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Investigation into the contractile response of melatonin in the guinea-pig isolated proximal colon: the role of 5-HT_4 and melatonin receptors

[1]A. Lucchelli, M.G. Santagostino-Barbone & *M. Tonini

Institute of Pharmacology, School of Pharmacy, University of Pavia, Viale Taramelli 14, I-27100 Pavia and *Department of Internal Medicine and Therapeutics, Division of Pharmacology and Toxicology, University of Pavia, Italy

1 The interaction of melatonin (N-acetyl-5-methoxytryptamine) with 5-hydroxytryptamine$_4$ (5-HT_4) receptors and/or with melatonin receptors (ML_1, ML_2 sites) has been assessed in isolated strips of the guinea-pig proximal colon. In the same preparation, the pharmacological profile of a series of melatonin agonists (2-iodomelatonin, 6-chloromelatonin, N-acetyl-5-hydroxytryptamine (N-acetyl-5-HT), 5-methoxycarbonylamino-N-acetyltryptamine (5-MCA-NAT)) was investigated.

2 In the presence of 5-$HT_{1/2/3}$ receptor blockade with methysergide (1 μM) and ondansetron (10 μM), melatonin (0.1 nM – 10 μM), 5-HT (1 nM – 1 μM) and the 5-HT_4 receptor agonist, 5-methoxytryptamine (5-MeOT: 1 nM – 1 μM) caused concentration-dependent contractile responses. 5-HT and 5-MeOT acted as full agonists with a potency ($-\log EC_{50}$) of 7.8 and 8.0, respectively. The potency value for melatonin was 8.7, but its maximum effect was only 58% of that elicited by 5-HT.

3 Melatonin responses were resistant to atropine (0.1 μM), tetrodotoxin (0.3 μM), and to blockade of 5-HT_4 receptors by SDZ 205,557 (0.3 μM) and GR 125487 (3, 30 and 300 nM). The latter antagonist (3 nM) inhibited 5-HT-induced contractions with an apparent pA_2 value of 9.6. GR 125487 antagonism was associated with 30% reduction of the 5-HT response maximum. Contractions elicited by 5-HT were not modified when melatonin (1 and 10 nM) was used as an antagonist.

4 Like melatonin, the four melatonin analogues concentration-dependently contracted colonic strips. The rank order of agonist potency was: 2-iodomelatonin (10.8) > 6-chloromelatonin (9.9) $\geqslant$ N-acetyl-5-HT (9.8) $\geqslant$ 5-MCA-NAT (9.6) > melatonin (8.7), an order typical for ML_2 sites. In comparison with the other agonists, 5-MCA-NAT had the highest intrinsic activity.

5 The melatonin ML_{1B} receptor antagonist luzindole (0.3, 1 and 3 μM) had no effect on the concentration-response curve to melatonin. Prazosin, an α-adrenoceptor antagonist possessing moderate/high affinity for melatonin ML_2 sites did not affect melatonin-induced contractions at 0.1 μM. Higher prazosin concentrations (0.3 and 1 μM) caused a non-concentration-dependent depression of the maximal response to melatonin without changing its potency. Prazosin (0.1 and 1 μM) showed a similar depressant behaviour towards the contractile responses to 5-MCA-NAT.

6 In the guinea-pig proximal colon, melatonin despite some structural similarity with the 5-HT_4 receptor agonist 5-MeOT, does not interact with 5-HT_4 receptors (or with 5-$HT_{1/2/3}$ receptors). As indicated by the rank order of agonist potencies and by the inefficacy of luzindole, the most likely sites of action of melatonin are postjunctional ML_2 receptors. However, this assumption could not be corroborated with the use of prazosin as this 'ML_2 receptor antagonist' showed only a non-concentration-dependent depression of the maximal contractile response to both melatonin and 5-MCA-NAT. Further investigation with the use of truly selective antagonists at melatonin ML_2 receptors is required to clarify this issue.

Keywords: Melatonin; melatonin receptor agonists; luzindole; prazosin; 5-HT_4 receptors; 5-HT_4 receptor agonists and antagonists; proximal colon

Introduction

The pineal hormone melatonin (N-acetyl-5-methoxytryptamine), the synthesis and secretion of which are circadian and influenced by photoperiod, has a regulatory role on a variety of physiological processes including biological rhythms and neuroendocrine functions. In fact, melatonin may be involved in the regulation of sleep-wake cycles (Wehr, 1991), puberty (Waldhauser & Steger, 1986), reproduction (Woordouw *et al*., 1992), metabolism and hair growth in photoperiodic species (Bartness & Goldmann, 1989), as well as in the circadian rhythmicity in reptiles, birds and mammals (Armstrong & Redman, 1993). Changes in melatonin rhythms or blood levels are also thought to occur in a number of conditions such as jet lag (Petrie *et al*., 1989) and seasonal affective disorders (SAD) (Wetterberg *et al*., 1990).

