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Mechanisms of melatonin-induced vasoconstriction in the rat tail artery: a paradigm of weak vasoconstriction

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1 Vasoconstrictor effects of melatonin were examined in isolated rat tail arteries mounted either in an isometric myograph or as cannulated pressurized segments. Melatonin failed by itself to mediate observable responses but preactivation of the arteries with vasopressin (AVP) reliably uncovered vasoconstriction responses to melatonin with maxima about 50% of maximum contraction. Further experiments were conducted with AVP preactivation to 5–10% of the maximum contraction.

2 Responses to melatonin consisted of steady contractions with superimposed oscillations which were large and irregular in isometric but small in isobaric preparations. Nifedipine (0.3 μM) reduced the responses and abolished the oscillations. Charybdotoxin (30 nM) increased the magnitude of the oscillations with no change in the maximum response.

3 Forskolin (0.6 μM) pretreatment increased the responses to melatonin compared to control and sodium nitroprusside (1 μM) treated tissues. The AVP concentration required for preactivation was 10 fold higher than control in both the forskolin and nitroprusside treated groups.

4 In isometrically-mounted arteries treated with nifedipine, melatonin receptor agonists had the potency order 2-iodomelatonin > melatonin > S20098 > GR196429, and the MT_2-selective antagonist luzindole antagonized the effects of melatonin with a low pK_B of 6.1 ± 0.1.

5 It is concluded that melatonin elicits contraction of the rat tail artery *via* an mt1 or mt1-like receptor that couples *via* inhibition of adenylate cyclase and opening of L-type calcium channels. Calcium channels and charybdotoxin-sensitive K^+ channels may be recruited into the responses *via* myogenic activation rather than being coupled directly to the melatonin receptors.

6 It is proposed that the requirement of preactivation for overt vasoconstrictor responses to melatonin results from the low effector reserve of the melatonin receptors together with the tail artery having threshold inertia. Potentiative interactions between melatonin and other vasoconstrictor stimuli probably also result from the threshold inertia. A simple model is presented and a general framework for consideration of interactions between weak vasoconstrictor agonists and other vasoconstrictor stimuli is discussed.

Keywords: Melatonin; mt1 receptors; MEL_1 receptors; cyclic AMP; potentiation; vasoconstriction; preactivation; rat tail artery

Abbreviations: AVP, arg[8]-vasopressin; ChTX, charybdotoxin; cyclic AMP, adenosine 3′:5′-cyclic monophosphate; cyclic GMP, guanosine 3′:5′-cyclic monophosphate; DMSO, dimethylsulphoxide; SNP, sodium nitroprusside

Introduction

Vascular melatonin receptors have been identified by specific 2-[^{125}I]-iodomelatonin binding in rat tail and cerebral vessels (Capsoni *et al*., 1994; Seltzer *et al*., 1992; Viswanathan *et al*., 1990; 1992; 1993) as well as in cerebral vessels from primates, including humans, (Stankov *et al*., 1993; Stankov & Fraschini, 1993). The vasoactive effects of melatonin have best been described in the rat tail artery where it has been shown to potentiate contractile responses of noradrenaline and sympathetic nerve stimulation (Krause *et al*., 1995; Ting *et al*., 1997; Viswanathan *et al*., 1990). However, direct smooth muscle contractions to melatonin have proved difficult to detect. For example, melatonin failed to cause contractions in arteries studied under isometric conditions (Krause *et al*., 1995; Viswanathan *et al*., 1990) while in pressurized arteries vasoconstriction occurred in only a minority of the tissues examined (Evans *et al*., 1992; Ting *et al*., 1997). The unreliability of vasoconstrictor responses to melatonin in the tail artery has not been adequately explained, particularly in light of the fact that melatonin seems to always enhance the responses to sympathetic nerve stimulation in that tissue.

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We noted that in all cases where melatonin mediated vasoconstriction the maximum effect of melatonin was small compared to noradrenaline, and thus we would classify melatonin vascular receptors as a weak vasoconstrictor system. We have previously found that slight preactivation of rat mesenteric arteries exposes otherwise invisible vasoconstriction responses to neuropeptide Y, an agonist that also has a small maximum effect compared to noradrenaline in most tissues (Lew *et al*., 1996). Thus the intention of this study was to determine whether preactivation could expose vasoconstrictor responses to melatonin in wire-mounted (isometric) and pressurized (isobaric) segments of rat tail artery and to characterize any responses obtained. The results led us to conclude that stimulus-response coupling pathways are stimulated both directly and indirectly during the responses to melatonin, and that the vasoconstrictor and potentiative effects of melatonin are the same. In those respects melatonin appears no different to other weak vasoconstrictor agonists that act at other receptor types. We propose a hypothesis about differences in the behaviours of weak and strong vasoconstrictor systems that appears to be generally applicable and predicts many of the patterns of interaction that have been previously reported as notable.

Methods

Tissue preparation

Male Sprague-Dawley rats aged between 8 and 10 weeks (250–300 g) were housed under a 12 h light dark cycle with food and water available *ad libitum*. Animals were anaesthetized with carbon dioxide (80% CO_2, 20% O_2) and killed by decapitation. The tail was removed and placed in cool Krebs solution (composition below). The proximal region of the tail artery was dissected out under a microscope and mounted either in an isometric myograph or as cannulated pressurized segments.

Wire-mounted preparation

Arteries were cut into 2 mm long segments and mounted on two 40 μm wires in a Mulvany-Halpern style isometric myograph (JP Trading, Aarhus, Denmark). The myograph chambers were filled with Krebs solution (37°C) and bubbled with 95% O_2 and 5% CO_2. Following a 30 min equilibration period, the diameter of the artery was set to be 90% of the diameter predicted for a distending pressure of 100 mmHg using standard calculations (Mulvany & Halpern, 1977). Following a 30 min stabilization period, vessels were contracted twice with a potassium depolarizing solution, KPSS (K^+ 120 mM substituted for Na^+) to assess tissue viability and to provide a reference contraction.

Cannulated preparation

Arteries were tied onto a glass cannula, gently flushed free of red blood cells, tied off at the distal end and pressurized to 55 mmHg using an elevated reservoir containing Krebs solution. The arteries were observed using a horizontally-mounted microscope (Olympus HSC) fitted with a video camera and the external diameter continuously measured using a computer with video capture card and software written by Dr T.O. Nield (Neild, 1989). Arteries were allowed to equilibrate for a minimum of 30 min before addition of KCl (160 mM) to assess tissue viability and to provide a reference contraction.

Experimental protocol

To expose contractile responses to melatonin, vasopressin (AVP) was applied in increasing concentrations until a contraction between 5 and 10% of the K^+ reference response was obtained. Cumulative melatonin concentration-response curves were constructed using 10 fold concentration increments in the continued presence of AVP. Single melatonin concentration-response curves were generated in wire-mounted arteries and two melatonin concentration-response curves were generated per tissue in cannulated arteries.

