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Melatonin receptors mediate potentiation of contractile responses to adrenergic nerve stimulation in rat caudal artery

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Abstract

The hormone melatonin potentiated contractile responses to adrenergic nerve stimulation in isolated ring segments of rat caudal artery. This effect was inhibited by the melatonin receptor antagonist luzindole but not by the serotonin 5-HT_2 receptor antagonist ketanserin. Melatonin had no direct effects on vascular tone. Melatonin agonists potentiated contractile responses with a relative order of potency (2-iodomelatonin, $EC_{50} = 0.6$ nM; melatonin, $EC_{50} = 4.7$ nM; *N*-acetylserotonin, $EC_{50} = 1.5$ μM) that is consistent with the melatonin ML_1 receptor subtype. Melatonin also potentiated contractions elicited by exogenous norepinephrine and produced its effects in the absence of an intact endothelium. These data suggest that melatonin acts on receptors in the smooth muscle. The caudal artery provides a useful functional assay for pharmacological analysis of melatonin receptors. Physiologically, melatonin may activate its receptors at night to influence thermoregulation in the rat by enhancing the effects of sympathetic input to the caudal artery.

Keywords: Melatonin receptor; Caudal artery, rat; Adrenergic nerve; Contraction

1. Introduction

The pineal hormone melatonin is involved in the adaptation of an animal's physiology and behavior to enhance survival under fluctuating environmental conditions. Elevated levels of melatonin circulate in the body during the hours of darkness and provide a signal as to the daily and seasonal state of the environment as well as to the rhythm of the internal circadian clock. Melatonin is thought to influence a variety of processes, including circadian physiology, neuroendocrine and immune function, and thermoregulation (Yu and Reiter, 1993), as well as having effects on cardiovascular function (Holmes and Sugden, 1976; Karppanen et al., 1973; Zanaboni and Zanaboni-Muciaccia, 1967).

Circadian variations are observed in a number of cardiovascular parameters such as blood pressure, heart rate, sympathetic reactivity and risk of acute cardiovascular disease (Panza et al., 1991; Quyyumi et al., 1992). A possible involvement of melatonin in cardiovascular regulation was first suggested by pinealectomy experiments in rats that resulted in hypertension (Karppanen et al., 1973; Zanaboni and Zanaboni-Muciaccia, 1967). The elevation in blood pressure was reversed by the administration of melatonin (Holmes and Sugden, 1976). Effects of melatonin on blood pressure could be mediated through central mechanisms, perhaps by inhibition of sympathetic output (Chuang et al., 1993). Several reports suggest a direct action of melatonin on blood vessels (Evans et al., 1992; Satake et al., 1991; Weekley, 1991, Weekley, 1993), but these studies are conflicting in that both vasodilation and vasoconstriction by melatonin have been reported. None of these vascular effects of melatonin were characterized pharmacologically however; and in some cases, relatively high concentrations of the hormone were used. An important issue relating to the interpretation of these observations is the specificity of the receptors involved. Melatonin may be acting via a specific melatonin receptor, or because of structural similarities, it could also affect other receptors in vascular tissue, e.g. serotonin receptors.

The characteristics of specific receptor sites for melatonin are beginning to be elucidated (Krause and

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Dubocovich, 1991). Much of the work to date has involved the characterization of binding sites with high affinity for the potent melatonin receptor agonist, 2-[^{125}I]iodomelatonin. Only a few functional assays for melatonin receptors, e.g. inhibition of either retinal dopamine release (Dubocovich, 1988) or cyclic AMP in neural or pituitary tissue (Carlson et al., 1989; Morgan et al., 1989; Iuvone and Gan, 1994), have been well-described. Luzindole is a selective melatonin receptor antagonist (Dubocovich, 1988; Iuvone and Gan, 1994) while the agonist *N*-acetylserotonin can be used to distinguish melatonin ML_1 and ML_2 receptor subtypes (Krause and Dubocovich, 1991).

Recently, specific 2-[^{125}I]iodomelatonin binding was demonstrated using quantitative autoradiography in rat caudal and cerebral blood vessels (Seltzer et al., 1992; Viswanathan et al., 1990, 1992) and cerebral vessels from primates and humans (Stankov et al., 1993). The pharmacological characterization of 2-[^{125}I]iodomelatonin binding sites in the caudal artery correlated with that for a specific melatonin receptor (Viswanathan et al., 1990). The latter study also demonstrated that melatonin potentiates norepinephrine-induced contraction; although the functional effects of melatonin were not characterized in detail. A more recent study, however, found only a small, but variable effect of melatonin in phenylephrine-constricted, adult rat caudal arteries (Evans et al., 1992).

