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Melatonin release from rat pineals in vitro is stimulated by both the α_2-adrenoceptor agonist medetomidine and the antagonist atipamezole

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Abstract

This study was done to clarify the role of α_2-adrenoceptors in the regulation of pineal melatonin synthesis. Rat pineal glands were incubated in oxygenated Krebs–Ringer solution in perifusion chambers, and perifused for 30 min with α_2-adrenoceptor ligands. The melatonin concentrations were measured from the perifusate by radioimmunoassay. Both medetomidine and atipamezole ($\geq 10^{-5}$ M) increased melatonin release. Yohimbine blocked the increase caused by medetomidine but not by atipamezole. The effects of medetomidine and atipamezole were also additive: the maximum response to atipamezole could be significantly increased by medetomidine. These results suggest that the two drugs stimulate the melatonin synthesis through different mechanisms: medetomidine through α_2-adrenoceptors and atipamezole possibly through nonadrenergic mechanisms. The results differ from previous in vivo experiments suggesting that α_2-adrenoceptor ligands affect melatonin synthesis both centrally and locally in the pineal gland. The local effects are most likely masked under the central regulatory systems in vivo.

Keywords: α_2-Adrenoceptor; Pineal gland; Melatonin; Medetomidine; Atipamezole; Yohimbine

1. Introduction

Melatonin (5-methoxy-*N*-acetyltryptamine), an indolic pineal hormone derived from serotonin, is acetylated by the rate limiting enzyme *N*-acetyltransferase, and further methylated by hydroxyindole-*O*-methyltransferase. The activation of *N*-acetyltransferase depends on the activity of the sympathetic nerves originating from the superior cervical ganglia. The pineal gland is innervated not only by these peripheral sympathetic, but also by parasympathetic and central commissural nerve fibers, coming through the pineal stalk (Korf et al., 1990; Møller et al., 1990; Simonneaux et al., 1996), although so far their functional importance has not been determined.

In the rat pineal gland, the formation and activation of *N*-acetyltransferase are regulated by both α_1- and β_1-adrenoceptors, located on postsynaptic structures (Reiter, 1991). The activation of α_1-adrenoceptors, having no effect alone, potentiates β-adrenergic stimulation of *N*-acetyltransferase. α_2-Adrenoceptors exist in the mammalian pineal gland (Simonneaux et al., 1991; Schaad and Klein, 1992), but their functions are still unclear. The α_2-adrenoceptor density measured in the rat pineal gland is a third of that in the total brain (Schaad and Klein, 1992).

Several pharmacological actions of α_2-adrenoceptor ligands are mediated through the activation of not only α_2-adrenoceptors, but also of imidazoline receptors. There is a very high concentration of imidazoline binding sites in the rat pineal gland (King et al., 1995). The two receptor classes have several common features, suggesting a similar relative orientation of important binding elements between the receptors (Hieble and Ruffolo, 1995). Various compounds with imidazoline moiety elicit a number of pharmacological effects through interactions with α_2-adrenoceptors and ion transport systems (Parini et al., 1996). Imidazoline receptors represent a heterogeneous family of proteins, currently grouped as imidazoline I_1 and I_2 sites, differing in their ligand recognition properties, tissue distribution, and probably their location within the cell (Regunathan and Reis, 1996).

Although the sympathetic innervation of the pineal gland predominates in controlling melatonin synthesis

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(Moore, 1996), there are many other receptors which are abundant in the pinealocytes and have functional significance. In addition to adrenergic receptors, there are receptors for serotonin, acetylcholine (Finocchiaro et al., 1990), and γ-aminobutyric acid as well as several neuropeptides (Drijfhout et al., 1996; Rekasi et al., 1998). Adrenergic stimulation might be modulated by the second messenger responses to neuropeptides and other regulatory substances, sharing the same limiting second messengers.

In the present study, the influence of α_2-adrenoceptor drugs on the melatonin synthesis in rat pineals was examined in vitro. Medetomidine, an α_2-adrenoceptor agonist, and atipamezole, an α_2-adrenoceptor antagonist, both have very high α_2/α_1 selectivity ratios in receptor binding experiments. Both drugs are imidazole derivatives, and their effects may be partly conveyed through nonadrenergic imidazoline receptors (Halme et al., 1995; Savola and Savola, 1996). Yohimbine was included in the experiments as the classical antagonist, and it is not an imidazole derivative. Experiments were carried out in order to determine whether the α_2-adrenoceptor drug effects in vitro differ from those found in our earlier in vivo experiments on melatonin synthesis at night (Mustanoja et al., 1997).

