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Calmodulin mediates melatonin cytoskeletal effects

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Abstract. In this article, we review the data concerning melatonin interactions with calmodulin. The kinetics of melatonin-calmodulin binding suggest that the hormone modulates cell activity through intracellular binding to the protein at physiological concentration ranges. Melatonin interaction with calmodulin may allow the hormone to modulate rhythmically many cellular functions. Melatonin's effect on tubulin polymerization, and cytoskeletal changes in MDCK and N1E-115 cells cultured with melatonin, suggest that at low concentrations (10^{-9} M) cytoskeletal effects are mediated by its antagonism to Ca^{2+}-calmodulin. At higher concentrations (10^{-5} M), non-specific binding of melatonin to tubulin occurs thus overcoming the specific melatonin antagonism to Ca^{2+}-calmodulin. Since the structures of melatonin and calmodulin are phylogenetically well preserved, calmodulin-melatonin interaction probably represents a major mechanism for regulation and synchronization of cell physiology.

Key words. Melatonin; calmodulin antagonist; mechanism of action; microtubules; N1E-115 cells; MDCK cells.

Introduction

The discovery of the pineal hormone, melatonin (MEL), by Lerner et al. in 1958[49] prompted major research efforts to elucidate its physiological role. A broad spectrum of metabolic and physiological effects of MEL has been recognized in a wide variety of species ranging from unicellular organisms and plants to higher vertebrates[24]. However, the precise physiological role that MEL plays in different species has yet to be established. Currently, it is accepted that the rhythmic synthesis and release of MEL is due to a circadian system that in mammals comprises the retina, the suprachiasmatic nucleus and the pineal gland[57]. Circulating plasma levels of MEL are thought to be a synchronizing signal that plays a central role in the control of the circadian rhythms of many species of reptiles, birds, and mammals (for review see ref. 27). It is accepted that exogeneous MEL is able to reset most rhythms in vertebratres[27] and to modify circadian rhythmic activities in plants[10] and unicellular organisms[8]. Although still controversial, in humans MEL may be involved in sleep regulation[4,34], affective disorders[69], ageing[64], and cancer[52].

Despite the knowledge gained over the years, basic questions about the mechanism of action of MEL remain unanswered. There is no general agreement about either the cell type that recognizes the MEL signal or the mechanism that triggers the metabolic cascade, resulting in a cellular response. Several anatomical and cellular sites and mechanisms of action have been proposed for the hormone (for reviews see refs 24 and 74). At present, many studies support the existence of specific MEL membrane receptors, mainly in the neurons of the Central Nervous System (CNS) associated with known MEL effects[46,74]. However, the multiple metabolic and cellular responses that have been described after MEL administration suggest that the hormone could act through other mechanisms. We have proposed that one such mechanism is through the interaction of the hormone with calmodulin (CaM). In the present paper we will review the evidence in support of this hypothesis. In order to demonstrate experimentally the existence of a hormone mechanism of action a step-by-step analysis of signal transduction from stimulus to response is required. We will review the available data following the same sequence.

Melatonin target cells

The first step in the recognition of a hormone signal is access of the signal to its target cells. The broad metabolic and physiological effects of MEL described in both plant and animal kingdoms suggest that the hormone has access to many kinds of cells. This is consistent with the chemical structure of the hormone. Melatonin (5-methoxy-N-acetyltryptamine) is a small, highly lipophilic molecule that would be expected to pass easily through the lipid bilayer of cellular membranes. We might expect MEL to behave more like other lipophilic hormones such as steroids than like hydrophilic ones such as proteins and peptides. Experimental evidence supports this hypothesis. Experiments with radioactive MEL (labelled with ^{3}H or ^{14}C), show that

