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). Anti-sense (but not sense) 32P-labeled oligonucleotide probes specific for 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 (Viswanathan et al., 1990; Evans et al., 1992; Krause et al., 1995; Mahle et al., 1997; Ting et al., 1997, 1999). The involvement of MT2 receptors in vasorelaxation in isolated rat caudal arteries, nanomolar concentrations of melatonin potentiating contraction to phenylephrine, suggesting activation of smooth muscle 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 (Kd 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 potentiating 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 vasodilation 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-
genhaupt et al., 1995). Available agonists generally show little discrimination between the melatonin receptor types; however, 6-chloromelatonin exhibits lower affinity than melatonin on the MT$_1$ receptor but equal affinity on the MT$_2$ receptor (Dubocovich et al., 1997). Luzindole, an often-used competitive melatonin receptor antagonist, shows 25 times higher affinity for MT$_2$ than MT$_1$ receptors. In contrast, the amidotetraline ligands 4P-ADOT and 4P-PDOT show at least 1000-fold higher affinity for the MT$_2$ compared with the MT$_1$ type and they act as competitive MT$_2$ 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 MT$_1$ 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 arterial (Dubocovich and Masana, 1998; Browning et al., 2000; Masana and Dubocovich, 2001; Ershahin et al., 2002).

It is now well accepted that activation of MT$_1$ 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 MT$_2$ 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 MT$_1$ and MT$_2$ melatonin receptors, suggest blockade of a receptor-mediating relaxation (Doolen et al., 1998). A role for MT$_2$ melatonin receptors in mediating vasorelaxation was also suggested in female Fisher rat caudal arteries (Doolen et al., 1999). However, the presence of MT$_2$ melatonin receptors and their role in vasodilation has been challenged by Ting et al. (1999). These authors were unable to demonstrate MT$_2$ melatonin receptor mRNA expression in male Wistar caudal arteries and reported that 4P-PDOT did not affect melatonin receptor mRNA expression in male Wistar caudal arteries (Dubocovich and Masana, 1998; Browning et al., 2000; Masana and Dubocovich, 2001). For the MT$_1$ receptor (accession no. U14449), one set of primers amplified a 431-base pair (bp) fragment: forward primer 5'-TAGGATATACAGTAACAAGAAT-3' and reverse primer 5'-CTAGCCACGAAGAGCCACT-3', corresponding to nucleotides 47 to 64, and reverse primer 5'-CTGAGGCCACAATAAGACC-3', corresponding to nucleotides 59 to 77, and reverse primer 5'-CACTGGGTCTCAGGCGTA-3', complementary to nucleotides 412 to 394. For the MT$_2$ receptor (accession no. U28218) one set of primers amplified a 310-bp fragment: forward primer 5'-TGGGACCTACCAACCGAGCC-3', complementary to nucleotides 21 to 39, and reverse primer 5'-TT(T/C)AA(A/G)AA(G/A)GGCGCCGCAA/AA(C/T)GGCA-3', a degenerate primer that can amplify MT$_1$ or MT$_2$ 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'-CCCTCTCATCACGGCTCTACA-3', corresponding to nucleotides 59 to 77, and reverse primer 5'-CAGGGGCCTCAGGGCTA-3', complementary to nucleotides 334 to 315 of the rat MT$_2$ 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 iso-pentane in dry ice. Antisense and sense oligonucleotide probes designed against specific sequences of MT$_1$ and MT$_2$ receptor mRNA (Hunt et al., 2001) were synthesized at the Northwestern University Biotechnology Facility. Probes were tail-spliced on the 3-prime end with $^{32}$P-tagged dATP (Amersham Biosciences, Piscataway, NJ). Each oligonucleotide probe was incubated with $^{32}$P-dATP in a 1:5 ratio in

**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.
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 it in D19 after 4 to 6 weeks. To determine the selectivity of the MT1 and MT2 melatonin receptors 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 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 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% 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.

