MT₂ Melatonin Receptors Are Present and Functional in Rat Caudal Artery

MONICA I. MASANA, SUZANNE DOOLEN, 1 CAGATAY ERSAHIN, WALID M. AL-GHOUL, 2 SUE PIPER DUCKLES, MARGARITA L. DUBOCOVICH, and DIANA N. KRAUSE

Departments of Molecular Pharmacology and Biological Chemistry (M.I.M., C.E., W.M.A., M.L.D.) and Psychiatry and Behavioral Science (M.L.D.), Northwestern Drug Discovery Program (C.E., M.L.D.), The Feinberg School of Medicine, Northwestern University, Chicago, Illinois; and Department of Pharmacology (S.D., S.P.D., D.N.K.), College of Medicine, University of California, Irvine, California

Received December 7, 2001; accepted February 9, 2002 This article is available online at http://jpet.aspetjournals.org

ABSTRACT

In rat caudal artery, contraction to melatonin results primarily from activation of MT₁ melatonin receptors; however, the role of MT₂ melatonin receptors in vascular responses is controversial. We examined and compared the expression and function of MT₂ receptors with that of MT₁ receptors in male rat caudal artery. MT₁ and MT₂ melatonin receptor mRNA was amplified by reverse transcription-polymerase chain reaction from caudal arteries of three rat strains (i.e., Fisher, Sprague-Dawley, and Wistar). Antisense (but not sense) 32P-labeled oligonucleotide probes specific to MT₁ or MT₂ receptor mRNA hybridized to smooth muscle, as well as intimal and adventitial layers, of caudal artery. In male Fisher rat caudal artery denuded of endothelium, melatonin was 10 times more potent than 6-chloromelatonin to potentiate contraction induced by either endogenous or exogenous vasoconstrictors. MT₁ melatonin receptors. The MT₁/MT₂ competitive melatonin receptor antagonist luzindole (3 μM), blocked melatonin-mediated contraction (0.1–100 nM) with an affinity constant (Kᵢ) value of 157 nM similar to that for the human MT₁ receptor. However, at melatonin concentrations above 100 nM, luzindole potentiated the contractile response, suggesting blockade of MT₂ receptors mediating vasorelaxation and/or an inverse agonist effect at MT₁ constitutively active receptors. The involvement of MT₂ receptors in vasorelaxation is supported by the finding that the competitive antagonists 4-phenyl 2-acetamidotetraline and 4-phenyl-2-propionamidotetraline, at MT₂-selective concentrations (10 nM), significantly enhanced contractile responses to all melatonin concentrations tested (0.1 nM–10 μM). We conclude that MT₂ melatonin receptors expressed in vascular smooth muscle mediate vasodilation in contrast to vascular MT₁ receptors mediating vasoconstriction.

Accumulating evidence indicates that the hormone melatonin regulates vascular tone; however, the nature of the response remains controversial. Both vasoconstrictor and vasodilator responses have been reported; however, data supporting the presence of melatonin receptors is found in some, but not all, vascular beds (Mahle et al., 1997). In isolated rat caudal arteries, nanomolar concentrations of melatonin potentiate contraction induced by either endogenous or exogenous vasoconstrictors (Viswanathan et al., 1990; Evans et al., 1992; Krause et al., 1995; Mahle et al., 1997; Ting et al., 1997; Geary et al., 1998). In rat cerebral arteries and arterioles, melatonin is a direct vasoconstrictor (Geary et al., 1997; Viswanathan et al., 1997). Melatonin-mediated contraction in caudal and cerebral arteries is blocked by melatonin receptor antagonists (Viswanathan et al., 1992; Krause et al., 1995; Geary et al., 1997; Ting et al., 1997, 1999).

Melatonin also dilates rat and rabbit aorta, iliac, renal, and basilar arteries (Satake et al., 1986; Shibata et al., 1989; Weekley, 1991); however, the nature of the receptor(s) involved is not known. The lack of specific 2-[125I]iodomelatonin binding in rat aorta (Viswanathan et al., 1990) suggests either that the melatonin-mediated vasodilation is not mediated by high-affinity melatonin receptors or that the sensitivity of the binding assay was not sufficient for detection of all melatonin receptor types (Dubocovich et al., 1998). In porcine pulmonary, coronary, and marginal colon arteries, high concentrations of melatonin and melatonin analogs may induce vasodilatation by a novel mechanism (Ting et al., 2000).