after its systemic administration to mice, despite its rapid rate of disappearance from blood (about 2 min), it is found in all tissues. Certain tissues, such as the adrenal gland and small intestine, retain the hormone after 30 min in a higher concentration than that of the plasma[45]. Because the remarkable effects of MEL on the CNS, the fate and regional distribution of ^{3}H-MEL in the brain is well known. After either intraperitoneal, intravenous, intracerebroventricular, or intracisternal administration of ^{3}H-MEL to rats[3,23,79], the hormone is found in all brain regions and highly concentrated in the midbrain and hypothalamus. Evidence that MEL enters the neurons was obtained by studying the intracellular distribution and fate of intracerebroventricularly administered ^{3}H-MEL[5]. At all times studied (from 1 min to 48 h) the major proportion of the radioactivity remained in the cytosol with steadily decreasing proportions in the purified nuclear fraction, mitochondrial fraction, and the microsomes. Moreover, unchanged ^{3}H-MEL was found associated with a cytosolic brain protein with a molecular weight lower than 29 K. The results concur with those reported by Cardinali[26] showing that in the rat CNS, ^{3}H-MEL binds with high affinity to mitochondrial, microsomal and nuclear fractions. They also agree with those of Cohen et al.[32] and Niles et al.[59], reporting a ^{3}H-MEL-specific binding to cytosol from various tissues of different mammalian species.

Immunohistological experiments showed that the hormone can be detected in the rat salivary gland[22], retina, cerebellum[20], and digestive system[21]. Although the mechanism (biosynthesis, uptake) accounting for the presence of MEL in the cells is not clear, some uptake is known to occur since cellular MEL content increases after hormone administration. Recently, evidence that MEL enters the cell was obtained by incubating MDCK cells with MEL and analyzing its intracellular distribution by a double immunofluorescence method. The hormone was not detected in control cells, but was found in MEL treated cells. As early as 3 h after incubation, MEL was detected as fluorescent spots beneath the plasma cell membrane, all over the cytoplasm and the nucleus (unpublished results). Thus, due to its high lypophilicity, MEL may enter different kinds of cells, crossing the cell membrane into the intracellular space. Once there, MEL is distributed to different cellular compartments.

Melatonin interaction with calmodulin

Although MEL enters into cells, the hormone target must recognize the signal and translate it into metabolic events that will result in a cellular response. Initially it was suspected that CaM could be involved in intracellular MEL signal recognition because changes in CaM activity could be compatible with the hormone effects found on the MDCK and N1E-115 cytoskeletal structure[14]. Other experiments had suggested a physical interaction between CaM and the pineal hormone[15]. However, it was not until recently that direct assessment of MEL-CaM binding as well as its kinetic constants was made[18]. When Ca^{2+}-CaM-dependent phosphodiesterase activity was measured in the presence of various MEL concentrations, basal phosphodiesterase activity was not modified but enzyme activation by CaM was inhibited by MEL in a dose-dependent manner[15]. In addition, electrophoretic separation of the products after the incubation of ^{3}H-MEL with CaM showed that ^{3}H-MEL co-migrates with CaM only if Ca^{2+} is present in the incubation mixture. Furthermore, when an excess of MEL (10^{-5} M) was incubated with CaM in the presence of Ca^{2+}, a change in the Ca^{2+}-CaM relative mobility (Ca^{2+}-CaM shift) was observed[15].

The most common method for assessing the kinetic constants of ligand binding to CaM is through equilibrium dialysis[50]; however, these constants are difficult to obtain due to the very low signal/noise ratio of the method. Recently we assessed CaM-MEL binding by a combination of CaM incorporation into liposomes and separation of free and bound ^{3}H-MEL by a rapid ultrafiltration method[58]. With the use of this system, it was found that MEL binding to CaM fulfills the major criteria for considering CaM as a receptor for MEL. The binding is saturable, reversible, Ca^{2+}-dependent, and of high affinity[18]. Saturation and association-dissociation studies revealed that ^{3}H-MEL binds to a single site with a Kd of 188 pM and a total binding capacity (Bmax) of 35 pM/ug of CaM[18]. The relative ability of some related compounds to displace ^{3}H-MEL binding was as follows: MEL > 6-Chloromelatonin > 6-Hydroxymelatonin > Luzindole > Trifluoperazine (TFP). Ki values were 0.193 ± 0.050, 2.3 ± 1.1, 7.1 ± 2, 2743 ± 415, >5000 nM, respectively[18]. Assessment of Ki values for serotonin, N-acetylserotonin and N-acetyltryptamine could not be obtained because of their hydrophilicity. Among other proteins examined (creatine phosphokinase, tubulin, bovine serum albumin, egg albumin and CaM), only CaM possessed a high affinity Ca^{2+}-dependent binding site for MEL.