There is evidence that affinity sites for melatonin are not confined to the brain (Krause & Dubocovich, 1991), but they are also present in a number of peripheral organs and tissues, including the retina (Dubocovich, 1983), the spleen (Poon & Pang, 1992), blood vessels (Persengiev, 1992; Krause *et al*., 1995), the vas deferens (Carneiro *et al*., 1994), the kidney and testes (Molinari *et al*., 1994; 1996). The alimentary canal of mammals and other vertebrates represents another important extrapineal source of melatonin (Bubenik *et al*., 1977; Holloway *et al*., 1980; Huether, 1993), where it is probably synthesized in the enterochromaffin cells (Raikhlin *et al*., 1978), and where melatonin binding sites have been identified (Lee & Pang, 1992; Bubenik *et al*., 1993). This suggests that melatonin interacts, in both the brain and periphery, with specific receptors, which have been classified into ML_1 and ML_2 types (see Hagan & Oakley, 1995; Dubocovich, 1995 for reviews). However, since melatonin is similar in structure to 5-methoxytryptamine (5-MeOT), a 5-HT_4 receptor agonist (Eglen *et al*., 1990), a

[1] Author for correspondence.

possible interaction between melatonin and 5-HT_4 receptors cannot be ruled out.

The aim of the present study was to examine whether melatonin (and a series of melatonin receptor analogues: 2-iodomelatonin, 6-chloromelatonin, N-acetyl-5-hydroxytryptamine (N-acetyl-5-HT)), 5-methoxycarbonylamino-N-acetyltryptamine (5-MCA-NAT) affects the contractile properties of the guinea-pig isolated proximal colon by interacting with 5-HT_4 receptors and/or with specific melatonin sites. The proximal colon was selected since it represents a reliable model for investigating 5-HT_4 receptor-mediated neurogenic excitatory responses in intestinal preparations (Elswood *et al*., 1991). The nomenclature for melatonin receptors recently proposed by Watson & Girdlestone (1996) will be used throughout the text.

Methods

Male Dunkin-Hartley guinea-pigs (400–500 g) were killed by CO_2 asphyxiation. A 10 cm long segment of the most proximal portion of the colon was removed and flushed intraluminally with modified Krebs-Henseleit solution (pH 7.4, 32°C). The composition of the solution was (mM): NaCl 118, KCl 5.6, $CaCl_2.2H_2O$ 2.5, $MgSO_4.7H_2O$ 1.19, NaH_2PO_4 1.3, $NaHCO_3$ 25 and glucose 10. It was continuously gassed with a mixture of 95% O_2 and 5% CO_2.

The colon was divided into three 2–3 cm-long segments, which were cut longitudinally to remove the mucosa by careful excision. The muscle strips were mounted in 10 ml organ baths containing Krebs-Henseleit solution added with methysergide (1 μM) and ondansetron (10 μM) to block 5-HT_1/5-HT_2 and 5-HT_3 receptors, respectively (Elswood *et al*., 1991). Tissues were placed under an initial tension of 1 g and allowed to equilibrate for at least 30 min with frequent washings before the experiment was started. Contractions were recorded by Mangoni isometric transducers connected to LNI chart recorders.

Protocol

A 'priming' maximal concentration of 5-HT (0.3 μM) was added to the bath since it has previously been shown that the first 5-HT administration causes a lower maximum than the second and the third administration (Elswood *et al*., 1991). After removal of the 'priming' 5-HT concentration, non cumulative concentration-response curves for 5-HT, the 5-HT_4 receptor agonist 5-MeOT, melatonin or the melatonin agonists 2-iodomelatonin, 6-chloromelatonin, N-acetyl-5-HT and the ML_2 receptor-selective 5-MCA-NAT (Molinari *et al*., 1996) were constructed. Agonists were administered as a 10 min dose-cycle and their exposure was dependent on the time the response had reached its maximum (usually 1–3 min). The redetermination of agonist concentration-response curves was carried out at 30 min intervals. For the second curve, the same or a different agonist (for between agonist comparisons) were used. When melatonin was the agonist, the first curve served as control, while the second curve was carried out either in the absence (time controls) or in the presence of one of the following pharmacological agents: atropine, a non selective muscarinic receptor antagonist, tetrodotoxin (TTX), a neuronal Na^+-channel blocker, pargyline, an irreversible monoamine oxidase inhibitor (Fowler *et al*., 1981), SDZ 205,557 and GR 125487, antagonists at 5-HT_4 receptors (Buchheit *et al*., 1992; Gale *et al*., 1994a), luzindole, a melatonin ML_1 receptor blocker (Sudgen, 1992; Dubocovich, 1988; 1995) that antagonizes the ML_{1B} receptor subtype (Watson & Girdlestone, 1996), prazosin, an α-adrenoceptor antagonist that has moderate/high affinity for melatonin ML_2 receptors without interacting with ML_1 sites (Dubocovich, 1995). Prazosin was also tested against contractile responses induced by 5-MCA-NAT. Antagonists (or other agents) were added to the reservoir containing Krebs-Henseleit solution and were allowed to equilibrate with the preparation for 30 min before the subsequent agonist curve was constructed. When pargyline was used, tissues were exposed to the drug for 30 min and then washed several times before agonist retesting. In each tissue, a single antagonist concentration was used. In some experiments, melatonin was tested as an antagonist towards 5-HT-induced contractions. Measurements were made at the plateau phase of contraction and agonists were washed out as soon as the peak response was reached. For between agonist comparisons, responses were expressed as a percentage of the maximal response to 5-HT.

