Pineal hyperactivity in spontaneously hypertensive rats: muscarinic regulation of indole metabolism

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SUMMARY

1. Choline acetyltransferase activity and [3H]quinuclidinyl benzylate-binding sites were detected in the pineal gland of normotensive Wistar-Kyoto rats and of spontaneously hypertensive rats.

2. In vitro, muscarinic activation by pilocarpine increased the pineal metabolic production of hydroxyindole derivatives up to 5-hydroxytryptamine and produced a less marked stimulation of melatonin biosynthesis.

3. Electrical field stimulation of pineal gland slices caused similar metabolic effects.

4. Muscarinic blockade with atropine inhibited the effects on hydroxyindole metabolism.

5. [3H]Quinuclidinyl benzylate-binding sites, indicative of muscarinic receptors, were more numerous, and basal 5-hydroxytryptamine and melatonin levels were higher, in the pineal gland of spontaneously hypertensive rats compared with Wistar-Kyoto rats.

6. The atropine-sensitive metabolic effects of pilocarpine and electrical field stimulation on the pineal gland were increased in spontaneously hypertensive rats compared with Wistar-Kyoto rats.

Key words: 5-hydroxytryptamine, melatonin, muscarinic activity, pineal gland, spontaneously hypertensive rats.

INTRODUCTION

The innervation of the mammalian pineal gland is mainly sympathetic and it modulates the activity of the enzymes of the indole pathway and the secretion of pineal hormones [1]. The possible existence of an additional cholinergic pineal innervation in various species is controversial. Schrier & Klein [2] were unable to detect choline acetyltransferase (ChAT) activity in pineal glands of rat or rabbit, whereas Rodriguez de Lores Arnaiz & Pellegrino de Iraldi [3] demonstrated that ChAT activity in the rat pineal gland was present even after bilateral superior cervical ganglionectomy (SCGx), suggesting that the cholinergic system did not originate in these ganglia. In addition, Moller & Korf [4] and Romijn [5] reported that pineal nerve terminals contain small clear vesicles staining intensely for ChAT. These structures did not disappear after SCGx and were considered to be cholinergic vesicles.

In fact, muscarinic binding sites detected in the pineal gland were not distinguishable from those identified in other tissues [6]. The number of sites did not decrease after SCGx, indicating that muscarinic receptors were not exclusively located in sympathetic terminals [6]. Using autoradiography, Schlumpf et al. [7] revealed binding of N-[3H]methylscopolamine to the fetal rat pineal gland and recently some reports have indicated that the binding sites are functional. Laitinen et al. [8] suggested that the activation of the muscarinic M_1-receptor by carbachol produces an accumulation of inositol monophosphates, a finding that confirms and extends a previous report by Basinska et al. [9].

There are also indications of an altered responsiveness to cholinergic agents in spontaneously hypertensive rats (SHR). A given dose of hemicholinium-3, administered by an intracerebroventricular injection, decreased arterial blood pressure in SHR but not in normotensive Wistar-Kyoto rats (WKY) [10]. Further, low doses of physostigmine that were inactive in WKY caused a pres-
sor response in SHR [11]. In addition, the pressor response to intravenously administered oxotremorine was greater in SHR than in WKY [12]. In a more direct approach, Helke et al. [13] showed that ChAT activity in specific brain areas of SHR underwent a significant age-related change compared with the corresponding activity in WKY. Moreover, there is evidence that the pineal gland function is involved in cardiovascular regulation [14]. Karppanen et al. [15, 16] reported that pinealectomy induces hypertension in rats, and Acuña et al. [17] proposed that the renin–angiotensin system can be modulated by the pineal gland through mineralocorticoid production.

The aim of this study was to determine whether the pineal cholinergic system is involved in the regulation of indole metabolism in the pineal gland of SHR and WKY. We thus investigated how the hydroxy- and methoxy-indole production was affected either by a cholinergic agonist or by electrical field stimulation and whether any difference may be attributable to changes in cholinergic muscarinic receptors or ChAT activity in SHR compared with WKY.

