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The role of calcium in the regulation of melatonin biosynthesis in the retina

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Abstract. Vertebrate retina rhythmically produces melatonin, a hormon involved in the regulation of several intraocular processes cued by environmental lighting conditions. Calcium ions play an important role in the induction process of serotonin N-acetyltransferase (NAT), a key regulatory enzyme in melatonin biosynthetic pathway. The physiological, i.e. nocturnal, increase of NAT activity in the retina depends on transmembrane transport of Ca^{2+} through the L-type of voltage-sensitive calcium channels. It is suggested that Ca^{2+} may regulate NAT activity indirectly, by affecting the intracellular cyclic AMP content, which is, in turn, critical in the regulation of melatonin biosynthesis. The mode and mechanisms of Ca^{2+} action on processes governing melatonin formation in the retina are discussed.

Key words: calcium ions, retina, melatonin biosynthesis

Mini-review

INTRODUCTION

Since its isolation and identification in the late 1950s by Lerner and coworkers (Lerner et al. 1958), melatonin (N-acetyl-5-methoxytryptamine) has attracted the attention of increasing number of scientists. Although it was once thought that melatonin is a unique product of pineal gland, numerous experimental data, accumulated mainly during the last decade, point to the presence of this compound in other parts of the central nervous system, notably in the retina (for review see Cahill et al. 1991, Zawilska and Nowak 1992). Thus, melatonin-like immunoreactivity has been found in retinas of several vertebrates, and the presence of authentic melatonin in chicken, rabbit, bovine and human retina has been established using HPLC with electrochemical detection and gas chromatography-mass spectroscopy (e.g. Bubenik et al. 1976, Hamm and Menaker 1980, Reppert and Sagar 1983, Leino 1984, Osol and Schwartz 1984, Hall et al. 1985, Nowak 1987, Zawilska and Iuvone 1989). Melatonin-like immunoreactivity in retina persists after pinealectomy (Hamm and Menaker 1980, Yu et al. 1981, Reiter et al. 1983), indicating that retinal melatonin is not of pineal origin but, rather, may be synthesized locally, within the eye. In line with this, it is now well documented that the retina contains the enzymatic machinery necessary for melatonin formation, and the synthesis in this tissue of radiolabeled melatonin from its precursors has been demonstrated (e.g. Cardinali and Rosner 1971, Gern and Ralph 1979, Nowak 1987, Redburn and Mitchell 1989; see also Zawilska and Nowak 1992). Although the ultimate evidence has not, so far, been provided, considerable data suggest that the melatonin biosynthesis and release in the retina are associated mainly with photoreceptor cells. Once released, melatonin acts as a biochemical and endocrine signal representing darkness, and exerts its actions at numerous target sites within the retina (for reviews see: Zawilska and Nowak 1989, 1992, Krause and Dubocovich 1990, Zawilska 1992).

The biosynthesis of melatonin proceeds *via* N-acetylation and subsequently O-methylation of

