Melatonin Reduces ^{3}H-Nitrendipine Binding in the Heart (43787)

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Abstract. **The effect of melatonin on cardiac and brain voltage-sensitive calcium channels, as measured by ^{3}H-nitrendipine binding, was examined in rats 3 hr after melatonin administration (either 0.5 or 1.0 mg/kg given subcutaneously). Scatchard analysis of the data on specific binding of ^{3}H-nitrendipine with crude cardiac membranes from melatonin treated rats revealed significant decreases ($P < 0.05$ and $P < 0.01$ for the 0.5 and 1.0 mg/kg melatonin doses, respectively) in the density of Ca^{2+} channels without a change in their affinity for the ligand. At doses of 0.5 and 1.0 mg/kg of melatonin, B_{max} values were 157 and 142 fmoles/mg protein, respectively, compared with a control value of 199 fmoles/mg protein in crude cardiac membranes. In brain, melatonin treatment did not statistically significantly influence either ^{3}H-nitrendipine binding or its affinity when compared with control animals. These results suggest that melatonin modulates the functional status of voltage sensitive calcium channels in the heart, changes that may have implications for normal cardiac physiology and for the pharmacological manipulation of the heart.** [P.S.E.B.M. 1994, Vol 207]

Both voltage and receptor-operated calcium channels located in the myocardial cell membrane are involved in permitting Ca^{2+} entry into the cells (1, 2). The functional status of the Ca^{2+} channels can be monitored by determining the specific binding of Ca^{2+} antagonists (3, 4). Alternations in the density of calcium channels in cardiac membranes has been associated with cardiomyopathy (3, 5). Generally, the status of Ca^{2+} channel in the myocardium seems to depend on the disease type. Intracellular calcium overload and deficiency are postulated to contribute to cardiac contractile failure and cell death through a number of distinct mechanisms (2). There is some evidence suggesting that calcium antagonists, such as diltiazem, may reduce intracellular calcium overload in dystrophic hamsters (6), and may have therapeutic value in cardiomyopathy which is accompanied by intracellular calcium overload.

The hormone melatonin, 5-methoxy-N-acetyltryptamine, is synthesized by pineal gland (7). The production and secretion of melatonin by the pineal occurs primarily during the dark phase of the light: dark cycle (8). In the rat, pineal and blood melatonin levels begin to increase shortly after onset of darkness, reaching a peak near mid-dark; during the latter half of the night, melatonin levels fall to daytime values about the time of light onset (9). This neural hormone participates in many important physiological functions including the control of seasonal reproduction (10, 11) as well as influences on the immune system (12) and suppression of the DNA-adduct formation induced by the carcinogen safrole (13). Recently, we reported that melatonin is a potent antioxidant in terms of its ability to scavenge the highly toxic hydroxyl radical; indeed, melatonin was much better than other well known free radical scavengers including glutathione and mannitol in quenching the hydroxyl radical (14). This action of melatonin may account for some of its effects on the cardiovascular system. The cardiovascular effects of melatonin include an action on the electrical activity of the heart and on the calcium pump in cardiomyocytes

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Received December 17, 1993. [P.S.E.B.M. 1994, Vol 207]
Accepted April 14, 1994.

0037-9727/94/2071–0034$10.50/0

(15–18). Herein, we report that melatonin also influences voltage-sensitive calcium channels in the heart.

Material and Methods

^{3}H-nitrendipine (specific activity = 77 Ci/mmole) was obtained from New England Nuclear, Boston, MA. Unlabeled nitrendipine was a generous gift from Miles Pharmaceuticals. GF/B glass fiber filters were obtained from Fisher Scientific, Houston, TX. All other reagents and chemicals were obtained from Sigma Chemical Co., St. Louis, MO.