**METHODS**

**Preparation of pineal slices**

Male SHR, 18–20 weeks of age, and matched normotensive WKY, housed and maintained under a 12 h light/12 h dark schedule and fed ad libitum with Purina chow and water, were decapitated at 17:00–19:00 hours. Systolic blood pressure, measured by the plethysmographic tail method, was 195 ± 7 mmHg in SHR and 140 ± 6 mmHg in WKY. Pooled pineal glands from 20–50 rats were carefully sliced and kept at 4°C in an enriched medium (RPMI 1640, Gibco Laboratories, Paisley, Scotland, U.K.) until drug or electrical stimulation was performed.

**[^H]Quinuclidinyl benzylate ([^H]-QNB)-binding assay**

Pooled pineal glands of at least ten rats were homogenized in 10 vol. of ice-cold 0.32 mol/l sucrose in a Potter–Elvehjem homogenizer. The binding assay was performed as previously described [18]. Briefly, 25–50 µl aliquots (10–20 µg/ml final protein concentration in the incubation medium) were incubated at 25°C with 2 ml of 0.05 mol/l phosphate buffer (pH 7.4) containing different concentrations (25–4000 pmol/l) of [^H]-QNB. After 60 min, incubation equilibrium was reached; 3 ml of ice-cold phosphate buffer was then added and the contents were passed through glass Whatman GF/B filters (Clifton, NJ, U.S.A.) under vacuum. The filters were washed three times with 3 ml of ice-cold buffer and placed in vials containing 10 ml of Triton X-100/Toluene-Phosphor, maintained at 25°C for 9–12 h, and the radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer (Rockville, MD, U.S.A.) at 30% counting efficiency. Specific binding was defined as total binding minus the binding in the presence of 1 µmol/l atropine, and represents 70–80% of total binding. Analysis of the [^H]-QNB binding was calculated by a computed non-linear least-square curve fitting procedure using a generalized model for ligand–receptor systems [19]. The calculated values (means ± SEM) for the maximal binding capacity (B_max) and the dissociation constant (K_d) of six experiments were compared for significant differences as described below.

**ChAT activity assay**

Tissues were homogenized (0.2%, w/v) in 15 mmol/l sodium phosphate buffer, pH 7.4, containing 0.3% (v/v) Triton X-100. Enzyme assays were run with substrate excess under V_max conditions as described by Fonnum [20]. The enzymatic product was confirmed as authentic acetylcholine by using a h.p.l.c. method [21].

**Drug stimulation**

Pineal gland slices were incubated at 37°C in specially designed microwells containing 200 µl of RPMI 1640 plus 50 µmol/l t-tryptophan with or without the addition of pilocarpine, atropine or nadolol (1 µmol/l) for 180 min. The enriched medium was bubbled with 5% CO_2 in O_2 just before being introduced into the microwells and was changed every 60 min. The incubation plates were placed in a metabolic waterbath shaker that maintained the temperature and atmosphere composition during the experiments.

**Electrical field stimulation**

Pineal gland slices, incubated in the medium described above, were subjected to 1.0 ms square pulses, at a rate of 2 Hz and at a supramaximal voltage of 20 V produced by a Grass 5-S stimulator (Quincy, MA, U.S.A.) by means of two platinum electrodes placed 5 mm apart on the bottom of the microwell chamber. In order to block a possible muscarinic-mediated effect, produced by electrical field stimulation, atropine, with or without nadolol (1 µmol/l), was added to the medium. Pineal gland slices were subjected to electrical field stimulation for 180 min with medium changes every 60 min. The conditions and the medium were the same as for drug stimulation.