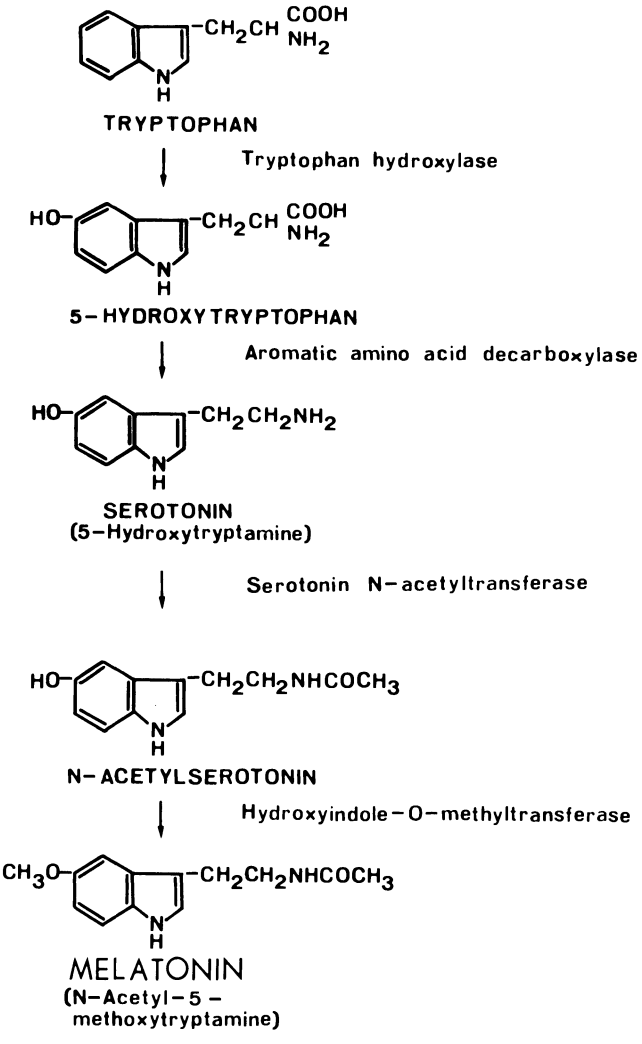

Fig. 1. Biochemical pathway for synthesis of melatonin from L-tryptophan.

serotonin (5-hydroxytryptamine). This two-step reaction involves sequential actions of two enzymes: serotonin N-acetyltransferase (NAT; EC 2.3.1.87) and hydroxyindole-O-methyltransferase (HIOMT; EC 2.1.1.4) (Fig.1). Melatonin is synthesized at night (or a dark phase of any imposed light-dark illumination cycle), following a circadian rhythm resulting from both endogenous mechanisms and environmental cues. The rhythmic formation of the hormone appears to be regulated primarily by distinct daily changes in the activity of NAT (which fluctuates in parallel to melatonin levels; Fig. 2),

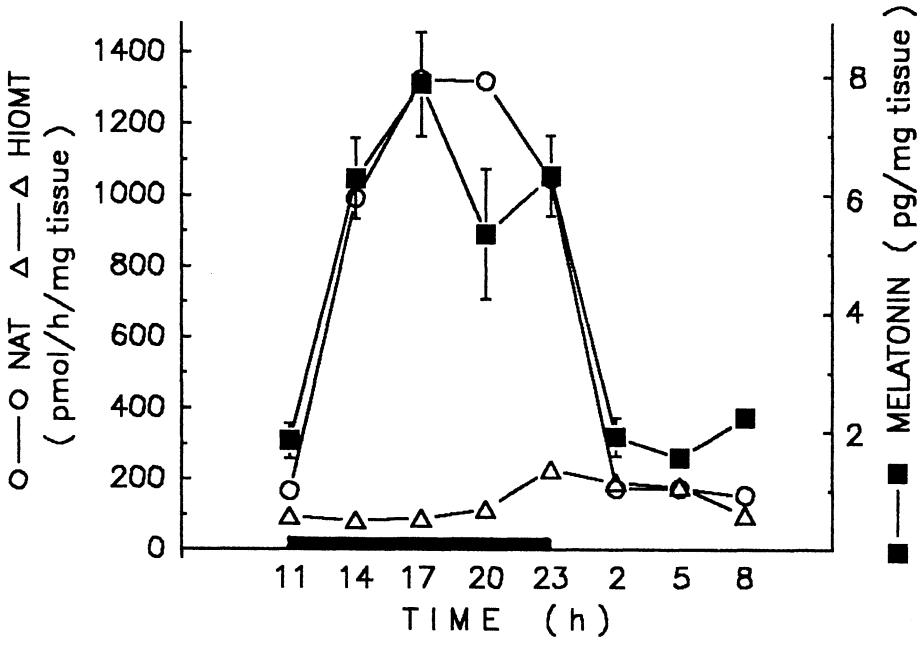

Fig. 2. Diurnal changes in serotonin N-acetyltransferase (NAT) activity, hydroxyindole-O-methyltransferase (HIOMT) activity, and melatonin content of chicken retina. Lights off between 23:00-11:00. The nocturnal elevation of NAT activity is closely correlated with the rhythm of melatonin. The enzymes' activities in tissue homogenates were determined by means of radioisotopic microassayes, as described in details by Nowak et al. (1989). Melatonin content was measured using a specific radioimmunoassay kit (DRG). Data shown are means ± SEM (n = 5-6/group for NAT and HIOMT, and n= 4/group for melatonin).

with an absolute amount of the synthesized melatonin being additionally dependent on serotonin availability during the whole period of high NAT activity. The nocturnal increase of both NAT activity and melatonin content of the retina are dramatically suppressed by acute light exposure. In contrast, the activity of HIOMT is only slightly affected by changes in the environmental illumination (Zawilska and Nowak 1992).

