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Review

Melatonin modulates microfilament phenotypes in epithelial cells: implications for adhesion and inhibition of cancer cell migration

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Summary. Cell migration and adhesion are cytoskeleton- dependent functions that play a key role in epithelial physiology. Specialized epithelial cells in water transport have specific microfilament rearrangements that make these cells adopt a polyhedral shape, forming a sealed monolayer which functions as permeability barrier. Also, specific polarized microfilament phenotypes are formed at the front and the rear of migratory epithelial cells. In pathological processes such as cancer, increased migration occurs in invasive cells driven by the formation of polarized and differential microfilament phenotypes. Melatonin, the main product secreted by the pineal gland during dark phase of the photoperiod, acts as a cytoskeletal modulator in normal and cancer cells. In this paper we will summarize evidence supporting that melatonin acts as a microfilament modulator in epithelial MDCK cells, and we will describe its effects on cytoskeleton organization involved in the mechanism by which melatonin synchronizes water transport. In addition, we will review recent data that indicate that melatonin is able to switch microfilament phenotypes in MCF-7 human mammary cancer cells, from invasive migratory cells to dormant microfilament phenotypes that occur in non- migratory cells. Moreover, we will discuss the implications of the cytoskeleton as therapeutic target for cancer cells.

Key words: Melatonin, Cytoskeletal, Calmodulin, Protein kinase C, Cancer

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Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is a lipophilic indolamine that crosses lipid bilayers (Costa et al., 1997). It is synthesized by the pineal gland during the dark phase of the photoperiod and it is rapidly released into the blood stream where the highest levels reach nanomolar concentrations (Reiter, 1993). Melatonin is a signal that conveys photoperiodic information to synchronize cell physiology with the dark-light cycle (Reiter, 1993) . Also, it has been described that melatonin and its metabolites, 3-cyclic melatonin and kynurenines, act as a free radical scavengers, neutralizing hydroxyl and peroxy radicals among others, preventing lipid membrane peroxidation and DNA damage (Reiter et al., 2000, 2007). Another important feature of melatonin is that it modulates the cytoskeletal structure organization. Microtubule polymerization is increased by nanomolar concentrations of the indole, while intermediate filaments are redistributed in N1E-115 neuroblastoma cells during neurite formation (Huerto Delgadillo et al., 1994; Benítez-King, 2000). Microfilaments, also participate in neurite formation elicited by melatonin. An increase in filamentous actin, organized in growth cones and lamellipodia, has been observed in N1E-115 cells cultured with melatonin concentrations similar to that observed in plasma and cerebrospinal fluid during the night (Bellon et al., 2007). In pathological conditions melatonin improves cell survival. In particular, the indole has oncostatic and scavenging properties (Blask et al., 2002) in cancer cells (Reiter et al., 2000). Also, it inhibits cell proliferation by different mechanisms including the binding to membrane receptors or by changing lipid metabolism, or by indirect mechanisms involving the immune system stimulation, inhibition of

estrogen production and through interaction of neuroendocrine axis down-regulation (Ram et al., 2002; Blask et al., 2003; Del Rio et al., 2004; Cos et al., 2006). In estrogen receptor alpha positive macrophage chemotactic factor human cancer breast cells (MCF-7 cells), nanomolar concentrations of melatonin inhibit cell proliferation and reduce invasiveness by increasing the expression of two cell surface adhesion proteins, E-cadherin and $ß_1$-integrin (Cos et al., 1998).

Melatonin radical scavenging ability is present in all organisms studied and may represent a phylogenetically well preserved early function, necessary to protect the organisms against adverse environmental conditions, allowing survival and the species preservation (Hardeland and Poeggeler, 2003). In this regard, the cytoskeleton is a phylogenetically well preserved structure (Jenkins et al., 2002) that allows the cell to have a well organized structure and allows it to have an optimal functionality. By contrast, in pathological processes such as cancer, an abnormal cytoskeletal rearrangement with a poor organization and structure is produced (Raz and Geiger, 1982). Thus, the capability of melatonin to modulate cytoskeletal organization may be another ancient mechanism for life preservation and to protect the organism against pathological damage.

In this paper we will summarize evidence that indicates that melatonin acts as a microfilament modulator in epithelial MDCK cells. Also, we will describe the effects of melatonin on cytoskeleton organization involved in the mechanism by which melatonin synchronizes water transport in kidney derived epithelial MDCK cells. In addition, we will review recent data that indicate that melatonin is able to switch microfilament phenotypes from invasive migratory cells to non-migratory and adherent cells in MCF-7 human mammary cancer cells. In addition, the melatonin signalling pathways involved in modulating the microfilament rearrangements in epithelial cells will be described. Moreover, we will discuss the implications of the cytoskeleton as a therapeutic target for cancer cells.

Microfilaments in transport epithelial cells

MDCK cells in culture maintain biochemical, physiological and structural features of kidney transporting epithelia, since they form epithelial cell monolayers that resemble the intercalated cells of renal cortical collecting ducts. These cells respond to extracellular signals, and naturally occurring activators (Lever, 1979; Taub and Saier, 1979). MDCK cell monolayers transport water and electrolytes from the apical to the basolateral side through both the paracellular and transcellular routes (Cereijido et al., 1978). A Na^+ K^+ adenosine triphosphatase (Na^+ K^+ ATPase) located in the basolateral domain participates in transcellular transport, creating a driving force for sodium vectorial transport from the apical to the basolateral direction across the cell (Contreras et al., 1989). Meanwhile, tight junctions formed between adjacent cells participate in selective ion and water transport through the paracellular pathway (Cereijido et al., 1978).

