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TETRAHYDROISOQUINOLINECARBOXYLIC ACIDS AND REGULATION OF PHENYLETHANOLAMINE *N*-METHYLTRANSFERASE IN CULTURED ADRENAL MEDULLA

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Abstract—Explants of rat adrenal medullae, cultured for up to 48 hr in the presence of 3′,4′-deoxynorlaudanosolinecarboxylic acid (DNLCA) or α-methyltyrosine, exhibited a 69% increase in phenylethanolamine *N*-methyltransferase (PNMT) activity as measured in dialyzed homogenates. A related tetrahydroisoquinoline, norlaudanosolinecarboxylic acid (NLCA), when added to the medium did not elevate PNMT activity. No increase in the amount of PNMT was detected by immunochemical titration of homogenates from DNLCA-treated cultured medulla, nor were there changes in rates of synthesis or degradation of the enzyme. Although DNLCA is an inhibitor of tyrosine 3-monooxygenase, it had no effect on PNMT activity when added directly to an incubation mixture *in vitro*. Kinetic analyses of dialyzed homogenates from explants cultured in the presence of DNLCA revealed that the V_{max} of PNMT was higher than that of control tissue. There was no decrease in K_m after DNLCA treatment. The increase in PNMT activity appears to be a compensatory response to depletion of medullary catecholamines by DNLCA or α-methyltyrosine.

Adrenal PNMT§ activity may be regulated by several different mechanisms. Changes in enzyme levels that arise by alteration in the rate of protein synthesis or degradation appear to be under the influence of adrenal cortical glucocorticoids, splanchnic nerve impulses, and adrenal epinephrine or *S*-adenosyl-L-methionine (SAM) levels [1–6]. A second mode of regulation occurs as an acute response of the enzyme to substrate and product inhibition [3, 7–11]. A third mechanism of regulation may entail the transformation of PNMT to more active forms of the enzyme. Multiple forms of PNMT have been detected [9–12], but have not been implicated in a regulatory mechanism.

In this paper, we report an increase in PNMT activity in rat adrenal medulla explants after exposure of the tissue to 3′,4′-deoxynorlaudanosolinecarboxylic acid (DNLCA), one of a group of tetrahydroisoquinolinecarboxylic acids (TIQCAs) capable of inhibiting tyrosine 3-monooxygenase [13, 14]. The effects of DNLCA on the activity, amount, rates of synthesis and degradation, and kinetics of PNMT from the explants have provided evidence for a previously unreported mechanism of regulation of PNMT activity.

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§ Abbreviations used: PNMT, phenylethanolamine *N*-methyltransferase; DNLCA, 3′,4′-deoxynorlaudanosolinecarboxylic acid; NLCA, norlaudanosolinecarboxylic acid; α-MT, α-methyltyrosine; TIQCA, tetrahydroisoquinolinecarboxylic acid; SAM, *S*-adenosyl-L-methionine; COMT, catechol *O*-methyltransferase; and MAO, monoamine oxidase.

MATERIALS AND METHODS

Normetanephrine, α-methyltyrosine and most other biochemicals were purchased from the Sigma Chemical Co. (St. Louis, MO). (±)TIQCAs were synthesized as previously described [15, 16]. L-[4,5-^{3}H(N)]Leucine, L-[^{14}C(U)]leucine and [methyl-^{14}C]SAM were purchased from the New England Nuclear Corp. (Boston, MA).

Explant culture. See the previous paper [16] for experimental details.

Enzyme assays. At the end of the culture period, the medullae were homogenized and dialyzed as previously described [17]. PNMT activity in a 50 μl aliquot of dialyzed homogenates was assayed by a modification of the method of Wurtman and Axelrod [1, 18]. Under these conditions, enzyme activity was linear for 45 min [18].

Assays of monoamine oxidase (MAO) in a 50 μl aliquot of dialyzed homogenates were performed by the method of Wurtman and Axelrod [19]. Catechol *O*-methyltransferase (COMT) activity was measured in 200 μl aliquots of dialyzed homogenates according to the method of Creveling and Daly [20], using 3,4-dihydroxybenzoic acid and SAM as substrates.