The MT₁ and MT₂ melatonin receptors are possible mediators of the physiological effects of melatonin (Dubocovich et al., 2000). They exhibit distinct structural, chromosomal, and pharmacological differences (Reppert et al., 1995, 1996; Slau-

ABBREVIATIONS: 4P-ADOT, 4-phenyl-2-acetamidotetraline; 4P-PDOTT, 4-phenyl-2-propionamidotetraline; RT-PCR, reverse transcription-polymerase chain reaction; PCR, polymerase chain reaction; bp, base pair(s); hMT, human melatonin receptor.
Increasing concentrations, may also act as MT1 competitive antagonists, show blockade of a 100-fold higher affinity for MT2 than MT1 receptors. In contrast, the amidotetraline ligands 4P-ADOT and 4P-PDOT show at least 100-fold higher affinity for the MT2 type and they act as competitive MT2 receptor antagonists in native tissues (Dubocovich et al., 1998; Hunt et al., 2001). Luzindole or the amidotetralines, when used at 0.1 μM or higher concentrations, may also act as MT2 competitive antagonists (Krause et al., 1995; Regrigny et al., 1999; Browning et al., 2000). Furthermore, luzindole and 4P-PDOT are inverse agonists in systems where receptors are constitutively active, including the rat caudal artery (Dubocovich and Masana, 1998; Browning et al., 2000; Masana and Dubocovich, 2001; Ersahin et al., 2002).

It is now well accepted that activation of MT1 melatonin receptors in caudal arteries facilitates adrenergic vasoconstriction (Krause et al., 1995; Ting et al., 1997, 1999; Bucher et al., 1999; Lew and Flanders, 1999). However, definitive demonstration of vascular MT2 receptors has been hampered by technical difficulties and discrepancies in technical approaches, rat strains and gender, drug types, and concentrations. Results from our laboratory suggest that the contractile responses to melatonin in male Fischer rat caudal arteries are mediated through activation of at least two distinct receptors (Doolen et al., 1998, 1999). The enhanced contractile responses to melatonin in the presence of 4P-ADOT, at a concentration that does not discriminate between MT1 and MT2 melatonin receptors, suggest blockade of a receptor-mediating relaxation (Doolen et al., 1998). A role for MT2 melatonin receptors in mediating vasorelaxation was also suggested in female Fisher rat caudal arteries (Dubocovich et al., 1999). However, the presence of MT2 melatonin receptors and their role in vasodilation has been challenged by Ting et al. (1999). These authors were unable to demonstrate MT2 melatonin receptor mRNA expression in male Wistar caudal arteries and reported that 4P-PDOT did not affect melatonin’s potentiation of electrically induced vasoconstriction.

To address these discrepancies, in this study we determined the expression of MT1 and MT2 melatonin receptor mRNA using highly sensitive reverse transcription-polymerase chain reaction (RT-PCR) and in situ hybridization techniques in caudal arteries of three rat strains. Furthermore, we reassessed the effects of selective MT2 melatonin receptor antagonists (Dubocovich et al., 1997, 1998) on melatonin-mediated potentiation of arterial contraction. We demonstrated that the MT2 melatonin receptor is indeed present in caudal artery and acts to attenuate MT1 melatonin receptor-mediated contraction.

**Materials and Methods**

**Animals.** Four-month-old Fisher-344, Wistar, or Sprague-Dawley male rats, obtained from Harlan (Indianapolis, IN), were maintained in temperature- (22 ± 1°C) and humidity-controlled rooms. Food and water were provided ad libitum. Animals were maintained in a 12-h light/dark cycle for at least 2 weeks before each experiment. Animals were sacrificed during the middle of the light cycle. All animal care and procedures were performed in accordance with institutional guidelines.