Receptor characterization

Using wire-mounted arteries, the receptors mediating the contractile responses to melatonin were investigated by examining the responses to a number of melatonin analogues as well as the effect of the MT_2-selective antagonist luzindole. Concentration-response curves to the melatonin analogues 2-iodomelatonin, S20098 and GR196429 were constructed following precontraction of the artery with AVP. The effect of luzindole (1.0, 3.2 or 10 μM) was tested by incubating the artery for 30 min before the start of the melatonin concentration-response curve. In both series of experiments arteries were pretreated with nifedipine (0.3 μM).

Stimulus-response mechanisms

Using cannulated segments of artery, the effect of the L-type calcium (Ca^{2+}) channel blocker nifedipine (0.3 μM) and the potassium channel blocker charybdotoxin (ChTX, 30 nM) on the responses to melatonin were examined. Time control experiments showed that at least two melatonin concentration-response curves could be generated without any effect on either the maximum response or potency, and therefore the effects of nifedipine and ChTX were examined using a within-tissue design. The first concentration-response curve always served as the control while the second concentration-response curve was performed in the presence of the channel blocker. Nifedipine and ChTX were added to the vessel chamber 30 min before constructing the melatonin concentration-response curve.

Forskolin treatment studies

To investigate a possible role for the inhibition of adenylate cyclase in the vasoconstrictor response to melatonin we performed experiments using forskolin which stimulates the production of cyclic AMP *via* activation of adenylate cyclase. In these experiments conducted using the cannulated arteries, control melatonin concentration-response curves were generated using the standard protocol and 30 min later the arteries were contracted with AVP to approximately 40% of the response to K^+. Once the response to AVP had stabilized, forskolin (0.6 μM) or sodium nitroprusside (SNP, 1 μM) was applied causing the tissue to relax to baseline levels. The tissues were reactivated to between 5 and 10% of the K^+ response with additional AVP and a melatonin concentration-response curve generated in the continued presence of the vasodilator and AVP.

Drugs and solutions

Drugs used were melatonin (Sigma Chemical Co., St Louis, MO, U.S.A.); 2-iodomelatonin (Research Biochemicals International, Natick, MA, U.S.A.); luzindole, GR196429 (N-(2-[2,3,7,8-tetrahydro-1H-furo{2,3-g}indolo-1-yl]ethyl)acetamide) and S20098 (N-(2-[7-methoxy-1-naphthalenyl]ethyl) acetamide) (gifts from Glaxo-Wellcome, Stevenage); Arg^8-vasopressin and charybdotoxin (Auspep, Parkville, Victoria, Australia); nifedipine (Bayer, Germany); forskolin (Cal Biochem-Nova Biochem Pty Ltd., Alexandria, New South Wales, Australia); and sodium nitroprusside (David Bull Laboratories, Mulgrave, Victoria, Australia). Melatonin, 2-iodomelatonin, GR196429, S20098, luzindole, nifedipine and forskolin were initially dissolved in DMSO to a concentration of 10 or 20 mM. All subsequent dilutions were made in water. AVP and ChTX were dissolved in water. Luzindole, GR196429, S20098 and forskolin stocks were stored at 4°C. All other drugs were made fresh daily. The Krebs solution was of the following composition (in mM): NaCl 119; KCl 4.69; $MgSO_4$ 1.17; KH_2PO_4 1.18; $NaHCO_3$ 25; CaCl 2.5; Glucose 11 and EDTA 0.026.

Data analysis

Contractile responses to melatonin are expressed as a percentage of the K^+ reference contraction, the precontraction level included in the response measurement. Individual agonist

concentration-response curves were fitted with a symmetrical logistic curve:

$$\text{Response} = a + \frac{b}{1 + e^{d(c+\log[A])}}$$

where a is the resting level of response, b is the response range, c is the $-\log_{10}$ of the molar concentration of agonist required to give a 50% maximum response (pEC_{50}, $-\log$ Molar), d is the slope and curvature parameter, e is the base of the natural logarithm and $[A]$ is the concentration of agonist. All values are expressed as the mean $\pm$ s.e.mean. For each experiment described, the n value was equal to the number of rats from which the arteries were obtained. Permutation tests (Ludbrook, 1994) were performed with the free software Permustat v 1.3 Randomized Test for Macintosh (Andrew F. Hayes), and analysis of variance (ANOVA) and Fishers tests were performed with InStat v 2.01 for Macintosh (GraphPad software). A P value of <0.05 was taken as significant.

The effect of luzindole was analysed using the method of Lew & Angus, (1995). Briefly, the effect of luzindole on the slope and maximum of the melatonin curve was assessed using one-way analysis of variance (no differences found). Non-linear regression of the pEC_{50} value against the antagonist concentration provided an estimate of the pK_B value of luzindole. These results were then displayed on a Clark plot. The points on the Clark plot are the mean log EC_{50} values from the agonist concentration-response curves at each concentration of antagonist plotted against the log ([luzindole] + K_B). A line representing the ideal interaction between the agonist and antagonist and allows easy comparison of the concentration-response curve spacing with the ideal for a simple competitive interaction.

Results

Vasoconstrictor effects of melatonin in wire-mounted arteries

Melatonin by itself failed to mediate an observable contractile response in wire-mounted tail artery segments but when the arteries were precontracted with AVP (10.23 ± 0.03 $-\log$ M) to 5–10% of the K^+ reference response, melatonin evoked concentration-dependent vasoconstriction in all tail arteries examined (Figure 1). Large irregular oscillations in active tension developed in response to suprathreshold concentrations of melatonin making quantitation of the melatonin concentration-response curve difficult. These oscillations were prevented by pretreatment with the L-type Ca^{2+} channel blocker nifedipine (0.3 μM). In the presence of nifedipine, the maximum response to melatonin was 13.5 ± 0.4% of the K^+-induced response with a pEC_{50} of 9.2 ± 0.1 (Figure 2).

The melatonin analogues 2-iodomelatonin, S20098 and GR196429 mediated concentration-dependent contractions in AVP precontracted wire-mounted arteries pretreated with nifedipine (Figure 2). 2-Iodomelatonin was approximately 5 fold more potent than melatonin and S20098 and GR196429 were approximately 4 and 100 fold less potent than melatonin respectively. The maximum responses elicited by 2-iodomelatonin and GR196429 were not different to that elicited by melatonin but the maximum response to S20098 was significantly smaller.

Pretreatment of wire-mounted arteries with the melatonin receptor antagonist luzindole (in the presence of nifedipine) caused a concentration-dependent rightward shift of the melatonin concentration-response curve with no change in slope and a small reduction of maximum that did not reach statistical significance (Figure 3, $P = 0.12$ ANOVA). A similar small reduction of maximum was found in vehicle control experiments with increasing concentrations of the luzindole vehicle (DMSO). A pK_B value of 6.1 ± 0.1 was calculated for luzindole using non-linear regression analysis. The results of the analysis are displayed in a Clark plot which shows the spacing of melatonin concentration-response curves to be consistent with a competitive interaction over the relatively limited concentration range used in this study (Figure 3).

