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Heteromeric MT₁/MT₂ Melatonin Receptors Modulate the Scotopic Electrophoretogram via PKC ζ in mice.

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Abstract

Melatonin plays an important role in the regulation of retinal functions, and previous studies have also reported that the action of melatonin on photoreceptors is mediated by melatonin receptor heterodimers. Furthermore, it has been reported that the melatonin-induced increase in the amplitude of the a- and b-wave is significantly blunted by inhibition of PKC. Previous work has also shown that PKC ζ is present in the photoreceptors, thus suggesting that PKC ζ may be implicated in the modulation of melatonin signaling in photoreceptors. To investigate the role PKC ζ plays in the modulation of the melatonin effect on the scotopic ERG, mice were injected with melatonin and with specific inhibitors of different PKC isoforms. PKC ζ knockout mice were also used in this study. PKC ζ activation in photoreceptors following melatonin injection was also investigated with immunocytochemistry. Inhibition of PKC ζ by PKC ζ -pseudosubstrate inhibitor (20 μ M) significantly reduced the melatonin-induced increase in the amplitude of the a- and b-wave. To further investigate the role of different PKCs in the modulation of the ERGs, we tested whether intra-vitreous injection of Enzastaurin (a potent inhibitor of PKC α , PKC β , PKC γ , and PKC ϵ) has any effect on the melatonin-induced increase in the a- and b-wave of the scotopic ERGs. Enzastaurin (100 nM) did not prevent the melatonin-induced increase in the amplitude of the a-wave, thus suggesting that PKC α , PKC β , PKC γ , and PKC ϵ are not involved in this phenomenon. Finally, our data indicated that, in mice lacking PKC ζ , melatonin injection failed to increase the amplitude of the a- and b-waves of the scotopic ERGs. An increase in PKC ζ phosphorylation in the photoreceptors was also observed by immunocytochemistry. Our data indicate that melatonin signaling does indeed use the PKC ζ pathway to increase the amplitude of the a- and b-wave of the scotopic ERG.

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Keywords

melatonin; retina; PKC ζ ; melatonin receptors; ERG

1. Introduction

In the mammalian retina, the hormone melatonin is synthesized by retinal photoreceptors during the night, and many studies have now demonstrated that melatonin plays a role in the regulation of many retinal functions (Summer & Wiechmann, 2009; Tosini et al., 2012) and can protect photoreceptors from apoptosis in mouse models of *retinitis pigmentosa* (Liang et al., 2001, Xu et al., 2017) or their viability during aging (Baba et al., 2009, Ganesini et al., 2016). A previous investigation has reported that melatonin acts on the photoreceptor via melatonin receptors' heterodimer (MT_{1/2h}) and is involved the activation of PKC, as a melatonin-induced increase in the amplitude of the a- and b-wave is significantly blunted by inhibition of PKC (Baba et al., 2013).

Among the several PKC isoforms that are expressed in the retina (Koike et al., 2005; Lee et al., 2007), PKC ζ and PKC λ are primarily expressed in the photoreceptors (Ghalayini et al., 1994; Lee et al., 2007; Omri et al., 2013). PKC λ is involved in photoreceptor development (Koike et al., 2005; Pizon-Guzman et al., 2011), and, because melatonin receptors knock-out mice show normal retinal development, we believe that this isoform is unlikely to mediate MT_{1/2h} signaling. Thus, we hypothesize that PKC ζ may be an isoform that is involved in the modulation of melatonin signaling in the photoreceptors. PKC ζ is an atypical isoform of PKC that is not activate by calcium or diacylglycerol, but it is believed to rely on a second messenger generated through the phosphoinositide 3-kinase (PI3-K).

Although PKC ζ is present in photoreceptors, its role is still not well defined. Lee et al. (2007) reported that, also in mice retinas, PKC ζ is mostly present in the inner and outer segments where it co-localizes with CaBP4 – a subfamily of neuronal Ca²⁺-binding proteins – and it is involved in the phosphorylation of CaBP4. A more recent study has implicated PKC ζ in the pathology of diabetic retinopathy; most specifically, it was reported that PKC ζ labeling in the cone photoreceptors was lost, and cone structure was altered in rats by hyperglycemia (Omri et al., 2013), thus suggesting that this PKC may represent a possible target to prevent the development of diabetic retinopathy. The aim of the present study was to investigate the role of PKC ζ in the modulation of the melatonin-induced increase in the amplitude of the a- and b-wave of the scotopic ERG in mouse.