Protein concentrations in aliquots of dialyzed tissue homogenates were measured by the method of Lowry *et al.* [21]. A unit of enzyme activity represents 1 nmole of product per hr.

Immunochemical titration of PNMT. Explants of

adrenal medullae were homogenized, dialyzed, and clarified by centrifugation for 10 min at 6000 *g*. The supernatant fraction was used for measurement of the relative concentrations of PNMT. Antibody to rat medullary PNMT was prepared [12] and used to titrate the amount of enzyme in the 6000 *g* supernatant fraction from homogenates, as previously described [17].

Measurement of rates of synthesis and degradation of PNMT. Medullae were cultured for 20 hr in the presence or absence of 0.5 mM DNLCA as previously described [16]. The medullae were then changed to fresh media of the same composition to which 40 μCi of L-[^{14}C(U)]leucine was added. After 4 hr tissue samples were removed and analyzed for [^{14}C]leucine incorporation into total protein (trichloroacetic acid precipitable) and PNMT. The incorporation of radioactive leucine into PNMT was determined by immunochemical precipitation of the enzyme with specific antibody to rat adrenal PNMT as previously described [6]. The remaining medullae were changed to fresh nonradioactive media of the same composition and maintained for an additional 20 hr. The tissue was then transferred to fresh medium containing 200 μCi of L-[4,5-^{3}H(N)]leucine. Four hours later the tissue was analyzed for [^{14}C]- and [^{3}H]leucine incorporation into total protein and PNMT. The relative rate of PNMT synthesis is the amount of tritiated leucine incorporated into PNMT divided by that incorporated into total protein. The rate of degradation is the percentage of [^{14}C]leucine remaining in PNMT after 48 hr.

Evaluation of the results. Statistical comparisons of means of two independent samples were made using Student's *t*-tests on samples meeting the requirements of homoscedasticity. Kinetic parameters were determined by least squares analyses.

RESULTS

Exposure of medullary tissue to DNLCA for 40 hr resulted in a 69% increase in PNMT activity (Table 1, experiments 1 and 2). It should be noted that this increase in PNMT is of the same magnitude as that produced by agents such as reserpine [22] and insulin [18], which also deplete medullary catecholamines *in vivo*. As seen in Table 1, some control values for PNMT varied considerably. Similar fluctuations in controls after hypophysectomy have been observed previously [23, 24]. These studies demonstrated that adrenal medulla PNMT activities of hypophysectomized rats will fall at different rates because of a differential removal from steroid hormone regulation. A possible reason for the variation in control PNMT activity in explants, then, may be their removal from the influence of steroid hormones. Since comparisons were made on matched halves of adrenal medulla in all experiments, the variability of control PNMT activity was of no consequence to the interpretation of the data. A structurally related TIQCA, norlaudanosolinecarboxylic acid (NLCA), produced no increase in PNMT activity (Table 1, experiment 3). Dose-dependency studies revealed that the elevation of PNMT was maximal at 0.2 mM DNLCA. The increase in PNMT activity was noted only after 40 hr; there was no change during the first 30 hr of culture in the presence of DNLCA (Table 1, experiment 4). The activities of two other enzymes of catecholamine metabolism, MAO and COMT, were not affected by inclusion of 0.5 mM DNLCA in the media (control: 26.2 ± 2.2 and 2.5 ± 0.14 vs DNLCA-treated: 23.7 ± 3.9 and 2.2 ± 0.29 units/mg protein respectively).

The availability of specific antibodies to rat medullary PNMT allowed us to determine whether this

Table 1. Effect of TIQCA and α-MT on enzyme activities in cultured adrenal medullary explants*

Expt.	Compound added	PNMT activity (units/mg protein ± S.E.M.)
1	None	29.38 ± 3.94
	0.05 mM DNLCA	34.77 ± 3.50
2	None	15.26 ± 2.09
	0.2 mM DNLCA	21.81 ± 2.55†
	0.5 mM DNLCA	25.84 ± 2.47‡
	1.0 mM DNLCA	22.51 ± 2.93†
3	None	14.97 ± 3.21
	0.5 mM NLCA	9.76 ± 1.80
4§	None	37.56 ± 6.36
	1.0 mM DNLCA	33.30 ± 4.53
5§	None	29.39 ± 5.93
	0.5 mM α-Methyltyrosine	31.94 ± 3.44
6	None	18.39 ± 3.04
	0.5 mM α-Methyltyrosine	26.24 ± 3.41‡

* Adrenal medullary tissue was cultured for 40 hr in medium 199 in the presence or absence of DNLCA at the concentrations indicated. A change to fresh medium of identical composition was made at 20 hr. Dialyzed homogenates of the tissue were assayed for PNMT as described in Materials and Methods. Each mean is derived from five replicate cultures.

