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Effects of Apomorphine Enantiomers and of Lisuride on 3,4-Dihydroxyphenylalanine Production in Striatal Synaptosomes

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SUMMARY

The effects of lisuride and of the *R*(−)- and *S*(+)-enantiomers of apomorphine were examined on 3,4-dihydroxyphenylalanine (DOPA) production by striatal synaptosomes and by crude, soluble striatal tyrosine hydroxylase. Due to their catechol structure, the enantiomers were almost equally effective in blocking soluble tyrosine hydroxylase (EC 1.14.16.2) (IC_{50} = 470 and 890 nM for *R*(−)- and *S*(+)-apomorphine, respectively), provided incubations were performed at pH 7.2 with 1 mM tetrahydrobiopterin as cofactor. The enantiomers were similarly effective in blocking synaptosomal DOPA production (IC_{50} = 410 and 970 nM for *R*(−)- and *S*(+)-apomorphine, respectively). As *S*(+)-apomorphine but not *R*(−)-apomorphine is considered to be a dopamine antagonist, these results support the assumption that the block of synaptosomal DOPA production by both apomorphine enantiomers is due to a direct inhibition of tyrosine hydroxylase. Lisuride at high concentrations (10–100 μM) blocked DOPA production in striatal synaptosomes; simultaneously, intrasynaptosomal dopamine was depleted. These data support the assumption that lisuride inhibits DOPA production indirectly, similar to reserpine. In accordance with this assumption, lisuride was without effect on DOPA production in dopamine-depleted synaptosomes. These results demonstrate that inhibition of synaptosomal DOPA production by at least some dopamine agonists may be explained by direct inhibitory effects on tyrosine hydroxylase.

INTRODUCTION

The regulation of DA[1] biosynthesis in dopaminergic nerve terminals is unclear. Most authors agree that extraneuronal DA (or other dopaminergic agonists) influences TH via presynaptic autoreceptors, as was originally suggested by Carlsson and Roth and their associates (1–3). In particular, the DA agonist, *R*(−)-apomorphine, is remarkably effective in blocking DOPA production in striatal slices and synaptosomes; these findings support the existence of DA receptors on the nerve terminal which modulate DA synthesis (4, 5).

Studies on the regulation of TH in catechol-synthesizing clonal cell lines showed that *R*(−)-apomorphine blocked DOPA production in these systems as effectively as in striatal slices and synaptosomes, but a DA receptor-mediated mechanism could not be demonstrated in these cell clones (6, 7). Reexamination of the effect of *R*(−)-apomorphine on striatal TH enzyme preparations revealed that *R*(−)-apomorphine—apparently due to its catechol structure—is a very effective direct blocker of TH, if physiological test conditions are used (8).

Apomorphine exists both in *R*(−)- and *S*(+)-enantiomeric forms. *S*(+)-Apomorphine, which contains a catechol moiety as does the *R*(−)-form, was shown to be a DA receptor antagonist in behavioral and biochemical assays (9, 10). Comparison of the effects of both enantiomers on the activity of soluble TH and DOPA production in striatal synaptosomes should, therefore, enable one to distinguish an autoreceptor-mediated effect on TH from a direct one.

Tissari *et al.* (11) recently reported that the ergoline derivative, LHM, can block DOPA production in striatal synaptosomes, but only at high concentrations. We observed that LHM blocks DOPA production in the pheochromocytoma cell clone PC-12 by depletion of DA from its vesicular stores into the cytoplasm.[2] Therefore, we

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[1] The abbreviations used are: DA, dopamine; TH, tyrosine hydroxylase (EC 1.14.16.2); DOPA, 3,4-dihydroxyphenylaline; LHM, lisuride hydrogen maleate; DOPAC, 3,4-dihydroxyphenylacetic acid; NSD 1055, 4-bromo-3-hydroxybenzyloxyamine dihydrogen phosphate; BH_4, (6-*R*, *S*)-L-erythro-5,6,7,8-tetrahydrobiopterin.

[2] M. Bräutigam, B. Kittner, and G. Laschinski, unpublished observation.

