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EVIDENCE FOR THE METHYLATION OF APOMORPHINE BY CATECHOL-*O*-METHYL-TRANSFERASE *IN VIVO* AND *IN VITRO*

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Abstract—Pretreatment with either pyrogallol, tropolone or 8-hydroxyquinoline enhanced markedly the mean stereotyped behavior scores after apomorphine treatment in the rat. Experiments *in vitro*, using rat liver or brain catechol-*O*-methyltransferase (COMT) preparations and ^{14}C-methyl-*S*-adenosyl-L-methionine, demonstrated that apomorphine was methylated by this enzyme system. The apparent K_m values for dopamine and apomorphine were $2{\cdot}6 \times 10^{-4}$ M and $1{\cdot}4 \times 10^{-3}$ M, respectively, for liver COMT. Pyrogallol and tropolone inhibited the methylation of apomorphine *in vitro* competitively when the apomorphine concentration was varied. These results suggest that methylation by COMT may represent an important metabolic pathway for the deactivation of apomorphine *in vivo*.

THE ANTI-PARKINSON activity of apomorphine, reported recently by Cotzias *et al.*,[1] has stimulated interest in the pharmacology of this compound. Apomorphine contains in its structure the elements of a catecholamine and possesses structural similarities to dopamine. The biochemical and pharmacological data accumulated thus far[2–7] support the original suggestion[8] that apomorphine selectively activates dopaminergic receptors.

The metabolic fate of apomorphine has not been extensively studied. Kaul *et al.*[9–12] concluded from studies in rodents that glucuronide formation was the major metabolic pathway for apomorphine. However, these authors could not account for 20–30 per cent of the total apomorphine administered, suggesting the existence of other metabolic pathways.

The participation of *S*-adenosyl-L-methionine:catechol-*O*-methyltransferase, EC 2.1.1.6 (COMT), in the metabolism of endogenous catecholamines is well documented. Furthermore, inhibition of COMT potentiates the biochemical effects of 3,4-dihydroxyphenylalanine[13,14] and the pharmacological effects of isoproterenol.[15] Since the apomorphine molecule contains a catechol moiety, it was rationalized that this agent might also be metabolized by COMT, and that inhibition of this enzyme should potentiate the pharmacological effects of apomorphine.

We have studied: (1) the effects of COMT inhibitors on apomorphine-induced stereotyped behavior in the rat and (2) the methylation of apomorphine by COMT *in vitro*. The results of this work have been reported in preliminary form.[16]

MATERIALS AND METHODS

Apomorphine-induced stereotyped behavior. Long Evans, male rats weighing 200–300 g were placed in circular, wire-mesh cages for 30 min or until normal exploratory activity had ceased. Control animals and animals pretreated with COMT inhibitors were given a subcutaneous injection of apomorphine and scored under blind conditions. The stereotyped behavior after apomorphine was quantitated using the following scoring system, sniffing, 1 point; licking and chewing at the wire, yet moving about the cage, 2 points; chewing mostly in one location, 3 points; and intensive gnawing on the wire, 4 points. All animals were scored at 10-min intervals for a maximum of 3 hr.

COMT inhibitors were injected intraperitoneally according to the following schedule: pyrogallol, 250 mg/kg and 150 mg/kg; tropolone, 100 mg/kg and 50 mg/kg; and 8-hydroxyquinoline, 100 mg/kg and 100 mg/kg; 30 min before and 1 hr after apomorphine respectively. The effects of these drugs on stereotyped behavior were tested against a wide range of doses of apomorphine in the case of pyrogallol, or against standard doses of 1 mg/kg and 5 mg/kg of apomorphine in animals pretreated with tropolone and 8-hydroxyquinoline.