Despite a continuous effort which has been devoted to establishing molecular mechanisms underlying the rhythmic melatonin production, our knowledge on this topic is still far from complete. Both in the pineal gland and retina the regulatory mechanisms for melatonin content are directed at the level of synthesis. NAT is an inducible enzyme, and an increase of intracellular cyclic AMP level seems to be an essential step in the induction and maintenance of high NAT activity (e.g. Morrissey and Lovenberg 1975, Deguchi 1979, Iuvone and Besharse 1983). In the pineal glands of rat and chick, and in the retinas of at least lower vertebrates, the cyclic AMP-dependent elevation of NAT activity and melatonin production requires protein synthesis and is probably regulated at the level of gene expression (Iuvone and Besharse 1983, Takahashi et al. 1989, Iuvone et al. 1990, Reiter 1991). Additionally, in the rat and chick pineal gland calcium influx appears to contribute significantly to the regulation of melatonin biosynthesis (e.g. Zatz and Mullen 1988, Sugden 1989, Zatz 1989, Zawilska and Nowak 1990, 1991). An important role of Ca^{2+} in regulation of the process of NAT induction has recently been postulated for the retina as well.

INVOLVEMENT OF CA^{2+} IN THE STIMULATION OF RETINAL NAT ACTIVITY *IN VITRO*

In studies performed on cultured eye cups of African clawed frog, *Xenopus laevis*, Iuvone and Besharse (1986) demonstrated for the first time that an

increase of the retinal NAT activity that occurs at night (in the dark) is mediated by a calcium-dependent process. Omitting $CaCl_2$ from the incubation medium completely blocked the increase of NAT activity in *Xenopus* eye cups incubated in darkness. It has been found that 10^{-4} - 10^{-3} M of free Ca^{2+} is required for the maximal stimulation of the enzyme activity in the dark. The effect of Ca^{2+} appears to be specific, since other divalent cations, i.e. Ba^{2+}, Sr^{2+} and Mn^{2+}, did not substitute for Ca^{2+}. Both organic (nifedipine and methoxyverapamil) and inorganic (Co^{2+} and Mg^{2+}) antagonists of voltage-sensitive calcium channels (VSCC) potently inhibited the nocturnal rise in NAT activity of *Xenopus* retina. On the basis of these studies, it has been suggested that an influx of Ca^{2+}, through VSCC, from the outside into the cytoplasm of the melatonin-synthesizing cells (i.e. photoreceptors), rather than an intracellular mobilization of these ions, is a necessary step to increase the retinal NAT activity.

The hypothesis that the transmembrane transport of Ca^{2+} is critical to melatonin synthesis in the retina is supported by several observations in other systems. The ability of forskolin and 3-isobutyl-1-methylxanthine (IBMX) to stimulate NAT activity in isolated hen and rat retina was highly attenuated and enhanced by omitting and increasing (from 1.3 to 3.9 mM) $CaCl_2$ concentration in the Krebs-Ringer incubation medium (Nowak 1990; J.Z. Nowak, personal communication). In photoreceptor-enriched monolayer cultures of chick embryo retinal cells K^+-evoked depolarization significantly increased NAT activity. The effect of high K^+ (35 mM) was completely blocked by an introduction to the incubation medium of EGTA, a chelator of Ca^{2+}, and significantly decreased by antagonists of VSCC, such as nifedipine, methoxyverapamil, Mn^{2+}, Mg^{2+} and Cd^{2+} (Avendano et al. 1990). Interestingly, a potent and selective VSCC agonist Bay K 8644, a 1,4-dihydropyridine that increases Ca^{2+} influx through the L-type of VSCC by promoting longer duration of channel openings (Hess et al. 1984), when added alone to the chick retinal cell cultures elevated the basal NAT activity in a nifedipine-sensitive manner. Bay K 8644 potentiated the K^+-evoked rise in the enzyme activity as well (Avendano et al. 1990). All these data strongly suggested that the effectiveness of K^+-induced depolarization of photoreceptor membrane to stimulate NAT activity in cultured retinal cells, is, in all likelihood, related to an influx of Ca^{2+} through the L-type of VSCC. Noteworthy, photoreceptors, a retinal cell compartment where melatonin biosynthesis primarily takes place (see Cahill et al. 1991, Zawilska and Nowak 1992), are depolarized in darkness and hyperpolarized in light-exposed retinas (e.g. Werblin and Dowling 1969, Hagins et al. 1970). Inner segments and synaptic terminals of photoreceptors contain VSCC (e.g. Fain et al. 1980, Corey et al. 1984) that are opened in darkness (i.e. when NAT activity and melatonin content are high) and closed as a consequence of light exposure (which decreases melatonin formation). These Ca^{2+} channels are blocked by Co^{2+}, Mg^{2+} and methoxyverapamil (Fain et al. 1980) at concentrations similar to those that inhibit an increase of NAT activity in the dark (Iuvone and Besharse 1986). Thus, it appears likely that in intact retinas, when photoreceptors depolarize in darkness, an increase in free Ca^{2+} concentration inside the inner segments of photoreceptors (resulting from Ca^{2+} influx through VSCC) activates the process of NAT induction, and consequently enhances the melatonin formation and level*. It remains to be established, however, whether Ca^{2+} by itself regulates the process of NAT induction, or, what seems more likely,

