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# Effect of melatonin in the rat tail artery: role of $K^+$ channels and endothelial factors

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**1** The role of endothelial factors and potassium channels in the action of the pineal hormone melatonin to potentiate vasoconstrictor responses was investigated in the isolated perfused tail artery of the rat.

**2** Melatonin (100 nM) potentiated contractile responses to both adrenergic nerve stimulation and $\alpha_1$-adrenoceptor stimulation by phenylephrine. After removal of the endothelium, melatonin no longer caused potentiation.

**3** The potentiating effect of melatonin was also lost when nitric oxide synthase was inhibited with L-NAME (10 nM). Thus potentiating effects depend on the presence of nitric oxide released by the endothelium. However, melatonin did not affect relaxation responses to acetylcholine in endothelium-intact arteries, nor did melatonin modulate relaxing responses to sodium nitroprusside in endothelium-denuded arteries. While melatonin does not appear to modulate agonist-induced release of nitric oxide nor its effect, melatonin may modulate nitric oxide production induced by flow and shear stress.

**4** When the $Ca^{2+}$-activated $K^+$ channel opener, NS 1619 (10 $\mu$M), was present, potentiating effects of melatonin were restored in endothelium-denuded vessels. However, addition of the opener of ATP-sensitive $K^+$ channels, cromakalim (3 $\mu$M), did not have the same restorative effect. Furthermore, addition of a blocker of $Ca^{2+}$-activated $K^+$ channels, tetraethylammonium (1 mM), significantly attenuated potentiating effects of melatonin. These findings support the hypothesis that melatonin inhibits the activity of large conductance $Ca^{2+}$-activated $K^+$ channels to produce its potentiating effects.

**5** Thus in the rat perfused tail artery, potentiation of constriction by melatonin depends on the activity of both endothelial factors and $Ca^{2+}$-activated $K^+$ channels. Our findings suggest that melatonin inhibits endothelial $K^+$ channels to decrease flow-induced release of nitric oxide as well as block smooth muscle $K^+$ channels to enhance vascular tone.

**Keywords:** Melatonin; rat tail artery; nitric oxide; endothelium; potassium channels

## Introduction

Secretion of the pineal hormone melatonin fluctuates with the daily light/dark cycle, with highest concentrations occurring shortly after midnight (Saarela & Reiter, 1993). Receptors selective for melatonin have been described and recently cloned, and there is evidence for several receptor subtypes (Dubocovich, 1995). In the vascular system, specific melatonin binding sites have been demonstrated in rat cerebral and tail arteries and in cerebral vessels from primates and man by use of quantitative autoradiography (Viswanathan *et al*., 1990, 1992; Stankov *et al*., 1993; Capsoni *et al*., 1994). If melatonin influences vascular reactivity, fluctuations in the blood concentration of melatonin could be related to circadian variations associated with stroke and other cardiovascular diseases. However, the role of melatonin in cardiovascular regulation is as yet undetermined.

Vascular effects of melatonin have been demonstrated in rat isolated cerebral and tail arteries. However, the nature of the contractile responses to melatonin differed depending on the preparation under study. In cerebral artery segments pressurized to physiological levels, melatonin induced a modest contraction (Geary *et al*., 1997). In helical strips or wire-mounted ring segments of tail artery, melatonin agonists had no direct effect. However, contractile responses to transmural adrenergic nerve stimulation and noradrenaline were potentiated (Viswanathan *et al*., 1990; Krause *et al*., 1995). In another study with pressurized tail arteries from juvenile rats, melatonin was shown to be a direct vasoconstrictor (Evans *et al*., 1992). After maturation, the direct effect was lost in pressurized tail arteries, but melatonin retained the ability to potentiate contractile responses to the $\alpha_1$-adrenoceptor agonist, phenylephrine (Evans *et al*., 1992). Contractile responses to melatonin in both rat tail and cerebral arteries were blocked by the melatonin receptor antagonist luzindole (Krause *et al*., 1995; Geary *et al*., 1997). The precise mechanisms by which melatonin affects the contractile state of the blood vessel have not been determined, although endothelium-independent and cyclic AMP-dependent mechanisms have been suggested (Capsoni *et al*., 1994; Krause *et al*., 1995).

The potentiating effects of melatonin seen in the rat tail artery are similar to those described for other vasoactive substances, such as neuropeptide Y (NPY), that also lack direct contractile effects in some tissues but potentiate contractions to other agonists. In the rat tail artery, NPY potentiates contractile responses to noradrenaline (Vu *et al*., 1989), and there is evidence that this action involves inhibition of $Ca^{2+}$-activated $K^+$ channels ($BK_{Ca}$) (Xiong & Cheung, 1994). The potentiating effects caused by NPY and melatonin are also similar to the effects of treatment with $K^+$ channel blockers (Cowan *et al*., 1993; Gustafsson & Nilsson, 1994). The hypothesis that the actions of agents such as melatonin and NPY involve closure of $K^+$ channels recently received further support by findings in rat isolated cerebral arteries. Direct contractile effects of melatonin are inhibited by the $BK_{Ca}$ channel blockers, charybdotoxin and tetraethylammonium, and melatonin decreases the vasodilator effect of the $BK_{Ca}$ channel opener, NS 1619 (Geary *et al*., 1997).

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There is evidence in the literature that the actions of potentiating agents may be modulated by endothelial factors, such as nitric oxide (NO). For example, potentiating effects of NPY have been shown to be abolished after removal of the endothelium (Daly & Hieble, 1987), although others have found that NPY-induced potentiation persists when endothelium is removed (Budai *et al*., 1989). We have previously demonstrated melatonin-induced potentiation in wire-mounted ring segments of rat tail artery that lack a functional endothelium (Krause *et al*., 1995). However, the possible influence of the endothelium on vascular responses to melatonin is not known. Endothelial factors, including NO, have been shown to modulate smooth muscle potassium channels (Taguchi *et al*., 1996). In different isolated vascular preparations such as wire-mounted, perfused, or pressurized arteries, there may be differences in the activity of NO synthase and/or potassium channels. Thus, depending on the type of preparation being studied, the role of the endothelium in responses to potentiating agents such as melatonin may differ. Interestingly, melatonin has recently been shown to inhibit NO synthase activity in homogenates of rat cerebellum (Pozo *et al*., 1994), suggesting that melatonin may directly modulate NO production.