Microfilaments are necessary for modulation of transepithelial permeability through both transcellular and paracellular routes. MDCK cells show a reticular pattern of very fine actin filaments tethered to the cell membrane, forming a cortical ring and extending laterally toward the basal side of the cells (Meza et al., 1980). Microfilament participation in the transcellular pathway of water and ion transport has been shown to occur through interaction with transmembrane proteins involved in vectorial transport, such as the band 3 anion exchanger (Drenckhahn et al., 1985), the epithelial Na^+ K^+ ATPase (Nelson et al., 1991), the Na^+ K^+ Cl- cotransporter (Jorgensen et al., 1984) and the Na^+ channel (Edelstein et al., 1988). Microfilaments also stimulate Na^+ K^+ ATPase activity by a mechanism that implicates a direct binding of actin to the enzyme (Cantiello, 1997). In addition, microfilaments associate with membrane components and by tight junctions to regulate the paracellular pathway of ion transport (Meza et al., 1980). Microfilament participation in tight junction sealing was demonstrated since in the presence of cytochalasin B, cytoplasmic and cortical microfilaments are disrupted, tight junctions lose their organization and the paracellular transport is lost (Meza et al., 1982).

Epithelial cells can be cultured on non-permeable supports. In this condition vectorial transported water is accumulated between the basolateral domain and the surface of the culture dish, forming blisters or domes (Lever, 1979). Microfilaments acquire different phenotypes in the dome and in the cells surrounding the dome. In these last cells, microfilaments form stress fibers that are assembled with phospho- vinculin, among other proteins, to constitute the adhesion plaques, which provide the necessary strength to support the dome architecture (Fig. 1). While in cells forming the dome, microfilaments form the cortical rings involved in thigh junction sealing to confer the strength for cell junctions that join adjacent cells. Together, this data indicate that specialized microfilament phenotypes play a crucial role for ion and water transport in epithelial cells.

Melatonin synchronizes water transport by microfilament reorganization in MDCK cells

MDCK cells have been used to study the melatonin effects on cytoskeletal organization. Chronic exposure of MDCK cells to 1 nM melatonin causes thicker microfilament stress fibres and enhanced actin staining at the cell borders (Benitez-King et al., 1990) associated with an increased number of domes. Also, melatonin has been tested in circadian cycles of melatonin incubation.

In vitro, it is possible to reproduce the cyclic changes of melatonin plasma circulating levels, and thereby to study cellular functions in response to a

rhythmic melatonin signalling. MDCK cells incubated for three consecutive cycles of 12 h with melatonin followed by 12 h without melatonin, showed a cyclic pattern of dome formation in synchrony with the melatonin signal (Ramirez-Rodriguez et al., 2003). A gradual increase in the number of domes was observed within the indole incubation, the maximum increment is reached after 6 h of melatonin incubation. However, the number of domes dropped gradually at longer times of incubation. After hormone withdrawal, the number of domes per field dropped even further, to the average basal number observed in monolayers cultured in regular media or in the presence of the vehicle. This pattern was observed in each of the three cycles. Moreover, melatonin induced an optimal increase in dome formation at a concentration similar to circulating levels of the hormone in plasma 1 nM (Reiter, 1993; Ramírez-Rodríguez et al., 2003). Thus, these results indicate that dome formation in the presence of melatonin followed a cyclic pattern with a similar profile to that of melatonin circulating in plasma. We corroborated that melatonin induced dome formation by increasing water transport, since a significant increase in the $[^3H]$-H_2O flux from the apical to the basal compartment was observed after 6 h of melatonin incubation. However, no significant differences were found in the vehicle incubated cells cultured for a 12 h cycle (Ramírez-Rodríguez et al., 2003). Thus, these data indicate that transported water is accumulated in domes since water flux from the apical to the basolateral domain in MDCK cell monolayers incubated with melatonin correlates with the temporal course of dome formation induced by the indole.

Cells that form part of a dome are detached from the surface and do not show an organized basal cytoskeleton (Castillo et al., 2002). While, cells surrounding the domes are firmly attached to the substratum through focal adhesion contacts formed by stress fibers assembled with phospho- vinculin, and integrins, among other proteins. Incubation of MDCK cell monolayers in cycles of 12 h with the vehicle, followed by a cycle of 12 h with 1 nM melatonin, showed that microfilament organization changes at both the apical and the basal sides of the cells surrounding the domes (Ramirez-Rodriguez et al., 2003). Actin microfilaments in cells incubated with the vehicle during 6 and 12 h were organized in typical cortical rings at the apical levels and in stress fibers at the basal level (Ramirez-Rodriguez et al., 2003). After 6 h of incubation with melatonin, abundant and thicker stress fibers are observed in cells that surround the dome and abundant focal adhesion contacts are formed (Fig. 2, panel B and D). In addition, the cortical rings are thicker than in the vehicle-incubated cells. Melatonin effects on microfilament organization are reversible since actin microfilaments recover basal organization in the vehicle-incubated cells (Ramírez-Rodriguez et al., 2003). Thus, data indicates that microfilament reorganization elicited by melatonin correlates with the cyclic pattern of water transport produced in synchrony with the melatonin signal. This set of observations might suggest that the modifications in urine production and osmolarity observed in the kidney during the dark phase, in which melatonin reaches the highest concentration, could be related to cytoskeletal modifications and permeability changes (Koopman et al., 1989; Richardson et al., 1992; Koopman et al., 1994).