2. Materials and methods

2.1. Animals

Adult male Wistar rats weighing about 300 g were used in all experiments. The rats were kept under controlled lighting conditions (12 h light/12 h dark) from birth, and they were synchronized with the lighting regime at least 2 weeks before each experiment. Illumination during the light period from 1500 to 0300 h was 100–150 lx at the level of the cages. During the dark period, there was dim red light of under 1 lx. Temperature and relative humidity were 23°C–26°C and 48%–60%, respectively. The rats were given food and water ad libitum. The animals were decapitated by guillotine in the middle of the dark period at 0900 h (illuminance <1 lx), and the pineal glands quickly removed and kept in oxygenated Krebs–Ringer solution in an iced waterbath until transferred into the perifusion chambers.

2.2. The perifusion system

The perifusion system consisted of a plastic chamber (Swinnex, Millipore, volume of 0.2 ml) connected to 3 mm silicone tubing, a thermostatic bath (Thermomix 1420, B.Braun, Espoo) that maintained the temperature at 37°C $\pm$ 0.5°C and a peristaltic pump (Desaga, Heidelberg), and a fraction collector (LKB 2211 SuperRac, Bromma). The perifusion chamber was filled with Krebs–Ringer solution (pH 7.4, gassed continuously with 100% O_2). The composition (mM) of the solution was as follows: NaCl, 140; KCl, 6; $MgCl_2$, 0.8; $CaCl_2$, 2; HEPES, 20 and glucose, 10.

In a preliminary experiment, the profile of the spontaneous melatonin secretion from perifused pineal glands was determined. Pineal glands were perifused with Krebs–Ringer solution in darkness at a constant flow rate of 0.2 ml/min (one gland/chamber). After discarding the first 10 min of effluent (equilibration period), the perifusate fractions were collected every 15 min in plastic tubes over a 13-h span (52 samples). Fig. 1 shows that after 30 min, the initial rate of melatonin release was very high ($\sim$ 300 pg/ml = 60 pg/min), and then decreased rapidly over the first 3 h remaining stable or decreasing slowly (around 30 pg/ml = 6 pg/min) for the remaining 10 h. The initial high rate of melatonin release from fresh perifused pineals is a constant finding (Simonneaux et al., 1989; Zhao and Touitou, 1993), although the origin of the phenomenon is not exactly known. It could, for instance, be related to transient noradrenaline release caused by cutting the central neural connections.

In the experiments, the glands were preincubated for 3 h in the perifusion chambers, then the baseline release was determined for every incubation as the average concentration of the six 15-min fractions. The pineal glands were then perifused with each drug for 30 min (starting at 4 h 30 min from the beginning of the incubation). When two drugs were used, the pineal glands were perifused in two consecutive 30-min periods. The effluent perifusate fractions were collected over an 8-h 45-min period (35 samples) and stored at -24°C until assayed for melatonin.

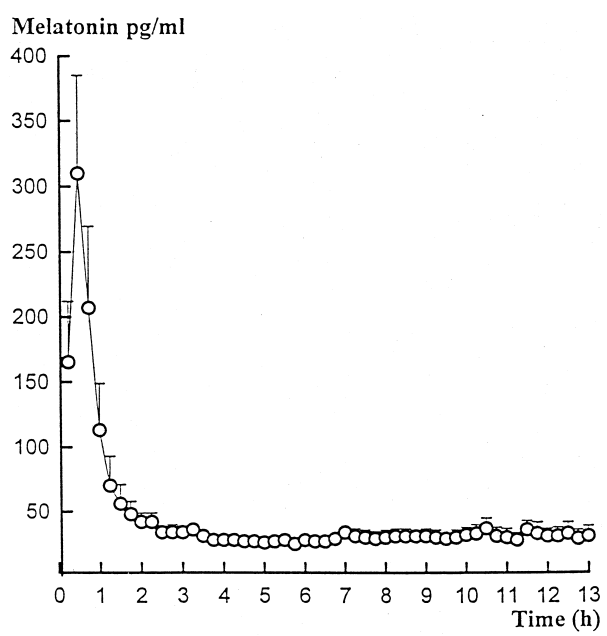


Fig. 1. Profile of melatonin release from perifused rat pineal glands in control conditions. The results are melatonin concentrations in 15-min fractions of collected incubation medium (flow 0.2 ml/min). The rats were decapitated in the middle of the dark period (12 h light/12 h dark) and the glands were incubated for 13 h. Means with S.E.M.s of eight glands (one gland/chamber) are shown.

