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Melatonin modulates rat myotube-acetylcholine receptors by inhibiting calmodulin

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Abstract

Melatonin, the pineal gland hormone, modulates α-bungarotoxin sensitive nicotinic acetylcholine receptors in sympathetic nerve terminals, cerebellum and chick retina imposing a diurnal variation in functional responses [Markus, R.P., Zago, W.M., Carneiro, R.C., 1996. Melatonin modulation of presynaptic nicotinic acetylcholine receptors in the rat vas deferens. J. Pharmacol. Exp. Ther. 279, 18–22; Markus, R.P., Santos, J. M., Zago, W., Reno, L.A., 2003. Melatonin nocturnal surge modulates nicotinic receptors and nicotine-induced [^{3}HI] glutamate release in rat cerebellum slices. J. Pharmacol. Exp. Ther. 305, 525-530; Sampaio, L.F.S., Hamassaki-Britto, D.E., Markus, R.P., 2005. Influence of melatonin on the development of functional nicotinic acetylcholine receptors in cultured chick retinal cells. Braz. J. Med. Biol. Res. 38, 603–613]. Here we show that in rat myotubes forskolin and melatonin reduced the number of nicotinic acetylcholine receptors expressed in plasma membrane. In addition, these cells expressed melatonin MT1 receptors, which are known to be coupled to G_i-protein. However, the pharmacological profile of melatonin analogs regarding the reduction in cyclic AMP accumulation and number of nicotinic acetylcholine receptors did not point to a mechanism mediated by activation of G_i-protein coupled receptors. On the other hand, calmidazolium, a classical inhibitor of calmodulin, reduced in a similar manner both effects. Considering that one isoform of adenylyl cyclase present in rat myotubes is regulated by Ca^{2+}/calmodulin, we propose that melatonin modulates the number of nicotinic acetylcholine receptors via reduction in cyclic AMP accumulation.
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Keywords: Melatonin; Nicotinic acetylcholine receptor; α-Bungarotoxin; Myotube culture; Cyclic AMP; Calmodulin

1. Introduction

Many biological processes follow diurnal rhythms related to the light–dark cycle. Melatonin, the hormone released at night by the pineal gland, is the marker of darkness (Simonneaux and Ribelayga, 2003). Darkness is associated with alterations in the number and function of α-bungarotoxin sensitive nicotinic acetycholine receptors in rat hypothalamus (Morley and Garner, 1990), cerebellum (Markus et al., 2003) and vas deferens (Carneiro et al., 1991). In the vas deferens, maximal contraction induced by activation of nicotinic acetycholine receptors increases at night, through a melatonin-dependent pathway (Carneiro et al., 1991, 1993). Administration of melatonin at night restores the diurnal rhythm response in the vas deferens from animals deprived of endogenous melatonin (Carneiro et al., 1994). In the rat cerebellum, stimulation of α-bungarotoxin sensitive nicotinic acetycholine receptors increases the noctunal release of glutamate which is abolished by procedures that block nocturnal surge of melatonin, such as constant lighting or inhibition of β adrenoceptors (Markus et al., 2003). Melatonin also modulates the numbers of α-bungarotoxin sensitive nicotinic acetylcholine receptors in the rat vas deferens (Markus et al., 1996), cerebellum (Markus et al., 2003) and in chick retina culture (Sampaio et al., 2005).

Distinct signaling transduction pathways, such as activation of G-protein coupled receptors (Dubocovich et al., 2000)

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and inhibition of Ca^{2+}/calmodulin interaction (Benitez-King et al., 1996) mediates melatonin effects. Melatonin and 2-iodomelatonin activate both G_i-protein coupled MT1 and MT2 receptors, while 4-phenyl-2-propionamidotetralin (4P-PDOT) is selective for the MT2 receptor (Dubocovich, 1995; Lotufo et al., 2001). Differently from the MT1 and MT2 receptors, the putative *MT3* melatonin receptors, characterized as a binding site for some analogs (Nosjean et al., 2000; Barrenetxe et al., 2004) shows a distinct pharmacological profile, with high affinity for *N*-acetylserotonin and high selectivity for the agonist 5-methoxycarbonylamino-*N*-acetyl-tryptamine (5-MCA-NAT) (Dubocovich, 1995). Luzindole blocks all these responses (Witt-Enderby et al., 2003).

In order to study the action mechanism of melatonin on the membrane expression of nicotinic acetylcholine receptors we used the rat cultured myotubes which express an homogeneous population of α-bungarotoxin sensitive receptors, localized in clusters associated with calmodulin-dependent nitric oxide synthase (Luck et al., 2000) and cyclic AMP-dependent protein kinase (PKA) (Perkins et al., 2001). In this model, appropriate expression of nicotinic acetylcholine receptors is modulated by cyclic AMP and calmodulin-dependent mechanisms (Kang et al., 2003; Tang et al., 2001).