**RT-PCR.** Fisher-344 (n = 8), Wistar (n = 8), and Sprague-Dawley (n = 8) male rats were sacrificed by decapitation and the caudal arteries immediately isolated and frozen at ~80°C. Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. The RNA pellet was dissolved in diethylpyrocarbonate-treated water and stored at ~80°C until use. To eliminate any residual contamination of DNA, duplicate tubes containing 1 μg of total RNA were pretreated with 1 unit of amplification grade DNase I (Invitrogen) in DNase I reaction buffer in the presence of 20 units of RNase inhibitor (PerkinElmer Life Sciences, Boston, MA) at room temperature. After 15 min, 1 μl of 25 mM EDTA solution, pH 8.0, was added to each tube, samples were heated for 10 min at 65°C to inactivate the DNase I, and thereafter immediately cooled on ice. Synthesis of first-strand cDNA and subsequent polymerase chain reaction (PCR) amplification were carried out using the Titan One Tube RT-PCR system (Roche Applied Science, Indianapolis, IN) in a PCR DNA thermocycler (PerkinElmer Life Sciences). DNase-treated RNA samples were reverse transcribed to cDNA in the presence of dNTP (each 2.5 mM) and AMV reverse transcriptase, using gene-specific primers. This procedure was followed by amplification in the same tube using an Expand High Fidelity enzyme blend (Taq polymerase and Pwo DNA polymerase) in the presence of 1.5 mM MgCl2 and 1 μM each of sense- and antisense-specific primers. The samples were incubated at 50°C for 30 min followed by thermocycling: one cycle at 94°C for 2 min; 10 cycles at 94°C for 30 s, 50°C for 30 s, and 68°C for 1 min; and 30 cycles at 94°C for 30 s, 50°C for 30 s, and 68°C for 1 min, plus elongation of 5 s for each cycle and a final elongation time of 10 min at 68°C. A reverse transcriptase negative reaction (enzyme inactivated at 94°C for 2 min) was run to determine whether the amplification product came exclusively from the RNA. RNA extraction, DNA reverse transcription, and PCR assays were conducted in a laboratory where melatonin receptor clones were never handled. Forward and reverse primer oligonucleotides were designed from the partial cDNA sequences derived from GenBank and by computer analysis using the PrimerSelect program in the LASERGENE Navigator software (DNASTar, Madison, WI) and synthesized at the Northwestern University Biotechnology Facility (Chicago, IL) (Fig. 1A). For the MT2 receptor (accession no. U144409), one set of primers amplified a 431-base pair (bp) fragment: forward primer 5'-TAG-GATATACTGAAGAAAGAT-3', corresponding to nucleotides 30 to 51, and reverse primer 5'-CTAGACGCAAGACCTC-3', complementary to nucleotides 460 to 442. Another set of nested primers amplified a 366-bp fragment: forward primer 5'-AGATTCCCTGT-GCTACG-3', corresponding to nucleotides 47 to 64, and reverse primer 5'-CTAGACGCAAGACCTC-3', complementary to nucleotides 412 to 394. For the MT2 receptor (accession no. U28218) one set of primers amplified a 310-bp fragment: forward primer 5'-TGGACCTACAGCGACC-3', corresponding to nucleotides 21 to 39, and reverse primer 5'-TCT(G/A)(G)CTCAGGCGCTG-3', degenerate primer that can amplify MT1 or MT2 sequences corresponding to nucleotides 814 to 791 of the human receptor sequence (accession no. U25341). The set of nested primers amplified a 264-bp fragment: forward primer 5'-CCCATGCT-CATCACGCTATA-3', corresponding to nucleotides 59 to 77, and reverse primer 5'-CCTCGGTTCAGGGCCA-3', complementary to nucleotides 334 to 315 of the rat MT2 melatonin receptor.

**In Situ Hybridization.** Sprague-Dawley rats were sacrificed during the light period, and the caudal artery was isolated, embedded in Tissue-Tek OCT compound, and immediately frozen in isopentane in dry ice. Antisense and sense oligonucleotide probes designed against specific sequences of MT1 and MT2 receptor mRNA (Hunt et al., 2001) were synthesized at the Northwestern University Biotechnology Facility. Probes were tailed on the 3-prime end with [32P]-tagged dATP (Amersham Biosciences, Piscataway, NJ). Each oligonucleotide probe was incubated with [32P]dATP in a 1.5 ratio in
the presence of terminal transferase for 1 h at 37°C and purified using Nick columns (Amersham Biosciences). Cryostat sections of caudal artery were hybridized with 33P-labeled oligoprobes following methods reported previously (Dubocovich et al., 1998; Hunt et al., 2001). The hybridization signal was detected either by apposing the artery sections on the slides to a Kodak film or by dipping them in NTB-2 emulsion (Eastman Kodak, Rochester, NY) and developing in D19 after 4 to 6 weeks.