2.3. Experiments

2.3.1. Experiment 1

The experiment was designed to determine whether the used α_2-adrenoceptor drugs have any effects on melatonin release from the pineal gland. Each incubation consisted of six chambers (one pineal gland/chamber) of which 1–2 were control chambers, where only Krebs–Ringer solution was used. Each perifusion was repeated 3–6 times for every drug and concentration. In the first series of incubations, there were seven different concentrations of medetomidine (10^{-9}, 10^{-7}, 10^{-6}, 10^{-5}, 3×10^{-5}, 10^{-4}, and 10^{-3} M), in the second series five concentrations of atipamezole (10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M), and in the third series two different concentrations of yohimbine (10^{-6} and 10^{-4} M).

2.3.2. Experiment 2

In this experiment, the combined effect of α_2-adrenoceptor antagonist atipamezole and α_2-adrenoceptor agonist medetomidine was studied. The drugs were added in two consecutive 30-min periods, first atipamezole (10^{-4} or 10^{-3} M) and then medetomidine (3×10^{-5} or 10^{-3} M). The concentrations had been tested in the preceding experiments.

2.3.3. Experiment 3

In this experiment, the ability of yohimbine to affect the increase of melatonin release, induced by the α_2-adrenoceptor agonist medetomidine and the α_2-adrenoceptor antagonist atipamezole, was studied. The pineals were first perifused with 10^{-4} M yohimbine for 30 min, and then either 3×10^{-5} M medetomidine or 10^{-4} M atipamezole for 30 min.

2.4. Measurement of melatonin

Melatonin was measured using a radioimmunological method described previously in detail (Vakkuri et al., 1984; Laakso et al., 1988). The unspecific binding in the assays was 5%–6%. The lower detection limit, defined as the apparent concentration at two standard deviations from the counts at maximum binding, was smaller than the lowest standard (19.5 pg/ml, extrapolated values in average 15 pg/ml, range 12–17 pg/ml). There was enough melatonin in the perifusate of a single gland for adequate radioimmunoassay sensitivity. Intra-assay variability was 4%–10%, and the interassay variability of 24 assays, including the assays of this study, during 24 months was 8%–10% depending on the concentration.

2.5. Drugs

Medetomidine and atipamezole hydrochloride were purchased from Farmos Group, Turku, Finland, and yohimbine hydrochloride from RBI, Natick, USA. The drugs were dissolved in Krebs–Ringer solution.

2.6. Calculations and statistics

Each time in the experiments, there was an individual control level (control chambers, where only Krebs–Ringer solution was used) and a basal level (the average value of the first six 15-min fractions after the stabilization of the incubation). The pineal glands varied in their capacity to secrete melatonin, and therefore the rate of melatonin secretion, during each 15-min period, was expressed as a percentage of the basal level. The mean percentages of control incubations were subtracted from corresponding percentages of the incubations with drugs. Then 100% was added to each value. The results are expressed as means and S.E.M.s of 3–6 similar incubations.

One- and two-way analyses of variance (ANOVA) and Tukey's multiple comparisons test were used in the statistical evaluations.