2. Materials and methods

2.1. Materials

Melatonin, *N*-acetylserotonin, 2-iodomelatonin, α-bungarotoxin, 3-isobutyl-1-methylxanthine, cytosine-B-D-arabinofuranoside hydrochloride (ARA-C), bovine serum albumin, 1-brome-3-chloropropane (BCP), sodium nitroprusside dehydrate (SNP) and isopropanol were purchased from Sigma Chemical Co., St. Louis, MO, USA; 4-phenyl-2-propionamidotetralin (4P-PDOT), luzindole (*N*-acetyl-2-benzytryptamine), 5-methoxy-carbonylamino-*N*-acetyl-tryptamine (5-MCA-NAT) and forskolin were from Tocris, Ballwin, MO, USA. Dulbecco's Modified Eagle Medium (DMEM), fetal calf serum, horse serum, collagenase, gentamicin reagent solution and Trizol™ Reagent were purchased from GIBCO BRL Products. Grand Island, NY, USA. 3-[^{125}I] iodotyrosyl α-bungarotoxin (specific activity 2000 Ci/mmol) was purchased from New England Nuclear, Boston, MA, USA. Pertussis toxin and calmidazolium chloride were from Calbiochem. Darmstadt, Germany. DEPC water and DNA-free kit were purchased from Ambion, Austin, TX, USA. Cyclic AMP and cyclic GMP kits were from BIOTRAK-Amersham Biosciences, Little Chalfont, Buckinghamshire, England.

2.2. Skeletal muscle culture

Primary skeletal muscles were obtained from hindlimb muscles of newborn rats as described by da Costa et al. (2001). Briefly, the hindlimbs were removed, minced and incubated for 2 h at 37 °C in a humidified atmosphere of 90% air and 10% CO_2 with collagenase (200 U/ml) diluted in Hank's balanced salt solution plus 3 mM $CaC1_2$. Then, the cell suspension was centrifuged (700 *g*, 5 min), the pellet was resuspended in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, 10% horse serum and 40 mg/l gentamicin, and the myoblasts (10^6 cells/well, 24 wells plate) were plated. The medium was replaced on the third day by DMEM supplemented with 8% horse serum, 2% fetal calf serum and 1 μM cytosine-B-D-arabinofuranoside hydrochloride.

On the fourth day, cells were incubated with a cytosine-B-D-arabinofuranoside hydrochloride free medium. All experiments were performed with 5–6-day-old differentiated cultured skeletal muscle cells.

2.3. [^{125}I]-α-Bungarotoxin binding sites

In this assay, 10^6 cells/well were incubated for 1 h in Krebs solution (mM, NaCl 116, KCl 4.6, $MgCl_2$ 1.5, $CaCl_2$ 2.5, $MgSO_4$ 1.0, $NaHCO_3$ 25, KH_2PO_4 0.8, glucose 11, pH 7.4, 37 °C) with 4 nM 3-[^{125}I] iodotyrosyl α-bungarotoxin in the absence, or presence of 0.1 μM unlabeled α-bungarotoxin. Cells washed three times with Krebs solution were resuspended in deionized water, immersed in scintillation cocktail (Ecolume, ICN Biomedicals, CO, USA) and radioactive was counted (Packard, TriCarb 2100TR, Downers Grove, Illinois, USA). Protein concentration was determined by the method of Bradford (1976) and the results were expressed as fmol/mg protein.

2.4. Cyclic AMP and cyclic GMP content

Cultured myotubes (10^6 cells/well) were rinsed two times with Krebs solution, pre-incubated with 1 mM 1 3-isobutyl-1-methylxanthine for 15 min and treated with melatonin (0.01–3 nM), *N*-acetylserotonin (0.1–1 nM), 4-P-PDOT (1–100 nM), or vehicle, in the presence or absence of forskolin (10 μm) for 5 min. When mentioned, the cells were incubated in pertussis toxin (0.5 μg/ml) overnight in a serum-free medium, before the addition of melatonin. Cyclic AMP or cyclic GMP from the samples was determined using the cyclic AMP or cyclic GMP Enzymeimmunoassay Biotrak (EIA) system. The results were expressed as a percentage of forskolin-induced increase in cyclic AMP or cyclic GMP basal levels shown as pmol/well.

2.5. Extraction of total RNA

Total RNA was extracted from cultured myotubes and pineal glands with Trizol™ Reagent. The samples received 134 μl of 1-brome-3chloropropane (BCP) and after centrifugation (12,000 x *g*, 15 min, 4 °C) the RNA present in the aqueous phase was precipitated with isopropanol. The pellet was rinsed twice in ethanol, resuspended in diethylpyrocarbonate (DEPC) water and treated with DNA-free kit (Ambion, Austin, TX, USA), for removal of DNA contamination. Reverse transcription-polymerase chain reaction (RT-PCR) was performed with 1 μg of total RNA, 0.5 μl of 0.2 μg/μl random primers, 1 μl of 10 mM deoxyribonucleotide triphosphate (dNTP) mix, 2 μl of 10 x PCR buffer, 2 μl of 10 mM dithiotreitol, 1 μl of 20 U/μl

ribonuclease inhibitor, and 1 μl of Superscript II reverse transcriptase (200 U, Invitrogen) for a final volume of 20 μl.