Microfilaments in epithelial cancer cells

In pathological conditions epithelial cells suffer neoplastic transformation, lose the focal contact adhesions, and abnormally proliferate. These cells have an aberrant microfilament organization and are resistant to "anoikis", a process of apoptosis that occurs in normal epithelial cells when they are detached from the substratum (Bharadwaj et al., 2005). By contrast, in malignant cells, a deficient stress fiber formation occurs, focal adhesion formation is decreased, the cells are

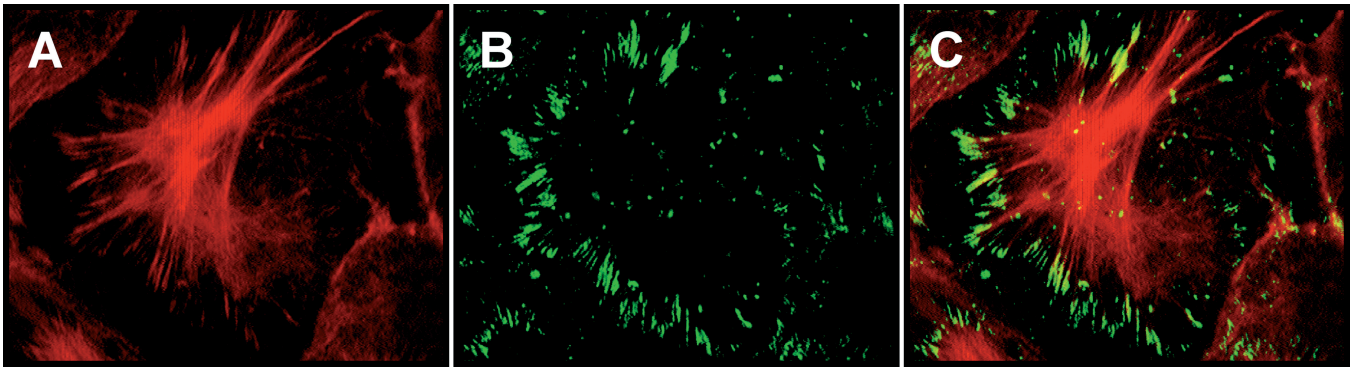

Fig. 1. Immunofluorescence staining of focal adhesion contacts. MDCK cells were simultaneously stained with rhodamine-phalloidin **(A)** and anti-vinculin antibody **(B)**. Overlay images of actin and vinculin forming the adhesion plaques are shown in **C**. Images illustrate the typical staining pattern of focal adhesion contacts in cells surrounding a dome. Microfilaments arranged in stress fibers are shown in red. Vinculin in adhesion plaques are shown in green. x 2500

detached, migration is produced and cell growth occurs in suspension (Wang, 2004).

Cell migration is generated by a driving force provided by dynamic cytoskeletal organization that takes place in opposite poles of the cells (Maidment, 1997; Friedl et al., 1998; Small and Kaverina, 2003). In cancer cells migration is enhanced during invasiveness, propitiating metastasis formation (Liotta and Stetler-Stevenson, 1991). Migrating cells protrude by microfilaments and microtubules arranged in membrane ruffles, lamellipodia and filopodial formations at the leading edge, while at the cell rear a retraction of these cytoskeletal structures occurs (Maidment, 1997; Raftopoulou and Hall, 2004). On the other hand, dormant cells are non- migratory and remain attached to the substratum by cell anchorage produced by a highly specialized microfilament organization in adhesion plaques (Barkan et al., 2008). Focal adhesion formation involves the microfilament arrangement in stress fibers (Zamir and Geiger, 2001), which interact with phospho-vinculin, among other proteins (Geiger et al., 1980).

Metastatic cells are characterized by poorly structured microfilaments and scarce anchorage to their substratum. By contrast, low invasive cells, such as the mammary cancer MCF-7 cells, attach to the substrate through focal adhesion contacts formed by actin stress fibers, and are joined together by adhesion proteins such as integrins and cadherins, among other proteins (Raz and Geiger, 1982). Although MCF-7 cells are slow metastatic cells, they form metastasis in experimental animal models and have been used to study the melatonin and estrogen mechanisms of action involved in the increased cell proliferation showed by these mammary cancer cells (Leonessa et al., 1992; Cos et al., 1998).

Abnormal microfilament organization is reverted by melatonin in cancer MCF-7 cells

The driving force for movement is mainly established by a dynamic structural balance between two kinds of cytoskeletal arrangements: microfilaments

