Heteromeric MT_1/MT_2 Melatonin Receptors Signal Via PKC-ζ in mice photoreceptors

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Keywords: melatonin, retina, PCKζ, melatonin receptors, ERG

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 PKCζ is present in the photoreceptors, thus suggesting that PCKζ may be implicated in the modulation of melatonin signaling in photoreceptors. To investigate the role PKCζ 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. PKCζ activation in photoreceptors following melatonin injection was also investigated with immunocytochemistry. Inhibition of PKCζ by PKCζ-pseudosubstrate inhibitor (20 μM) 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-vitreal injection of Enzastaurin (a potent inhibitor of PKCβ, PCKα, PKCγ, and PKCε) 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 PKCβ, PCKα, PKCγ, and PKCε are not involved in this phenomenon. Finally, our data indicated that, in mice lacking PKCζ, melatonin injection failed to increase the amplitude of the a- and b-waves of the scotopic ERGs. An increase in PKCζ phosphorylation in the photoreceptors was also observed by immunocytochemistry. Our data indicate that melatonin signaling does indeed use the PKCζ pathway to increase the amplitude of the a- and b-wave of the scotopic ERG.

1. Introduction

In the mammalian retina, the hormone melatonin is synthetized 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, Gianesini 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), PKCζ and PKCλ are primarily expressed in the photoreceptors (Ghalayini et al., 1994; Lee et al., 2007; Omri et al., 2013). PKCλ 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 PKCζ 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 PKCζ is present in photoreceptors, its role is still not well defined. Lee et al. (2007) reported that, also in mice retinas, PKCζ is mostly present in the inner and outer segments where it co-localizes with CaBP4 – a subfamily of neuronal Ca^{2+}-binding proteins – and it is involved in the phosphorylation of CaBP4. A more recent study has

implicated PKCζ in the pathology of diabetic retinopathy; most specifically, it was reported that PKCζ 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 PKCζ 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-$f^{+/+}MT_1$ knockout (KO, $MT_1^{-/-}$) and C3H-$f^{+/+}MT_2^{-/-}$ KO ($MT_2^{-/-}$) 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 and lights off (ZT 12) at 18:00 hours Eastern Standard Time (EST). 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). PKCζ KO mice in a C57/Bl6 genetic background were kindly donated by Dr. Huganir (Department of Neuroscience, John Hopkins University). 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. PKCζ 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 µM solution. The volume of 1 µl was intravitreally injected in one eye (final concentration of 20 µM) using a 10 ul 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 µM working solution. The volume of 1 µl was intravitreally injected into one eye (final concentration of 100 nM) using a 10 ul 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 EST) 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 µm 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).

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

3. Results

3.1. Inhibition of PKCζ 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 PKCζ by PKCζ-pseudosubstrate inhibitor (20 μM) 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-vitreal injection of Enzastaurin (a potent inhibitor of PKCβ, PCKα, PKCγ, and PKCε) 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 PKCβ, PCKα, PKCγ, and PKCε are not involved in the modulation of the melatonin-induced increase of the a-wave of the scotopic ERG. The

Commentato [KB1]: Do we need to say b-wave is downstream of a-wave and other PKCs might involve photo signal transduction? Statement may be confusing just looking at the data unless reader knows ERG.

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 PKCζ 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 PKCζ KO mice. The scotopic ERG of PKCζ KO mice did not differ from that of control mice (two-way ANOVA, $p>0.01$), and – consistently with the result obtained with the PKCζ-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 PKCζ phosphorylation in the photoreceptor. Figure 4 shows representative micrographs of retinal sections. Melatonin injection (i.p., 1mg/kg) induced a significant increase in PKCζ phosphorylation in the photoreceptors (Figure 4A). No increase in the PKCζ phosphorylation was observed in MT_1 and MT_2 KO mice 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 PKCζ because inhibition of PCKζ activity via intravitreal injection of PKCζ-pseudosubstrate inhibitor or by genetic ablation of PKCζ completely abolished the increase of a-wave of the scotopic ERG. The observation that the amplitude of the a-wave at 7 ms is already significantly affected by PKCζ inhibition or removal together with the localization PKCζ