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have reinvestigated LHM-induced inhibition of synaptosomal DOPA production, monitoring the synaptosomal DA and DOPAC content.

MATERIALS AND METHODS

DOPA production in striatal synaptosomes. Male Wistar rats (150–250 g) were obtained from Winkelmann (Borchen, FRG). The striatum was dissected after decapitation; the crude synaptosomal fraction (P_2) was prepared as described by Kapatos and Zigmond (12). The synaptosomes were suspended in 500 μl of sodium phosphate buffer, pH 7.4 (10 mM)/pair of striata. The buffer contained 122 mM NaCl, 0.9 mM $CaCl_2$, 1.2 mM $MgSO_4$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM ascorbic acid, and 12 mM glucose.

Measurement of DOPA production after blockade of aromatic L-amino acid decarboxylase. The assay was performed in a total volume of 500 μl of the above buffer, containing 100 μl of the synaptosomal suspension (corresponding to about 1 mg of protein), tyrosine (100 μM), and drugs or solvents as indicated. The experiments were started by addition of NSD 1055 to a final concentration of 100 μM. Incubations were carried out at 37° and were usually stopped after 20 min by addition of perchloric acid (final concentration 0.4 M). The pH was checked regularly and found to be between 7.0 and 7.3. Synaptosomes were sonicated in the incubation buffer, centrifuged at 10,000 × *g* for 10 min, and the supernatant was assayed for DOPA, DA, and DOPAC. Protein was determined according to the method of Lowry *et al.* (13).

Measurement of [^{3}H] catechol production. In order to validate the results obtained by determining DOPA production after inhibition of the aromatic L-amino acid decarboxylase, synaptosomal [^{3}H]catechol production was measured as described by Iversen *et al.* (18). Before each experiment, L-[2,6-^{3}H] tyrosine was purified from catechol impurities on aluminum oxide. The assay conditions were as described above (without NSD 1055); after preincubation for 10 min at 37°, the experiments were started by adding 0.25 μCi of L-[2,6-^{3}H]tyrosine (per test tube) diluted in nonradioactive L-tyrosine (final concentration, 5 μM). Incubation was terminated after 10 min by the addition of perchloric acid as described above. Labeled catechols were isolated from unreacted L-[2,6-^{3}H]tyrosine by adsorption to aluminum oxide and quantified by liquid scintillation spectrometry. Typical blank and control values were 400 and 4000 dpm/assay tube, respectively. Absolute synaptosomal catechol production was calculated under the assumption that the intrasynaptosomal radioactive concentration of L-[2,6-^{3}H] tyrosine corresponds to its concentration in the incubation buffer without correction for recovery of [^{3}H]catechols over aluminum oxide.

Activity of striatal TH. Preparation of crude soluble striatal TH and assay conditions were as recently described (8). Tests reported in this paper were performed by 5 mM Tris-HCl, pH 7.2, containing 30 μM tyrosine and 1 mM BH_4. Incubation time was 30 min.

DA-depleted synaptosomes. To analyze the effect of LHM, striatal synaptosomes were prepared as described above and divided into two fractions. One fraction was incubated with 10 μM reserpine, and the other one was incubated with the appropriate control solution for 20 min at 37°. This incubation time was sufficient to deplete DA from the reserpine-treated synaptosomes. Subsequently, both fractions were centrifuged at 1000 × *g* for 1 min, the supernatant was decanted, and the synaptosomes were resuspended in fresh incubation buffer. The effects of 100 μM LHM and 1 μM *R*(−)-apomorphine were tested on both fractions.

Catechol assay. Catechols were assayed as described previously after a purification step with aluminum oxide by high pressure liquid chromatography and subsequent electrochemical detection (7). α-Methyl-DOPA and adrenaline were used as internal standards for DOPA and DA, respectively. Recovery of DOPAC was determined in separate samples by addition of a known amount of DOPAC; the obtained recovery (~50%) was used to correct the DOPAC measurements.