**Tryptophan and hydroxy- and methoxy-indole determination**

After a 3 h incubation with drugs or under electrical field stimulation, slices were quickly washed with ice-cold 150 mmol/l NaCl solution. The slices were homogenized in 200 µl of ice-cold 0.1 mol/l perchloric acid and the medium was adjusted to a perchloric acid concentration of 0.1 mol/l. Media and homogenates were independently centrifuged at 10000 g for 10 min at 4°C and the supernatants were separated by h.p.l.c. Indole metabolites were determined as described by Finocchiaro et al. [22].

Results were expressed as the amount (mean ± SEM, n = 4) of the pineal substance released plus its tissue content.
The production of each indole metabolite showed a particular pattern during the first 3 h of incubation, e.g. the production of some metabolites was greater by the second hour while that of others reached maximum by the third hour. For longer incubation times (overnight) indole production fell to low values, suggesting a derangement of pineal function. Thus, for the sake of simplicity, comparisons were performed using the sum of the first 3 h of release and the tissue content of each indole metabolite. In general terms, the amount of metabolites released after stimulation represented 70–99% of the total production.

Protein determination

Protein was determined by the method of Lowry et al. [23].

Drugs

Pilocarpine (hydrochloride), atropine (sulphate), 1,5-hydroxytryptophan, 5-hydroxytryptamine (5-HT; hydrochloride), N-acetyl-5-hydroxytryptamine (NAc-5-HT), 5-hydroxyindole 3-acetic acid (5-HIAA) (free acid) and melatonin were obtained from Sigma Chemical Company (St Louis, MO, U.S.A.). Nadolol was kindly provided by Byk Liprandy (Buenos Aires, Argentina).

Statistical analysis

Analysis of variance with repeated measurements of the same elements and Tukey’s test for individual mean differences were performed [24].

RESULTS

Binding of [3H]QNB to pineal cell membranes and ChAT activity

[3H]QNB binding to pineal membranes was saturable and linear up to 100 μg of membrane protein/assay. The binding curves of [3H]QNB in the pineal gland were as expected for a homogeneous population of antagonist binding sites. The SHR pineal membranes had a 10-fold increased density \((P<0.01)\) of muscarinic receptors \(B_{\text{max}}\) (270 ± 60 fmol/mg of protein) when compared with WKY pineal membranes \(B_{\text{max}}\) (22 ± 3 fmol/mg of protein), without any significant difference in \(K_d\) (41 ± 18 and 28 ± 13 pmol/l, respectively).

Pineal cell homogenates showed ChAT activity that was higher, but not significantly so, in SHR than in WKY \((0.73 ± 0.20 \text{ versus } 0.45 ± 0.17 \text{ nmol min}^{-1} \text{ mg of protein}^{-1}, \text{respectively}).

Effects of drug and electrical field stimulation on methoxy- and hydroxy-indole production by pineal gland slices

SHR had higher basal levels of 5-HT (four-fold) and melatonin (two-fold) when compared with WKY (Fig. 1d). Whereas 5-HIAA levels showed no differences between the rat strains, NAc-5-HT levels in SHR pineal gland slices were about 50% of those in WKY pineal gland (Figs. 1b and 1c).

When pineal gland slices were incubated with pilocarpine, an almost three-fold increase in 5-HT levels \((P<0.01)\) in WKY but no change in SHR 5-HT levels, and a significant increase in 5-HIAA levels \((P<0.05)\) but no change in NAc-5-HT levels in either strain (Fig. 1), were observed.

The specific muscarinic antagonist atropine decreased the pilocarpine-induced effect on 5-HT \((P<0.01)\) and 5-HIAA \((P<0.05)\) in both SHR and WKY. In WKY, atropine significantly decreased \((P<0.01)\) NAc-5-HT production in the presence of pilocarpine even below the basal value (Fig. 1).

