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Research Article

Evaluation of signal transduction pathways mediating the nuclear exclusion of the androgen receptor by melatonin

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Abstract. The intracellular signaling pathways mediating the nuclear exclusion of the androgen receptor (AR) by melatonin were evaluated in PC3 cells stably transfected with the AR. The melatonin-induced nuclear exclusion of the AR by melatonin (100 nM, 3 h) was blocked by LY 83583 (an inhibitor of guanylyl cyclases). 8-Bromo-cGMP (a cell-permeable cGMP analog), mimicked the effect of melatonin, as did ionomycin (a calcium ionophore) and PMA [an activator of protein kinase C (PKC)], and their effects were blocked by GF-109203X (a selective PKC inhibitor). BAPTA (an intracellular calcium chelator) blocked the effects of melatonin and 8-bromo-cGMP but not of PMA. Inhibition or activation of the protein kinase A pathway did not affect basal or melatonin-mediated AR localization. We conclude that the melatonin-mediated rise in cGMP elicits AR nuclear exclusion via a pathway involving increased intracellular calcium and PKC activation. These results define a novel signaling pathway that regulates AR localization and androgen responses in target cells.

Key words. Melatonin; protein kinase C; protein kinase A; protein kinase G; cGMP; androgen receptor; nuclear localization.

The androgen receptor (AR) is a member of the steroid/thyroid hormone receptor gene superfamily that function as ligand-dependent transcription factors [1]. The AR mediates the androgen-dependent development, differentiation, and maintenance of male reproductive function, support of sexually dimorphic functions including cognitive activities, and enhancement of prostate cancer growth [2]. Unliganded AR is found in the nucleus in some target tissues and in the cytosol and nucleus in others [3, 4]. Nuclear localization is a hallmark of AR activity. Upon ligand binding, the AR binds to hormone response elements in the promoter region of inducible androgen-dependent genes, thus controlling their transcription [1]. Moreover, mutations in the DNA-binding domain of the AR lead to nuclear exclusion of the receptor and result in loss of androgen sensitivity [5]. In addition, nuclear localization of the aggregated AR mutant is thought to promote spinal bulbar muscular atrophy (SBMA, Kennedy's disease) pathology [6, 7]. This raises new awareness of the significance of the intracellular localization of the AR in health and disease.

Melatonin, the hormone secreted at night by the pineal gland, inhibits the growth of human benign (BPH) and tumor (LNCaP) prostate cells [8–10]. The effects of melatonin on AR localization, level, and activity have recently been assessed in human prostate cancer LNCaP cells, which express innate AR, and in human androgen-insensitive prostate carcinoma PC3 cells stably transfected with a wild-type AR-expressing vector (PC3-AR). In both cell types, the AR was localized in the cell nucleus, and melatonin (1–100 nM, 1 and 24 h) caused its robust exclusion [11, 12; Rimler et al., unpublished data]. The nuclear export inhibitor, leptomycin B, prevented this process. No such effect of melatonin has been found on

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the nuclear localization of type α estrogen receptors (ERα) in these cells, suggesting AR specificity of this effect [Rimler et al., unpublished data].

AR nuclear exclusion by melatonin was associated with a marked decrease in androgen-induced reporter gene activity in both cell types [11, 12; Rimler et al., unpublished data]. The decrease was most probably due to the nuclear exclusion and not to AR inactivation because the AR level as well as androgen-binding capacity in the two cell types were not suppressed but, rather, enhanced [11, 12; Rimler et al., unpublished data]. The signal transduction pathways involved in the nuclear exclusion of the AR have not been elucidated.

Melatonin has been shown to activate specific G-protein coupled receptors [13] which subsequently activate a number of intracellular signal transduction pathways. Among these are inhibition of forskolin-induced cAMP formation, enhancement as well as suppression of cGMP levels, and activation of protein kinase C (PKC) [13-17]. In the present study, we utilized a pharmacological approach to examine the role of these assorted signaling pathways in mediating the nuclear exclusion of the AR by melatonin in PC3-AR cells.