2.6. Polymerase chain reaction

Oligonucleotide primers were designed using Primer 3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi/primer3_www.cgi), based on the sequences obtained from the GenBank database accession numbers AF130341 (MT1) and AF141863 (MT2) (*National Center for Biotechnology Information National Library of Medicine National Institutes of Health*, www.ncbi.nlm.nih.gov,) as follows: receptor MT1; 5′-AAC.CTG.CTG.GTC.ATC.CTG.TC-3′ (sense), 5′-GAC.ACT.CAG.GCC.CAT.TAG.GA-3′ (anti-sense) and receptor MT2: 5′-CAT.CCA.CTT.CTT.CTT.TCC.AA-3′ (sense), 5′-TGC.AAG.GCC.AAT.ACA.GTT.GA-3′ (anti-sense). In the polymerase chain reaction, 1 μl of cDNA, 0.3 μl of AmpliTaq Gold DNA polymerase (5 U/μl, Amersham Biosciences, Buckinghamshire, England), 4 μl of 1.25 mM dNTP mix, 2 μl of each primer (10 pmol/μl), 2.5 μl of 10 x PCR buffer, and 1.5 μl of 25 mM $MgCI_2$ (all Invitrogen) were used for a final volume of 25 μl. The PCR reaction started with 6 min denaturation at 95 °C, followed by 35 cycles of 30 s denaturation at 95 °C, 45 s annealing at 60 °C, 1 min extension at 72 °C, ending with a final cycle of 10 min at 72 °C. The PCR was performed with the iCYCLER system (Biorad, Hercules, CA, USA). The size of the PCR products was verified in 1% ethidium bromide-stained 1.5% agarose gels, the band corresponding to the expected size (201 bp for MTl, primer location 123–142 bp and 323–304 bp and 202 bp for MT2, primer location 204–223 bp and 384–405 bp) was excised, and the fragment extracted by centrifugation in 50 μ1 filter tips (USA Scientific Inc.). Gel image was captured and digitalized by GEL PRO IMAGE system (Fotodyne Inc., Hartland, USA).

2.7. Cloning and sequencing

The PCR products were then passed through Millipore columns for salt and enzyme removal, eluted with PCR buffer and cloned in pCR II TOP0 vector (TOP0 TA Cloning kit, Invitrogen). The vector was then inserted into competent cells and the bacteria containing the DNA selected by ampicillin resistance and screened by PCR with T7 primer. The purified DNA (Mini-Prep kit, Promega, Madison, WI, USA) was quantified and sequenced in a ABI Prism sequencer (mod 310, ABI, Foster City, CA, USA). The nucleotide sequences were compared to the database of the *National Center for Biotechnology Information National Library of Medicine National Institutes of Health* (www.ncbi.nlm.nih.gov) site, using BLAST software.

2.8. Statistical analysis

B_{max} and K_d values were calculated by the GRAPHPAD program (Intuitive Software for Science, San Diego, CA, USA). The differences between experimental groups were compared by Student's *t* test (comparison of two means) or analysis of variance followed by Newman–Keuls (comparison of more than two means). The number of experiments refers to the number of different cultures.

3. Results

3.1. Determination of mRNA for MT1 and MT2 receptors

Rat myotube and pineal gland (control) PCR products presented bands of the expected size for MT1 receptor (201 bp), while the size for MT2 receptor followed the pattern only in the pineal gland. The band obtained with myotubes cDNA presented a larger size (Fig. 1). When PCR products were cloned and sequenced, the comparison to the GenBank database confirms a 100% of homology (accession number AFl30341) for MT1 mRNA from rat myotubes and MT1 and MT2 receptors from the pineal gland. Therefore, no effect can be mediated by MT2 melatonin receptor, as it is not expressed in this model.

3.2. Effect of melatonin and analogs on the number of α-bungarotoxin binding sites

The binding of [^{125}I]-α-bungarotoxin to cultured myotubes was saturable and presented a high specificity (B_{max} = 645 fmol/mg protein, and K_d = 3.2 nM) (Fig. 2). Treatment of cultures with melatonin (0.1–30 nM) for 8 h reduced the number of sites in a dose-dependent manner (Fig. 3A). The IC_{50} value for melatonin effect was 0.19 nM (confidence limit (CL) 0.03–1.24 nM), and the maximal reduction in the number of binding sites was 56.4 ± 5.67%. Incubation of cultures with melatonin (100 nM) for 1, 4 or 6 h did not interfere with the number of nicotinic acetylcholine receptors (data not shown). On the other hand, incubation of melatonin (0.1–100 nM) for 48 h resulted in a 50% reduction in the number of [^{125}I]-α-bungarotoxin binding sites in all doses tested (Fig. 3A). Thus, melatonin is able to reduce by 50% the membrane expression of nicotinic acetylcho1ine receptors, in a time dependent manner which commences after 8 h.

