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EFFECTS OF APOMORPHINE *IN VITRO* ON THE UPTAKE AND RELEASE OF CATECHOLAMINES IN CRUDE SYNAPTOSOMAL PREPARATIONS OF RAT STRIATUM AND HYPOTHALAMUS

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Abstract—It is currently believed that apomorphine exerts its pharmacological effects primarily by a direct stimulatory action on post-synaptic dopaminergic receptors. In the present study, uptake of ^{3}H-dopamine into crude synaptosomal preparations of rat striatum and uptake of ^{3}H-norepinephrine into crude synaptosomal preparations of rat hypothalamus were inhibited 50 per cent by concentrations of apomorphine of $3{\cdot}6 \pm 0{\cdot}5 \times 10^{-5}$ M and $3{\cdot}2 \pm 0{\cdot}2 \times 10^{-5}$ M respectively. Lineweaver–Burk plots of ^{3}H-catecholamine uptake data revealed that apomorphine exhibited mixed inhibition kinetics for dopamine uptake into striatum ($K_i = 1{\cdot}91 \pm 0{\cdot}19 \times 10^{-5}$ M) and mixed inhibition kinetics for norepinephrine uptake into hypothalamus ($K_i = 2{\cdot}0 \pm 0{\cdot}17 \times 10^{-5}$ M). In crude synaptosomes of rat striatum, apomorphine elicited a dose-dependent release of ^{3}H-dopamine during a 5-min incubation period at 37°. In contrast, the drug failed to produce a significant release of ^{3}H-norepinephrine from crude synaptosomes obtained from rat hypothalamus. While these results suggest that apomorphine in high concentrations is capable of inhibiting the uptake of catecholamines and stimulating the release of dopamine at nerve endings, it is unlikely that these events contribute significantly to the behavioral effects elicited in animals after administration of the drug, since such effects occur at much lower doses.

Most of the biochemical and pharmacological data accumulated thus far would appear to support the contention that apomorphine exerts its primary effects in the central nervous system by a direct stimulatory action on the post-synaptic dopaminergic receptors [1–6]. Thus, the decreased rate of dopamine synthesis, a pre-synaptic event, has been explained on the basis of a negative feedback mechanism initiated by stimulation of the post-synaptic dopamine receptor by apomorphine [7, 8].

More recent evidence also suggests that apomorphine can inhibit dopamine synthesis and elicit changes in dopamine turnover by stimulation of pre-synaptic, inhibitory, dopaminergic receptors [9–13].

Apomorphine can inhibit the biosynthesis of labeled dopamine from labeled tyrosine in striatal slices at concentrations which are lower than those required to inhibit preparations of tyrosine hydroxylase obtained from bovine adrenal glands [14]. The more effective inhibition of labeled dopamine biosynthesis in striatal slices could be due to feedback control of dopamine synthesis resulting from stimulation of pre-synaptic dopamine receptors or could be due to accumulation of the drug in dopamine-containing neurons with its subsequent inhibition of tyrosine hydroxylase [15]. These observations, together with the fact that the drug has a catechol as a part of its structure, suggest the possibility that apomorphine may also have the potential to inhibit the uptake and/or modify the release of biogenic amines in pre-synaptic nerve endings. Alteration of one or both of these pre-synaptic processes could conceivably account for at least part of the behavioral effects elicited in animals after administration of apomorphine. The present studies were designed, therefore, to determine whether apomorphine was capable of altering the uptake and/or release of dopamine (DM) and norepinephrine (NE) in nerve endings obtained from striatum and hypothalamus.

