S W. Graves, Dr. C. D. Thulin, and Dr. Noel Owen for their time, effort and cooperation. I would thank my wife, Erica Rae N. Okere for the support and comfort she has been to me during my studies.
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ABBREVIATIONS

<table>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAPTA</td>
<td>2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester</td>
</tr>
<tr>
<td>CaMK</td>
<td>Ca(^{2+})/calmodulin-dependent protein kinase</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanine monophosphate</td>
</tr>
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<td>D2</td>
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</tr>
<tr>
<td>D4</td>
<td>D4 subtype of the dopamine receptor site family</td>
</tr>
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<td>DP</td>
<td>Dopamine</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetracetae</td>
</tr>
<tr>
<td>G(_{i})(\alpha)</td>
<td>G protein (\alpha) subunit inhibitory subtype</td>
</tr>
<tr>
<td>G(_{q})</td>
<td>G protein (\alpha) subunit q subtype</td>
</tr>
<tr>
<td>G(_{16})</td>
<td>G protein (\alpha) subunit 16 subtype</td>
</tr>
<tr>
<td>G(\beta\gamma)</td>
<td>G protein (\beta\gamma) subunit dimer</td>
</tr>
<tr>
<td>HEPES</td>
<td>[N-(2-Hydroxyethyl)piperazine-N’-2-ethanesulfonic Acid]</td>
</tr>
<tr>
<td>MT</td>
<td>Melatonin</td>
</tr>
<tr>
<td>MT1</td>
<td>MT1 subtype of the melatonin receptor family</td>
</tr>
<tr>
<td>MT2</td>
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<tr>
<td>Pdc</td>
<td>Phosducin</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP dependent protein kinase</td>
</tr>
<tr>
<td>PP1</td>
<td>Type 1 protein phosphatase</td>
</tr>
<tr>
<td>PP2A</td>
<td>Type 2A protein phosphatase</td>
</tr>
<tr>
<td>PTX</td>
<td>Pertussis toxin</td>
</tr>
<tr>
<td>S54</td>
<td>Serine 54</td>
</tr>
<tr>
<td>S73</td>
<td>Serine 73</td>
</tr>
<tr>
<td>14-3-3</td>
<td>Phosphoserine binding protein</td>
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CHAPTER 1
INTRODUCTION TO THE ROLE OF PHOSDUCIN IN PHOTOTRANSDUCTION

Vertebrate vision is initiated by a canonical G protein cascade in the outer segment of photoreceptor cells of the retina, involving the receptor rhodopsin with its 11-cis retinal chromophore, the photoreceptor G protein transducin (G\(_t\)), cGMP phosphodiesterase and cGMP-gated-cation channels (Arshavsky et al., 2002). The light-induced decrease in cGMP closes the channels, resulting in a decrease in Na\(^+\) and Ca\(^{2+}\) in the outer segment and a hyperpolarization of the photoreceptor cell plasma membrane. This hyperpolarization closes voltage-gated Ca\(^{2+}\) channels in the inner segment and synaptic terminus, blocking glutamate release into the post-synaptic cleft and thereby initiating the neuronal response.

The light-induced decrease in intracellular Ca\(^{2+}\) triggers a number of molecular events that decrease the sensitivity of the photoreceptor to light. These include an increase in guanylyl cyclase activity which restores cGMP concentrations, an increase in the binding affinity of the cation channels for cGMP which reopens the channels and an increase in rhodopsin phosphorylation which leads to arrestin binding and rhodopsin inactivation (Fain et al., 2001). The ability of photoreceptors to light adapt is essential in maintaining their responsiveness over the broad range of light intensities normally encountered. In addition to Ca\(^{2+}\)-dependent mechanisms, it has been recently proposed that light-induced translocation of G\(_t\) contributes to longer term light adaptation (Sokolov
et al., 2002). In the dark, $G_i$ is found almost exclusively in the outer segment, associated with the disc membranes; but in the light $G_i$ redistributes throughout the photoreceptor cell with the majority in the inner segment as would be expected for a soluble protein (Sokolov et al., 2002). This redistribution of $G_i$ is associated with a reduction in light sensitivity (Sokolov et al., 2002). The mechanism of translocation is unknown, but may involve the interaction of the $G_i\beta\gamma$ dimer with phosducin (Pdc) since the translocation of both $G_i\alpha$ and $G_i\beta\gamma$ was disrupted in mice in which the phosducin gene was deleted (Sokolov et al., 2004).