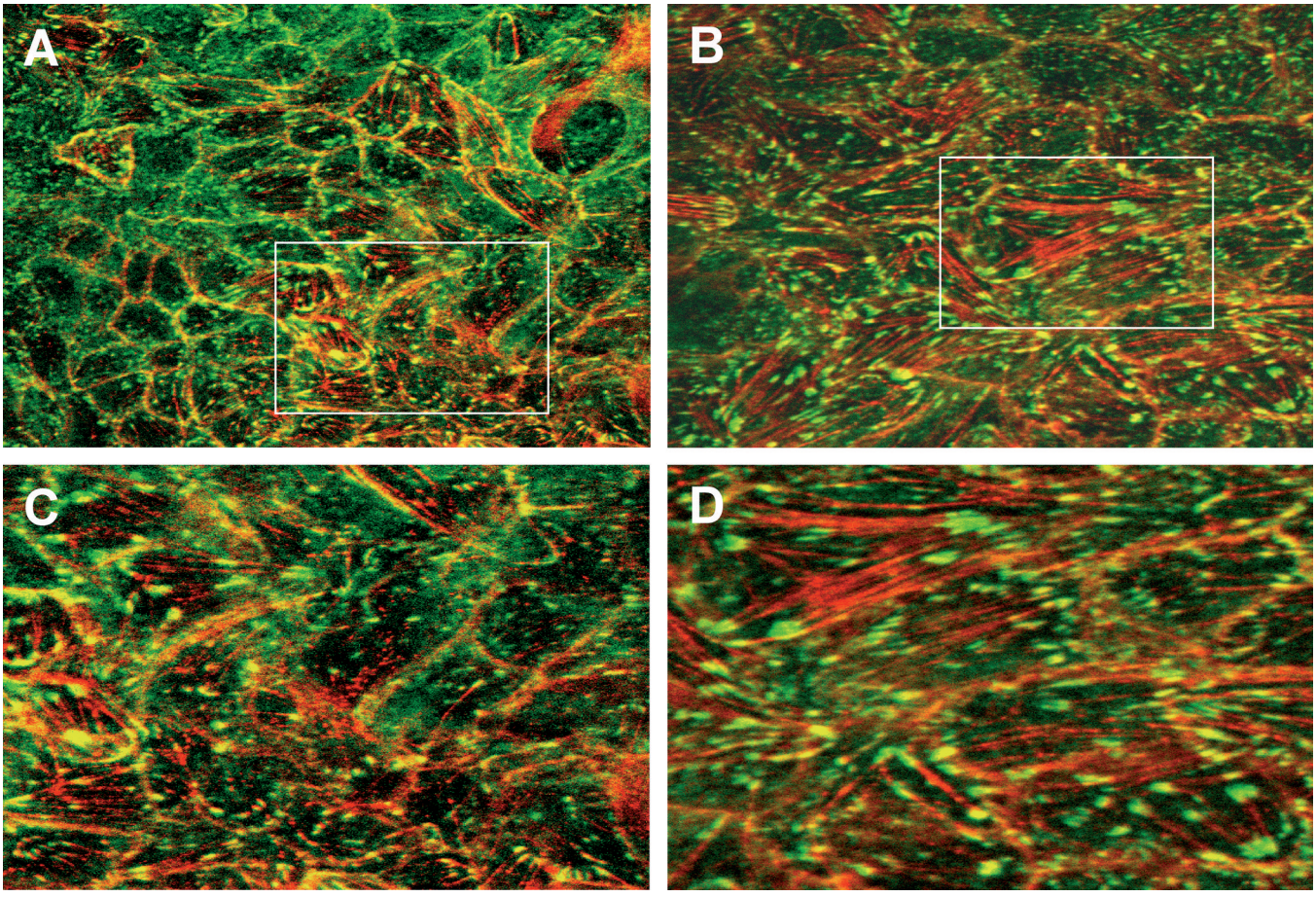

Fig. 2. A. Melatonin induces stress fibers and focal adhesion contact formation in MDCK cells. A. MDCK cells incubated with the vehicle for 6 h show stress fibers (red) and vinculin (green) labelling. **B.** Melatonin incubated-MDCK cells show more abundant areas with thicker stress fibers and wider vinculin labelling in comparison with the vehicle incubated cells. **C and D.** Higher magnification of focal adhesions shown in panel A and B. A, B, x 1,000; C, D, x 1,600

organized in ruffles and lamellipodia with small point contacts of 0.1-1 µm located at their edge (Nobes and Hall, 1995), and focal adhesions formed by elongated streak-like structures of 3-10 µm in length, associated with actin stress fibers, located in the cell body (Burridge et al., 1987; Zamir et al., 2000; Zaidel et al., 2003). In cancer cells this structural balance is lost. The cells are detached from the substratum, migration is enhanced and invasion and metastasis to other tissues are produced.

Diversity of microflament phenotypes elicited by melatonin was studied in migratory neoplastic MCF-7 cells by a wound healing assay. Disruption of an epithelial cell monolayer by an injury causes an immediate repair that involves cell migration and anchorage (Omelchenko et al., 2003; Farooqui and Fenteany, 2005). Migration of epithelial cells can be stimulated by the ROCK inhibitor Y-27633 and after 24 h incubation the wound is almost healed in the presence of this compound (Omelchenko et al., 2003). However, melatonin inhibits wound healing in the presence of Y-27632 compound (Ortíz-López et al., 2009), indicating that cell migration is decreased with the nocturnal plasma concentration of melatonin (1 nM).

Leader cells form a flat lamella extending toward the wound and have microfilaments organized in membrane ruffles, lamellipodia and microspikes. These cells prompt migration in the wound healing assay by moving on the free substrate, pulling their neighbour cells after them, so that eventually multi- cellular outgrowths are formed (Omelchenko et al., 2003). Leader cell number is increased in the presence of melatonin, and microfilament phenotypes observed in migratory leader cells are changed in the presence of the indoleamine (Ortíz-López et al., 2009). Migratory phenotypes are structured in the presence of the Y-27632 compound and the number of ruffles is increased. While, in the presence of melatonin the number of ruffles at the margin edges diminished and microfilaments are arranged in extended lamellipodia (Ortíz-López et al., 2009).

In experimental conditions to measure cell anchorage (Fig. 3), melatonin increases the number of focal adhesion contacts in MCF-7 cells, and microfilaments are arranged in thicker bundles of stress fibers assembled with phospho- vinculin to form focal adhesion contacts (Ortíz-López et al., 2009). These results strongly suggest that melatonin inhibits cancer cell invasion and metastasis formation by changing microfilament phenotypes of migratory cells (ruffles and lamelipodia) to stress fibers that are microfilament phenotypes of attached cells. The stress fibers converge in a migration/anchorage switch, inhibiting cancer cell invasion and by this mechanism melatonin might inhibits metastasis.

Signalling pathways involved in microfilament organization modulated by melatonin: Implications of Calmodulin

Protein kinase C (PKC) was initially demonstrated to be involved in the mechanisms by which melatonin elicits stress fiber formation in epithelial cells (Ramirez-Rodriguez et al., 2003). Evidence that supports this notion was obtained using the PKC inhibitor bisindolylmaleimide and the PKC agonist, the phorbol 12,13 myristate-acetate in MDCK and MCF-7 cells cultured with melatonin (Ramirez-Rodriguez et al., 2007; Ortíz-López et al., 2009). The PKC inhibitor abolished the increased number and thickening of stress fibers, as well as the augmented number of focal adhesion contacts elicited by the indole in both cell lines (Ramirez-Rodriguez et al., 2007; Ortíz-López et al., 2009). Also, the PKC agonist resembled the microfilament organization induced by melatonin and increased the number of stress fibers and their thickness (Ramirez-Rodriguez et al., 2007; Ortíz-López et al., 2009).

