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PHARMACOLOGICAL ANALYSIS OF THE NEUROTRANSMITTER MECHANISMS REGULATING PHENYLETHANOLAMINE *N*-METHYLTRANSFERASE IN THE ADRENAL GLAND

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Abstract—The i.p. administration of reserpine daily for 4 days to rats brought about an increase of adrenal phenylethanolamine *N*-methyltransferase (PNMT) activity. However, the combination of the systemic administration of *p*-chlorophenylalanine (PCPA) and reserpine for 3 days produced an earlier increase in this adrenal enzyme. The effect was reduced significantly in the denervated gland. Prior administration of 5,7-dihydroxytryptamine (DHT) i.c.v. to rats greatly potentiated the inducing effect of reserpine. On the other hand, the depletion of catecholamines by giving rats alpha-methyl-*p*-tyrosine (AMPT) i.p. or 6-hydroxydopamine (6-OHDA) i.c.v. did not alter the action of reserpine on adrenal PNMT. PCPA, DHT, AMPT and 6-OHDA did not have any effect by themselves on adrenal PNMT, but the combination of PCPA and AMPT, each given i.p., caused increased adrenal PNMT activity. The administration of dopamine agonists, a treatment that increases adrenal TH, did not modify adrenal PNMT. We conclude that the induction of PNMT by reserpine involves depletion of catecholamines and serotonin, the depletion of serotonin having the more powerful effect. A monoaminergic (serotonergic) inhibitory pathway is involved in the central regulation of adrenal PNMT activity.

The activities of adrenal tyrosine hydroxylase (TH†, EC 1.14.16.2), dopamine beta-hydroxylase (DBH, EC 1.14.2.1), and phenylethanolamine *N*-methyltransferase) PNMT, EC 2.1.1.28) are increased by stressors and by certain drug treatments [1–4]. The response of adrenal TH to such treatments has been studied extensively in this laboratory and elsewhere in order to elucidate the role of central dopaminergic [5], noradrenergic [6], serotonergic [7], and cholinergic [8] systems that regulate the increases. Less is known about neurotransmitters concerned with DBH activity of the adrenal gland of the rat, but cholinergic [3] and monoaminergic fibres [9] are certainly involved. We now examine the regulation of the activity of a third adrenal enzyme involved in catecholamine synthesis, viz. PNMT. For these studies we have used rats given pharmacological agents that affect monoaminergic functions.

MATERIALS AND METHODS

Male Sprague–Dawley rats (200 ± 10 g or 290 ± 10 g) were purchased from Canadian Breeding Farms and Laboratories Ltd., St. Constant, Quebec. The rats were kept in individual cages under controlled lighting (12 hr on/12 hr off) and were fed *ad lib*.

Animals submitted to surgery were anesthetized with 300 mg/kg, i.p., of chloral hydrate (Fisher Scientific Co., Montreal, Quebec). Splanchnicotomy was performed on the left side as previously described [9]. The stereotaxic coordinates for the intracerebroventricular (i.c.v.) injection of neurotoxins were P 1.0, L 1.5 and V 3.5 mm. The ventricular target site was confirmed by injection of methylene blue. A Hamilton syringe with a 26-gauge needle was used for the injections. 6-Hydroxydopamine hydrochloride (6-OHDA) and 5,7-dihydroxytryptamine creatinine sulfate (DHT) (Sigma, St. Louis, MO) were injected i.c.v. in 20 μl of 0.1% ascorbic acid in saline. Doses of the neurotoxins are given as the weight of the salt. Reserpine (Sigma, St. Louis, MO) was dissolved in a solution of 0.5% methyl-cellulose. Alpha-methyl-*p*-tyrosine hydrochloride (AMPT) and *p*-chlorophenylalanine hydrochloride (PCPA) were obtained from Sigma. Apomorphine was from F. E. Cornell & Co., Montreal, Quebec; piribedil from Laboratoires Servier, Neuilly-sur-Seine, France; and clonidine hydrochloride from Boehringer Ingelheim, Inc., Burlington, Ontario. Drug dosage schedules are fully described in the tables.