3. Results

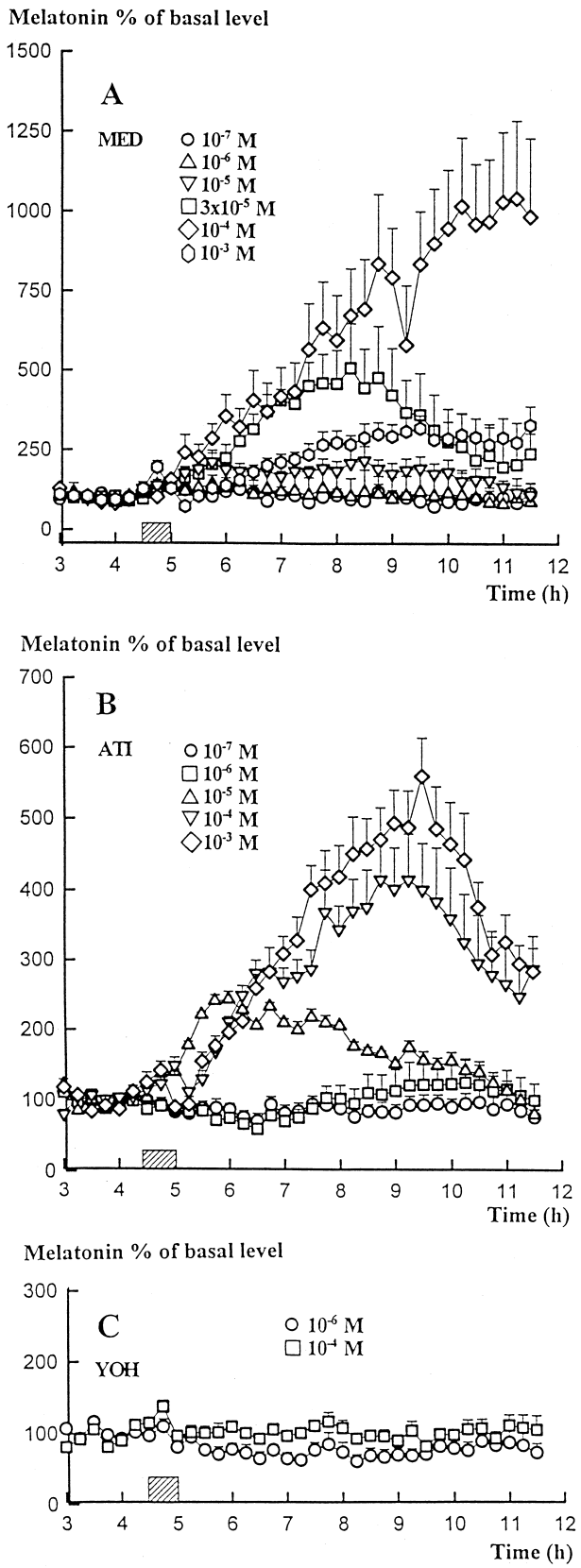
3.1. Experiment 1

Both medetomidine and atipamezole increased melatonin release in vitro from perifused rat pineal glands collected during the dark period. Medetomidine increased the melatonin level significantly with concentrations $10^{-5}\ldots10^{-3}$ M, while lower concentrations (10^{-6}, 10^{-7} and 10^{-9} M) had no effect (Fig. 2A). At concentrations from 10^{-5} to 10^{-4} M, the response increased concentration dependently up to 10-fold, while the highest concentration 10^{-3} M, caused only a 3-fold increase (two-way ANOVA suggested a significant difference between the effects of the concentrations 10^{-4} and 10^{-3} M: treatment $P<0.05$, time $P<0.001$, interaction $P<0.001$). The maximum melatonin release caused by the 10^{-4} M concentration remained throughout the experiment.

The highest concentration of atipamezole (10^{-3} M) increased the release of melatonin 6-fold in 4–5 h, and was 3-fold at the end of the experiment (Fig. 2B). The second highest concentration (10^{-4} M) was almost equally effective (two-way ANOVA suggested no significant difference between the treatments). The 10^{-5} M concentration caused still a 2.5-fold increase in 1 h, lasting for 3.5 h, before decreasing to the basal level. The smaller concentrations (10^{-6}, 10^{-7} M) had no significant effect on melatonin release.

The different concentration response relationships for medetomidine and atipamezole are shown in Fig. 3. The maximum melatonin release rate increased quite linearly with increasing the atipamezole concentration, while a peak response for medetomidine was reached at the

10^{-4} M concentration. The millimolar concentration of medetomidine was clearly less effective.



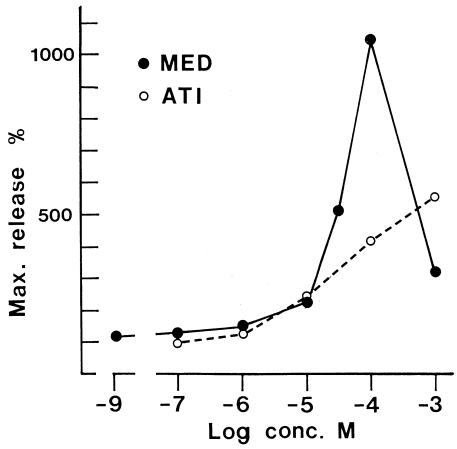


Fig. 3. The maximum release of melatonin from perifused rat pineal glands at various concentrations of medetomidine and atipamezole. The results are maximum responses in percentages taken from means of 3–6 chambers in Fig. 2A and B.