The present study was designed to explore further the mechanism of action of melatonin in the rat isolated, perfused tail artery. Specifically, two questions have been addressed. The first is whether potentiation by melatonin of contractile responses is influenced by the presence of endothelial factors. The second is whether melatonin potentiates contractile responses by modulating the activity of potassium channels. Since the tail artery has been shown to possess melatonin receptors and the endothelium is functional in the isolated perfused artery, we have used this preparation to address these questions.

## Methods

### *Tissue preparation*

Male Sprague Dawley rats weighing between 250–300 g were killed by decapitation, and segments of tail artery were removed and placed in Krebs solution. The 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, ascorbic acid 0.3 and glucose 11.5. In some arteries the endothelium was removed by gently threading a 5 cm segment of 6-0 silk through the artery lumen followed by 3 min luminal perfusion with distilled $H_2O$.

### *Measurement of perfusion pressure*

A proximal, 4 cm segment of tail artery was cannulated at both ends, suspended vertically in a tissue bath of 0.5 ml capacity and perfused and superfused using a constant volume perfusion system with Krebs solution saturated with 95% $O_2$-5% $CO_2$ at 37°C at a rate of 2.0 ml $min^{-1}$. Smooth muscle contractions were measured as changes in perfusion pressure monitored with a Statham P23 Ac transducer. The resulting electrical signals were digitized by a MacLab analogue-digital converter and recorded by a Macintosh computer. Transmural nerve stimulation (TNS) was delivered with a Grass S48 stimulator through platinum electrodes placed 5 cm apart at either end of the tissue. Stimulation parameters were 60 V, 1 ms pulse duration, with various frequencies delivered cumulatively (0.25–64 Hz) for 60 s at each frequency or with a single frequency, 4 Hz for 3 min. After an initial 60 min equilibration period, arteries were stimulated with various frequencies delivered cumulatively (1–8 Hz; 60 s/frequency) until responses were consistent (three to five times). Arteries were then perfused and superfused for an additional 20 min before the experiment was begun.

### *Vasoconstrictor responses*

Vasoconstrictor responses to TNS or phenylephrine in endothelium-intact or denuded arteries were obtained before and during exposure to melatonin. In some experiments, $N^G$-nitro-L-arginine methyl ester (L-NAME) was added to the perfusate 30 min before stimulation. Vasoconstrictor responses were expressed as either developed pressure (mmHg) or as a % of the response of the artery segment to 10 $\mu$M methoxamine plus 120 mM KCl (standard response).

### *Vasodilator responses*

Vasodilator responses were measured in arteries exposed to a constrictor agent or TNS, and results expressed as a % of pressure developed during the precontraction. In these experiments, the presence of functional endothelium was determined by testing the action of acetylcholine (1 $\mu$M) in arteries precontracted with phenylephrine (0.3 $\mu$M). Endothelium-intact arterial segments relaxed to acetylcholine by $59 \pm 4\%$ of the precontracted pressure ($n = 11$), while there was no significant relaxation ($5 \pm 4\%$; $n = 11$) when acetylcholine was added to endothelium-denuded segments. In endothelium-intact arterial segments, vasodilator responses to acetylcholine during either 4 Hz TNS or phenylephrine treatment were obtained before and during exposure to melatonin. Vasodilator responses to sodium nitroprusside, cromakalim, or NS 1619 were determined during either 4 Hz TNS or constriction with phenylephrine (0.3–1 $\mu$M) in endothelium-denuded arteries before and during exposure to melatonin.

### *Drugs and solutions*

Acetylcholine chloride, cromakalim, tetraethylammonium, melatonin, $N^G$-nitro-L-arginine methyl ester (L-NAME), methoxamine HCl, phenylephrine and sodium nitroprusside were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). NS 1619 (1-(2′-hydroxy-5′-trifluoromethylphenyl) trifluoromethyl-2-(3H) benzimidazolone) was purchased from RBI (Natick, MA, U.S.A.).

Drugs were dissolved in double distilled water, with the following exceptions. NS 1619 was first dissolved in 100% dimethyl sulphoxide to produce a concentrated stock solution (20 mM) from which final bath dilutions were made. Melatonin was dissolved in 20% ethanol and then diluted in double distilled water to 10 mM stock concentration. Melatonin was tested at 100 nM, the concentration which was previously determined to give maximal effects in arterial ring segments (Krause *et al*., 1995). Addition of vehicles alone had no significant effects.

### *Statistical analysis*

Results are expressed as mean ± s.e.mean. Statistical significance was determined by either paired Student's $t$ test or analysis of variance with a Bonferroni/Dunn *post hoc* comparison, as appropriate. Levels of $P < 0.05$ were taken to indicate statistical significance.

## Results

### *Effect of melatonin on endothelium-intact and denuded arteries*

Adrenergic nerve stimulation (0.25 to 64 Hz, 60 s) of endothelium-intact, perfused tail arteries caused perfusion pressure to increase in a frequency-dependent manner (Figures 1a and 2a). Control responses to nerve stimulation did not change over the course of each experiment, but were abolished by 1 $\mu$M tetrodotoxin, confirming dependence on nerve activation (data not shown). Addition of melatonin (100 nM) to the perfusate did not change the resting pressure. However, contractile responses to the lower frequencies of nerve stimulation (1 to 8 Hz) were significantly increased in the presence of melatonin (Figures 1a and 2a).