Our results implicated the melatonin-induced elevation of cGMP in the nuclear exclusion of the AR and defined the cascade of events underlying this response. These data provide new insights into the role of intracellular signaling pathways in AR intracellular trafficking.

Materials and methods

Materials

RPMI-1640 medium (RPMI), RPMI-1640 medium without phenol red (RPMI-P), fetal calf serum (FCS), charcoal-stripped FCS (FCSC), L-glutamine, and antibiotics were obtained from Biological Industries (Beit Haemek, Israel). Melatonin, bovine serum albumin (BSA), phorbol 12-myristate 13-acetate (PMA), Hoechst 33258 (2′[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bi-1H-benzimidazole), 8-bromo-cAMP (8-bromo-adeno sine-3′:5′-cyclic monophosphate), 8-bromo-cGMP (8-bromoguanosine 3′5′ cyclic monophosphate), H-89 (N-(-2-[methylamino]ethyl)-5-isoquinoline-sulfonamide), ionomycin, GF109203X-HCl (bisindolylmaleimide hydrochloride), BAPTA (1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid), and EGTA (ethyleneglycol-bis (β aminoethyl 1 ether) N,N,N′,N′-tetraacetic acid) were obtained from Sigma (St. Louis, Mo.). KT5823 and LY 83583 were purchased from Calbiochem (San Diego, Calif.). Polyclonal (rabbit) anti-AR antibodies (prepared against a synthetic peptide corresponding to the N-terminal 21 amino acids of mouse, rat, and human AR), and the neutralizing synthetic peptide for these antibodies were purchased from Affinity Bioreagents (USA). The ECL chemiluminescence kit was obtained from Amersham (UK).

PC3-AR cell culture

Human PC3 cells, stably transfected with wild-type AR cDNA [18], were donated by Dr. A. Cato (Karlsruhe, Germany). The cells were cultured in RPMI medium containing 10% FCS, 50 units/ml penicillin, 50 µg/ml streptomycin, and 600 ng/ml G418. For each experiment, the cells were grown in RPMI-P containing 2 mM glutamine and 5% FCSC for 4 days at 37°C in a humidified atmosphere with 5% CO_2.

AR nuclear exclusion assay

Cells were plated on glass coverslips (No. 1, 22 mm; Corning) (35,000 cells/coverslip) inserted into six-well culture dishes and grown for 2 days in RPMI-P medium containing 2 mM glutamine and 5% FCSC. Various pharmacological agents (for 3 h), melatonin (100 nM), their combination or vehicle were then added and incubation resumed for 3 h. The cells, attached to the coverslips, were then washed three times with HBSS/HEPES buffer (0.02 M HEPES, pH 7.4) at room temperature, fixed (methanol, 5 min), and permeabilized (acetone, 2 min), washed twice with HBSS/HEPES buffer, and once with HBSS/HEPES containing 1% BSA pH 7.4. The coverslips were then incubated with 200 µg/ml normal goat IgG in HBSS/HEPES for 30 min to block non-specific absorption sites, washed three times in HBSS/HEPES and then incubated for 1 h with 4 µg/ml polyclonal (rabbit) anti-AR antibodies in HBSS/HEPES, in the absence or presence of the neutralizing synthetic peptide for these antibodies, washed three times in HBSS/HEPES, and then incubated in HBSS/HEPES with Cy3-conjugated goat anti-rabbit IgG (1.5 µg/ml, 1 h) and washed in HBSS/HEPES. Nuclei were stained with Hoechst 33258 (10 µg/ml, 5 min), the slides were washed again in HBSS/HEPES and dried. The cells were photographed at 40-fold magnification in a fluorescence microscope (to visualize AR), and then in UV light (to visualize cell nuclei).

