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In Vivo and In Vitro Interaction of Flunarizine With D-Fenfluramine Serotonergic Effects

T. MENNINI,[1] M. GOBBI, D. CRESPI, M. CINQUANTA, E. FRITTOLI, P. GIORCELLI, M. ANELLI AND S. CACCIA

Istituto di Ricerche Farmacologiche Mario Negri, Milano, Italy

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MENNINI, T., M. GOBBI, D. CRESPI, M. CINQUANTA, E. FRITTOLI, P. GIORCELLI, M. ANELLI AND S. CACCIA. *In vivo and in vitro interaction of flunarizine with D-fenfluramine serotonergic effects.* PHARMACOL BIOCHEM BEHAV **53**(1) 155–161, 1996.—Flunarizine (35 mg/kg), but not haloperidol and trifluperazine, counteracted the initial indole depletion induced by D-fenfluramine (dF) in vivo (5 mg/kg), without affecting ex vivo [^{3}H]-serotonin (5-HT) uptake by synaptosomes or changing the brain concentrations of the parent drug and its main active metabolite, D-norfenfluramine (dNF). The long-term indole depletion induced by repeated doses of dF (5 mg/kg, b.i.d. for 4 days) was also reversed by flunarizine pretreatment. Flunarizine, methiothepin, and trifluperazine, but not haloperidol, reduced in vitro the Ca^{2+}-dependent [^{3}H]5-HT release stimulated by 0.5 μM dF and dNF from superfused synaptosomes. At the concentrations used in release experiments the drugs were not active on [^{3}H]5-HT uptake nor on the calcium-calmodulin protein kinase activity, thus excluding an effect on the uptake carrier or on phosphorylation of synaptic proteins involved in exocytosis, respectively. The drugs did not consistently affect [^{3}H]5-HT release induced by depolarization, or dNF-induced [^{3}H]dopamine release in vitro. The fact that flunarizine, as methiothepin and 5-HT uptake inhibitors, counteract dF-induced indole depletion in vivo suggests a relation between the reduction of the Ca^{2+}-dependent release of [^{3}H]5-HT induced by dF in vitro and the protective effect on the short- and long-lasting depletion of indoles induced in vivo by high doses of dF.

Flunarizine Trifluperazine Methiothepin D-Fenfluramine D-Norfenfluramine Indole levels and release Dopamine release Calcium-calmodulin protein kinase activity

D-FENFLURAMINE (dF) is an anorectic agent structurally related to amphetamine (see Ref. 11, for review). It selectively enhances serotonin (5-HT) transmission in the brain (10,11) by inhibiting 5-HT reuptake in nerve endings and stimulating its release from a vesicular pool (2,14,27). The release induced by low concentrations of dF is, like that induced by depolarization, Ca^{2+}-dependent (14,15); whereas at higher concentrations it is mainly Ca^{2+}-independent (14).

Its main active metabolite, D-norfenfluramine (dNF), also inhibits 5-HT reuptake and enhances 5-HT release from isolated synaptosomes, from both the vesicular and cytoplasmic pool (2,27). In addition, differently from its parent compound, dNF enhances [^{3}H]dopamine ([^{3}H]DA) release from rat striatal synaptosomes (10) and binds to 5-HT_{1C} receptors with appreciable affinity in various animal species (26).

At doses higher than those required to produce anorexia, dF in rodents (4,5,19,21) and nonhuman primates (30) causes a marked and long-lasting decrease in brain content of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), which is related to the initial decrease of brain indoles induced by the drug. As with other amphetamine derivatives, these effects are accompanied by reductions of other 5-HT markers, like its synthetic enzyme and the uptake carrier.

The mechanisms underlying the indole-depleting effect of dF are not clear, but it can be prevented by pretreatment with 5-HT uptake inhibitors (13,33,35), which also antagonize the 5-HT release induced by the drug in vitro (14,25). Methiothepin also prevents the lasting indole depletion induced in vivo by high dF doses (12).

The long-lasting reduction in 5-HT and tryptophan hydroxylase activity induced by 3,4-methylenedioxy-methamphetamine (MDMA) can be prevented by flunarizine through a mechanism unrelated to its Ca^{2+} channel antagonist activity (8,20).