Catechol-O-methyltransferase in vitro. To demonstrate that apomorphine is methylated by COMT, experiments *in vitro* were conducted using either rat liver or brain as an enzyme source. The enzyme was partially purified by a modification of the method used by Axelrod and Tomchick.[17] The crude homogenate was filtered through cheesecloth, centrifuged and then filtered through glass wool to remove fat particles. After centrifugation at 100,000 *g*, the supernatant was dialyzed against 1 mM sodium phosphate buffer, pH 7·0, and then slowly adjusted to pH 5·3 with 1 N acetic acid. After centrifugation at this pH, the supernatant was neutralized and fractionated with ammonium sulfate. The pellet obtained between 30 and 55% ammonium sulfate was resuspended in 1 mM sodium phosphate buffer and stored at −65°.

The enzyme assay procedure was essentially that of Axelrod,[18] in which ^{14}C-methyl-*S*-adenosyl-L-methionine (^{14}C-SAM) was the methyl donor, and the ^{14}C-methylated products were extracted into 10 ml of a mixture of toluene and isoamyl alcohol (3:2, v/v). After a 5-min centrifugation at 600 *g*, 5 ml of the organic layer was placed in a scintillation vial with 12 ml of a scintillation mixture (4 g Omnifluor per liter of a 2:1 toluene–Triton X-100 mixture) and counted in a Packard Tri-Carb scintillation spectrometer. In a typical assay, components in a total volume of 0·42 ml, pH 7·8, were as follows: ^{14}C-SAM, 0·60 mM, 0·1 mCi/m-mole; catecholamine substrate varied from 0·07 to 0·7 mM; Mg^{2+}, 3 mM; Tris, 50 mM; and 1–4 mg of extract protein. The reaction was started by adding the substrate, ^{14}C-SAM, incubated at 37° for 30 min, and terminated by adding 0·5 ml of half molar borate, pH 10. Blank assays, containing extract but no catechol substrate, were incubated at the same time and gave between 50 and 70 cpm with different extract preparations. Blank values were subtracted from counts obtained in the assays. Assays were performed in duplicate or triplicate with a maximum deviation of 3 per cent. Stock solutions of catecholamines were freshly prepared in oxygen-free water and protected from light.

For paper chromatography of incubation products, aliquots were spotted on Whatman No. 1 paper and eluted with a mixture of *n*-butyl alcohol–glacial acetic acid–ethanol (7:2:2, v/v/v). Paper strips were scanned using a Nuclear Chicago Actigraph III.

RESULTS

Apomorphine-induced stereotyped behavior. Doses of apomorphine ranging from 0·5 mg/kg to 20 mg/kg produced consistent and dose-related increases in mean behavioral scores (Fig. 1a). The duration of stereotyped behavior also increased with increasing dose (Fig. 1, b and c). A dose of 1 mg/kg produced stereotyped behavior lasting approximately 1 hr, whereas 20 mg/kg induced stereotyped behavior for 3 hr.

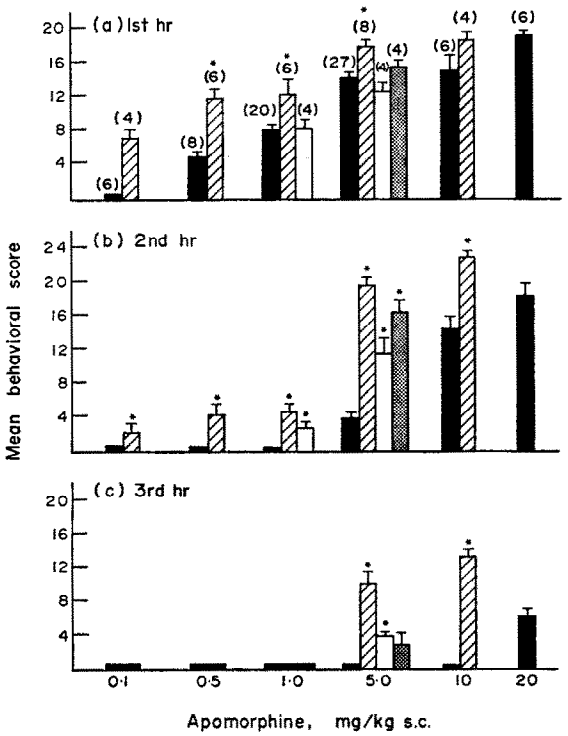

FIG. 1. Mean stereotyped behavior scores after apomorphine treatment. (a), (b) and (c) show the mean scores during the first, second and third hr respectively. (■) Control animals receiving only apomorphine; (▨) pyrogallol pretreated; (□) tropolone pretreated (▩) 8-hydroxyquinoline pretreated. Asterisk indicates significantly different ($P < 0·05$) from control animals for that dose of apomorphine. P values were calculated using Student's *t*-test.