CaM is an ubiquitous Ca^{2+} binding protein that relays Ca^{2+} signals to many target proteins[31]. Ca^{2+}-CaM acts both directly by interacting with key target enzymes, e.g. brain adenylate cyclase and phosphodiesterase[70,72], and structural proteins, e.g. microtubule binding proteins (MAPs) and tubulin[48], and indirectly via specific protein kinases[44]. CaM activates at least five protein kinases and a single protein phosphatase, calcineurin[44], and thereby can modulate the activities of the many substrates of these enzymes. In the absence of Ca^{2+} the CaM polypeptide chain has a random structure that undergoes pronounced conformational changes and adopts a fixed and well-defined structure

following Ca^{2+} binding. This is the active form (Ca^{2+}-CaM)[9]. Upon Ca^{2+} activation, CaM exposes hydrophobic regions where CaM target proteins may bind[76]. Inhibitors bind to CaM at, or near, the binding sites of target enzymes and thus prevent their association with CaM[77]. Unlike known CaM antagonists (peptides and drugs) which bind CaM in the micromolar range, MEL binds to the protein in the picomolar range[18]. The high affinity binding of MEL to CaM suggests that the hormone is able to modulate cell activity by intracellularly binding to CaM at physiological concentration ranges.

Because of the chemical structure of MEL, we suspect that the hormone will share physicochemical and pharmacological properties with CaM antagonists such as phenothiazines and naphthalensulfonamides. Much attention has been devoted to the Ca^{2+}-dependent interaction of CaM with these drugs, which have been very useful in elucidating the interaction of CaM and its physiological targets. In addition, they have a potential therapeutic use as CaM antagonists. These drugs are hydrophobic compounds and they interact with the strongly hydrophobic alpha helix segment of Ca^{2+}-CaM[50,77]. A direct relationship between CaM affinity and ability to inhibit CaM-dependent enzymes has been reported for phenothiazines[50], especially TFP. The pharmacological effects of these phenothiazines are mediated by binding to D2 receptors[71] and by inhibition of protein kinase C[2], and not through binding calmodulin. It is probable that the 5-methoxy group, which makes MEL hydrophobic, may interact with the strongly hydrophobic alpha helix segment of Ca^{2+}-CaM as phenothiazines do[50]. MEL inhibits CaM-dependent phosphodiesterase activity at nanomolar concentrations contrast to its picomolar affinity for CaM[15,18]. However, unlike phenothiazines, MEL does not bind to D2 receptors[30] and does not inhibit protein kinase C (unpublished result). Therefore, MEL could be considered as selective CaM antagonist at physiological concentrations.

Melatonin cytoskeletal effects

Since MEL has both intracellular access to and high affinity for CaM, the next step in the analysis comprises two questions: 1) Does MEL binding to CaM occur in the cell? and 2) What are the biochemical events that follow intracellular MEL-CaM binding and result in a cellular response? The answers to both questions are hindered by lack of information, and also because CaM modulates multiple metabolic pathways. Since major cellular processes regulated by CaM include protein phosphorylation and dephosphorylation[44], cyclic nucleotide metabolism[33,40], cell proliferation[67], Ca^{2+} transport[75], and cytoskeletal organization[38], we might suspect that most described actions of MEL could be explained as CaM antagonist effect. To our knowledge, besides the isolated observation that MEL inhibits the CaM-dependent $Ca^{2+}Mg^{2+}$-ATPase activity in synaptosomes[6], the only direct experimental data about intracellular MEL-CaM interactions has been described in MDCK and N1E-115 cell lines.