In the present study we checked whether flunarizine pretreatment counteracted the 5-HT-depleting action of high dF

[1] Requests for reprints should be addressed to Tiziana Mennini, Istituto di Ricerche Farmacologiche Mario Negri, Via Eritrea 62, 20157 Milano, Italy.

doses in rats. Because, among other effects, flunarizine also has antidopaminergic activity (7) and inhibits calmodulin (23), we compared its effect with haloperidol and trifluperazine.

To verify a possible relationship between the lasting reduction of 5-HT levels and neurotransmitter release in vitro, we also studied the effect of flunarizine, haloperidol, trifluperazine, and methiothepin in vitro on dF-induced [^{3}H]5-HT release and on the stimulation of [^{3}H]5-HT and [^{3}H]DA release induced by dNF.

METHODS

Animals

Male CD-COBS rats weighing 150–175 g (Charles River, Italy) were used. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (39) and international laws and policies (40,41).

Drug Treatment

At the start of the study the rats were randomly divided into groups of five. In the acute study rats received a single intraperitoneal injection (IP) of 5 mg/kg dF hydrochloride or saline, 15 min (or 60 min in some experiments) after a single IP injection of flunarizine (35 mg/kg, as free base), trifluperazine (1 mg/kg) or haloperidol (1 mg/kg), or their corresponding vehicle. The doses and pretreatment times were mostly derived from the literature (8,20). All animals were killed by decapitation 4 h after the injection of dF or saline.

In the subchronic study rats were given dF alone or after flunarizine, trifluperazine, or haloperidol pretreatment as above, at approximately 12 h intervals for 4 days and killed 1 week later.

Brains were immediately removed, blotted with paper to remove excess surface blood, and quickly frozen in dry ice. The cortex was dissected and stored at −20°C until assayed.

Chemical Analysis

Concentrations of 5-HT and 5-HIAA in cortex were measured by high performance liquid chromatography with electrochemical detection (1). Brain concentrations of dF and dNF were analyzed by an electron capture gas chromatographic procedure described previously (3).

Statistical Analysis

Statistical analysis included one-way and two-way analysis of variance (ANOVA) with posthoc comparisons using Duncan's multiple *t*-test and the Mann–Whitney *U*-test. Probabilities *(p)* less than 0.05 were considered statistically significant.

[^{3}H]5-HT and [^{3}H]DA Release from Superfused Synaptosomes

Rat brains were rapidly dissected and their hippocampus (for 5-HT release) were homogenized in 40 volumes of ice-chilled 0.32 M sucrose, pH 7.4, in a glass homogenizer with a Teflon pestle. The homogenates were centrifuged at 1000 × *g* for 5 min and the supernatants centrifuged again at 12,000 × *g* for 20 min to yield the crude synaptosomal pellet (P_2) (17). The synaptosomes were resuspended in about 20 volumes of Krebs-Henseleit buffer with the following composition (mM): NaCl (125); KCl (3); $CaCl_2$ (1.2); $MgSO_4$ (1.2); NaH_2PO_4 (1); $NaHCO_3$ (22); glucose (10); gassed with 95% O_2 and 5% CO_2; pH 7.2–7.4.

The suspension was then added to an equal volume of the same buffer containing [^{3}H]5-HT (Amersham, 11 Ci/mmol, final concentration 0.06 μM). After 15 min incubation at 37°C, the solution was diluted with fresh buffer and 5 ml aliquots (about 5 mg initial tissue) were distributed onto 0.65 μm cellulose nitrate filters (Societa' Italiana di Microfiltrazione) in a 16-chamber superfusion apparatus held thermostatically at 37°C (29).

The synaptosomes were layered onto the filters by aspiration from the bottom under moderate vacuum. Superfusion was started (t = 0 min) at a rate of 0.5 ml/min with standard medium; after an equilibration period of 42 min, fractions were collected every 2 min until t = 60 min. The filters were put into scintillation vials and counted for radioactivity, as the fractions, in 8 ml of Ultima-Gold (Packard). dF and dNF were present in the superfusion medium from t = 47 to t = 50 min. All the other compounds were present from t = 40 min to t = 50 min.

The overflow was also induced by depolarizing the synaptosomes, replacing the normal medium from t = 47 to t = 50 with one containing a higher KCl concentration (15 mM instead of an equimolar concentration of NaCl). The radioactivity released by 15 mM K^+ and by 0.5 μM dF consists mainly of unmetabolized [^{3}H]5-HT (14).