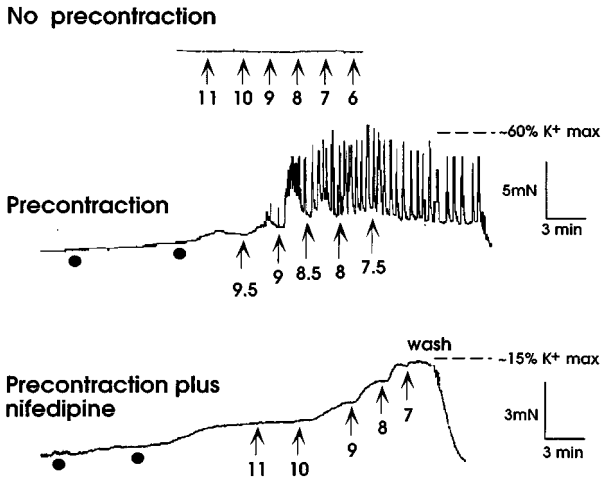

Figure 1 Chart records showing cumulative melatonin concentration-response curves in three wire-mounted segments of rat tail arteries. Numbers are melatonin concentrations ($-\log$ M). The upper panel shows the inability of melatonin to mediate observable contractions when administered alone. Preactivation with AVP (●), (middle and lower panels) unveiled a vasoconstrictor effect of melatonin. The oscillations in force generated by melatonin (middle panel) were prevented by pretreatment with nifedipine, 0.3 μM (lower panel).

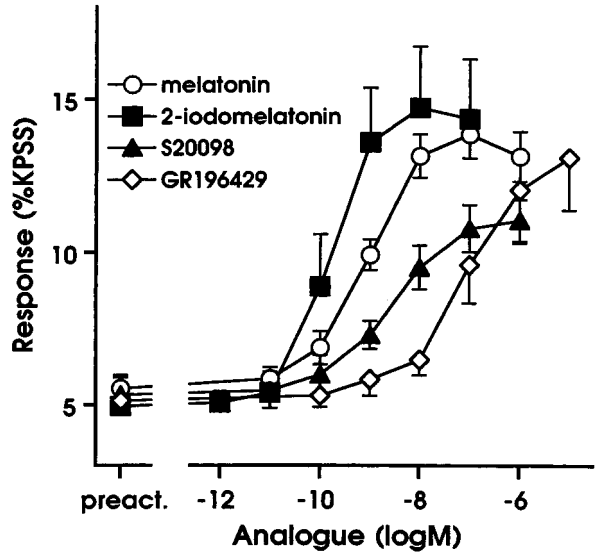

Figure 2 Cumulative concentration-response curves to melatonin ($n = 6$) and the melatonin analogues 2-iodomelatonin ($n = 4$), S20098 ($n = 5$) and GR196429 ($n = 5$) in wire-mounted tail arteries pretreated with nifedipine (0.3 μM). Arteries were contracted with AVP prior to constructing each concentration-response curve. Responses have been expressed as a percentage of the maximum contraction induced by K^+ (120 mM) and are shown as the mean $\pm$ s.e.mean.

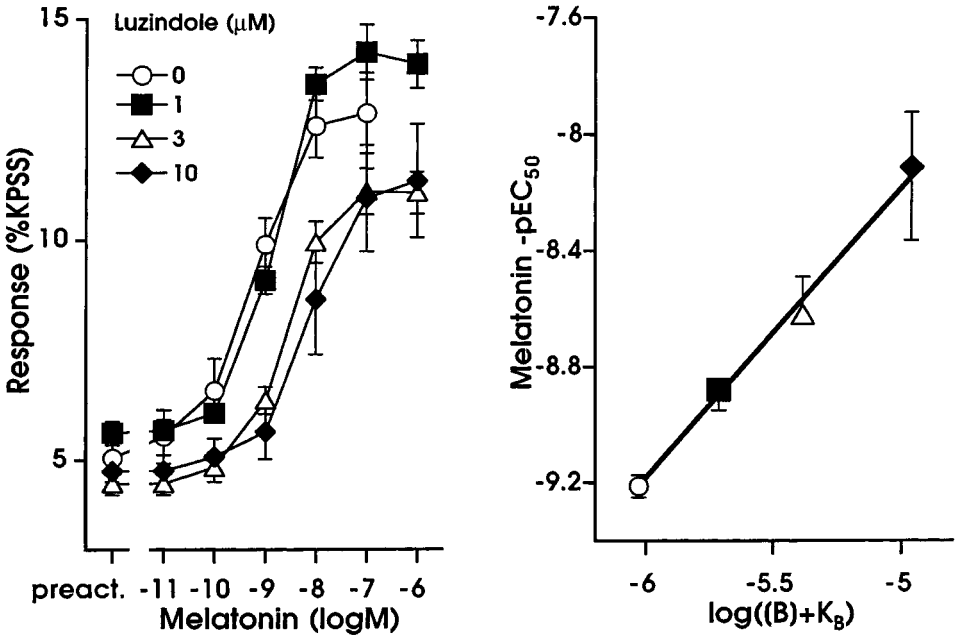

Figure 3 Effect of the melatonin analogue luzindole on melatonin concentration-response curves in wire-mounted tail arteries. Left: Melatonin concentration-response curves in the absence and presence of luzindole at the concentrations indicated ($n=4-6$ one curve per tissue). Right: Clark plot from the data in the left panel after non-linear regression analysis for pK_B estimation. The points on the Clark plot represent the mean $-pEC_{50}$ values and the error bars provide an estimate of the confidence band around the line, obtained as two times the standard error of the difference between the observed and predicted $-pEC_{50}$ values.