2. Materials and Methods

2.1. Animals

C3H-f^{+/+} (C3H), C3H-f^{+/+}MT₁ knockout (KO, MT₁^{-/-}) and C3H-f^{+/+}MT₂^{-/-} KO (MT₂^{-/-}) mice (three to four months old) were used in this study. Mice were maintained in 12 hours light and 12 hours dark with lights on (zeitgeber time [ZT] 0) at 06:00 A.M. and lights off (ZT 12) at 6:00 P.M. The light was supplied with fluorescent tubes and the light intensity ranged from 200 to 400 lux at cage level. The genotypes were determined according to the

protocols previously described (Baba et al., 2013). We have previously reported that although C57/BL/6 mice are unable to synthesize melatonin (Ebihara et al., 1986) administration of exogenous melatonin still increases the amplitude of the a- and b-wave of the scotopic ERG via activation of MT_{1/2h} (Baba et al., 2013). Therefore, instead to produce a PKC ζ KO mouse in a C3H-F^{+/+}, we decided to investigate the effect that administration of exogenous melatonin will produce of the amplitude of the scotopic ERG in mice lacking PKC ζ in a C57/BL6J genetic background.

All the experimental procedures were carried out in accordance with Association for Assessment of Laboratory Animal Care policies and approved by the Morehouse School of Medicine Animal Care and Use Committee.

2.2. Scotopic electroretinogram (ERG)

The scotopic ERGs were performed on anesthetized (ketamine: 80 mg/kg; and xylazine: 16 mg/kg) mice as described in our previous publications (Baba et al., 2009; Baba et al., 2013). To determine the photoreceptors' response to the different experimental treatments we measured the amplitude of the a-wave 7 ms after the initial flash of light (Peachey et al., 2012; Baba et al., 2013).

Melatonin (Sigma, St Luis, MO) was dissolved in ethanol and then diluted with sterilized PBS. Melatonin was administered by intraperitoneal (1 mg/kg i.p.) or intravitreal (i.v. in various dosages) injection. PKC ζ -pseudosubstrate (ApexBio Tech, Houston, TX) was dissolved with 150 ml of acetonitrile and 350 ml of ddH₂O. The solution was diluted with PBS to make a 400 μ M solution. The volume of 1 μ l was intravitreally injected in one eye (final concentration of 20 μ M) using a 10 μ l Hamilton syringe (Hamilton Company, Reno Nevada). The same volume of vehicle (30% acetonitrile) was injected into the other eye. Enzastaurin (ApexBio Tech, Houston, TX) was first dissolved in DMSO to make a 10 mM solution and diluted with PBS to make a 2 μ M working solution. The volume of 1 μ l was intravitreally injected into one eye (final concentration of 100 nM) using a 10 μ l Hamilton syringe (Hamilton Company, Reno Nevada). The same volume of vehicle (0.2% DMSO) was injected into the other eye (see Baba et al., 2013 for further details). All melatonin injections were given at ZT5 (i.e., 11:00 A.M.) when the endogenous melatonin level was low and ERGs were performed at ZT 6.

2.3. Immunohistochemistry

Eyes for immunohistochemistry were isolated and fixed in paraformaldehyde (PAF) 4% for 1 h and cryoprotected in sucrose 30% overnight at 4°C. Cryosections (14 μ m thick) were washed three times for 10 min in PBS and then incubated for 45 min in 1% bovine serum albumin (BSA) and 0.4% Triton-X100 in PBS to permeabilize membranes and block unspecific binding. Sections were incubated overnight at 4°C with primary antibodies diluted in 1% BSA and 0.03% Triton-100 (see Table 1), washed in PBS, and then incubated for 2 h at room temperature in secondary antibody (anti-rabbit conjugated with Alexa Fluor 488, 1:1000 Molecular Probes) diluted in 1% BSA in PBS. Nuclear staining was obtained with propidium iodide. After washing in PBS, they were cover-slipped with Vectashield

(Vector Laboratories). Retinal sections were visualized with a confocal microscope (Zeiss LSM700); files were processed with image manipulation software (GIMP2.8.18).