*Calcium ions seem to regulate different aspects of photoreceptor physiology. Thus, for example, Ca^{2+} are involved in the regulation of melatonin biosynthesis, which occurs mainly in inner segments of photoreceptive cells. Additionally, Ca^{2+}, by affecting the level of cyclic GMP in outer segments of photoreceptors, function as a key factor in the regulation of the process called photoreceptor light adaptation (i.e. changes in sensitivity of photoreceptors reciprocal to background illumination). This phenomenon enables the vertebrate visual system to efficiently operate over a large range of light intensities. A rise in Ca^{2+} lowers the cyclic GMP, and a fall in Ca^{2+} raises the cyclic GMP content. Photoexcited rhodopsin triggers a cascade of cellular events leading to the stimulation of cyclic nucleotide phosphodiesterase (PDE) activity, with a subsequent drop in the level of cyclic

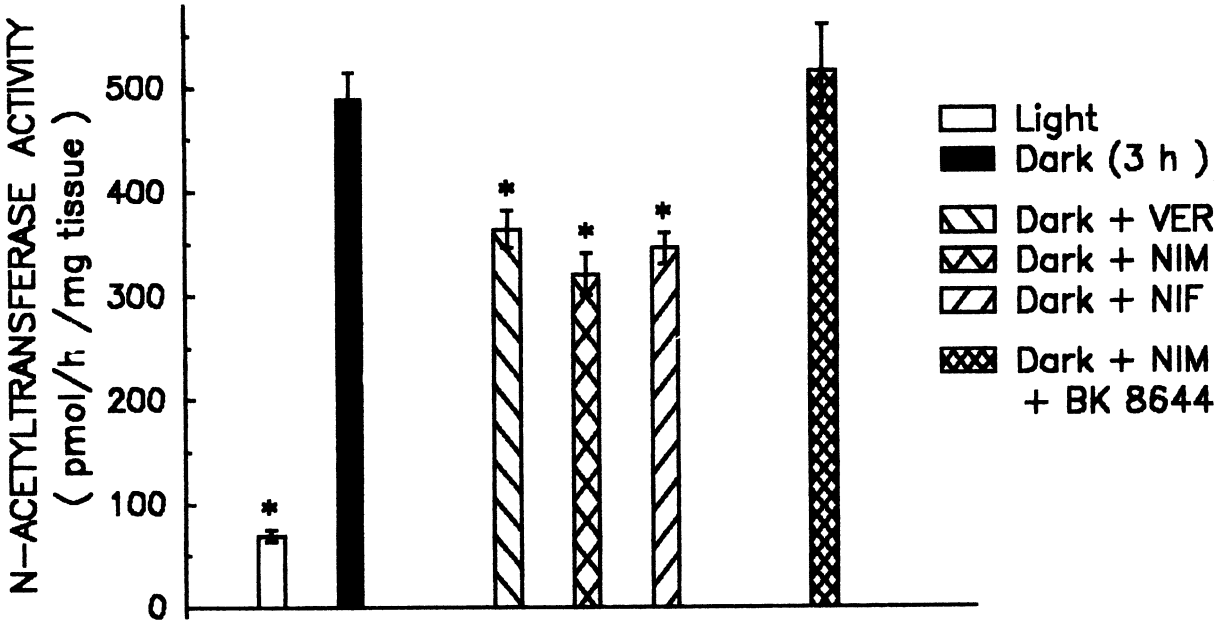

Fig. 3. VSCC antagonists inhibit *in vivo* the nocturnal increase of NAT activity of chicken retina. Chickens were maintained on a 12:12 h light-dark illumination schedule. Verapamil (VER), nimodipine (NIM) and nifedipine (NIF), each at a dose of 10 mg/kg, were administerd i.p. at the end of the light phase. Bay K 8644 (BK; 1 mg/kg, i.p.) was given 30 min after NIM injection. Chickens were kept in darkness, and were sacrificed 3 h after the administration of VSCC antagonists. Retinas were isolated, frozen and assayed for NAT activity (as described by Nowak et al. 1989). "Light" represents measurements on tissues that were dissected and frozen at the time of light-offset of the light-dark cycle. "Dark" refers to the enzyme activity in tissues isolated from chickens injected with vehicle and sacrificed at approximately the same time as drug-treated animals. Values are means ±SEM (n = 5-6/group). $^{*}P$ <0.05 vs. Dark. Note that Bay K 8644 abolishes the suppressive effects of NIM on the night time NAT activity of chicken retina.