Data analysis

Agonist potency values are expressed as $-\log EC_{50}$ (negative logarithm of molar agonist concentration inducing 50% of the maximum effect), which were estimated by non-linear curve fitting. Apparent affinity estimates (pA_2) from single antagonist concentrations were calculated by the Gaddum (1957) equation. All data are expressed as means ± s.e.mean. Differences between means were analysed by analysis of variance with Bonferroni adjustment. Values of $P<0.05$ were taken as statistically significant.

Drugs

5-Hydroxytryptamine hydrochloride, melatonin, N-acetyl-5-hydroxytryptamine, tetrodotoxin, atropine sulphate, pargyline hydrochloride were obtained from Sigma Chemical Co.; luzindole, 2-iodomelatonin, 6-chloromelatonin, and 5-methoxycarbonylamino-N-acetyltryptamine (5-MCA-NAT/GR 135,531) from Tocris Cookson; 5-methoxytryptamine hydrochloride and prazosin hydrochloride from Research Biochemical Incorporated; methysergide hydrogen maleate and SDZ 205,557 (2-methoxy-4-amino-5-chlorobenzoic acid 2-(diethyl-amino)-ethyl ester hydrochloride) were a generous gift from Sandoz; ondansetron (1,2,3,9-tetrahydro-9-methyl-3-(2-methylimidazol-1)methyl carbazole-4-one hydrochloride hydrate and GR 125487 ([1-[2(methylsulphonylamino)ethyl]4-piperidinyl]methyl-5-fluoro-2-methoxy-1H-indole-3-carboxylate hydrochloride) were kindly donated by Glaxo. With the exception of luzindole and 2-iodomelatonin, all drugs were dissolved in distilled water and administered in volumes not exceeding 1% v/v of the bath volume. Stock solutions of luzindole were prepared in 1:10 v/v ethanol/water. 2-Iodomelatonin was dissolved (2 mg ml^{-1}) in dimethylsulphoxide (DMSO). Further dilutions were in water. The ethanol/water and DMSO/water solutions were without effect on the contractile activity of the proximal colon.

Results

In the guinea-pig proximal colon, 5-HT (1 nM–1 μM), 5-MeOT (1 nM–1 μM) and melatonin (0.1 nM–10 μM) produced concentration-dependent, monophasic contractile curves (Figures 1 and 2). Compared to 5-HT (or 5-MeOT), the response to melatonin was slow in onset; the peak response being reached within 3 min (Figure 1). Potency values for 5-HT and 5-MeOT were 7.8 ± 0.01 and 8.0 ± 0.04, respectively. Both compounds caused the same maximum response. The potency value for melatonin was 8.7 ± 0.03 and the maximum effect achieved was 58 ± 4% of that induced by 5-HT. 2-Iodomelatonin (0.001 nM–0.1 μM), 6-chloromelatonin (0.01 nM–1 μM), N-acetyl-5-HT (0.01 nM–1 μM) and 5-MCA-NAT (0.01 nM–0.1 μM) also concentration-dependently contracted the intestine. Their maximum effects were 61 ± 23%, 68 ± 9%, 65 ± 14% and 90 ± 24% of that induced by 5-HT, while their potency values were 10.8 ± 0.73, 9.9 ± 0.62, 9.8 ± 0.62 and 9.6 ± 0.11, respectively (Figure 3). In time control experiments, no evidence of desensitization was obtained for all the agonists provided there were frequent solution changes (every 5–10 min) and a 30 min recovery period between subsequent agonist concentration-response curves.