The fractional release rate (FRR) was calculated as 100 times the amount of radioactivity released into each 2-min fraction over the total radioactivity present on the filter at the start of that fraction. The FRR found before the stimulus (t = 44–46), expressed as a percentage in 2 min, are reported as basal outflow. The overflow (%) was calculated as the difference between the FRR in the presence (t = 48–56) and absence (mean values of t = 44–48 and t = 56–60) of the drug.

[^{3}H]DA release was studied in similar way, using synaptosomes from rat striatum loaded with 0.06 μM [^{3}H]DA (NEN, 40.9 Ci/mmol).

[^{3}H]5-HT Uptake from Synaptosomes

[^{3}H]5-HT uptake was investigated as previously described (2) incubating hippocampal synaptosomes for 5 min at 30°C with 0.06 μM [^{3}H]5-HT in the absence and presence of 1 μM citalopram to determine passive diffusion. In vitro flunarizine, methiothepin, or trifluperazine were added at different concentrations (10 nM–10 μM) during a preincubation period of 5 min. Ex vivo the effect of flunarizine was studied in synaptosomes obtained from cortex or hippocampus of rats treated IP with 35 mg/kg of flunarizine or vehicle, and killed 15 min later.

Endogenous Ca^{2+}-Calmodulin Dependent Protein Kinase-II Activity

Rat brain hippocampus was rapidly dissected and homogenized in 20 volumes of ice-chilled buffer (10 mM Tris HCl (pH 7.4), 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride, 2 mM benzamidine, and 0.1 mg/ml bacitracin). The homogenate was centrifuged at 150,000 × *g* for 30 min and the pellet was resuspended in the original volume of cold standard buffer by vigorous vortexing, centrifuged a second time, then resuspended and kept on ice until analysis.

Endogenous phosphorylation was assayed at 30°C in a reaction mixture (final volume 90 μl) containing 25 mM Tris HCl (pH 7.4), 6 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1.5 mM $CaCl_2$, 2 μM [γ^{32}P]ATP in the absence or presence of 1 μg of calmodulin. The reaction was started

by the addition of 100 μg tissue proteins and stopped after 15 s by the addition of 45 μl of a solution containing 3% sodium dodecyl sulphate (SDS), 5% 2-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue in 0.12 M Tris HCl, pH 6.8 (final concentrations). The samples (60 μl) were subjected to discontinuous SDS-polyacrylamide gel electrophoresis. The gels were fixed, stained, destained, dried, and autoradiographed. Ca^{2+}-calmodulin-dependent protein kinase-II (Ca^{2+}/CaM PK-II) activity was evaluated as incorporation of ^{32}P into synapsine-Ia by analysis of the autoradiographic film (37). The specific Ca^{2+}/CaM PK-II activity was calculated as the difference between the activity with and without calmodulin.

RESULTS

5-HT Concentrations in Cortex

A single injection of dF hydrochloride (5 mg/kg, IP) reduced the content of 5-HT and its main metabolite 5-HIAA in particularly sensitive brain regions such as the cortex and hippocampus by, respectively, 70–80% and 30–40% compared to vehicle-treated rats, 4 h after dosing. Table 1 shows the results in the cortex, where it was possible to concomitantly determine the drug concentrations. Flunarizine pretreatment (35 mg/kg, 15 min before dF), significantly attenuated the depletion of 5-HT (45% vs. 76% reduction, $p < 0.01$), and 5-HIAA (18% vs. 39% reduction, $p < 0.01$) induced by dF. In contrast, trifluperazine [1 mg/kg, given either 15 min (Table 1) or 60 min (not shown) before dF] and haloperidol (1 mg/kg, 15 min before dF) did not appreciably alter the indole-depleting effects compared to dF treatment alone. Flunarizine, trifluperazine, and haloperidol had no effect on 5-HT and 5-HIAA or on the cortical concentrations of dF and its active metabolite dNF (Table 1). The indole-depleting effect of acutely injected dF was rapidly reversible (4), whereas a repeated schedule of the same dose of dF b.i.d. for 4 days still reduced mean cortical 5-HT (35%, $p < 0.01$) and 5-HIAA (−46%, $p < 0.01$) content 1 week after the last dose (Fig. 1). Again, this effect was reversed (5-HT: $p < 0.01$, 5-HIAA: $p < 0.05$, vs. dF-treated rats) by pretreatment with flunarizine. Flunarizine alone did not significantly affect 5-HT, but slightly lowered 5-HIAA (−27%, $p < 0.05$ compared to vehicle-treated animals). In the same experimental conditions, trifluperazine (1 mg/kg, b.i.d. for 4 days) had no effect on the long-lasting indole-depleting action of dF (data not shown).