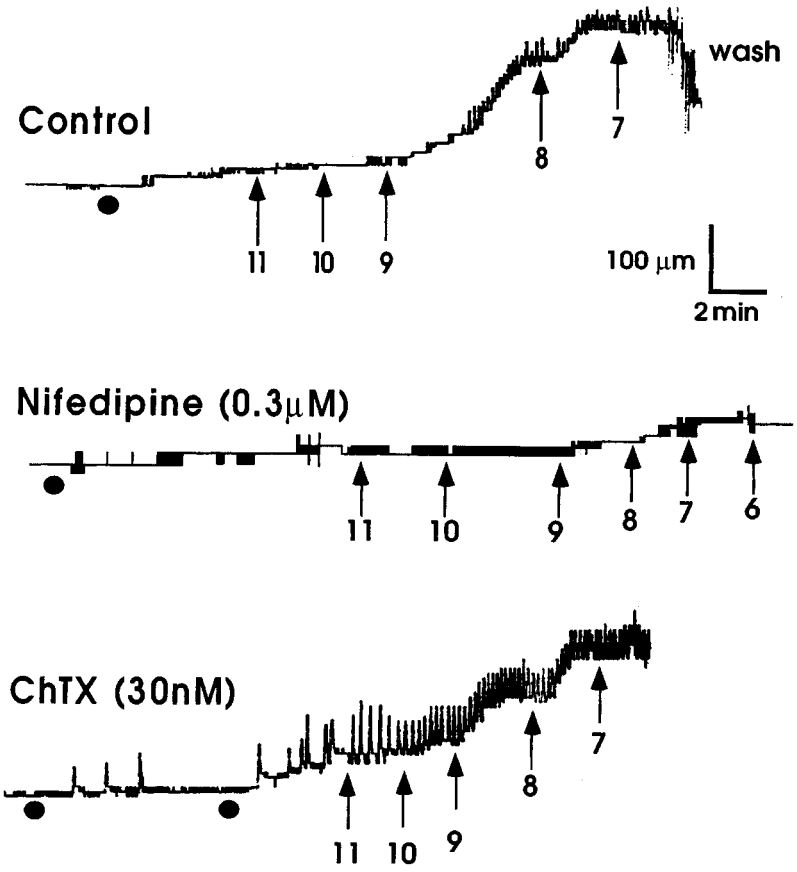

Figure 4 Chart records of concentration-response curves to melatonin in three different cannulated segments of rat tail artery. All arteries were precontracted with AVP (●) before applying melatonin (melatonin concentrations indicated by the numbers, −log M). Upper panel: Control responses to melatonin. Middle panel: Responses to melatonin in the presence of nifedipine (0.3 μM). Nifedipine treatment inhibited the oscillations in diameter which developed in association with the responses to melatonin. Lower panel: Responses to melatonin in the presence of charybdotoxin (ChTX, 30 nM). ChTX treatment caused an increase in the amplitude of the oscillations to melatonin as well as causing oscillations to develop in response to AVP.

Vasoconstrictor effects of melatonin in cannulated arteries

Melatonin failed to elicit any measurable vasoconstriction response in the three cannulated arteries tested in the absence of preactivation, but after precontracted with AVP (10.8 ± 0.05, $-\log$ M), melatonin elicited concentration-dependent contraction in all arteries examined. The responses to melatonin had an oscillatory component, but in contrast to the responses of wire-mounted artery segments the oscillations were small (to a maximum of $5.6 \pm 1.0\%$ of the K^+ reference contraction, see Figure 4). The melatonin concentration-response curve had a maximum of $38 \pm 2\%$ of the K^+ reference contraction, and a pEC_{50} of 9.3 ± 0.1. It was possible to generate two successive melatonin concentration-response curves at 30 min intervals without any change in the sensitivity or maximum response to melatonin (Figure 5) so subsequent experiments were conducted using a within-tissue control design.

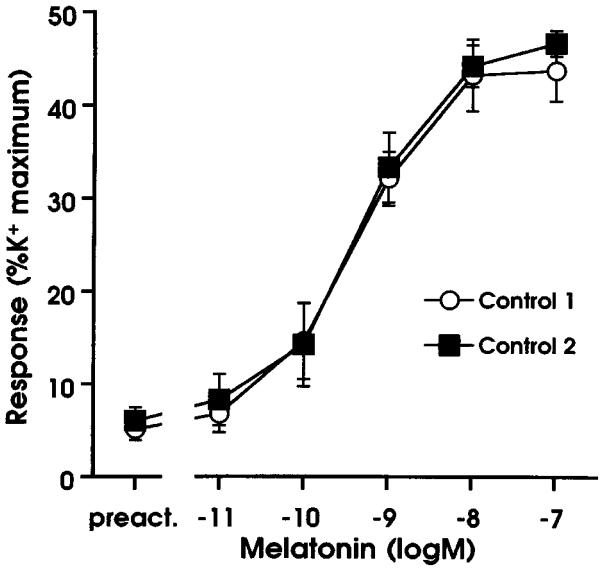

Figure 5 Time control melatonin concentration-response curves in cannulated tail arteries. Arteries were contracted with AVP prior to constructing each concentration-response curve and the second concentration-response curve was constructed 30 min after the first. Responses have been expressed as a percentage of the maximum contraction induced by K^+ (160 mM) and are shown as the mean $\pm$ s.e.mean.

Nifedipine (0.3 μM) reduced the maximum response to melatonin by approximately 60% (Figure 6) and caused a rightward shift of approximately 3 fold ($\Delta pEC_{50} = 0.57 \pm 0.15$ vs 0.04 ± 0.01 for time controls, $P = 0.016$ permutation test). The oscillatory component of the responses to melatonin were abolished by nifedipine (Figure 4, $P = 0.008$ Fishers exact test). ChTX (30 nM) alone had no effect on resting tone of the artery but did cause a small leftward shift of the melatonin concentration-response curve ($\Delta pEC_{50} = -0.13 \pm 0.04$ vs 0.04 ± 0.01 for time controls, $P = 0.005$ permutation test) with no change in the maximum response (Figure 6). The effect of ChTX treatment on the amplitude of the response-associated diameter oscillations was quite pronounced, increasing to $14.4 \pm 1.4\%$ of the K^+ reference contraction (Figure 4, $P < 0.0001$ permutation test). In the presence of ChTX, diameter oscillations were associated with the AVP preactivation as well as the responses to melatonin (Figure 4). Neither nifedipine nor ChTX affected the concentration of AVP required for precontraction (10.8 ± 0.1 and 10.7 ± 0.1, $-\log$ M, respectively).