The purpose of the present study was to define the functional effects of melatonin in the rat caudal artery and to characterize the pharmacology of this response. The caudal artery serves as a model for vascular melatonin receptors, and we have found it provides a useful functional assay for pharmacological analysis of melatonin receptors.

2. Materials and methods

2.1. Tissue preparation

Adult male Sprague-Dawley rats weighing 200–300 g were housed under a 12 h light/12 h dark cycle with food and water available ad libitum. Animals were killed by decapitation on the morning of the experiment. The caudal artery was dissected out and the proximal region cut into ring segments, 3 mm in length. Each segment was mounted through the lumen on two platinum wires in a 50 ml tissue bath filled with oxygenated Krebs solution at 37°C. Composition of the Krebs solution was (in mM): NaCl, 118; KCl, 4.8; $CaCl_2$, 1.6; KH_2PO_4, 1.2; $NaHCO_3$, 25; $MgSO_4$, 1.2; disodium EDTA, 0.027; and glucose, 11. Tissues were equilibrated for 1 h and then stretched slowly to a resting tension of 1.0 g, which was previously determined to be optimal for force development. The tissues were equilibrated for an additional 1 h and then washed once with Krebs solution before starting the experimental protocol.

2.2. Measurement of vasoconstrictor responses

Isometric contractions of arterial segments were recorded using Gould Staham UC2 force transducers with microscale accessory and a MacLab analog to digital converter system (World Precision Instruments, New Haven, CT).

Perivascular nerves were electrically stimulated via two electrodes placed on either side of the tissue, 1 cm apart. Every 2 min, a Grass S48 Stimulator delivered a 5 s train of electrical pulses (15 V, 0.3 ms in duration) at a frequency of 1–2 Hz. When consistent baseline contractile responses were obtained, one concentration of melatonin (10^{-10}–10^{-6} M) was added to each arterial segment. When the effect of melatonin on contraction reached a maximum, the tissues were washed with Krebs solution until the contractions returned to baseline levels. Melatonin agonists were tested in a similar manner. Antagonists were added to the baths either 9 min (luzindole) or 25 min (ketanserin) prior to the addition of an agonist. For each experiment, an untreated arterial segment was run in parallel to serve as a control for possible time-related effects.

Direct contractile responses to norepinephrine and serotonin (5-HT) were obtained by adding these agents to the baths of arteries which did not receive transmural nerve stimulation. Following measurement of the response to norepinephrine, the tissue was washed with Krebs solution, and then treated with melatonin for 2 min before norepinephrine was added again. Following washout, the response to norepinephrine alone was again measured.

2.3. Data analysis

Contractile responses were measured as the peak g of force developed above the resting level. The effects of melatonin were expressed as % potentiation, i.e., the peak contraction in the presence of drug as % of the control contraction measured just prior to drug exposure. For some experiments, the duration of contraction also was measured as the time from the beginning of contraction to the point where the tissue had relaxed to 20% of the peak contraction (Glenn and Duckles, 1994).

In agonist studies, the concentration which produced half-maximal effects (EC_{50}) was determined. For antagonist experiments, a concentration ratio (CR) was calculated from the agonist EC_{50} values determined in the absence and in the presence of the antagonist. Estimates of the apparent dissociation con-

stant (K_B) for the antagonist were calculated according to the equation: $pK_B = \log (CR - 1/[\text{antagonist}])$.

All results are expressed as mean ± S.E.M. Statistical significance was determined using Student's paired *t*-test.

2.4. Drugs

Melatonin, *N*-acetylserotonin, norepinephrine, 5-hydroxytryptamine (5-HT) and acetylcholine were all obtained from Sigma Chemical Co. (St. Louis, MO). 2-Iodomelatonin was purchased from Research Biochemicals (Natick, MA), and ketanserin was supplied by Janssen Pharmaceuticals (Beerse, Belgium). Luzindole was a gift from Dr. Margarita Dubocovich, Northwestern University (Chicago, IL). All drugs were dissolved in water except for melatonin and 2-iodomelatonin which were dissolved initially in ethanol and further diluted in water to the desired final concentration.