Pdc is a 28 kDa photoreceptor protein that binds the $G_i\beta\gamma$ complex in the light and dissociates in the dark (Lee et al., 2004; Sokolov et al., 2004). When bound, Pdc blocks the interaction of $G_i\beta\gamma$ with $G_i\alpha$ and with lipid membranes (Yoshida et al., 1994). The Pdc-$G_i\beta\gamma$ interaction is controlled by phosphorylation of Pdc at serines 54 (S54) and 73 (S73) which is in turn controlled by light. In the dark when Ca\textsuperscript{2+} and cAMP concentrations are high, S54 is phosphorylated by a Ca\textsuperscript{2+}/calmodulin-dependent protein kinase (CaMK) and S73 is phosphorylated by cAMP-dependent protein kinase (PKA) (Lee et al., 2004; Lee et al., 1990). Phosphorylation at both sites creates a binding site for the phospho-serine binding protein 14-3-3 (Thulin et al., 2001). In its phosphorylated and 14-3-3 bound form, Pdc can not interact with $G_i\beta\gamma$ (Thulin et al., 2001). Light exposure decreases Ca\textsuperscript{2+} and cAMP in the photoreceptor (Cohen and Blazynski, 1990; Krizaj and Copenhagen, 2002; Nir et al., 2002), inhibiting the activity of CaMK and PKA. Moreover, light increases the rate of dephosphorylation of serine 54 by PP2A by a yet to be determined mechanism, while dephosphorylation of serine 73 by PP1 occurs in both the light and dark (Lee et al., 2004). As a result, Pdc is dephosphorylated in
response to light, which permits a high affinity interaction with G_{i}\beta\gamma. Once bound, Pdc may facilitate G_{i}\beta\gamma translocation to the inner segment or retain G_{i}\beta\gamma in the inner segment. In this manner, Pdc may participate in long term light adaptation processes.

Dopamine and melatonin are neuromodulators that coordinate light and dark adaptation in the retina. Light and circadian day cause a decrease in the synthesis and release of melatonin from photoreceptor cells (Besharse and Iuvone, 1983; Pang et al., 1980), which in turn results in an increase in dopamine release from dopaminergic cells in the inner plexiform layer of the retina (Witkovsky, 2004). The increase in retinal dopamine concentration contributes to a number of light-adaptive mechanisms in photoreceptor cells, including the switch from rod to cone-mediated vision (Krizaj and Witkovsky, 1993; Manglapus et al., 1999). The opposite effects are observed in the dark and during the circadian night. Thus, melatonin is high at night in the dark and vision is rod-mediated, while dopamine is high during the day in the light and vision is cone-mediated (Cahill et al., 1991; Manglapus et al., 1999).

The molecular mechanisms by which dopamine and melatonin exert their effects on photoreceptor cells are not well established. In the case of dopamine, it is known to activate D4-type receptors coupled to G_{i} in mammalian photoreceptor cells resulting in a decrease in cAMP concentration (Nir et al., 2002). This decrease reduces cAMP-dependent protein kinase (PKA) activity, which is believed to be a primary cause of dopamine effects on photoreceptor cells (Witkovsky, 2004). Melatonin on the other hand activates MT_{1} and MT_{2} receptors that can couple to multiple G-proteins of the G_{i} and G_{q} families (Dubocovich et al., 2004). In the retina, MT_{1} receptors localize primarily to the dopaminergic cells and other cell types in the inner retina (Scher et al., 2002).
Localization of MT$_1$ receptors to rod photoreceptors has been shown in humans (Savaskan et al., 2002; Scher et al., 2002), but not in guinea pigs or rats (Fujieda et al., 1999; Fujieda et al., 2000). Thus, melatonin may exert its effects on photoreceptor cells directly through MT$_1$ receptors or indirectly by controlling dopamine release in the inner retina.

The fact that dopamine decreases cAMP levels in photoreceptor cells suggests that it may regulate phosducin phosphorylation and thereby impact G$_{\beta\gamma}$ translocation and phototransduction. Similarly, melatonin could also affect phosducin phosphorylation by controlling cAMP or Ca$^{2+}$ levels. To test this possibility, the effects of dopamine and melatonin on the phosphorylation of S54 and S73 of phosducin were measured in intact bovine retina. The results show that dopamine and melatonin reciprocally regulate the phosphorylation of S54 and S73, indicating that by modulating cAMP and Ca$^{2+}$ concentration in photoreceptor cells, they can control the interactions of Pdc with its binding partners, G$_i$$\beta\gamma$ and 14-3-3.
CHAPTER 2
EXPERIMENTAL PROCEDURES