Drug comparisons were performed in the same tissue preparation to avoid problems with variations in synaptosomal activity on different days. All tests were performed in triplicate and the arithmetic mean ± SD is given in the figures. The IC_{50} values were determined by at least 12 determinations, using at least four different concentrations.

Materials. *R*(−)-Apomorphine was obtained from Sigma Chemie (Taufkirchen, FRG); *S*(+)-apomorphine was obtained from Research Biochemicals Inc. (Wayland, MA). Both compounds were dissolved in 0.9% NaCl containing 0.1% $Na_2S_2O_5$. LHM was a gift of Dr. W. Kehr (Schering AG, Berlin, FRG). NSD 1055 was obtained from Smith & Nephew Research Ltd. (Harlow, Essex, U.K.).

BH_4 was from Dr. B. Schircks Laboratorien (Iona, Switzerland). BH_4 solutions always contained 10 mM dithioerythritol. Reserpine was from Fluka AG (Buchs, Switzerland). Reserpine was dissolved in glacial acetic acid at 128 mg/ml; dilutions were carried out in 0.9% NaCl. L-[2,6-^{3}H]Tyrosine (specific activity 41 Ci/mmol) was obtained from Amersham-Buchler (Braunschweig, FRG).

RESULTS

Our method to estimate DOPA production in striatal synaptosomes allows the simultaneous assessment of synaptosomal DA and DOPAC contents. The formation of DOPA was linear over at least 20 min (Fig. 1A); therefore, this time interval was used to test the effects of various drugs on synaptosomal DOPA production.

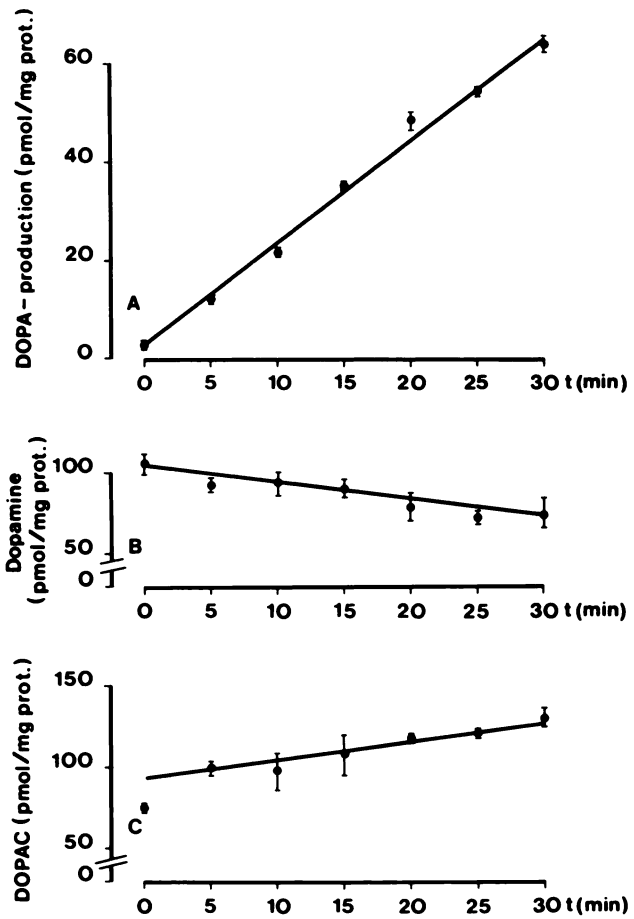

FIG. 1. *Time courses of synaptosomal DOPA, DA, and DOPAC content*

Rat striatal synaptosomes were incubated at pH 7.2 and 37° for various lengths of time. Experiments were started by addition of NSD 1055 (time point 0) and stopped by perchloric acid addition. After centrifugation, catechols were determined in the supernatant fluid. DOPA production, and DA and DOPAC content are indicated in A, B, and C, respectively. Each point represents the arithmetic mean ± SD of three observations.

Synaptosomal DA decreased during the incubation period; obviously some of the DA was metabolized to DOPAC, as a corresponding increase in DOPAC was found (Fig. 1, B and C).