Following removal of the endothelium, control responses to TNS were significantly greater than those of intact arteries (Figures 1b and 2b). For example with 2 Hz TNS, transmural pressure increased $5 \pm 1$ mmHg in endothelium-intact arteries, while in endothelium-denuded arteries there was an increase in pressure of $13 \pm 2$ mmHg following TNS at 2 Hz ($n=7$; $P<0.005$; ANOVA). In endothelium-denuded arteries, responses to TNS were no longer significantly affected by the presence of melatonin (Figures 1b and 2b).

### *Effect of melatonin on phenylephrine-induced contraction*

Contractile responses to the $\alpha_1$-adrenoceptor agonist, phenylephrine (1 $\mu$M), were also significantly increased following addition of 100 nM melatonin ($60 \pm 15$% potentiation; $n=6$). After removal of the endothelium, contractile responses to 1 $\mu$M phenylephrine were significantly increased compared to endothelium-intact arteries ($71 \pm 11$ mmHg in endothelium-intact arteries and $113 \pm 17$ mmHg in endothelium-denuded arteries; $n=10$; $P<0.01$). In endothelium-denuded arteries, 100 nM melatonin did not produce any significant potentiation of contractile responses to phenylephrine.

### *Effect of L-NAME on melatonin-induced potentiation*

L-NAME (10 $\mu$M), an inhibitor of nitric oxide synthase, was used to determine if endothelial production of nitric oxide might be involved in the effect of melatonin on endothelium-intact arteries. By itself, L-NAME had no effect on basal perfusion pressure, but L-NAME did significantly increase contractile responses to TNS (Figure 3). However, in the presence of L-NAME, melatonin no longer significantly potentiated contractile responses to TNS (Figure 3). The order of exposure to L-NAME and melatonin did not influence the final response to TNS of the endothelium-intact artery (data not shown). As expected, L-NAME had no effect on contractile responses to TNS in arteries when the endothelium was removed ($n=5$).

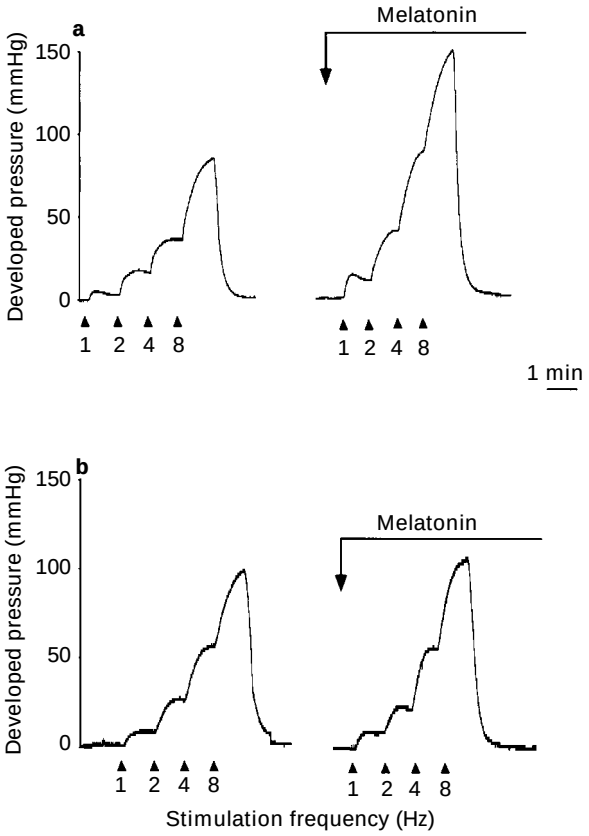


**Figure 1** Representative tracings of the effect of 100 nM melatonin on vasoconstrictor responses to various frequencies of transmural nerve stimulation (TNS) in (a) endothelium-intact and (b) endothelium-denuded perfused tail arteries of the rat. Developed pressure is shown in mmHg, and frequencies of TNS are indicated by the arrowheads.

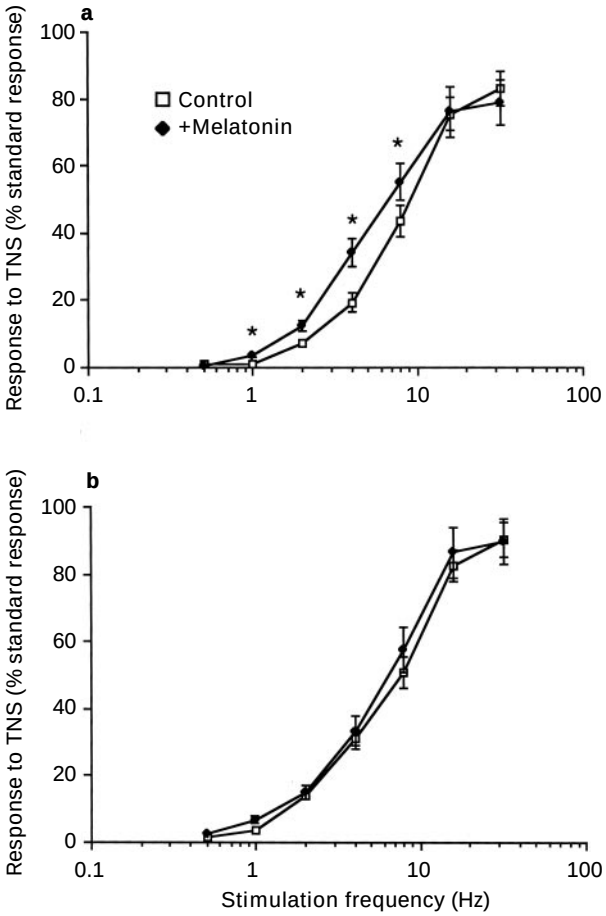


**Figure 2** Comparison of effects of melatonin on vasoconstrictor responses to nerve stimulation in (a) endothelium-intact and (b) endothelium-denuded arteries. Frequency response curves to nerve stimulation (60 s) in the presence and absence of melatonin (100 nM) are shown. Perfusion of melatonin through the arteries began 2 min before nerve stimulation. Changes in pressure are expressed as % of the response of the tissue to methoxamine (10 $\mu$M) plus KCl (120 mM) (standard response). *Significantly dierent ($P<0.05$) from responses to TNS without melatonin by Student's paired $t$ test. Values are means and vertical lines show s.e.mean, $n=6$.