Measuring the effects of dopamine and melatonin on Pdc phosphorylation – Bovine eyes were taken 5 min. post mortem from a local abattoir between 7:00 - 7:30 A.M. Retinas were removed immediately and cut into four approximately equal pieces in the light. Two pieces were dark adapted at 23°C in 1.0 ml of HEPES/Ringer's buffer (10 mM HEPES pH 7.5, 120 mM NaCl, 0.5 mM KCl, 0.2 mM CaCl₂, 0.2 mM MgCl₂, 0.1 mM EDTA, 10 mM glucose and 1 mM dithiothreitol) with or without 100 µM dopamine-HCl (SigmaAldrich) or 100 µM melatonin (SigmaAldrich) for one hr in the case of S73 phosphorylation or two hrs in the case of S54 phosphorylation. This amount of dark adaptation has been previously shown to give maximal Pdc phosphorylation at each site (Lee et al., 2004). The two remaining retinal pieces were light adapted for 30 min. under a 60W bulb at 30 cm distance at 23°C. After adaptation, all samples were homogenized in hypotonic buffer (20 mM HEPES pH 7.5, 10 mM EDTA, 1 mM dithiothreitol, 1 µM microcystin LR and 0.2 mM PMSF) using a tissue tearer (Dremel). This procedure blocked further phosphorylation/dephosphorylation reactions by diluting the cellular ATP, chelating the Mg²⁺ with EDTA and inhibiting phosphatases with microcystin. The phosphorylation state of Pdc was stable in this buffer for at least 2 hrs (Lee et al., 2004). Tissue debris was removed by centrifugation at 30,000 x g for 10 min, after which the supernatants were collected and protein concentrations were determined using the
Coomassie Plus Protein Assay Reagent (Pierce). Twelve µg of total protein from each sample were separated by SDS-PAGE on 12% gels and immunoblotted with a 1:1000 dilution of rabbit primary antibody specifically recognizing either phosphorylated S54 or phosphorylated S73 (Lee et al., 2004) and a 1:2000 dilution of goat anti-rabbit IgG peroxidase-conjugated secondary antibody (Calbiochem) as described previously (Lee et al., 2004). The blots were developed with the ECL Plus chemiluminescence reagent (Amersham), and the band intensity was quantified using a Storm 860-D phosphorimager (GE Healthcare) in chemiluminescence mode.

The dopamine concentration-dependence data were fitted by a non-linear least squares algorithm to an equation for inhibition of phosphorylation: 

\[ P = \frac{P_{\text{max}} - P_{\text{min}}}{1 + \left( \frac{[\text{dopamine}]}{IC_{50}} \right)} + P_{\text{min}}, \]

where \( P \) is the level of phosphorylation at a given dopamine concentration, \( P_{\text{max}} \) is the maximal level of phosphorylation at zero dopamine, \( P_{\text{min}} \) is the minimal level of phosphorylation at saturating dopamine and \( IC_{50} \) is the dopamine concentration at half-maximal inhibition. Similarly the melatonin concentration-dependence data were fitted to an equation for activation of phosphorylation: 

\[ P = \frac{P_{\text{max}} - P_{\text{min}}}{1 + \left( \frac{[\text{melatonin}]}{EC_{50}} \right)} + P_{\text{min}}, \]

where \( P \) is the level of phosphorylation at a given melatonin concentration, \( P_{\text{max}} \) is the level of phosphorylation at saturating melatonin, \( P_{\text{min}} \) is the level of phosphorylation at zero melatonin and \( EC_{50} \) is the melatonin concentration at half-maximal activation.

**Determining the effects of dopamine and melatonin antagonists on Pdc phosphorylation**

– Retinas were dark adapted as described above with or without 100 µM dopamine and 900µM of the D2 receptor-specific antagonist L-741,626 (Tocris) or 700 µM of the D4
receptor-specific antagonist L-745,870 trihydrochloride (Tocris). Control samples contained 0.33% DMSO which served as the vehicle for the D2 and D4 antagonists. In the case of melatonin, retinas were dark adapted with or without 100 µM Melatonin and 10 µM of the MT1 and MT2 receptor antagonist luzindole (Tocris). Control samples contained HEPES-Ringer’s buffer as the vehicle for the luzindole. After dark adaptation, the samples were immunoblotted to determine S54 and S73 phosphorylation as described above.

Measuring the contribution of $G_i$ and $Ca^{2+}$ signaling to the changes in Pdc phosphorylation induced by dopamine and melatonin – To determine if dopamine and melatonin were acting through $G_i$ signaling, retinas were treated with or without 5 µg/ml pertussis toxin (PTX) in HEPES/Ringer’s buffer in the presence and absence of 100 µM dopamine or melatonin during the 1hr (S73) or 2 hrs (S54) dark-adaptation period. After which, the samples were immunoblotted to determine S54 and S73 phosphorylation as described above.

