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Reevaluation of the Regulation of β-Adrenergic Receptor Binding by Desipramine Treatment

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SUMMARY

Treatment of rats with desipramine (DMI) has been shown to down-regulate β-adrenergic receptor-stimulated adenylate cyclase and reduce the B_{max} of β-adrenergic receptors in some brain areas. Recent reports have indicated that the down-regulation in the number of β-adrenergic receptors following DMI treatment does not occur if the serotonin system has been impaired following parachlorophenylalanine (PCPA) or 5,7-dihydroxytryptamine injection. We have previously shown that [^{3}H]dihydroalprenolol ([^{3}H]DHA), the most commonly used radioligand to measure central nervous system β-adrenergic receptors, labels another site under normal experimental procedures, in addition to the β-adrenergic receptors. This second site has some pharmacological characteristics of the 5-hydroxytryptamine$_{1B}$ receptor. The depletion of serotonin following PCPA injection was indeed able to prevent the down-regulation of [^{3}H]DHA binding sites after DMI injection. However, PCPA alone increased the density of [^{3}H]DHA binding sites. If the nonlinear, least squares, curve-fitting program LIGAND was allowed to define [^{3}H]DHA nonspecific binding or if the more selective β-adrenergic receptor radioligand [^{3}H]CGP-12177 was used, the B_{max} of β-adrenergic receptors was not changed after PCPA injection. Importantly, PCPA did not prevent β-adrenergic receptor down-regulation following DMI treatment. The blockade of 5-hydroxytryptamine$_2$ receptors, via ketanserin administration, during DMI treatment did not change the response of β-adrenergic receptors. Furthermore, if LIGAND was used to define the nonspecific binding of [^{3}H]DHA, the down-regulation of β-adrenergic receptors was significant 24 hr after a single DMI injection. The same rapid down-regulation was demonstrated with [^{3}H]CGP-12177. However, if [^{3}H]DHA was used to label β-adrenergic receptors in the "typical" manner (nonspecific binding defined by 10 μM alprenolol), a decrease in the number of β-adrenergic receptors was significant only after seven daily DMI injections. These data demonstrate that the use of [^{3}H]DHA to measure β-adrenergic receptors can be misleading, because changes in its second binding site can conceal the changes occurring in β-adrenergic receptors. Moreover, these results suggest that a similarity in the time course of action of DMI cannot be used to support the hypothesis that its therapeutic antidepressant action is related to β-adrenergic receptor down-regulation.

Many authors have reported that a common effect of the chronic treatment of rats with antidepressant therapies is the desensitization of the β-adrenergic receptor stimulation of adenylate cyclase, coupled with a reduction in the number of β-adrenergic receptors in some brain regions. These effects have become a cornerstone in the development of the biogenic amine hypothesis of depression (1), in part because of the apparent similar time course for these biochemical effects and the gradual development of a therapeutic response to antidepressant treatment (1–4). Tricyclic antidepressants, such as DMI, inhibit the reuptake of both norepinephrine and serotonin. A number of recent papers have suggested that the decrease in β-adrenergic receptors is dependent on the integrity of serotonergic as well as noradrenergic synaptic transmission. For example, depletion of serotonin with the tryptophan hydroxylase inhibitor PCPA (5, 6) or lesion of serotonergic neurons with the neurotoxin 5,7-dihydroxytryptamine (7, 8) is reported to block the ability of desipramine to decrease β-adrenergic receptors measured with [^{3}H]DHA. However, some authors have reported a dissociation between the blocking effect of the serotonin lesion on DMI-induced β-adrenergic receptor down-regulation and the development of the desensitization of the β-adrenergic receptor-stimulated adenylate cyclase system after chronic DMI administration (8, 9).

In the accompanying paper, we have demonstrated that the most widely used β-adrenergic receptor radioligand, [^{3}H]DHA,

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ABBREVIATIONS: DMI, desmethylimipramine HCl; [^{3}H]DHA, [^{3}H]dihydroalprenolol hydrochloride; CGP-12177, (−)-4-(3-*tert*-butylamino-2-hydroxypropoxy)-benzimidazol-2-one; CGS-12066B, 7 trifluoromethyl-4-(4-methyl-1-piperazinyl)-pyrrolo[1,2a]quinoxaline dimaleate salt; ICI 89406, 1-(2-cyanophenoxy)-3-β-(3-phenylureido)ethyl-amino-2-propanolol; PCPA, *para*-chlorophenylalanine methyl ester HCl; 5HT, 5-hydroxytryptamine; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-hydroxypropanesulfonic acid.

labels a second binding site, with some pharmacological similarities to the $5HT_{1B}$ receptor (10). Thus, modifications of the serotonergic systems could potentially alter the binding of [^{3}H]DHA to its second binding site rather than, or as well as, to β-adrenergic receptors, complicating the interpretation of the experiments.