Atropine by itself reproduced the pattern observed with pilocarpine plus atropine (Fig. 1) for 5-HT (WKY, 45 ± 5 pmol/mg of protein; SHR, 129 ± 13 pmol/mg of protein), 5-HIAA (WKY, 138 ± 14 pmol/mg of protein; SHR, 146 ± 15 pmol/mg of protein), NAc-5-HT (WKY, 41 ± 4 pmol/mg of protein; SHR, 39 ± 3 pmol/mg of protein) and melatonin (WKY, 26 ± 3 pmol/mg of protein; SHR, 42 ± 5 pmol/mg of protein).

Electrical field stimulation increased the levels of all the assayed indoleamines in both strains, except NAc-5-HT and 5-HIAA in WKY, and 5-HT in SHR (Fig. 2). Significantly higher levels of 5-HT \((P<0.05)\) and melatonin \((P<0.01)\) and no differences in levels of 5-HIAA and NAc-5-HT were found in SHR compared with WKY after electrical field stimulation. The presence of atropine in the incubation media blocked the electrical field stimulation of 5-HIAA levels in both SHR and WKY (Fig. 2b). The effect of atropine on 5-HT levels was greater in both strains, driving them below the basal levels (Fig. 2a). Under electrical field stimulation the inhibition of 5-HT production induced by atropine was more effective \((P<0.05)\) in SHR (a reduction of 199 ± 13 ng/mg of protein) than in WKY (a reduction of 137 ± 10 ng/mg of protein). On the other hand, the electrically stimulated melatonin production was not significantly reduced by atropine in both strains (Fig. 2d). In the presence of nadolol plus atropine, the electrical field-stimulated 5-HT production was almost completely abolished in both strains, reaching similar values \((\text{SHR } 6 ± 1 \text{ ng/mg of protein}; \text{WKY, } 9 ± 2 \text{ ng/mg of protein}). The simultaneous presence of both blocker agents inhibited \((P<0.05)\) the effects of electrical field stimulation on melatonin production in both strains \((\text{SHR, } 34 ± 4 \text{ ng/mg of protein}; \text{WKY, } 18 ± 2 \text{ ng/mg of protein})\) but a significant difference between them still persisted \((P<0.05)\).

In WKY, when pineal gland slices were stimulated in the presence of atropine, production of NAc-5-HT was decreased with respect to the basal \((P<0.01)\) and electrically stimulated \((P<0.01)\) values. This effect was not observed in SHR.

Essentially the same profile was observed when data for each metabolite were expressed either as its concentration \((\text{ng/mg of protein})\) or as the total amount produced by a whole pineal gland \((\text{ng per pineal gland})\).
DISCUSSION

An increasing body of information supports the hypothesis that centrally mediated cholinergic activity is implicated in the development of hypertension. In SHR, intravenous physostigmine potentiated hypertension [25]. Similarly, Hoffman et al. [26] reported that carbachol also potentiated the pressor response in SHR compared with WKY. The involvement of postsynaptic cholinergic sites is upheld by the results of Caputi et al. [27], who found an age-related hypotensive effect on atropine in SHR but not in WKY. It is therefore plausible to speculate that activation of the cholinergic system in the central nervous system could produce the hypertensive state in SHR [28].

These results agree with the current view that the cholinergic system is quiescent in normotensive but not in hypertensive animals [29] and may be indicative of an enhanced turnover of acetylcholine in the cerebral nuclei of SHR [30].

Our results indicate that [3H]QNB-binding sites are present in the pineal gland. These muscarinic receptors might have functional activity, since their stimulation by pilocarpine increased the pineal gland metabolic production of hydroxyindole derivatives up to 5-HT, both in WKY and SHR, with a slight effect on melatonin biosynthesis. Pilocarpine and electrical field stimulation of pineal gland slices caused similar metabolic effects. These effects were inhibited by muscarinic blockade with atropine but not when the specific β-adrenergic blocker nyalolol was added (data not shown). These results suggest that acetylcholine is the neurotransmitter involved. Therefore, cholinergic activity may regulate indole metabolism in the pineal gland of both strains. These data confirm the findings of a previous study on the muscarinic regulation of tryptophan metabolism in the rat pineal gland [22].