Yohimbine did not affect melatonin release in this system. The melatonin concentrations stayed close to the basal level for the following 7 h 30 min (Fig. 2C). Significant differences among the melatonin levels was suggested by one-way ANOVA when the lower concentration of yohimbine was applied. Tukey's test, however, did not disclose any differences between pretreatment level (fraction 6) and the post-treatment levels.

3.2. Experiment 2

The α_2-adrenoceptor antagonist atipamezole at the concentration 10^{-4} M did not counteract the effect of the α_2-adrenoceptor agonist medetomidine at the concentration 3×10^{-5} M (Fig. 4A). When applied consecutively, their effects appeared to be additive. The increase was approximately the same as the sum of the increases in melatonin profiles caused by atipamezole and medetomidine separately.

When 10^{-3} M concentrations of atipamezole and medetomidine were applied successively, a slight initial

Fig. 2. The effect of various concentrations of medetomidine (A), atipamezole (B), and yohimbine (C) on melatonin release from perifused rat pineal glands. The release is expressed in 15-min fractions as percentages of basal release (the average concentration of six fractions preceding the perifusion of drugs) and corrected according to concomitant control incubations without drugs (see Section 2). The results are means with S.E.M.s of 3–6 chambers. The rectangle indicates the interval of drug application; the concentrations are shown in the figure. For clarity, the results at 10^{-9} M concentration have been omitted in A. One-way ANOVAs: medetomidine (A) 10^{-5} M $P < 0.005$, higher concentrations $P < 0.001$; atipamezole (B) 10^{-5} M and higher concentrations $P < 0.001$. For two-way ANOVAs, see the text.

increase was followed by a slight decrease of melatonin release (Fig. 4B), before returning to the control level. The