N-acetylserotonin, an agonist that presents higher affinity for *MT3* than for MT1 or MT2 receptors, also reduced the number

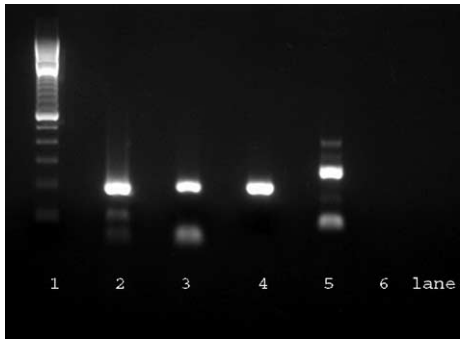

Fig. 1. RT-PCR analysis of MT1 and MT2 melatonin receptor mRNA expression in rat myotubes (lanes 3 and 5) and pineal gland (lanes 2 and 4), respectively. Lane 1—molecular weight standard; Lane 6—negative control, no template added. Band sizes are correct for rat myotube MT1 and for pineal gland MT1 and MT2.

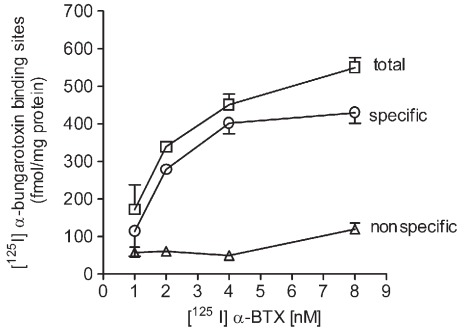

Fig. 2. Saturation curve to [^{125}I]-α-bungarotoxin in cultured myotubes. We seeded cells in culture dishes of 24 wells (10^6 cells/well) and kept in culture for 6 days, as described in Materials and methods. The saturation curve was obtained incubating the labeled ligand in the absence (square) or in the presence (triangle) of 0.1 μM nonlabeled α-bungarotoxin. Specific binding (circle) was calculated as the difference between total and non-specific binding. B_{max} value = 645 ± 164, and K_d value = 3.2 ± 1.9 nM. Each point represents the mean ± S.E.M. of 3–6 different cultures.

of binding sites (Fig. 3B). The IC_{50} value for *N*-acetylserotonin was 2.12 nM (CL: 0.2–24.1 nM) and the maximal reduction of receptors number was 59.57 ± 11.9%. Luzindole (10 μM), an antagonist of both MT1 and MT2 receptors, blocked melatonin effect (Fig. 3C). Surprisingly, 4P-PDOT, considered a selective ligand for MT2 receptor (Dubocovich, 1995; Lotufo et al., 2001), promoted per se a reduction in the number of α-bungarotoxin binding sites (Fig. 3D), in a system devoid of this receptor.

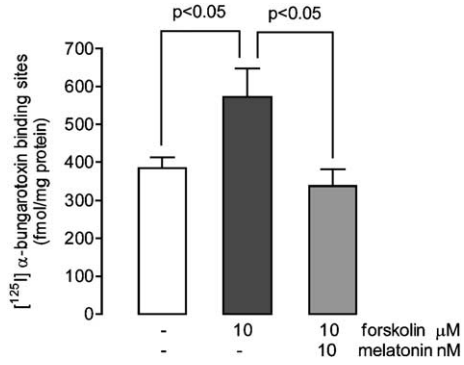

Fig. 4. Effect of forskolin on the number of α-bungarotoxin binding sites. Forskolin (10 μM, 8 h) increased the number of [^{125}I]-α-bungarotoxin binding sites in cultured rat myotubes, while melatonin (10 nM) reversed this effect. Data are shown as mean ± S.E.M. of 3 cultures. *P < 0.05.

2-iodomelatonin, a high affinity agonist for MT1 and MT2 receptors, which does not cross the membrane (Paul et al., 1999), and 5-MCA-NAT, a selective agonist for *MT3* binding sites, (Molinari et al., 1996; Nosjean et al., 2001) did not change the number of α-bungarotoxin binding sites (data not shown).

Considering that: a) only MT1 receptors are expressed in myotubes, b) 2-iodomelatonin, an agonist highly selective for MT1 receptors, has no effect on this system, c) 4P-PDOT, a selective ligand for MT2 receptors, mimics melatonin and *N*-acetylserotonin response, we concluded that the effect of melatonin on nicotinic acetylcholine receptors was not mediated by G-protein coupled receptors.

