Melatonin potentiates NE-induced vasoconstriction without augmenting cytosolic calcium concentration

C. VanDeputte, P. Giummelly, J. Atkinson, P. Delagrange, E. Scalbert, and C. Capdeville-Atkinson. Melatonin potentiates NE-induced vasoconstriction without augmenting cytosolic calcium concentration. Am J Physiol Heart Circ Physiol 280: H420–H425, 2001.—Because little is known of the intracellular mechanisms involved in the vasoconstrictor effect of melatonin (Mel), we examined the in vitro effects of Mel by using perfused cylindrical segments of the rat tail artery loaded with the intracellular Ca\(^{2+}\)-sensitive fluorescent dye, fura 2. Mel (10\(^{-14}\) to 10\(^{-4}\) M) had no effect on baseline perfusion pressure or [Ca\(^{2+}\)]\(_i\), but increased, at submicromolar concentrations, the vasoconstrictor effect of norepinephrine (NE) (P = 0.0029). Mel did not modify NE-induced [Ca\(^{2+}\)]\(_i\) mobilization, and thus the [Ca\(^{2+}\)]\(_i\) sensitivity of NE-induced contraction increased in the presence of Mel. Mel consistently increased KCl-induced vasoconstriction and [Ca\(^{2+}\)]\(_i\) sensitivity of contraction, but differences were not statistically significant. In conclusion, Mel increases the [Ca\(^{2+}\)]\(_i\) sensitivity of vasoconstriction evoked by NE suggesting that Mel may amplify endogenous vasoconstrictor responses to sympathetic outflow.

tail artery; intracellular calcium; norepinephrine

A primary vasoconstrictor effect at nanomolar concentration would be in agreement with its cerebrovascular constrictor effect in vivo. However, a direct vasoconstrictor effect of Mel is difficult to obtain in vitro (8). Ting et al. (26) suggested that Mel constricts pressurized, cylindrical segments but not arterial rings. We tested whether Mel vasoconstricts perfused, pressurized cylindrical segments of the rat tail artery.

Vandeputte, C., P. Giummelly, J. Atkinson, P. Delagrange, E. Scalbert, and C. Capdeville-Atkinson. Melatonin potentiates NE-induced vasoconstriction without augmenting cytosolic calcium concentration. Am J Physiol Heart Circ Physiol 280: H420–H425, 2001. –Because little is known of the intracellular mechanisms involved in the vasoconstrictor effect of melatonin (Mel), we examined the in vitro effects of Mel by using perfused cylindrical segments of the rat tail artery loaded with the intracellular Ca\(^{2+}\)-sensitive fluorescent dye, fura 2. Mel (10\(^{-14}\) to 10\(^{-4}\) M) had no effect on baseline perfusion pressure or [Ca\(^{2+}\)]\(_i\), but increased, at submicromolar concentrations, the vasoconstrictor effect of norepinephrine (NE) (P = 0.0029). Mel did not modify NE-induced [Ca\(^{2+}\)]\(_i\) mobilization, and thus the [Ca\(^{2+}\)]\(_i\) sensitivity of NE-induced contraction increased in the presence of Mel. Mel consistently increased KCl-induced vasoconstriction and [Ca\(^{2+}\)]\(_i\) sensitivity of contraction, but differences were not statistically significant. In conclusion, Mel increases the [Ca\(^{2+}\)]\(_i\) sensitivity of vasoconstriction evoked by NE suggesting that Mel may amplify endogenous vasoconstrictor responses to sympathetic outflow.

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MELATONIN (Mel) produces vasodilatation (6) or vasoconstriction (4, 17), or has no effect (1) in vivo. Some in vitro experiments show vasodilatation at micromolar concentration (21–23, 25), whereas others show vasoconstriction at nanomolar concentrations (7, 8, 10, 14, 15, 29).

A primary vasoconstrictor effect at nanomolar concentration would be in agreement with its cerebrovascular constrictor effect in vivo. However, a direct vasoconstrictor effect of Mel is difficult to obtain in vitro (8). Ting et al. (26) suggested that Mel constricts pressurized, cylindrical segments but not arterial rings. We tested whether Mel vasoconstricts perfused, pressurized cylindrical segments of the rat tail artery.