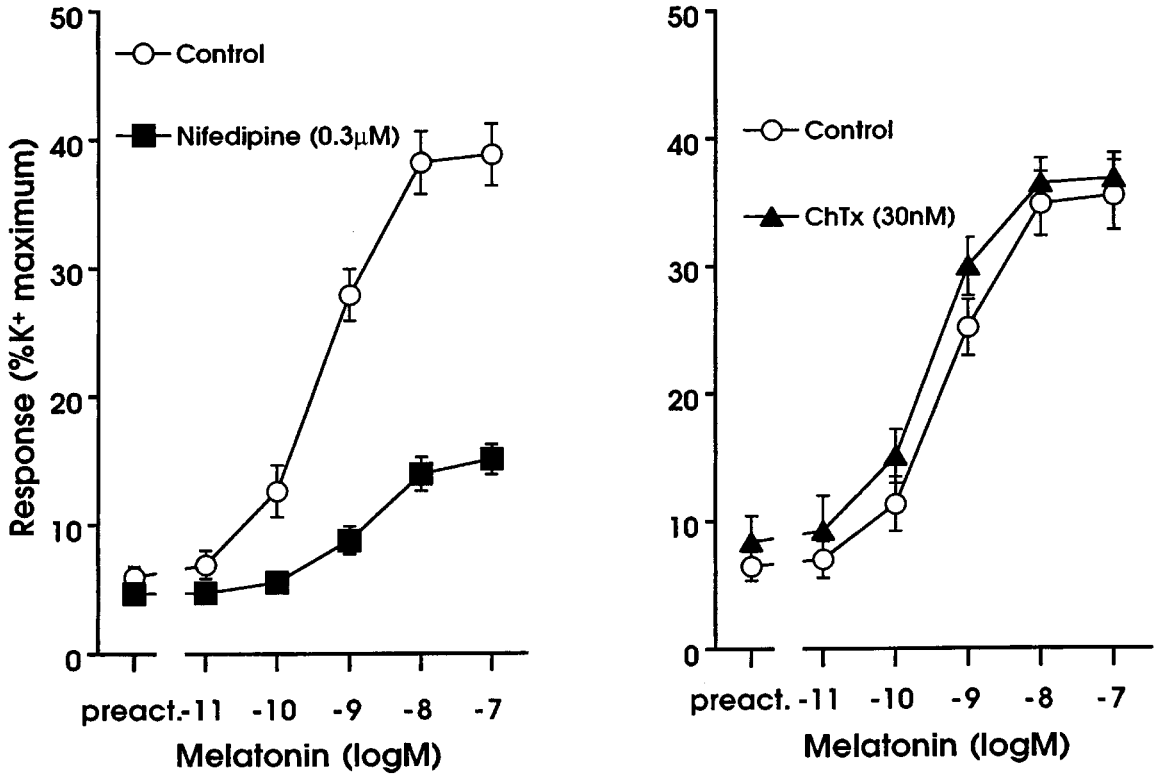

Figure 6 Effect of nifedipine (left, $n = 5$) and charybdotoxin (ChTX, right, $n = 6$) on concentration-response curves to melatonin in cannulated tail arteries precontracted with AVP. Nifedipine caused a marked attenuation of the responses to melatonin whilst ChTX treatment caused a small leftward displacement of the melatonin concentration-response curve. Responses have been expressed as a percentage of the maximum contraction induced by K^+ (160 mM) and are shown as the mean $\pm$ s.e.mean.

Forskolin

Control melatonin concentration-response curves were constructed with normal preactivation (i.e. 5–10% contraction elicited by AVP) and then after washout the tissues were precontracted with AVP to about 40% and either forskolin (0.6 μM) or SNP (1 μM) applied. Each elicited virtually complete relaxation of the artery so additional AVP was applied to obtain the normal level of preactivation for a melatonin concentration-response curve in the continued presence of the vasodilators. The concentration of AVP needed for the final preactivation was identical in the forskolin and SNP groups; 10 fold higher than in the control curve. The maximum response to melatonin was significantly larger than control in the presence of forskolin (Figure 7, $54\pm4\%$ vs $34\pm3\%$ $P=0.031$ permutation test) but SNP decreased it slightly from 35 ± 2 to $29\pm3\%$ (Figure 7, $P=0.091$ permutation test).

Discussion

Receptors

The NC-IUPHAR subcommittee on Melatonin Receptors has recently revised the nomenclature of the three known melatonin receptors so that the receptors formerly known as MEL_{1A} (or Mel_{1a} or ML_{1A}) are now mt1 receptors, MEL_{1B} (or Mel_{1b} or ML_{1B}) are now MT_2 and the uncloned ML_2 receptors are now called MT_3 receptors (Alexander & Peters, 1998). This new nomenclature is used in this paper with the former receptor names added parenthetically to avoid confusion.

Our experiments characterizing the melatonin receptors in the rat tail arteries were all conducted in wire-mounted tissues, preactivated with AVP to expose the vasoconstrictor effect of melatonin, and pretreated with nifedipine to eliminate the large force oscillations that otherwise confounded quantitation of the melatonin concentration-response curves in that preparation. Neither of these treatments is likely to have altered the properties of the melatonin receptors in these arteries, and indeed our conclusion regarding the nature of the receptors is the same as that previously made in this tissue by others without either nifedipine or preactivation (Krause *et al*., 1995; Ting *et al*., 1997). Melatonin and agonists non-selective for mt1 and MT_2 receptors (MEL_{1A} and MEL_{1B} receptors) elicited concentration-dependent contractions of wire-mounted tail artery segments giving the potency order 2-iodomelatonin > melatonin > S20098 > GR196429. Others have reported the same agonist potency sequence and the same low intrinsic activity of S20098 for the enhancement of sympathetic nerve stimulation responses in this artery (Ting *et al*., 1997). Luzindole antagonized the vasoconstrictor effects of melatonin in an apparently competitive manner with a pK_B of 6.1, similar to that reported by others for melatonin receptor-mediated enhancement of responses to sympathetic nerve stimulation in this artery (Krause *et al*., 1995) but substantially less than has been reported for other tissues, e.g. $pK_B=7.7$ in the rabbit retina (Dubocovich, 1988) 7.3 in CHO cells expressing mt1 (MEL_{1A}) receptors (Witt-Enderby & Dubocovich, 1996) and 6.9 in chick retinal cell cultures (Iuvone & Gan, 1994). The variable potency of luzindole suggests heterogeneity of melatonin receptor subtypes or a variable mixture of subtypes in these different tissues; luzindole has only modest selectivity for MT_2 (MEL_{1B}) receptors compared to the mt1 (MEL_{1A}) receptors and so a mixed receptor population might not result in obviously non-competitive behaviour when a non-selective agonist like melatonin is used (Kenakin, 1992). Based on the current classification of melatonin receptor subtypes, our results indicate the melatonin receptor mediating contractions of the rat tail artery is probably an mt1 or mt1-like receptor.

The similarities of agonist and antagonist properties obtained when the measured responses are vasoconstriction of preactivated arteries (present study) and melatonin receptor-mediated enhancement of responses to sympathetic nerve stimulation (Krause *et al*., 1995; Ting *et al*., 1997) suggest that vasoconstriction mediated by melatonin and the potentiation by melatonin of other vasoconstriction responses are mediated by the same receptors.

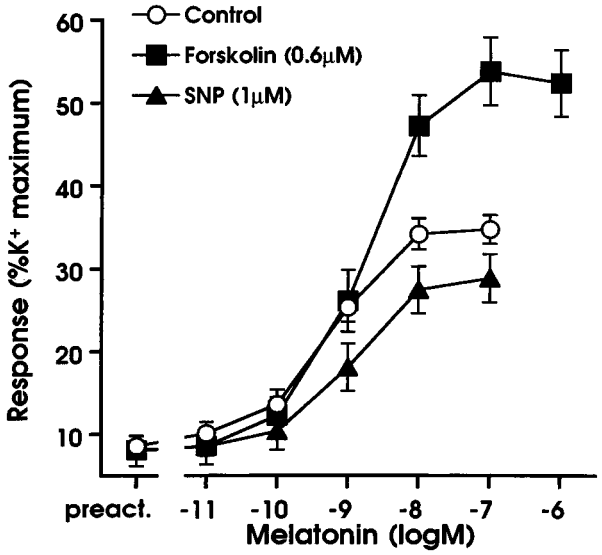

Figure 7 Effect of forskolin ($n=6$) and SNP ($n=6$) treatment on concentration-response curves to melatonin in rat cannulated tail artery. Control curves ($n=12$) from both sets of tissues were not different and are grouped together. Forskolin caused a marked enhancement of the maximum response to melatonin whilst SNP caused a small reduction. Responses have been expressed as a percentage of the maximum contraction induced by K^+ (160 mM) and are shown as the mean±s.e.mean.