3. Results

3.1. Potentiation of vasoconstriction by melatonin

Melatonin had no direct effect on the resting tone of rat caudal artery segments. Transmural nerve stimulation at a frequency of 1–2 Hz caused a vasoconstriction of 0.1–0.2 g in the isolated caudal artery segments. Addition of melatonin to the bath enhanced the contractile response to nerve stimulation (Figs. 1A, 2). Melatonin increased the peak of the contraction but did not significantly alter its duration. (Duration of control contractions was 0.38 ± 0.01 min vs. 0.40 ± 0.01 min for contractions in the presence of 10^{-7} M melatonin, $n = 3$.) The potentiating effect of melatonin was usually observed within the first 1–2 min, and the contractions remained potentiated for as long as melatonin was present. The effect of melatonin was reversible, and the contractions induced by nerve stimulation returned to baseline levels within 5–20 min following wash of the tissue with Krebs solution (Fig. 1A).

Melatonin also significantly potentiated the contractile response produced by exogenous norepinephrine (Figs. 1B, 2). This effect was concentration-dependent and similar in magnitude to the percent potentiation by melatonin of contractions induced by nerve stimulation (Fig. 2).

In several experiments, the integrity of the endothelium was assessed by measuring the vasodilating effect of acetylcholine (7×10^{-6} M) following induction of tone by norepinephrine (2×10^{-6} M). Acetylcholine had no effect, indicating the lack of a functional endothelium, which is often the case after mounting the caudal artery ring segments on intraluminal wires. Papaverine, a direct smooth muscle dilator, however, completely relaxed the artery segments back to baseline (data not shown). These same artery segments also responded to melatonin with a potentiation of contraction similar to that shown in Fig. 1.

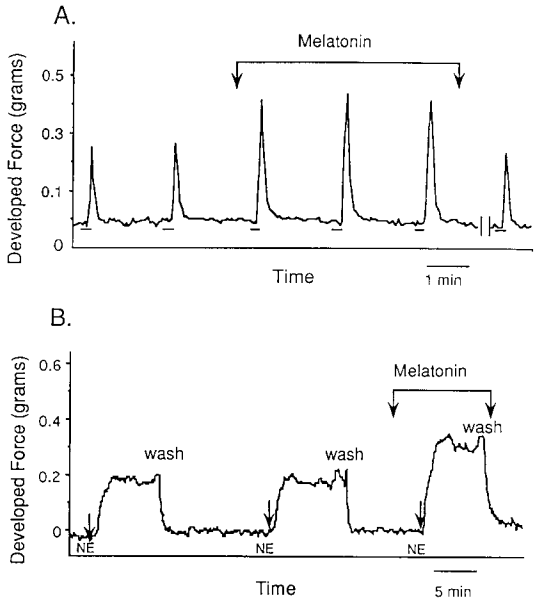

Fig. 1. Effect of melatonin on contractile responses to transmural nerve stimulation (A) and norepinephrine (B) in the rat caudal artery. Representative tracings are shown. (A) Each contraction was elicited by a brief train of transmural nerve stimulation (2 Hz, 10 pulses, 5 s in duration) which was applied every 2 min (–). Melatonin (3×10^{-8} M) was present in the bath during the time indicated and then washed out using fresh Krebs solution. The final contraction shown was measured 10 min after the start of the wash. (B) Each contraction was elicited by the addition of norepinephrine (NE; 3×10^{-8} M). Melatonin (10^{-7} M) was added at the time indicated and was present throughout the final norepinephrine exposure.

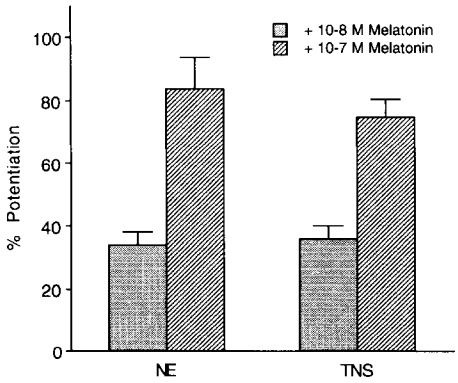

Fig. 2. Potentiation by melatonin of contractions induced by either transmural nerve stimulation (TNS) or norepinephrine (NE) is concentration-dependent and similar in magnitude. Values shown are the means ± S.E.M. for three to nine separate experiments.