Rats were deeply anesthetized with Brietal (sodium methohexital), 65 mg/kg given intraperitoneally (i.p.), and then the adrenals were removed and placed on ice. The glands were homogenized with a Teflon homogenizer in 1 ml of 0.05 M Tris buffer,

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† Abbreviations: TH, tyrosine hydroxylase; ANOVA, analysis of variance; PCPA *p*-chlorophenylalanine; DBH, dopamine beta-hydroxylase; DHT, dihydroxytryptamine; 6-OHDA, 6-hydroxydopamine; i.c.v., intracerebroventricular; i.p., intraperitoneal; AMPT alpha-methyl-*p*-tyrosine; and PNMT, phenylethanolamine *N*-methyltransferase.

pH 7.4, containing 0.1% Triton X-100 for DBH assay and in 1 ml of 0.05 M phosphate buffer, pH 7.4, containing 0.15 KCl, 0.1 mM dithiothreitol (DTT) and 1 mM EDTA for PNMT assay. Homogenates were centrifuged at 10,000 *g* for 10 min. The supernatant fraction was kept at −70° for up to 4 days prior to assay.

Adrenal PNMT was determined by the method of Yu [10] with some modifications: supernatant fraction, 20 μl, was added to 30 μl of the incubation medium. The final concentrations of the reactants in this medium were: Tris buffer, pH 8.6, 0.1 M; dithiothreitol, 3 μM; octopamine, 1 mM; and [^{14}C]-*S*-adenosylmethionine, 0.4 nmole (58.2 mCi/mmole). The reaction was carried out at 37° for 30 min and was followed by the addition of 100 μl of borate buffer, pH 10, 0.5 M. The methylated product was isolated from the mixture by extraction into 500 μl of toluene–isoamyl alcohol (3:2, v/v). After centrifugation, 200 μl of the organic phase was evaporated, dissolved in 1 ml of ethanol, and mixed with 10 ml of Liquifluor for radioactive counting. Dialysis of crude preparations of adrenal PNMT was carried out at 4° for 18 hr against 1 mM phosphate buffer at pH 7.4, containing 1 mM EDTA and 0.1 mM DTT [11]. The supernatant fractions of the adrenal gland homogenates from control and reserpine-treated rats were divided each into two portions, one for dialysis at 4° and the other for maintenance at that temperature during the dialysis period.

Catecholamines and serotonin were determined by reversed-phase high performance liquid chromatography with electrochemical detection according to the procedures of Felice *et al.* [12] and Anderson *et al.* [13] respectively.

Each value represents a mean ± standard error. Adrenal PNMT activity is expressed as nmoles of synephrine formed per 30 min per adrenal. Significance of the differences between means was calculated by Student's *t*-test. Analysis of variance (ANOVA) was done in indicated experiments [14].

RESULTS

Effects of reserpine and AMPT on adrenal and brain catecholamine levels. Reserpine, given i.p. daily for 4 days in a dose of 2.5 mg/kg, decreased adrenal noradrenaline (NA), adrenaline (AD) and dopamine (DA) by 66, 45 and 48%, and brain NA, AD and DA by 53, 90 and 67% of corresponding control respectively. AMPT, 200 mg/kg, i.p., given daily in two doses for 4 days, decreased adrenal NA, AD and DA by 68, 38 and 58%, and brain by 58, 90 and 71% respectively. The combination of the two drugs produced decreases of 82, 59 and 79% in adrenal NA, AD and DA, and of 81, 91 and 84% in the brain respectively. Denervation of the adrenal did not affect catecholamine concentration by day 4 after the operation, which confirms previous reports of this laboratory [15].

Effects of reserpine, PCPA and DHT on brain serotonin levels. We showed previously that the administration of PCPA i.p. and DHT i.c.v., in a way that potentiates the action of reserpine on adrenal DBH and PNMT activities, decreases serotonin in the raphe area and forebrain [9]. Reserpine in our experimental conditions decreased brain serotonin by 55%. PCPA and DHT given separately produced decreases of 75 and 48% in this monoamine in the brain respectively. The combination of reserpine and PCPA or reserpine and DHT decreased brain serotonin by 97 and 88% respectively.

