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European Journal of Pharmacology 401 (2000) 59–62

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Short communication

Purinergic and noradrenergic cotransmission in the rat pineal gland

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Received 6 June 2000; accepted 9 June 2000

Abstract

ATP is coreleased with noradrenaline in several noradrenergic synapses, and P2-like receptors were shown to be present in rat pineal glands. A new method of functional investigation was developed to assess the importance of both transmitters (noradrenaline and ATP) in eliciting the synthesis of melatonin and its precursor *N′*-acetyl-5-hydroxytryptamine (*N′*-acetyl-5-HT) through transmural electrical field stimulation of cultured pineal glands. Incubation with the β-adrenoceptor antagonist propranolol ($>10^{-7}$ M) blocked almost completely the production of *N′*-acetyl-5-HT, whilst the P2 receptor antagonists pyridoxalphosphate-6 azophenyl-2′,4′-disulfonic acid (PPADS, $>3\times10^{-6}$ M) and suramin ($>10^{-6}$ M) blocked it partially. These findings indicate a physiologically relevant role for the purinergic cotransmission in this system. © 2000 Published by Elsevier Science B.V.

Keywords: Pineal gland; Melatonin; *N′*-acetyl-5-hydroxytryptamine (*N′*-acetyl-5-HT); Suramin; P2-receptor

1. Introduction

The pineal gland conveys information concerning light–dark cycles to the body's physiology for the organization of seasonal and circadian rhythm, through a rhythmic secretion of *N′*-acetyl-5-methoxytryptamine, known as melatonin, which is synthesized from 5-hydroxytryptamine (5-HT) via *N*-acetylation by arylalkylamine-*N*-acetyltransferase to *N′*-acetyl-5-hydroxytryptamine (*N′*-acetyl-5-HT) and *O*-methylation by hydroxyindole-*O*-methyltransferase to melatonin (Klein et al., 1983). Both melatonin and *N′*-acetyl-5-HT are released in the blood stream (Pang et al., 1980). The gland is innervated primarily by peripheral sympathetic tract (Kappers, 1960). Noradrenaline triggers the nocturnal peak of melatonin by inducing arylalkylamine-*N*-acetyltransferase synthesis and activation.

In the 1930s, due to the pioneering studies performed by Sir Henry Dale and his colleagues (Sneddon et al., 1996), sympathetic neurons were thought to release only noradrenaline as neurotransmitter. Forty years later, Burnstock (1976) proposed that some nerve cells release ATP as a noradrenaline cotransmitter. Now cotransmission is considered the rule rather than the exception in the autonomic nervous system.

There are two main families of nucleotide receptors: adenosine or P1 receptors coupled to G-protein, and P2 receptors primarily subdivided into two main subtypes: ligand-gated ion channels (P2X receptors) and G-protein coupled receptors (P2Y receptors) (Fredholm et al., 1994). Both P1 and P2 receptors are present in the pineal gland. Stimulation of P1 receptors elevates adenosine 3′,5′-cyclic monophosphate (cyclicAMP) (Sarda et al., 1989) and promotes an increase in *N′*-acetyl-5-HT and melatonin production (Vacas et al., 1989; Ferreira et al., 1994). We have previously shown that ATP and the non-hydrolizable analog, adenyl-imidodiphosphate, were able to potentiate *N′*-acetyl-5-HT production induced by noradrenaline. This effect, but not the effect of adenosine, was blocked by suramin, an antagonist selective to P2 receptors (Ferreira et al., 1994). Considering the presence of P2 receptors in a sympathetic innervated organ, we raised the hypothesis of purinergic and noradrenergic cotransmission in the rat pineal gland.

In the present paper, we established an experimental model for ''in vitro'' stimulation of pineal glands and showed that nerve released ATP increases the synthesis of

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0014-2999/00/$ - see front matter © 2000 Published by Elsevier Science B.V.
PII: S0014-2999(00)00416-7

N′-acetyl-5-HT and melatonin by acting on P2-like receptors.