The inhibition of synaptosomal DOPA production by reserpine and LHM is shown in Fig. 2A. Reserpine is known to induce a temporary block of DOPA production by blocking DA uptake into vesicles, resulting in a transient increase in cytosolic DA, and ultimately, depletion of vesicle stores of this amine. This is indicated by the observed decrease in synaptosomal DA and its recovery as DOPAC (Fig. 2, B and C). The transient increase in free cytosolic DA will result in the inhibition of the activity of TH, which is a cytosolic enzyme. LHM at high concentrations seems to have a reserpine-like effect on striatal synaptosomes as well. There was no effect of LHM (up to 100 μM) on soluble striatal TH (not shown). To elucidate the mechanism of action of LHM on synaptosomal DOPA production, the synaptosomes were depleted of DA by preincubation with 10 μM reserpine. Fig. 3 shows that LHM was ineffective in DA-depleted synaptosomes, whereas the *R*(−)-apomorphine-induced inhibition was unchanged.

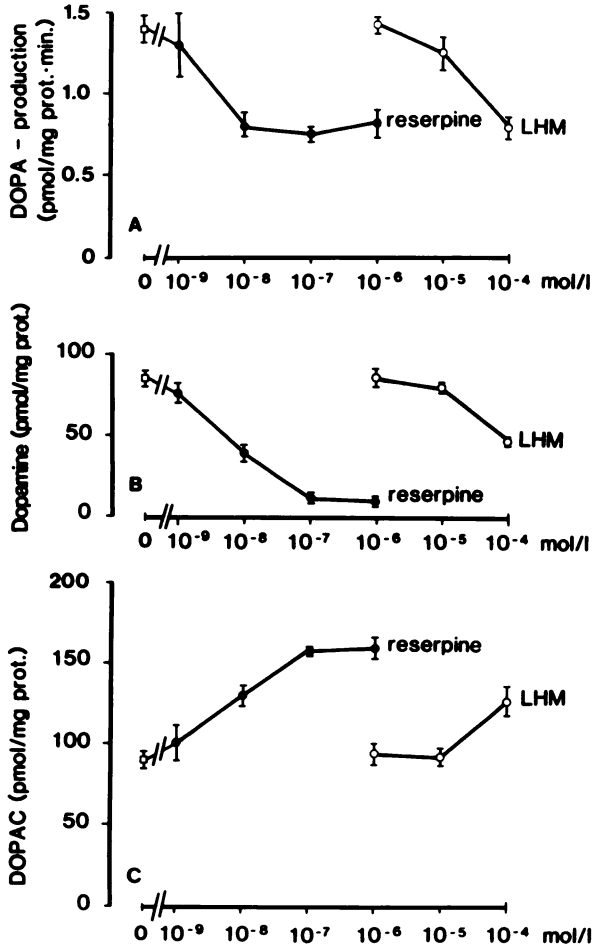

FIG. 2. *Effects of reserpine and LHM on synaptosomal DOPA production, and DA and DOPAC content*

Synaptosomes were incubated with reserpine or LHM at various concentrations for 20 min. DOPA production, and DA and DOPA content are indicated in A, B, and C, respectively. Each point represents the arithmetic mean ± SD of three observations.