A semi-quantitative determination of the cellular localization of the AR (cytoplasm and nucleus) was obtained by determining the intensity of AR fluorescence per square millimeter in the cytoplasm and nucleus of the various cells in the field and calculating the mean ratio (cytoplasmic/nuclear staining intensity) between these values across all cells in the field. The mean ratio $\pm$ standard deviation from a minimum of six fields per treatment condition were enumerated.

Pharmacological treatments

Cells were treated for 3 h with the following agents**:** 8-bromo-cGMP (100 nM), PMA (100 ng/ml), ionomycin (10 µM), 8-bromo-cAMP (0.1 mM), GF109203X-HCl (500 nM), KT5823 (1 µM), LY 83583 (50 µM), BAPTA

(20 µM), EGTA (1 mM), or H-89 (50 nM). Immunocytochemical analysis was then performed as described above.

Statistical evaluations

All data presented in the figures are representative of two to three experiments. The semi-quantitative evaluations are expressed as the mean±SD and differences between treatment groups were judged to be statistically significant at $p<0.05$, as determined by analysis of variance (ANOVA) followed by post hoc Student-Neumann-Keuls analyses.

Results

The role of several potential melatonin-signaling pathways in the nuclear exclusion of the AR was investigated using indirect immunofluorescence microscopy. This was pursued by evaluating the effects of a variety of pharmacological agents, known to either activate or inhibit these pathways, on AR localization in the absence or presence of melatonin.

As expected, most of the AR staining in the control PC3-AR cells was associated with the cell nuclei (fig. 1 A, B), whereas in cells treated with melatonin (100 nM, 3 h), a substantial amount of AR appeared in the cell cytoplasm (fig. 1 C, D). No labeling was observed in the absence of the AR antibody, indicating specificity of the cytochemical staining (not shown).

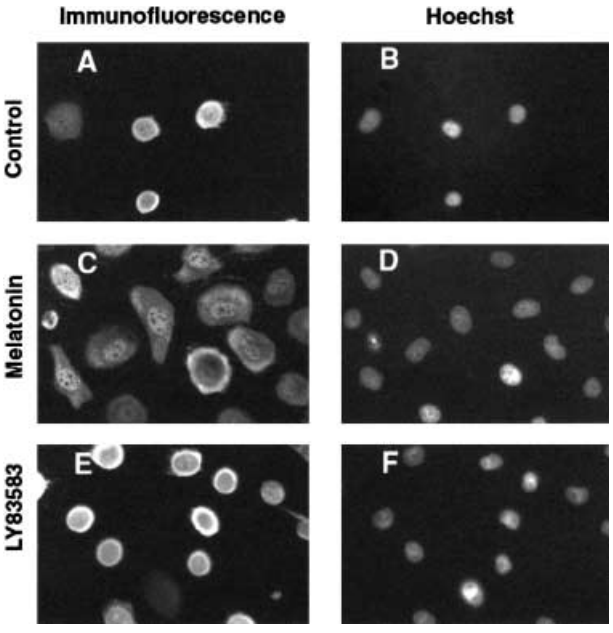

Figure 1. Effect of melatonin treatment on AR localization and its blockade by LY83583. Cells were grown in RPMI-P medium containing 5% FCSC and then treated for 3 h with vehicle (*A, B*) or 100 nM melatonin without (*C, D*) or with (*E, F*) LY83583 (50 µM), and subjected to indirect immunofluorescence for AR detection (*A, C, D*). Cell nuclei were stained with Hoechst (*B, D, F*).