MATERIAL AND METHODS

Crude synaptosomal preparations. Male albino rats of the Sprague–Dawley strain weighing from 200 to 300 g were killed by decapitation, and their brains were rapidly removed and dissected on an ice-filled Petri dish. The hypothalamus and striatum (including caudate nucleus, putamen and globus pallidus) were immediately weighed and homogenized in 20 vol. of 0·32 M sucrose containing 25 mM Tris buffer, pH 7·4. All homogenizations were performed with ten up-and-down strokes with a pestle revolution of 840 rev/min. The Teflon pestle (A. H. Thomas Co., Philadelphia, Pa.) was ground to obtain a clearance of 0·025 cm between it and the glass vessel. The nuclei and cell debris were separated by centrifugation at 1000 *g* for 10 min. The supernatant was gently separated from the 1000 *g* pellet and thoroughly mixed to yield a uniform suspension. This supernatant served as the source of the crude synaptosomal preparations.

Uptake studies. The standard incubation medium contained: NaCl, 145 mM; KCl, 3 mM; $CaCl_2$, 1·28 mM; $MgCl_2$, 1·19 mM; glucose, 11 mM; Tris buffer (pH 7·4), 25 mM; ascorbate, 1 μM; iproniazid, 10 μM; and 0·2 μM ^{3}H-dopamine (0·08 μCi/ml) or 0·2 μM ^{3}H-(±)-norepinephrine (0·08 μCi/ml) and 0·5 ml of the crude synaptosomal preparation in the

final volume of 3·0 ml. All incubations were conducted at 37° for 5 min. The uptake at 0° was also studied for each experiment in order to determine the amount of tritiated amines accumulated by non-specific processes. Unless specified otherwise, the amount of ^{3}H-amine accumulated by the temperature-insensitive process (uptake at 0°) was subtracted from the total uptake at 37° to yield the uptake by the temperature-sensitive process only (37° − 0°). All drugs used in the study were dissolved in the incubation medium and were preincubated with the crude synaptosomal preparations for 5 min at 37° or 0° before the addition of the labeled substrate. Because of the ease of oxidation of apomorphine, the drug was always prepared immediately before the incubation procedures were to begin. After the addition of the labeled catecholamine, the incubations were allowed to proceed for an additional 5 min under an atmosphere of 95% O_2–5% CO_2. The reaction was stopped by adding 2·0 ml of ice-cold 0·32 M sucrose containing 25 mM Tris buffer, pH 7·4, to each incubation tube and then cooling the tubes in ice. After centrifugation at 49,600 *g* for 10 min, the supernatant was discarded, and the pellet was washed by gentle resuspension in 5·0 ml of 0·9% NaCl by means of a thin glass rod. The suspension was again centrifuged at 49,600 *g* for 10 min, the supernatant was discarded, and the pellet was resuspended in 2·0 ml of 0·4 N perchloric acid. The precipitated protein was removed by centrifugation, and a 1·0-ml aliquot of the supernatant was added to 9·0 ml scintillation fluid (2:1 mixture of toluene and Triton X-100 containing 2,5-diphenyloxazole and 1,4-bis-[2-(5-phenyloxazolyl)]benzene as fluors) and counted in a Packard Tri-Carb liquid scintillation counter, model 3320.

The IC_{50} values were determined by incubating the crude synaptosomal preparations from hypothalamus and striatum in the presence of 0·2 μM ^{3}H-dopamine or 0·2 μM ^{3}H-(±)-norepinephrine and various concentrations of the inhibitor. At least four different concentrations of the drug, producing from 30 to 70 per cent inhibition of uptake of the catecholamines, were used to determine the IC_{50} values. The results were plotted on semilogarithmic paper, and the line was determined by connecting the points on the graph.

Kinetic analysis of the uptake data for ^{3}H-(±)-norepinephrine and ^{3}H-dopamine was determined with a General Electric 265 computer with the aid of the FORTRAN program of Cleland [16] for enzyme kinetic data. Results were graphically represented by means of the typical Lineweaver–Burk plots.