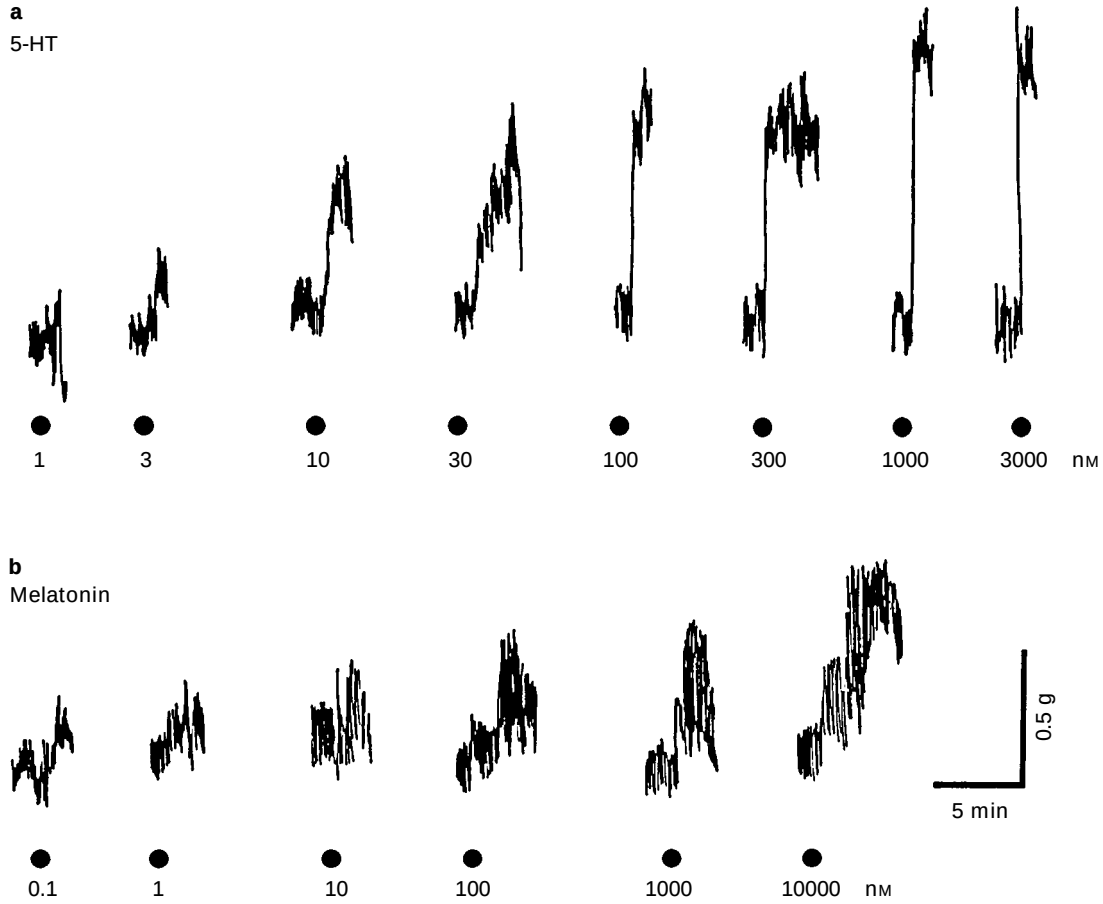

Figure 1 Tracing showing contractile responses caused by increasing concentrations of 5-hydroxytryptamine (5-HT) (a) and melatonin (b) in isolated strips of guinea-pig proximal colon.

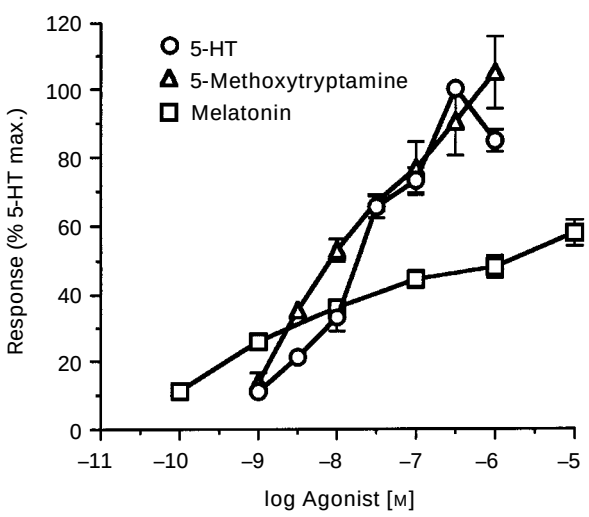

Figure 2 Concentration-response curves for the contractile response of 5-hydroxytryptamine (5-HT), 5-methoxytryptamine and melatonin in isolated strips of guinea-pig proximal colon. Results are mean and vertical lines show s.e.mean, $n=14$.