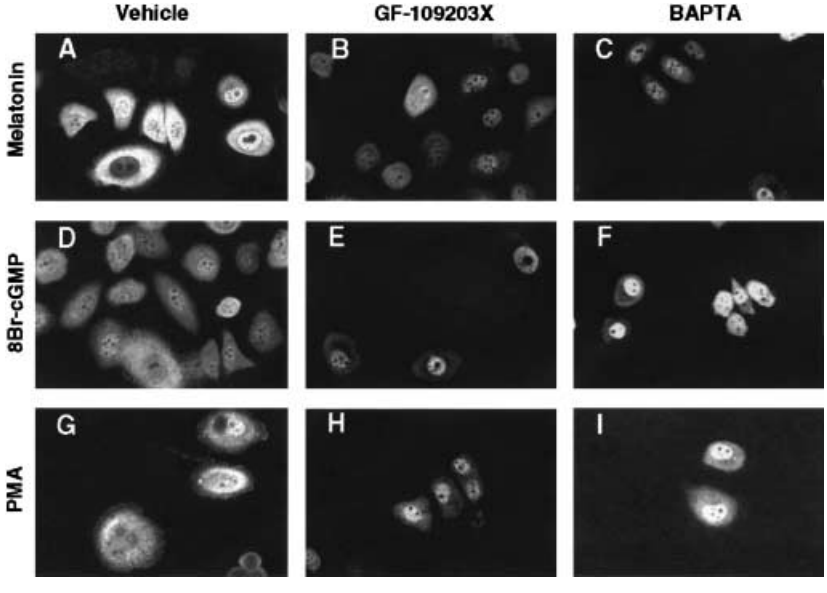

Figure 2. Effect of pharmacologic activators and inhibitors of potential signal transduction pathways on AR localization. Cells were grown in RPMI-P medium containing 5% FCSC and then treated for 3 h with various agents and subjected to indirect immunofluorescence for AR detection**.** Nuclear exclusion of the AR induced by melatonin (*A*), 8-bromo-cGMP (*D*), or PMA (*G*) was abrogated by the PKC inhibitor GF-109203X (*B, E, H,* respectively). Nuclear exclusion of the AR by melatonin (*A*) or 8-bromo-cGMP (*D*) but not PMA (*G*) treatment was reversed by the intracellular calcium-chelating agent BAPTA (*C, F, I,* respectively).

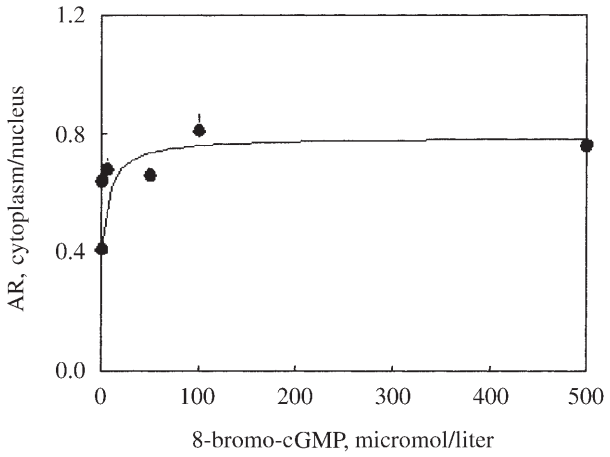

Figure 3. Dose-dependent effect of 8-bromo-cGMP treatment on AR localization. Cells were grown in RPMI-P medium containing 5% FCSC and then treated for 3 h with various concentrations of 8-bromo-cGMP and subjected to indirect immuno-fluorescence for AR detection. The cellular localization of the AR (cytoplasm and nucleus) is expressed as the mean ratio between the intensity of AR fluorescence per square millimeter in the cytoplasm and nucleus of the various cells in the field. *$p < 0.01$, Student t test compared to control cells treated with vehicle.