To determine if dopamine and melatonin were acting via changes in intracellular $Ca^{2+}$, retinas were treated with 50 µM of the cell-permeant $Ca^{2+}$ chelator BAPTA-AM (SigmaAldrich) or the 0.33% DMSO vehicle in HEPES/Ringer buffer without $Ca^{2+}$ in the presence or absence of 100 µM dopamine or melatonin during the dark-adaptation period. After which, the samples were immunoblotted to determine S54 and S73 phosphorylation as described above.
Dopamine inhibits phosphorylation of Pdc at S54 and S73: The effects of dopamine on Pdc phosphorylation at S54 and S73 were determined by treating bovine retinas with dopamine in both the light and dark-adapted state and measuring the degree of phosphorylation by immunoblotting with phosphorylation site-specific antibodies (Lee et al., 2004). As reported by Lee et al. (2004), Pdc phosphorylation at both S73 and S54 was 4-fold less in light-adapted retina compared to dark-adapted retina (Fig. 1). These differences have been shown to correspond to phosphorylation stoichiometries between 15-20% in the light and 55-70% in the dark for both sites (Lee et al., 2004). Dopamine had no effect on the low level of phosphorylation in the light, but it caused a 40% decrease in phosphorylation of both S73 and S54 in the dark (Fig. 1). The decrease in S73 phosphorylation in response to dopamine is consistent with the reported decrease in cAMP in photoreceptor cells induced by dopamine (Nir et al., 2002). This decrease in cAMP would lead to a decrease in PKA-mediated phosphorylation of S73 (Lee et al., 2004; Lee et al., 1990). In contrast, the decrease in S54 phosphorylation in response to dopamine could not have been predicted from previous data. Phosphorylation at S54 is strictly controlled by intracellular Ca\(^{2+}\), through a CaMK (Lee et al., 2004; Thulin et al., 2001). Therefore, it appears that dopamine is causing a decrease in intracellular Ca\(^{2+}\) in photoreceptors which in turn results in a decrease in S54 phosphorylation.
The concentration dependence of the inhibition of S73 and S54 phosphorylation by dopamine was also determined (Fig. 1C). Dopamine inhibited S73 phosphorylation with an IC50 of 1µM and reached a maximal inhibition of 50%.

Figure 1. Effect of dopamine on Pdc S73 and S54 phosphorylation in light and dark-adapted retinas. Bovine retinas were light or dark-adapted with or without 100 µM dopamine as described in Methods. Soluble proteins were extracted from the retina and equal amounts of protein were separated by SDS-PAGE and immunoblotted with an antibody specific to S73 (A) or S54 (B). The Pdc band intensities were quantified and normalized to that of the dark-adapted sample without dopamine. The concentration dependence of the dopamine-induced inhibition of S73 and S54 phosphorylation in dark-adapted retinas is shown in (C). Bars and symbols in the graphs represent the average ± standard error from at least three separate experiments. The line in (C) represents a non-linear least squares fit of the data to an inhibition equation as described in Methods. The statistical significance relative to the dark sample without dopamine was determined by a paired t-test (** P < 0.01). Representative immunoblots are shown below the graphs.
The IC50 value is in the same concentration range as the estimated dopamine concentration in the retina in the light (Witkovsky et al., 1993) and the concentration range required for activation of dopamine receptors (Deary et al., 1991), indicating that the dopamine-induced changes in S73 phosphorylation are occurring within a physiologically significant concentration range. In the case of S54, a higher concentration of dopamine was required to inhibit S54 phosphorylation. The IC50 was 50 \( \mu \text{M} \) and the maximal inhibition was 70%. The reason for the IC50 difference between S54 and S73 may be the metabolism of the exogenously added dopamine during the longer dark-adaptation period for S54 than for S73 (2 hr versus 1 hr, see Methods), making the actual concentration less than the concentration added.

*Dopamine exerts its effects on Pdc phosphorylation through the D4 receptor:* Dopamine receptors of the D2 family are expressed in photoreceptor cells and mediate decreases in cAMP in response to dopamine. The D4 subtype has been shown to be the receptor responsible for dopamine signaling in the mouse and rat (Nir et al., 2002; Patel et al., 2003), but in other species it is not known whether D2 or D4 receptors are involved (Witkovsky, 2004). To determine whether D2 or D4 receptors mediate the decrease in Pdc phosphorylation in bovine photoreceptors, retinas were treated with the D2-specific antagonist L-741,626 or the D4-specific antagonist L-745,870 trihydrochloride in the presence of dopamine. S73 phosphorylation was unchanged by the D2-specific antagonist (Fig. 2A). In contrast, the D4-specific antagonist not only blocked the dopamine-induced decrease in S73 phosphorylation, it also caused a 40% increase in S73 phosphorylation over the unstimulated level (Fig. 2A). These results show that dopamine
is acting through the D4 receptor and not the D2 receptor to inhibit Pdc S73 phosphorylation in bovine photoreceptor cells. The increase in S73 phosphorylation above basal levels upon treatment with the D4-antagonist suggests that either L-745,870 is functioning as an inverse agonist on D4 receptors or that a residual amount of endogenous dopamine remains in these retinas during the 1 hr dark adaptation period. L-745,870 has not been reported to be an inverse agonist for D4 receptors (Patel et al., 1997; Pillai et al., 1998). Moreover, retinal dopamine levels have been shown to remain high early in the circadian subjective day in the absence of light (Manglapus et al., 1999) and all of these experiments were performed early in the day.