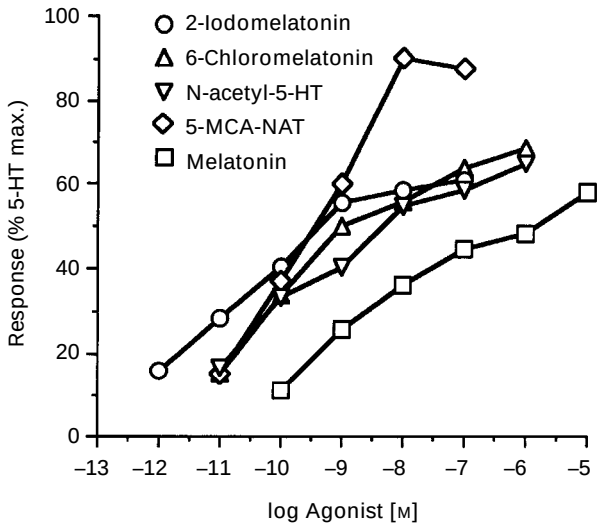

Figure 3 Concentration-response curves for the contractile response of the melatonin analogues 2-iodomelatonin, 6-chloromelatonin, N-acetyl-5-HT, 5-MCA-NAT (and melatonin) in isolated strips of guinea-pig proximal colon. Results are the mean of 7 determinations (for clarity the s.e.mean values have been omitted), with the exception of melatonin for which $n=14$.

The contractile response to melatonin was not affected by pargyline (100 μM), thus ruling out any involvement of the monoamine oxidase system in the degradation of the molecule, but was slightly, not significantly, reduced by both atropine (0.1 μM) and tetrodotoxin (TTX, 0.3 μM) (data not shown). SDZ 205,557 (0.3 μM) and GR 125487 (3–300 nM) did not affect the contractile response to melatonin (Figure 4). Conversely, GR 125487 (3 nM) antagonized, with an apparent pA_2 value of 9.6 ± 0.05, the contractile response to 5-HT and reduced its maximum effect to $70\pm7\%$ (Figure 5a). Melatonin (1, 10 nM) was ineffective as an antagonist of 5-HT-induced contractions (Figure 5b). After 30 min incubation with melatonin, the tone of the preparation was similar to that observed in the absence of the drug.

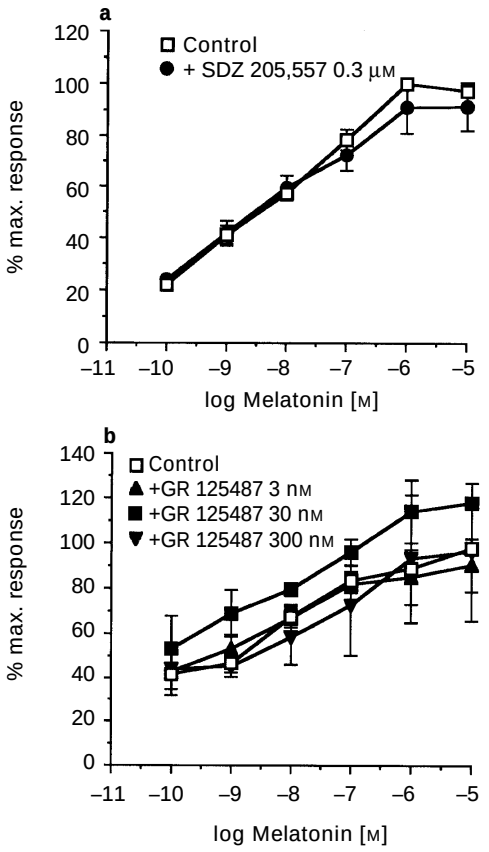

Figure 4 Effect of SDZ 205,557 (a), and GR 125487 (b) on the concentration-response curves to melatonin in isolated strips of guinea-pig proximal colon. Results are mean and vertical lines show s.e.mean, $n=4-12$.