Male Sprague-Dawley rats weighing about 250 g were obtained and housed in a 14:10-hr light:dark cycle (lights on at 06:00 hr) with water and food provided *ad libitum* following the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Experimental rats were subcutaneously injected with one of two doses of melatonin (i.e., either 0.5 or 1.0 mg/kg). Control rats received a subcutaneous injection of alcoholic saline only. Melatonin was dissolved in ethanol and then diluted with saline (1:100). Injections were carried out at 10:00 hr. Animals were sacrificed by decapitation 3 hr later (at 01:00 hr). Hearts and brains were removed and rinsed with cooled saline. Blood was collected and serum was obtained by centrifugation; sera were stored at −80° C until assay.

The large vessels and connective tissue were carefully removed from the hearts and brains. Cardiac ventricles and cerebral cortices were processed for preparation of crude membranes as described by Wagner *et al.* (3). Cardiac tissue was homogenized in 50 m*M* Tris-HCl, pH 7.4 (15 ml/g tissue) with a Tekmar TR-10 homogenizer; brain tissue was homogenized with a Polytron PT-20 in the same buffer. The resulting homogenates were centrifuged at 1000*g* for 10 min and the pellet was discarded. The supernatant was centrifuged at 48,000*g* for 20 min. The resulting pellet was resuspended and centrifuged twice in same buffer at the same speed, again for 20 min. The final pellet was resuspended in 50 m*M* Tris-HCl, pH 7.4. Protein concentration was determined by the method of Bradford (19) using bovine serum albumin (BSA) as a standard.

^{3}H-nitrendipine binding to calcium channels was estimated using the method of Glossmann and Ferry (4). Crude cardiac and cortical membranes (100–150 μg) were incubated with 0.05–2.00 n*M* ^{3}H-nitrendipine in the absence or presence of 5 μ*M* cold nitrendipine for 1 hr at 22°–23°C. The assay was terminated by addition of 3.0 ml ice-cold 0.1 *M* Tris-HEPES buffer, pH 7.4 containing 20% (w/v) polyethlene glycol. Reaction media were passed through GF/B, glass fiber filters. Filters were washed three times with 2.5 ml ice-cold buffer (0.1 *M* Tris-HEPES, containing 8.5% polyethylene glycol). Following filtration, the filters were placed into vials and counted in the Beckman Model LS 5000 CE liquid scintillation counter. The specific activity of ^{3}H-nitrendipine was calculated by substracting nonspecific binding from total binding. Total and nonspecific ^{3}H-nitrendipine bindings were determined in the absence and presence of 5 μ*M* unlabeled nitrendipine. Melatonin in the serum was measured by direct radioimmunoassay (10).

Statistical significance between groups was determined using an analysis of variance (ANOVA) followed by the Student-Newman-Keuls test for multiple comparisons. P value <0.05 was taken to reflect a significant difference.

Results

Serum melatonin levels, after either saline or melatonin injection, are shown in Figure 1. Melatonin injections of either 0.5 or 1.0 mg/kg resulted in large increases in serum melatonin levels. Serum melatonin levels in control animals and in rats 3 hr after treatment with either 0.5 or 1.0 mg/kg melatonin were 189 ± 47, 804 ± 325, and 1392 ± 610 pg/ml, respectively.

Specific binding of ^{3}H-nitrendipine at different concentrations of radioligand was reduced in crude cardiac membranes after melatonin treatment when compared with that of saline injected animals (Fig. 2). Scatchard analysis of the data from the melatonin injected animals revealed a significant decrease in B_{max} without any change in the K_d value.

To determine whether melatonin also influenced ^{3}H-nitrendipine binding in the brain, ^{3}H-nitrendipine binding to cerebral cortex was determined. In crude