Figure 2. Effect of D2 and D4 receptor-specific antagonists on the dopamine-induced decrease in Pdc S73 and S54 phosphorylation. Retinas were dark-adapted with 100 µM dopamine and 900 µM of the D2-specific antagonist L-741,626 or 700 µM of the D4-specific antagonist L-745,870 as indicated. Phosphorylation of S73 (A) and S54 (B) were determined as in Fig. 1. Bars represent the average ± standard error from at least three separate experiments. The statistical significance relative to the dark sample without dopamine was determined by a paired t-test (P < 0.01). Representative immunoblots are shown below the graphs.

Therefore, it appears that a residual amount of endogenous dopamine is found in these retinas, causing S73 phosphorylation to be reduced. In the case of S54 phosphorylation, the D2-specific antagonist again had no effect, while D4-specific antagonist completely
blocked the dopamine-induced decrease, but it did not cause an increase above the basal level as was observed with S73 phosphorylation (Fig. 2B). Together, these antagonist data show that dopamine is acting via a D4 receptor and not a D2 receptor to decrease S54 and S73 phosphorylation.

**Figure 3.** Effect of PTX on the dopamine-induced decrease in Pdc S73 and S54 phosphorylation. Retinas were treated with 5 µg/ml PTX during the dark adaptation period with dopamine as indicated. Phosphorylation of S73 (A) and S54 (B) were determined as in Fig. 1. Bars represent the average ± standard error from at least three separate experiments. The statistical significance relative to the dark sample without dopamine was determined by a paired t-test (**) *P* < 0.01. Representative immunoblots are shown below the graphs.

Investigation of the down-stream signaling pathways involved in the dopamine-dependent decrease in Pdc phosphorylation: The principal signaling mechanism for the D4 receptor is through G\textsubscript{i} inhibition of adenylyl cyclase activity. To determine if G\textsubscript{i} is required for the observed effects of dopamine on Pdc phosphorylation, retinas were pretreated with pertussis toxin (PTX), which specifically blocks coupling of G\textsubscript{i} to receptors as a result of ADP-ribosylation of the G\textsubscript{iα} subunit (Bokoch et al., 1983; Codina et al., 1983; Katada and Ui, 1982). PTX blocked the dopamine-induced decrease in both S73 and S54
phosphorylation (Fig. 3), demonstrating that G_i is involved in the dopamine response. G_i-mediated inhibition of S73 phosphorylation is consistent with the classical mechanism of G_i signaling: inhibition of adenylyl cyclase activity resulting in a decrease in cAMP concentration and PKA activity. However, the mechanism of G_i-dependent inhibition of S54 phosphorylation is less obvious and more intriguing. As mentioned above, S54 phosphorylation is strictly dependent on Ca^{2+} (Lee et al., 2004). Thus, activation of G_i by the D4 receptor must somehow cause a decrease in photoreceptor Ca^{2+}.

To further assess the role of Ca^{2+} in the dopamine-dependent decreases in S73 and S54 phosphorylation, retinas were pretreated with the Ca^{2+} chelator BAPTA-AM, prior to the addition of dopamine. In the case of S73, Ca^{2+} chelation had little effect on S73 phosphorylation, either in the presence or absence of dopamine (Fig. 4A), consistent with recent data showing no Ca^{2+} dependence of S73 phosphorylation in intact retina (Lee et al., 2004).

Figure 4. Effect of the Ca^{2+} chelator BAPTA-AM on the dopamine-induced decrease in Pde S73 and S54 phosphorylation. Retinas were treated with 50 µM BAPTA-AM during the dark adaptation period with dopamine as indicated. Phosphorylation of S73 (A) and S54 (B) were determined as in Fig. 1. Bars represent the average ± standard error from at least three separate experiments. The statistical significance relative to the dark sample without dopamine was determined by a paired t-test (* P < 0.05 ** P < 0.01). Representative immunoblots are shown below the graphs.
In the case of S54, Ca\textsuperscript{2+} chelation decreased phosphorylation as expected. In addition, Ca\textsuperscript{2+} chelation blocked any further reduction in phosphorylation by dopamine (Fig. 4B), indicating that the effects of dopamine on S54 phosphorylation required a change in Ca\textsuperscript{2+}. This result confirms the observation that dopamine is decreasing S54 phosphorylation by decreasing Ca\textsuperscript{2+}. A potential mechanism by which dopamine may lower photoreceptor Ca\textsuperscript{2+} is presented below (discussed in Chapter 4).