**Fig. 1.** RT-PCR amplification of MT1 and MT2 melatonin receptors from rat caudal artery. A, alignment of the nucleotide sequences for the rat melatonin receptors MT1 and MT2, showing the position of the oligonucleotide primers used for the PCR amplification. Solid arrow denotes sequence of MT1 primers; dotted arrow denotes sequence of MT2 primers. B, agarose gel electrophoresis of PCR-amplified products from Fisher-344, Sprague-Dawley, and Wistar rat caudal artery mRNA. The single bands at approximately 366 bp for the MT1 and 264 bp for the MT2 melatonin receptors correspond to the expected length of the cDNA products produced by the oligonucleotide primers for these receptors after a second amplification using nested primers.
MT2 oligonucleotide probes used to hybridize to target mRNAs, 32P-labeled antisense oligonucleotide probe (MT1, and MT2) was hybridized to tissue sections under three experimental conditions: first, alone to determine the total hybridization signal; second, in the presence of 100 times excess of nonlabeled heterologous sense probe to block cross-hybridization; and third, in the presence of 100 times excess of nonlabeled homologous sense probes to determine the extent of nonspecific hybridization (Hunt et al., 2001).

Measurement of Contractile Force. Four-month-old Fisher-344 male rats were sacrificed and caudal arteries removed. Proximal segments of the arteries (3 mm in length) were denuded of endothelium by intimal rubbing and then mounted on platinum wires in an oxygenated 37°C Krebs' solution: 122 mM NaCl, 5.2 mM KCl, 1.6 mM CaCl2, 1.2 mM KH2PO4, 25.5 mM NaHCO3, 1.2 mM MgSO4, 0.027 mM disodium EDTA, and 11.5 mM glucose. Isometric contractions were recorded using FT 10 force transducers and MacLab analog to digital converter system (World Precision Instruments, New Haven, CT). After 1 h, tissues were stretched to 1-g resting tension. Maximum contraction of each tissue was determined by exposure to 100 μM phenylephrine. Arterial segments were precontracted to approximately 10 to 20% of this maximum using 0.1 to 1 μM phenylephrine and then increasing concentrations of melatonin (0.1 nM–10 μM) were added cumulatively. Responses were measured as grams of contraction above the initial control response to phenylephrine. Some tissues were incubated with 10 μM luzindole (32P-labeled), 10 nM and 3 μM 4P-ADOT, and 10 nM and 3 μM 4P-PDOT for 10 min before and during exposure to melatonin. The absence of functional endothelium was verified in each segment by the lack of relaxation to 1 μM acetylcholine (<15%) after precontraction with 0.1 μM phenylephrine.

Data Analysis. In some cases, the contractile responses in grams were normalized as a percentage of the maximum response observed with melatonin (100%). Theoretical values for EC50 and maximum response (Emax) were calculated from sigmoidal concentration-response curves by nonlinear regression using GraphPad Prism (GraphPad Software, San Diego, CA). The mean EC50 values with corresponding 95% confidence intervals were determined from values obtained in individual experiments. The concentration ratio of Emax values for melatonin in the presence and absence of antagonist was determined to calculate the apparent dissociation constant (Kb) of the antagonist according to the equation pKb = log (concentration ratio – 1) – log [antagonist] (Dubocovich et al., 1997). Unless otherwise indicated, results are expressed as mean ± S.E.M., and n refers to the number of animals tested. Statistical significance was determined using Student’s unpaired t test with P < 0.05.