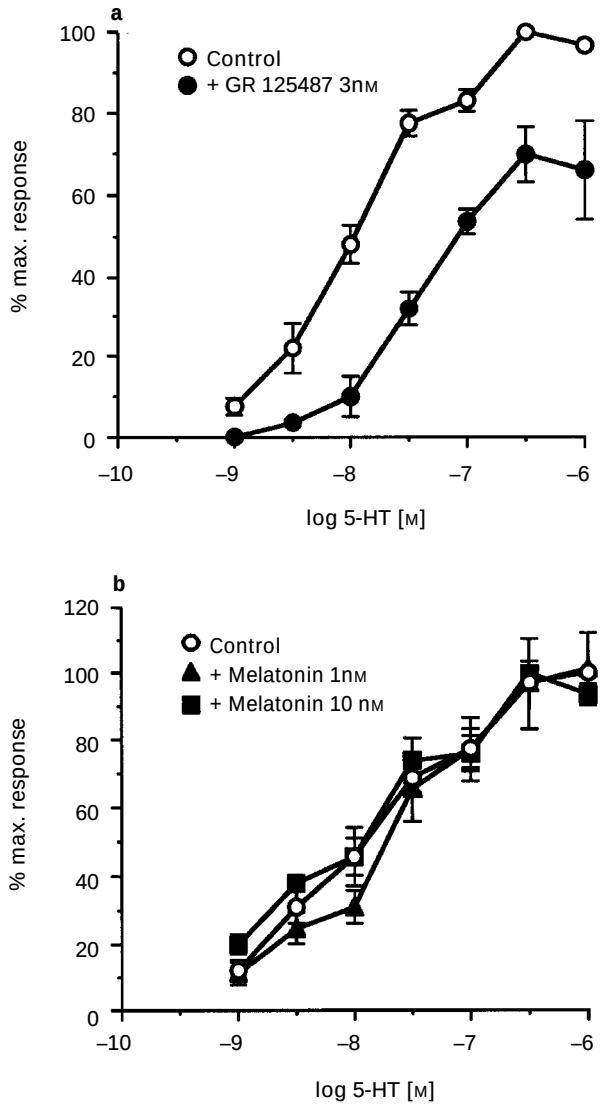

Figure 5 Effect of GR 125487 (a), and melatonin (b) on the concentration-response curves to 5-HT in isolated strips of guinea-pig proximal colon. Results are mean and vertical lines show s.e.mean, $n=4$.

Luzindole (0.3, 1 and 3 μM) had no effect on melatonin-induced contractions (Figure 6). Prazosin (0.1 μM) did not affect the concentration-response curve to melatonin. Higher prazosin concentrations (0.3 and 1 μM) caused a significant depression of the melatonin response in the concentration range of 0.1 to 10 μM (Figure 7a). However, the observed depression (approximately 30%) was not concentration-dependent and was not associated with significant changes of the melatonin potency values (8.8 ± 0.17 and 8.7 ± 0.17 in the presence of 0.3 and 1 μM prazosin, respectively). Prazosin (0.1 and 1 μM) significantly inhibited 5-MCA-NAT-induced contractile responses in the concentration range of 1 to 100 nM (Figure 7b). Similar to melatonin, prazosin-induced inhibition (approximately 30%) was neither concentration-dependent nor associated with changes of the agonist potency values (9.5 ± 0.15 and 9.4 ± 0.22 in the presence of 0.1 and 1 μM prazosin, respectively).

Discussion

In this study we found that melatonin (N-acetyl-5-methoxytryptamine), in spite of some structural resemblance with the 5-HT_4 receptor agonist 5-MeOT, induces contractile responses in the guinea-pig isolated proximal colon which are not mediated by activation of 5-HT_4 receptors.

In the presence of 5-$HT_{1/2/3}$ receptor blockade, 5-HT was found to contract the guinea-pig proximal colon with a potency value comparable to those obtained previously (Elswood *et al*., 1991; Gale *et al*., 1994b). Like 5-HT, the 5-HT_4 receptor agonist 5-MeOT behaved as a full agonist and was almost equipotent with 5-HT. This is at variance with the findings of Elswood *et al*. (1991) and Gale *et al*. (1994b), who found 5-MeOT behaved as a partial agonist and was 25 times less

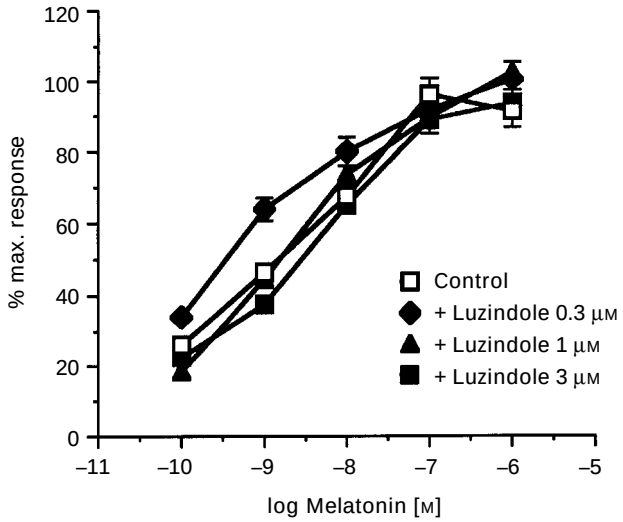

Figure 6 Effect of luzindole on the concentration-response curve to melatonin in isolated strips of guinea-pig proximal colon. Results are mean and vertical lines show s.e.mean, $n=4-12$.