it affects some mechanisms (e.g. synthesis or inactivation) related more with the cyclic AMP system (see below).

CA^{2+} INFLUX THROUGH VSCC REGULATES *IN VIVO* NAT ACTIVITY IN THE RETINA

Various compounds that bind to and block the L-type of VSCC, i.e. verapamil and 1,4-dihydropyridines such as nimodipine, nifedipine and nitrendipine, have recently been found to effectively suppress *in vivo* the nocturnal NAT activity in the vertebrate retina (Fig. 3)(Zawilska and Nowak 1990, Zawilska et al. 1992). The tested drugs displayed similar potency in decreasing the night-driven enzyme activity when they were administered to chickens prior to the light offset and during the dark phase of an imposed light-dark illumination cycle. This inhibitory effect of the VSCC antagonists on the retinal NAT activity was apparently related to their Ca^{2+}-blocking properties, since the agonist of VSCC, Bay K 8644, abolished the decline in the nighttime rise of the enzyme activity produced by nimodipine and nifedipine. Furthermore, Bay K 8644 given to chickens at the end of the light phase of the light-dark cycle, markedly

GMP. Light-sensitive (cyclic GMP-gated) ionic channels in outer segments of photoreceptors, therefore, close down causing a hyperpolarization of the plasma membrane, and a decrease of an internal Ca^{2+} concentration (for a review see Yau and Baylor 1989). The decline in Ca^{2+}, in turn, stimulates guanylate cyclase, and probably also inhibits PDE, helping to restore the concentration of cyclic GMP to the dark level (Fig. 5)(e.g. Kawamura and Murakami 1989, Koch and Stryer 1988, Matthews et al. 1988, Nakatani and Yau 1988; see also Tamura et al. 1991, Yau and Baylor 1989). This effect is expected to shorten the "visual excitation" triggered by a photon absorption.