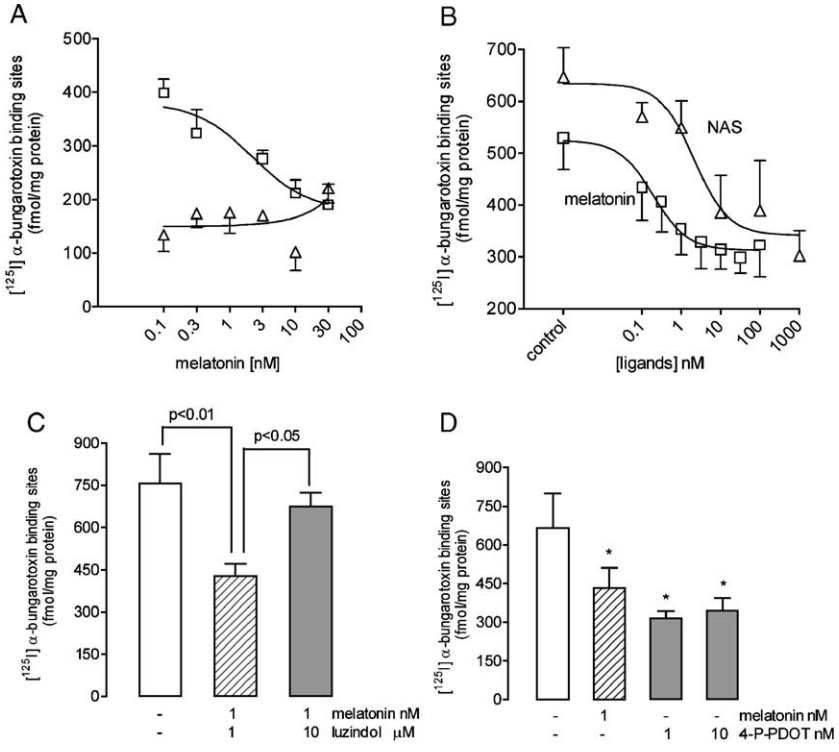

Fig. 3. Effect of melatonin and analogs on the number of [^{125}I]-α-bungarotoxin binding sites. (A) Melatonin incubated for 8 (squares) or 48 h (triangles) reduced in a time-dependent manner [^{125}I]-α-bungarotoxin B_{max}. (B) Melatonin (squares, n = 8) and *N*-acetylserotonin (NAS, triangles, n = 4–12) reduced in a dose-dependent manner [^{125}I]-α-bungarotoxin B_{max}. (C) Luzindole (10 μM) reversed the effect of melatonin (n = 3). (D) 4P-PDOT promoted the same effect of melatonin (n = 4). *P < 0.05. Therefore in this system luzindole is acting as an antagonist and 4P-PDOT as an agonist.

3.3. Effect of forskolin on the number of α-bungarotoxin binding sites

Forskolin (10 μM, 8 h) increased the number of [^{125}I]-α-bungarotoxin binding sites in cultured rat myotubes, while melatonin (10 nM) reversed this effect (Fig. 4).

3.4. Effect of melatonin and analogs on the production of cyclic AMP

Forskolin (10 μM, 5 min) increased cyclic AMP from 0.84±0.09 pmol/well (n=7) to 7.7±1.3 pmol/well. Melatonin (0.01–10 nM) and *N*-acetylserotonin (0.1–10 nM), incubated for 5 min with forskolin, inhibited cyclic AMP accumulation in a dose-dependent manner (melatonin IC_{50} value=2.96 nM; CL: 1.89 to 4.62 nM; *N*-acetylserotonin IC_{50} value=54.3 pM; CL: 27.4–107 pM). Interestingly, the MT2 antagonist 4P-PDOT (1–100 nM) also inhibited cyclic AMP production (IC_{50} value=1.47 nM; CL: 1.23–1.76 nM) (Fig. 5). Although luzindole (10 μM) blocked the effect of melatonin (data not shown), the pharmacological profile of melatonin analogs was not compatible with an action on MT1-melatonin receptors.

Treatment of cells with pertussis toxin (0.5 μg/ml, overnight) did not change inhibitory effect of melatonin on the production of cyclic AMP induced by forskolin (data not shown); suggesting that melatonin signaling transduction does not involve G_i-protein.

3.5. Effect of melatonin on cyclic GMP production

In myotubes, calmodulin activates nitric oxide synthase with a downstream stimulation of guanylyl cyclase and formation of cyclic GMP (Stamler and Meissner, 2001). Melatonin (1 nM) and calmidazolium (50 μM), a classical inhibitor of calmodulin, block KCl (30 mM)-induced cyclic GMP accumulation (Fig. 6). On the other hand, neither melatonin nor calmidazolium impaired the effect of sodium nitroprussiate (30 μM), a nitric oxide donor, which stimulates guanylyl cyclase, in a nitric oxide synthase independent manner.

