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Tryptophan hydroxylase is modulated by L-type calcium channels in the rat pineal gland

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Abstract

Calcium is an important second messenger in the rat pineal gland, as well as cAMP. They both contribute to melatonin synthesis mediated by the three main enzymes of the melatonin synthesis pathway: tryptophan hydroxylase, arylalkylamine *N*-acetyltransferase and hydroxyindole-*O*-methyltransferase. The cytosolic calcium is elevated in pinealocytes following α_1-adrenergic stimulation, through IP_3-and membrane calcium channels activation. Nifedipine, an L-type calcium channel blocker, reduces melatonin synthesis in rat pineal glands in vitro. With the purpose of investigating the mechanisms involved in melatonin synthesis regulation by the L-type calcium channel, we studied the effects of nifedipine on noradrenergic stimulated cultured rat pineal glands. Tryptophan hydroxylase, arylalkylamine *N*-acetyltransferase and hydroxyindole-*O*-methyltransferase activities were quantified by radiometric assays and 5-hydroxytryptophan, serotonin, *N*-acetylserotonin and melatonin contents were quantified by HPLC with electrochemical detection. The data showed that calcium influx blockaded by nifedipine caused a decrease in tryptophan hydroxylase activity, but did not change either arylalkylamine *N*-acetyltransferase or hydroxyindole-*O*-methyltransferase activities. Moreover, there was a reduction of 5-hydroxytryptophan, serotonin, *N*-acetylserotonin and melatonin intracellular content, as well as a reduction of serotonin and melatonin secretion. Thus, it seems that the calcium influx through L-type high voltage-activated calcium channels is essential for the full activation of tryptophan hydroxylase leading to melatonin synthesis in the pineal gland.

Keywords: L-type calcium channel; Melatonin; Arylalkylamine *N*-acetyltransferase; Tryptophan hydroxylase; Hydroxyindole-*O*-methyltransferase; Pineal gland

Introduction

Melatonin is the main hormone synthesized and released from the pineal gland following sympathetic stimulation during the night. Norepinephrine interacts with α-and β-adrenoceptors in the pinealocyte membranes and, consequently, elevates intracellular cAMP and calcium levels (Sugden et al., 1986; Sugden, 1989). These two second messengers participate in some way in the synthesis and activation of the three most important enzymes in the melatonin biosynthetic pathway (Sugden et al., 1986; Ehret et al., 1989, 1991; Klein et al., 1970; Ribelayga et al., 1997, 1999; Yu et al., 1993). Tryptophan hydroxylase (TPH, EC 1.14.16.4) is the first enzyme, catalyzing the transformation of tryptophan into 5-hydroxytryptophan, and is the rate-limiting step in the serotonin synthesis. Serotonin accumulates in the pineal gland during the day and, only at night, due to the activation of the enzyme arylalkylamine *N*-acetyltransferase (AA-NAT; EC 2.3.1.87), it is converted to *N*-acetylserotonin, that is then transformed into melatonin by the catalysis of hydroxyindole-*O*-methyltransferase enzyme (HIOMT, EC 2.1.1.4) (Sugden, 1989; Simonneaux and Ribelayga, 2003).

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In rat pineal gland, TPH synthesis and activity are stimulated by norepinephrine. cAMP, through the activation of cAMP-dependent protein kinase A (PKA), phosphorylates the transcription factor cAMP response element-binding protein (CREB), which starts the enzyme synthesis (Ehret et al., 1991). In addition, enzyme phosphorylation by PKA, protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase and its further association with 14-3-3 protein promotes its activation (Ehret et al., 1989; Banik et al., 1997; Ichimura et al., 1987; Johansen et al., 1995). It was demonstrated that the predominant TPH isoform in the pineal gland, that shows circadian rhythm peaking at night, is TPH1. THP2 is also present, but in much less quantity and does not show the typical daily pattern (Sugden, 2003; Patel et al., 2004).

AA-NAT has its synthesis and activity stimulated by norepinephrine at night. Differently from TPH, however, AA-NAT activity is elevated by 150 times (whereas TPH activity is increased twice at night), and although the same cAMP/PKA/CREB pathway (Maronde et al., 1999; Roseboom and Klein, 1995; Roseboom et al., 1996) is involved in the synthesis of both enzymes, for the AA-NAT this transcriptional process is a pre-requisite for its activation. This, in turn, is accomplished by PKA and PKC phosphorylation, and the subsequent complex formation with 14-3-3 protein (Ganguly et al., 2001).