Action of reserpine on PNMT activity. It has been shown that three injections of reserpine given over a period of 6 days lead to about a 20% increase of PNMT in the adrenal gland [1]. The experimental period could be shortened by administration of this monoamine depletor to rats daily for 4 days, with

Table 1. Effects of PCPA, DHT, 6-OHDA and reserpine on adrenal PNMT activity

Expt.	Treatment	PNMT activity (nmoles synephrine formed per 30 min per adrenal) N	Without reserpine	N	With reserpine
A	Control	5	6.16 ± 0.34	7	8.00 ± 0.52*
	6-OHDA	5	7.53 ± 0.55	5	8.70 ± 0.62
B	Control	5	6.67 ± 0.39	5	6.57 ± 0.38
	PCPA	5	7.79 ± 0.39	5	9.36 ± 0.67†
C	Control	3	3.79 ± 0.55	4	4.99 ± 0.23*
	DHT	3	4.46 ± 0.36	5	7.61 ± 0.58‡

Each value is a mean ±S.E. Experiment A: 6-OHDA was given i.c.v. in a dose of 200 μg in 20 μl of 0.1% ascorbic acid in saline 4 days before the first of four daily injections of reserpine. Experiment B: PCPA was administered in a dose of 300 mg/kg once 24 hr before the first of three daily injections of reserpine. Experiment C: DHT was administered i.c.v., 175 μg in 20 μl of 0.1% ascorbic acid in saline, 30 min after 20 mg/kg of desimipramine and 4 days prior to the first of four daily injections of reserpine. Reserpine was given i.p. in a dose of 2.5 mg/kg, and the animals were killed 24 hr after the last injection.

* $P < 0.025$ with respect to control without reserpine.

† $P < 0.05$ with respect to control with or without reserpine, as well as with respect to PCPA without reserpine.

‡ $P < 0.01$ with respect to DHT without reserpine and control with reserpine.

an increase of 30% ($P < 0.025$) in the activity of adrenal PNMT (Table 1, Expts. A and C). However, three daily injections were insufficient to induce the enzyme (Table 1, Expt. B). Because of the difference in catecholamine content of PNMT preparations from control and reserpine-treated rats, enzyme activity was determined after dialysis. An increase in PNMT activity of 22% was observed in non-dialyzed and dialyzed preparations, suggesting that the elimination of catecholamines in the assay mixture of PNMT from reserpine-treated rats does not interfere with the *in vitro* determination of the activity. A similar result was reported previously in insulin-treated rats [16].

Effects of drugs affecting brain serotonin. The systemic administration of a single large dose of PCPA (300 mg/kg, i.p.), an inhibitor of tryptophan hydroxylase [17], to rats did not produce a statistically significant increase of adrenal PNMT activity (Table 1, Expt. B). The injection of DHT (175 μg, i.c.v.), in order to produce central serotonergic denervation [18], also did not affect adrenal PNMT activity significantly (Table 1, Expt. C). However, when either of these treatments was combined with the administration of reserpine (see legend of Table 1 for the dose schedule), there was potentiation of the effects of that drug (Table 1, Expts. B and C).

