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Melatonin inhibits the development of tolerance to U-50,488H analgesia via benzodiazepine–$GABA_A$ergic mechanisms

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Abstract

Melatonin, a primary secretory product of pineal gland, is known to produce many of its pharmacological actions via benzodiazepine–γ-aminobutyric $acid_A$ ($GABA_A$)ergic mechanisms. Recently, we showed that benzodiazepine–$GABA_A$ergic mechanisms play an important role in U-50,488H (U50) analgesia and its tolerance. Hence, in the present study, the effect of melatonin on U50 analgesia and its tolerance was investigated. Furthermore, the possible role of benzodiazepine–$GABA_A$ergic mechanisms in the actions of melatonin on U50 analgesia was investigated. All experiments were performed using the radiant tail-flick test for mice. Melatonin [0.2, 1 and 5 mg/kg, intraperitoneal (i.p.)] neither produced analgesia nor affected the acute U50 (40 mg/kg, i.p.) analgesia. Tolerance to U50 analgesia was induced by administering U50 (40 mg/kg, i.p.) twice daily over 6 days. Treatment with melatonin (1 and 5 mg/kg, i.p) 15 min prior to each dose of U50 inhibited the development of tolerance, whereas a low dose of melatonin (0.2 mg/kg, i.p.) did not. The inhibition of U50 tolerance by melatonin was reversed by the chronic treatment with flumazenil (0.1 mg/kg), a benzodiazepine receptor antagonist and picrotoxin (1 mg/kg), a $GABA_A$-gated chloride channel blocker. Flumazenil and picrotoxin neither affected tail-flick latencies nor altered acute U50 analgesia and its tolerance. Interestingly, chronic 6-day melatonin treatment in a vehicle (U50-naive) group did not alter U50 analgesia measured on day 7. Together, these findings suggest that melatonin interferes with the neural mechanisms involved in the development of tolerance to U50 analgesia. The inhibition of U50 tolerance by melatonin was reversed by flumazenil and picrotoxin treatment, suggesting that benzodiazepine–$GABA_A$ergic mechanisms play an important role in the development of tolerance to U50 analgesia and that melatonin inhibits the development of U50 tolerance via benzodiazepine–$GABA_A$ergic mechanisms.

Keywords: Melatonin; U-50,488H; Analgesia; Tolerance; Benzodiazepine–$GABA_A$ergic mechanisms

1. Introduction

Opioid drugs are widely used for treating pain of moderate to severe intensity, such as the pain in carcinoma, in biliary or renal colic and after surgery. However, the clinical usefulness of opioid such as morphine is limited because of their tendency to produce tolerance, respiratory depression and constipation (Foley, 1991). Opioid drugs act on three major receptor types in the brain and spinal cord: delta, kappa and mu (Dhawan et al., 1996). It is thought that the analgesics acting on the kappa and delta receptors might be helpful in preventing the problems encountered with the use of mu receptors agonists. The development of alternative opioid analgesics has focused primarily on selective kappa receptor compounds such as U-50,488H (U50), particularly because these drugs show little or no respiratory depressant activity and produce little or no physical dependence or produce very mild degree of dependence (Castillo et al., 1986; Pazos et al., 1983; Szmuszkovicz, 1999). However, chronic administration of U50 also leads to the development of tolerance to its analgesic response.

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Hence, the search for agents which can inhibit tolerance to opioid analgesia and the mechanisms involved in the inhibition of tolerance by these agents has been an important area of research (Vanderah et al., 2001).

Melatonin (*N*-acetyl-methoxy tryptamine) is the primary secretory product of the pineal gland. Several biochemical and behavioural studies indicate that melatonin mimics some of the actions of diazepam, a benzodiazepine receptor agonist (Marangos et al., 1981; Coloma and Niles, 1988; Niles and Peace, 1990; Golombek et al., 1991a; Golombek et al., 1992; Pierrefiche et al., 1993). Diazepam was shown to attenuate morphine and U50 analgesia via benzodiazepine–γ-aminobutyric acid$_A$ ($GABA_A$)-ergic mechanisms (Huong et al., 1997). Furthermore, it has been suggested that diazepam inhibit tolerance to morphine possibly via benzodiazepine–$GABA_A$ergic mechanisms (Sheu et al., 1995). Recently, we showed that diazepam inhibit tolerance to U50 analgesia via benzodiazepine–$GABA_A$ergic mechanisms (Nemmani and Ramarao, 2000). Melatonin is also reported to attenuate morphine analgesia (Datta et al., 1982). Melatonin analgesia was blocked by flumazenil, indicating that melatonin actions involve benzodiazepine receptor mechanisms (Golombek et al., 1991b). Taking into consideration all these points, we hypothesised that melatonin may affect tolerance to kappa-opioid-induced analgesia via benzodiazepine–$GABA_A$ergic mechanisms. Hence, in the present study, the effect of melatonin on tolerance to U50 analgesia was investigated using the mice tail-flick test. Furthermore, the role of benzodiazepine–$GABA_A$ergic mechanisms in the effect of melatonin on tolerance to U50 analgesia was studied by using flumazenil, a benzodiazepine receptor antagonist and picrotoxin, a $GABA_A$gated chloride channel blocker.