2. Materials and methods

2.1. Pineal gland and transmural electrical field stimulation

Pineal glands were obtained from male and female Wistar rats (2–3 months) kept under light–dark cycle of 12:12 h with food and water ad libitum and killed by decapitation between 11 and 14 h. The glands were rapidly dissected and placed in an ice-cold chemically defined BGJb medium (Biggers et al., 1961), supplied with 2 mM glutamine, 0.1 mg ml^{-1} ascorbic acid, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 1 mg ml^{-1} bovine serum albumin. Immediately following extraction, four glands were transferred to a 24-multiwell plate (one gland and 300 μl of BGJb medium per well). The remaining wells were filled with distilled water, in order to keep the relative air humidity. The plate was half immersed in 35°C water. The lid of the plate was adapted to support four pairs of platinum electrodes positioned in parallel (one pair per well, 1 cm apart, glands centered between each pair of electrodes) plus a needle for 95% O_2, 5% CO_2 perfusion (Fig. 1). Each pair was connected to a NARCO SI-10 electrical stimulator (rectangular pulses of 30 V, 0.5 ms, frequency as shown in results). The glands were maintained in BGJb medium for 15 min before beginning the experiments.

2.2. Protocols

2.2.1. Frequency-response curve

Frequency-response curve (1, 3, 10 and 30 Hz) was constructed maintaining the stimulation for 20 min. Between each frequency, an interval of 10 min was allowed, during which the medium was collected, the wells were twice washed with 300 μl of medium and the stimulators were adjusted to the next frequency. Noradrenaline content in the medium was determined by high performance liquid chromatography (HPLC) and electrochemical detection.

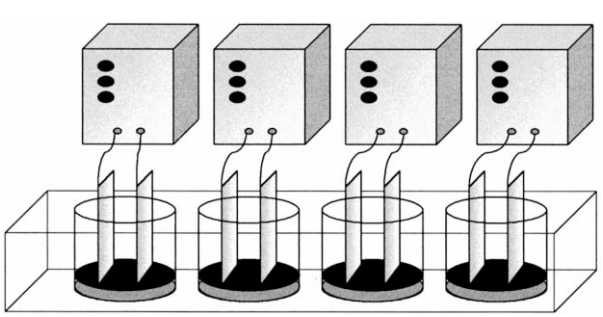

Fig. 1. Depiction of the apparatus for pineal gland transmural electrical field stimulation (see text for description).

2.2.2. Time-response curve

Time-response curve was determined by stimulation (10 Hz, optimal frequency as shown in results) during 45, 90 and 180 min, followed by 195, 150 and 60 min, respectively of resting time, in order to complete 4 h to allow production of *N′*-acetyl-5-HT and melatonin. Independent of the stimulation schedule no *N′*-acetyl-5-HT or melatonin was detectable before 3 h of incubation. *N′*-acetyl-5-HT and melatonin contents in the medium were determined by HPLC and electrochemical detection.

2.2.3. Effect of antagonists

Effects of the β-adrenoceptor antagonist propranolol, and the P2 antagonists PPADS and suramin, incubated 15 min prior the beginning of the electrical stimulation and maintained throughout the experiment, were determined in glands stimulated for 4 h (10 Hz, 0.5 ms, 30 V). One or two control glands were assayed in parallel.

2.3. Determination of noradrenaline, N′-acetyl-5-HT and melatonin by HPLC

Amines contents in the incubation medium were determined according to Mazzacoratii et al. (1990) and Ferreira et al. (1994). The chromatographic system (Shimadzu, Kyoto, Japan) was isocratically operated. For noradrenaline, the mobile phase (0.02 M dibasic sodium phosphate; 0.02 M citric acid, 0.12 mM EDTA, 556 mg l^{-1} heptanesulfonic acid, 2% methanol, pH 2.64) flowed at a rate of 0.5 ml min^{-1} through a 5-μm Resolve C_{18} reversed-phase column (150 × 3.9 mm i.d., Waters), with detector potential adjusted to +0.80 V. For *N′*-acetyl-5-HT and melatonin detection, the mobile phase consisted of 0.1 M sodium acetate, 0.1 M citric acid, 0.15 mM EDTA, methanol (10% for *N′*-acetyl-5-HT and 25% for melatonin), pH 3.7, flow rate of 1.0 ml min^{-1}, same column. The detector potential was adjusted to +0.90 V (vs. Ag/AgCl reference electrode).