The Rho associated protein kinase (ROCK) participates in this mechanism downstream of the PKC pathway. This conclusion was reached because the increased migration elicited by the ROCK inhibitor Y-27632 in MCF-7 cells is abolished by melatonin, while stress fiber thickening and the increased focal adhesion formation caused by melatonin in MDCK and MCF-7 cells is prevented by the ROCK inhibitor and by bisindolylmaleimide, the PKC inhibitor (Ramirez-Rodriguez et al., 2007; Ortíz-López et al., 2009). Also, in an *in vitro* enzymatic assay it was demonstrated that melatonin stimulates ROCK activity, downstream of the PKC pathway. The reconstituted enzymatic mixture was formed by a cell homogenate obtained from either of the cell lines to provide ROCK and PKC, and the peptide S6 utilized as specific substrate for ROCK. In this system, the ROCK inhibitor Y-27632 and the PKC inhibitor blocked the ROCK stimulation elicited by melatonin (Ramirez-Rodriguez et al., 2007). Besides, evidence suggesting that calmodulin (CaM) is also involved in the melatonin modulation of microfilament phenotypes in epithelial cells has been published.

Calmodulin is a ubiquitous, highly phylogenetically conserved acidic protein which functions as an intracellular Ca^{2+} sensor (Klee and Vanaman, 1982; Chin and Means, 2000). It binds four Ca^{2+} molecules and exposes an alpha helix region rich in hydrophobic amino acid residues that interact with structural and enzymatic sites in protein targets (Klee and Vanaman, 1982). CaM is involved in a broad spectrum of cellular functions (Klee and Vanaman, 1982; Chin and Means, 2000). Among these, modulation of cytoskeletal organization by CaM is important for cell motility and microfilament organization in stress fibers (Klee and Vanaman, 1982; Chin and Means, 2000). Subcellular distribution of CaM and its sorting to microfilaments has been implicated in the regulation of cellular contractility (McGinnis et al., 1998). CaM showed a fiber-like distribution pattern in the cytosol of PtK2 kidney epithelial cells, which resembles actin distribution in stress fibers (Yuan et al., 2008). Additional evidence from the co-immunoprecipitation analysis confirmed that CaM interacts with actin directly or indirectly (Yuan

et al., 2008). Nevertheless, it is not known whether or not CaM-associated to actin is in a non-phosphorylated or phosphorylated state. However, it is known that phosphorylation of CaM in serine and threonine residues affects the interaction of this protein with its target proteins (Quadroni et al., 1998) or its Ca^{+2}-binding capability (Aiuchi et al., 1991).

Stress fibers are formed by actin and myosin and their contractility is regulated by both Ca^{2+}-dependent and Ca^{2+}-independent mechanisms (Katho et al., 2001). The phosphorylation of regulatory myosin light chains by the Ca^{2+}/CaM dependent enzyme myosin light chain kinase (MLCK) has been shown to be essential for stress fiber contraction (Goeckeler and Wysolmerski, 1995). CaM is associated with MLCK and stimulates the myosin light chain phosphorylation, prompting myosin/actin contraction. Also, ROCK controls actomyosin contractility and stress fiber assembly through myosin light chain phosphorylation in the absence of Ca^{2+} (Katho et al., 2001). This evidence has suggested that different categories of stress fibres exist, since differential and rapid and extensive stress fiber contraction was induced by MLCK than was by ROCK (Katho et al., 2001). When the activity of ROCK but not MLCK was inhibited, cells not only lost their stress fibers and focal adhesions but also appeared to lose cytoplasmic tension. Furthermore, the disposition of actin and myosin II undergoes striking reorganization. F-actin initially forms a fine network of filaments that fills the cytoplasm and then reorganizes into prominent stress fibers. Myosin II rapidly forms discrete aggregates, associated with the actin network and by 2.5 min assumes a distinct periodic distribution along the stress fibers (Goeckeler et al., 2008). Thus evidence suggests that Ca^{2+} is used to generate rapid contraction, whereas Rho-kinase plays a major role in maintaining sustained contraction in cells (Katho et al., 2001).