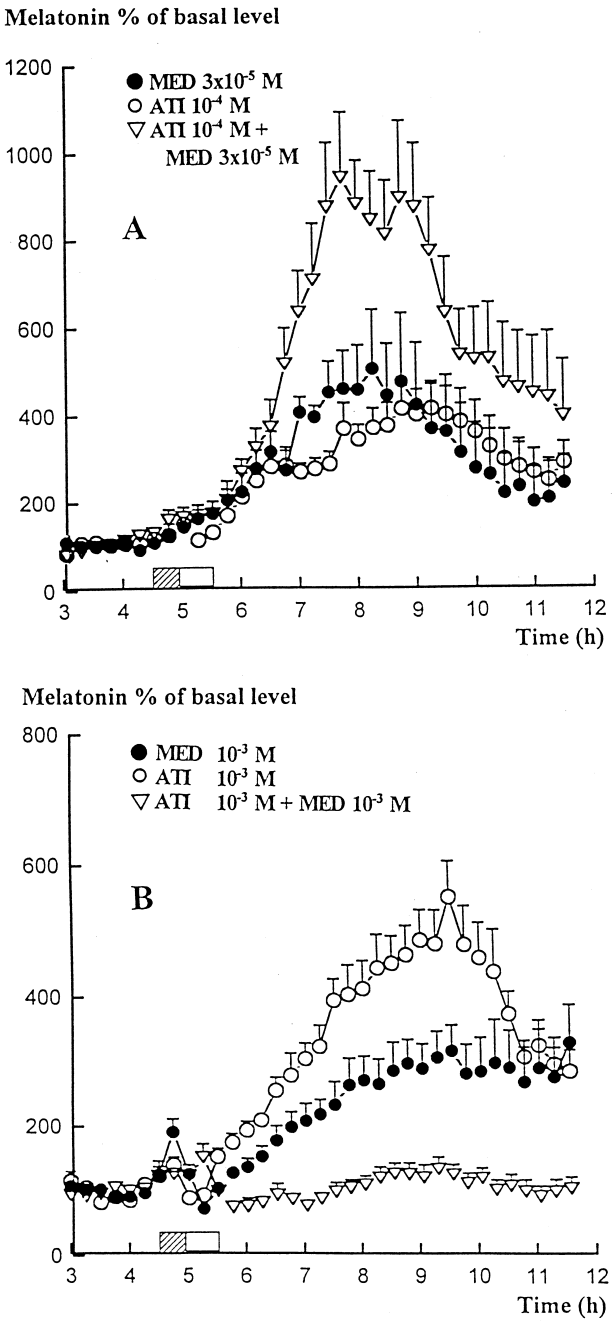


Fig. 4. The combined effect of atipamezole (ATI) and medetomidine (MED) on melatonin release from perifused rat pineal glands compared to the effects of MED and ATI alone. Two combinations of the concentrations were used: A and B. The release is expressed in 15-min fractions as percentages of basal release (details in Fig. 2). The results are means with S.E.M.s of 3–5 chambers. The rectangle indicates the interval of drug application; ATI was first perifused for 30 min, then MED for 30 min. The symbols are explained in the figure. One-way ANOVAs: ATI 10^{-3} + MED 10^{-3} $P < 0.002$, all other treatments $P < 0.001$. Two-way ANOVAs for A: ATI vs. ATI + MED, treatment NS, time $P < 0.001$, interaction $P < 0.001$; MED vs. ATI + MED, treatment NS, time $P < 0.001$, interaction $P < 0.001$. For B: ATI vs. ATI + MED, treatment $P < 0.001$, time $P < 0.001$, interaction $P < 0.001$; MED vs. ATI + MED, treatment $P < 0.05$, time $P < 0.001$, interaction $P < 0.001$.

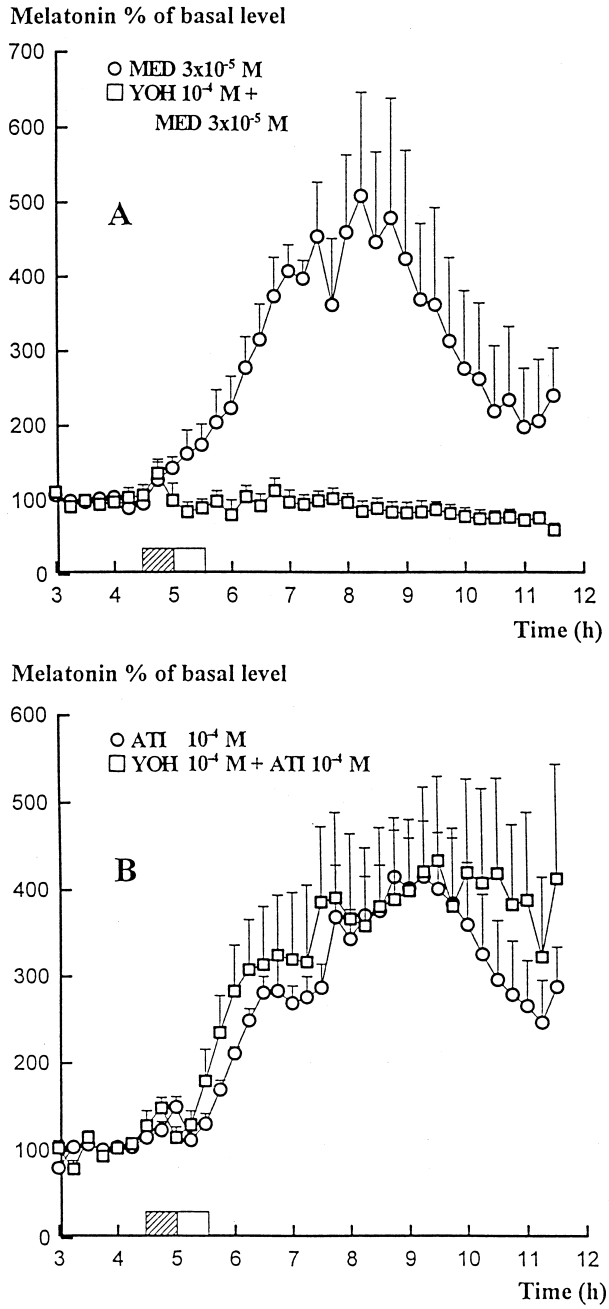


Fig. 5. The combined effect of yohimbine (YOH) and medetomidine (A) or atipamezole (B) on stimulated melatonin release from perifused rat pineal glands compared to the effects of medetomidine (MED) or atipamezole (ATI) alone. The release is expressed in 15-min fractions as percentages of basal release (details in Fig. 2). The results are means with S.E.M.s of 3–4 chambers. The rectangle indicates the interval of drug application; YOH was first perifused for 30 min, then MED or ATI for 30 min. The symbols are explained in the figure. One-way ANOVAs: MED $P < 0.001$, YOH + MED $P < 0.005$, ATI $P < 0.001$, YOH + ATI $P < 0.001$. Two-way ANOVA for A: MED vs. YOH + MED, treatment $P < 0.02$, time $P < 0.01$, interaction $P < 0.01$. For B: ATI vs. YOH + ATI, treatment NS, time $P < 0.001$, interaction NS.

combined effect of the drugs was not additive at very high concentrations, and it was lower than the separate effects of the drugs.