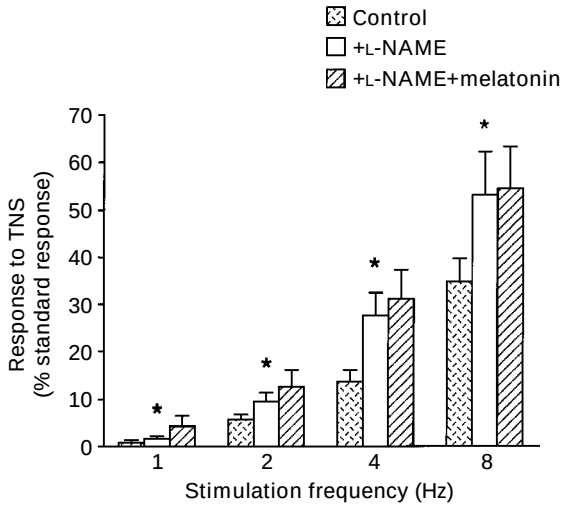


**Figure 3** Effect of L-NAME (10 $\mu$M) or L-NAME plus melatonin (100 nM) on vasoconstrictor responses to various frequencies of transmural nerve stimulation (TNS) in endothelium-intact tail arteries. Results are expressed as % of standard response to 10 $\mu$M methoxamine plus 120 mM KCl. Values are means $\pm$ s.e.mean, $n=6$. *$P<0.05$ for responses in the presence of L-NAME compared to control responses by Student's paired $t$ test. Responses were not significantly different when treatment with L-NAME was compared to treatment with L-NAME and melatonin.

### *Effect of melatonin on responses to acetylcholine and sodium nitroprusside*

The possible influence of melatonin on either the synthesis of nitric oxide or its effect on smooth muscle was further explored by use of acetylcholine and sodium nitroprusside, respectively. In the first set of experiments, the effect of acetylcholine (0.01 – 1.0 $\mu$M) on responses to adrenergic nerve stimulation (4 Hz) was determined in endothelium-intact arteries (Figure 4a). Acetylcholine caused a concentration-dependent decrease in the perfusion pressure induced by adrenergic nerve stimulation. As expected contractile responses to nerve stimulation were elevated in the presence of melatonin (100 nM); for example at 4 Hz the control contractile response to nerve stimulation was $81 \pm 9$ mmHg, compared to $106 \pm 9$ mmHg in the presence of melatonin ($P<0.001$, $n=16$; paired $t$ test). However, the % decrease in perfusion pressure caused by acetylcholine was not significantly different from that observed in the absence of melatonin, regardless of the acetylcholine concentration used (Figure 4a).

In a parallel set of experiments, the exogenous nitric oxide donor sodium nitroprusside (0.1 – 10 $\mu$M) was tested in endothelium-denuded arteries to determine if melatonin blocks vascular smooth muscle relaxation to nitric oxide. As shown in Figure 4b, sodium nitroprusside caused a concentration-dependent decrease in perfusion pressure during TNS (4 Hz) that was not significantly altered by the presence of 100 nM melatonin.

Because of possible prejunctional effects of acetylcholine when tissues are contracted by TNS, vasodilatation to acetylcholine was also measured with phenylephrine as the preconstriction stimulus. Acetylcholine (1 $\mu$M) caused a $36 \pm 6$% reduction in the developed pressure induced by phenylephrine. In the presence of L-NAME (10 $\mu$M), acetylcholine had little effect ($7 \pm 5$% decrease in pressure, $n=5$; $P<0.01$ compared to the absence of L-NAME; paired $t$ test), suggesting that acetylcholine-induced vasodilatation is mediated primarily by nitric oxide. In the presence of melatonin, contractile responses to phenylephrine (0.3 $\mu$M) were markedly increased,