Current evidence indicates that melatonin in

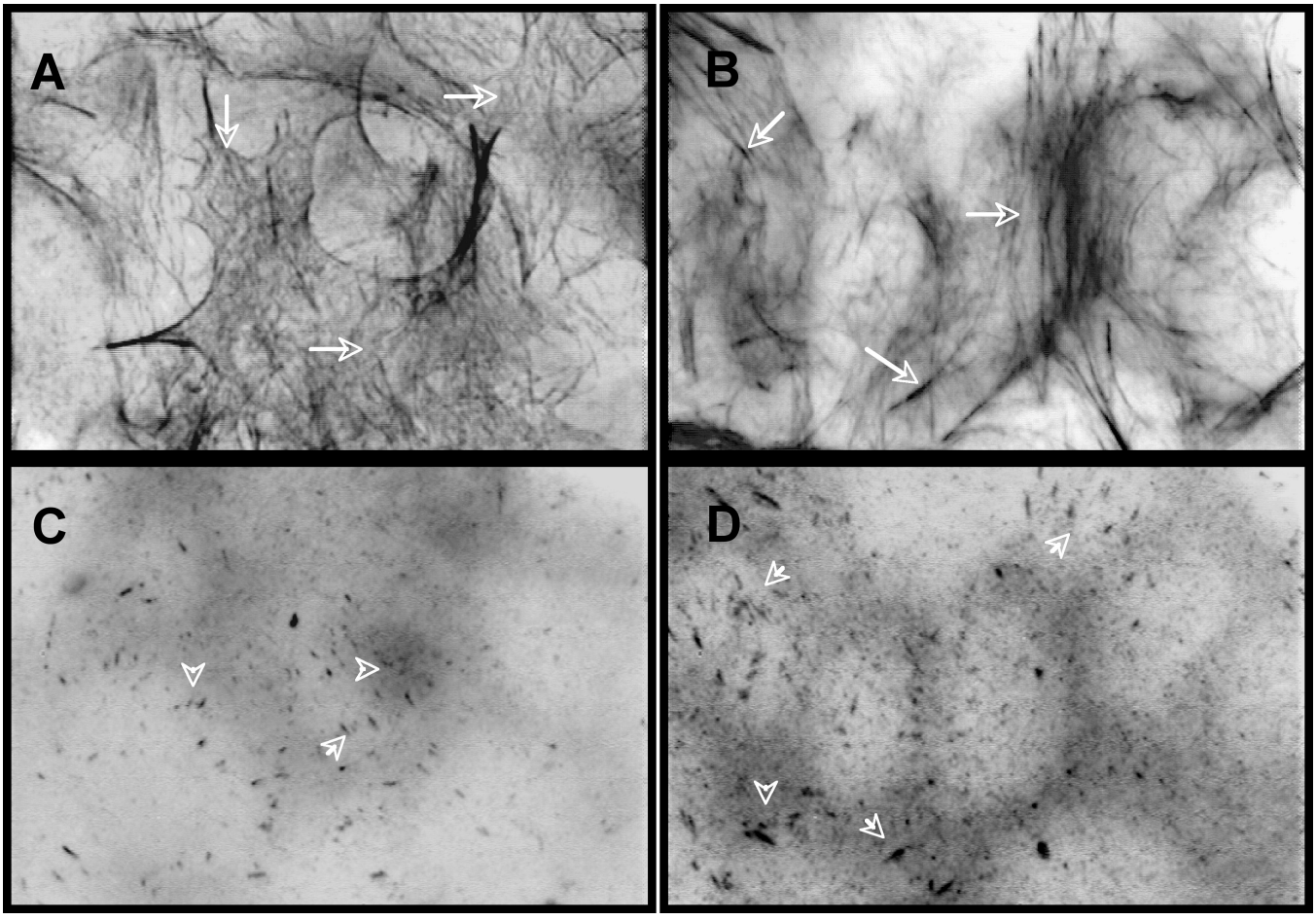

Fig. 3. Focal adhesion contacts in cancer derived MCF-7 cells. **A.** Micofilament organization in MCF-7 cells incubated with the vehicle show thinner stress fibers. **B.** In melatonin incubated-MCF-7 cells stress fibers are thicker and larger than those observed in the vehicle incubated cells. **C.** Thin vnculin spots (0.5-1.5 μm in length) are observed in the vehicle treated cells. **D.** Melatonin induces more abundant and wider vinculin labelling (<1.8 μm in length) indicating the presence of focal adhesion contacts in MCF-7 cells. x 2,500

epithelial cells modulates stress fiber formation by Ca^{2+}/CaM and through ROCK (Benítez-King, 2006; Ramírez-Rodriguez, et al., 2007). Melatonin binds to CaM and antagonizes its activity (Benítez-King et al., 1993). In MDCK cells the CaM antagonist ophiobolin resembles the effects of melatonin, causing the thickening of stress fibers associated with an increased water transport, measured as dome formation. Also, in MDCK cells, melatonin induced CaM redistribution from the cytosol to the membrane cytoskeletal fraction and in the nucleus of MDCK cells (Benitez-King et al., 1991). This effect is reversible, since after melatonin withdrawal, CaM is redistributed to the cell periphery. This data suggests that melatonin might induce stress fiber formation by recruiting CaM in microfilaments that will associate with MLCK and myosin phosphorylation.

Recently, we have demonstrated that melatonin induces CaM phosphorylation by the alpha isoform of PKC in MDCK cells and that this post-translational modification of CaM is associated with its subcellular redistribution to the cytoskeleton (Soto-Vega et al., 2004) (Fig. 4A,B). This evidence has suggested that phosphorylation is a signal to change the CaM distribution in a spatio-temporal manner, which consequently exerts its functions in different cellular compartments. In fact, Dai (2002) demonstrated that CaM is redistributed from the particulate fraction to the cytosol in MCF-7 cells treated with melatonin (Dai et al., 2002). Thus, data strongly suggest that phosphorylation is a signal elicited by melatonin to concentrate