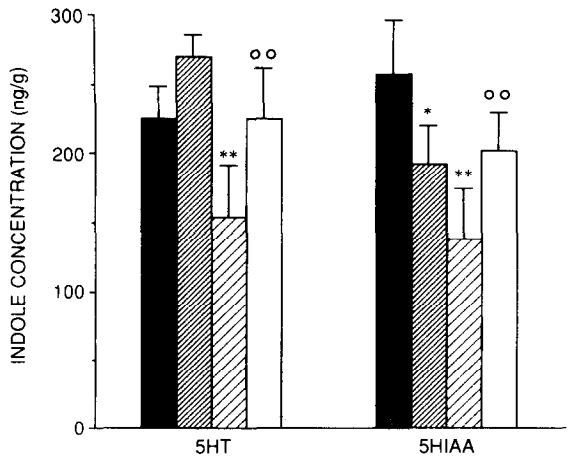

FIG. 1. Effect of flunarizine on the long-term depletion of cortical indoles induced by repeated doses of dF hydrochloride. The rats received dF (5 mg/kg, IP) or vehicle at approximately 12 h intervals for 4 days. Flunarizine (35 mg/kg, IP) was given 15 min before the injection of dF or vehicle. All the animals were killed 1 week after the last dose of dF hydrochloride. Symbols: [■] control; [▨] flunarizine; [▨] dF; [□] flunarizine + dF. The results are means ± SD of five animals. (**) $p < 0.01$; (*) $p < 0.05$ vs. repeated vehicle regimen; (°°) $p < 0.01$; (°) $p < 0.05$ vs. dF alone, ANOVA and Duncan's test.

[^{3}H]5-HT Uptake from Synaptosomes

When added in vitro, the IC_{50} of methiothepin, flunarizine, and trifluperazine for the inhibition of specific [^{3}H]5-HT up-

TABLE 1

EFFECTS OF FLUNARIZINE, TRIFLUPERAZINE, AND HALOPERIDOL ON THE SHORT-TERM DEPLETION OF INDOLES INDUCED BY D-FENFLURAMINE IN RAT CORTEX

Treatment (mg/kg,IP)	Indole Content (% of control)		Drug Concentration (μg/g)	
	5-HT	5-HIAA	dF	dNF
dF (5)[a]	24 ± 6*	61 ± 10*	3.3 ± 1.4	3.8 ± 0.5
Flunarizine + dF	55 ± 7*‡	82 ± 12§	2.4 ± 0.9	3.5 ± 1.0
Flunarizine (35)	96 ± 10	94 ± 9	–	–
Trifluperazine + dF	26 ± 4*	65 ± 6*	2.8 ± 0.2	3.7 ± 0.6
Trifluperazine (1)	87 ± 17	109 ± 16	–	–
Haloperidol + dF	29 ± 5*	64 ± 10*	3.3 ± 0.4	4.1 ± 0.4
Haloperidol (1)	90 ± 9	101 ± 7	–	–

Flunarizine, trifluperazine, and haloperidol were injected 15 min before dF (5 mg/kg) or vehicle. Rats were killed 4 h after D-fenfluramine. Each value is the mean ± SD of five rats ([a]mean of all experiments).

dF: D-fenfluramine, dNF: D-norfenfluramine.

*$p < 0.01$, vs. control (vehicle-treated rats); ‡$p < 0.01$, §$p < 0.05$ vs. dF alone; F for interaction: $p < 0.01$; ANOVA and Duncan's test.

The indole content in control rats was: 5-HT = 0.35 ± 0.03 μg/g; 5-HIAA = 0.20 ± 0.03 μg/g).

take into synaptosomes were: 2.2 ± 0.3 μM, 5.1 ± 0.6 μM, and >10 μM. At 1 μM the three drugs inhibited specific [^{3}H]5-HT uptake by 24%, 25%, and 13%, respectively. Drugs concentrations higher than 1 μM were not used in release experiments to avoid interference with the uptake carrier.