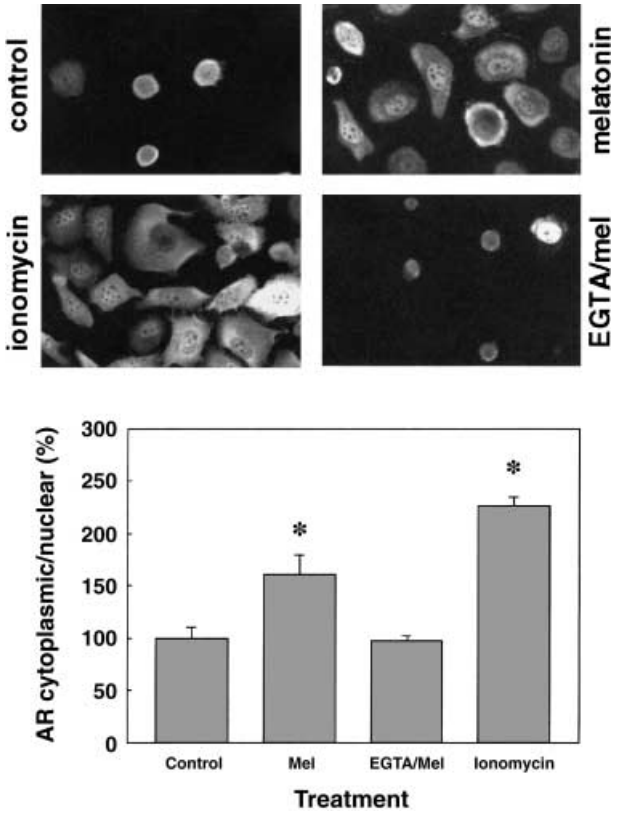

Figure 4. Effects of ionomycin, melatonin, and EGTA treatments on AR localization. Cells were grown in RPMI-P medium containing 5% FCSC and then treated for 3 h with vehicle, melatonin (100 nM) without or with EGTA (1 mM), and ionomycin (10 µM), and subjected to indirect immunofluorescence for AR detection (upper panels). The cellular localization of the AR (cytoplasm and nucleus) is depicted in a bar graph as the mean ratio between the intensity of AR fluorescence per square millimeter in the cytoplasm and nucleus of the various cells in the field (as % of the control cells). *$p < 0.01$ compared to control cells treated with vehicle (ANOVA followed by Student-Neumann-Keuls test).

Since melatonin enhances cGMP levels in PC3-AR cells [Rimler et al., unpublished data], the effects of LY 83583, a competitive inhibitor of soluble guanylyl cyclase [19] on the melatonin-mediated nuclear exclusion of the AR was investigated. LY 83583 did not affect the basal nuclear localization of the AR (not shown) but completely abolished the effect of melatonin (fig. 1E, F).

Following this finding, the ability of a cell-permeable cGMP analog (8-bromo-cGMP) to mimic the melatonin-mediated nuclear exclusion of the AR was studied (fig. 2). Like melatonin, 8-bromo-cGMP (100 nM, 3 h) promoted a marked nuclear exclusion of the AR (fig. 2D). The effect of 8-bromo-cGMP was significant and concentration dependent (IC_{50} around 8 µM; fig. 3).

KT5283 (1 µM), a cell-permeable inhibitor of protein kinase G [20], did not affect AR localization on its own and did not prevent the nuclear exclusion induced by 100 nM of either melatonin or cGMP (data not shown).

Melatonin has been found to inhibit cAMP formation in a number of systems including the parent cell line PC3 [13, 15]. The involvement of cAMP in AR nuclear exclusion was thus investigated. The cell-permeable cAMP analog, 8-bromo-cAMP, which would presumably compensate for a decrease in cAMP, had no effect on its own on AR localization and did not prevent its melatonin-mediated nuclear exclusion (data not shown). Moreover, H-89, a selective inhibitor of protein kinase A (PKA), which would presumably block cAMP downstream processes, did not promote nuclear localization of the AR and had no measurable effect on the melatonin-mediated nuclear exclusion of the AR (data not shown).

Involvement of the PKC pathway in the nuclear exclusion was investigated. In cells treated with PMA (100 ng/ml), a potent PKC activator [21], the AR appeared in the cell cytoplasm (fig. 2G). The PKC inhibitor GF-109203X [22] prevented AR nuclear exclusion regardless of whether it was promoted by melatonin, 8-bromo-cGMP, or PMA (fig. 2B, E, H).