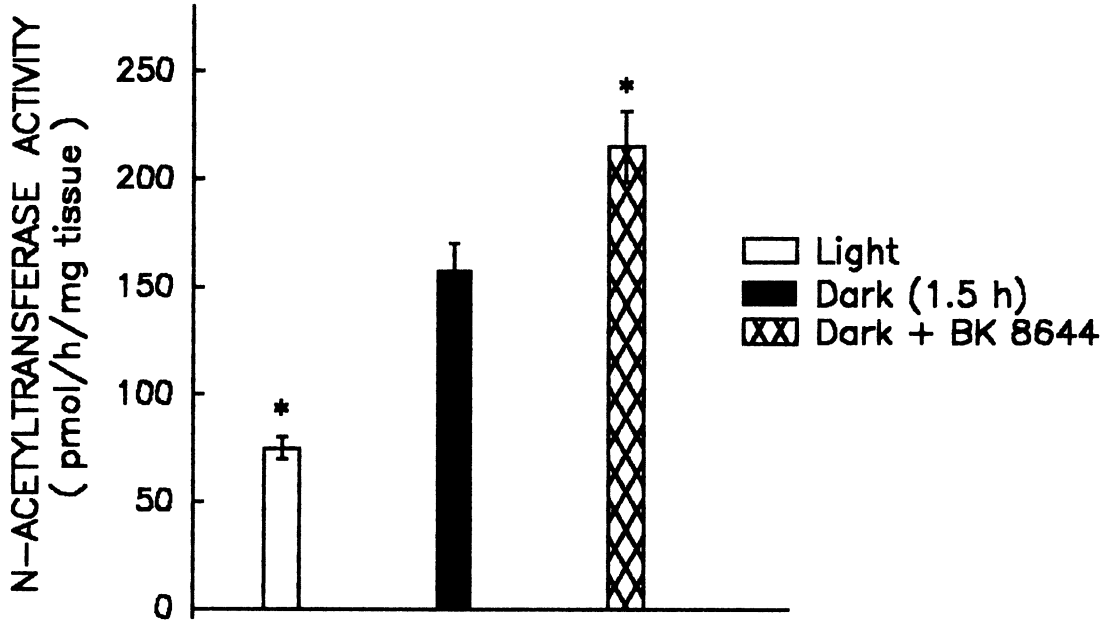

Fig. 4. VSCC agonist, Bay K 8644, enhances the nocturnal increase of NAT activity in chicken retina. Bay K 8644 was administered i.p. just before the end of the light phase of the light-dark illumination cycle at a dose of 1 mg/kg. Chickens were sacrificed after 1.5 h in darkness. Values are means ±SEM (n = 5-6/group) $^{*}P$ vs. Dark. For methodological details see Fig. 3.

potentiated the nocturnal increase of NAT activity in the retina (Fig. 4). Similar observations have been made for pineal glands of hen (Zawilska and Nowak 1990) and rat (Zawilska and Nowak 1991). The inhibitory and stimulatory effect of the VSCC antagonists and agonist, respectively, on the nocturnal increase of the retinal (as well as pineal) NAT activity in living animals gives an additional support to the idea that the transmembrane transport of Ca^{2+} into the cytoplasm of the melatonin-synthesizing cells is a physiological signal involved in a complex process of NAT induction.

DOES CA^{2+} REGULATES MELATONIN PRODUCTION IN THE RETINA THROUGH THE CYCLIC AMP SYSTEM?

The results of the studies discussed above set the stage for a search of molecular mechanism(s) underlying the stimulatory action of Ca^{2+} on processes governing melatonin formation in the retina. Although the ultimate evidence has not been provided thus far, accumulating data suggest that Ca^{2+} may regulate NAT activity indirectly, by affecting an intracellular cyclic AMP content, which is, in turn, critical in the regulation of melatonin biosynthesis (see Zawilska and Nowak 1992). It has been demonstrated that in the rat pineal gland, the β-adrenoceptor-induced increase in cyclic AMP level and NAT activity is potentiated by activation of α_1-adrenoceptors (Auerbach et al. 1981, Klein et al. 1983), in a mechanism that involves α_1-adrenoceptor-stimulated Ca^{2+} influx into pinealocytes (Sugden et al. 1986). In cultured chick pineal cells Ca^{2+} influx through the L-type of VSCC, acting *via* cyclic AMP, potentiates melatonin biosynthesis and release (Zatz 1989). Consistent with this, the K^{+}-evoked depolarization stimulates cyclic AMP accumulation and NAT activity in photoreceptor-enriched chick retinal cell cultures in a process that involves activation of the L-type of VSCC (Avendano et al. 1990, Iuvone et al. 1991a,b). The increases of both cyclic AMP accumulation and NAT activity elicited by K^{+} were synergistically enhanced by PDE inhibitors, and blocked by MDL 12330A, an adenylate cyclase inhibitor (Iuvone et