Transduction mechanisms

MT_2 and mt1 receptors couple to inhibition of adenylate cylcase *via* $G_{i/o}$ proteins (Alexander & Peters, 1998; Dubocovich, 1995; Morgan *et al*., 1994) and cloned human mt1 (MEL_{1A}) and MT_2 (MEL_{1B}) receptors have been shown to inhibit forskolin-induced accumulation of cyclic AMP in HEK293 cells and CHO cells (Conway *et al*., 1997; Witt-Enderby & Dubocovich, 1996). Our experiments with forskolin were designed to test for evidence of melatonin receptor stimulus-response coupling through inhibition of adenylate cyclase in the rat tail artery, and the results are consistent with such coupling; the maximum response to melatonin was increased in the presence of forskolin. Inhibition of adenylate cyclase is a stimulus for vasoconstriction by disinhibiting vasoconstrictor tone that is otherwise inhibited by cyclic AMP (see Thomas *et al*., 1993). The forskolin treatment caused a strong vasodilator stimulus by activation of adenylate cyclase so a higher concentration of AVP was applied to achieve the normal level of arterial preactivation. Under those conditions the vasoconstrictor stimulus that could be disinhibited by inhibition of adenylate cyclase was larger than control, therefore so was the vasoconstrictor effect of melatonin. The SNP treatment was an equally effective vasodilator stimulus to the forskolin

treatment, but acting *via* elevation of cyclic GMP rather than cyclic AMP. SNP failed to increase the size of the responses to melatonin, and even decreased them slightly. This suggests the effect of forskolin on the responses to melatonin was not solely a consequence of the higher AVP concentration because the AVP needed for the normal preactivation was identical in the SNP and forskolin treated groups. The effect of forskolin was presumably a consequence of the combination of the higher AVP concentration combined with higher adenylate cyclase activity and therefore we interpret these experiments as evidence for the melatonin receptors coupling to vasoconstriction *via* inhibition of adenylate cyclase. However, we do note that these experiments do not address the question of whether the melatonin receptors can initiate a contractile response in the absence of adenylate cyclase stimulation. Melatonin receptors in non-vascular tissues (Ebisawa *et al*., 1994) and other receptors that couple *via* $G_{i/o}$ in vascular smooth muscle have been shown to decrease stimulated but not basal cyclic AMP accumulation rate, e.g. NPY receptors (Fredholm *et al*., 1985) and α_2-adrenoceptors (Wright *et al*., 1995) so it is possible that the activation of $G_{i/o}$ coupled receptors does not affect basal adenylate cyclase activity. However, there are many different isoforms of adenylate cyclase with different regulatory properties so it is possible that the activated and basal rates of cyclic AMP accumulation are the products of different enzymes. Further, it is not clear that adenylate cyclase activity would ever be completely unstimulated under physiological circumstances or in our experiments where the tail arteries are preactivated, so the inability to inhibit cyclic AMP production under other circumstances may not preclude melatonin receptors from mediating vasoconstriction by inhibiting adenylate cyclase. Further experiments are needed to address these issues.

The role of adenylate cyclase inhibition in vascular responses to melatonin might be more directly demonstrated with measurements of cyclic AMP concentrations. However, while there are many published experiments where the effects of melatonin on cyclic AMP levels in cultured cells and brain and eye tissues have been measured (see Krause & Dubocovich, 1991), few experiments of that type have been performed using vascular tissue and the results are inconsistent. Melatonin has been shown to decrease forskolin-induced stimulation of cyclic AMP accumulation in rat cerebral arteries by about 25% (Capsoni *et al*., 1994), or by a significant but unspecified amount (Viswanathan *et al*., 1997). In the only published experiment using rat tail arteries (Viswanathan *et al*., 1990), melatonin had no effect on forskolin-stimulated accumulation of cyclic AMP in the presence of the phosphodiesterase inhibitor IBMX. However, there are several problems with interpreting those results. First, both studies for which details are available employed only single concentrations of melatonin (10 nM) and forskolin (either 1 or 10 μM). It remains to be seen whether melatonin may have been more effective at inhibiting the activity of adenylate cyclase stimulated by a lower concentration of forskolin, or indeed whether higher concentrations of melatonin might have been more effective. Secondly, the time over which tissues were exposed to forskolin and melatonin prior to measurement of cyclic AMP (20 or 30 min) in both studies seems excessive in light of the fact that melatonin-induced vasoconstriction develops quite rapidly and is not always well maintained (see Figure 1, also Ting *et al*., 1997). One might expect that the effect of melatonin on cyclic AMP levels would be maximal only a few minutes after the application of melatonin and wane thereafter. An integrated measurement of cyclic AMP formed during 20 or 30 min is unlikely to be a sensitive measurement of the effect of melatonin. We suggest that the direct measurements of cyclic AMP concentrations neither support nor refute the hypothesis that inhibition of adenylate cyclase is an important part of the coupling of melatonin receptors to vasoconstriction. Direct characterization of the effects of melatonin on cyclic AMP concentrations awaits better designed studies.

Inhibition of adenylate cyclase is not the only stimulus-response coupling mechanism involved in the responses to melatonin in these arteries. Inhibition of L-type calcium channels with nifedipine greatly depressed the melatonin concentration-response curve in the cannulated arteries (Figure 6). Both L-type calcium channels and ChTX-sensitive K^+ channels are involved in the oscillations elicited by melatonin because they were abolished by nifedipine and enhanced by ChTX. This suggests that the oscillations consisted of a L-type calcium channel-mediated contraction and a K^+ channel-mediated relaxation. The persistence of the oscillations in the presence of ChTX indicate that ChTX-insensitive inhibitory mechanisms are also involved, possibly but not necessarily K^+ channels. The exact nature of the K^+ channels that are involved cannot be decided from our data because although ChTX is selective for large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}), other K^+ channels that could repolarize the artery and cause a relaxation are also sensitive to that toxin (e.g. delayed rectifier K^+ channels, K_v, Alexander & Peters, 1998). Thus the oscillations could result from the interplay of smooth muscle cell contractile activation *via* L-type calcium channels and inactivation by either or both Ca^{2+}- and voltage-gated K^+ channels. It is possible that melatonin is able to modulate the opening of potassium channels by inhibiting adenylate cyclase and thereby decreasing protein kinase A activity (Ren *et al*., 1996). Since many different vasoconstrictor agonists are able to elicit responses with an oscillatory component in the rat tail artery (e.g. AVP, neuropeptide Y, noradrenaline, methoxamine and endothelin) we feel that it is quite likely that the oscillations result from general properties of arterial activation rather than from particular properties of melatonin receptor signal transduction.

The fact that the oscillations were far more pronounced in the wire-mounted arteries than the pressurized arteries gives additional insight into their genesis. The important difference between the preparations is that activation of the wire-mounted arteries leads to an unopposed increase in smooth muscle cell tension, whereas in the pressurized preparation the artery constricts upon activation and so the increased smooth muscle cell tension is attenuated by a decrease in overall wall tension. Thus the stimulus for myogenic depolarization upon activation of the wire-mounted arteries is greater than for an equivalent degree of activation in the pressurized preparation. There is evidence from rat small mesenteric arteries that most of the noradrenaline-induced intracellular Ca^{2+} increase results from depolarization-induced opening of calcium channels (Nilsson *et al*., 1994). Depolarization is less in cannulated arteries than in wire-mounted arteries (Schubert *et al*., 1996), so the overall result is myogenic amplification of vasoconstrictor responses being much more pronounced in isometric circumstances than in isobaric (VanBavel & Mulvany, 1994). We suggest an analogous role of myogenic amplification (*via* depolarization and L-type calcium channel opening) of responses of the rat tail artery to melatonin.