MDCK cells are normal cells that in culture form functional monolayers similar to natural epithelia[28]. These cells display different morphologies during growth[13] until they reach confluence and become functional[29]. MDCK cells transport water that accumulates between the basolateral domain and the solid surface of the petri dish. Since the cells have tight junctions, the fluid pumped under the monolayer detaches them from the support and forms blisters or domes[29]. Both cell morphology and dome formation depend on microfilament rearrangements[53]. N1E-115 cells are neoplastic neurons derived from a murine neuroblastoma. They display many of the characteristics of normal neurons, such as functional receptors and neurite outgrowth[73]. There is evidence in N1E-115 cells that cytoskeleton is the major internal structure defining neuron morphology[51], and that neurite formation is due mainly to microtubule enlargement[42].

In MDCK and N1E-115 cells, MEL at a physiological concentration induces morphological, structural and physiological responses. Both cell lines, cultured with 10^{-9} M of the hormone, form a fine network with contacts between adjacent cells. A twofold increase in the number of N1E-115 cells with neurites and MDCK cells with cytoplasmatic elongations is observed[14]. Moreover, when N1E-115 cells treated with MEL, are stained with an antitubulin antibody, long neurite processes connecting cells to each other (neurite outgrowth) become evident. When MDCK MEL treated cells are stained with an anti-actin antibody, thicker fibers beneath the plasma membrane and in the nuclear region are seen, together with an increase in dome formation[14]. Evidence that MEL-CaM binding occurs in the cell was obtained by the simultaneous localization of MEL and CaM by a double immunofluorescence method. Subconfluent MDCK cells were incubated for 12 h with vehicle or 10^{-9} MEL. They were sequentially stained with an anti-melatonin antibody elicted in rabbit, followed by an anti-calmodulin antibody elicited in sheep. Specific second antibodies coupled to FITC or RITC were used against the first antibodies. In MEL-treated cells, CaM distribution changes. The hormone and protein are both localized at the same sites: at the cell periphery, all over the cytoplasm, and in the nuclear region[16]. Since MEL and CaM are in the same intracellular compartments it is possible that they are bound to each other.

In MDCK and N1E-115 MEL-treated cells, structural and metabolic changes related to MEL-CaM interactions are also found. A CaM compartmentalization

occurs in MDCK cells cultured for 6, 12, 24 h, or 4 days with 10^{-9} M MEL. The CaM accessible to the specific anti-calmodulin antibody was stained in vehicle-treated cells as fluorescent spots located at the cell periphery. On the other hand in MEL-treated cells, the protein is distributed all over the cell as a fine reticular lattice[16]. The distribution of CaM was confirmed by assessing CaM concentration in subcellular fractions. CaM was quantified by RIA in subcellular fractions obtained by differential centrifugation of MDCK cells. Cells treated for 4 days with 10^{-9} M MEL showed an increase of 40% in membrane bound CaM content while cytosolic CaM decreased to 40%[16]. No changes in the nuclear fractions were observed. Twelve h after MEL withdrawal, the subcellular CaM distribution returned to the normal pattern as observed by immunofluorescence[16]. Similar changes in CaM compartmentalization also occurred in N1E-115 cells. In these cells CaM is normally distributed all over the cell, while in MEL-treated cells CaM appeared to be concentrated as a fine meshwork at the cell periphery (unpublished observations). Both MDCK and N1E-115 MEL-treated cells showed an increase in cell proliferation as well as a twofold CaM increase in the logarithmic growth phase[15,17]. In the stationary phase, CaM concentration drops almost 40% and cell growth is inhibited by 50%[15,17]. CaM concentration at a given time depends on its synthesis/degradation ratio. The results suggest that this ratio is different at different cell growth stages and/or different exposure times to the hormone. Preliminary experiments in MDCK cells treated for up to 6 days with 10^{-9} MEL suggest that both CaM synthesis and degradation are modified. There is a twofold increase in CaM mRNA level in the logarithmic phase and a return to normal levels in the stationary phase[60]. Pulse-chase experiments with ^{35}S-methionine show that there is an increase in CaM synthesis in the logarithmic phase with return to normal values in the stationary phase. Nevertheless the half-life of ^{35}S-CaM changed from 19 h in control cells to 16 h[17]. Thus, MEL may increase CaM synthesis by enhancing the translation of calmodulin mRNA. MEL binding to CaM may also increase the rate of CaM degradation. CaM translocation from the cytosol to the membrane as well as changes in cellular content will in turn affect specific cellular responses modulated by CaM[75]. Therefore, both changes in CaM cellular levels and CaM translocation may represent additional mechanisms by which the hormone modulates cell responses.