A second aspect of our study involved the intracellular transduction mechanism by which Mel potentiates vasoconstriction. Whereas some reports suggest that the effect of Mel involves intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), others suggest that the effect may be independent of changes in [Ca\(^{2+}\)]\(_i\). Regarding the first hypothesis Weekley (31) suggested that Mel constricted the pig coronary artery (preconstricted with KCl) by releasing norepinephrine (NE) from perivascular nerves because this effect was abolished by prior treatment with 6-hydroxydopamine, which destroys the structure of sympathetic nerve terminals. NE released by Mel would then stimulate a postsynaptic \(\alpha_1\)-adrenoceptor and produce an increase in [Ca\(^{2+}\)]\(_i\). Further evidence suggesting an involvement of [Ca\(^{2+}\)]\(_i\) was provided by Geary et al. (10). They showed that Mel reduced the diameter of pressurized rat cerebral arteries and this effect was abolished by the G protein inhibitor, pertussis toxin, and was attenuated by bradykinin calcium channel blockers. They suggested Mel acts via a G\(_s\) or G\(_i\) protein-coupled receptor and activation of the latter leads to inhibition of the large conductance of Ca\(^{2+}\)-activated K\(^+\) channels that regulates membrane potential and thus Ca\(^{2+}\) entry.

Other experiments suggest that changes in [Ca\(^{2+}\)]\(_i\) may not be necessary. Krause et al. (14) showed Mel potentiated the vasoconstrictor response of the rat tail artery to NE and suggested this could be explained by a postsynaptic mechanism by which Mel lowers production of the vasodilator cAMP. The latter effect, reported by Capsoni et al. (5), provided evidence that nanomolar concentration of Mel inhibits forskolin-stimulated cAMP in rat circle of Willis arteries.

One way of resolving the above problem is to measure vasomotion and [Ca\(^{2+}\)]\(_i\) simultaneously in the same preparation. In our paper, therefore, Mel-induced changes in the [Ca\(^{2+}\)]\(_i\) sensitivity of vasoconstriction were evaluated in segments of the

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rat tail artery loaded with the Ca$_{2+}$-sensitive dye, fura 2.

A final aspect of our study is the use of high KCl and NE as vasoconstrictor agents. Several authors (12) have shown that electromechanical stimulation with high KCl involves low [Ca$^{2+}$]$_i$ sensitivity of contraction, whereas the latter is amplified in pharmacomechanical stimulation with NE. Furthermore, we have shown that NE-induced vasoconstriction of the rat tail artery has a substantial [Ca$^{2+}$]$_i$-independent component (20). Were Mel to act on the latter, then it could be expected that Mel would potentiate NE-induced, but not KCl-induced, changes in [Ca$^{2+}$]$_i$, sensitivity of contraction.

Fig. 1. Baseline perfusion pressure (mmHg) of perfused rat tail artery segments (n = 6 per group) measured at 7-min intervals in the presence or absence of melatonin (Mel). PSS, physiological salt solution.

Fig. 2. A: Mel potentiation of nonepinephrine (NE, 10$^{-6}$ M)-induced vasoconstriction of the perfused rat tail artery segment presented as differences in the amplitudes between NE-induced vasoconstriction [% KCl (80 mM)] in the presence (n = 6) or absence (PSS + ethanol, n = 6) of Mel. *P < 0.05 vs. Mel (10$^{-14}$ M).

B: increase in intracellular Ca$^{2+}$ ([Ca$^{2+}$]$_i$) concentration sensitivity of NE (10$^{-6}$ M)-induced vasoconstriction produced by Mel. [Ca$^{2+}$]$_i$ sensitivity of NE-induced vasoconstriction is expressed as ΔP/Δ[Ca$^{2+}$]$_i$. The increase in [Ca$^{2+}$]$_i$ sensitivity of NE-induced vasoconstriction is expressed as % of response in absence of Mel, i.e., [ΔP/Δ[Ca$^{2+}$]$_i$ in presence of Mel]/(ΔP/Δ[Ca$^{2+}$]$_i$ in absence of Mel) × 100.

Fig. 3. Typical recordings of changes in perfusion pressure produced in perfused rat tail artery segments by KCl (20–120 mM) and NE (10$^{-7}$ to 3 × 10$^{-5}$ M) in physiological saline + ethanol (A) or Mel (10$^{-7}$ M) (B).
METHODS

The tail arteries of male Wistar rats (8–9 mo, 544 ± 17 g, Iffa Credo, L’Arbresle, France) were dissected between 9 and 10 AM [nadir of the Mel cycle (18)] and experiments performed between 10 AM and 2 PM. The technique for measurement of vasoconstriction and [Ca\(^{2+}\)]\(_i\), mobilization has been previously described (2, 3, 20, 27). Cannulated segments of the tail artery were perfused with a physiological salt solution (PSS) (mM: 140 NaCl, 5 KCl, 1.5 CaCl\(_2\), 1 MgCl\(_2\), 6 glucose, and 10 HEPES; in 100% O\(_2\); 7.40 ± 0.01 pH; 37°C; at a rate of 1.5 ml/min) and placed in a spectrophotometer cuvette (Fluorolog F1 T11, SPEX, Edison, NJ). The endothelium was removed by brief coperfusion of air (27).