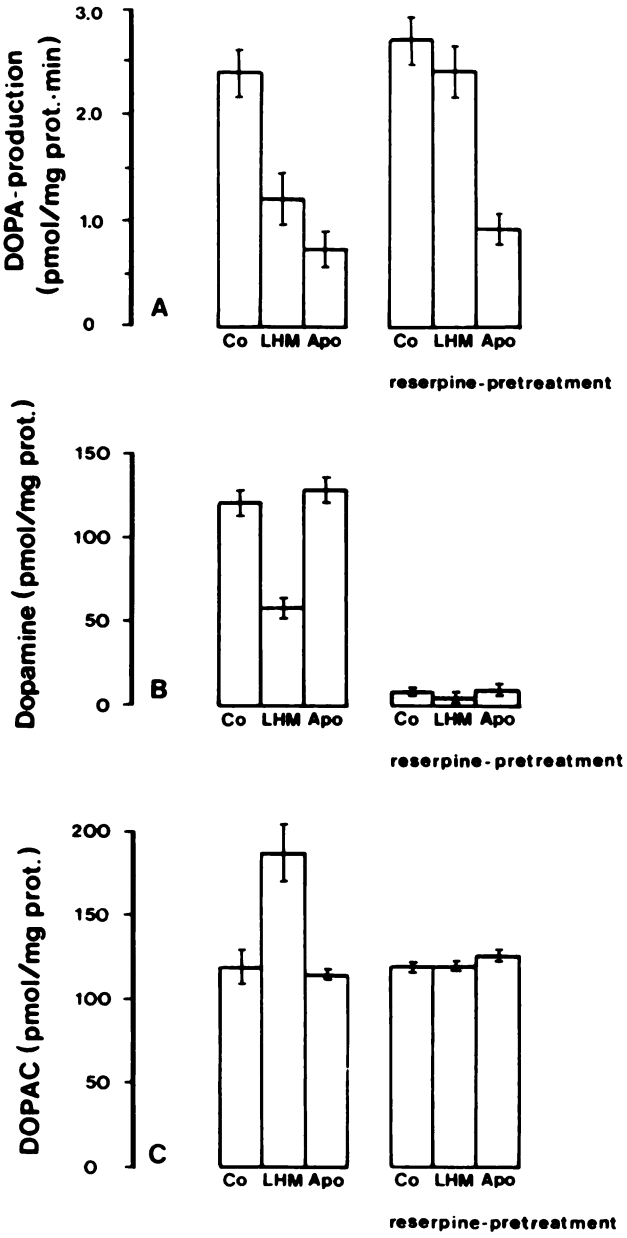

FIG. 3. *Effects of LHM and R(−)-apomorphine on DOPA production, and DA and DOPAC content in DA-depleted synaptosomes*

Synaptosomes were depleted of DA by pretreatment with 10 μM reserpine. The effect of 100 μM LHM and 1 μM *R*(−)-apomorphine was tested on untreated synaptosomes (*left* group of columns) and synaptosomes depleted of DA (*right* group of columns). DOPA production, and DA and DOPAC content are indicated in A, B, and C, respectively. Each point represents the arithmetic mean ± SD of three observations.

R(−)-Apomorphine is known to block DOPA production in striatal synaptosomes. *S*(+)-Apomorphine, which is a DA antagonist with low affinity for DA receptors (10), was almost equally potent as the *R*(−)-enantiomer in blocking the DOPA production in striatal synaptosomes (Fig. 4A). IC_{50} values for inhibition of synaptosomal DOPA production were 410 and 970 nM for the *R*(−)- and *S*(+)-enantiomers, respectively. In a second set of experiments, respective IC_{50} values of 630 and 950 nM were obtained. Synaptosomal DA and DOPAC did not change considerably by incubation with *R*(−)- or

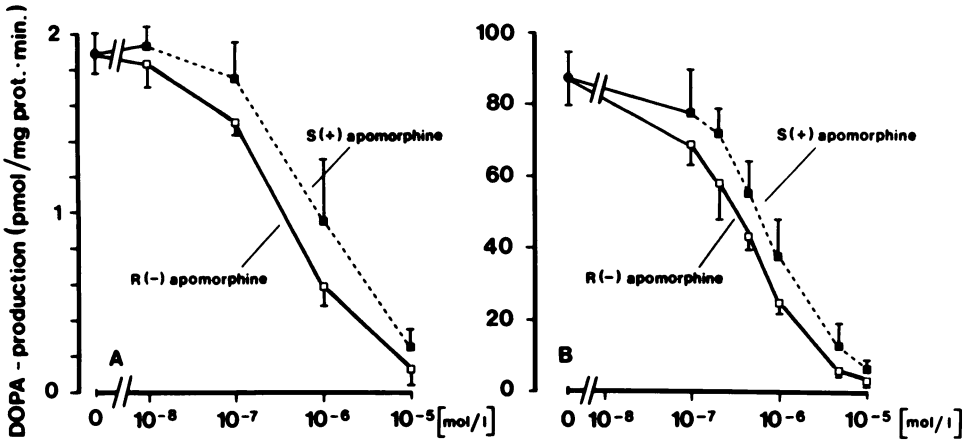

FIG. 4. *Inhibition of synaptosomal DOPA production and TH activity by R(−)- and S(+)-apomorphine*