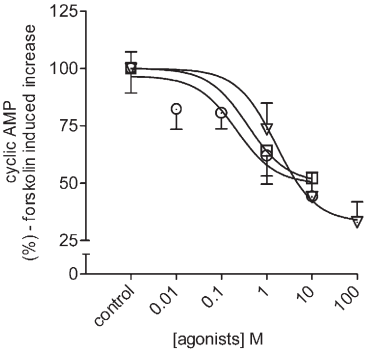

Fig. 5. Effect of melatonin and analogs on the cyclic AMP level in myotubes stimulated with forskolin (10 μM). Melatonin (circles), *N*-acetylserotonin (squares), and 4P-PDOT (triangles) inhibit in a dose-dependent manner forskolin-induced cyclic AMP production. Forskolin (10 μM) induced a production of 7.7±1.3 pmol/10^6 cells (n=7). Data are shown as mean±S.E.M. of 4–6 cultures.

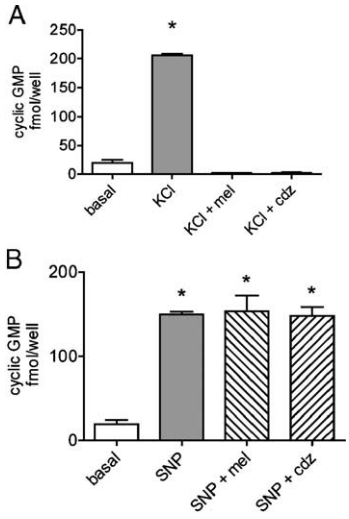

Fig. 6. Effect of melatonin (mel, 1 nM) and calmidazolium (cdz, 50 μM) on the production of cyclic GMP induced by KC1 (30 mM) (A), or sodium nitroprussiate (SNP, 30 μM) (B). Data are expressed as mean±S.E.M. of 3 cultures. *P<0.05 comparing to basal.

3.6. Inhibition of calmodulin mimicked melatonin effect on cyclic AMP production and reduction of the number of nicotinic acetylcholine receptors

The experiments described above strongly suggest that melatonin blocks calmodulin in rat myotubes. Here we tested if calmidazolium (50 μM) is able to mimic melatonin in reducing the number of [^{125}I]-α-bungarotoxin binding sites and cyclic AMP accumulation. Fig. 7 shows that similarly to melatonin, preincubation of calmidazolium (50 μM) for a short interval (5 min) reduces cyclic AMP accumulation, and a larger interval (8 h) resulted in a reduction of the number of [^{125}I]-α-bungarotoxin binding sites.

4. Discussion

Time keeping in mammals involves the decodification of diurnal cyclic information delivered by the alternation between

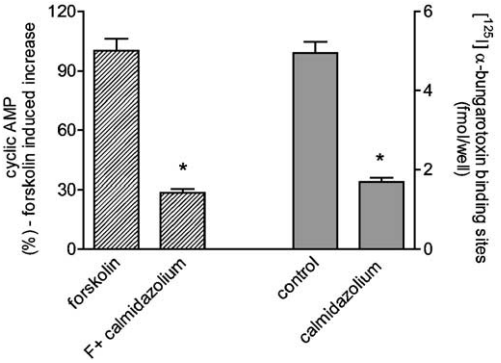

Fig. 7. Calmidazolium effect on forskolin-induced cAMP production and the number of α-bungarotoxin binding sites. The left axis shows the percentage of forskolin (F)-induced production of cyclic AMP in the presence or absence of calmidazolium (n=4). The right axis shows the number of [^{125}I]-α-bungarotoxin binding sites expressed as fmol/well (n=5). Data are shown as mean±S.E.M. *P<0.05.

day and night. In this process, melatonin is secreted only during the dark phase of the day, which makes this hormone the hallmark of darkness (Simonneaux and Ribelayga, 2003). Melatonin signal is translated at the cell level in order to synchronize biological responses. Our previous studies showed that melatonin, at a concentration compatible to nocturnal surge (nM range), modulates neuronal α-bungarotoxin sensitive nicotinic acetylcholine receptors in rat sympathetic nerve terminals (Zago and Markus, 1999), chick retina (Sampaio et al., 2005) and rat cerebellum (Markus et al., 2003). In contrast, modulation of α-bungarotoxin nonsensitive nicotinic acetylcholine receptor occurs only at mM range of melatonin (Schiller et al., 2003). In order to study the mechanism responsible for melatonin modulation of α-bungarotoxin sensitive nicotinic acetylcholine receptors, we used rat myotubes. This culture expresses a homogenous population of nicotinic receptors clustered in association to adenylyl cyclase and nitric oxide synthase (Froehner, 1991), allowing the evaluation of melatonin effect on cyclic AMP and cyclic GMP production and their influence on nicotinic receptor expression.

Our results showed that melatonin reduced the number of nicotinic acetylcholine receptors in a dose- and time-dependent manner. Incubating melatonin for 8 h resulted in a dose-dependent effect (0.1–10 nM), while after 48 h a maximal reduction was attained even with the lower melatonin concentration (0.1 nM). This time-course suggests that melatonin triggers a multi-step cascade, and promotes a cumulative effect.