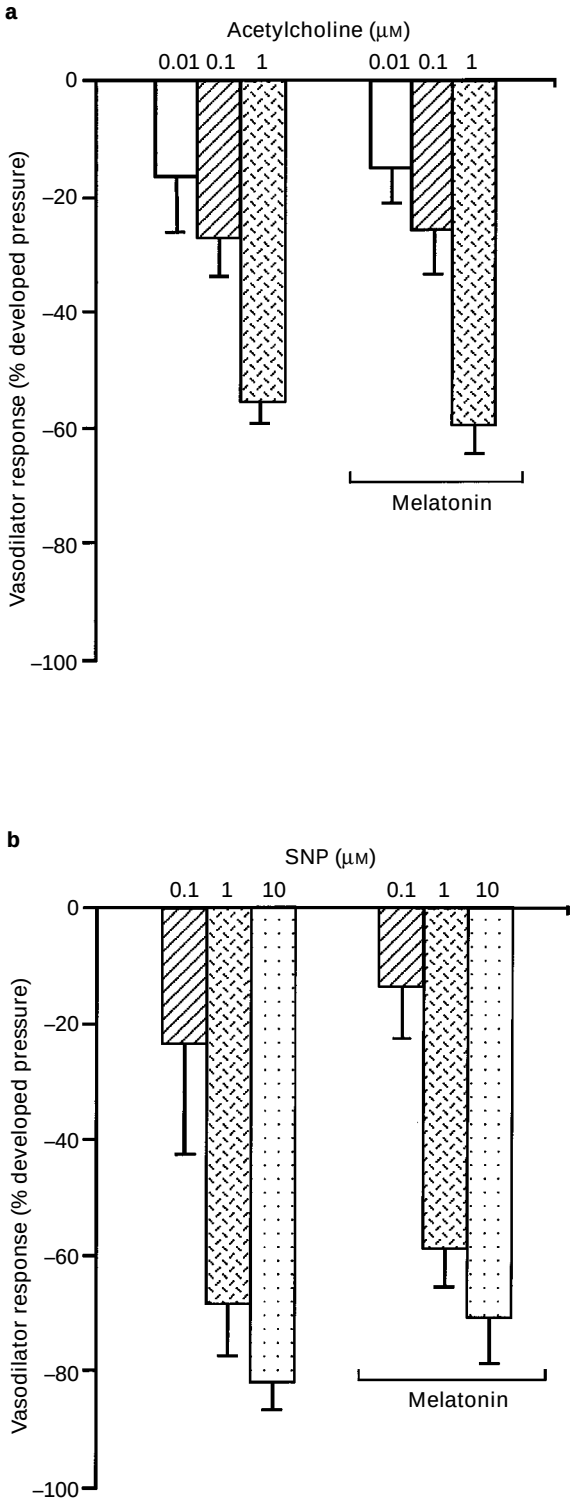


**Figure 4** Effect of melatonin on vasodilator responses to (a) acetylcholine in endothelium-intact arteries or (b) sodium nitroprusside (SNP) in endothelium-denuded arteries. Acetylcholine (0.01 – 1 $\mu$M) or sodium nitroprusside (0.1 – 10 $\mu$M) were added to the perfusate at the peak of each response to maintained nerve stimulation (4 Hz). Melatonin (100 nM) was added 2 min preceding nerve stimulation. Vasodilator responses are expressed as a percentage of the developed pressure produced by nerve stimulation in the absence of vasodilator. Values are means $\pm$ s.e.mean, $n=6$.

and the % relaxation to acetylcholine was reduced (Figure 5 and Table 1). To control for the marked increase in contractile responses to phenylephrine in the presence of melatonin, a higher concentration of phenylephrine (1 – 3 $\mu$M) was used to

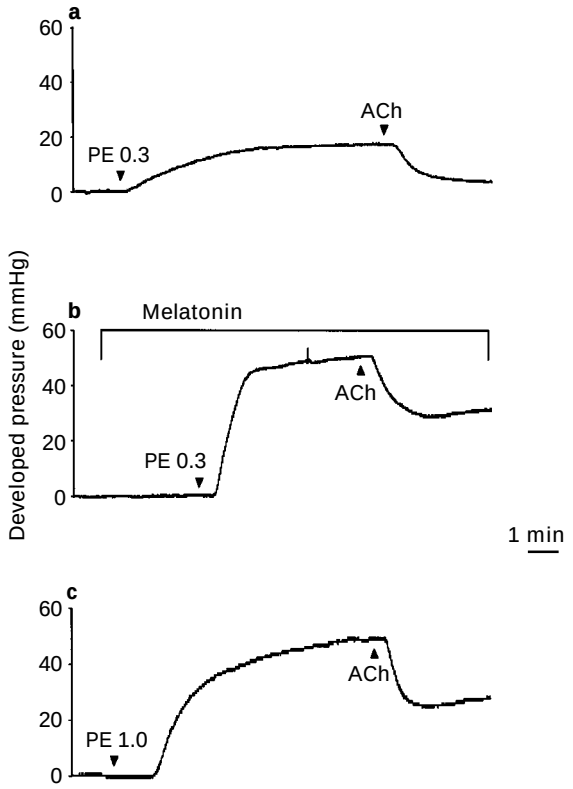


**Figure 5** Representative tracings of the vasodilator effects of acetylcholine (1 $\mu$M) in arteries pre-contracted with phenylephrine (PE); (0.3 or 1 $\mu$M) or phenylephrine plus melatonin (100 nM). (a) Control vasodilator response to acetylcholine (ACh) is shown. (b) Vasodilator response to acetylcholine in artery precontracted with melatonin and 0.3 $\mu$M phenylephrine. (c) Response to acetylcholine in artery pre-contracted with a higher concentration of phenylephrine (1 $\mu$M) to match response to phenylephrine (0.3 $\mu$M) in the presence of melatonin (pressure-matched).

attain a level of perfusion pressure similar to that caused by phenylephrine (0.3 $\mu$M) in the presence of melatonin (Figure 5c). Under these pressure-matched conditions, the vasodilator effect of acetylcholine was not significantly different with and without melatonin (Table 1).

### *Effect of potassium channel openers*

NS 1619 (10 $\mu$M), which opens large-conductance $Ca^{2+}$-activated $K^+$ channels ($BK_{Ca}$) (Holland *et al.*, 1996), decreased transmural pressure in phenylephrine-contracted, endothelium-denuded arteries (59$\pm$10%, $n=5$) (Figure 6b). The vasodilator effect of NS 1619 was inhibited by the $K^+$ channel blocker tetraethylammonium (TEA), confirming an action on $K^+$ channels in this tissue. The control vasodilator response to 10 $\mu$M NS 1619 was 41$\pm$7 mmHg in contrast to 27$\pm$7 mmHg when TEA (1 mM) was present ($P<0.008$, $n=9$; paired $t$ test). As previously shown, melatonin had no effect on endothelium-denuded, phenylephrine-contracted arteries (Figure 6a). However, when NS 1619 was present, addition of melatonin (100 nM) now caused a marked increase of transmural pressure (Figures 6b and 7).

Cromakalim has been shown to enhance $K^+$ conductance through ATP-sensitive $K^+$ channels ($K_{ATP}$) (Nelson, 1993). As shown in Figure 6c, cromakalim (3 $\mu$M) caused vasodilation in endothelium-denuded arteries similar in magnitude to that produced by NS 1619 (53$\pm$13% decrease in developed pressure, $n=6$). In contrast to findings with NS 1619, addition

**Table 1** Effect of melatonin on vasoconstrictor responses to phenylephrine and vasodilator responses to acetylcholine (1 $\mu$M) in rat endothelium-intact, perfused tail artery

| *Constrictor* | *Developed pressure* (mmHg) | *Relaxation to ACh* (% developed pressure) |
|---|---|---|
| Phenylephrine (0.3 $\mu$M) | 32$\pm$3 | $-$42$\pm$4 |
| Phenylephrine (0.3 $\mu$M) + melatonin (100 nM) | 113$\pm$14 | $-$27$\pm$5* |
| Phenylephrine; 1–3 $\mu$M (pressure-matched) | 138$\pm$13 | $-$34$\pm$5 |

Relaxation to acctylcholine (ACh) is expressed as percent of pressure developed in response to constrictor. Values are means$\pm$s.e.mean, $n=6$.
* $P<0.05$ compared to phenylephrine (0.3 $\mu$M) alone (paired $t$-test).