3. Results

3.1. Inhibition of PKC ζ blocks the melatonin-induced increase in the a- and b-wave of the scotopic ERG.

Figure 1 shows the results obtained by inhibiting different PKC isoforms. Inhibition of PKC ζ by PKC ζ -pseudosubstrate inhibitor (20 μ M) blocked the melatonin-induced increase in the amplitude of the a- and b-wave (two-way ANOVA, $p < 0.01$). To further investigate the role of different PKCs in the modulation of the ERGs, we tested whether intra-vitreous injection of Enzastaurin (a potent inhibitor of PKC α , PKC β , PKC γ , and PKC ϵ) has any effect on the melatonin-induced increase in the a- and b-wave of the scotopic ERGs. Enzastaurin (100 nM) did not prevent the melatonin-induced increase in the amplitude of the a-wave (Figure 2, two-way ANOVA, $p > 0.05$), thus suggesting that inhibition of these isoforms PKC α , PKC β , PKC γ , and PKC ϵ are not involved in the modulation of the melatonin-induced increase of the a-wave of the scotopic ERG. The amplitude of the b-wave was significantly decreased in the mice-treated Enzastaurin (Figure 2, two-way ANOVA, $p < 0.01$).

3.2. Administration of melatonin in PKC ζ KO mice does induce and increase in the amplitude of a- and b-wave of the scotopic ERG.

Figure 3 shows the result obtained with PKC ζ KO mice. The scotopic ERG of PKC ζ KO mice did not differ from that of control mice (two-way ANOVA, $p > 0.01$), and – consistently with the result obtained with the PKC ζ -pseudosubstrate inhibitor – melatonin injection (1 mg/kg) failed to induce an increase the amplitude of the a- and b-waves of the scotopic ERGs (Figure 3, two-way ANOVA, $p > 0.01$).

3.3. Administration of exogenous induces PKC ζ phosphorylation in the photoreceptor.

Figure 4 shows representative micrographs of retinal sections. Melatonin injection (i.p., 1 mg/kg) induced a significant increase in PKC ζ phosphorylation in the photoreceptors (Figure 4A). No increase in the PKC ζ phosphorylation was observed in MT₁ and MT₂ KO mice (B, C) or in mice that received the vehicle injection.

4. Discussion

Our previous work has shown that melatonin signaling via MT_{1/2h} plays an important role in the modulation of the scotopic ERGs (Baba et al., 2009; Sengupta et al., 2011; Baba et al., 2013). The data presented in this study indicate that MT_{1/2h} signaling involves

PKC ζ because inhibition of PKC ζ activity via intravitreal injection of PKC ζ -pseudosubstrate inhibitor or by genetic ablation of PKC ζ completely abolished the increase of a-wave of the scotopic ERG (see Figure 1-3). The observation that the amplitude of the a-wave at 7 ms is already significantly affected by PKC ζ inhibition or removal together with the localization PKC ζ immunoreactivity within the inner and outer segments of the

photoreceptors indicate that PKC ζ acts at this level to modulate the action of melatonin. Our data also suggest that the other PKC isoforms are not implicated in the modulation of melatonin signaling at the level of the photoreceptors (i.e., a-wave), but a significant effect was observed at the levels of the b-wave. This result suggests that one of these PKC isoforms may be implicated in the regulation of the scotopic ERGs at the level of the inner retina. Further studies will be required to identify the specific PKC isoform that is responsible for such a phenomenon.

The means by which melatonin may increase the photosensitivity of the photoreceptor is not known, but recent work in the frog *Xenopus laevis* has indicated that PLC-PKC signaling is involved in the light-dependent translocation of Arrestin1 in rod photoreceptors (Orisme et al., 2010) and our studies indicate that MT_{1/2h} does indeed signal via PLC-PKC ζ ; thus we would like to suggest that melatonin may increase photoreceptor photosensitivity by affecting the translocation of arrestin from the outer to the inner segments.

Our previous study shows that melatonin signaling is also involved in the modulating of photoreceptor viability during aging (Gianesini et al., 2016). The mechanism by which melatonin signaling supports photoreceptor health involves activation during the night of the AKT-FOXO1 pathway with the consequent downregulation of the Fas-FasL signaling (Gianesini et al., 2016; Bretagno-Sanchez et al., 2017). Because PKC ζ is known to be involved in the phosphorylation of AKT (Bandyopadhyay et al., 2002), it is possible to speculate that the decrease in the number of cones observed in MT₁^{-/-} and MT₂^{-/-} mice (Gianesini et al., 2016) may be a direct consequence of the lack of nocturnal activation of this PKC isoform. Indeed, a previous study has reported that downregulation of PKC ζ may lead to change in cone structure and premature death of these cells (Omri et al., 2013). In conclusion, our study indicates that increase in the amplitude of the a- and b-wave of the scotopic ERGs that follow the administration of exogenous melatonin is mediated by PKC ζ , as its inhibition of removal completely prevents the increase of the amplitude of the a- and b-wave. Further studies will be needed to identify the substrates activated by melatonin signaling via PKC ζ in mice photoreceptors and the mechanism by which melatonin increase. In this context, it is important to mention that our laboratory has recently shown that melatonin receptors are present and functional in a photoreceptor-like cell line, 661W (Bretagno-Sanchez et al., 2017), where they seem to activate similar pathways of those activated in mice rods and cones. Hence, the study of the substrate activated by PKC ζ in photoreceptors may be facilitated by the use of these cells.