Pyrogallol treatment (250 mg/kg plus 150 mg/kg) caused a marked potentiation of the effects of apomorphine. First, pyrogallol lowered the threshold for the induction of stereotyped behavior from 0·5 mg/kg to 0·1 mg/kg of apomorphine (Fig. 1a). Second, the mean behavioral scores at all doses of apomorphine were significantly increased during the first hr observation period (Fig. 1a). Furthermore, the duration of action of all doses of apomorphine was approximately doubled (Fig. 1, b and c). Lastly, the second-hr mean scores after 5 and 10 mg/kg of apomorphine were increased from $4 \pm 0·08$ and $14·7 \pm 1·4$ to $19·8 \pm 1·0$ and $23·1 \pm 0·9$ respectively (Fig. 1b).

Treatment with tropolone (100 mg/kg plus 50 mg/kg) or 8-hydroxyquinoline (100 mg/kg plus 100 mg/kg) did not produce statistically significant increases in mean

behavioral scores during the first-hr observation period (Fig. 1a). However, both treatments produced a marked prolongation of stereotyped behavior and significant increases in the mean scores during the second-hr scoring period (Fig. 1, b and c).

In the absence of apomorphine, none of the COMT inhibitors produced stereotyped behavior.

Catechol-O-methyltransferase in vitro. The specific activities of the partially purified extracts of COMT, using the maximum velocities obtained with dopamine as substrate, were 5 and 0·9 nmoles of *O*-methylated product/min/mg of protein. The apparent K_m values for dopamine were $2{\cdot}6 \times 10^{-4}$ M for liver enzyme and $2{\cdot}3 \times 10^{-4}$ M for brain enzyme. These are similar to the K_m values reported for norepinephrine[19,20] with liver extract and somewhat lower than that reported for dopamine.[21]

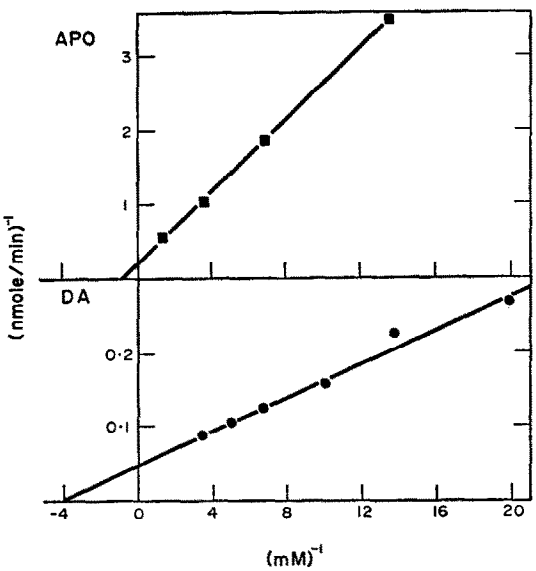

FIG. 2. Substrate kinetics for apomorphine or dopamine methylation by rat liver COMT. Ordinate is reciprocal of initial velocity expressed as nanomoles of *O*-methylated product formed per min. Abscissa represents reciprocal of substrate concentration: (■), apomorphine; (●) dopamine. Each assay contained approximately 4 mg of extract protein. Assays were performed in duplicate with maximum variation of < 3 per cent.

When the COMT extracts were incubated with ^{14}C-SAM and apomorphine, a ^{14}C-methylated product was obtained. The formation of this product was a function of the initial apomorphine concentration (Fig. 2). The apparent K_m value for apomorphine found with various liver enzyme preparations was $1{\cdot}4 \pm 0{\cdot}3$ mM. There was no significant difference between results obtained with liver and brain extracts.