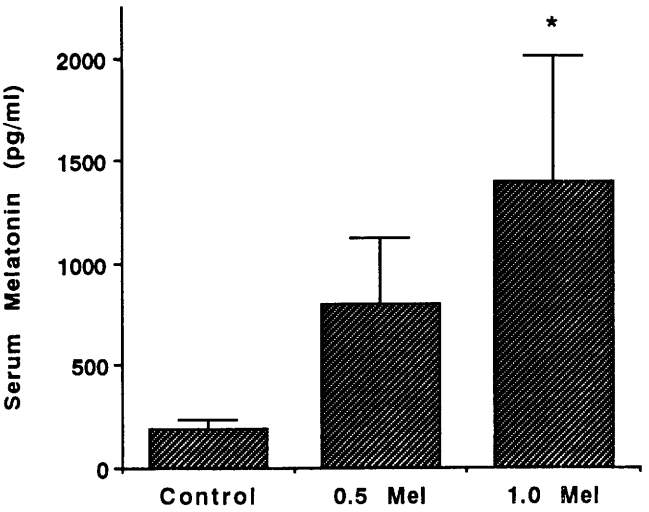

Figure 1. Concentration of melatonin in sera collected at the end of the study. Con, saline injected group; 0.5 Mel, animals injected with 0.5 mg/kg 3 hr before blood was collected; 1.0 Mel, rats injected with 1.0 mg/kg melatonin. Melatonin in the serum was measured by direct radioimmunoassay. The values are mean ± SEM of six to eight individual rats. *$P < 0.05$ compared with control group.

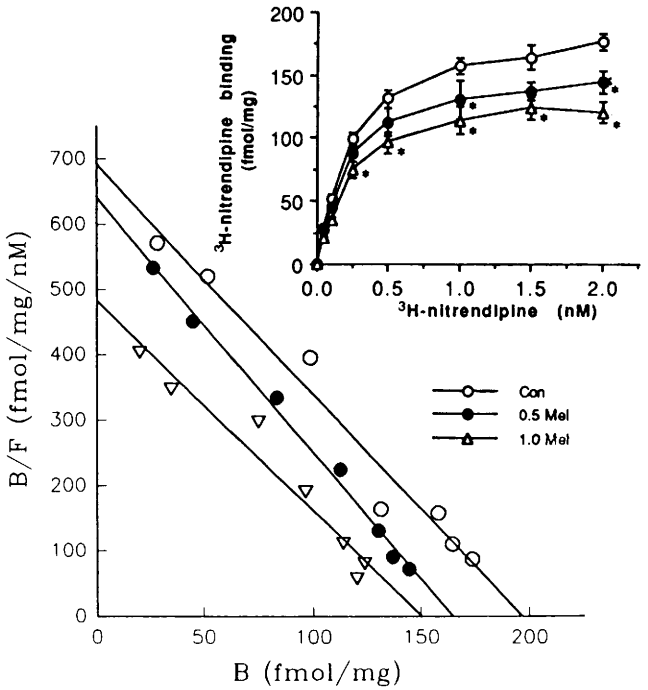

Figure 2. Scatchard analysis of data for specific ^{3}H-nitrendipine binding with crude cardiac membranes from control animals and from animals injected with one of two doses of melatonin. ^{3}H-nitrendipine binding was assayed with 100–150 μg protein per tube in presence of 5 μ*M* cold nitrendipine. The concentration of ^{3}H-nitrendipine varied between 0.05 and 2.0 n*M*. The assay was performed in duplicate. For each group (control and two melatonin treated groups), the experiments were performed either four, five or six times. $^*P < 0.05$ compared with control group.

brain membranes, the density of ^{3}H-nitrendipine receptor sites was not significantly different in terms of either B_{max} or K_d when compared with those in control animals, although there were tendencies for increased values in melatonin treated groups. The B_{max} and K_d values for cardiac and brain membranes in the three groups of rats are summarized in Table I.

Table I. Summary of Nitrendipine Binding Kinetic Constants in Crude Membranes from Heart and Brain

	K_d (n*M*)	B_{max} (fmoles/mg protein)
Heart		
Control (6)	0.27 ± 0.03	199.0 ± 8.1
0.5 mg/kg melatonin (4)	0.22 ± 0.02	156.7 ± 18.6*
1.0 mg/kg melatonin (5)	0.25 ± 0.01	142.2 ± 12.6**
Brain		
Control (4)	0.29 ± 0.03	88.0 ± 9.8
0.5 mg/kg melatonin (4)	0.31 ± 0.06	97.1 ± 14.3
1.0 mg/kg melatonin (4)	0.28 ± 0.06	111.6 ± 12.8