2. Methods

2.1. Animals

The present studies were conducted on male Swiss albino mice weighing 20–25 g (Central Animal Facility, NIPER, India). The mice were housed six per cage at a controlled temperature (22±1 °C), humidity (50±10%) and light (0600–1800 h) in a room. Food and water were made available ad libitum. The analgesic responses to various treatments in the whole study were determined starting at 1000 h. The institutional animal ethics committee approved all the experimental protocols.

2.2. Drugs

U-50,488H (Pharmacia and Upjohn, USA) in saline, melatonin (Dabur Pharmaceuticals, India) in 0.5% ethanol–water, flumazenil (F.Hoffmann La Roche & Co., Switzerland) in 1% DMSO–water and picrotoxin (Sigma, USA) in 1% ethanol–water were used in the present study. All agents were administered by intraperitoneal (i.p.) route in a volume of 10 ml/kg of body weight. Our pilot studies showed that none of the vehicles (solvents) used in preparing the compounds affected tail-flick latencies significantly.

2.3. Measurement of analgesic response

Analgesic response was determined by tail-flick test (radiant heat) as described previously (Nemmani and Ramarao, 2000). The tail-flick latencies to thermal stimulation (radiant heat) were determined twice in a gap of about 30 s at 0, 30, 60, 90, 120 and 180 min. Mean basal latencies were found to be 4–6 s. A cut-off point was fixed at 20 s to prevent any injury to the tail of the animal. Percentage maximum possible effect (% MPE) was determined by using the formula

$$\%\ MPE = \{(Post\ latency\text{-}Basal\ latency)/ \times (Cutoff\ latency\text{-}Basal\ latency)\} \times 100.$$

A graph was plotted as % MPE vs. time. The area under curve ($AUC_{0-180\ min}$) was calculated from the % MPE vs. time graph, using trapezoidal method, and the data were subjected to statistical analysis.

2.4. Treatment schedule

To study the effect of acute melatonin (0.2, 1 and 5 mg/kg) on U50 (40 mg/kg) analgesia, mice were treated with melatonin 15 min prior to vehicle or U50 administration, and tail-flick latencies were determined as described above. To investigate the chronic effect of melatonin on acute U50 (40 mg/kg) analgesia, mice were treated with melatonin twice daily for 6 days in the morning and evening and on day 7 in the morning. Vehicle or U50 was administered 15 min after the last dose of melatonin. Mice were administered U50 (40 mg/kg) twice daily over 6 days to induce tolerance to U50 analgesia, as described previously (Nemmani and Ramarao, 2000). On day 7, the analgesic response was assessed in separate groups of mice administered with U50 (40 mg/kg). To determine the effect of melatonin on U50 tolerance, melatonin (0.2, 1 and 5 mg/kg) was administered 15 min prior to each dose of vehicle (U50-naive) or U50 (U50-tolerant) for 6 days. Possible involvement of benzodiazepine–$GABA_A$ergic mechanisms in the effect of melatonin (1 mg/kg) on tolerance to U50 analgesia was studied by administering flumazenil (0.1 mg/kg) and picrotoxin (1 mg/kg) 1 h prior to each dose of U50. The analgesic response to U50 (40 mg/kg) was determined on day 7.