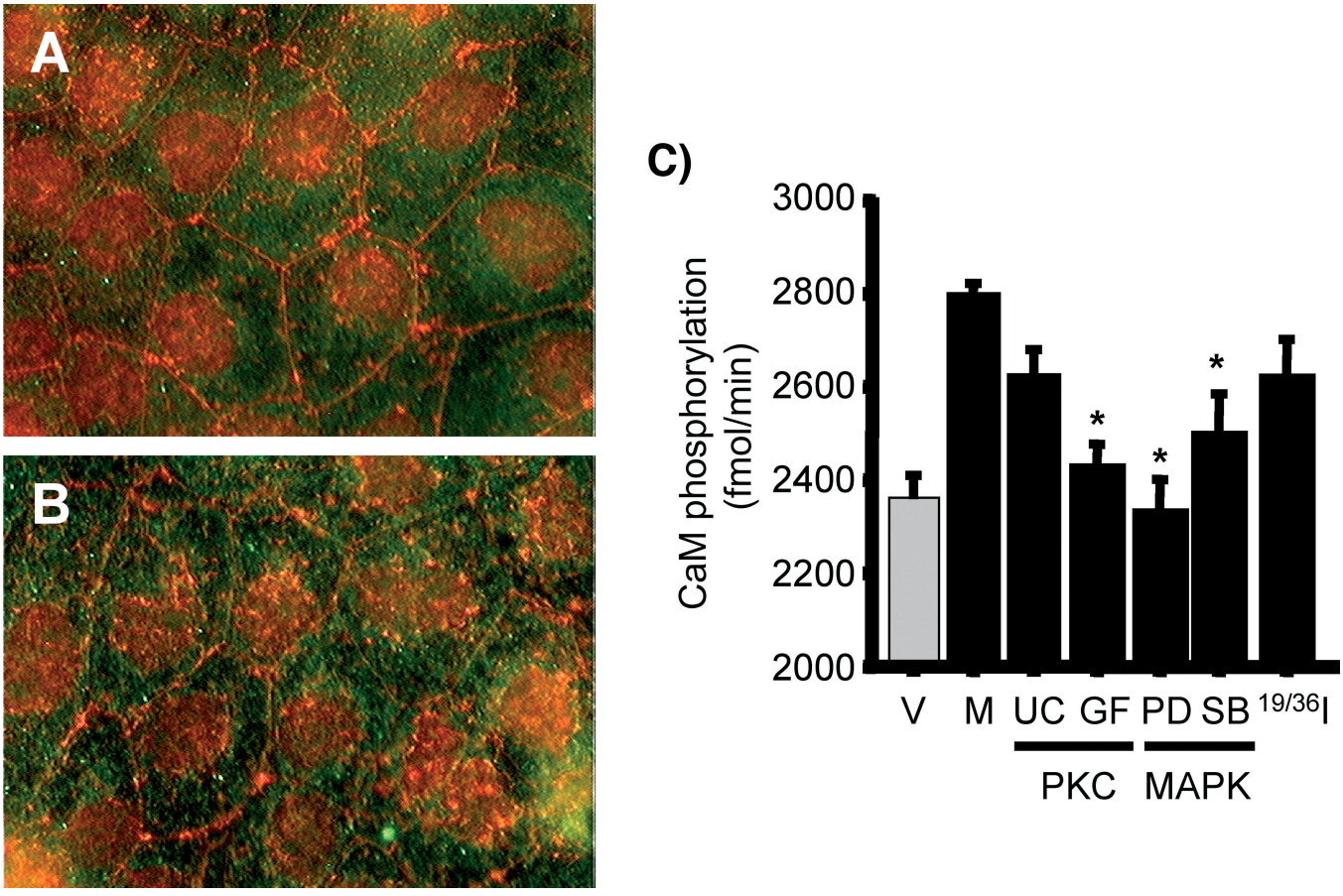

Fig. 4. Colocalization of calmodulin with phosphoserine/phosphothreonine residues in MDCK cells treated with melatonin. MDCK cell monolayers were incubated with either **(A)** the vehicle, or **(B)** 10^{-9} M melatonin for 5 h. After incubation, MDCK cells were fixed and simultaneously stained with anti-CaM (green), anti-phosphoserine and anti-phosphothreonine (red) antibodies. Phospho-CaM was detected by image overlay. Images shown phospho-CaM (orange) distributed in the membrane, in the nucleus. CaM (green) was distributed in spots, as well as in thin filaments all over the cells. Quantitative effects of melatonin on CaM phosphorylation are shown in **(C)**. CaM was used as substrate and MCF-7 cell extracts as a kinase source. CaM phosphorylation was measured by the P^{32} incorporation and [λ--^{32}P]-ATP was used as phosphate donor. Bars indicate total incorporation without substraction of basal phosphorylation. In the presence of 10^{-5} M melatonin, an increased CaM phosphorylation is observed. While in the presence of either the PKC inhibitors, calphostin (UC), bisindolylmaleimide (GF), the PKC (19-36) peptide pseudosubstrate; or the MAPK and ERK inhibitors, (PD098059 and SB203580, respectively), decreased CaM phosphorylation triggered by melatonin occurred. Results represent the mean ±SEM of one out of three experiments done in quadruplicate. Asterisks indicate significant differences compared with the vehicle. ($p<0.05$). A, B, x 1600

CaM in the cytosol or in the cytoskeleton according to the cell type. Moreover, the results indicate that melatonin has bimodal opposite effects on CaM activity, since in a space of minutes melatonin acts as CaM antagonist, while over a longer period the indole acts as a CaM agonist by recruiting this protein in specific subcellular compartments, one of which is the cytoskeleton structure. CaM phosphorylation induced by melatonin through PKC is also produced in cancer derived MCF-7 cells. In these cells, melatonin produces a redistribution of CaM and phospho- CaM, as can be seen by simultaneous immunoflorescence staining of CaM and phosphoserine/phosphothreonine residues (Fig. 4A,B). In addition, in cell homogenates of MCF-7 cells used as a source of PKC, melatonin stimulated the phosphorylation of exogenously added purified CaM. CaM phosphorylation stimulated by melatonin was inhibited either by bisindolylmaleimide, the PKC inhibitor, or the MAPK inhibitors (Fig. 4C). Thus, these results indicate that phosphorylation of CaM induced by melatonin is modulated by the PKC and MAPK signalling pathways. Since both MAPK and ERKs are downstream kinases in the PKC signalling pathway (Rigot et al., 1998), another possibility could be that MAPK and ERKs can be activated by melatonin downstream of the PKC signalling pathway.