Agents that rise intracellular levels of cyclic AMP, such as forskolin, increase the number of cell surface nicotinic acetylcholine receptors in mouse fibroblasts containing stably integrated *Torpedo* nicotinic acetylcholine receptors (Green et al., 1991; Ross et al., 1991). Maximal forskolin-induced increase in membrane-expressed receptors was obtained only after 48 h incubation (Jayawickreme et al., 1994). Here we show that forskolin, incubated for the same interval, also increases the number of membrane-expressed nicotinic acetylcholine receptors in rat myotubes. Interestingly, melatonin reverses both forskolin effects (accumulation of cyclic AMP and increase in the number of membrane expressed nicotinic acetylcholine receptors). The long interval between cyclic AMP signaling (5 min) and receptor reduction (8–48 h) favors a multi-step process.

In spite of a recent report showing that human melatonin MT1 receptors expressed in neuronal cells promotes cyclic AMP accumulation (Schuster et al., 2005), most commonly melatonin MT1 and MT2 receptors are coupled to G_i-proteins, which reduce cyclic AMP accumulation (Simonneaux and Ribelayga, 2003). RT-PCR determination of mRNA and the cloning of the resulting products from rat myotubes showed the presence of melatonin MT1 receptors mRNA, discarding any involvement of myotube melatonin MT2 receptors in the responses here studied.

In order to evaluate the contribution of melatonin MT1 and the putative *MT3* receptor we analyzed the pharmacological profile of melatonin analogs. The absence of 5- MCA-NAT effect, as well as, the high potency of *N*-acetylserotonin discarded the involvement of the putative melatonin *MT3* receptor. Melatonin MT1 receptor contribution is also questionable assuming its low affinity to *N*-acetylserotonin and is not sensitive to 4P-PDOT, and its high affinity to 2-iodomelatonin (Masana et al., 2003). Here we observed a high affinity to *N*-acetylserotonin and 4P-PDOT, and no response to 2-iodomelatonin. We reinforced this conclusion showing that pertussis toxin does not block melatonin-induced reduction in cyclic AMP. Therefore, the present data exclude the participation of G_i-protein coupled melatonin receptors in the reduction of forskolin-induced cyclic AMP production. An important conclusion, derived from the present data, is that 4P-PDOT, considered a selective antagonist or partial agonist of melatonin MT2 receptors (Dubocovich, 1995; Lotufo et al., 2001), mimics melatonin effect despite the absence of melatonin MT2. Therefore, this melatonin analog interacts with other melatonin signaling pathways, besides melatonin MT2 receptor.

Several melatonin effects are mediated by inhibition of calmodulin (Benitez-King et al., 1993), which plays a role in the maintenance of nicotinic acetylcholine receptors clusters in rat myotubes and activates some isoforms of adenylyl cyclase. In cultured myotubes, nicotinic acetylcholine receptors form aggregates with scaffold proteins and muscle constitutive nitric oxide synthase (Luck et al., 2000). This enzyme, activated by calmodulin (Fulton et al., 2001), is essential for cluster formation (Peng, 1984; Tai and Connolly, 1986). Regarding adenylyl cyclase, at least nine closely related isoforms have been cloned and characterized in mammals (Sinnarajah et al., 2001), some of them being modulated by Ca^{2+}/calmodulin and/or calcium. Skeletal muscle presents adenylyl cyclase isoforms II, VI, VII and IX (Defer et al., 2000), adenylyl cyclase VI being positively modulated by Ca^{2+}/calmodulin (Beazely and Watts, 2005).

Melatonin binds to calmodulin with high affinity (nM range), promoting several functional effects, such as tubulin polymerization, cytoskeletal rearrangement, inhibition of estrogen receptors, etc. (Benitez-King and Anton-Tay, 1993; Romero et al., 1998; del Rio et al., 2004). In addition, luzindole inhibits [^{3}H]-melatonin binding to calmodulin with a K_i value of 2.7 μM (Benitez-King et al., 1993). Therefore, the concentrations of melatonin and luzindole here tested are compatible with a calmodulin-dependent effect. In the present report we show evidences for an inhibitory effect of melatonin on nitric oxide-induced cyclic GPM accumulation, a well-known Ca^{2+}/calmodulin activated function relevant to organization of rat myotube (Stamler and Meissner, 2001). Melatonin and the calmodulin blocker, calmidazolium, reduce KCl-induced cyclic AMP accumulation. The effect of the nitric oxide donor, sodium nitroprusiate, was not affected by melatonin. A similar result was obtained in rat cerebellum, where melatonin reduces nitric oxide activity and binds calmodulin (Pozo et al., 1997).