2.5. Statistical analysis

The data were analyzed by one-way analysis of variance (one-way ANOVA) followed by a post hoc multiple

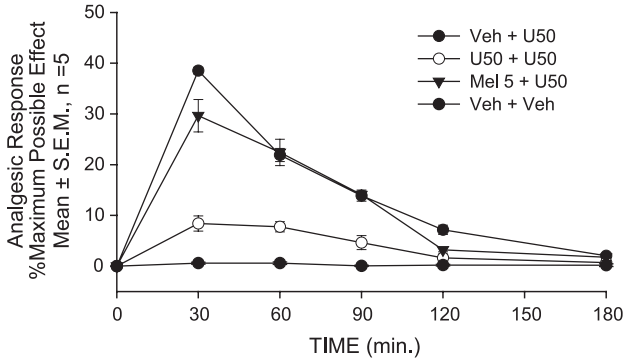

Fig. 1. The time course of % maximum possible effect of melatonin (5 mg/kg, i.p.) on tolerance to U50-induced analgesia. Mice were rendered tolerant to U50 by twice daily administration of U50 (40 mg/kg, i.p.) for 6 days. Melatonin (5 mg/kg, i.p.) was treated twice daily 15 min prior to U50 injection. The analgesic response to U50 (40 mg/kg, i.p.) was determined on day 7. All the values are expressed as mean±S.E.M.

comparison test. A value of $p<0.05$ was considered significant.

3. Results

3.1. Effect of melatonin on U50 analgesia

The time course of U50 analgesia is shown in Fig. 1. The peak analgesic effect of U50 was observed at 30 min, and the analgesic response lasted almost up to 120 min after its administration. Melatonin (0.2, 1 and 5 mg/kg) per se did not produce analgesia (Table 1). On the other hand, treatment with either acute or chronic melatonin did not modify the peak maximal effect or duration of U50 analgesic response (Table 2).

3.2. Effect of melatonin on tolerance to U50 analgesia

Twice daily administration of U50 for 6 days produced tolerance to U50, as shown by decrease in the analgesic response (expressed as $AUC_{0–180\ min}$) (Table 3). The analgesic response to U50 (40 mg/kg) was decreased to 25.8%±5.2 in U50-tolerant mice when compared to that of U50-naive mice (Fig. 1). The decrease in the analgesic response in U50-tolerant mice is mainly due to decrease in the peak analgesic response of U50. Melatonin (1 and 5 mg/kg) inhibited the development of tolerance to U50 analgesia.

Table 1
Effect of melatonin (0.2, 1 and 5 mg/kg i.p.) on tail-flick latencies in mice

Treatment group (mg/kg)	Analgesic response AUC (0–180 min), n=5–6
Vehicle	100.6±27.45
Melatonin (0.2)	61.72±5.57
Melatonin (1)	72.48±11.45
Melatonin (5)	61.67±8.51

Mice were given melatonin (0.2, 1 and 5 mg/kg, i.p.), and then tail-flick latencies were measured as described in the methodology.

Table 2
Effect of acute and chronic melatonin on acute U50 analgesia

Treatment (mg/kg)	Analgesic response	
	AUC (0–180 min), Mean±S.E.M., n=6	
	Acute	Chronic
Vehicle	51.95±2.5	107.8±25.64
U50 (40)	2434.0±77.10*	2385.97±81.85*
Melatonin (0.2)+U50 (40)	2271.7±149.9*	2325.14±106.5*
Melatonin (1)+U50 (40)	2331.2±67.2*	2261.65±148.47*
Melatonin (5)+U50 (40)	2365.1±154.2*	2364.5±107.96*

For acute study, mice were treated with melatonin (0.2, 1 and 5 mg/kg, i.p.) 15 min prior to U50 administration, and the analgesic response was determined. For chronic study, mice were treated with melatonin twice daily for 6 days and on day 7 in the morning. U50 was administered 15 min after last dose of melatonin, and then tail-flick latencies were measured at various time points.

* $p<0.05$ vs. vehicle group.

On the other hand, similar treatment with melatonin (0.2, 1 and 5 mg/kg) did not alter the U50 analgesia in U50-naive mice (Table 3). Melatonin (1 and 5 mg/kg) treatment restored analgesic response to U50 to 71.1%±2.2 and 83.5%±4.7 when compared to that of U50-naive mice, respectively. However, low dose of melatonin (0.2 mg/kg) failed to inhibit the development of tolerance to U50 analgesia (Table 3).