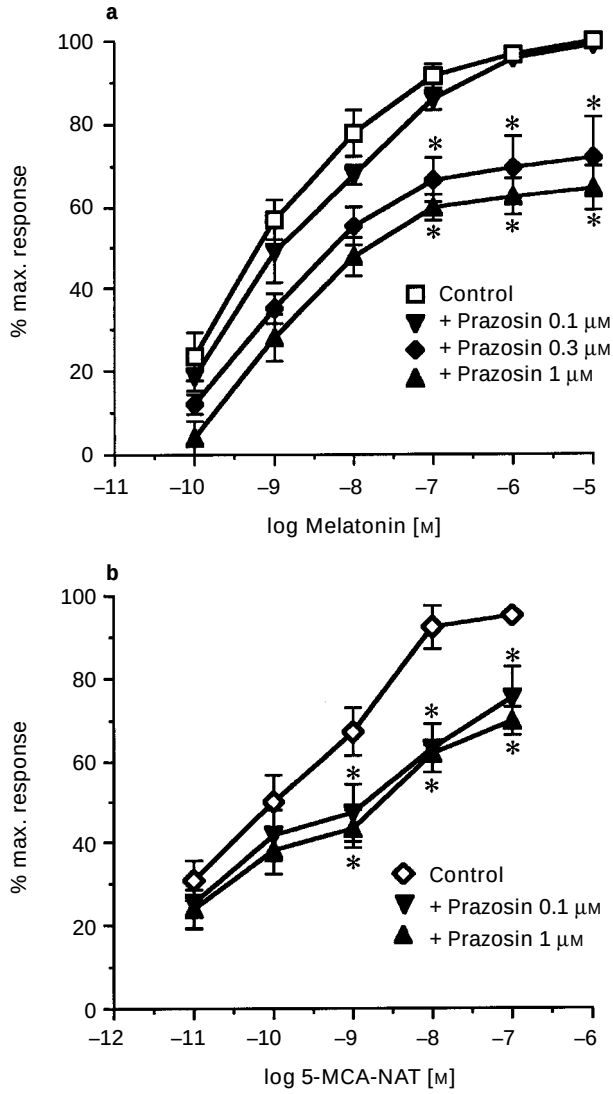

Figure 7 Effect of prazosin on the concentration-response curves to melatonin (a) and 5-MCA-NAT (b) in isolated strips of guinea-pig proximal colon. Results are mean and vertical lines show s.e.mean, $n=4-12$. $^*P<0.05$ compared to the corresponding value in the control curve.

potent than 5-HT. The discrepancy between our results and those mentioned above is difficult to reconcile, since our experimental protocol was apparently similar to that used by the above mentioned authors. However it should be noted that different potency values have been obtained with 5-MeOT at 5-HT_4 receptors in another preparation (i.e. the rat isolated oesophagus) by Reeves *et al.* (1991) and Baxter *et al.* (1991).

In the presence of 5-$HT_{1/2/3}$ receptor blockade, melatonin caused the guinea-pig proximal colon to contract. The degree of maximal contraction induced by melatonin was lower than that induced by 5-HT, but melatonin was almost 10 times more potent than 5-HT. The effect of melatonin was not mediated either by activation of muscarinic receptors, since it was unaffected by atropine, or by activation of nerve pathways, since it was resistant to TTX. Therefore, the most likely site of action of melatonin is at receptors located on the effector cells.

Since all experiments were carried out in the presence of methysergide and ondansetron, theoretically, only (excitatory) 5-HT_4 receptors could have been activated by melatonin, if the latter has affinity for 5-HT receptors. However, in the guinea-pig proximal colon 5-HT_4 receptors have a neural distribution and mediate 5-HT-induced contractions through stimulation of cholinergic nerves (Elswood *et al.*, 1991). Therefore, the evidence that the contractile effect of melatonin was resistant to atropine and TTX rules out any participation of 5-HT_4 receptors in this response. These findings were further substantiated by the findings that the 5-HT_4 receptor antagonists SDZ 205,557 (Buchheit *et al.*, 1992) and GR 125487 (Gale *et al.*, 1994a) failed to antagonize melatonin-induced contractions. The latter compound is an unsurmountable antagonist, which is 10,000 times more selective for the 5-HT_4 receptor over other types of 5-HT receptor. The unsurmountable nature of GR 125487 antagonism has been also confirmed in this study, where it was shown to inhibit 5-HT-induced contractions with an apparent affinity value of 9.6, a value similar to that (apparent pK_B: 10) obtained by Gale *et al.* (1994a) in the same preparation.