When [^{3}H]5-HT uptake was studied ex vivo, in synaptosomes obtained from rats treated 15 min before with 35 mg/kg of flunarizine, no significant inhibition could be observed either in cortical (control: 815 ± 150, flunarizine: 864 ± 80 fmol/min/mg protein) or hippocampal (control: 739 ± 120, flunarizine: 745 ± 80 fmol/min/mg protein) synaptosomes.

[^{3}H]5-HT Release from Superfused Synaptosomes

Effect of drugs on basal outflow. The basal outflow of tritium (FRR) from superfused rat hippocampal synaptosomes preloaded with [^{3}H]5-HT amounted to 1.8 ± 0.3 (percent per 2 min) of the total synaptosomal radioactivity in the first fraction collected (t = 44–46). This was only slightly increased in the presence of 1 μM methiothepin (2.5 ± 0.4, percent per 2 min), and 1 μM trifluperazine (2.3 ± 0.3), but not by 1 μM flunarizine (1.9 ± 0.3). At 0.3 μM or lower drug concentrations, basal outflow was unaffected (data not shown).

Effect of drugs on evoked overflow. Exposure of synaptosomes from t = 47 to t = 50 to 15 mM K^+, 0.5 or 10 μM dF, and 0.5 μM dNF resulted in tritium overflow amounting, respectively, to 7.6 ± 1.0, 6.8 ± 0.7, 11.6 ± 1.8, and 6.2 ± 0.4 (percent per 8 min). The radioactivity released by K^+, 0.5 μM dF, and 0.5 μM dNF consists mainly of unmetabolized [^{3}H]5-HT (Ref. 14 and unpublished data); this proportion slightly decreased at highest dF concentrations (14).

Methiothepin, flunarizine, and trifluperazine (Fig. 2), but not haloperidol (not shown), added in the superfusion medium 7 min before the releasing stimulus (from t = 40 min) reduced the [^{3}H]5-HT overflow stimulated by 0.5 μM dF and dNF. The most active compound was methiothepin, for which a complete dose-response curve could be calculated with IC_{50} 26 nM and 107 nM for dF and dNF, respectively (Fig. 3). Equiactive concentrations of flunarizine and trifluperazine (giving about 40% inhibition) were 0.3 μM and 1 μM, respectively (Fig. 2). Haloperidol was tested at 0.1 μM, because at higher concentrations (1 μM) it enhances tritium overflow by a Ca^{2+} independent mechanism (data not shown).

Figure 2 also shows that concentrations of methiothepin, flunarizine, and trifluperazine active in inhibiting the dF- and dNF-induced [^{3}H]5-HT overflow were less potent on the [^{3}H]5-HT overflow induced by depolarization with 15 mM K^+. Only flunarizine had a slight but significant, not dose-dependent effect on K^+-induced [^{3}H]5-HT overflow. However, at 1 μM, flunarizine reduced the effect of depolarization by 20% and that of dF by 60% ($p < 0.01$). Flunarizine was also the only drug that, at 1 μM, slightly reduced (35%) the tritium overflow induced by 10 μM dF.