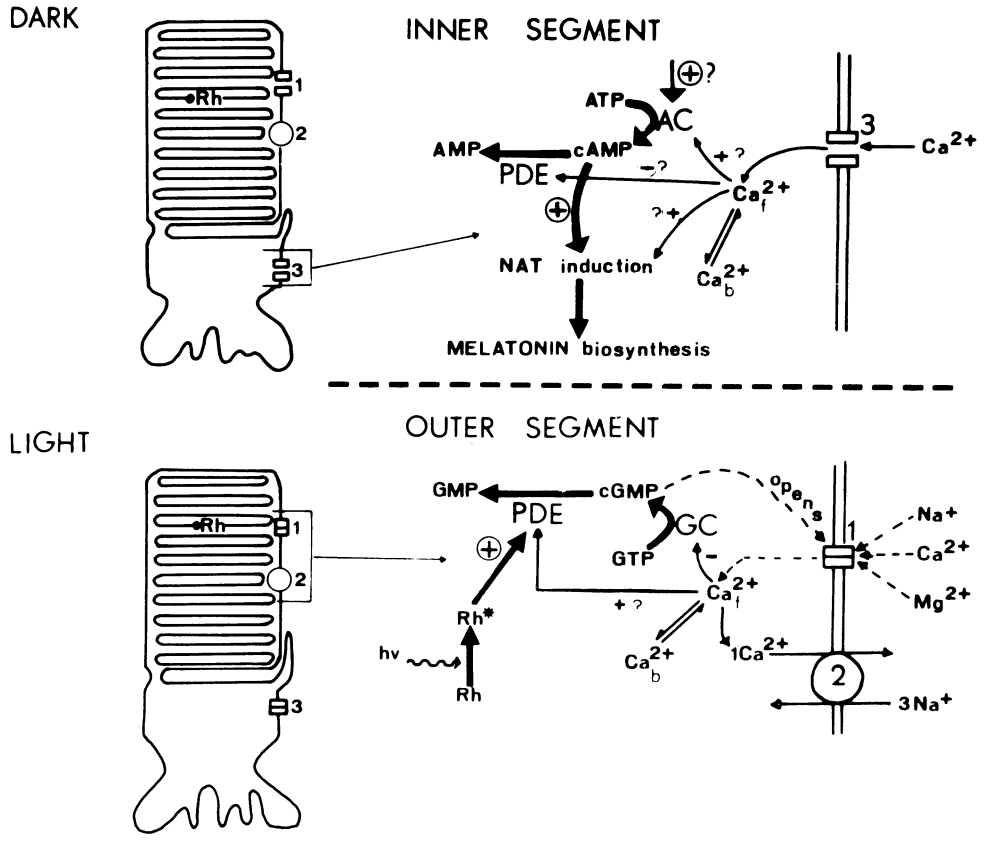

Fig. 5. Model for mechanisms underlying the regulatory roles of calcium ions in photoreceptor physiology. Symbols: 1, light-sensitive (cGMP-gated) ionic channels; 2, Na^+-Ca^{2+} exchange carrier; 3, voltage-sensitive calcium channel; Ca^{2+}_f, free calcium ions; Ca^{2+}_b, bound calcium ions; AC, adenylate cyclase; PDE, cyclic nucleotide phosphodiesterase; GC, guanylate cyclase; Rh rhodopsin; Rh^*, photoexcited rhodopsin; hv, photon. In darkness, VSCC localized to membrane of inner segment of a photoreceptor cell, are opened, allowing Ca^{2+} influx from the outside into the cytoplasm. An increase in cytoplasmic concentration of free Ca^{2+} is an important stimulatory signal in a cascade of biochemical events leading to a cAMP-dependent NAT induction, with a consequent rise in melatonin biosynthesis. The physiological stimulus accounting for a rise of intracellular cAMP level in photoreceptors remains unknown. The molecular mechanisms underlying the regulatory role of Ca^{2+} in the formation of retinal melatonin are poorly understood. In the dark, when intraphotoreceptor concentration of cGMP is relatively high, the light-sensitive (cGMP-gated) ionic channels in the plasma membrane of outer segment of the photoreceptor cell are also opened, allowing a steady "dark current" to enter the interior of the cell. The inward current keeps the photoreceptor partially depolarized. In darkness, there is a balance of Ca^{2+} influx and Ca^{2+} efflux; Ca^{2+} enters the inner segment of the cell through the light-sensitive ionic channel and leaves it by means of Na^+-Ca^{2+} exchange pump. Excited by flashes of light rhodopsin, Rh^*, triggers a cascade of biochemical processes leading to the stimulation of PDE. The activated PDE rapidly hydrolyzes cGMP. A fall in the intracellular concentration of cGMP causes a closure of the light-sensitive (cGMP-gated) ionic channels, with concomitant hyperpolarization of the plasma membrane. As Ca^{2+} efflux continues, the net concentration of free (internal) Ca^{2+} decreases. The decline in Ca^{2+}, in turn, appears to restore the intraphotoreceptor cGMP level to the dark level, by stimulating the acticity of GC, and probably also by inhibiting the activity of PDE.

al. 1991a,b). The concept that a requirement of Ca^{2+} in the process of NAT induction in the retina may be primarily at the level of cyclic AMP production or/and degradation received an additional support from *in vivo* studies performed on chickens. Under light conditions the basal NAT activity of the chicken retina is low. However, the treatment of the animals kept during a subjective dark phase of a light-dark cycle in an illuminated environment with various compounds capable of increasing intracellular cyclic AMP levels, i.e. (1) non-hydrolyzable analogs of the nucleotide, dibutyryl-cyclic AMP