2.4. Drugs

PPADS, suramin, propranolol, *N′*-acetyl-5-HT, noradrenaline, melatonin, BGJb medium and bovine albumin were purchased from Sigma, St Louis, USA; ascorbic acid from Hoechst, Brazil; citric acid, EDTA, sodium acetate, sodium bisulphite, methanol, perchloric acid and acetic acid from Merck, Brazil.

2.5. Data analysis

Results of noradrenaline, melatonin and *N*′-acetyl-5-HT content are expressed as ng well^{-1}. All data are presented as mean ± S.E.M.

3. Results

Glands which were not stimulated during the experimental time (4 h), did not produce detectable *N*′-acetyl-5-HT and melatonin. In time-response experiments, one gland was always maintained with no stimulation.

The first goal was to evaluate if noradrenaline was released in a frequency-dependent manner. The concentration of noradrenaline released in the culture medium after stimulation of the glands (0.5 ms, 30 V) at the frequencies 1, 3 and 10 Hz was 10.2 ± 3.3 ($n = 4$), 65.6 ± 8.6 ($n = 4$), 109.9 ± 17.7 ($n = 4$) ng well^{-1}, respectively. Increasing the frequency to 30 Hz does not promote further increase in the amount of noradrenaline released. Thus, the frequency used in our experiments was 10 Hz.

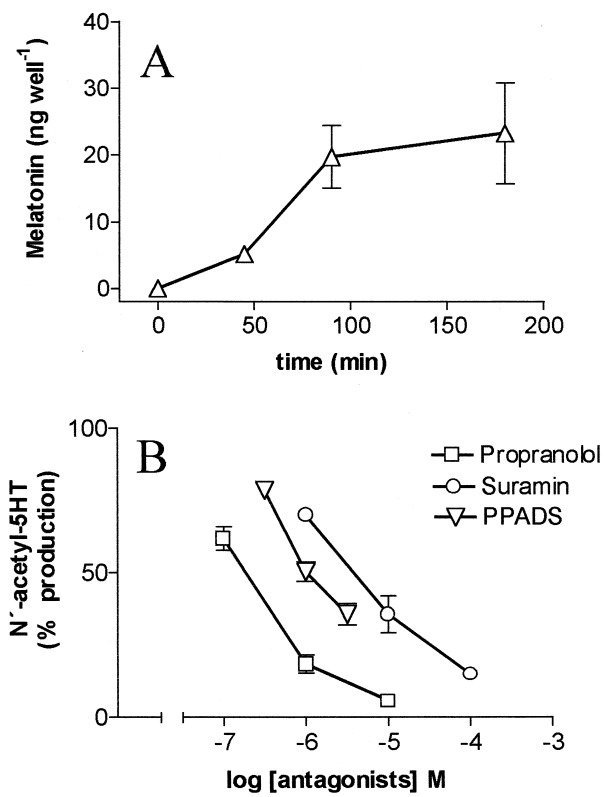

Fig. 2. Production of melatonin and *N*′-acetyl-5-HT by field-stimulated pineal glands. (A) Time-course of melatonin production by transmural field-stimulated isolated pineal glands. The glands were stimulated (10 Hz, 0.5 ms, 30 V) for different times, as shown in the abscissa. Melatonin production was assessed 4 h after the beginning of the stimulation. (B) Inhibition of *N*′-acetyl-5-HT production induced by transmural field-stimulation by propranolol (□), suramin (○) or PPADS (▽). The data shown are expressed as percentage of *N*′-acetyl-5-HT produced by controls (30.4 ± 2.8 ng well^{-1}, $n = 26$). Controls and antagonist incubated glands were tested simultaneously. Data are shown as mean ± S.E.M. of four glands.