The possible role of intracellular calcium ions (Ca^{2+}) in PKC activation and subsequently AR nuclear exclusion was studied. If Ca^{2+} were involved in melatonin action, an increase in intracellular Ca^{2+} by independent means should promote the nuclear exclusion of the AR whereas agents that decrease intracellular Ca^{2+} would prevent these effects. Compatible with these notions, in cells treated with ionomycin (10 µM), a Ca^{2+} ionophore [23], the AR was mostly excluded from the nucleus (fig. 4). The effect of ionomycin was essentially similar or greater than that of melatonin. Lowering extracellular Ca^{2+} with

EGTA prevented AR nuclear exclusion by melatonin (fig. 4). In addition, the intracellular calcium chelating agent BAPTA (20 μM) prevented the melatonin- as well as the 8-bromo-cGMP-induced nuclear exclusion of the AR (fig. 2C, F) but not that induced by PMA (fig. 2I).

Discussion

The results obtained in this study strongly suggest that the elevation of cGMP induced by melatonin induces AR nuclear exclusion. This is supported both by the melatonin-mimicking effect of the cGMP analog 8-bromo-cGMP, as well as by the complete blockade of the melatonin effect by LY 83583, a potent inhibitor of the soluble guanylate cyclase. The blockade by LY 83583 may imply that melatonin enhances cGMP in the PC3-AR cells via the soluble guanylate cyclase pathway (directly or indirectly, e.g., via the nitric oxide pathway). Melatonin has been shown to stimulate cGMP formation in amphibia via Mel-1c melatonin receptors but the expression of these receptors in mammalian tissues has not been demonstrated. On the other hand, MT_2 melatonin receptors, expressed in mammalian HEK293 cells, have been shown to induce suppression rather than enhancement of cGMP levels via the soluble guanylyl cyclase pathway [24, 25]. Other melatonin receptor subtypes that may mediate cGMP responses have not been described. However, the enhancement of cGMP level by melatonin may not be due to activation of soluble guanylyl cyclase. In such a case, the effect of LY 83583 may be explained by a general lowering of cellular cGMP that abrogates cGMP elevation by melatonin in the cells.

The results also show that activation of PKC is a critical event in the nuclear exclusion of the AR because (i) the effects of melatonin as well as cGMP on AR localization are all blocked by inhibiting the PKC pathway and (ii) direct activation of PKC by PMA promotes AR nuclear exclusion. These data also imply that PKC activation occurs downstream of the melatonin-cGMP cascade. Activation of PKC by melatonin has been demonstrated in benign prostate epithelial cells, in LNCaP cells that express innate MT1 melatonin receptors [26], in NIH 3T3 cells stably expressing the human mt1 receptor and in *Xenopus* oocytes transfetcted with the human mt1 receptor [10, 16, 17, 27]. Activation of phosphoinositide hydrolysis, directly or indirectly, by melatonin has been demonstrated in some of these systems, potentially leading to PKC activation [17, 27].

Another possibility for PKC activation by melatonin is by increasing intracellular Ca^{2+}. Effects of melatonin on calcium homeostasis have been demonstrated in a number of systems. In rat hypothalamic tissue and synaptosomes and in prostate LNCaP cells, melatonin effected a sustained in-