Our data show that vasoconstrictor responses to melatonin involve not only inhibition of adenylate cyclase, but also the opening of L-type calcium channels and ChTX-sensitive K^+ channels. While these experiments do not allow comparison of the relative roles of these three mechanisms, it is possible that inhibition of adenylate cyclase is the primary mechanism

whereby the melatonin receptors initiate responses. The conclusion that potassium channels are opened during vasoconstriction responses to melatonin contrasts strongly with that of Geary *et al.* (1997; 1998) who suggest that the effect of melatonin on the rat cerebral artery and tail artery are mediated by closure of potassium channels. However, in those studies less attention was paid to the level of arterial tone pertaining before melatonin was applied, and no correction was made for the effects of interventions on that tone. Further, the constant flow pump perfused preparation used by those authors would have resulted in high shear stress on the endothelium and thus likely a much higher level of nitric oxide than in our study where there was no shear stress on the endothelium. This may have altered the importance of different signal transduction pathways. However, the differing conclusions may be the result of erroneous interpretation rather than differences between tissues and preparations.

†It has been implied by other authors that the smooth muscle cell tension decreases upon activation under isobaric conditions (Schubert *et al.*, 1996; VanBavel & Mulvany, 1994). This is unlikely to be correct because although the total wall tension will decrease during vasoconstriction approximately according to the law of Laplace (tension = pressure*radius), an increasing fraction of the total wall tension is transferred from passive elements in the wall to the active elements in the smooth muscle cell. This allows the smooth muscle cell tension to increase with vasoconstriction even as the total wall tension decreases.

Preactivation

Melatonin increases the size of responses to the sympathetic nerves and to noradrenaline consistently in every rat tail artery segment examined (Krause *et al.*, 1995; Ting *et al.*, 1997; Viswanathan *et al.*, 1990) but the vasoconstrictor effects of melatonin are somewhat fugitive. We did not see any vasoconstriction when melatonin was applied alone, and others have reported that only a subset of rat tail artery segments display overt vasoconstriction responses to melatonin (Evans *et al.*, 1992; Ting *et al.*, 1997; Viswanathan *et al.*, 1990). Modest preactivation of the arteries reliably exposed vasoconstriction responses to melatonin. We chose to preactivate the arteries in this study using AVP because it provided a relatively stable and easily-titrated level of vasoconstriction. However, preactivation with any vasoconstrictor agonist would probably expose the responses to melatonin. We have found that both endothelin 1 and the α_1-adrenoceptor agonist methoxamine work equally well (data not shown) while others have used preactivation by noradrenaline or phenylephrine (Doolen *et al.*, 1998; Viswanathan *et al.*, 1993). Arteries that develop spontaneous tone do not require preactivation to reliably express vasoconstrictor responses to melatonin (Geary *et al.*, 1997) or to NPY (Lew *et al.*, 1996). Even with preactivation the maximum vasoconstriction response to melatonin is always less than the tissue maximum response, 40 – 50% in this study. In contrast AVP and noradrenaline can elicit sufficient stimulus in the rat tail artery for responses well in excess of the tissue maximum when functional antagonism is used to prevent the tissue maximum from acting as a ceiling, 170% and nearly 400% respectively (Lew, 1995). Clearly the stimulus for vasoconstriction mediated by melatonin receptors is weak. The phenomenon of responses to other weak vasoconstrictor agonists being exposed by preactivation with another vasoconstrictor is well known: sumatriptan and neuropeptide Y (NPY) are two examples (Cocks *et al.*, 1993; Grundemar & Hogestatt, 1992; Lew *et al.*, 1996; MacLennan *et al.*, 1993). The relevant receptors for those agonists, like mt1 receptors, couple to vasoconstriction *via* inhibition of adenylate cyclase so the indirectness of reduced cyclic AMP as a vasoconstrictor stimulus might be taken as the explanation for the effect of preactivation on responses to those weak agonists. However, partial agonists acting at differently coupled receptors also display strong sensitivity to preactivation (e.g. α_1-adrenoceptors, Lues & Schümann, 1984; Purdy & Weber, 1988) so we need a more general explanation for the sensitivity of weak vasoconstrictors to preactivation. We suggest that the need for preactivation to reliably record vasoconstrictor responses to melatonin is the combined consequence of mt1 receptors being only weakly coupled to vasoconstriction and the rat tail artery requiring a significant degree of activation to reach the threshold for vasoconstriction responses, a condition that can be described as threshold inertia and that will similarly affect other weakly coupled vasoconstrictor systems.

Threshold inertia

We propose a theory that not only can explain the effect of preactivation on the responses to melatonin in the rat tail artery, but also has general relevance to vasoconstriction responses in isolated arteries. The theory is not completely novel, but its overall implications have not been explicitly stated before. We theorize that arteries *in vitro* frequently have a basal state whereby a substantial amount of vasoconstrictor stimulus is required to reach the threshold for any overt mechanical vasoconstriction response. This condition, that we refer to as threshold inertia, affects strong and weak vasoconstrictor systems unevenly and we hypothesize that it accounts both for the need for preexisting tone to expose weak system responses and for the ability of weak systems to potentiate the effects of strong systems. Because a tissue state exactly at the threshold for a mechanical response is unlikely, any artery that does not have basal active tone probably has some degree of threshold inertia. Thus the theory has wide relevance to studies of arteries *in vitro*.

In discussing the implications of this theory we will explore the most general case, ignoring the issues of different mechanisms of stimulus-response coupling and concentrate instead on different strengths of agonist concentration-response coupling. We define strong vasoconstrictors as agonists that can elicit intracellular stimulus in excess of that needed to maximally activate the artery and weak vasoconstrictors as agonists that cannot maximally activate the artery. It is important to note that weak vasoconstrictors can be either full agonists (i.e. have high intrinsic efficacy) at receptors that can mediate only weak activation (presumably the case for melatonin), or partial agonists acting at receptors that can mediate strong activation. Thus the strength of a vasoconstrictor system (agonist and its receptors) is more related to 'effector reserve' as discussed previously (Lew, 1995) than to receptor reserve.