*Melatonin increases phosphorylation of Pdc at S54 and S73:* Melatonin exerts many of the opposite effects of dopamine on photoreceptor physiology (Dubocovich et al., 2004). Hence, it was of interest to determine if melatonin treatment would change the phosphorylation of Pdc. Intact retinas were treated with melatonin under light and dark-adaptation conditions and phosphorylation of Pdc at S73 and S54 was measured using the phosphorylation site-specific antibodies. Melatonin treatment during light adaptation caused no significant change in the low level of S73 or S54 phosphorylation. On the other hand, melatonin treatment during dark adaptation increased phosphorylation of both S73 and S54 by 1.5-fold (Fig. 5). This increase in S73 and S54 phosphorylation approaches 100 % phosphorylation at these sites because previous studies showed that S73 and S54 were phosphorylated 55-70 % under similar dark-adaptation conditions in the absence of melatonin (Lee et al., 2004). A 1.5-fold increase in phosphorylation in the presence of melatonin would result in 80-100 % phosphorylation of S73 and S54. Unlike dopamine, the half-maximal effect of melatonin occurred at approximately the same concentration for both S73 and S54 phosphorylation (10 µM, Fig. 5C). In addition, effects of melatonin were completely blocked by the MT1 and MT2 receptor antagonist
luzindole (Fig. 6), indicating the melatonin-induced increase in S54 and S73 occurs specifically through melatonin receptors. Thus, melatonin is again opposing the effects of dopamine on another physiological property of photoreceptor cells.

Figure 5. Effect of melatonin on Pdc S73 and S54 phosphorylation in light and dark-adapted retinas. Retinas were light or dark-adapted with or without 100 µM melatonin as described in Methods. Phosphorylation of S73 (A) and S54 (B) were determined as in Fig. 1. The concentration dependence of the melatonin-induced increase in S73 and S54 phosphorylation in dark-adapted retinas is shown in (C). Bars and symbols represent the average ± standard error from at least three separate experiments. The line in (C) represents a non-linear least squares fit of the data to an activation equation as described in Methods. The statistical significance relative to the dark sample without melatonin was determined by a paired t-test (** P < 0.01). Representative immunoblots are shown below the graphs.
Figure 6. Effect of a melatonin receptor-specific antagonist on the dopamine-induced decrease in Pdc S73 and S54 phosphorylation. Retinas were dark-adapted with 100 µM melatonin and 10 µM of the MT1 and MT2 receptor-specific antagonist luzindole as indicated. Phosphorylation of S73 (A) and S54 (B) were determined as in Fig. 1. Bars represent the average ± standard error from at least three separate experiments. The statistical significance relative to the dark sample without melatonin was determined by a paired t-test (** P < 0.01). Representative immunoblots are shown below the graphs.

Investigation of the down-stream signaling pathways involved in the melatonin-dependent increase in Pdc phosphorylation: Melatonin receptors have been shown to exert their effects through multiple G proteins, including Gi, Gq and G16 (Dubocovich et al., 2004). Therefore, it was important to determine which G protein subtype was involved in the melatonin-induced increase in S73 and S54 phosphorylation. To assess the role of Gi, retinas were pretreated with PTX prior to addition of melatonin. The melatonin-induced increase in S54 and S73 phosphorylation was completely insensitive to PTX (Fig. 7), very much in contrast to what was observed with dopamine.
Figure 7. Effect of PTX on the melatonin-induced decrease in Pdc S73 and S54 phosphorylation.
Retinas were treated with 5 µg/ml PTX during the dark adaptation period with melatonin as indicated. Phosphorylation of S73 (A) and S54 (B) were determined as in Fig. 1. Bars represent the average ± standard error from at least three separate experiments. The statistical significance relative to the dark sample without melatonin was determined by a paired t-test (** P < 0.01). Representative immunoblots are shown below the graphs.

This result shows that G_i is not involved in melatonin signaling in photoreceptors and it suggests that melatonin is acting via G_q or G_16 to enhance Pdc phosphorylation.