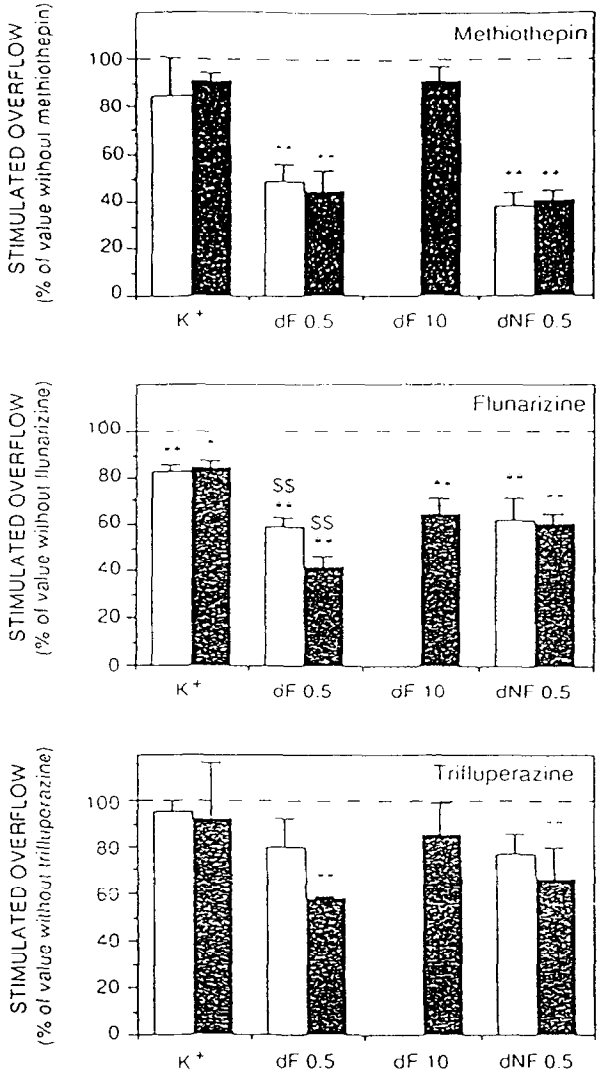

FIG. 2. Effects of methiothepin, flunarizine, and trifluperazine on the tritium overflow stimulated by 15 mM K^+, 0.5 or 10 μM dF (dF), or 0.5 μM dNF (dNF) from superfused rat hippocampal synaptosomes preloaded with [^{3}H]5-HT. Methiothepin, flunarizine, and trifluperazine were added in the superfusion medium at a concentration of 0.3 μM (open bars) or 1 μM (filled bars), 7 min before the releasing stimulus. Each value is the mean ± SD of 3–4 replications and represents the tritium overflow in the presence of the drug, expressed as a percentage of the overflow in its absence. (**) $p < 0.01$; (*) $p < 0.05$ different from control (ANOVA and Tukey's test); (§) $p < 0.01$, different from dF (ANOVA and Tukey's test).

[^{3}H]DA Release from Superfused Synaptosomes

dNF was more potent than dF in inducing [^{3}H]DA release: 3 min exposure of synaptosomes (from t = 47 to t = 50) to 5 μM of the two drugs, resulted in tritium overflow amounting to 3.0 ± 0.1 (dF) and 9.0 ± 0.8 (dNF). The releasing activity of dNF was dose-dependent, the overflow (percent per 8 min) being 4.9 ± 0.2, 13.9 ± 0.5, and 20.1 ± 0.5 at 3, 10, and 20 μM, respectively.

The basal outflow of tritium from superfused rat striatal synaptosomes preloaded with [^{3}H]DA was slightly increased by 1 μM trifluperazine, flunarizine, or methiothepin (Fig. 4). Methiothepin, flunarizine, and trifluperazine, tested at 1 μM, did not affect the [^{3}H]DA overflow induced by 5μM dNF (Fig. 4).

Endogenous Ca^{2+}/CaM PK-II Activity

At the concentrations active on [^{3}H]5-HT release, none of the drugs employed significantly inhibited Ca^{2+}/CaM PK-II activity. They were inactive up to 3 μM; maximal inhibition could be obtained with 30 μM trifluperazine and methio-

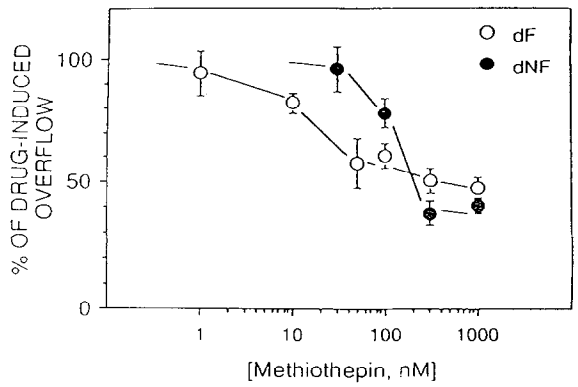

FIG. 3. Inhibitory activity of methiothepin on the [^{3}H]5-HT overflow induced by 0.5 μM D-fenfluramine (dF) or by 0.5 μM D-norfenfluramine (dNF). Methiothepin was added in the superfusion medium at the concentrations indicated 7 min before dF or dNF. Each value is the mean ± SD of 3-4 replications and represents the tritium overflow in the presence of methiothepin, expressed as a percentage of the overflow in its absence.

thepin, whereas 30 μM flunarizine produced only 60% inhibition (data not shown).