Drugs. Melatonin (N-acetyl-5-methoxytryptamine), 6-chloromelatonin, phenylephrine hydrochloride, and acetylcholine chloride were purchased from Sigma-Aldrich (St. Louis, MO). Luzindole (2-benzyl-N-acetyltryptamine) and 4-phenyl-2-propionamidotetraline (4P-PDOT) were purchased from Tocris Cookson (St. Louis, MO). 4-Phenyl 2-acetamidotetraline (4P-ADOT) was synthesized in the Department of Medicinal Chemistry, University of Groningen (Groningen, The Netherlands). Luzindole, 4P-ADOT, and 4P-PDOT are melatonin receptor-specific (Dubocovich et al., 1998). Their affinities on the hMT1 and hMT2 melatonin receptors expressed in mammalian cell lines were verifed for each batch. Drugs were dissolved in double distilled water unless otherwise indicated. Stock solutions (10 mM) of melatonin and 6-chloromelatonin were made in 20% ethanol and then diluted in Krebs' solution to appropriate concentrations. Luzindole (1 mM), 4P-ADOT (1 mM), and 4P-PDOT (1 mM) were prepared in 50% ethanol and then diluted in Krebs' solution to appropriate concentrations. The final ethanol concentrations in the tissue bath did not affect contractility.

Results

MT1 and MT2 Melatonin Receptor mRNA Expression in Rat Caudal Artery. The expression of melatonin receptors mRNA in caudal arteries was determined by RT-PCR and in situ hybridization autoradiography using MT1 and MT2 melatonin receptor-specific oligonucleotides. Figure 1A shows the nucleotide sequence of the MT1 and MT2 melatonin receptors, as well as the specific sequences of the forward and reverse primers used for the first and second (nested primers) round of amplification. PCR products of the expected size, corresponding to the cDNA sequence of both receptors were amplified from Fisher-344, Sprague-Dawley, and Wistar rat caudal arteries after two rounds of amplification (Fig. 1B). For the MT1 receptor, a band of 431 bp was observed after the first amplification, and the corresponding band of 366 bp was observed after reamplification with the nested primers. For the MT2 receptor, no visible bands were observed after the first round, but nested primers, specific for the rat MT2 receptor, amplified a product of the expected size (264 bp). Nucleotide sequence of the PCR products confirmed that they are 100% homologous to the published rat MT1 and MT2 melatonin receptor sequences (accession nos. U14409 and U28218, respectively).

We next localized melatonin receptor mRNA in the Sprague-Dawley rat caudal artery by in situ hybridization using 32P-labeled oligonucleotide probes complementary to target mRNA sequences of either the rat MT1 or MT2 melatonin receptors. Figure 2 shows the specificity of the oligonucleotides used as probes in the rat caudal arteries determined by in situ hybridization autoradiography using 32P-labeled oligonucleotides in the presence of an excess of unlabeled sense homologous or heterologous probes as described previously (Hunt et al., 2001). The hybridization signal observed with antisense 32P-labeled MT1 (Fig. 2A) or MT2 probes (Fig. 2B) was not altered in the presence of a 100-fold excess of nonlabeled heterologous sense MT2 (Fig. 2C) or MT1 (Fig. 2D) oligonucleotides, respectively. In contrast, a 100-fold excess of nonlabeled homologous sense MT1 (Fig. 2E) or MT2 (Fig. 2F) oligonucleotides completely blocked hybridization of the corresponding labeled probe. Hybridization signal with sense oligonucleotide probes was negligible (data not shown). Together, these results confirm the specificity of the antisense MT1 and MT2 oligonucleotide probes for their mRNA targets and the lack of cross-hybridization.

We next determined the localization of the hybridization signal in cross sections of rat caudal arteries by emulsion autoradiography (Fig. 3). MT1 melatonin receptor oligonucleotide probes hybridized primarily to the muscle layer (media > intima > adventitia) of the rat caudal artery (Fig. 3A). Antisense oligonucleotides specific for the MT2 melatonin receptor mRNA hybridized to all three layers of the rat caudal artery (Fig. 3B).

Melatonin Receptor-Mediated Contractile Responses in Rat Caudal Artery. In male Fisher rat caudal artery segments, melatonin by itself did not affect the resting tension (data not shown). However, melatonin significantly enhanced vascular tone when added cumulatively to tissues precontracted with phenylephrine (Fig. 4). The vasorelaxant responses were maximal within 3 to 5 min of melatonin addition and sustained throughout the duration of melatonin exposure.

Pharmacological characterization of the melatonin receptor-mediated arterial contraction was conducted using melatonin and 6-chloromelatonin. Melatonin and 6-chloromelatonin (0.1 nM–10 μM) potentiated phenylephrine-induced.
contraction in a concentration-dependent manner (Fig. 4) with potencies (EC50) of 1.6 and 17 nM, respectively.

