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SHORT COMMUNICATIONS

Inhibition of phenylethanolamine-*N*-methyltransferase by human cerebrospinal fluid—a sodium chloride effect

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One of the current radiochemical methods for measuring dopamine-beta-hydroxylase (DBH) activity is the coupled enzymatic assay described by Molinoff *et al.* [1]. This procedure utilizes tyramine as substrate and involves the enzymatic beta-hydroxylation of tyramine to form octopamine (DBH step). The octopamine formed by the DBH is enzymatically *N*-methylated to radioactive *N*-methyl octopamine (synephrine) by phenylethanolamine-*N*-methyl transferase (PNMT) by the transfer of a labeled methyl group from *S*-adenosylmethionine (SAM). To quantify the octopamine formed during the DBH step, standard curves are constructed by the addition of variable amounts of octopamine to the entire reaction mixture, and the formation of radioactive synephrine is determined.

While performing assays of human cerebrospinal fluid DBH activity [2], it was observed that at the octopamine (0.08 to 0.64 μM) and SAM (1 μM) concentrations usually employed, there was a 50–60 per cent inhibition of the PNMT step by the cerebrospinal fluid (CSF), when compared with the activity obtained when the CSF was replaced either by distilled H_2O or 5 mM Tris–HCl, pH 7.4 (Table 1). The degree of inhibition produced by the CSF did not vary from patient to patient (n = 4).

To determine if the inhibitor(s) was generated during the DBH step and to characterize the nature of the inhibition, the activity of PNMT was assayed separately, maintaining the same ratios of enzyme (PNMT), substrates and CSF employed in the full DBH assay.

Bovine adrenal PNMT was purified by differential centrifugation, ammonium sulfate precipitation and gel filtration through Sephadex G-200 [1]. A typical reaction mixture contained 1.0 μl of enzyme preparation (34.0 μg protein); 18.8 μl of 1 M Tris–HCl buffer, pH 8.6; 0.2 μl [^{3}H]-methyl-SAM (New England Nuclear Corp.; sp. act. 12.6 Ci/m-mole; 0.1 μCi/assay); 10 μl of nonradioactive SAM dissolved in distilled H_2O (final SAM concentration ranged from 1 to 47.4 μM); and 40 μl of either 5 mM Tris–HCl, pH 7.4, distilled H_2O or CSF. The reaction was initiated by addition of 10 μl of the substrate, octopamine (final concentration ranged from 0.08 to 320 μM). After 20 min of incubation at 37°, the reaction was stopped with 0.1 ml of 0.5 M borate buffer, pH 10, and the [^{3}H]synephrine was extracted in a mixture of toluene–isoamylalcohol (3:2, v/v). After drying the samples at 80°, the residue was dissolved in 7.5 ml of a mixture containing 1000 ml toluene, 150 ml methanol and 5 g of 2,5-diphenyloxazole (PPO) and counted by liquid scintillation spectrometry. Blank values were obtained by omitting the substrate from the incubation mixture.

As can be seen in Fig. 1A, the omission of the DBH

Table 1. Effect of human CSF on the PNMT step of the DBH assay*

	Internal standards octopamine (pmoles)	Activity minus blank (cpm)	% Inhibition by CSF
H_2O†	6.25	6,465 ± 702	63.2 ± 5.1
CSF	6.25	2,383 ± 191	
H_2O†	12.5	12,902 ± 1,165	59.2 ± 4.2
CSF	12.5	5,134 ± 625	
H_2O†	25.0	20,925 ± 1,468	56.5 ± 5.1
CSF	25.0	8,895 ± 973	

* Aliquots of 40 μl CSF or distilled H_2O were used for each assay and incubated at 37° in a reaction mixture containing 4 μl of 1 M sodium acetate, pH 5.0; 5 μl of 0.5 M sodium fumarate; 2 μl of 12 mM pargyline; 0.4 μl catalase (Boehringer) containing 8 μg enzyme (1500 units); 2 μl of 30 mM tyramine; 3 μl of 113 mM sodium ascorbate, pH 5.0, and 3.6 μl of H_2O. After 2 hr of incubation, the pH of the reaction mixture was raised by adding to each tube 20 μl of a solution containing 17.2 μl of 1 M Tris–HCl buffer, pH 8.6; 1 μl of purified bovine adrenal phenylethanolamine-*N*-methyl transferase (PNMT); 1.5 μl of H_2O and 0.3 μl [^{3}H]methyl-*S*-adenosylmethionine ([^{3}H]SAM; sp. act.: 5.35 Ci/m-mole; final concentration: 1 μM). A total of 100 nCi [^{3}H]SAM was used per assay. The octopamine concentrations employed were 0.08, 0.16 and 0.32 μM. Forty-five min later, the PNMT reaction was stopped by the addition of 0.1 ml of 0.5 M borate buffer, pH 10. The labeled synephrine was extracted into 1.5 ml toluene–isoamylalcohol (3:2, v/v). After centrifugation at 1000 *g* for 10 min, 1 ml of the organic phase was transferred to a counting vial, and the samples were dried overnight in a chromatography oven at 80°. After it was dried, the residue was dissolved in 7.5 ml of a mixture containing 1000 ml toluene, 150 ml methanol and 5 g PPO and counted in a scintillation counter. To prevent the formation of octopamine during the DBH step, the CSF samples were maintained at 2° before the addition of the PNMT. Assays where the CSF was replaced either by distilled H_2O or 5 mM Tris–HCl were also incubated at 2° during the DBH step. Blanks averaged 970 ± 93 cpm.