† $P < 0.05$

‡ $P < 0.01$.

§ In experiments 4 and 5 the medullae were assayed after 30 hr.

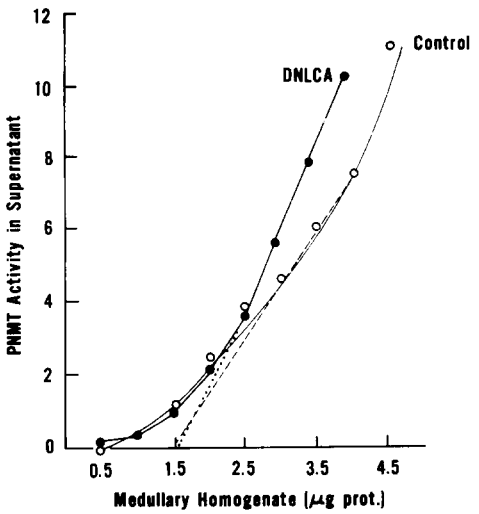

Fig. 1. Immunotitration of PNMT from rat adrenal medullae cultured in the presence or absence of DNLCA. Tissue equivalent to six medullae was placed in a dish containing medium 199 with or without 0.5 mM DNLCA. At the end of the incubation period, the tissue was treated as outlined under Materials and Methods. The resulting preparation was added to anti-PNMT antibody, and the activity of the enzyme remaining unprecipitated was determined by the usual assay procedure. Activity is expressed as dpm $\times 10^{-2}$ after 30 min.

change in PNMT activity was due to a change in the amount of enzyme. The immunotitration curve shows a shift to the left in PNMT activity from DNCLA-treated tissue, indicating an increase in activity (Fig. 1). However, the equivalence points, as noted by the intercept of the curves on the abscissa, are essentially the same. Thus, the increase in PNMT activity was not accompanied by an increase in amount of enzyme protein. To further confirm this point, we measured the rates of synthesis and degradation of PNMT and total protein after exposure of medullae to 0.5 mM DNLCA and radioisotopically labeled leucine. These results showed that there was no significant change in the relative rate of PNMT synthesis (i.e. 1.56 in DNLCA-treated medullae and 1.45 in controls). Of the [^{14}C]leucine incorporated into PNMT after 24 hr, 87% was detected at 48 hr in both DNLCA-treated and control tissue.

Although adrenal homogenates were dialyzed before they were assayed for PNMT, we could not rule out the possibility that DNLCA might be tightly bound and acting as an allosteric modulator of the enzyme. Thus, DNLCA was added directly to incubation mixtures of dialyzed homogenates. The concentrations of DNLCA chosen for this experiment were in the range of that found to be present in medullary tissue after exposure to 0.5 mM DNLCA in culture [16]. DNLCA in concentrations up to 0.5 mM did not increase PNMT activity when added directly to the incubation mixture (12.54 ± 0.16 for DNLCA-treated vs 13.33 ± 0.47 units/adrenal pair for control homogenates).

The DNLCA-induced increase in PNMT activity (Table 1) seemed to correlate well with our previous findings of a depletion of dopamine content of medullae exposed to DNLCA after 30 hr and of all three medullary catecholamines after 40 hr [16]. Further support for this notion was gained by the finding that NLCA, a structurally related TIQCA which failed to depress medullary catecholamine content after 40 hr of culture [16], failed to elevate PNMT activity (Table 1, experiment 3). To substantiate the hypothesis that the increased PNMT activity was related to the depletion of the medullary norepinephrine and epinephrine rather than to a specific effect of DNLCA, medullae were cultured in the presence of 0.5 mM α-methyltyrosine, and PNMT activity was measured. We have shown previously that exposure of this tissue to this known tyrosine hydroxylase inhibitor results in a marked depletion of dopamine at 30 hr and in a total catecholamine deficit at 40 hr [16]. Comparable to the elevation elicited by DNLCA, there was a 43% increase in PNMT activity after medullae were cultured in the presence of α-methyltyrosine at 40 hr but not at 30 hr (Table 1, experiment 6).