In vitro studies have been helpful in understanding the cytoskeletal effects of MEL. Tubulin polymerization in vitro depends on GTP[43]. In the presence of MAPs, the inhibitory effect of Ca^{2+} on microtubule polymerization is increased by exogenously added CaM[19,47]. Polymerization of bovine brain twice cycled microtubule protein was monitored after the addition of GTP at different melatonin concentrations. A complete reversal of Ca^{++}-CaM inhibition of microtubule polymerization was observed with 10^{-9} M MEL[6]. This effect was similar to the inhibition produced by the well known CaM antagonists TFP at 10^{-5} M and compound 48/80 at 30 ug/ml[62]. In the range of 10^{-7} to 10^{-5} M, MEL decreased microtubule formation in a dose-dependent manner[6]. Reversible Ca^{2+}-CaM inhibition of microtubule polymerization was also observed when MDCK detergent extracted cytoskeletons were treated with 10^{-9} M MEL. The hormone protects microtubule networks against Ca^{++}/CaM-induced disruption. Microtubules in MEL-treated cytoskeletons are thicker and longer than control cytoskeletons when microtubules are disrupted by Ca^{2+} activation of endogeneous CaM[6].

It is known that CaM enhances the inhibitory effects of Ca^{2+} on microtubule polymerization by the formation of Ca^{++}/calmodulin/tubulin or Ca^{++}/calmodulin/MAPs complexes[47], and that TFP prevents this inhibition by binding to Ca^{2+}-CaM, thus blocking the formation of both complexes[62]. Moreover, fluorometric and circular dichroism studies of the interaction of TFP (10^{-4} M) and tubulin suggested that TFP changes tubulin secondary structure[7]. Although further experiments are needed, it is probable that high melatonin concentrations induce a change in the tubulin secondary structure since as well as its non-specific binding to tubulin, the hormone displaces ^{3}H-colchicine-binding and increases GTP binding to the protein[63,78].

Taken together these data suggest that MEL effects on microtubule polymerization depend on the MEL concentration present in the assay. At physiological concentrations (10^{-9} M) MEL binds to CaM, blocking both MAPs/CaM and tubulin/CaM complex formation. As a result a net gain in microtubule size occurs. In the absence of active calmodulin, or at pharmacological concentrations (10^{-5} to 10^{-7} M), MEL binds directly to tubulin which alters its secondary structure, thus preventing further tubulin assembly and favoring microtubule disruption. This hypothesis provides a plausible explanation for the reported contradictory microtubule changes induced by the hormone.

MEL effects at the cytoskeletal level have been described both in different species and under different experimental conditions. Thus it is difficult to interpret the results. Several factors must be taken into account: the amount of MEL present in the assay, the accessibility of the hormone to the experimental system, the actual concentrations reached in the cells, the presence of CaM, as well as the Ca^{2+} concentration. Despite these restrictions, the effects on microtubule assembly in relation to MEL concentration can be grouped into two general categories. As summarized in table 1, microtubule enlargement in different experimental systems is produced at low concentrations of the hormone (10^{-9} M or less). Higher concentrations (10^{-6} to 10^{-4} M) produce micro-