immunoreactivity within the inner and outer segments of the photoreceptors indicate that PKCζ 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 has indicated that PLC-PKC signaling is involved in the light-dependent translocation of arrestin via PLC-PKC signaling (Orisme et al., 2010) and our studies indicate that $MT_{1/2h}$ does indeed signal via PLC-PKCζ; 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 PKCζ 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 $MT_1^{-/-}$ and $MT_2^{-/-}$ 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 PKCζ 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

PKCζ, 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 PCKζ 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 PKCζ in photoreceptors may be facilitated by the use of these cells.

Acknowledgments

This work was supported by: grants from the National Institutes of Health Grants EY022216, EY026291, GM116760 to K.B. and by 5U54NS083932, S21MD000101, G12-RR03034, U54RR026137 to Morehouse School of Medicine.

References

Baba, K., Benleulmi-Chaachoua, A., Journe, A.S., Kamal, M., Guillaume, J.L.,Dussaud, S., Gbahou, K., Yettou, C., Liu, C., Contreras-Alcantara, S., Jockers, R., Tosini, G., 2013. Heteromeric MT1/MT2 Melatonin receptor modulate photoreceptor function. Sci. Signal. 6, ra89.

Baba, K., Pozdeyev, N., Mazzoni, F., Contreras-Alcantara, S., Liu, C., Iuvone, P.M., Tosini, G., 2009. Melatonin modulates visual function and cell viability in the mouse retina via the MT1 melatonin receptor. Proc. Natl. Acad. Sci. USA 106, 15043-15048.

Bandyopadhyay, G., Sajan, M.P., Kanoh, Y., Standaert, M.L., Quon, M.J., Lea-Currie, R., Sen, A., Farese, R.V., 2002. PKC-zeta mediates insulin effects on glucose transport in cultured preadipocyte-derived human adipocytes. J. Clin. Endocrinol. Metab. 87, 716-723.

Ghalayini, A.J., Koutz, C.A., Wetsel, W.C., Hannun, Y.A., Anderson, R.E., 1994. Immuno-localization of PKC zeta in rat photoreceptor inner segments. Curr. Eye Res.13, 145-150.

Gianesini, C., Hiragaki, S., Laurent, V., Hicks, D., Tosini, G., 2016. Cone Viability Is Affected by Disruption of Melatonin Receptors Signaling. Invest. Ophthalmol. Vis. Sci. 57, 94-104.

Koike, C., Nishida, A., Akimoto, K., Nakaya, M.A., Noda, T., Ohno, S., Furukawa, T., 2005. Function of atypical protein kinase C lambda in differentiating photoreceptors is required for proper lamination of mouse retina. J. Neurosci. 25,10290-10298.

Lee, A., Jimenez, A., Cui, G., Haeseleer, F., 2007. Phosphorylation of the Ca2+-binding protein CaBP4 by protein kinase C zeta in photoreceptors. J. Neurosci. 27, 12743-12754.

Liang, F.Q., Aleman, T.S., Zaixin, Y., Cideciyan, A.V., Jacobson, S.G., Bennett, J., 2001. Melatonin delays photoreceptor degeneration in the rds/rds mouse. Neuroreport 12,1011-1014.

Omri, S., Behar-Cohen, F., Rothschild, P.R., Gélizé, E., Jonet, L., Jeanny, J.C., Omri, B., Crisanti, P., 2013. PKCζ mediates breakdown of outer blood-retinal barriers in diabetic retinopathy. PLoS One 8, e81600.

Orisme, W., Li, J., Goldmann, T., Bolch, S., Wolfrum, U., Smith, W.C., 2010. Light-dependent translocation of arrestin in rod photoreceptors is signaled through a phospholipaseC cascade and requires ATP. Cell Signal. 22, 447-456.