3.3. Experiment 3

Yohimbine blocked the increase of melatonin release caused by medetomidine, but not the increase caused by atipamezole. When yohimbine (10^{-4} M) and medetomidine (3×10^{-5} M) were applied consecutively, the melatonin level stayed at the control level (Fig. 5A). Atipamezole (10^{-4} M), on the contrary, increased the melatonin level to the same 4-fold level despite the earlier application of yohimbine at 10^{-4} M (Fig. 5B).

4. Discussion

Both medetomidine and atipamezole increased the melatonin level in perifused rat pineal glands collected during the dark period. Yohimbine, a non-imidazole derivative, blocked the increase of melatonin release caused by medetomidine, but not the increase caused by atipamezole. The effects of medetomidine and atipamezole were also additive: the response to 10^{-4} M atipamezole could be significantly increased by medetomidine, but not by increasing the concentration of atipamezole. The concentration response relationships of the two drugs were different. These results suggest that medetomidine and atipamezole must stimulate the melatonin synthesis through different mechanisms; medetomidine through α_2-adrenoceptors and atipamezole possibly through some nonadrenergic mechanisms.

Medetomidine has a very high α_2/α_1 selectivity ratio in receptor binding experiments, and has no subtype selectivity for α_2-adrenoceptors, or affinity for β-adrenoceptors, histamine, serotonin, muscarine, dopamine, tryptamine, γ-aminobutyric acid, opiate, or benzodiazepine receptors (Virtanen et al., 1988). It is an imidazole derivative having markedly less affinity for imidazoline I_1- or I_2-receptors than for the α_2-adrenoceptors (Wikberg et al., 1991; Ernsberger et al., 1997). In addition to this, dexmedetomidine has high affinity for nonadrenergic receptors, distinct from imidazoline I_1 and I_2 mivazerol binding sites in the human striatum (Flamez et al., 1997) and H_2-receptor antagonist cimetidine binding sites in the rat spinal cord (Savola and Savola, 1996). In the rat pineal glands, however, the complete inhibition of the response to medetomidine by yohimbine suggests that medetomidine acted through α_2-adrenoceptors.

The α_2-adrenoceptor ligands in the pineal gland have a single binding site belonging to the α_{2D}-adrenoceptor subtype according to saturation experiments (Simonneaux et al., 1991; Schaad and Klein, 1992). The presence of functional α_2-adrenoceptors has been shown at the pineal nerve endings, where by inhibiting the noradrenaline release (Pelayo et al., 1977; Drijfhout et al., 1996) they can decrease melatonin synthesis. On the other hand, postsynaptic α_2-adrenoceptors have been reported to stimulate melatonin synthesis in mammals potentiating cAMP induced *N*-acetyltransferase activity (Schaad and Klein, 1991). In another experiment, exposure of rat pineal glands to α_2-adrenoceptor agonist clonidine in vitro also directly increased *N*-acetyltransferase activity (Alphs et al., 1980). The pineal glands in our experiments are likely to contain both pre- and postsynaptic elements at 4 h 30 min when the drug stimulus is given, because nerve endings stay intact at least for 5 h (Parfitt and Klein, 1976) and are still quite well preserved at 48 h, although most of the vesicles have then lost their dense core (Karasek, 1973). Because medetomidine, however, caused a stimulation of melatonin release in our experiments, the effect is probably postsynaptic.

The best known α_2-adrenoceptor signaling results in the inhibition of the adenylate cyclase, although the following decrease in the formation of cAMP does not explain all physiological effects of α_2-adrenoceptor activation (Aantaa et al., 1995). In endogenous and recombinant systems, α_2-adrenoceptors activation decreases cellular cAMP levels at low agonist concentrations, and increases it at higher concentrations (Jasper et al., 1998). The biphasic cAMP concentration responses of α_2-adrenoceptors may be partially a result of coupling to multiple G-proteins. It has been suggested that adenylate cyclase activation by α_2-adrenoceptors could be mediated by the same mechanism that mediates β-adrenergic stimulation of cAMP formation (Eason et al., 1992). If medetomidine in our experiments had increased cAMP levels, it could have led to the activation of *N*-acetyltransferase.