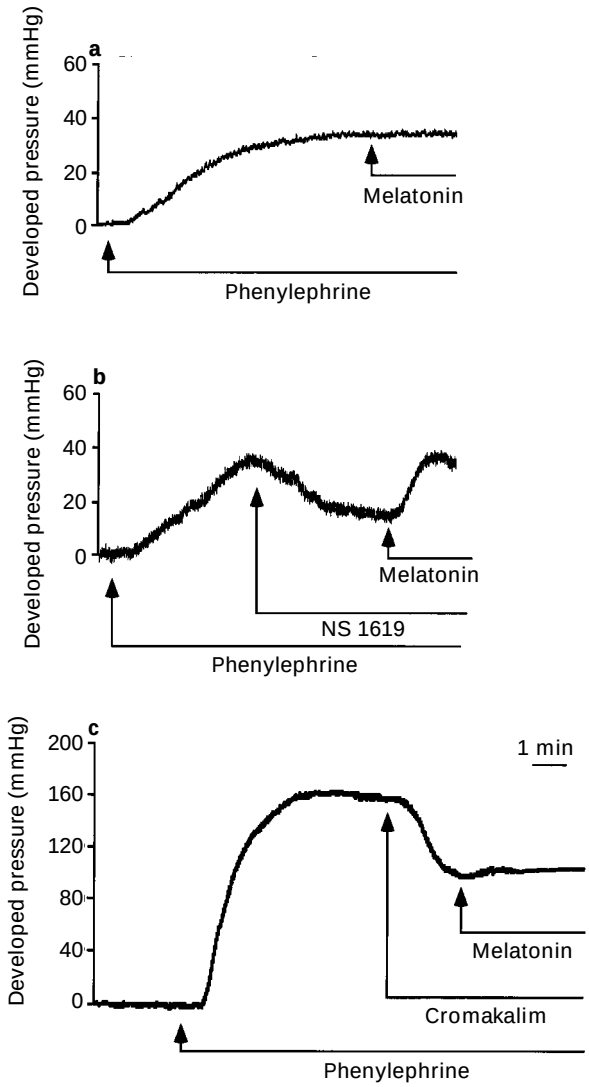


**Figure 6** Representative tracings showing effect of melatonin (100 nM) in endothelium-denuded, phenylephrine-treated arteries in the absence and presence of K channel openers, NS 1619 or cromakalim. (a) Effect of melatonin (100 nM) in artery pre-contracted with phenylephrine (1 $\mu$M). (b) Effect of melatonin in the presence of both NS 1619 (10 $\mu$M) and phenylephrine (1 $\mu$M). (c) Effect of melatonin in the presence of both cromakalim (3 $\mu$M) and phenylephrine (1 $\mu$M).

of melatonin (100 nM) in the presence of cromakalim had little or no contractile effect (Figures 6c and 7). Interaction between cromakalim and melatonin was also tested by assessing

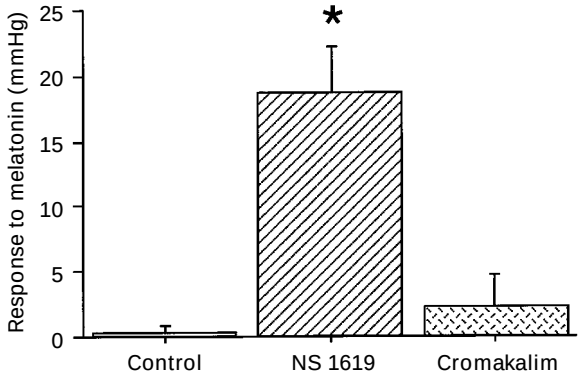


**Figure 7** Vasoconstrictor response to melatonin in the presence of $K^+$ channel openers, NS 1619 (10 $\mu$M) or cromakalim (3 $\mu$M), in the absence of endothelium. Endothelium-denuded arteries were precontracted with phenylephrine (1 $\mu$M), $K^+$ channel openers were added, and responses to melatonin were then tested. Values are means $\pm$s.e.mean, $n=6$. *Significantly different from control melatonin response, $P<0.05$, as tested with Student's paired $t$ test.

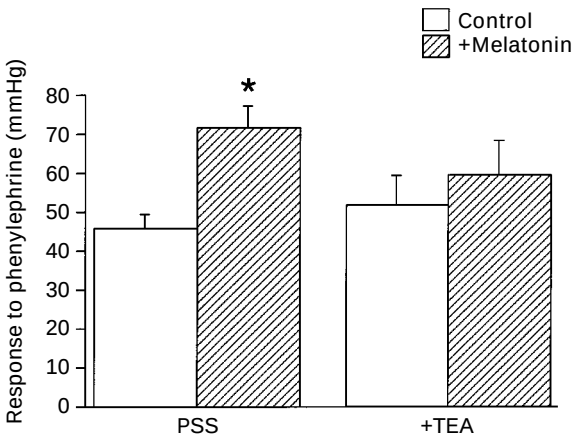


**Figure 8** Effect of tetraethylammonium (TEA; 1 mM) on potentiation induced by 100 nM melatonin. Responses to phenylephrine in the absence and presence of melatonin are given in mmHg. Phenylephrine concentrations in the presence of TEA were adjusted so that contractile responses would be similar to those in the presence of physiological salt solution (PSS) alone. Phenylephrine concentration in PSS was 1 $\mu$M, while 600 $\mu$M phenylephrine was used in the presence of TEA. Values are means $\pm$s.e.mean, $n=6$. *Significantly different from control by paired Student's $t$ test.

whether vasodilator responses to cromakalim would be influenced by the prior addition of melatonin. In endothelium-denuded arteries activated by TNS at 4 Hz, vasodilator responses to 10 $\mu$M cromakalim were $46\pm11\%$ of developed pressure in the absence of melatonin compared to $40\pm16\%$ in the presence of 100 nM melatonin ($n=6$).