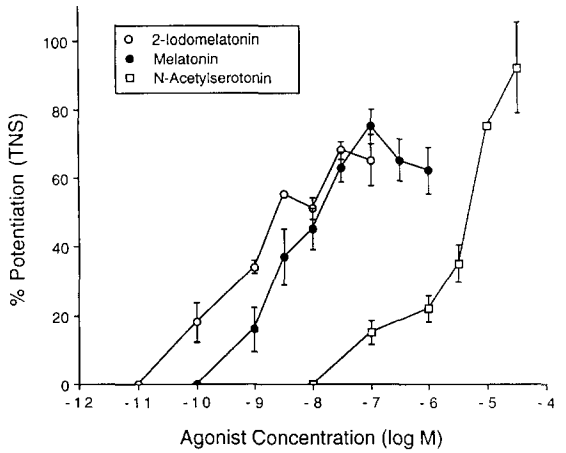

Fig. 3. Potentiation of contractile responses to adrenergic nerve stimulation (TNS) by melatonin and two melatonin agonists, 2-iodomelatonin and *N*-acetylserotonin. Concentration-response curves are shown. Values represent the mean ± S.E.M. for three to ten experiments.

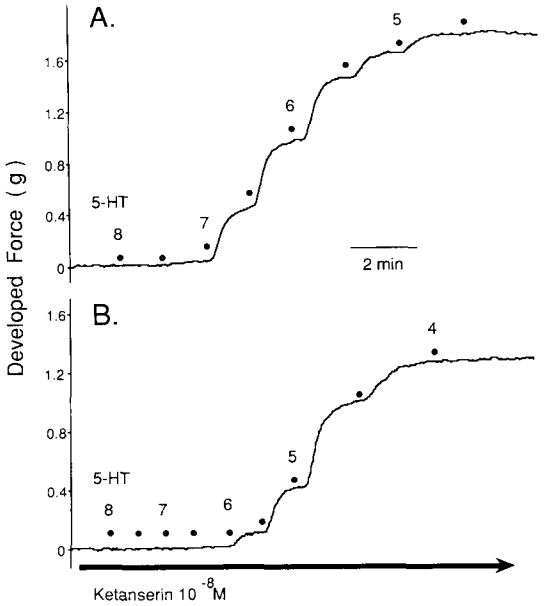

Fig. 4. Serotonin (5-HT) directly contracts the rat caudal artery. Representative tracings are shown of the responses to 5-HT, applied cumulatively in 0.5 log M increments. Concentrations of 5-HT are indicated as −log M. (A) 5-HT alone and (B) in the presence of the 5-HT_2 receptor antagonist, ketanserin (10^{-8} M).

3.2. Potency of melatonin agonists

Melatonin (10^{-9}–10^{-6} M) acted in a concentration-dependent manner to potentiate contractile responses to transmural nerve stimulation (Figs. 2, 3). A maximal effect of 75 ± 5% potentiation was observed at 10^{-7} M melatonin, and the EC_{50} value for melatonin was 4.7×10^{-9} M. 2-Iodomelatonin, a potent melatonin agonist, enhanced stimulation-evoked contractions in a similar manner, with an EC_{50} of 6.3×10^{-10} M. *N*-Acetylserotonin, the metabolic precursor to melatonin, also potentiated the contractile response; however this substance was three orders of magnitude less potent ($EC_{50} = 1.5 \times 10^{-6}$ M) and produced a greater maximum potentiation as compared with melatonin. In contrast to melatonin, serotonin (10^{-8}–10^{-5} M) directly contracted the rat caudal artery in the absence of another constrictor (Fig. 4) with an EC_{50} of 2.6×10^{-7} M, as expected from the literature (Feniuk and Humphrey, 1989).

3.3. Selective antagonism of the effects of melatonin

Preincubation of the tissue with luzindole, a known melatonin receptor antagonist, significantly inhibited the effect of melatonin on contractions evoked by transmural nerve stimulation (Figs. 5, 6A). Luzindole, at a concentration of 2×10^{-6} M, had no effect on its own; but it shifted the melatonin concentration-response curve to the right in a competitive-like manner and increased the EC_{50} value almost 10-fold (26.0×10^{-9} M) (Fig. 5). An estimated K_B value of 3.7×10^{-7} M was calculated for luzindole.