Table 1. Microtubule enlargement effects of melatonin

Effect	System	[MEL]
Increased birefringence of mitotic spindle	Frog melanocytes[41]	2.3 p.p.m.
	Haemanthus Katherinae Baker plant cells	23.0 p.p.m.
Pigment aggregation	Retinal pigment[61] ephithelium and coroid cells	4.3×10^{-9} M[a]
		4.3×10^{-8} M
		4.3×10^{-7} M
	Xenopus embryo[56] cells	1.0×10^{-15} M
		1.0×10^{-13} M
	Frog dermal melanocytes[54]	4.3×10^{-10} M
	Acanth. kuhliisumatranus[68]	2.1×10^{-7} M[b]
	Botia macracanthus	2.1×10^{-5} M
	Nannostomus ocellatus	
	Hyphess. herbertaxelrodi	
	Nannostomus marginatus	
	Grynochelius aymonieri	
	Aequidens pulcher	
	Hyphessobrycon serpae	
	Hyphessobrycon scholzei	
Antagonism of colchicine effects on pigment dispersion	Frog dermal[53] melanocytes	5.0×10^{-5} M
Increased number of microtubules	Rat pineal[36] gland	25–200 μg/Kg[c]
Antagonism of MSH effects	Frog skin[1]	4.3×10^{-10} M
Antagonism of colchicine-induced mitotic arrest	HeLa cells[35]	1.0×10^{-4} M
Neurite outgrowth	N1E-115 cells[14]	1.0×10^{-9} M
Cytoplasmatic elongations	MDCK cells	

Melatonin concentrations were recalculated from the original data. References are supplied. Melatonin administration was: [a]intraocularly perfused, [b]in the swimming water, and [c]subcutaneously injected.

tuble disruption (table 2). Besides the data summarized in both tables, short term experiments report a lack of MEL effects on microtubules: no inhibition of microtubule polymerization in vitro, no inhibition of neurite outgrowth in neuroblastoma cells, no induction of mitotic arrest in CHO cells, and no ^{3}H-colchicine binding to tubulin[66]. Also, 10 different fish species did not show changes in pigment aggregation or dispersion[68]. Clearly, further data on MEL-CaM interactions are needed.

As mentioned, CaM is a multifunctional protein modulator. Therefore, the question of how MEL can modify a specific cellular function arises. According to our data, MEL not only modulates a specific cellular function by the kinetics of its binding to CaM and the genetic expression of a given cell type; modulation also depends on the location of the different protagonists with respect to one another (Ca^{2+} concentration increase, MEL, CaM and target enzymes) in a given compartment of the cell at a given time. Thus, MEL-specific cellular responses and cellular specificity can be expected.

Concluding remarks

Classically, hormones act either by stimulating the production of other hormones or by exerting permissive effects in order to preserve cell function and homeostasis. Unlike these, MEL acts by antagonizing the intra-

Table 2. Microtubule depolymerizing effects of melatonin

Effect	System	[MEL]
Arrest of granule movement	B6 mouse[37] melanoma cells	4.3×10^{-5} M
		4.3×10^{-4} M
Inhibition of microtubule reassembly	Sciatic nerve[65] of toad	8.6×10^{-4} M[a]
Inhibition of colchicine binding to tubulin	Purified fetal[63] brain tubulin	1.0×10^{-5} M
		1.0×10^{-4} M
Increases to GTP binding to tubulin		5.0×10^{-4} M
Melanin granule dispersion	*Moenkhausia oligolepis*[68]	2.1×10^{-5} M[b]
	Hemigrammus ocellifer	
	Nannostomus ocellatus	
	Hyphess. herbertaxelrodi	
Disruption of mitotic apparatus	Onion root tips[12]	8.0×10^{-4} M
Oral band regeneration delay	*Stentor coeruleus*[11]	200×10^{-6} M
Microtubule crystaloid and tubular formation	Rat median[25] eminence	1.5 μg[c]
Inhibition of rapid axonal flow	Retinal ganglion cells	15.0 μg

Melatonin concentrations were recalculated from the original data. References are supplied. Melatonin administration was: [a]intracardiac injected, [b]in the swimming water, and [c]intravitreally injected.

cellular Ca^{2+} receptor calmodulin, thus modulating cell activity. Accordingly, the rhythmic release of MEL will impose on its target cells rhythmic oscillation of their activity; the cell activity set-point will follow rhythmic MEL variations. Thus, MEL could be considered as a rheostatic hormone. Finally, since most of our knowledge is based on cytoskeletal studies, modifications of the many other CaM-regulated pathways should be explored.

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