In a tissue with sufficient threshold inertia a weak agonist will be unable to cause enough stimulus to mediate a measured response; it will merely decrease the inertia. If that tissue is preactivated the total effect of the weak agonist can be expressed as response because the inertia has been overcome in the process of preactivation. In that way preactivation has a profound effect on the concentration-response curves of weak agonists. In contrast, a strong agonist can easily overcome the inertia and mediate a large response by itself, thus preactivation only causes a non-parallel leftward shift of the curve. These patterns are shown in Figure 8 which models a system

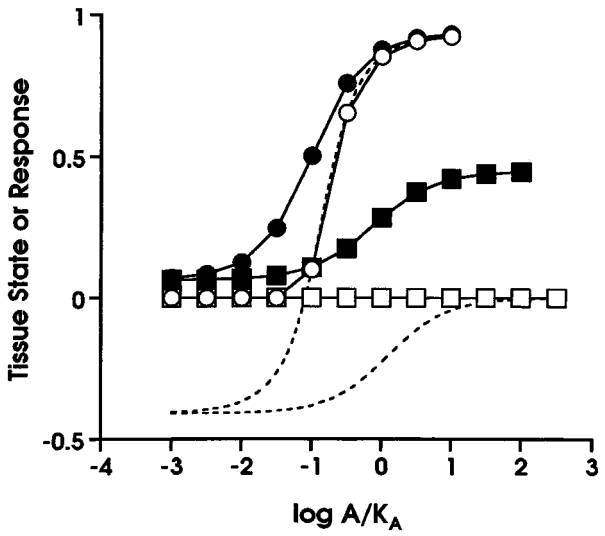

Figure 8 Theoretical concentration-response curves for a strong agonist (circles) and a weak agonist (squares) with (closed symbols) and without (open symbols) preactivation in a tissue with threshold inertia. Without preactivation the weak agonist is unable to raise the tissue state above the threshold for a response (i.e. 0) and so the concentration-response curve is a flat line. The strong agonist is able to elicit enough activation to overcome the inertia and mediate a maximal response. Preactivation exposes the responses to the weak agonist by raising the full concentration-response curve of the weak agonist above the threshold, and causes a non-parallel leftward shift of the strong agonist concentration-response curve. Dashed lines represent the concentration-tissue state curves without preactivation, with preactivation the tissue state curves and response curves are coincident. The model used is

$$\text{tissue state} = \frac{2}{\pi}\text{atan}(S + I)$$

where S is stimulus (the product of the concentration of bound receptors and agonist efficacy, [AR].e) and I is the inertia term. The efficacy term (e) is the product of the total receptor number and the agonist intrinsic efficacy, and was 10 for the strong agonist and 0.75 for the weak. I was -0.75 to provide threshold inertia and 0.1 for preactivation. Response was assumed to be 0 where tissue state was <0. The relatively steep slope of the strong agonist control curve (open circles) is very similar to the slope of the AVP concentration-response curve in the rat tail artery (Lew, 1995).

where tissue state can have any value between -1 and 1 but the measured responses can only occur between 0 and 1. This simple model can explain why preactivation exposes otherwise silent effects of melatonin and other weak vasoconstrictors. Furthermore, the model explains why threshold activation with a strong agonist causes a left shift of the concentration-response curve for another strong agonist. This phenomenon has been known for many years: Ariëns *et al.* (1960) concluded from the effects of activating agonists that threshold inertia means 'facilitation by subthreshold doses may be expected', and others later described that facilitation as 'threshold synergism' (Stupecky *et al.*, 1986). Of course a threshold concentration of a strong agonist is equivalent in the model to a high concentration of a weak agonist, and thus the threshold synergism idea also explains the ability of melatonin to enhance responses to other vasoconstrictors and to sympathetic nerve stimulation, even where direct responses to melatonin are invisible.

Melatonin is well known for its ability to increase the size of responses to sympathetic nerve stimulation and to noradrenaline (Evans *et al.*, 1992; Ting *et al.*, 1997; Viswanathan *et al.*, 1990) and again other weak systems such as NPY receptors share this ability (Lundberg *et al.*, 1985; Neild, 1987) as do partial and full agonists acting at more strongly coupled receptors (Purdy & Weber, 1988). We suggest that the ability of both weak and strong vasoconstrictors to potentiate other vasoconstrictor stimuli is the result of it activating the tissue to a state close to the threshold for mechanical responses. It is unnecessary to postulate different mechanisms of vasoconstriction and enhancement of nerve-mediated responses. Positive evidence for the similarity of mechanisms in the case of melatonin is provided by the observations that luzindole has the same potency at inhibiting the vasoconstrictor and the potentiating effects of melatonin, and S20098 has a smaller maximal effect than melatonin both as a vasoconstrictor and as a potentiator (this study and Ting *et al.*, 1997).

The threshold inertia is probably mediated at least partly by basally-released nitric oxide (NO). In the rat aorta removal of the endothelium or treatment with haemoglobin to sequester NO caused a preferential enhancement of vasoconstrictor responses to a weak agonist (clonidine) over the responses to strong agonists (noradrenaline, phenylephrine and 5-HT) (Martin *et al.*, 1986). When the receptor reserve (and hence the effector reserve) for phenylephrine was reduced by partial irreversible antagonism of the α-adrenoceptors with dibenamine, the effect of endothelium removal on the responses to phenylephrine was similar to that on clonidine in the control tissues. The authors concluded that basally-released NO depressed the responsiveness of the artery to vasoconstrictors, and that depression was more significant for the partial agonist clonidine than for full agonists that have receptor reserve. Both that conclusion and the pattern of the data are consistent with threshold inertia being mediated by basal NO production. In the rat tail artery responses to sympathetic nerve stimulation are substantially enhanced by inhibition of nitric oxide synthase (Lew & Angus unpublished data) and by removal of the endothelium (Geary *et al.*, 1998) so there is evidence for basally-released NO in the tissue used in this study as well. Geary *et al.*(1998) also showed that potentiation by melatonin of the responses to sympathetic nerve stimulation in the perfused tail artery was only seen when the endothelium was present and concluded that melatonin exerts its effect *via* inhibition of endothelial NO production. However, the inability of melatonin to potentiate sympathetic nerve responses that were already potentiated by removal of the endothelium is a predictable result where the mechanism of potentiation for both cases being removal of baseline inertia. Thus it is not necessary to ascribe an endothelial cell site for the potentiative action of melatonin.

Under normal circumstances *in vivo* most small arteries are partially contracted under the influence of a wide range of vasoactive factors and their own myogenic responsiveness and so no threshold inertia is possible. This not only explains why responses to weak vasoconstrictor agonists are readily observed *in vivo*, but it also leads to the conclusion that the behaviour of melatonin and other weak vasoconstrictor agonists in normal *in vitro* assays may be a poor predictor of their actions *in vivo*. It is tempting to suggest that arterial assays that do not develop spontaneous tone should be preactivated slightly to yield a more physiological reactivity. However, we should point out that in the rat mesenteric artery melatonin does not mediate significant vasoconstriction even after preactivation because preactivation cannot overcome the absence of specific receptors.

Conclusions

The results of this study demonstrate that under appropriate conditions melatonin elicits vasoconstriction of the rat isolated tail artery. This effect of melatonin is mediated *via* activation

of specific mt1-like receptors which are probably coupled to vasoconstriction *via* inhibition of adenylate cyclase. The opening of L-type Ca^{2+} channels also appears to be involved in mediating the contractile response to melatonin, although this may be an indirectly stimulated opening of the channels *via* a myogenic response to increased active smooth muscle tension rather than direct coupling to melatonin receptor activation. Melatonin potentiates the responses to sympathetic nerve stimulation in all segments of rat tail artery but only reliably causes overt vasoconstriction response when the segments are preactivated. It is hypothesized that this pattern of activity is a direct consequence of the tissue having threshold inertia combined with the weak coupling of the mt1-like receptors to vasoconstriction responses. This hypothesis can be extended to weak vasoconstrictors generally and has important consequences for the interpretation of the interactions between vasoconstrictor agonists.

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