Release studies. In studies designed to determine both the spontaneous and drug-induced release of either ^{3}H-(±)-norepinephrine or ^{3}H-dopamine from crude synaptosomal preparations of hypothalamus or striatum, the following procedure was employed. Homogenates were prepared as previously described. A crude synaptosomal pellet was obtained by centrifuging the 1000 *g* supernatant at 49,600 *g* for 10 min. The 49,600 *g* pellet was resuspended in 20 vol. of the previously described incubation medium for every g of original wet weight of fresh tissue. An aliquot (5·0 ml) was incubated in the presence of 0·2 μM ^{3}H-dopamine or 0·2 μM ^{3}H-(±)-norepinephrine for a period of 5 min at 37° with agitation in an atmosphere of 95% O_2–5% CO_2. The uptake of the labeled amines was stopped by cooling on ice. The crude synaptosomal preparation was centrifuged at 49,600 *g* and the pellet was washed with 5·0 ml of 0·9% NaCl to remove any loosely bound ^{3}H-amine. The wash was discarded after centrifugation, and the crude synaptosomal pellet was resuspended with incubation medium to its original volume. A 0·5-ml aliquot of this suspension was added to 2·5 ml fresh incubation medium and the release of either ^{3}H-dopamine or ^{3}H-(±)-norepinephrine was studied at 37° in the presence and absence of the drug. All release studies were conducted for a period of 5 min in an atmosphere of 95% O_2–5% CO_2 with agitation. The per cent release in the absence of the drug (spontaneous release) was subtracted from the per cent release in the presence of the drug to yield the percentage of ^{3}H-amine released by the drug alone (drug-induced release). The rest of the procedure was identical to that already described for the uptake studies.

Dopamine-2-^{3}H (8·02 Ci/m-mole; radiochemical purity > 97 per cent) and ±-norepinephrine-7-^{3}H (10·1 Ci/m-mole; radiochemical purity > 98 per cent) were obtained from New England Nuclear (Boston, Mass.). Apomorphine was obtained from S. B. Penick & Co. (New York, N.Y.).

RESULTS

Effect of apomorphine on uptake of catecholamines into striatum and hypothalamus. The effect of various concentrations of apomorphine on the 5-min uptake of 0·2 μM solutions of ^{3}H-(±)-norepinephrine and ^{3}H-dopamine into crude synaptosomal preparations of rat hypothalamus and striatum is shown in Figs. 1 and 2. Apomorphine was an equipotent inhibitor of dopamine uptake into striatum and norepinephrine uptake into hypothalamus. The IC_{50} for apomorphine as an inhibitor of dopamine uptake into crude striatal synaptosomes was 3·6 ± 0·5 × 10^{-5} M in four separate experiments, while the IC_{50} for the drug as an inhibitor of norepinephrine uptake into crude hypothalamic synaptosomes was 3·2 ± 0·2 × 10^{-5} M in four experiments.

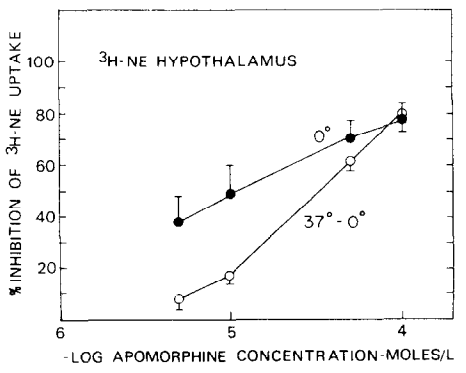

Fig. 1. Effect of apomorphine on the 5-min uptake at 0° and 37° − 0° of 0·2 μM ^{3}H-(±)-norepinephrine into crude synaptosomal preparations of rat hypothalamus. Data are expressed as per cent of the respective controls. Control uptake at 0° was 805 ± 285 cpm. Temperature-dependent uptake (37° − 0°) was calculated as described in Methods. Each point represents the mean ± S. E. of four separate experiments.