Baseline perfusion pressure (mmHg) and tissue autofluorescence (AF) (excitation 360 nm, emission 510 nm) were measured, and the arterial segment was perfused with a high K\(^+\) solution (80 mM KCl, 2 min, 3×) followed by NE (10\(^{-6}\) M, 2 min). Segments whose vasoconstrictor responses to KCl or NE were <30 or >50 mmHg were discarded (6/48 = 10%). This initial vasoconstrictor response to KCl (80 mM) was used to normalize subsequent vasoconstrictor responses. Fura 2 was loaded by perfusing PSS containing fura 2-acetoxymethyl (AM) ester (5 × 10\(^{-6}\) M) for 90 min, followed by washout with PSS (20 min); isomissive fluorescence was measured before and after fura 2 loading to calculate loading efficiency.

Excitation (300 to 400 nm, emission 510 nm) and emission fluorescence spectra (480 to 530 nm, excitation 340 or 380 nm) and absorption (340, 380, and 510 nm) of Mel (10\(^{-9}\) M) in PSS in the absence of proteins (11). R\(_{340/380}\) was determined. Mel fluorescence was undetectable at concentrations <10\(^{-4}\) M. At 10\(^{-4}\) M fluorescence was 10 times less than that of the fura 2-loaded segment. Absorption was insignificantly low (<0.02).

Segments were illuminated alternatively (1 s) at excitation wavelengths of 340 and 380 nm (emission 510 nm). Background-corrected (see below) changes in fluorescence at 340 nm were divided by those at 380 nm (R\(_{340/380}\)). The formula of Grynkiewicz et al. (11) as modified by Scanlon et al. (24), is often used to calculate [Ca\(^{2+}\)]\(_i\) (in nM)

\[
[\text{Ca}^{2+}] = K_a \times \frac{[R'_{340/380} - R'_{\text{min}}]/(R'_{\text{max}} - R'_{340/380})]}{\times \beta'}
\]

where \(K_a\) represents the dissociation constant, which is 224 nM in PSS in the absence of proteins (11). R\(_{\text{max}}\) is the fluorescence ratio at saturating calcium (4 mM CaCl\(_2\)) plus the calcium ionophore, ionomycin (10\(^{-5}\) M, 5 min); R\(_{\text{min}}\) is the fluorescence ratio in calcium-free PSS containing EGTA (10\(^{-2}\) M) and ionomycin (10\(^{-5}\) M, 7 min); \(\beta'\) was the ratio of R\(_{340/380}\) at 0 and saturating calcium concentration. The ratio of R\(_{\text{max}}\) over R\(_{\text{min}}\) was used as an indication of the extent of hydrolysis of the fura 2-AM ester in each group. Background fluorescence was measured following perfusion with MnCl\(_2\) (10\(^{-3}\) M) and ionomycin (10\(^{-5}\) M) for 3 min.

The \(K_a\) for fura 2 may be higher than 224 nM in smooth muscle cytoplasm (13). For instance, \(K_a\) can be increased by as much as 300% by adding proteins to the calibration solutions (28). Therefore, we used the simpler formula to estimate [Ca\(^{2+}\)]\(_i\) (in arbitrary units (a.u.))

\[
[\text{Ca}^{2+}] = \frac{[R'_{340/380} - R'_{\text{min}}]/(R'_{\text{max}} - R'_{340/380})]}{\times \beta'}
\]

Signals for perfusion pressure and fura 2 fluorescence were converted into a digital form at a sampling rate of 1 Hz.

In a first experiment, arteries were stimulated six times with NE (10\(^{-6}\) M, 2 min, 5 min washout between each stimulation) in the presence or absence of increasing concentra-
Mel was dissolved in ethanol (100%) then diluted with PSS (final ethanol concentration, 1% vol/vol). Results are expressed as means ± SE. Vasoconstrictor (ΔP) and change in \([Ca^{2+}]_i\) (Δ[Ca\(^{2+}\)]\(_i\)) responses were presented as differences between baseline pressure and \([Ca^{2+}]_i\). Differences between means were determined by ANOVA followed by the Bonferroni test. The null hypothesis was rejected at \(P < 0.05\).

**RESULTS**

A baseline perfusion pressure of 18 ± 1 mmHg was stable throughout the experiment. Neither ethanol nor Mel had any effect on baseline perfusion pressure (\(P > 0.05\) for both, Fig. 1). Mel potentiated NE-induced (10\(^{-6}\) M) contraction in a bell-shaped fashion (Fig. 2A). Mel had no effect on NE-evoked \([Ca^{2+}]_i\) mobilization (for example, Δ[Ca\(^{2+}\)]\(_i\) were +0.25 ± 0.02 and +0.26 ± 0.04 a.u. in the absence and presence of 10\(^{-6}\) M Mel, \(P = 0.6427\)). Mel also increased the \([Ca^{2+}]_i\) sensitivity of vasoconstriction in a bell-shaped fashion (Fig. 2B).