Rat striatal synaptosomes were incubated at pH 7.2 and 37° for 20 min with *S*(+)- or *R*(−)-apomorphine added at various concentrations (A). Crude soluble TH from rat striatum was incubated at pH 7.2 and 37° for 30 min with 30 μM tyrosine and 1 mM BH_4 with *S*(+)- or *R*(−)-apomorphine added at various concentrations (B). Each point represents the arithmetic mean ± SD of three observations.

S(+)-apomorphine when compared to untreated controls.

When the effects of both enantiomers were tested in soluble TH preparations, IC_{50} values for the inhibition of TH were 470 and 890 nM for the *R*(−)- and the *S*(+)-enantiomers, respectively (Fig. 4B). In a second set of experiments, respective IC_{50} values of 420 and 890 nM were obtained.

Table 1 shows the effect of haloperidol on the inhibition of DOPA production from striatal synaptosomes, induced by the two enantiomers of apomorphine. In the NSD 1055 assay, haloperidol had only marginal effects on the *R*(−)-apomorphine-induced inhibition. Haloperidol alone induced a small decrease in DOPA production; simultaneously, the synaptosomal DA content was decreased to 82% and DOPAC content was increased to 113% as compared to the controls.

In the [^{3}H]catechol assay, the effect of 1 μM haloperidol alone on DOPA production was much more pronounced. Haloperidol at 1 μM yielded a slight decrease in the *R*(−)-apomorphine-induced inhibition and a slight increase in the *S*(+)-apomorphine-induced inhibition of synaptosomal [^{3}H]catechol production. At this haloperidol concentration, the assessment of the haloperidol-induced reversal critically depended on the 100% value used. At a concentration of 100 nM, haloperidol had only marginal effects on DOPA production alone and on apomorphine-induced inhibition; at 10 nM, haloperidol was ineffective (not shown). Both apomorphine enantiomers are effective inhibitors of DOPA production in the [^{3}H]catechol assay as well (Table 1).

DISCUSSION

Hirata *et al.* (14) and Haycock and Patrick (15) recently demonstrated that determination of DOPA accumulation after blocking the aromatic L-amino acid decarboxylase can be used as an indirect measure of TH activity in rat brain slices and synaptosomes. Our direct measurements of catechols allow the assessment of synaptosomal DA and DOPAC. Simultaneous monitoring of these catechols easily detects drugs which deplete DA from vesicular stores, resulting in a transient elevation of cytosolic DA and a consequent inhibition of synaptosomal TH.

While the accumulation of DOPA may eventually lead to a feedback inhibition of TH, the latter does not appear to occur during the time course of the incubations. This indicates that either not enough DOPA has accumulated to inhibit the enzyme or DOPA is released into the incubation buffer. From our experiments with cell cultures we know that DOPA easily leaves the cells (7, 16). Ip *et al.* (17) showed that considerable amounts of DOPA are also released by rat superior ganglia if the aromatic L-amino acid decarboxylase is blocked. Thus, determination of synaptosomal DOPA accumulation appears to be a valid assay to measure TH activity in striatal synaptosomes.

Our studies show that *R*(−)-apomorphine blocks DOPA production in striatal synaptosomes with an IC_{50} of 410–630 nM. This value is in accordance with the findings of other authors in striatal synaptosomes (5, 11, 18–22) and slices (4, 23–26). *S*(+)-Apomorphine, first

TABLE 1

Antagonism between haloperidol and the two apomorphine enantiomers regarding DOPA production of striatal synaptosomes

The results obtained with the NSD 1055 assay and with the [^{3}H]catechol assay are shown. The data represent the arithmetic mean ± SD of five determinations.