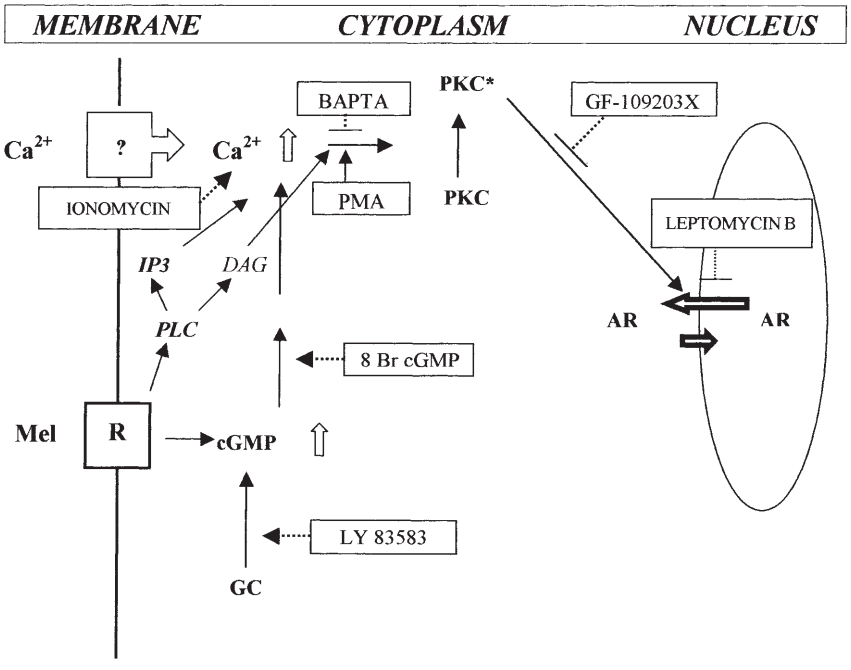

Figure 5. Hypothetical scheme by which melatonin may effect AR nuclear exclusion**.** Stimulation of the melatonin receptor elicits an increase in cGMP that triggers an increase in intracellular Ca^{2+}, leading to PKC activation. Active PKC promotes cellular changes within the prostate cell resulting in nuclear exclusion of the AR. LY 83583, BAPTA, and GF 109203X (inhibitors of guanylate cyclase, intracellular Ca^{2+}, PKC and nuclear export, respectively) inhibit AR exclusion, whereas 8-bromo-cGMP, ionomycin, and PMA (enhancers of cGMP, intracellular Ca^{2+}, and PKC respectively) promote AR exclusion. A putative activation by melatonin of phospholipase C (PLC) to generate inositoltriphosphate (IP3) and diacylglycerol (DAG) is also presented. DAG, in the presence of Ca^{2+}, may activate PKC.

hibition of stimulated calcium influx [28–31]. However, there are also several examples demonstrating a transient increase in intracellular Ca^{2+} by melatonin, e.g., in single amphibian pigment cells, in rat hypothalami and synaptosomes, in HEK 293 cells stably expressing mt1 receptors, and in primary cultures of ovine pars tuberalis cells endogenously expressing MT1 receptors [29, 32–34]. Thus melatonin likely induces elevation of intracellular Ca^{2+} in the PC3-AR cells leading to PKC activation.

Compatible with this notion is the observation that BAPTA, a chelating agent that lowers intracellular Ca^{2+}, was able to inhibit AR nuclear exclusion induced by melatonin, as well as cGMP, but did not prevent the exclusion induced by PKC activation. This strongly suggests that elevation of Ca^{2+} is induced by cGMP in the cells and this precedes the activation of PKC leading to nuclear exclusion of the AR. Moreover, these data imply that elevation of Ca^{2+} is necessary for the activation of PKC and subsequent nuclear exclusion of the AR. This is further corroborated by the fact that introduction of Ca^{2+} into the cells via a calcium ionophore was sufficient to cause AR nuclear exclusion.

How the elevation of cGMP links melatonin to enhancement of intracellular Ca^{2+} is not yet clear. The PKG inhibitor, KT5283, failed to prevent the cGMP or melatonin response suggesting that PKG is not involved in this process. However, no definite conclusion can be drawn at this point, since the PKG inhibitors, while potent in vitro, seem to be less efficacious in whole cells [35].

The following simplified mechanism (fig. 5) may be proposed, on the basis of these findings, to explain AR nuclear exclusion by melatonin. Melatonin elicits an increase in cGMP that triggers an increase in intracellular Ca^{2+}, leading to PKC activation. Active PKC promotes cellular changes within the prostate cell resulting in nuclear exclusion of the AR. The possibility for dual action of melatonin on Ca^{2+} and phosphoinositide metabolism cannot be excluded at present.

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