### *Effect of potassium channel blocker*

Tetraethylammonium blocks $BK_{Ca}$ channels when used at appropriate concentrations (Nelson & Quayle, 1995). Therefore we tested whether potentiation produced by melatonin would be influenced by the presence of tetraethylammonium (1 mM. Tetraethylammonium, itself, enhanced the effect of phenylephrine on intact arteries. In order to match contractile responses to phenylephrine in the absence and presence of tetraethylammonium, a lower concentration of phenylephrine was used in the presence of tetraethylammonium. As shown in Figure 8, in endothelium-intact arteries contracted by 100 nM phenylephrine, melatonin did not cause any significant potentiation in the presence of tetraethylammonium. The effect of the $BK_{Ca}$ channel blocker also was tested on endothelium-denuded vessels. However, tetraethylammonium had no significant effects on the contractile response to 1 $\mu$M phenylephrine ($122\pm7$ mmHg, control, versus $129\pm17$ mmHg with tetraethylammonium).

## Discussion

The present study confirms earlier observations that melatonin potentiates contractile responses of the rat tail artery to adrenergic stimulation (Viswanathan *et al*., 1990; Evans *et al*., 1992; Krause *et al*., 1995). In our previous study, this effect was shown to be mediated by melatonin receptors on vascular smooth muscle (Krause *et al*., 1995). The current findings demonstrate additional roles for $Ca^{2+}$-activated potassium ($BK_{Ca}$) channels and endothelial-derived nitric oxide (NO) in the mechanism by which melatonin enhances vasoconstriction. Both factors are critical for eliciting a response to melatonin in isolated perfused rat tail arteries, although the requirement for endothelium appears to depend on the membrane potential of the vascular smooth muscle. Melatonin does not appear to interfere directly with agonist-induced production of NO or the action of NO on the smooth muscle cells; but melatonin may modulate flow-induced NO production, possibly through inhibition of endothelial potassium channels. However, when the vascular smooth muscle is more depolarized, inhibition of smooth muscle $BK_{Ca}$ channels also appears to be a component of the contractile response to melatonin. Given these insights into the mechanism by which melatonin affects vascular tone, it is apparent that various factors such as smooth muscle membrane potential, intracellular calcium levels, arterial perfusion, stretch and pressure can all modulate the contractile effects of melatonin.

Melatonin increased the responsiveness of endothelium-intact arteries to adrenergic nerve stimulation as well as to the $\alpha_1$-adrenoceptor agonist, phenylephrine, indicating that melatonin acts postjunctionally to modulate the contractile response. However, following removal of the endothelium, melatonin no longer potentiated contractile responses to either adrenergic stimulus. This finding suggests that the endothelium may be a target for melatonin action. Endothelium-dependent potentiation by melatonin could be achieved through inhibition of the release of vasodilator substances from the endothelium (NO, endothelium-derived hyperpolarization factor, prostacyclin ($PGI_2$)) or inhibition of the effects of these substances on vascular smooth muscle cells. Alternatively, melatonin could cause endothelium-dependent potentiation by enhancing the release of contractile factors such as endothelin from the endothelium (Yanagisawa *et al*., 1988). However, because the effect of melatonin in endothelium-intact perfused arteries was abolished in the presence of an inhibitor of NO synthase, L-NAME, it appears that NO is the major factor involved in the endothelial modulation of the actions of melatonin.

One explanation of our results would be that melatonin blocks the conversion of L-arginine to NO by an action on NO synthase. In fact inhibition of NO synthesis by melatonin has been demonstrated in the rat cerebellum (Pozo *et al*., 1994). If melatonin acts in the tail artery by inhibiting NO synthase, then melatonin should have attenuated the vasodilator effects of acetylcholine, a known stimulator of endothelial NO production. Since it did not, another possibility is that melatonin interferes with the effect of NO on smooth muscle

cells, for example by preventing the activation of guanylate cyclase. If this were true, then melatonin should alter the vasodilator effects of sodium nitroprusside. However, vasodilatation to sodium nitroprusside in arteries denuded of endothelium was unaffected by the presence of melatonin. Thus, neither agonist-induced release of NO nor vasodilator effects of NO were altered by melatonin. In what manner, then, do the actions of melatonin depend on the endothelium?

One possibility is that melatonin may selectively inhibit shear-induced release of NO. In perfused, unstretched blood vessels, flow and shear stress act as persistent stimuli for the release of endothelial factors, particularly NO (Moncada *et al*., 1991; Griffith, 1994). In fact the ability of the NO synthase inhibitor, L-NAME, to increase significantly contractile responses of the perfused tail artery demonstrates that NO is being released in this perfused preparation. The action of shear stress to release NO would increase as the perfused vessel constricts in response to adrenergic nerve stimulation or $\alpha_1$-adrenoceptor agonists. In the constant volume perfusion system that we have used, vessel diameter is not fixed and decreases progressively during stimulation. Since flow rate is inversely related to vessel radius by Poiseuille's law, reduction in lumen diameter greatly increases vascular resistance and shear stress along the luminal surface, leading to even greater release of NO (Tesfamariam & Cohen, 1988; Snow *et al*., 1994).