Peachey, N.S., Ray, T.A., Florijn, R., Rowe, L.B., Sjoerdsma, T., Contreras-Alcantara, S., Baba, K., Tosini, G., Pozdeyev, N., Iuvone, P.M., Bojang, P. Jr., Pearring, J.N., Simonsz, H.J., van Genderen, M., Birch, D.G., Traboulsi, EI., Dorfman, A., Lopez, I., Ren, H., Goldberg, A.F., Nishina, P.M., Lachapelle, P., McCall, M.A., Koenekoop, R.K., Bergen, A.A., Kamermans, M., Gregg, R.G., 2012. GPR179 is required for depolarizing bipolar cell function and is mutated in autosomal recessive complete congenital stationary night blindness. Am. J. Hum. Genet. 90, 331-339.

Pinzon-Guzman, C., Zhang, S.S., Barnstable, C.J., 2011. Specific protein kinase C isoforms are required for rod photoreceptor differentiation. J. Neurosci. 31, 18606-18617.

Sánchez-Bretaño, A., Baba, K., Janjua, U., Piano, I., Gargini, C., Tosini, G., 2017. Melatonin partially protects 661W cells from H_2O_2-induced death by inhibiting Fas/FasL-Caspase-3. Mol. Vis. 23, 844-852.

Sengupta, A., Baba, K., Mazzoni, F., Pozdeyev, N.V., Strettoi, E., Iuvone, P.M., Tosini, G., 2011. Localization of melatonin receptor 1 in mouse retina and its role in the circadian regulation of the electroretinogram and dopamine levels. PLoS One 6, e24483.

Tosini, G., Baba, K., Hwang, C.K., Iuvone, P.M., 2012. Melatonin: an underappreciated player in retinal physiology and pathophysiology. Exp. Eye Res. 103, 82-89.

Wiechmann, A. F., Summers, J.A., 2008. Circadian rhythms in the eye: the physiological

significance of melatonin receptors in ocular tissues. Prog. Retin. Eye Res. 27, 137-160.

Xu, X.J., Wang, S.M., Jin, Y., Hu, Y.T., Feng, K., Ma, Z.Z., 2017. Melatonin delays photoreceptor degeneration in a mouse model of autosomal recessive retinitis pigmentosa. J. Pineal Res. 63, e12428.

Caption for Figures

Figure 1. *Effect of PKCζ inhibition on the scotopic ERG*. C3H mice were injected with melatonin (i.p. 1mg/kg); then one eye was injected (i.v.) with PKCζ-pseudosubstrate inhibitor (i.v. 20 μM), while the other eye was injected with vehicle. PKCζ-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 PKCζ-pseudosubstrate inhibitor-treated eyes; white circles indicate vehicle-treated eyes. Each point represents the mean ± standard error of the mean (SEM, $n=6$). Representative ERG traces for PKCζ-pseudosubstrate inhibitor treated (Black) and vehicle treated eyes (Gray) are shown in D.

Figure 2. *Effect of PCKα, PKCβ, PKCγ, and PKCε 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 ± SEM ($n=4$). Representative ERG traces for Enzastaurin treated (Black) and vehicle treated eyes (Gray) 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 PKCζ KO mice.* PCKζ 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 ± SEM ($n=5$). Representative ERG traces for $PKCζ^{-/-}$ mice injected with melatonin (Black) or vehicle (gray) are shown in D.

Figure 4. *Melatonin induces the phosphorylation of PKCζ.* C3H (A), $MT_1^{-/-}$ (B), and $MT_2^{-/-}$ (C) panels show the staining (green) obtained with anti-PKCζ (upper) and anti-pPKCζ (bottom) antibodies. Red staining represents the nuclei labeled with propidium iodide. (A) In C3H mice, dark adaptation increases the levels of p-PKCζ, and this process is augmented after the treatment with exogenous melatonin. In melatonin receptors KO mice (B and C), there is no increase in phosphorylation of PKCζ ($n=3$).