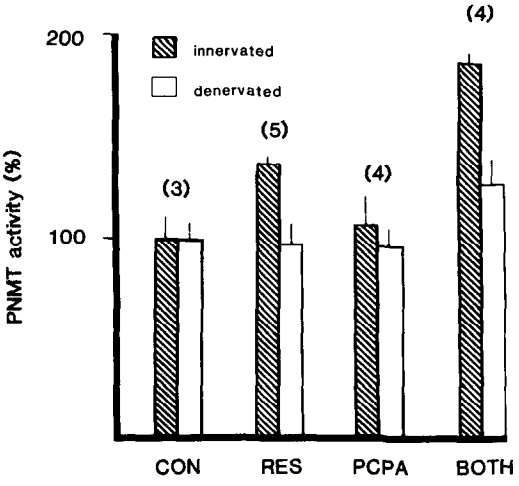

Fig. 1. Effects of hemisplanchnicotomy, reserpine and PCPA on adrenal PNMT activity. Hemisplanchnicotomy was carried out 5 days before starting the treatment. PCPA was given in a single dose of 300 mg/kg, i.p. 2 days before the first injection of reserpine. Reserpine was given daily in a dose of 2.5 mg/kg, s.c., for 4 days. Animals were killed 24 hr after the last injection. ANOVA was carried out with extraction of sum of squares for splanchnicotomy (1 degree of freedom), treatments (3), interaction (3) and remainder (17). Significance of differences between means was calculated from the mean square for remainder (0.3138). Probabilities are as follows: Both intact vs denervated side <0.005, and reserpine intact vs reserpine denervated side <0.01. Other probabilities are:

Difference between	Intact	Denervated
Control vs reserpine	<0.05	NS
Reserpine vs PCPA	<0.05	NS
Both vs reserpine	<0.025	=0.05
Both vs PCPA	<0.005	NS
Both vs control	<0.005	NS

PCPA in combination with reserpine given for 4 days was further tested in rats that had been hemisplanchnicotomized. For the intact adrenal, the effects on PNMT activity (Fig. 1) were similar to those obtained previously. However, in the case of the denervated gland, reserpine either alone or with PCPA no longer caused an increase of PNMT activity.

Effects of drugs affecting brain catecholamines. Two treatments affecting catecholaminergic mechanisms were tested with respect to adrenal PNMT activity. 6-OHDA, given by i.c.v. injection in a dose of 200 μg, in order to bring about central catecholaminergic denervation [19], had no significant effect on adrenal PNMT activity nor did it alter the effect of reserpine given to rats for 4 days (Table 1, Expt. A). The second treatment was the administration of AMPT, an inhibitor of TH that produces a great decrease in catecholamine levels in the central nervous system and the periphery [20]. AMPT was given i.p. in a daily dose of 200 mg/kg, divided into two injections, for 4 days. The adrenal PNMT activity of control rats was 3.78 ± 0.45 nmoles per 30 min per adrenal (N = 3) and that of AMPT-treated animals was 4.37 ± 0.25 (N = 3) nmoles per min per adrenal. The difference between these means (13%) was not statistically significant.

The effects of two dopamine agonists, piribedil and apomorphine, were also tested. These drugs were given in dosage schedules that invariably result in large increases of adrenal TH activity [5, 7]. Thus, piribedil was administered i.p. in a dose of 50 mg/kg, twice a day, for 4 days. Apomorphine was given s.c. in doses of 3 and 10 mg/kg, respectively, in two separate experiments, four times a day for 4 days. Apomorphine (10 mg/kg) was also administered to rats that received a prior injection of PCPA, 300 mg/kg, i.p. Similarly, clonidine, a noradrenergic agonist that increases adrenal TH activity [6], was tested in doses of 15 μg/kg and 2 mg/kg, given intraperitoneally twice a day for 4 days. None of these catecholamine agonists significantly modified adrenal PNMT activity in the rat adrenal.

Monoaminergic interaction. Because of the non-discriminating action of reserpine in releasing monoamine neurotransmitters, an experiment was carried out in which PCPA and AMPT were administered separately in order to test their depleting actions on serotonin and catecholamines respectively (Table 2). As before, neither drug had an effect. However, their combination increased adrenal PNMT by 44% above control value ($P < 0.01$). Thus, both types of monoamine, catecholamines and serotonin, must undergo depletion in order to produce induction of PNMT in the adrenal gland of the rat.

DISCUSSION

Reserpine is a very well known inducer of adrenal TH, DBH and PNMT, acting mainly through a neural mechanism [21], but there are distinct differences in the forms of response of these enzymes. Thus, 24 hr after a single injection of reserpine there is already an increase in adrenal TH [22]. However, a longer period to time, up to 4 days in the present study, is necessary to bring about an increase of

adrenal PNMT. It is known that all three adrenal enzymes are under neural control, and that DBH and PNMT are additionally subject to humoral regulation [23], and humoral control is especially prominent in the case of PNMT.