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Melatonin plays an important role in the modulation of retinal function.

The action of melatonin on photoreceptor is mediated by melatonin receptors heterodimers.

Melatonin action on the mouse photoreceptors is mediated the PKC ζ .

Melatonin signaling may be involved in the translocation of arrestin.

Figure 1. Effect of PKC ζ inhibition on the scotopic ERG.

C3H mice were injected with melatonin (i.p. 1mg/kg); then one eye was injected (i.v.) with PKC ζ -pseudosubstrate inhibitor (i.v. 20 μ M), while the other eye was injected with vehicle. PKC ζ -pseudosubstrate inhibitor completely blocked the melatonin-induced increases in the amplitude of the 7 msec a-wave (A), a-wave (B), b-wave (C, two-way ANOVA $p < 0.01$). Black circles indicate PKC ζ -pseudosubstrate inhibitor-treated eyes; white circles indicate vehicle-treated eyes. Each point represents the mean $\pm$ standard error of the mean (SEM, $n=6$). Representative ERG traces for PKC ζ -pseudosubstrate inhibitor (black) and vehicle (gray) treated eyes are shown in D.

Figure 2. Effect of PKC α , PKC β , PKC γ , and PKC ϵ inhibition on the scotopic ERG.

Mice were injected with melatonin (i.p. 1mg/kg); then one eye was injected (i.v.) with Enzastaurin (100 nM), while the other eye was injected with vehicle. Enzastaurin did not block the melatonin-induced increase in the amplitude a-wave (A and B, two-way ANOVA $p > 0.05$), but Enzastaurin blocked the increase in the amplitude of b-wave (C, two-way ANOVA $p < 0.01$). Black circles indicate Enzastaurin-treated eye; white circles indicate vehicle-treated eyes. Each point represents mean $\pm$ SEM ($n=4$). Representative ERG traces for Enzastaurin (black) and vehicle (grey) treated eyes are shown in D.

Figure 3. Melatonin administration does not induce an increase in the amplitude of the a- and b-wave of the scotopic ERG in PKC ζ KO mice.

PKC ζ KO mice were injected with melatonin (i.p. 1mg/kg) or vehicle. Melatonin did not induce an increase in the amplitude of the a- and b-wave of the scotopic ERG (C, two-way ANOVA $p > 0.05$). Black circles indicate melatonin-treated mice; white circles indicate vehicle-treated mice. Each point represents the mean $\pm$ SEM ($n=5$). Representative ERG traces for PKC ζ KO mice injected with melatonin (black) or vehicle (grey) are shown in D.

Figure 4. Melatonin induces the phosphorylation of PKC ζ .

CH3 (A), MT $_1^{-/-}$ (B), and MT $_2^{-/-}$ (C) panels show the staining (green) obtained with anti-PKC ζ (upper) and anti-pPKC ζ (bottom) antibodies. Red staining represents the nuclei labeled with propidium iodide. (A) In CH3 mice, dark adaptation increases the levels of p-PKC ζ , and this process is augmented after the treatment with exogenous melatonin (A, middle and right panels). In melatonin receptors KO mice (B and C), there is no increase in phosphorylation of PKC ζ . OS = Outer segments; IS = inner Segments; ONL = outer nuclear layer; OPL = Outer plexiform layer; INL = inner nuclear layer; IPL = inner plexiform layer; GCs = ganglion cells layer. Micrographs (Magnification 40 X, Scale bars = 50 μ M) are representative of results obtained from at least 3 mice for each experimental condition.

Table 1.

List of antibodies used in this study.

Antibody	Host	Dilution	Supply	Application
Protein Kinase ζ	rabbit	1:250	Cell Signaling	IF
p-Protein Kinase ζ	rabbit	1:250	AbCam	IF

IF: Immunofluorescence