Independently of cAMP, activation of α_2-adrenoceptors can lead to other types of intracellular signaling (Aantaa et al., 1995). For example, modulation of ion channel activity and increased availability of intracellular calcium ions can augment *N*-acetyltransferase activity similarly to α_1-adrenoceptor mediated augmentation (Reiter, 1991). Dexmedetomidine has been shown to increase cytosolic calcium in astrocytes (Zhao et al., 1992). In a recent study, α_2-adrenoceptor mediated increase of intracellular calcium was suggested to occur even in pineal cells (Venkataraman et al., 1998).

Atipamezole has been described as a potent, specific and selective α_2-adrenoceptor antagonist (Scheinin et al., 1988; Virtanen et al., 1989), with no subtype selectivity for α_2-adrenoceptors (Sjöholm et al., 1992; Halme et al., 1995). In binding studies and studies with isolated organs, atipamezole had no effect on β-adrenoceptors, serotonin, muscarine, dopamine, tryptamine, γ-aminobutyric acid, opiate and benzodiazepine receptors (Virtanen et al., 1989). It has high affinity to imidazoline receptors (Sjöholm and Scheinin, 1994; Sjöholm et al., 1992; Halme et al., 1995), which are densely distributed in the pineal gland (King et al., 1995). Furthermore, atipamezole has binding sites distinct from both classical α_2-adrenoceptors and imidazoline I_1 and I_2 receptors, at least in neonatal rat lung membranes and human striatum (Sjöholm et al., 1992; Flamez et al., 1997), having similarities to the cimetidine

binding sites mentioned above, found for dexmedetomidine in the rat spinal cord (Savola and Savola, 1996).

Because yohimbine did not inhibit the increase in the melatonin release caused by atipamezole, we assume its effect is through nonadrenergic receptors or by some non-receptor mechanism. The most probable receptor site is the one in which rilmenidine binds, because these are abundant in the pineal gland (King et al., 1995). A possible mechanism might be an atipamezole induced transient increase of noradrenaline release from degenerating presynaptic nerve endings (directly or indirectly). This could explain the transitory and smaller effect of atipamezole compared to the sustained and bigger effect caused by medetomidine, possibly through postsynaptic mechanisms.

The inhibition of monoamino-oxidase is a possible non-receptor mechanism by which atipamezole might increase melatonin synthesis in the pineal glands. Several imidazoline derivatives possess such properties (Raddatz et al., 1997), although this has not been studied with either atipamezole or medetomidine. If the elimination of the spontaneously released noradrenaline is inhibited, this can produce inreased melatonin synthesis. Further study is needed to show whether the effect of atipamezole is mediated through imidazoline receptors, other nonadrenergic receptors or through non-receptor mechanisms.

The combined effects of medetomidine and atipamezole were additive when lower concentrations were added, while at millimolar concentrations, the combined effect was lower than the seperate effects of the drugs. It cannot be concluded, however, that atipamezole counteracted the effect of medetomidine, because very high concentrations combined can have non-spesific influences on the glands, as suggested also by the decreased response to the highest concentration of medetomidine alone.

Earlier in our in vivo experiments on rats, medetomidine decreased the pineal melatonin level, and atipamezole, having no effect alone, counteracted this effect (Mustanoja et al., 1997). This finding was in agreement with other studies in vivo in which the α_2-adrenoceptor agonist clonidine decreased serum melatonin level in humans and pineal *N*-acetyltransferase activity in rats (Alphs et al., 1980; Lewy et al., 1986), and the α_2-adrenoceptor antagonist Org 3770 increased the human serum melatonin level (Palazidou et al., 1989). The stimulatory effects of α_2-adrenoceptor ligands in the pineal found in the present study are most likely masked by the central effects in intact animals. The inhibition of the melatonin synthesis in vivo was thought to be caused either by presynaptic mechanisms at the pineal level, or in the areas of the central nervous system containing pathways to the pineal gland, because medetomidine did not reduce isoprenaline-sitmulated melatonin synthesis (Mustanoja et al., 1997). The present results provide direct evidence that pineal postsynaptic α_2-adrenoceptors do not participate in the inhibition of the melatonin synthesis caused by medetomidine in rats.

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