We next compared the ability of the competitive antagonists 4P-ADOT, 4P-PDOT, and luzindole, at concentrations selective for either the MT2 (10 nM) or both the MT1 and MT2 (3 μM) receptors (Dubocovich et al., 1997, 1998), to potentiate or block the contraction elicited by a single melatonin (10 nM) concentration in phenylephrine-treated caudal arteries. 4P-ADOT and 4P-PDOT, when tested alone did not affect arterial tone or phenylephrine-induce contraction, excluding a possible partial agonistic effect on either melatonin receptor (Browning et al., 2000). However, in the presence of a 10 nM concentration of either 4P-ADOT or 4P-PDOT, melatonin (10 nM) markedly potentiated the phenylephrine-induced arterial contraction (Fig. 5A). In contrast, concentrations of either 4P-ADOT (3 μM) or luzindole (3 μM) that block MT1 and MT2 melatonin receptors nonselectively (Dubocovich et al., 1997, 1998) significantly inhibited the contractile effect of 10 nM melatonin (Fig. 5B). This result is consistent with blockade of contraction mediated by MT1 melatonin receptors.

To further support a role for MT2 receptor blockade in vascular relaxation, we determined the effect of a MT2-selective concentration of 4P-PDOT (10 nM) on melatonin-mediated, concentration-dependent contractile responses (Fig. 6). Melatonin (0.1 nM–10 μM) potentiated phenylephrine-mediated contractions in caudal arteries in a concentration-dependent manner with an pEC50 value of 8.6 and a maximal effect at 10 nM (Fig. 6A; Table 1). 4P-PDOT at 10
Herein, we report for the first time expression of vascular 
MT₂ melatonin receptor mRNA using caudal arteries of three 
rat strains, i.e., Fisher-344, Sprague-Dawley, and Wistar. 
MT₁ melatonin receptor mRNA from Wistar rat caudal 
arteries was previously detected by RT-PCR (Ting et al., 1997), 
but these authors were unable to amplify mRNA for the MT₂ 
melatonin receptor. The discrepancy with our results could 
be attributed to differences in experimental conditions. First, 
our RT-PCR studies used RNA isolated from caudal arteries 
pooled from six to eight rats, providing a higher number of 
MT₂ mRNA copies. In the previous study, RNA was isolated 
and reverse transcribed from arteries of individual animals 
(Ting et al., 1997). Second, in the present study, the sensitiv-
ity of mRNA detection was increased by using the One-
Step PCR technique (Limbach et al., 1999). Detection of 
residual genomic DNA can be excluded because samples were 
DNase-treated, and no amplification was observed in nega-
tive controls consisting of mRNA samples that were not re-
verse transcribed, or in samples from a rat tissue that does 
not express MT₁/MT₂ receptors (i.e., mammary gland; data 
not shown). Amplification of MT₁ and MT₂ cDNAs from three 
strains of rats further supports the validity of the assay.

Hybridization of complementary oligonucleotide probes to 
both MT₁ and MT₂ melatonin receptor mRNAs also indicate 
expression of the both receptor types in the artery. MT₁ and 
MT₂ melatonin receptor mRNAs were localized by in situ 
hybridization to distinct layers of the arterial wall using 
³²P-oligonucleotide probes (this study) and digoxigenin-la-
beled probes (data not shown). The hybridization of both 
antisense oligonucleotide probes was specific as determined 
using sense probes and competition of ³²P-labeled antisense 
probes with homologous and heterologous unlabeled oligonu-
cleotides. Using the same techniques, MT₁ and MT₂ mRNA 
was localized to human cerebellum (Al-Ghoul et al., 1998) 
and mouse (Dubocovich et al., 1998) and rat (Hunt et al., 
2001) suprachiasmatic nuclei. The high level of mRNA ob-
served by in situ hybridization studies compared with the 
need for extensive rounds of amplification to demonstrate 
expression of MT₂ receptors by RT-PCR is intriguing. The 
high sensitivity of our in situ hybridization procedures could 
be attributed to the use of small oligonucleotide probes in-
stead of riboprobes and to the immediate preparation and 
processing of arterial sections for in situ hybridization 
(Al-Ghoul et al., 1998).