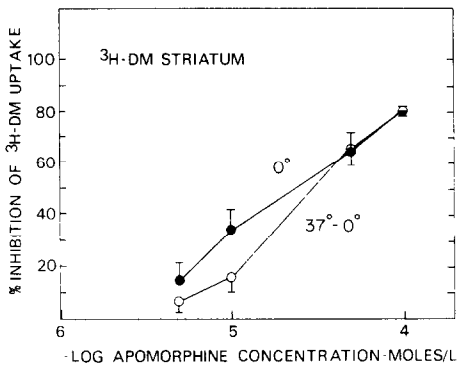

Fig. 2. Effect of apomorphine on the 5-min uptake at 0° and 37° − 0° of 0·2 μM ^{3}H-dopamine into crude synaptosomal preparations of rat striatum. Data are expressed as per cent of the respective controls. Control uptake at 0° was 2,130 ± 261 cpm. Control uptake at 37° was 44,303 ± 2,892 cpm. Temperature-dependent uptake (37° − 0°) was calculated as described in Methods. Each point represents the mean ± S. E. of four separate experiments.

Not only did apomorphine block the temperature-dependent uptake of the ^{3}H-amines in a dose-dependent manner, but it also blocked the temperature-insensitive uptake (uptake at 0°) in a similar manner. However, the inhibition by apomorphine of catecholamine uptake at 0° was variable, as indicated by the large standard errors of the means in Figs. 1 and 2. It should be noted that the uptake of ^{3}H-catecholamine at 0° contributes less than 25 per cent of the total uptake observed for ^{3}H-catecholamine at 37°.

Effects of apomorphine on the release of ^{3}H-catecholamines from striatum and hypothalamus. Table 1 demonstrates that in crude synaptosomal preparations of striatum, apomorphine (APO) elicited a dose-dependent release of ^{3}H-dopamine during a 5-min incubation at 37°. In contrast, apomorphine produced a slight non-dose-dependent release of ^{3}H-(±)-norepinephrine from crude synaptosomal preparations of hypothalamus.

Kinetic analysis of the uptake of ^{3}H-catecholamines in the presence and absence of apomorphine. In order to determine the nature of the inhibition of catecholamine uptake by apomorphine, the 5-min uptake of a wide range of concentrations of ^{3}H-(±)-norepinephrine in hypothalamus and ^{3}H-dopamine in striatum was studied at 37° and 0° in the presence and absence of apomorphine. The rate of uptake of the ^{3}H-catecholamine was rapid but linear for at least 5 min of incubation at all substrate concentrations used (0·05 μM to 0·5 μM) in both brain areas. The uptake at 0° was subtracted from the uptake at 37° and the data were analyzed by means of the typical Lineweaver–Burk plots. The results are shown in Figs. 3 and 4. Apomorphine exhibited mixed inhibition kinetics for dopamine uptake into striatum and mixed inhibition kinetics for norepinephrine uptake into hypothalamus. The K_m for dopamine uptake in striatum was $4{\cdot}2 \pm 0{\cdot}25 \times 10^{-7}$ M, while the K_i for apomorphine as an inhibitor of dopamine uptake was $1{\cdot}91 \pm 0{\cdot}19 \times 10^{-5}$ M in three separate experiments. In hypothalamus, the K_m for norepinephrine uptake was $4{\cdot}2 \pm 0{\cdot}31 \times 10^{-7}$ M and the K_i for apomorphine as an inhibitor of norepinephrine uptake was $2{\cdot}0 \pm 0{\cdot}17 \times 10^{-5}$ M, in three separate experiments.

Additional studies. For comparison purposes, morphine in concentrations ranging from 10^{-8} to 10^{-4} M was studied in conjunction with apomorphine to determine if the opiate could block the uptake of catecholamines in crude synaptosomal preparations of hypothalamus and striatum. At a concentration of 10^{-4} M, morphine only produced a 17 ± 4 per cent