	DOPA production (pmol/mg protein · min)					
	NSD 1055 assay		[^{3}H]catechol assay			
	0[a]	1 μM	0	0.1 μM	0	1 μM
Controls	3.30 ± 0.27	2.95 ± 0.15	2.65 ± 0.12	2.38 ± 0.12	3.66 ± 0.07	2.16 ± 0.08
R(−)-Apomorphine 1 μM	0.96 ± 0.26	1.17 ± 0.13	0.37 ± 0.03	0.56 ± 0.07	0.46 ± 0.02	0.64 ± 0.02
S(+)-Apomorphine 1 μM	1.40 ± 0.48	1.50 ± 0.28	0.86 ± 0.06	0.93 ± 0.08	1.34 ± 0.13	1.01 ± 0.05

[a] Haloperidol concentrations.

synthesized by Saari and King (27), was found to be a DA antagonist both *in vivo* and *in vitro* (9, 10, 28). If inhibition of synaptosomal DOPA production by *R*(−)-apomorphine, a D_2-agonist (10), involves a dopaminergic receptor, then *S*(+)-apomorphine, a D_2-antagonist (10), should be unable to inhibit synaptosomal DOPA production and should be able to reverse the effect of *R*(−)-apomorphine. The observations concerning the autoreceptor regulation of DA release are in accordance with this idea: Lehmann *et al.* (28) observed no effect of *S*(+)-apomorphine on electrically evoked release of DA from striatal slices, and *S*(+)-apomorphine was able to antagonize the *R*(−)-apomorphine-induced effect.

In contrast to these results on dopaminergic regulation of DA release, *S*(+)-apomorphine was an effective blocker of synaptosomal DOPA synthesis (Fig. 4). These findings indicate that inhibition of synaptosomal DOPA production by *R*(−)-apomorphine may be explained by its direct inhibitory effect on TH. Goldstein *et al.* (4) reported in 1970 that, due to its catechol structure, *R*(−)-apomorphine is a direct blocker of soluble striatal TH. Whereas IC_{50} values above 10 μM were determined (4, 18, 20), we recently showed that the inhibitory potency of *R*(−)-apomorphine on soluble TH critically depended on the incubation conditions used; at pH 7.2 and with the natural cofactor BH_4, the IC_{50} was 200–600 nM, depending on the cofactor concentration used (8).

Recently, Shen *et al.* (29) reported that *R*(−)-apomorphine and other catechols can block the dihydropteridine reductase (EC 1.6.99.10). The relevance of this finding for the inhibition of DOPA production remains to be established. We demonstrated blockade of DOPA production by *R*(−)-apomorphine ($IC_{50} < 1$ μM) in cell cultures with no changes of cellular BH_4 content (16). Moreover, *R*(−)-apomorphine (10 mg/kg, intraperitoneally) did not affect striatal BH_4 content in rats.[3] The effects of the apomorphine enantiomers on TH activity in our assay system must have been direct. Even if all tyrosine (30 μM) in the assay system were converted to DOPA, this would hardly change the BH_4 concentration (1 mM). In accordance with this consideration, omission of dihydropteridine reductase did not affect the IC_{50} of *R*(−)-apomorphine (data not shown).

It has been pointed out by Waggoner *et al.* (20) that efficient blockade of DOPA production *in vitro* is obtained only by DA agonists with catechol structure. Noncatechols like ergots and pergolide (19, 22, 26) showed only weak or no effects on DOPA production *in vitro*. Moreover, several groups (30–32) showed that DA agonists like 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene or DA, which are subject to the DA uptake system, lose their inhibitory effects on synaptosomal DOPA production, if their uptake into the synaptosomes is prevented. From these observations, Cerrito and Raiteri (30) and Maura and Raiteri (31) suggested that it is the DA newly taken up that regulates DOPA synthesis in striatal nerve endings.