In contrast, ketanserin (10^{-8} M), a well known 5-HT_2 receptor blocker, did not significantly inhibit the action of melatonin (Fig. 6A). We confirmed that ketanserin (10^{-8} M) effectively inhibits the contractile effects of serotonin in this tissue (Fig. 4) with an estimated K_B value of 7.2×10^{-10} M, similar to published values (Feniuk and Humphrey, 1989). Ketanserin (10^{-8} M) also partially inhibited the effects of higher concentrations of *N*-acetylserotonin ($\geq 3 \times 10^{-5}$

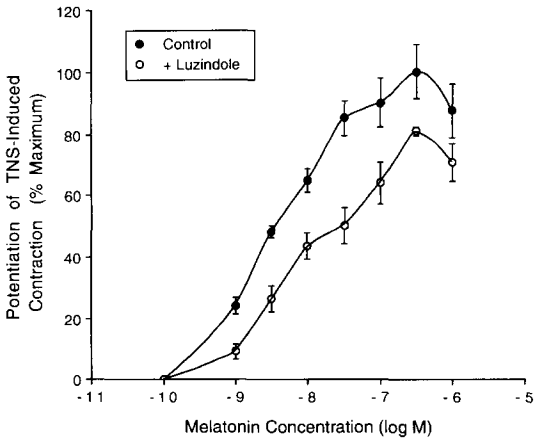

Fig. 5. Effect of luzindole on the melatonin concentration-response curve. Potentiation by melatonin of contractile responses to transmural nerve stimulation (TNS) was determined in the absence and presence of 2×10^{-6} M luzindole. Data are expressed as the % of the maximum response to melatonin and represent the means ± S.E.M. for three to five experiments.

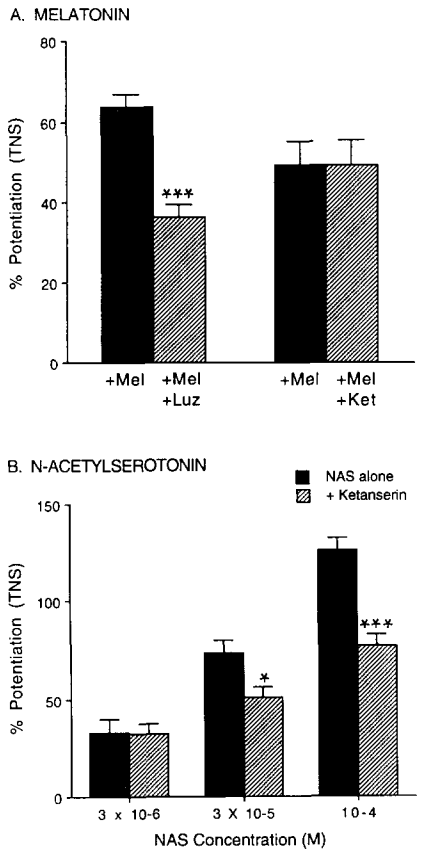

Fig. 6. Effect of luzindole and ketanserin on potentiation by (A) melatonin and (B) *N*-acetylserotonin of contractile responses to transmural nerve stimulation (TNS). (A) Potentiation by melatonin (3×10^{-8} M) alone and in the presence of either luzindole (Luz; 2×10^{-6} M) or ketanserin (Ket; 10^{-8} M). Data represent the mean ± S.E.M. for 3–12 experiments. (B) Potentiation by *N*-acetylserotonin (NAS) in the absence and presence of ketanserin (10^{-8} M). Data represent the mean ± S.E.M. for four experiments. * $P < 0.05$, *** $P < 0.001$.

M), but had no effect on the potentiation induced by lower concentrations of *N*-acetylserotonin (Fig. 6B).

4. Discussion

We have demonstrated that melatonin acts through specific receptors in the rat caudal artery to potentiate contractile responses to sympathetic nerve stimulation. Using isolated ring segments of the densely innervated, proximal portion of the caudal artery, we found a consistent, reversible potentiation by melatonin of neurogenic vasoconstriction. The effect of this hormone appears to be post-junctional, because melatonin had similar effects on the contractions elicited by exogenous norepinephrine. Viswanathan et al. (1990) also reported a small, but significant, potentiation by melatonin of norepinephrine-induced contractions of helical strips of the caudal artery, although this response was not characterized in detail. In contrast, Evans et al. (1992) studied the distal portion of adult rat caudal artery, which receives less innervation (Sittiracha et al., 1987); but they found little or no effect of melatonin on either preconstricted ring segments or pressurized artery segments preconstricted with 3 μM phenylephrine. It is not clear if the latter result reflects a gradient of melatonin receptors along the artery or technical differences between the studies.

Enhancement of contraction by melatonin appeared to be independent of the endothelium, suggesting that melatonin is acting on receptors in the smooth muscle. This is consistent with the localization by autoradiography of specific 2-[^{125}I]iodomelatonin binding sites in the smooth muscle layer of the caudal artery (Viswanathan et al., 1990; D.N. Krause, unpublished observations). Melatonin had no direct effect on smooth muscle tone, however, in agreement with previous studies (Evans et al., 1992; Viswanathan et al., 1990).