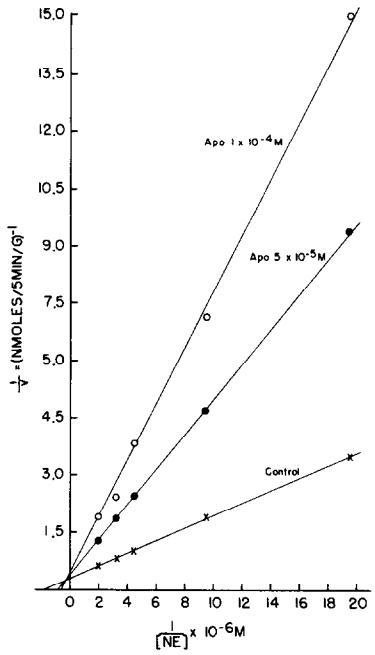

Fig. 3. Lineweaver–Burk plot showing the effect of apomorphine on the 5-min, temperature-dependent uptake of concentrations of ^{3}H-(±)-norepinephrine ranging from 0·5 μM to 0·05 μM into crude synaptosomal preparations of rat hypothalamus. Each point is the mean of three separate experiments.

Table 1. Apomorphine-induced release of dopamine and norepinephrine from crude synaptosomal preparations of rat striatum and hypothalamus

Condition	Striatum				Hypothalamus			
	No. of expt.	^{3}H-DM in pellet (cpm ± S. E.)	Total release (%)	Drug-induced release (%)	No. of expt.	^{3}H-NE in pellet (cpm ± S. E.)	Total release (%)	Drug-induced release (%)
0 Time	4	49,528 ± 3,491			4	8,789 ± 561		
5-min Spontaneous release	4	40,951 ± 2,791	17		4	7,523 ± 436	14	
APO 5×10^{-6} M	4	41,781 ± 1,794	16	0	4	7,324 ± 345	17	2
APO 1×10^{-5} M	4	39,289 ± 2,268	21	3	4	7,240 ± 408	18	3
APO 5×10^{-5} M	4	32,381 ± 1,976	35	17*	4	7,308 ± 191	17	2
APO 1×10^{-4} M	4	26,477 ± 2,307	47	29*	4	6,975 ± 542	21	6

* Significantly different from the 5-min spontaneous release value, $P < 0{\cdot}05$.

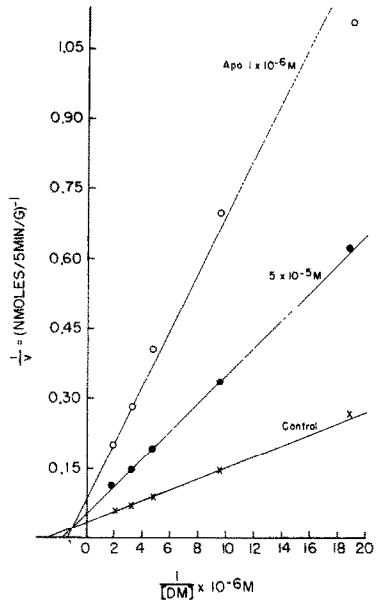

Fig. 4. Lineweaver–Burk plot showing the effect of apomorphine on the 5-min, temperature-dependent uptake of concentrations of ^{3}H-dopamine ranging from 0·5 μM to 0·05 μM into crude synaptosomal preparations of rat striatum. Each point is the mean of three separate experiments.

inhibition of catecholamine uptake in both tissues in four separate studies. While the inhibition produced by morphine was small, it was significantly different from control values, $P < 0{\cdot}05$.

The effect of apomorphine (10^{-7} M to 3×10^{-3} M) on the 5-min uptake of two different concentrations of ^{3}H-tyrosine (5×10^{-5} and 5×10^{-7} M) was also studied in crude synaptosomal preparations of hypothalamus and striatum. In both tissue preparations, apomorphine, at all concentrations studied, failed to inhibit the 5-min uptake of 5×10^{-5} M or 5×10^{-7} M ^{3}H-tyrosine, in four separate studies.