† Similar results were obtained when distilled H_2O was replaced by 5 mM Tris–HCl, pH 7.4.

step did not modify the CSF inhibition of PNMT. These results suggested that the inhibitor(s) is a pre-existing species in the CSF and it is not generated during the incubation of the DBH step.

Dilution of the CSF with distilled H_2O (1:2, v/v) resulted in a proportional reduction of the inhibition (Fig. 1A). Furthermore, the inhibitor(s) was not destroyed by heating the CSF at 95° for 10 min, but it was removed by dialyzing the CSF against either distilled H_2O or 5 mM Tris–HCl, pH 7.4 (Fig. 1A). Storage of the CSF samples at −4° or at 2° for 24–48 hr failed to modify the CSF–PNMT inhibitory effect.

The substrate-velocity relationships for PNMT are shown in Fig. 1B. The apparent K_m for octopamine was 7.5 μM. Employing octopamine as the variable substrate and SAM at a fixed concentration (47.4 μM), an inhibition of the enzyme activity was observed at high octopamine concentrations, as previously described [3]. Under the same experimental conditions, an inhibition of PNMT activity was obtained in the presence of the CSF (Fig. 1B). This inhibition was reversed when high octopamine concentrations were employed (Fig. 1B). In another set of experiments (n = 3) in which SAM was the variable substrate (4.7 to 47.4 μM) and octopamine was held at a fixed saturating concentration (32 μM), the CSF failed to modify the activity of PNMT.

The thermal stability and the readily dialyzable nature of the inhibitor(s) suggested that the effects of the CSF on PNMT activity could be mediated by ions. Due to the high concentration of NaCl in the CSF (150 mM), experiments were carried out in which the CSF was dialyzed against 5 mM Tris–HCl with and without 150 mM NaCl. As can be seen in Fig. 1A, the addition of NaCl to the dialysis solution restored the inhibitory effect of the CSF on the PNMT reaction.

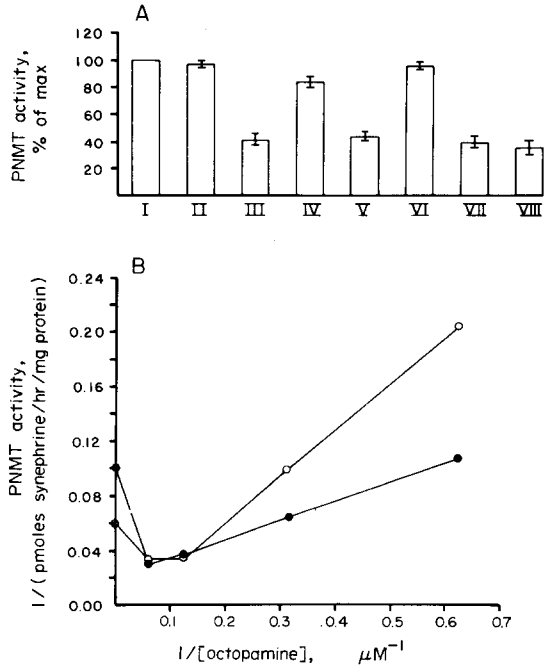

Fig. 1. Inhibition of PNMT activity by human CSF. (A) Effects of dilution, temperature, dialysis and addition of NaCl. Assays were performed as described in the text. PNMT activity was measured in the presence of distilled H_2O (I), 5 mM Tris–HCl (II), CSF (III), CSF diluted 1:2 (v/v) with distilled H_2O (IV), CSF heated at 95° for 10 min (V), CSF after dialysis against 5 mM Tris–HCl, pH 7.4 (VI), CSF after dialysis against 5 mM Tris–HCl, pH 7.4, containing 150 mM NaCl (VII), and H_2O or 5 mM Tris–HCl, pH 7.4, containing 150 mM NaCl (VIII). The concentrations of octopamine and SAM employed were 1.6×10^{-7} M and 1.0 μM respectively. (B) Lineweaver–Burk plot plot of the effects of human CSF on bovine adrenal PNMT. SAM concentration: 47.4 μM. Key: (●) distilled H_2O; and (○) CSF.

Moreover, when NaCl was added to the reaction mixture (75 mM final concentration), the degree of PNMT inhibition was found to be similar to that produced by the CSF (Fig. 1A). More recently it has been demonstrated that the kinetics of inhibition of PNMT activity by NaCl is similar to that obtained with the CSF (L. Cubeddu X. and M. Gimenez, manuscript submitted for publication). These results indicate that the inhibition of PNMT activity is mediated by the high NaCl concentration present in the CSF.

The high NaCl content of the extracellular fluid reduces the sensitivity of the coupled enzymatic assay for determinations of DBH activity, particularly when the low enzymatic activity makes it mandatory to work with undiluted samples and the amount of octopamine formed is very small, as in the case of the human CSF [2]. A similar problem arises for determinations of the nerve stimulation mediated release of DBH activity in preparations perfused by or incubated in solutions with a high NaCl content (Krebs–Ringer bicarbonate or Krebs–Ringer phosphate) [4–6]. Many of the assays which ultimately utilize a SAM-dependent *N*-methylase to measure endogenous biogenic amines levels [7] or enzyme activity [1] do so at substrate concentrations well below saturation [3–5]. In all these cases, the concentrations of the *N*-methyl acceptor substrate were one order of magnitude lower than its apparent K_m (7.5 μM present study). Therefore, unusual salt, pH, and other effects not observed with isolated purified enzyme experiments at high levels of substrate(s) should be considered as likely possibilities when using those assays.

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