However, it is not clear whether melatonin is acting directly on the photoreceptor cells or whether it exerts its effect indirectly by inhibiting dopamine release from the dopaminergic cells in the inner retina (discussed in Chapter 4). To investigate the Ca^{2+} dependence of the melatonin-mediated increase in S54 and S73 phosphorylation, retinas were pretreated with the Ca^{2+} chelator BAPTA-AM prior to addition of melatonin. Ca^{2+} chelation had no effect on the melatonin-induced increase in S73 phosphorylation, demonstrating that melatonin was acting on S73 by a Ca^{2+}-independent process (Fig. 8A). S73 phosphorylation is mediated by PKA in a cAMP-dependent manner. Consequently,
it can be concluded that melatonin treatment of the retina must in some manner cause an increase in cAMP in the photoreceptor cells. In the case of S54, Ca\(^{2+}\)-chelation inhibited phosphorylation as in Figure 4 and completely blocked the increase in phosphorylation caused by melatonin (Fig. 8B). This result indicates that the melatonin-induced increase in S54 phosphorylation is Ca\(^{2+}\)-dependent. Hence, melatonin must be inducing an increase in Ca\(^{2+}\) in photoreceptor cells. Possible mechanisms by which melatonin increases both photoreceptor cAMP and Ca\(^{2+}\) are discussed in Chapter 4.

Figure 8. Effect of the Ca\(^{2+}\) chelator BAPTA-AM on the melatonin-induced decrease in Pdc S73 and S54 phosphorylation. Retinas were treated with 50 µM BAPTA-AM during the dark adaptation period with dopamine as indicated. Phosphorylation of S73 (A) and S54 (B) were determined as in Fig. 1. Bars represent the average ± standard error from at least three separate experiments. The statistical significance relative to the dark sample without melatonin was determined by a paired t-test (** *P* < 0.01). Representative immunoblots are shown below the graphs.
Dopamine and melatonin exert multiple effects on photoreceptor cells that contribute to the process of light and dark adaptation, respectively (Dubocovich et al., 2004; Witkovsky, 2004). The results presented in this study indicate that dopamine and melatonin help control the phosphorylation state of Pdc. Pdc had been shown to be phosphorylated at S54 and S73 in the dark and dephosphorylated in the light (Lee et al., 2004). In the dark, Pdc had been shown to bind 14-3-3 protein when S54 and S73 are phosphorylated (Thulin et al., 2001), while in the light Pdc binds G\textsubscript{i}βγ when S54 is dephosphorylated (Lee et al., 2004). The binding of Pdc to G\textsubscript{i}βγ is believed to increase the solubility of G\textsubscript{i}βγ, increasing its movement from the outer segment to the inner segment of the rod photoreceptor cell in response to light (Sokolov et al., 2004). The movement of G\textsubscript{i} to the inner segment in the light has been shown to decrease the sensitivity of rods to light, thus contributing to the process of light-adaptation (Sokolov et al., 2002). Retinal dopamine concentration increases during the day and decreases during the night in a circadian-driven manner, whereas retinal melatonin concentration varies in precisely the opposite manner (Manglapus et al., 1999). Thus, it is possible that one way in which dopamine contributes to light adaptation is by decreasing the phosphorylation of Pdc, thereby increasing its binding to G\textsubscript{i}βγ and decreasing the amount of G\textsubscript{i}βγ in the outer segment. In contrast, melatonin may contribute to dark adaptation by increasing the
phosphorylation of Pdc, thereby decreasing its binding to G_{i\beta\gamma} and increasing the amount of G_{i\beta\gamma} in the outer segment. In addition, Pdc shows significant localization in the synaptic terminal region of photoreceptors (Chen et al., 2005). However, its function there is unknown. It is possible that dopamine and melatonin could regulate certain synaptic events by changing the phosphorylation state of Pdc.

The results presented here provide insight into the mechanism by which dopamine and melatonin control Pdc phosphorylation. The data from Fig. 2 indicate that dopamine is functioning through a D4 receptor. D4 receptors have been localized to the photoreceptor cells in mice and rats (Nir et al., 2002; Patel et al., 2003), suggesting that dopamine exerts its effect directly on the photoreceptor cells by binding to D4 receptors on their surface. In the case of S73, dopamine would decrease phosphorylation via a classical G_{i} pathway. The dopamine-liganded D4 receptor would activate G_{i} which would inhibit adenylyl cyclase, decreasing the concentration of cAMP. This decrease in cAMP would decrease PKA activity resulting in a decrease in S73 phosphorylation. This hypothesis is supported by the data of Fig. 3, showing that the dopamine-dependent decrease in S73 phosphorylation is inhibited by PTX treatment.

The mechanism by which dopamine decreases S54 phosphorylation appears more complex. S54 has been shown to be phosphorylated in a strictly Ca^{2+} dependent manner by a CaMK (Lee et al., 2004; Thulin et al., 2001). Moreover, Pdc is only found in the cytosol of photoreceptor cells and is not localized to any other cell types in the retina (Chen et al., 2005; Lee et al., 1988; Thulin et al., 1999). In light of these facts, dopamine must cause a decrease in intracellular Ca^{2+} in photoreceptor cells. Current understanding of Ca^{2+} handling in photoreceptors suggests only one way by which dopamine could
cause a decrease in Ca\(^{2+}\) concentration: through the effects of G\(_i\) on voltage-gated Ca\(^{2+}\) channels (Krizaj and Copenhagen, 2002). L-type Ca\(^{2+}\) channels are the predominant voltage-gated Ca\(^{2+}\) channels expressed in photoreceptor cells (Krizaj and Copenhagen, 2002). These channels can be inhibited by G\(_i\) indirectly by a decrease in PKA-dependent phosphorylation of the channels (Carbone et al., 2001; van der Heyden et al., 2005).