and 8-bromo-cyclic AMP, (2) forskolin, a diterpene which directly stimulates adenylate cyclase, or (3) PDE inhibitors, aminophylline and IBMX, resulted in a marked elevation of the retinal NAT activity. Interestingly, an additional treatment of chickens with VSCC agonist (Bay K 8644), and antagonists (nimodipine, nifedipine, nitrendipine, and verapamil), did not significantly affect the increases in NAT activity of the retina produced by the examined cyclic AMP "promoters" (Zawilska et al. 1992). The inability of VSCC blockers to modify NAT activity stimulated by dibutyryl-cyclic AMP and 8-bromo-cyclic AMP suggests that these drugs most probably do not affect the process of NAT induction directly. On the other hand, the lack of effect of the VSCC drugs on increases in the enzyme activity produced by forskolin and by PDE inhibitors may favour an idea that Ca^{2+}-channel antagonists decreased the nocturnal NAT activity in the retina by either inhibiting the activity of adenylate cyclase (and, thus, slowing down the cyclic AMP production), or by activating cyclic AMP breakdown (and, thus, lowering the cyclic AMP level).

Details on the mechanism by which an increased level of Ca^{2+} inside photoreceptors, and pinealocytes, affects the cyclic AMP generating or/and inactivating system are largely unknown, and will no doubt be a subject of an intense investigation in the nearest future. Hypotheses that have recently been considered include the activation of Ca^{2+}-phospholipid-dependent protein kinase, i.e. protein kinase C (PKC), and the activation of calmodulin. An involvement of PKC in the discussed action of Ca^{2+} is strongly suggested by two pieces of evidence. Firstly, in the rat pineal gland the stimulation of α_1-adrenoceptors, by increasing an intracellular content of Ca^{2+} and diacylglycerol, appears to translocate and activate PKC, which in turn amplifies the β-adrenoceptor-mediated stimulation of cyclic AMP formation (e.g. Ho et al. 1988, Sugden and Klein 1988). Secondly, it has been demonstrated that PKC inhibitors, H7 and staurosporine, blocked the K^+-evoked induction of NAT activity in photoreceptor-enriched retinal cell cultures. Moreover, pretreatment of the cells with phorbol esters (which directly activate PKC) down-regulated PKC, and as a consequence of this, inhibited the K^+-induced stimulation of NAT activity (Iuvone et al. 1991b). On the other hand, it has been shown that the nocturnal increase of melatonin synthesis and release from chicken pineal gland is inhibited by antagonists of calmodulin , calmidazolinum, W-7 and W-13, an observation indicating a possible role of calmodulin in this cyclic AMP-dependent phenomenon (Takahashi et al. 1989). It should be also noted that Ca^{2+} entry through VSCC have recently been found to increase an intracellular cyclic AMP level in bovine chromaffin cells, an effect presumably resulting from the activation of Ca^{2+}/calmodulin sensitive isozyme of adenylate cyclase (Keogh and Marley 1991).

CONCLUDING REMARKS AND SOME EMERGING PRACTICAL CONSIDERATIONS

The discussed here data indicate that Ca^{2+} is an important physiological factor contributing to the nocturnal generation of melatonin in the vertebrate retina (and pineal gland). Although the precise mechanism of action of Ca^{2+} still remains uncovered, most of the relevant findings favour the idea that these ions affect melatonin biosynthesis by influencing primarily the formation of cyclic AMP, an intracellular messenger that directly controls the process of NAT induction. Therefore, Ca^{2+}, entering the photoreceptors through the L-type of VSCC (which are opened in darkness, i.e when photoreceptors' membrane is depolarized), play a role of a signal which activates the cascade of biochemical events responsible for the formation of melatonin.

The discussed results, especially those coming from *in vivo* experiments with the use of VSCC antagonists, e.g. verapamil, nimodipine or nifedipine, might have some practical implications, and, therefore, seem worth mentioning. Several VSCC blockers, including derivatives of 1,4-dihydropyridine, have found broad appeal as therapeutic agents. Such drugs, being used in some cardiovascular and neuropsychiatric diseases, effectively alleviate

various pathological symptoms in suffering patients. However, following their application (especially a long-term one), they could lead to a blockade of melatonin biosynthesis. At present, the consequences of melatonin deficiency in humans, and particularly in the retina, are not recognized, and until then the problem may be "invisible" for practical medicine. Yet, it may appear profitable to be aware of such a possibility, simply in order to better understand the profile of these drugs' action.

ABBREVIATIONS

HIOMT	hydroxyindole-O-methyltransferase
HPLC	high performance liquid chromatography
IBMX	3-isobutyl-1-methylxanthine
NAT	serotonin N-acetyltransferase
PDE	cyclic nucleotide phosphodiesterase
PKC	protein kinase C
VSCC	voltage-sensitive calcium channels

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