Note. Each value is a mean ± SEM of four to six experiments (number in parentheses). Values for both the K_d and the B_{max} were obtained by Scatchard analysis. ^{3}H-nitrendipine binding was assayed with 100–150 μg protein/tube of the crude membranes from heart and brain in the presence of 5 μ*M* cold nitrendipine. The concentration of ^{3}H-nitrendipine varied between 0.05 and 2.00 n*M*. The assay was carried in duplicate. $^*P < 0.05$, $^{**}P < 0.01$ versus control value.

Discussion

This study is the first to show an effect of melatonin on nitrendipine binding to cardiac membranes. The results indicate that melatonin can block voltage-sensitive calcium channels as measured by the ^{3}H-nitrendipine binding assay. The nonspecific binding in our experiment was less than 30% when cold nitrendipine concentrations were 5000× greater than that of ^{3}H-nitrendipine. This indicates the binding is highly specific. In this study, we choose to collect tissues 3 hr after melatonin treatment based on data from preliminary experimental results (data not shown). These studies showed that melatonin exerts its optimal effect at this time.

That melatonin may be functionally related to the calcium channel also comes from the observations of Kumar *et al.* (20). They reported that dihydropyridine binding in the Harderian gland of female hamsters was much lower than that of the male (20). It is known that the melatonin levels in female hamster Harderian gland are characteristically much higher than those of the male (21). These findings, coupled with those presented herein, suggest that melatonin may block calcium channels in the Harderian gland as well.

Presently, the mechanism by which melatonin reduces nitrendipine binding in heart is not known. The modulation of L-type Ca^{2+} channels may be mediated by cAMP-dependent kinase and probably involves the phosphorylation of the channel (22). Recently, three additional pathways have been postulated to modulate the 1,4-dihydropyridine sensitive Ca^{2+} channel. It is known that melatonin inhibits cAMP activity in the pituitary gland (23). Thus, it is possible that melatonin may directly bind to the subunit of calcium channel protein or that it may indirectly influence the channel thereby changing its function as reflected by a modulation of cAMP in cardiac myocytes.

We also investigated melatonin's effect on the calcium channel in the cerebral cortex. The results show that melatonin has no statistically significant effect on ^{3}H-nitrendipine binding in the brain cortex, although it revealed a tendency toward an increase in the nitrendipine binding density. It is possible that the brain may exhibit a different sensitivity to melatonin. According to reports by Lee *et al.* (24, 25), the ability of any factor to influence calcium channels will vary among organs. Thus, the differential responses of calcium channels to melatonin in heart and brain was not unexpected.

Some reports have shown that melatonin also lowers heart rate (15). Using interval microprocessor analysis of the electrocardiogram, Wynn and Arendt (18)

showed that melatonin had the effect of lengthening the RR interval and delaying the onset of the R wave. These changes may relate to melatonin's action on cardiac calcium channels. Calcium entry into myocytes occurs through voltage-sensitive calcium channels, which are blocked by calcium channel antagonist drugs such as nitrendipine (3). When melatonin decreases nitrendipine binding to cardiac membranes, it reduces the intracellular myocyte calcium levels and may slow heart rate. While the doses of melatonin used in this experiment produced nonphysiological levels in the blood, melatonin, because of its high lipophilicity and nontoxic nature (26), may have applicability in clinical medicine as a pharmacological agent.

The peak time for heart attacks is in the morning (27). At this time, melatonin levels have just dropped after their elevation at night (7, 8). The circadian rhythm in melatonin secretion may have a relationship with myocardial infarction because of its effect on both calcium channels and Ca^{2+}-pumping ATPase. Previous studies in our laboratory have shown that melatonin stimulates Ca^{2+}-pumping ATPase in cardiac sarcolemmal membrane (14). Because of this combination of effects at the level of the cardiomyocyte, this neural hormone may offer protection from heart attacks.

This research work was supported by NSF Grant IN 9121262 to R. J. R.

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