Further evidence for the enzymatic methylation of apomorphine is illustrated in Fig. 3a, which shows tracings of radioactivity on paper chromatograms obtained from incubation mixtures of liver COMT and ^{14}C-SAM. The activity at the origin is that of the substrate, ^{14}C-SAM. Apomorphine also remained at the origin in this system. However, when both ^{14}C-SAM and apomorphine were present, a ^{14}C-methyl-labeled product appeared with an R_f of 0·87. This product was also fluorescent under

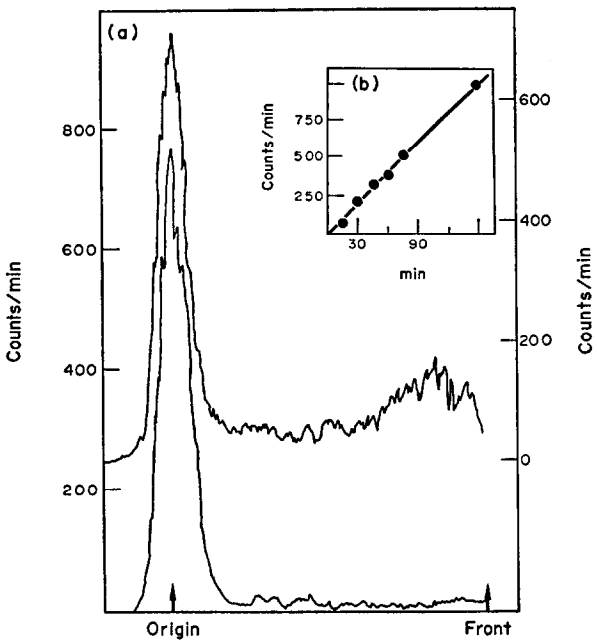

FIG. 3. Chromatography of products after incubation of apomorphine with liver COMT. (a) One mg of extract protein was incubated with 0·6 mM ^{14}C-methyl-SAM (1 mCi/m-mole) in the absence (lower scan) or presence (upper scan) of 1 mM apomorphine. After 1 hr at 37°, 40-μl aliquots were chromatographed on Whatman no. paper 1, using as developing solvent a mixture of butanol–glacial acetic acid–ethanol (7:2:2, v/v/v). The paper chromatographic strips were counted in a Nuclear Chicago strip scanner. (b) Results of a similar experiment in which the incubation was terminated at various times and, after chromatography, the area of the chromatogram containing the product was counted in a Packard Tri-Carb scintillation spectrometer.

u.v. light. Its formation was dependent on the presence of the enzyme; therefore, a non-enzymatic reaction between apomorphine and SAM did not occur. The small plot in Fig. 3b shows that the formation of the methylated product of apomorphine was linear with time for at least 2 hr.

Since known inhibitors of COMT potentiated the activity of apomorphine *in vivo*, as described in the previous section, the effect of COMT inhibitors on apomorphine

TABLE 1. COMPETITIVE INHIBITION OF APOMORPHINE (APO) METHYLATION BY KNOWN COMT INHIBITORS

Inhibitor	K_i (APO)* (μM)	K_i (NE)† (μM)
Tropolone	10	12
Pyrogallol	11	17

* K_i was calculated from slopes of reciprocal plots obtained by varying apomorphine concentration in the absence and presence of inhibitors. Liver enzymes were used.

† NE (norepinephrine) data are from Belleau and Burba.[22]

methylation was studied *in vitro*. In Table 1 inhibition constants obtained using apomorphine as substrate for the liver enzyme are compared with values reported by other workers who used norepinephrine as substrate. Pyrogallol, which is itself an alternate substrate for COMT, appeared to compete with apomorphine at concentrations as low as 2 μM. The apparent inhibition constant for pyrogallol competition with apomorphine was 11 μM. In addition, it was found that the mechanism of tropolone inhibition was competitive (Fig. 4).