Although treatments (PCPA, i.p.; DHT, i.c.v.) that result in extensive depletion of cerebral serotonin in the raphe area and forebrain [9] bring about significant increases of adrenal TH activity [7], they did not influence adrenal PNMT (Table 1) or DBH [9]. However, such treatments given prior to reserpine potentiated the action of that inducer (Table 1), just as was observed previously for adrenal DBH [9]. The results with catecholamine depletion show that, as with serotonin-depleting agents, neither 6-OHDA (Table 1, Expt. A) nor AMPT (Table 2) affected adrenal PNMT activity. In contrast to drugs that cause depletion of serotonin, 6-OHDA did not influence the action of reserpine at all (Table 1). These results emphasize the importance of specifically reducing the serotonergic component for induction of adrenal PNMT by reserpine through the neural route.

Comparison of the actions of DHT and PCPA with reserpine shows that the former was more effective under our experimental conditions. It is possible that the difference lies in the release by DHT (given i.c.v.) of additional modulators from serotonergic nerve endings. This actually occurs after central administration of DHT, in that there is depletion of substance P and thyrotropin releasing hormone, in parallel with serotonin, from the ventral spinal cord [24].

The experiments with individual monoamine-depleting agents show that cerebral loss of serotonin or catecholamines alone is not sufficient to induce adrenal PNMT. Earlier we found that cerebral catecholamine depletion does not increase adrenal TH [5] or DBH [9]. The failure of AMPT to affect PNMT by itself illustrates that the depletion of catecholamines alone, either centrally or peripherally, is not able to affect this adrenal enzyme. Thus, neither a local effect *in vivo* brought about by depleting catecholamines, nor an effect *in vitro* by reducing the amount of catecholamines in the enzyme assay is responsible for the observed increased in PNMT activity. However, the simultaneous decrease of serotonin and catecholamines, achieved by administering AMPT to rats that previously had received a single injection of PCPA, produced a significant increase in adrenal PNMT activity (Table 2), just as in the case of DBH [9]. These results indicate (i) that in order for reserpine to act as inducer of adrenal DBH and PNMT, depletion of both types of monoamine was necessary (Fig. 1); and (ii) that this response was neurally mediated (Fig. 1, Expt. C). This neutral mediation may be (a) more or less direct, through central mechanisms regulating adrenal functions; or (b) indirect through actions on other autonomic activities that themselves influence splanchnic discharge. Nevertheless, it is clear that a central serotonergic pathway exerts a net inhibitory effect in the control of adrenal PNMT activity, just as has been recognized previously for TH [7] and DBH [9]. But it cannot be concluded on the basis of the present evidence that the serotonergic tracts are the same in all three cases.

Present attempts to induce adrenal PNMT with dopaminergic agents alone were unsuccessful. Similarly, the use of clonidine by itself did not provide evidence for a noradrenergic mechanism in regard to this enzyme. The failure of these types of catecholaminergic stimulation to affect adrenal PNMT contrasts with the importance of these mechanisms, especially the dopaminergic function, in the regulation of adrenal TH [5, 15] and, hence, points up the specificity and differential control of the central pathways involved in the regulation of catecholamine-synthesizing enzymes in the adrenal gland.

Table 2. Effects of PCPA and AMPT on adrenal PNMT activity

Treatment	N	PNMT activity (nmoles synephrine formed per 30 min per adrenal)
Control	4	5.45 ± 0.32
PCPA	3	4.59 ± 0.70
AMPT	3	4.91 ± 0.17
Both	4	7.83 ± 0.62

Each value is a mean ±S.E. PCPA was administered in a dose of 300 mg/kg, i.p., once 48 hr before the first injection of AMPT, which was given i.p. in a dose of 200 mg/kg per day in two injections for 4 days. Animals were killed 18 hr after the last injection. ANOVA was carried out with extraction of sum of squares for AMPT (1 degree of freedom), for PCPA (1 df), interaction (1 df) and remainder (7 df). Significance of the difference between means was calculated from the mean square for remainder (0.7302). Probabilities are as follows: control vs both < 0.01; AMPT vs both < 0.005; PCPA vs both < 0.05.

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