Mechanisms involved in shear-induced and agonist-induced release of NO may differ (Olesen *et al*., 1988; Cooke *et al*., 1991; Griffith, 1994). For example, $K^+$ channel blockers have been shown to inhibit selectively shear-induced release of NO while leaving acetylcholine-induced dilatation unaltered (Hutcheson & Griffith, 1994). Indeed, one possibility would be that melatonin inhibits endothelial $K^+$ channels activated by shear. An action of melatonin on endothelial cell $K^+$ channels controlling release of NO would be consistent with our data showing that melatonin is unable to block either agonist-induced or exogenous NO-dependent vasodilatation. Furthermore, our observation that actions of melatonin are inhibited by the $K^+$ channel blocker, TEA, is also consistent with a primary action of melatonin on $BK_{Ca}$ channels.

In the present study, we have also shown that melatonin-induced potentiation is restored in endothelium-denuded arteries in the presence of NS 1619, a known activator of $BK_{Ca}$ channels (Armstead, 1997). This did not occur following activation of $K_{ATP}$ channels by cromakalim, even though levels of dilatation produced by NS 1619 and cromakalim were comparable. TEA inhibited vasodilator responses to NS 1619, consistent with activation of $BK_{Ca}$ channels. However, the blockade by 1 mM TEA was incomplete. It is possible that NS 1619 has additional effects in the perfused rat tail artery, such as blockade of voltage-sensitive $Ca^{2+}$ channels (Edwards *et al*., 1994; Holland *et al*., 1996). However, in rat cerebral arteries, we have shown that melatonin, TEA and charybdotoxin, a selective blocker of $BK_{Ca}$ channels, all reverse NS 1619-induced vasodilatation (Geary *et al*., 1997). Combined with our present findings, the preponderance of evidence suggests that the action of melatonin is to reduce the activity of $BK_{Ca}$ channels in either vascular smooth muscle or endothelial cells. Interestingly, in pressurized rat cerebral arteries studied in the absence of flow or shear stress where tonic NO release may be minimal, L-NAME did not inhibit the constrictor effects of melatonin (Geary *et al*., 1997). In this preparation, where melatonin effects are apparently not dependent on NO synthase activity, however, contractile responses to melatonin were significantly inhibited by either TEA or charybdotoxin, but were unaltered by apamin, which blocks the small-conductance $K_{Ca}$ channels. Thus in the pressurized rat cerebral artery preparation, effects of melatonin may be predominantly mediated by actions on smooth muscle $BK_{Ca}$ channels.

If melatonin inhibits $BK_{Ca}$ channels on either smooth muscle or endothelial cells, what determines the relative importance of these actions in a particular tissue or preparation? In our previous study with rat tail artery segments stretched between platinum wires (Krause *et al*., 1995), we found that melatonin caused potentiation of constriction in the absence of functional endothelium, as demonstrated by a lack of relaxation to acetylcholine. In contrast to the perfused arteries used here, wire-mounted arteries are stretched to optimize contractile responses, and the smooth muscle cells would be depolarized as compared to cells in an unstretched perfused vessel (Bülbring & Kuriyama, 1963; Davis *et al*., 1992). In fact, smooth muscle cells of unstretched segments of endothelium-intact, rat tail arteries have been shown to be 27 mV more hyperpolarized as compared to stretched, endothelium-denuded, wire-mounted segments (Jobbling & McLachlan, 1992; Chen & Rembold, 1996). The lack of stretch-induced depolarization in the perfused preparation would be expected to decrease the open-state probability of smooth muscle cell $K^+$ channels (Nelson & Quayle, 1995) as compared to the wire-mounted preparation. Thus, in the perfused preparation, an action of melatonin on smooth muscle $K^+$ channels may not be apparent due to the low open-state probability of these channels. This hypothesis is consistent with our finding that the $BK_{Ca}$ channel blocker, TEA, was also unable to potentiate the contractile effects of phenylephrine in the endothelium-denuded perfused preparation.

In cannulated arteries used in the perfusion system, the endothelium remains intact and functional, in contrast to a vessel in which wires, inserted into the lumen, damage the endothelial layer. Thus endothelial factors, including NO, would be expected to have a significant influence in the perfused artery. In some tissues it has been suggested that NO acts directly by enhancing smooth muscle $K^+$ channel activity (Bolotina *et al*., 1994; Nelson & Quayle, 1995). However, in the rat tail artery the actions of NO appear to result primarily from alterations in the relationship between intracellular $Ca^{2+}$ and force development (Cheung & MacKay, 1985; Chen & Rembold, 1996). Thus we hypothesize that, in rat perfused tail arteries, melatonin blocks endothelial $K^+$ channels causing a decrease in shear-induced release of NO and subsequent potentiation of contractile responses. In contrast, the major action of melatonin in wire-mounted, endothelium-denuded arteries is most probably mediated by an effect on smooth muscle $BK_{Ca}$ channels, which are more likely to be in an open state in this preparation.

In conclusion, these data indicate that, in the rat perfused tail artery, melatonin potentiates contractile responses to adrenergic nerve stimulation and $\alpha_1$-adrenoceptor agonists. The use of an unstretched, endothelium-intact vessel along with tonic, flow-induced release of endothelium-derived NO combine to be critical variables responsible for melatonin-induced potentiation. However, in situations where vascular smooth muscle $BK_{Ca}$ channels show a high open-state probability, such as in the presence of NS 1619 or in a wire-mounted preparation, melatonin may cause potentiation by inhibiting $BK_{Ca}$ channels on smooth muscle cells. Our data suggest that the open-state probability of endothelial and smooth muscle cell $BK_{Ca}$ channels may be an important determinant of the vasoconstrictor action of melatonin.

Although there remains much to be learned regarding the vascular effects of melatonin, it is becoming apparent that the

response to melatonin may depend on the state of the artery. The degree to which melatonin modulates vascular contractility may be influenced by pressure, flow and endothelial shear stress as well as the pre-existing level of smooth muscle tone due to the vasoactive agents present. A better understanding of the critical factors influencing the action of melatonin will lead to more consistency among experimental studies, as well as to the elucidation of important variables affecting the physiological consequences of activating vascular melatonin receptors.

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