In contrast, Waggoner *et al.* (20) and Tissari *et al.* (11) showed that 7-hydroxy-N,N-di-n-propyl aminotetralin and LHM (both without catechol structure) have a partial inhibitory effect on synaptosomal DOPA production. In contrast to *R*(−)-apomorphine, both drugs exhibited a rather flat dose-response curve, and high concentrations (100 mM) caused only 50% maximal inhibition of DOPA production. We only examined LHM, and our data indicate that the effect of LHM on synaptosomal DOPA production can be explained by a reserpine-like mechanism, i.e., via depleting DA from its vesicular stores into the cytoplasm (Fig. 2). In accordance with this hypothesis, LHM—but not *R*(−)-apomorphine—loses its effect on DOPA production in DA-depleted synaptosomes (Fig. 3). The effects of reserpine (or lisuride) on DOPA production are transient: if there exists no DA which can be released, DOPA production will once more increase. This is indicated by the restored level of DOPA production after pretreatment with reserpine (controls, Fig. 3).

In support of a receptor-mediated regulation of DA biosynthesis *in vitro*, many authors reported a reversal of the apomorphine-induced inhibition of synaptosomal DOPA production by neuroleptics. However, this reversal was only weak and was not observed by others (4, 19, 20). In the NSD 1055 assay, we only observed a marginal antagonism between *R*(−)-apomorphine and haloperidol (1 μM) (see Table 1). In the [^{3}H]catechol assay, the antagonism seemed to be more pronounced, especially if the effect of *R*(−)-apomorphine in the presence of haloperidol was assessed by comparison with the value obtained by incubation with haloperidol alone (e.g., Ref. 18). If this procedure for calculating the antagonism between haloperidol and apomorphine is chosen, there also exists an antagonism between *S*(+)-apomorphine and haloperidol at a concentration of 1 μM. With haloperidol at a concentration of 100 nM, the antagonism leveled off and was no longer detected at a concentration of 10 nM.

Whether this marginal reversal of the *R*(−)-apomorphine-induced inhibition of DOPA production by haloperidol at high concentrations suffices to assume an autoreceptor-mediated control of DOPA synthesis in this model may remain to be discussed. Comparing the results obtained *in vitro* on the regulation of DA synthesis and DA release, the evidence for a common mechanism seems to be rather weak: *R*(−)-apomorphine blocked electrically evoked DA release in striatal slices with an IC_{50} well below 100 nM; ergots and pergolide also were effective blockers of DA release; different classes of neuroleptics facilitated DA release (at nanomolar concentrations) and completely reversed the inhibition by DA agonists (for reviews see Refs. 33 and 34).

Recently, Compton and Johnson (35) and Fowler *et al.* (36) suggested that an autoreceptor control of synaptosomal TH activity cannot be established *in vitro*. Therefore, it may be worthwhile to reconsider the validity of this model for testing drugs with respect to their DA autoreceptor-stimulating or -blocking activities.

Haloperidol at a concentration of 1 μM caused a pronounced inhibition of TH in the [^{3}H]catechol assay, which was in accordance with the findings of others (e.g., Refs. 5 and 18). Delanoy and Dunn (24) suggested that

[3] M. Bräutigam, B. Kittner, and H. Herken, unpublished observation.

this inhibition was due to a reserpine-like effect if haloperidol was applied at high concentrations. The direct measurement of a decrease in DA and an increase in DOPAC in the NSD 1055 assay supported this concept, although the observed blockade of DOPA production was lower in the NSD 1055 assay, and other unknown mechanisms possibly contributed to the apparent decrease of [^{3}H]catechol production. Since, in the [^{3}H]catechol assay, the production of all catechols was measured (i.e., besides that of DOPA, at least also that of DA and DOPAC), haloperidol might interfere with the synthesis of one of the latter products. Conversely, haloperidol may interfere with the transport of labeled tyrosine into the synaptosomes.

Our studies allow the following conclusions. 1) If there exists an autoreceptor-mediated regulation of DA synthesis, a reliable method for its demonstration *in vitro* remains to be established. 2) DA agonists with catechol structure are apparently not good tools for this purpose because of a pronounced direct effect on TH. Comparison of both apomorphine enantiomers may distinguish direct from receptor-mediated effects on TH. 3) Simultaneous measurement of synaptosomal DA and DOPAC may reveal indirect drug actions, as demonstrated for LHM.

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