Because the increased activity could not be explained by either a direct effect of DNLCA on PNMT or by an increase in the amount of enzyme, we investigated the possibility that there was a conversion of the enzyme to a form having different kinetic characteristics. Kinetic analysis of PNMT activity *in vitro* indicates that there was an increase in V_{max} (Fig. 2) when SAM was the variable substrate. Least squares analyses in three separate experiments revealed that the V_{max} of PNMT from DNLCA-treated adrenals (114 ± 3.1 units/mg protein) was

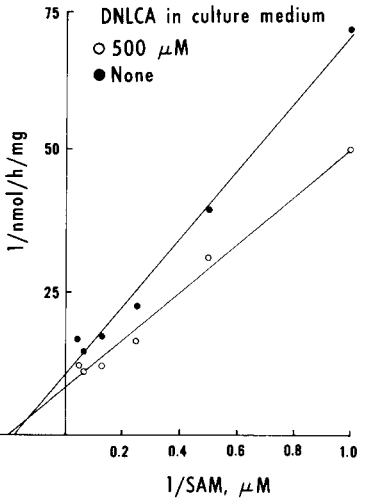

Fig. 2. Lineweaver–Burk plot of PNMT activity from rat adrenal medullae cultured in the presence or absence of DNLCA. Incubation mixtures contained from 1 to 24 μM [methyl-^{14}C]SAM in 4 mM phosphate, pH 7.9, 850 μM normetanephrine and PNMT from dialyzed homogenates of cultured medullae. Upon incubation for 30 min at 37°, the reaction was terminated, and PNMT was assayed as described under Materials and Methods. Estimated kinetic parameters: for control tissue, K_m of SAM = 5.6 μM, V_{max} = 94 units/mg protein; for DNLCA-treated tissue, K_m of SAM = 5.0 μM, V_{max} = 119 units/mg protein.

significantly greater than that of controls (92.7 ± 7.2 units/mg protein, $P < 0.05$). However, no significant decrease in K_m was observed (Fig. 2).

DISCUSSION

Several lines of evidence suggest that in the adrenal medulla the levels of both substrates and products of PNMT play a role in regulating this enzyme. Such regulatory influences appear to be expressed by changes in steady-state concentrations of PNMT [1, 3, 5, 6] as well as by direct inhibition of the enzyme [3, 8–11]. It is now known that multiple forms of PNMT can be isolated from bovine and rabbit adrenal medulla, and these forms possess distinctly different kinetic parameters [9–12]. In analogy with regulatory mechanisms of other enzymes, we propose that PNMT can also be controlled by interconversion to activated molecular forms as a result of changes in substrate or product levels in the adrenal medulla. The results reported herein provide preliminary evidence to support such a mechanism of regulation of PNMT.

First, two established inhibitors of tyrosine 3-monooxygenase have been shown to deplete catecholamine levels of adrenal medulla explants and cause an elevation of PNMT activity in a time-dependent manner. The timing of the PNMT activation relative to the onset of catecholamine decrease is consistent with a causal relationship. Increases in PNMT activity of a similar magnitude have also been noted *in vivo* after depletion of catecholamines by other agents [18, 22]. Furthermore, a structurally related TIQCA, NLCA, had no effect on medullary catecholamines and did not increase PNMT activity.

Second, the DNLCA-mediated increase in PNMT activity was not due to a change in the amount of enzyme protein either through alteration in the rate of synthesis or degradation. Furthermore, there was no direct effect of DNLCA on PNMT activity *in vitro*. There was, however, an increase in the V_{max} of PNMT and no decrease in K_m after exposure of medullae to DNLCA. This shift in enzyme kinetics correlates well with the findings of Lee *et al.* [9–11] who demonstrated the existence of multiple forms of PNMT having, in some cases, similar K_m values but different V_{max} values in rabbit. These results are consistent with the possibility of a similar multiplicity for rat adrenal PNMT.

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