The HIOMT regulation is different from that of TPH and AA-NAT. HIOMT gene expression is partly activated by norepinephrine at night. In addition HIOMT activity is further stimulated by neuropeptide Y (NPY) that promotes intracellular calcium elevation (Ribelayga et al., 1999; Simonneaux et al., 1999).

The cytosolic calcium level in the pinealocyte is increased by α_1-adrenergic activation, acting in both mobilization from the endoplasmic reticulum by IP_3-stimulation and through the store-operated calcium channels ("SOCs") (Gomperts et al., 2002; Lee et al., 2006; Parekh and Putney, 2005). Moreover, high voltage-activated calcium channels are also present in the rat pineal gland (Afeche et al., 2006; Chik et al., 1995, 1997; Chin et al., 1992) and these seem to be activated either by acetylcholine or norepinephrine (Afeche et al., 2006; Darvish and Russell, 1998; Letz et al., 1997). We have recently reported that the blockade of L-type calcium channel with nifedipine, a specific L-type calcium channel antagonist belonging to dihydropyridine class, reduced melatonin synthesis (Afeche et al., 2006), but the mechanisms involved were not resolved.

With the purpose of clarifying the modulation of melatonin synthesis and secretion by the L-type calcium channels, we studied the effects of nifedipine, in noradrenergic stimulated cultured rat pineal glands. The results showed that both melatonin synthesis and secretion stimulated by norepinephrine were impaired by nifedipine and that TPH activity is the main step involved. Neither AA-NAT nor HIOMT activities were affected by nifedipine. The indoles 5-hydroxytryptophan (5-HTP), serotonin (5-HT) and *N*-acetylserotonin (NAS) were all reduced in pinealocytes by nifedipine, probably as a consequence of TPH activity reduction.

Materials and methods

Animals

Young male Wistar rats were kept under a 12:12 light–dark cycle (lights on at 7:00 am) in a temperature-controlled (21 ± 2 °C) room with water and food ad libitum.

All procedures were approved by the Institute of Biomedical Sciences (University of São Paulo, Ethical Committee for Animal Research, Brazil; CEEA) and are in agreement with the ethical principles in animal research adopted by the Brazilian College of Animal Experimentation (COBEA).

Pineal gland culture

The animals (weighing 150–180 g) were decapitated and their pineal glands were removed and immediately placed in ice-cold Biggers, Gwatkin, Judah medium with Fitton–Jackson modification (BGJ_b — Gibco, Grand Island, NY, USA), with the addition of BSA (1 mg/ ml), 2 mM glutamine, 0.1 mg/ ml ascorbic acid, and penicillin (100 U/ ml)–streptomycin (100 μg/ml). Pineal glands were cultured as described by Parfitt et al. (1976). Briefly, pineal glands were incubated (37 °C; 95% O_2–5% CO_2) in BGJ_b medium on 24-well plates (2 glands/well; 200 μl/well) for 48 h (the time needed for the occurrence of total degeneration of the presynaptic elements) before treatments and the medium was changed after the first 24 h. After 48 h of culture, the glands were placed in fresh medium for 1 h and then submitted to one of the treatments described below. After 5 h of treatment, the glands were collected, frozen in dry ice and kept at −85 °C until assayed.

Pinealocyte culture

Pineal glands were dissociated by papain digestion (Papain Dissociation System, Worthington Biochemical Corporation, Freehold, NJ). The glands were isolated (from rats with approximately 100 g) and immediately placed in ice-cold Dulbecco's Modified Eagle's Medium (DMEM) (glucose: 1000 mg/l, HEPES: 5.9 g, sodium bicarbonate: 3.7 g) (St. Louis, MO, USA). Then, the tissue was incubated at 37 °C for 50 min in papain (0.01%) and DNase (0.01%) solution. After removal of papain and its blockade with ovomucoid (2 mg/ml), the pinealocytes were mechanically dispersed and resuspended in DMEM supplemented with 10% fetal calf serum and 1% penicillin–streptomycin to obtain the concentration of 2×10^5 cells/ml. Five ml of cells in a culture medium were cultivated in 25 cm^2 culture flasks, at 37 °C, in 5% CO_2/95% air, for 24 h.

Melatonin and indole assays

Melatonin, NAS, 5-HT and 5-HTP contents were determined by HPLC with electrochemical detection (Waters System, Milford, MA, USA). The indolamines were separated on a Resolve C18 column (5 μm, 3.9 × 150 mm). The chromatographic