We found that the basal production in SHR was four times greater than in WKY, whereas melatonin levels were only twice as great. These results may indicate that in SHR an increased production of 5-HT exists with respect to that of melatonin when compared with WKY. Since atropine by itself decreased the basal 5-HT level in SHR, it may be suggested that the muscarinic hyperactivity is the cause of the significantly greater 5-HT production observed in this strain.

On the other hand, electrical field stimulation produced a significant increase in 5-HT levels in WKY, whereas SHR production was not significantly enhanced. Atropine treatment not only blocked this effect on 5-HT yield but also decreased it to below the basal value in both SHR and WKY.

However, a significantly disparate 5-HT production in SHR and WKY still persisted. The atropine-induced decrease in basal and electrically stimulated 5-HT production was significantly greater in SHR when compared with WKY. Furthermore, electrical field stimulation increased melatonin levels in both SHR and WKY with-
out any significant blocking effect of atropine in SHR, implying that most of the electrically stimulated melatonin production in these rats is not cholinergic. Melatonin levels in SHR reached higher values than those of the WKY controls. All these results suggest the presence of a functional cholinergic muscarinic system which is more active in SHR than in WKY, acting almost exclusively on 5-HT production. As demonstrated in certain nuclei of the central nervous system (31) and peripherally (32), the cholinergic system would partly act through 5-HT release. In addition, we found a tenfold increase in the density of muscarinic binding sites in the SHR pineal gland with respect to that of WKY, without significant changes in ChAT activity, although this is not a sufficient condition to exclude presynaptic mechanisms, which might explain the differences between SHR and WKY particularly observed under electrical field stimulation. These differences would depend on the endogenous neurotransmitter release. In fact, an enhanced turnover of acetylcholine in the cerebral nuclei of SHR has been reported (12, 30, 33). Therefore, an increased cholinergic activity in the SHR pineal gland is not sufficient to explain the differences observed in the 5-HT and melatonin levels of SHR and WKY. In fact, only the addition of atropine plus nadolol, a selective β-adrenergic antagonist, blocked the electrical field-stimulated production of 5-HT in pineal glands of SHR and WKY, driving it to similar values in both strains. On the other hand, the simultaneous presence of both blocker agents inhibited the effects of electrical field stimulation on melatonin production in either strain without affecting the differences between them, indicating that other factors may be involved. Our results provide evidence of: (i) the release of acetylcholine and noradrenaline by electrical field stimulation and (ii) the existence of a basal muscarinic stimulation in SHR that does not allow a further stimulation by an electrical field. Hence, it is suggested that a higher cholinergic and noradrenergic activity in the pineal gland is present in SHR. The cholinergic hyperactivity observed in the pineal gland of SHR may be a particular instance of a more widespread phenomenon. In a preliminary report, we showed an increase in the number of muscarinic receptors and some indication of a high turnover of acetylcholine in the lateral septal area (33). Similar findings were reported by Hershkowitz et al. (34) in the posterior hypothalamus in SHR of different ages. Other authors have failed to demonstrate an increased density of postsynaptic muscarinic receptors in SHR (35).

It is difficult to establish whether the tryptophan metabolism in SHR participates in the development or maintenance of hypertension or if it is merely an adaptive compensatory phenomenon. This last possibility seems more likely, since pinealectomy induces hypertension (15) probably through the withdrawal of an inhibitory control of renin and mineralocorticoid release (16, 17).

Two major conclusions can be drawn from our findings: (i) the cholinergic system may regulate 5-HT levels and, to a lesser extent, affect melatonin production in the
pineal gland of SHR and WKY, and (ii) in the SHR pineal gland there is an enhanced cholinergic postsynaptic response to stimuli.

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