MT₁ receptor mRNA was localized primarily in the smooth 
muscle layer of the caudal artery, whereas MT₂ receptor 
mRNA appeared more evenly distributed throughout the ves-
sel wall. Expression of receptor protein in vascular smooth 
muscle is indicated by specific binding of 2-[¹²⁵I]iodomelato-
nin, a nonselective MT₁/MT₂ radioligand, in the medial layer 
of caudal and cerebral arteries (Viswanathan et al., 1990, 
1992). This binding, however, likely reflects MT₁ melatonin 
receptors because the density of endogenous MT₂ receptors 
is generally too low in mammalian tissues to be detected by 
receptor autoradiography (Liu et al., 1997; Dubocovich et al., 
1998). Although in the adventitia we found primarily mRNA 
for MT₂ receptors, Savaskan et al. (2001) observed immuno-
reactivity in the adventitia layer of human superficial and 
intrahippocampal arteries using an antibody directed 
against MT₁ melatonin receptors. The function of putative 
melatonin receptors in the adventitia is not known; however, 
it is interesting that levels of immunoreactivity were signif-

Discussion

This study demonstrates expression of MT₂ melatonin re-
ceptor mRNA and establishes MT₂-mediated functional 
responses in the rat caudal artery. The mRNA for both the MT₁ 
and MT₂ melatonin receptors was localized in the smooth 
muscle layer. This finding is consistent with the presence of 
pharmacologically distinct melatonin receptors (MT₁ and 
MT₂) that modulate contractile responses to melatonin in 
caudal artery segments denuded of endothelium. These re-
results suggest that in the arterial smooth muscle, activation of 
the MT₂ melatonin receptor mediates vasodilation, whereas 
activation of the MT₁ receptor enhances vasoconstriction.

Fig. 5. Effect of the competitive melatonin receptor antagonists 4P-
ADOT, 4P-PDOT, and luzindole on melatonin-mediated contraction in 
male rat caudal arteries. The effects of melatonin (10 nM) in the presence 
and absence of the antagonist 4P-ADOT (10 nM and 3 µM), 4P-PDOT (10 
nM), and luzindole (3 µM) are expressed as percentage of the maximal 
melatonin response attained in each individual experiment. The antag-
onists were present 10 min before and during exposure to melatonin (10 
nM). Values represent means ± S.E.M. of five to seven experiments. * , 
P < 0.05 compared with melatonin alone.

Fig. 6. Effect of the melatonin receptor antagonists, luzindole and 4P-
PDOT on melatonin-mediated contraction in male rat caudal arteries. 

nM shifted to the left the melatonin concentration-effect 
curve and increased the maximal response to melatonin with 
no change in potency (Fig. 6A; Table 1). On the contrary, the 
nonselective MT₁/MT₂ antagonist luzindole (3 µM) shifted 
the melatonin concentration-response curve to the right in a 
competitive manner, significantly decreasing the potency of 
melatonin to contract the artery (Table 1), with an estimated 
affinity constant (Kᵦ) of 151 nM (pKᵦ = 6.8). Luzindole also 
increased the maximal response to melatonin (Fig. 6B; Table 1).
The presence of functional MT$_2$ melatonin receptors in caudal arteries denuded of endothelium is suggested by the effects of the selective MT$_2$ receptor antagonists. 4P-ADOT and 4P-PDOT, at MT$_2$-selective concentrations, significantly enhanced contractile responses to melatonin. The potency of melatonin to elicit contraction was not altered, as expected for an MT$_1$-mediated response (Table 1). The enhancement of melatonin-mediated contraction in the presence of MT$_2$ antagonists could be explained if activation of MT$_1$ melatonin receptors were to mediate vasorelaxation. Similar results were obtained with 4P-PDOT in caudal arteries of female Fisher-344 rats; and in fact, the effect of the MT$_2$ antagonist was increased after in vivo estrogen exposure (Doolen et al., 1999). The MT$_1$/MT$_2$-nonselective antagonist luzindole also potentiated melatonin-induced contraction, which is consistent with blockade of MT$_2$ receptors-mediating dilatation. Unfortunately, without selective MT$_2$ antagonists to block melatonin-induced vasoconstriction, it is not possible to directly measure the postulated MT$_2$-mediated vasorelaxation in the caudal artery. It should be noted that potentiation of contraction by high concentrations (100 nM or above) of the mixed antagonist luzindole or the MT$_2$-selective amidotetra- alanines could result in part from their inverse agonist properties at MT$_1$, constitutively active melatonin receptors in caudal artery (Browning et al., 2000; Masana and Dubocovich, 2001; Ersahin et al., 2002). Inverse agonists such as luzindole or 4P-PDOT could shift the equilibrium of constitutive active receptors in caudal artery (Ersahin et al., 2002) to the uncoupled form, making more receptors available for activation by high concentrations of melatonin leading to potentiation of vasoconstriction. It is unlikely that the potentiation observed at higher melatonin concentrations is due to 4P-PDOT partial agonist properties at the MT$_1$ receptor (Ting et al., 1999), because a partial agonist effect of 4P-PDOT in this model should have been more evident at lower concentrations of melatonin. Furthermore, 4P-PDOT at 10 nM shows no affinity for the MT$_1$ melatonin receptor (Dubocovich et al., 1998).