Alternatively, L-type channels have been reported in some cellular systems to be inhibited by G\(_i\) directly (Carbone et al., 2001), probably via G\(\beta\gamma\) inhibition as is the case with N-type and P/Q type voltage-gated Ca\(^{2+}\) channels (Herlitze et al., 1996; Ikeda, 1996). Either way, dopamine acting through the D4 receptor and G\(_i\) would inhibit L-type Ca\(^{2+}\) channels, resulting in a decrease in intracellular Ca\(^{2+}\) concentration. This decrease in Ca\(^{2+}\) may contribute to the dopamine-dependent desensitization of photoreceptors, given the multiple events that decrease photoreceptor sensitivity to light upon a decrease in cytosolic Ca\(^{2+}\).

In the case of melatonin, it could increase Pdc phosphorylation by binding to MT receptors expressed in photoreceptor cells, or it could do so indirectly by binding to MT receptors expressed in dopaminergic cells in the inner retina and inhibit dopamine release from those cells. MT receptors have not been localized in the bovine retina, but in other species they are found in the horizontal, amacrine and ganglion cells of the inner retina (Fujieda et al., 1999; Fujieda et al., 2000; Savaskan et al., 2002; Scher et al., 2002). They have also been found in the photoreceptor cells of the human retina (Savaskan et al., 2002; Scher et al., 2002), but not in the photoreceptors of rat or guinea pig (Fujieda et al., 1999; Fujieda et al., 2000). Thus, it is not clear whether bovine photoreceptors have melatonin receptors. MT receptors have been shown to couple to the G\(_i\) and G\(_q\) families
of G-proteins (Dubocovich et al., 2004). Thus, the Ca\(^{2+}\)-dependent increase in S54 phosphorylation caused by melatonin (Fig. 7 and 8) could result from activation of MT receptors on the photoreceptor cells working through G\(_q\) to increase Ca\(^{2+}\). However, the cAMP-dependent increase in S73 phosphorylation does not appear to result from direct activation of MT receptors. In order to increase cAMP over the one hour time course of these experiments, MT receptors would have to couple to G\(_s\), but they do not normally activate G\(_s\). MT receptors have only been shown to couple to G\(_s\) in heterologous over-expression experiments (Chan et al., 2002).

There appears to be more evidence for an indirect effect of melatonin on Pdc phosphorylation through the inhibition dopamine release from cells in the inner retina. For example, the effects of melatonin treatment were exactly the opposite of those caused by the addition of dopamine, as would be expected if the retinal dopamine concentration were decreased by melatonin. Moreover, the increase in S73 phosphorylation in the presence of the D4-receptor antagonist L-745,870 suggests that the retinas contained residual amounts of dopamine. These bovine retinas were collected and processed during the morning hours, a time when retinal dopamine concentration is increasing, even during dark-adaptation (Manglapus et al., 1999). Furthermore, a melatonin-induced decrease in dopamine could account for the increase in S73 phosphorylation that can not be explained by a direct effect of melatonin on the photoreceptors themselves. However, the melatonin-mediated increase in S73 phosphorylation was insensitive to PTX treatment and Ca\(^{2+}\) chelation, which would block signaling of MT receptors through both of their established G protein partners, G\(_i\) and G\(_q\). It is possible that melatonin could inhibit
dopamine release via a G-protein independent pathway such as β-arrestin-mediated activation of mitogen-activated protein kinases (Lefkowitz and Shenoy, 2005).

Although the detailed mechanisms of action remain to be resolved, this study clearly shows that dopamine inhibits the Pdc phosphorylation at S54 and S73 in intact retina and that melatonin reverses that effect. Given the close coupling of S54 phosphorylation to Ca\(^{2+}\) concentration and S73 phosphorylation to cAMP concentration, it can also be concluded that dopamine decreases Ca\(^{2+}\) and cAMP in the cytosol of the photoreceptor cell while melatonin increases their concentration. A dopamine-dependent decrease in cAMP has been shown (Nir et al., 2002), but the dopamine-mediated decrease in Ca\(^{2+}\) was previously unknown, as were the melatonin-induced increases in cAMP and Ca\(^{2+}\). It seems likely that most of the adaptive effects of dopamine and melatonin on photoreceptor cells are mediated through changes in the concentration of these important second messengers.
REFERENCES