DISCUSSION

The observation in the present study that apomorphine is an inhibitor of dopamine uptake into crude synaptosomal preparations of striatum and an inhibitor of norepinephrine uptake into crude synaptosomal preparations of hypothalamus suggests that apomorphine can block the transport of catecholamines across neuronal membranes. However, the mechanism whereby apomorphine inhibits the uptake process is rather complex. Apomorphine itself is apparently bound to neuronal membranes, since it is capable of blocking not only the active transport (uptake at 37° − 0°) but also the passive diffusion (uptake at 0°) of catecholamines in a concentration-dependent manner. Lineweaver–Burk plots of catecholamine uptake data revealed that apomorphine produced mixed inhibition kinetics for dopamine uptake into striatum and mixed inhibition kinetics for norepinephrine uptake into hypothalamus. Mixed inhibition kinetics predict that an inhibitor may act on both the reaction velocity and substrate affinity, giving a mixture of competitive and non-competitive effects [17]. Since apomorphine is apparently bound in a concentration-dependent manner to neuronal membranes and since it contains a catechol group, we would postulate that it competes with catecholamines for binding to the carrier present in neuronal membranes, thus giving rise to the competitive component of the mixed inhibition kinetics. The non-competitive component could arise as a result of the alteration by apomorphine of either the "movement" of the carrier across the membrane or of the dissociation of the carrier–substrate complex on the inside of the neuronal membrane.

Other investigators have demonstrated that apomorphine is capable of inhibiting tyrosine hydroxylase *in vitro* as well as inhibiting the conversion of ^{14}C-tyrosine to ^{14}C-dopamine in rat striatal slices [14]. Since the striatal preparations were more sensitive to the inhibitory effects of apomorphine than the cell-free preparations, the authors suggested that apomorphine either accumulates in dopaminergic neurons and, as a consequence, inhibits tyrosine hydroxylase, or the drug can stimulate the dopamine receptors resulting in a feedback control of dopamine biosynthesis [14, 15]. Additional studies by these investigators suggest the latter mechanism probably accounts for the greater sensitivity of the striatal slices to apomorphine [15]. However, the possibility also exists that apomorphine may block the uptake of labeled tyrosine at the nerve membrane and this phenomenon could account for at least part of the inhibition of the biosynthesis of labeled dopamine seen after the addition of apomorphine to these preparations. This concept was negated in the present study with the demonstration that the drug failed to inhibit the uptake of ^{3}H-tyrosine into homogenates of hypothalamus and striatum. Moreover, these results also demonstrate a certain degree of specificity of apomorphine for inhibition of catecholaminergic pumps.

The possibility still existed that apomorphine could be accumulated by nerve endings and thus modify the release of biogenic amines from their intraneuronal stores. In order to test this hypothesis, the effects of various concentrations of apomorphine were studied on the 5-min release of labeled dopamine from striatum and labeled norepinephrine from hypothalamus. A dose-dependent release of ^{3}H-dopamine from striatum was elicited by apomorphine, while the drug only produced a trivial non-dose-dependent release of ^{3}H-norepinephrine from hypothalamus. These results would suggest that apomorphine can be accumulated in nerve endings and can preferentially release catecholamines from storage sites present in dopaminergic neurons. This phenomenon, together with the ability of apomorphine to block the re-uptake mechanism at the pre-synaptic striatal neuronal membrane, could result in an elevation of dopamine concentration in the synaptic cleft which, in turn, could lead to stimulation of dopaminergic receptors present on either pre- or post-synaptic membranes.

The question then arises as to whether the concentrations of apomorphine necessary to produce the effects observed *in vitro* can be achieved *in vivo*. While no data are available on the concentration of apomorphine present in rat brain, recent studies have shown that concentrations of apomorphine of 0·9 μg/g can be achieved in mouse brain 20 min after an intraperitoneal injection of 3 mg/kg of the drug [18].

Using these values and assuming that apomorphine is evenly distributed throughout the brain, it can be calculated that the concentration of the drug is approximately 3×10^{-6} M. Presumably, similar concentrations of apomorphine can be achieved in rat brain. Thus the effects *in vitro* elicited by apomorphine in the present study may be anticipated to occur *in vivo* at doses of the drug higher than 3 mg/kg.

However, the behavioral effects observed in animals *in vivo* can be elicited at much lower doses of the drug. Therefore, it is unlikely that these behavioral effects arise as a consequence of apomorphine's ability to modify the uptake and release of dopamine at dopaminergic nerve endings.

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