In conclusion, both MT$_1$ and MT$_2$ melatonin receptors are present and functional in vascular tissue. Melatonin seems to modulate vascular smooth muscle tone via activation of MT$_1$ melatonin receptors that mediate contraction and MT$_2$ receptors that mediate vasorelaxation. Although the functional significance of this finding remains to be elucidated, the presence of multiple receptor subtypes with opposing actions is a common scheme in the vasculature. Because regulation of blood flow in the tail is an important mechanism for thermoregulation in the rat, the ability to regulate two different melatonin receptors with opposing vascular actions could provide greater flexibility in adapting body temperature to circadian and seasonal changes. In humans, melatonin selectively vasodilates (and therefore increases heat loss) in distal skin regions having mainly thermoregulatory functions, such as fingertips and toes, whereas it does not affect proximal regions, such as thorax and abdomen (Krauchi et al., 2000). In this way, responses to melatonin may be in-

---

**TABLE 1**

MT$_2$ and MT$_1$/MT$_2$ antagonists on efficacy and maximum contractile effect of melatonin

Data from cumulative concentration-response curves for melatonin in precontracted segments of caudal arteries from male Fisher-344 rats. Mean and 95% confidence interval (CI) given for pEC$_{50}$ and mean ± S.E.M. for maximum contraction ($E_{\text{max}}$) in grams of contractile force.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>pEC$_{50}$ (C.I.)</th>
<th>$E_{\text{max}}$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melatonin</td>
<td>8.6 (9.2–8.0)</td>
<td>0.115 ± 0.013</td>
<td>6</td>
</tr>
<tr>
<td>+ 10 nM 4P-PDOT</td>
<td>8.8 (9.2–8.3)</td>
<td>0.170 ± 0.005*</td>
<td>6</td>
</tr>
<tr>
<td>Melatonin</td>
<td>8.7 (9.2–8.2)</td>
<td>0.101 ± 0.003</td>
<td>6</td>
</tr>
<tr>
<td>+ 10 nM 4P-ADOT*</td>
<td>157 nM) that corresponds to the affinity of luzindole or 4P-PDOT to block the melatonin-mediated contraction were 157 and 302 nM, respectively.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* $p < 0.05$ when compared with melatonin alone.
volved in the fine-tuning of vascular tone in selective vascular beds, as circulating melatonin levels rise and fall throughout the night.

Acknowledgments

We thank Dr. Linda Van Eldik for the use of laboratory facilities to perform the RNA isolation and RT-PCR of melatonin receptors.

References


Dubocovich ML, Masana MI, Jacob S, and Sauri DM (1997) Melatonin receptor antagonists that differentiate between the human Mel1a and Mel1b recombinant subtypes are used to assess the pharmacological profile of the rabbit retina Mel1 presynaptic heteroreceptor. Naunyn-Schmiedeberg’s Arch Pharmacol 355:365–375.


Address correspondence to: Dr. Margarita L. Dubocovich, Department of Molecular Pharmacology and Biological Chemistry (S-215), the Feinberg School of Medicine, Northwestern University, 303 E. Chicago Ave., Chicago, IL 60611. E-mail: mdubo@northwestern.edu