Microfilament phenotypes elicited by melatonin in MDCK cells are mediated by an independent membrane receptor pathway. This is supported by the fact that microfilament rearrangements are observed in the presence of a wide range of concentrations of luzindole, the mt1 and MT2 melatonin membrane receptor antagonist. Also, thickening of stress fibers and increased focal adhesion formation are observed in preparations of microfilaments in situ. This preparation contains filamentous actin, the kinases and the microenvironment involved in microfilament organization, but lacks of plasma membranes, membrane proteins and receptors (Lenk et al., 1977; Benítez-King et al., 2001). Thus, this preparation allowed the demonstration of microfilament rearrangements in conditions where membrane receptors were removed. Additionally, intracellular actions of melatonin non-mediated by membrane receptors are supported by the fact that this indole is a highly lipophilic molecule which crosses the lipid bilayers of cellular membranes (Costa et al., 1997) and which is accumulated intracellularly (Menendez-Pelaez and Reiter, 1993). By contrast, in

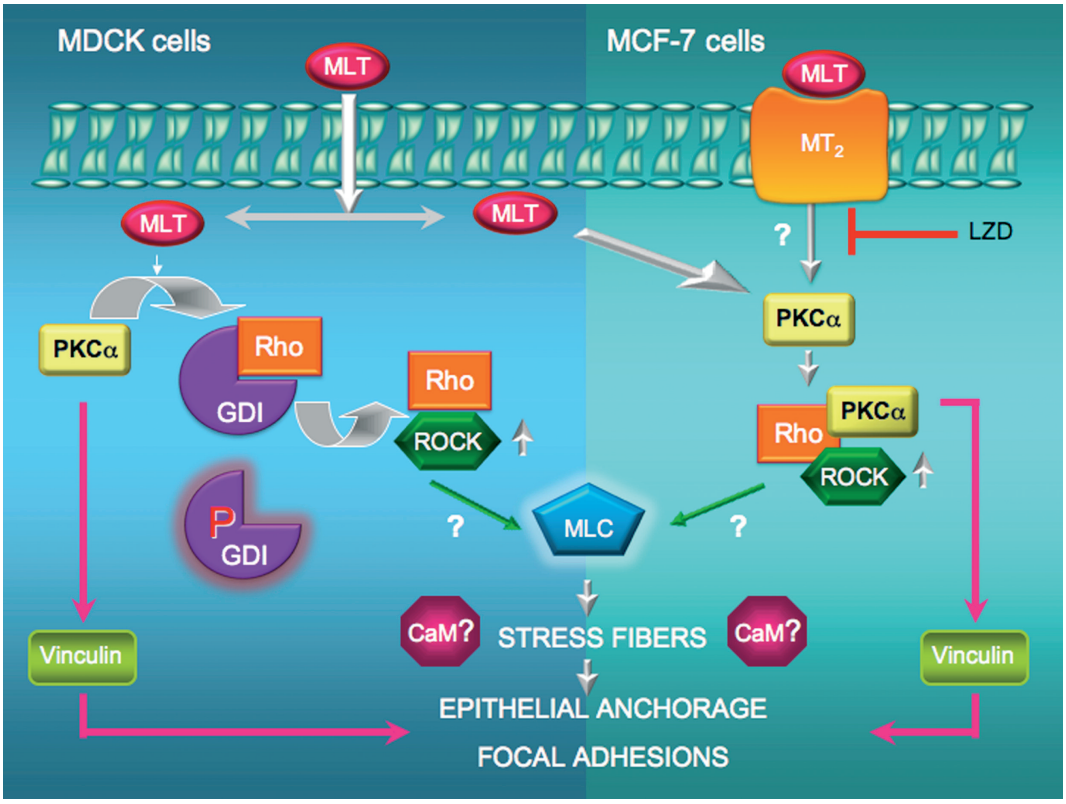

Fig. 5. Representative scheme of the mechanisms by which melatonin induces stress fiber formation and elicits focal adhesion contact formation in transport epithelial cells (MDCK) and in cancer derived cells (MCF-7). On the left side melatonin (MLT, red) crosses the plasma membrane of MDCK cells to activate the alpha isoform of PKC (PKC, yellow) that in turn activates Rho (orange). Rho interacts with Rho-dependent kinase (ROCK, green), which is activated to induce stress fiber formation through myosin- light chain kinase phosphorylation (MLC, blue). Phospho-MLC phospho-rylates myosin, favouring the stress fiber actin or inducing actin polimerization. In addition, melatonin through PKC, activation induces vinculin phosphorylation (vinculin, green) which will form the focal adhesion contacts supported by stress fibers. On the right side in MCF-7 cells melatonin through its membrane receptor activation induces PKC activation to cause stress fiber formation and vinculin phosphorylation, to finally form focal adhesion contacts which are important for epithelial anchorage. This effect is inhibited by luzindole, the melatonin receptor antagonist (LZD). Finally, calmodulin (CaM, pink) may associate with microfilaments to induce stress fiber contraction by activation of MLCK and myosin light chain phosphorylation.

MCF-7 cells, melatonin signal is transduced by ROCK downstream of PKC pathway and melatonin receptors to inhibit the cytoskeletal phenotype of migratory cells and to stimulate cell anchorage. This notion is in agreement with the reported participation of melatonin receptors in the melatonin oncostatic actions in MCF-7 cells and is supported by the fact that in the presence of luzindole the organization of microfilament phenotypes elicited by melatonin in MCF-7 cells is blocked. Thus, data indicate that differential regulation of microfilament phenotypes by melatonin is mediated by membrane receptors in MCF-7 cells and that is non-mediated by membrane receptors in MDCK cells (Fig. 5).

Concluding remarks

Melatonin, the main product synthesized by the pineal gland, acts as a modulator of the cytoskeletal structure. In MDCK epithelial cells, the indole modulates water transport by changing the stress fiber structure and increasing focal adhesion formation. In cancer MCF-7 mammary breast cancer cells, melatonin changes the microfilament phenotypes from highly migratory to an anchorage phenotype. Our results suggest that according to the cell type, melatonin will produce cytoskeletal phenotypes involved in important cellular functions for life, for instance cell movement, through membrane receptors or by direct intracellular interactions. Finally, it is important to mention that our results strongly suggest that the cytoskeleton can be a target for therapeutical actions of melatonin, since the results and the studies discussed here support that melatonin induces a well organized cytoskeleton. It also induces a differentiated cell structure that may participate in the melatonin oncostatic effects.

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