NE induced a monophasic increase in perfusion pressure and a biphasic increase in \([Ca^{2+}]_i\), whereas KCl induced a monophasic increase in \([Ca^{2+}]_i\) (Figs. 3 and 4). Initial vasoconstrictor responses to KCl (ΔP, 80 mM) were +67 ± 9 and +61 ± 4 mmHg in the presence or absence of Mel, respectively. Mel (10\(^{-7}\) M) had no significant effect on KCl-induced vasoconstriction (Figs. 3 and 5). Taking, for example, an increase in perfusion pressure of +80 mmHg (produced by 100 mM KCl or 3 × 10\(^{-5}\) M NE, see Fig. 5) Mel had a slight, nonsignificant effect in the presence of KCl but increased the response to NE by 76%. Mel induced a similar increase in the vasoconstrictor responses to all concentrations of NE ≥10\(^{-6}\) M. Mel had no effect on the vasoconstrictor response to NE at 3 × 10\(^{-7}\) M [27 ± 7 to 32 ± 7% KCl (80 mM)] but potentiated (a 110% increase) vasoconstrictor response to 10\(^{-6}\) M NE [51 ± 10 to 106 ± 19% KCl (80 mM)]. Mel had no effect on NE-induced \([Ca^{2+}]_i\) mobilization (Figs. 3–5). Thus Mel increased \([Ca^{2+}]_i\) sensitivity of NE-induced vasoconstriction. Taking, for example, a value of Δ[Ca\(^{2+}\)]\(_i\) (a.u.) of +0.4, using the data presented in Fig. 5, calculated increases in perfusion pressure (ΔP in mmHg) were 49 ± 15 and 23 ± 13 mmHg (\(P > 0.05\)) for KCl, and 134 ± 20 and 71 ± 13 mmHg (\(P < 0.05\)) for NE, in the presence and absence of Mel (10\(^{-7}\) M). Maximal fluorescence in the presence of KCl (R\(_{340/380}\) = 2.40 ± 0.13) or NE (R\(_{340/380}\) = 1.87 ± 0.15) was far less than that produced by ionomycin (R\(_{max}\) = 6.21 ± 0.37).

**DISCUSSION**

Mel has no effect on baseline or KCl-induced increases in perfusion pressure of the rat tail artery but potentiates vasoconstriction induced by NE. The lack of effect of Mel on baseline perfusion pressure, in our experiment, suggests there may be some threshold pressure below which Mel does not increase perfusion pressure, because Ting et al. (26) showed that Mel decreases lumen diameter in pressurized (60 mmHg) rat tail artery segments. Furthermore, in our experiment, Mel had no effect on the vasoconstrictor response...
to NE at $3 \times 10^{-7}$ M but potentiated that to $10^{-6}$ M NE.

Although, there is a consistent effect of Mel on KCl-induced vasoconstriction with a shift to the left in the concentration-response curve, this is not significant and is far less marked than the effect of Mel on NE-induced contraction. For a given $([\text{Ca}^{2+}])$ mobilization ($\Delta([\text{Ca}^{2+}])$ of 0.4 a.u. vasoconstriction produced by NE was three times greater than that induced by KCl. This suggests that the potentiating effect of Mel depends on the cellular mechanism of contraction involved.

An explanation is that a substantial component of the vasoconstrictor response to NE comes from intracellular amplifying mechanisms downstream or independent of $([\text{Ca}^{2+}])$, mobilization such as $([\text{Ca}^{2+}])$ -independent modulation of phosphorylation/dephosphorylation events via diacylglycerol-protein kinase C and other pathways (3, 12, 19, 20, 30). A minor component of the contraction induced by KCl involves NE release from nerve endings (9). It is possible that the slight potentiation of KCl-induced vasoconstriction by Mel involves KCl-induced NE release. A final argument is that at higher levels of $([\text{Ca}^{2+}])$, mobilization, vasoconstriction does not appear to be related to $([\text{Ca}^{2+}])$, mobilization, and at such levels, Mel potentiates NE-induced vasoconstriction but not KCl-induced vasoconstriction. This may be an artifact due to flattening off of the response of $([\text{Ca}^{2+}])$ -fura complex, although maximal fluorescence in the presence of KCl or NE was far less than that produced by ionomycin.

In conclusion, we suggest that Mel potentiates the vasoconstrictor response to NE at some event downstream or independent of $([\text{Ca}^{2+}])$, mobilization, increasing $([\text{Ca}^{2+}])$, sensitivity of constriction. This may be of physiological relevance because the effect occurs at nanomolar Mel concentrations which are attained at the peak of the melatonin circadian rhythm (16).

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REFERENCES