It is interesting to note that in the rat cerebellum, melatonin also reduces the number of α-bungarotoxin sensitive nicotinic acetylcholine receptors, imposing a diurnal rhythm in cholinergic function (Markus et al., 2003; Reno et al., 2004). The diurnal variation of acetylcholine-induced glutamate release is lost in constant light, a procedure that impairs nocturnal melatonin surge. The rhythmic release of glutamate is restored

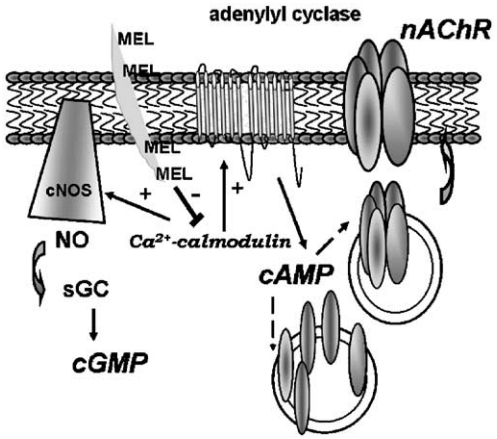

Fig. 8. Myotubes clusters co-localize nicotinic acetylcholine receptors (nAChR), adenylyl cyclase and constitutive nitric oxide synthase (cNOS), among other proteins (Luck et al., 2000). Inhibition of calmodulin by melatonin (MEL) results in a reduction of cyclic GMP (cGMP) and cyclic AMP (cAMP). The decrease in cyclic AMP that mediates membrane expression of nicotinic acetylcholine receptors, probably by signaling phosphorylation of proteins assisting at the level of folding or assembly of the receptor in the Golgi, will result in a diminution of the number of nicotinic acetylcholine receptors in plasma membrane, i.e., a reduction in the number of [^{125}I]-α-bungarotoxin binding sites.

by nocturnal administration of melatonin. On the other hand, nicotinic acetylcholine receptors in rat and chick myotubes have a half-life of 24 h, being therefore susceptible to melatonin synchronization (Devreotes and Fambrough, 1975).

The data described, so far, suggest that melatonin diffuses through the plasma membrane and inhibits calmodulin, which in turn reduces Ca^{2+}/calmodulin nitric oxide synthase activity, and cyclic GMP accumulation (Fig. 8). Finally, we showed that calmidazolium was able to simulate both melatonin reduction in number of nicotinic acetylcholine receptors and cyclic AMP accumulation. Therefore, we suggest that melatonin reduction in cyclic AMP accumulation is a result of the inhibition of a Ca^{2+}/calmodulin-dependent adenylyl cyclase. This is a fast process that triggers a multi-step cascade that in the long-term reduces the number of nicotinic acetylcholine receptors expressed in the plasma membrane.

Receptors traffic into and out the membrane involves some steps that are regulated by cyclic AMP-dependent protein kinases. It enhances the desensitization and decreases the resensitization rate of *Torpedo* and skeletal muscle receptors, but does not elicit internalization (Nishizaki and Sumikawa, 1998; Paradiso and Brehm, 1998). Changes in receptor sensitivity modify the functionality of the receptor or binding affinity, but cannot explain the reduction in receptor number. Only a small fraction of internalized receptors are re-incorporated the membrane, the larger fraction comes from newly synthesized receptors (Devreotes and Fambrough, 1975). Intracellular cyclic AMP accumulation increases the incorporation of new receptors to the plasma membrane. However, deletion of nicotinic acetylcholine receptor serines that can be phosphorylated by cyclic AMP-dependent protein kinase A does not interfere with receptor membrane incorporation. Otherwise, cyclic AMP-dependent phosphorylation is relevant to proteins that assist folding and assembly of the receptor (Jayawickreme et al., 1994). This is a long-term process, and maximal forskolin efficiency is attained after 48 h. Interestingly, this period coincides with that necessary for maximal reduction of membrane expressed nicotinic acetylcholine receptors by melatonin in myotubes. Although it is out of our scope to measure phosphorylation of Golgi proteins, the most plausible pathway involved in the melatonin-mediated reduction in the number of nicotinic acetylcholine receptors may be a decreased cyclic AMP accumulation and reduced incorporation of new receptors (Fig. 8). We also observed that melatonin decreases cyclic GMP accumulation, opening the possibility that melatonin may interfere with the process of fusion of myoblasts to form myotubes, a process highly regulated by Ca^{2+}/calmodulin dependent nitric oxide synthase (Stamler and Meissner, 2001).

In summary, this study clearly shows that melatonin-dependent reduction of nicotinic acetylcholine receptors is not due to activation of the G_i-protein coupled MT1 or MT2 receptors, or to the putative *MT3* receptor. Our data indicate that calmodulin is the target for melatonin effects herein described. Melatonin inhibits calmodulin and, as a consequence reduces cyclic AMP and cyclic GMP accumulation. The reduction of cyclic AMP accumulation mediates the decrease in the number of receptors. Since the effects here described were obtained with concentrations compatible with nocturnal melatonin surge, and nicotinic acetylcholine receptors in plasma membrane has a turnover of 24 h, this opens the possibility for a diurnal modulation in skeletal muscle physiology, based on nocturnal melatonin surge from the pineal gland.

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