The rank order of potency of agonists has long been used as a means of characterizing receptors. In our hands, a series of melatonin agonists gave the following rank order of potency: 2-iodomelatonin > 6-chloromelatonin ⩾ N-acetyl-5-HT ⩾ 5-MCA-NAT > melatonin, an order similar to that obtained in binding studies for ML_2 sites in brain and peripheral tissues from the hamster (Molinari *et al.*, 1996). Two melatonin receptors have been characterized so far, namely ML_1 (subdivided into ML_{1A} and ML_{1B}, but see Reppert *et al.*, 1996, for ML_{1C} (Mel_{1c}) receptors) and ML_2 sites. 2-Iodomelatonin is the most potent agonist at both sites (see Watson & Girdlestone, 1996), while 5-MCA-NAT has micromolar affinity for ML_1 and nanomolar affinity for ML_2 sites. With regard to melatonin, it has picomolar and nanomolar affinity for ML_1 and ML_2 receptors, respectively (see Dubocovich, 1995; Hagan & Oakley, 1995 for reviews). Therefore, the potency value of 8.7 found for melatonin (and 9.6 for 5-MCA-NAT) in the guinea-pig proximal colon would also be consistent with activation of ML_2 receptors. Additionally, it is well known that these sites are functionally coupled to phosphoinositide hydrolysis (Eison & Mullins, 1993; Popova & Dubocovich, 1995), a mechanism which could account for the contractile effect of melatonin. In an attempt to strengthen the hypothesis of ML_2 receptor involvement, prazosin, a compound better known for its α-adrenoceptor blocking properties, was used as a pharmacological tool to block ML_2 receptors (Dubocovich, 1995; Watson & Girdlestone, 1996). This compound has previously been found to antagonize N-acetyl-5-HT- and 2-iodomelatonin-induced phosphoinositide hydrolysis in sirian hamster RPMI 1846 melanoma cells (Eison & Mullins, 1993), and to inhibit the binding of 2-[^{125}I]-iodomelatonin and 2-[^{125}I]-MCA-NAT at central and peripheral ML_2 sites (affinity range: 7.6–8.1) (Dubocovich, 1995; Molinari *et al.*, 1996). Under our experimental conditions, prazosin failed to cause a rightward shift of the melatonin and 5-MCA-NAT curves, but rather produced a non-concentration-dependent depression of the E_{max} to both agonists. This evidence casts some doubt on the presence of excitatory ML_2 receptors in the guinea-pig proximal colon.

On the other hand, the participation of ML_{1B} sites in the effect of melatonin was ruled out due to the lack of any effect of luzindole. This was a rather expected finding since ML_{1B} receptors (as well as ML_{1A} and ML_{1C} receptors) are linked to inhibition of adenylate cyclase via $G_{i/o}$ proteins (Watson & Girdlestone, 1996; Reppert *et al.*, 1996). Under basal conditions, stimulation of ML_1 receptors, as well as receptors coupled to the same transduction mechanism like M_2 muscarinic receptors, should not cause any contractile effect in the guinea-pig intestine unless adenosine 3′:5′-cyclic monophosphate (cyclic AMP) production is previously enhanced by appropriate pharmacological treatment (e.g. isoprenaline administration: Thomas *et al.*, 1993). Based on our evidence, the resistance of melatonin and 5-MCA-NAT responses to prazosin and the inefficacy of luzindole suggest that mechanisms unrelated to ML_1/ML_2 (as well as 5-$HT_{1/2/3/4}$ receptors) contribute predominantly to the whole contractile response of melatonin. This is particularly evident for the concentration-response curve of melatonin which is shallow and extends over more than 5 log concentration units, suggesting that more than one receptor/mechanism is at work simultaneously.

When used as an antagonist, melatonin did not modify 5-HT-induced contractions in the guinea-pig proximal colon. Therefore, this preparation behaves differently from the rat isolated duodenum, in which melatonin was found to antagonize the excitatory response to 5-HT (Quastel & Rahamimoff, 1965). It is worth remembering that in the duodenum, the stomach (Fioretti *et al*., 1972), the ileum (Bubenik, 1986) and the colon (Harlow & Weekley, 1986) of the rat, melatonin causes a reduction of the tone and of spontaneous phasic motility. Currently, the (local) functional role of melatonin in the intestine is still rather obscure, although in *in vivo* studies melatonin was found to be involved in cholecystokinin-induced changes of ileal motility in rats (Benouali-Pellisier, 1994), and to decrease food transit time in mice (Bubenik & Dhanvantari, 1989).

In conclusion, in the guinea-pig proximal colon melatonin causes the preparation to contract via mechanisms involving neither ML_1 nor 5-$HT_{1/2/3/4}$ receptors. While the rank order of agonist potencies would suggest the participation of ML_2 receptors, the ineffectiveness of prazosin on melatonin- and 5-MCA-NAT-induced contractions questions the involvement of these sites. To ascertain this, further experiments with the use of truly selective antagonists at the melatonin ML_2 receptor subtype are required.

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