DISCUSSION

A main finding of this study is that flunarizine counteracts the indole depletion induced by dF in vivo, without changing the brain concentrations of the parent drug and its main active metabolite. A protective effect of flunarizine, unrelated to its Ca^{2+} channel antagonist activity, has also been reported for the long-lasting reduction in 5-HT and its synthetic enzyme induced by MDMA (8,20). The protective effect of flunarizine is unrelated to its antidopaminergic activity, because haloperidol fails to reduce the indole depletion in vivo. Our results, at variance with recently published data (8), are in agreement with the reported failure of haloperidol to counteract the reduction in 5-HT and its synthetic enzyme induced by MDMA (20). In vivo trifluperazine, a Ca^{2+}-calmodulin inhibitor, also failed to prevent the depletion of cortical indoles induced by dF. However, preliminary studies indicated that 4 h after injection of 1 mg/kg, the drug was present in rat brain at a concentration of about 0.5 μM, lower than required in vitro to appreciably inhibit Ca^{2+}/CaM PK-II activity (>3 μM). Higher doses of trifluperazine were not considered in these studies because of its strong cataleptic activity.

To find an explanation of the in vivo protective effect of flunarizine on indole depletion induced by dF, we considered some possible mechanisms in vitro.

One mechanism by which substituted amphetamines induce long-lasting 5-HT depletion is through DA release (18,34). Because dNF, differently from its parent compound, enhances [^{3}H]DA release in vitro (10), we checked whether flunarizine interacts with dNF-induced [^{3}H]DA release from rat striatal synaptosomes. Like methiothepin and trifluperazine, flunarizine did not inhibit this release in vitro, thus excluding that the protection seen in vivo could be related to antagonism of dNF's effect on DA release.

Another possibility was an inhibitory effect of flunarizine on 5-HT uptake. In fact 5-HT uptake inhibitors prevent the indole-depleting effect of dF (13,33,35). However, the [^{3}H]5-HT uptake by synaptosomes was not significantly reduced by flunarizine, either in vitro or ex vivo, thus excluding the possibility of an effect on the 5-HT carrier.

Then we studied flunarizine effect on dF and dNF-induced [^{3}H]5-HT release. The mechanism of dF-induced [^{3}H]5-HT release is dual: that induced by low concentration is Ca^{2+}-dependent, exocytotic-like; whereas at higher doses the tritium overflow is mainly Ca^{2+}-independent and carrier mediated (14). In vitro flunarizine did reduce the [^{3}H]5-HT release induced by low concentrations of dF and its metabolite, being less active on that induced by the highest dF concentration. These effects of flunarizine in vitro are unlikely to be related to its Ca^{2+} channel antagonism because it only slightly reduced depolarization-induced [^{3}H]5-HT release, whereas ω-conotoxin and ω-agatoxin, selective blockers of N- and P-type Ca^{2+} channels, had similar inhibitory potency (about 20 and 50%, respectively) both on dF- and K^+-induced release (9,15). It is also unrelated to antidopaminergic effects, Because in vitro haloperidol was unable to change dF- and dNF-induced [^{3}H]5-HT release.

Trifluperazine in vitro reduced only the [^{3}H]5-HT release induced by low dF and dNF concentrations, although with three times lower potency than flunarizine. This could explain the lack of protection by trifluperazine on dF-induced indole depletion in vivo, possibly related, as discussed before, to the low brain concentrations obtained after this treatment.

The concentrations of flunarizine and trifluperazine used for the present study in vitro did not affect endogenous Ca^{2+}/CaM PK-II activity, thus excluding the possibility that the