Fig. 2A shows the production of melatonin induced by 45-, 90- and 180-min stimulation. The production of *N*′-acetyl-5-HT and melatonin induced by transmural field-stimulation was dependent on the total time of stimulation. Stimulation for 45 min was not sufficient to promote maximal production of melatonin, which was attained at 90 and 180 min.

The production of *N*′-acetyl-5-HT was inhibited by propranolol, suramin and PPADS (Fig. 2B). The last two are antagonists of P2-like receptors, and the concentrations chosen were around the IC_{50} values found in the literature (Ralevic and Burnstock, 1998).

4. Discussion

The aim of this paper was to show that nerve terminals of rat isolated pineal glands could be transmurally field-stimulated and that ATP and noradrenaline are coreleased. The protocols allowed us to obtain a frequency-dependent release of noradrenaline, indicating that the method was feasible to evaluate purinergic–noradrenergic cotransmission. The ability of released noradrenaline to promote *N*′-acetyl-5-HT and melatonin production was evaluated: detection of measurable *N*′-acetyl-5-HT or melatonin was observed after 3 h of stimulation and the maximal synthesis, measured 4 h after the beginning of the stimulation, occurred when glands were stimulated for at least 90 min. In other words, 90 or 180 min of stimulation resulted in the same amount of *N*′-acetyl-5-HT produced 4 h after the beginning of the stimulation. Hence, before observing the effect of transmural stimulation, a minimum time necessary for the transcription and translation of arylalkylamine-*N*-acetyltransferase must be allowed (Parfitt et al., 1976).

In order to evaluate the effect of endogenous released noradrenaline and ATP on the production of *N*′-acetyl-5-HT, the interaction of the transmitters with specific receptors was blocked. Either the β-adrenoceptor antagonist, propranolol, or the inhibitors of P2-like receptors, suramin and PPADS, inhibited *N*′-acetyl-5-HT production induced by transmural stimulation, strongly suggesting that ATP is coreleased with noradrenaline. Although stimulation of P1 receptors has been shown to induce the synthesis of melatonin in the pineal gland (Sarda et al., 1989, Ferreira et al., 1994), the effect herein described is probably mediated by P2-like receptors as it is blocked by suramin. This hypothesis was reinforced by the effect of PPADS, since this inhibitor, although not selective among P2-receptor subtypes, does not antagonize P1 receptors (Ralevic and Burnstock, 1998).

Therefore, as both antagonists were able to block *N*′-acetyl-5-HT transmural-stimulation induced production, we suggest that the P2-receptors present in the pineal gland (Ferreira et al., 1994) are endogenously stimulated by

nerve-released ATP. Neuronal-released ATP could also act as a modulator of the spread of action potential in clusters of action-potential producing pinealocytes (Schenda and Vollrath, 1999). Both neurotransmitters probably act through different signal transduction pathways. Noradrenaline is known to activate adenylyl cyclase and protein kinase C, via β_1- and α_1-adrenoceptors, respectively. P2-receptors act by increase of intracellular Ca^{2+} and activation of protein kinase C (Ferreira and Markus, personal communication). A crosstalk between the purinergic and the noradrenergic pathways probably modulates the production of melatonin (Nikaido and Takahashi, 1996). The center of the gating mechanism is the adenylyl cyclase of type 1, which is controlled by cyclicAMP and Ca^{2+} signals in the pineal gland (Tzavara et al., 1996).

In summary, our results show that the purinergic and noradrenergic cotransmission is present in the rat pineal gland and both contribute to promote *N′*-acetyl-5-HT and melatonin synthesis.

Acknowledgements

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, No. 96/04497-0). RPM and EJMB are research fellow and undergraduate fellow of Conselho Nacional de Pesquisa Científica e Tecnológica (CNPq). The technical assistance of Débora Aparecida Moura is gratefully acknowledged.

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