3.3. Effect of flumazenil and picrotoxin on melatonin-induced inhibition of tolerance to U50 analgesia

The analgesic response to U50 was decreased by about threefold in U50-tolerant mice compared to that of U50-naive mice. As shown in Fig. 2, melatonin treatment (1 mg/kg) inhibited the development of tolerance to U50 analgesia. Flumazenil (0.1 mg/kg, 1 h prior) and picrotoxin (1 mg/kg, 1 h prior) treatment did not alter the tolerance to U50

Table 3
Effect of melatonin on U50 analgesia in U50-naive and U50-tolerant mice

Treatment groups[a]	Analgesic response to U50 ($AUC_{0–180\ min}$)
U50-naïve	
Vehicle	2613.0±62.0
Melatonin (0.2)	2630.0±84.7
Melatonin (1)	2437.5±217.9
Melatonin (5)	2548.9±51.2
U50-tolerant	
Vehicle	675.8±135.3*
Melatonin (0.2)	775.6±102.5
Melatonin (1)	1857.2±59.5[#]
Melatonin (5)	2183.3±125.0[#]

[a] Mice were rendered tolerant to U50 by twice daily administration of U50 (40 mg/kg) for 6 days. Melatonin (0.2, 1 and 5 mg/kg) was treated 15 min prior to each dose of vehicle (U50-naïve) or U50 (U50-tolerant) for 6 days. The analgesic response to U50 (40 mg/kg) was determined on day 7. All values were expressed as mean±S.E.M., n=5.

* $p<0.05$ vs. vehicle group.

[#] $p<0.05$ vs. U50-tolerant group.

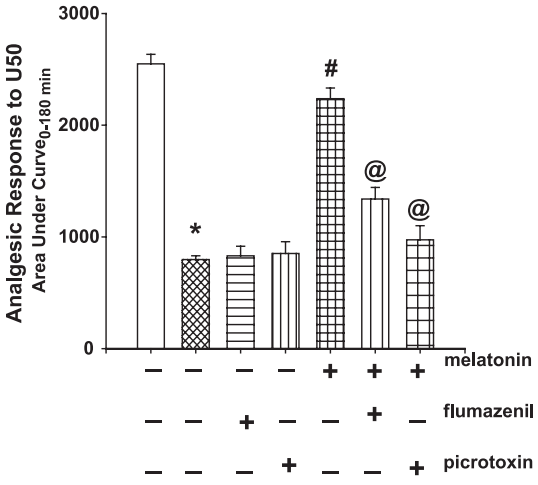

Fig. 2. Effect of flumazenil (0.1 mg/kg) and picrotoxin (1 mg/kg) on melatonin-induced inhibition of tolerance to U50 analgesia. Mice were rendered tolerant to U50 by twice daily administration of U50 (40 mg/kg) for 6 days. Melatonin (1 mg/kg) was treated 15 min prior to each dose of U50, whereas flumazenil and picrotoxin was treated 1 h prior to each dose of U50. The analgesic response to U50 (40 mg/kg) was determined on day 7. All values were expressed as mean±S.E.M., n=5. Open bar indicates control; hatched bar indicates U50-tolerant. + indicates that the respective drugs were treated, and − indicates no treatment, *p<0.05 vs. control; #p<0.05 vs. U50-tolerant+vehicle; @p<0.05 vs. U50-tolerant+melatonin.

analgesia but blocked the melatonin-induced inhibition of tolerance to U50 (Fig. 2).

4. Discussion

The isolation of the primary secretory product of the pineal gland melatonin by Lerner et al. (1958) marked the beginning of the modern era in pineal research. There is now evidence that melatonin may have a role in the biological regulation of circadian rhythm, sleep, mood, reproduction, tumor growth, aging and pain. In this study, for the first time, we provide evidence that melatonin treatment neither produced analgesia nor affected U50 analgesia. However, chronic melatonin inhibits the development of U50 tolerance. Earlier studies indicate that melatonin (20–40 mg/kg i.p.) produces analgesia (Golombek et al., 1991b) and attenuates opioid analgesia (Datta et al., 1982). In this present study, we found that melatonin (0.2–5 mg/kg) neither produced analgesia nor did it affect U50 analgesia (Tables 1 and 2). Furthermore, we found that chronic treatment of melatonin also did not produce analgesia or altered U50 analgesia (Table 2). However, recent findings show that melatonin produces analgesia by releasing endogenous opioids (Yu et al., 2000a,b). The authors used very high doses (30–120 mg/kg, i.p.) of melatonin to elicit analgesia. Thus, low doses (0.2–5 mg/kg) used in the study could explain the discrepancy in findings. In agreement with our study, Pang et al. (2001) reported that there was no significant difference in nociceptive response in formalin test when melatonin was injected intraperitoneally at doses of 0.1, 5 and 20 mg/kg body weight to the mice. We preferred low doses of melatonin (0.2–5 mg/kg) over high doses as high doses of melatonin cause motor impairment (Golombek et al., 1991a) and may interfere with the measurement of analgesia. In this study, we observed that chronic melatonin treatment inhibit the tolerance to U50 analgesia. On the other hand, chronic treatment of melatonin to U50-naive mice did not alter U50 analgesia. These findings suggest that melatonin interferes with the pharmacological phenomena involved in the development of tolerance to U50 analgesia. In our preliminary studies, it was observed that melatonin at doses used in the study did not alter motor activity in the rota-rod test. Thus, it can be deduced that the inhibition of U50 tolerance by melatonin does not involve its sedative effect. Furthermore, in our studies, we noted that chronic treatment of melatonin neither produced analgesia nor altered the acute U50 analgesia. Thus, these findings suggest that even chronic treatment of low doses of melatonin does not cause release of endogenous opioids, thereby ruling out the possibility that opioid mechanisms play a role in the inhibition of tolerance to U50 analgesia by melatonin.