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 EC50 values for melatonin in the presence and absence of antagonist was determined to calculate the apparent dissociation constant (Ki) 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-2-acetamidotetraline) and 4-phenyl-2-acetyltryptamine) and 4-phenyl-2-propionamidotetraline (4P-PDOT) were purchased from Tocris Cookson (St. Louis, MO). Luzindole, 4P-ADOT, and 4P-PDOT are melatonin receptor-specific (Dubocovich et al., 1998). Their affinities for the hMT1 and hMT2 melatonin receptors expressed in mammalian cell lines were verified for each new 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 (EC\textsubscript{50}) 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 MT\textsubscript{2} (10 nM) or both the MT\textsubscript{1} and MT\textsubscript{2} (3 \mu M) 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 \mu M) or luzindole (3 \mu M) that block MT\textsubscript{1} and MT\textsubscript{2} 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 MT\textsubscript{1} melatonin receptors.

To further support a role for MT\textsubscript{2} receptor blockade in vascular relaxation, we determined the effect of a MT\textsubscript{2}-selective concentration of 4P-PDOT (10 nM) on melatonin-mediated, concentration-dependent contractile responses (Fig. 6). Melatonin (0.1 nM–10 \mu M) potentiates phenylephrine-mediated contractions in caudal arteries in a concentration-dependent manner with a pEC\textsubscript{50} 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 MT<sub>2</sub> melatonin receptor mRNA using caudal arteries of three rat strains, i.e., Fisher-344, Sprague-Dawley, and Wistar. MT<sub>2</sub> 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 MT<sub>2</sub> 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 MT<sub>2</sub> 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 sensitivity 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 negative controls consisting of mRNA samples that were not reverse transcribed, or in samples from a rat tissue that does not express MT<sub>1</sub>/MT<sub>2</sub> receptors (i.e., mammary gland; data not shown). Amplification of MT<sub>1</sub> and MT<sub>2</sub> cDNAs from three strains of rats further supports the validity of the assay.

Hybridization of complementary oligonucleotide probes to both MT<sub>1</sub> and MT<sub>2</sub> melatonin receptor mRNAs also indicate expression of the both receptor types in the artery. MT<sub>1</sub> and MT<sub>2</sub> melatonin receptor mRNAs were localized in situ hybridization to distinct layers of the arterial wall using <sup>33</sup>P-oligonucleotide probes (this study) and digoxigenin-labeled probes (data not shown). The hybridization of both antisense oligonucleotide probes was specific as determined using sense probes and competition of <sup>33</sup>P-labeled antisense probes with homologous and heterologous unlabeled oligonucleotides. Using the same techniques, MT<sub>1</sub> and MT<sub>2</sub> mRNA was localized to human cerebellum (Al-Ghoul et al., 1998) and mouse (Dubocovich et al., 1998) and rat (Hunt et al., 2001) supraoptic nuclei. The high level of mRNA observed by in situ hybridization studies compared with the need for extensive rounds of amplification to demonstrate expression of MT<sub>2</sub> 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 instead of riboprobes and to the immediate preparation and processing of arterial segments for in situ hybridization (Al-Ghoul et al., 1998).

MT<sub>1</sub> receptor mRNA was localized primarily in the smooth muscle layer of the caudal artery, whereas MT<sub>2</sub> receptor mRNA appeared more evenly distributed throughout the vessel wall. Expression of receptor protein in vascular smooth muscle is indicated by specific binding of 2-<sup>[125]I</sup>iodomelatonin, a nonselective MT<sub>1</sub>/MT<sub>2</sub> radioligand, in the medial layer of caudal and cerebral arteries (Viswanathan et al., 1990, 1992). This binding, however, likely reflects MT<sub>1</sub> melatonin receptors because the density of endogenous MT<sub>2</sub> 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 MT<sub>2</sub> receptors, Savaskan et al. (2001) observed immunoactivity in the adventitia layer of human superficial and intrahippocampal arteries using an antibody directed against MT<sub>1</sub> melatonin receptors. The function of putative melatonin receptors in the adventitia is not known; however, it is interesting that levels of immunoreactivity were signifi-
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 $MT_1$ antagonists to block melatonin-induced vasorelaxation, 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 amidotetralines 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 vasorelaxation. It is unlikely that the potentiation observed at higher melatonin concentrations is due to 4P-PDOT partial agonist properties at the $MT_2$ 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-
volved in the fine-tuning of vascular tone in selective vascular beds, as circulating melatonin levels rise and fall throughout the night.

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