The effect of melatonin is similar to that of other potentiators of smooth muscle contraction such as neuropeptide Y. Both agents increase the peak tension of the contractile response of caudal artery to nerve stimulation, however neuropeptide Y also prolongs the duration of the contraction (Glenn and Duckles, 1994). This suggests some difference in the underlying mechanisms of potentiation for melatonin and neuropeptide Y. Like neuropeptide Y, melatonin is known to inhibit the production of cyclic AMP (Capsoni et al., 1994; Carlson et al., 1989; Iuvone and Gan, 1994; Morgan et al., 1989), which causes vasodilation (Lincoln and Cornwell, 1991). Whether this mechanism is important in the potentiation of adrenoceptor-mediated contraction has yet to be determined.

In the caudal artery, melatonin appears to be acting through specific receptors with the pharmacological characteristics of an ML_1-like melatonin receptor. Known melatonin agonists potentiated the contractile response to nerve stimulation with an order of potency similar to that found for 2-[^{125}I]iodomelatonin binding sites in this artery (Viswanathan et al., 1990) and in other tissues such as the brain and retina (Krause and Dubocovich, 1991). Compared with melatonin, *N*-acetylserotonin has been shown to be equipotent at melatonin ML_2 receptor sites but about a 1000-fold less potent at melatonin ML_1 receptors (Krause and Dubocovich, 1991). The receptor in the caudal artery is consistent with the melatonin ML_1 receptor profile.

Selective receptor antagonists were also used to determine the specificity of the melatonin response. The effects of melatonin were inhibited by the selective melatonin receptor antagonist, luzindole (Dubocovich, 1988; Krause and Dubocovich, 1991). This finding is consistent with the action of melatonin on specific melatonin receptors. The dissociation constant calculated for luzindole in the caudal artery is similar to that reported recently for melatonin receptors in chick

retina that inhibit cyclic AMP accumulation (Iuvone and Gan, 1994). However, the apparent K_B value for the arterial melatonin receptor is almost 20-fold higher than that reported for presynaptic melatonin receptors that inhibit dopamine release in the rabbit retina (Dubocovich, 1988).

Receptors for melatonin's metabolic precursor, serotonin, are well-described in the vasculature (Feniuk and Humphrey, 1989); and we have confirmed a direct contractile effect of serotonin in the caudal artery that is blocked competitively by the 5-HT_2 receptor antagonist, ketanserin. In contrast, melatonin had no direct effect on vessel tone nor were its effects blocked by ketanserin. This indicates that melatonin acts on a receptor that is distinct from the vascular 5-HT_2 receptor. Interestingly, *N*-acetylserotonin, which is related in structure to both serotonin and melatonin, appeared to affect melatonin receptors at lower concentrations and serotonin receptors at higher concentrations. In the presence of ketanserin, the maximal potentiating effect of *N*-acetylserotonin was similar to that produced by melatonin.

As of yet, few functional assays for melatonin receptors have been described (Krause and Dubocovich, 1991). The rat caudal artery offers not only a convenient test system for potential melatonin agonists and antagonists, but also presents a model for investigating the function of vascular melatonin receptors. The caudal artery represents a vessel heavily innervated with adrenergic nerves which plays a major role in thermoregulation (O'Leary et al., 1985). Interestingly, melatonin is suggested to cause a generalized decrease in sympathetic tone (Chuang et al., 1993) and has been reported to have hypotensive effects in vivo (Holmes and Sugden, 1976; Karppanen et al., 1973; Zanaboni and Zanaboni-Muciaccia, 1967). While these effects of the hormone appear to be opposite to the increase in sympathetic action observed here in the isolated caudal artery, the answer may lie in the apparently highly restricted localization of vascular melatonin receptors to the caudal and cerebral arteries (Krause et al., 1994; Viswanathan et al., 1990). Indeed, preliminary results in our laboratory suggest that, in the rat, vessels such as the femoral and mesenteric arteries do not show the potentiating responses to melatonin that we have observed in the caudal artery (Krause et al., 1994). In particular vascular beds, it may be important physiologically to run counter to the prevailing decrease in sympathetic tone, and thus melatonin receptors are poised to respond by enhancing sympathetic input in these arteries only. The rat, in particular, is a nocturnal animal which may need to conserve heat when exposed to the cooler temperatures at night. Enhancement of heat conservation via vasoconstriction of the caudal artery would be consistent with the thermoregulatory role proposed for melatonin (Badia et al., 1993).

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