The benzodiazepines and GABA are generally considered to produce inhibitory effects in the central nervous system. Opioid and $GABA_A$ receptors are coexpressed by neurons in rat brain. These findings provide anatomical evidence that GABAergic and opioidergic systems are closely linked, and activity of the same neuron may be regulated directly by both GABA and opioids (Kalyuzhny et al., 2000). In agreement with neuroanatomical evidence of possible interaction, pharmacological studies showed that diazepam attenuates kappa-opioid-induced analgesia, and the attenuation effect was blocked by flumazenil, a benzodiazepine receptor antagonist and $GABA_A$gated chloride channel blocker. This suggests the involvement of benzodiazepine–GABAergic mechanisms in attenuation of kappa-opioid-induced analgesia (Huong et al., 1997; Nemmani and Ramarao, 2002). Furthermore, several lines of studies indicate that GABAergic mechanisms play a role in the modulation of mu- and kappa-opioid-induced analgesia (Huong et al., 1997; Mantegazza et al., 1982; Palaoghu and Ayhan, 1986).

Melatonin like diazepam was shown to have affinity for benzodiazepine receptors labelled by [^{3}H]diazepam (Marangos et al., 1981) to enhance $GABA_A$ binding (Coloma and Niles, 1988) and to inhibit t-butyl bicyclophosphorothionate (TBPS) binding in the brain (Niles and Peace, 1990). Furthermore, it has been demonstrated that anticonvulsant, antianxiety and motor activity impairment activity by melatonin were inhibited by flumazenil, indicating the involvement of benzodiazepine receptors in pharmacological actions of melatonin (Golombek et al., 1991a; Golombek et al., 1992; Pierrefiche et al., 1993). It is well known that benzodiazepines such as diazepam act as allosteric modulators of $GABA_A$ergic activity by enhancing the binding of GABA to $GABA_A$ receptor complex, thereby increasing the frequency at which the associated chloride ion channel is opened (Haefely and Polc, 1986). This

produces antianxiety, anticonvulsant, motor activity impairment and myorelaxation (Rudolph et al., 1999). Consistent with the literature, we reported that diazepam inhibits tolerance to U50 analgesia, which was blocked by flumazenil, a benzodiazepine receptor antagonist and picrotoxin, a $GABA_A$gated chloride channel blocker, suggesting the involvement of benzodiazepine–$GABA_A$ergic mechanisms in the development of U50 tolerance (Nemmani and Ramarao, 2002). Therefore, it is likely that inhibition of tolerance to U50 analgesia by melatonin is mediated via benzodiazepine–$GABA_A$ergic mechanisms. The idea that benzodiazepine–$GABA_A$ergic mechanisms contribute to melatonin-induced inhibition of tolerance to U50 analgesia is supported by the finding that flumazenil and picrotoxin blocked the inhibition of tolerance to U50 analgesia by melatonin in mice.

5. Conclusion

At present, it is not clear how melatonin is different from diazepam in altering acute U50 analgesia. It remains to be seen whether high doses of melatonin also attenuate U50 analgesia similar to diazepam. Nevertheless, present findings clearly indicate that melatonin inhibits U50 tolerance without altering acute U50 analgesia. The inhibition of tolerance to U50 analgesia by melatonin involves benzodiazepine–$GABA_A$ergic mechanisms.

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