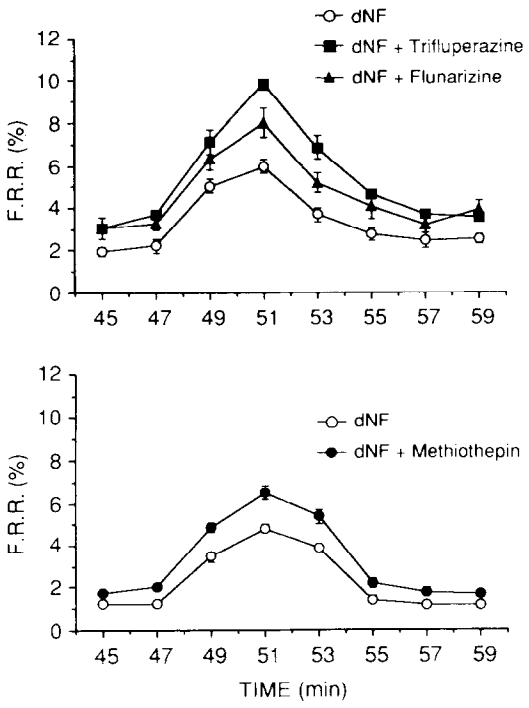

FIG. 4. Effects of methiothepin, flunarizine, and trifluperazine on the tritium overflow stimulated by 5 μM dNF from superfused rat striatal synaptosomes preloaded with [^{3}H]DA. dNF (5 μM) was present from t = 47 to t = 50. Methiothepin, flunarizine, and trifluperazine were added in the superfusion medium at a concentration of 1 μM, 7 min before the releasing stimulus. Each point is the mean FRR value (percent) ± SD of 3-4 replications.

inhibition of [^{3}H]5-HT release induced by low dF concentration was due to inhibition of Ca^{2+}/CaM-related phosphorylation of synaptic proteins involved in exocytosis (22). This is further supported by the fact that depolarization-induced [^{3}H]5-HT release was slightly or not modified by flunarizine and trifluperazine, respectively.

Another drug that selectively reduced [^{3}H]5-HT release induced by low dF and dNF concentrations in vitro, as well as the long-term indole decrease induced in vivo by high doses of dF (12), is methiothepin. Like flunarizine and trifluperazine, the effect of methiothepin was not related to the inhibition of Ca^{2+}-calmodulin-mediated processes. Its action in vitro also seems unrelated to its antagonism of presynaptic 5-HT_{1B} autoreceptors, because in our experimental conditions dF-induced [^{3}H]5-HT release was also reduced (unpublished results) with CP-93129, a 5-HT_{1B} agonist (24). Moreover, methiothepin antagonized the effect of CP-93129 on depolarization-induced [^{3}H]5-HT release (unpublished results).

As already discussed for flunarizine, the concentrations of trifluperazine and methiothepin effective on [^{3}H]5-HT release do not appreciably inhibit 5-HT uptake, a mechanism known to interfere both with Ca^{2+}-dependent and Ca^{2+}-independent dF-induced [^{3}H]5-HT release in vitro (14,25) and in vivo (31).

Having excluded antidopaminergic and anti-Ca^{2+} calmodulin activity, and an effect on 5-HT carrier, we can only speculate on the mechanism by which flunarizine, trifluperazine, and methiothepin inhibit the calcium-dependent [^{3}H]5-HT release induced by low dF concentration in vitro. Specific [^{3}H]dF binding has been described for brain membranes in vitro (28) and in vivo (16), for which flunarizine, trifluperazine, and methiothepin all have affinity (IC^{50} 144, 151, and 2140 nM, respectively; Mennini, unpublished data). However, this does not seem to be related to the antagonism of dF-induced [^{3}H]5-HT release in vitro, because the relative rank order of potency in the two tests is not correlated. Moreover, haloperidol, which has no effect on [^{3}H]5-HT release induced in vitro by dF, is among the most potent inhibitors of [^{3}H]dF binding (IC_{50} 1 nM).

Flunarizine (36) and trifluperazine (38) have affinity for sigma receptors, a proposed target for neuroprotective drugs (36), with a rank order fitting well with our results on [^{3}H]5-HT release in vitro. Although dF and other inhibitors of 5-HT uptake have recently been reported to bind to rat sigma-1 receptors (6), the high affinity of haloperidol in the same tests (6,38) and the fact that methiothepin has no appreciable affinity for the sigma-1 subtype (6) leaves doubt about the contribution of sigma receptors.

In conclusion we found that flunarizine, trifluperazine, and methiothepin inhibited dF- and dNF-induced [^{3}H]5-HT release in vitro, although the precise mechanism of interaction is still not clear. This effect was specific, because the drugs did not significantly affect [^{3}H]5-HT release induced by depolarization, or dNF-induced [^{3}H]DA release in vitro.

The fact that flunarizine and methiothepin, as well as 5-HT uptake inhibitors, counteract dF-induced indole depletion in vivo suggests a relation between the reduction of the vesicular, Ca^{2+}-dependent release of [^{3}H]5-HT induced by dF and dNF in vitro and the protective effect on the long-lasting depletion of indoles induced in vivo by high doses of dF.

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