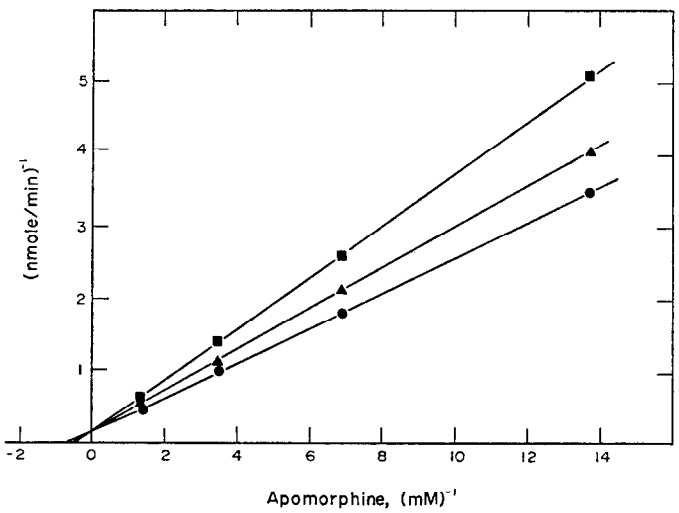

FIG. 4. Tropolone as inhibitor of apomorphine methylation. Reciprocal of liver enzyme activity is plotted against reciprocal of concentration of the substrate, apomorphine, in the absence and presence of tropolone. (●) Control; (▲) 2·5 μM tropolone; (■) 8·0 μM tropolone. Details of the enzyme assay are in Methods.

DISCUSSION

The potentiation of apomorphine by COMT inhibitors is consistent with the results of other authors who have shown that the pharmacological effects of other catechols, isoproterenol[15,23] and L-3,4-dihydroxyphenylalanine (L-dopa),[14,23] are potentiated *in vivo* by prior treatment with a COMT inhibitor. This potentiation of apomorphine suggests that apomorphine is metabolized by COMT in both liver and brain. Inhibition of liver COMT would be expected to elevate blood levels of apomorphine, resulting in larger amounts reaching the brain. However, both pyrogallol and tropolone, at the doses used in this study, effectively inhibit brain COMT in the rat.[14,23,24] Baldessarini and Chace[14] have concluded that at least part of the potentiating effects of pyrogallol on the accumulation of ^{3}H-L-dopa and its catecholamine products in the brain is a result of brain COMT inhibition. Apomorphine, unlike endogenous catecholamines, is not potentiated by monoamine oxidase inhibitors[25] (and unpublished observations), suggesting that apomorphine does not undergo significant deamination *in vivo*. Therefore, methylation may represent an important metabolic route for the inactivation of apomorphine, particularly in brain tissue, and inhibition of brain COMT alone could account for the potentiation of apomorphine observed in this study.

The data clearly show that apomorphine is methylated *in vitro* and that this methylation is dependent upon the presence of ^{14}C-SAM and the enzyme catechol-*O*-methyltransferase. However, since the end product of this enzymatic reaction was not chemically identified, one may only speculate as to the site of methylation. The only conceivable sites for methylation would be on one or both of the hydroxyl groups of apomorphine. It is known that, although liver COMT can probably methylate most other catechols in either the *para-* or *meta*-position, the enzyme methylates catecholamines predominantly in the *meta*-position.[21,26] Thus, it is presumed that apomorphine would be methylated at the 11-hydroxy group.

The methylation of apomorphine *in vitro* was competitively inhibited by both tropolone and pyrogallol. It has been reported that both of these COMT inhibitors competitively inhibit the *O*-methylation of norepinephrine,[22] with inhibition constants very similar to those found in this study with apomorphine as substrate (Table 1). Most authors[22,24,27–29] report competitive inhibition for tropolone and pyrogallol although a relatively small non-competitive component has been proposed for pyrogallol.[30] The similarities between the inhibition kinetics observed using either norepinephrine or apomorphine as substrate (Table 1) suggest that apomorphine binds to the same enzyme form as does norepinephrine and other catecholamines.

It is concluded that apomorphine is metabolized *in vivo* and *in vitro* by liver and brain COMT. Potentiation of the centrally mediated behavioral effects of apomorphine by known COMT inhibitors suggests that methylation by COMT may represent an important pathway for the metabolism of apomorphine *in vivo*.

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