For this study, we have examined the influence of serotonin depletion following PCPA treatment or the effect of prolonged $5HT_2$ receptor blockade on the ability of DMI to down-regulate β-adrenergic receptors, using a selective ligand for β-adrenergic receptors, [^{3}H]CGP-12177, and utilizing computer analysis of [^{3}H]DHA binding with the program LIGAND (11), which enables the selective labeling of β-adrenergic receptors by [^{3}H]DHA to be quantified (10). In these studies, we also determined the time course of the down-regulation of β-adrenergic receptors by DMI, because there is some controversy in the literature as to the ability of the acute versus chronic administration of DMI to down-regulate β-adrenergic receptors (3, 4, 12, 13), which could have resulted from a differential effect of DMI on the β-adrenergic receptor and "serotonergic" components of [^{3}H]DHA binding.

Materials and Methods

Animals and tissue preparation. Adult male Sprague-Dawley rats (Charles River, Wilmington, MA), weighing 200–300 g, were used throughout the experiments; they were housed in group cages under standard conditions (12-hr light-dark cycle) with free access to food and water. The animals were killed by decapitation (between 10 a.m. and 1 p.m.) and the brains were immediately removed into ice-cold saline. Brain regions were rapidly dissected, placed into plastic vials, frozen on dry ice, and stored at −70° until the day of the experiment. Rat cerebral cortices, hippocampi, or striata were homogenized using a Tekmar Tissuemizer (setting of 6 for 15 sec) in 50 volumes of cold 50 mM Tris·HCl (pH 7.7 at 25°) and were centrifuged three times at 35,000 × *g* for 20 min.

Drug treatments. In time course experiments, DMI was injected at a dose of 15 mg/kg, intraperitoneally, daily. When the interaction of DMI-induced β-adrenergic receptor down-regulation and $5HT_2$ receptor blockade was studied, DMI was injected intraperitoneally, at 10 mg/kg, twice a day, whereas ketanserin tartrate was either injected intraperitoneally at 5 mg/kg, twice a day, or released by Alzet osmotic minipumps at a dose of 0.34 mg/kg/hr. When minipumps were used, the rats were anesthetized with methoxyflurane (Metofane; Pitman-Moore Inc.) and Alzet osmotic minipumps (model 2001) were placed subcutaneously in the scapular region of the animal. When the influence of serotonin depletion was investigated, the dosage of PCPA was 350 mg/kg, intraperitoneally, daily for the first 3 days and 125 mg/kg every other day from day 4 through day 11. Control animals received an injection of saline solution (0.9% NaCl). DMI was administered (15 mg/kg, intraperitoneally, daily) for 8 days to either saline- or PCPA-treated rats, starting from day 4. All animals were killed 24 hr after the last injection.

β-Adrenergic receptor binding. Two different ligands were used to measure β-adrenergic receptor binding, [^{3}H]DHA (specific activity, 52.3–95 Ci/mmol; New England Nuclear, Boston, MA) and [^{3}H]CGP-12177 (specific activity, 53 Ci/mmol; Amersham Corporation, Arlington Heights, IL). Binding was performed in borosilicate disposable tubes. The final assay volume of 2 ml consisted of 100 μl of radiolabeled ligand, 100 μl of alprenolol (final concentration, 10 μM) to measure the "nonspecific" binding, competing drug as required, or incubation buffer (50 mM Tris·HCl, pH 7.7 at 25°), 800 μl of incubation buffer, and 1 ml of membrane suspension containing 6 mg of wet weight tissue, which was added at the start of the incubation.

The test tubes were incubated for 30 min at 37°, then filtered under vacuum through Whatman GF/C filters, and washed three times with 5 ml of ice-cold Tris buffer, using a modified Brandel cell harvester. Filters were placed in plastic min-scintillation vials and 5 ml of Ecolume (ICN Biomedicals, Inc.) were added. Radioactivity trapped on the filters was counted using a Beckman LS 5000 TD scintillation counter at an efficiency of 43%.

In saturation experiments, [^{3}H]DHA was usually incubated in a concentration range of 0.2 to 6.0 nM, except in time course experiments, where it was incubated in a concentration range of 0.2 to 8.0 nM. Saturation curves of [^{3}H]CGP-12177 were performed in a concentration range of 0.025 to 2.0 nM, whereas in competition studies 0.2 nM [^{3}H]CGP-12177 was used.

$5HT_2$ receptor binding. [^{3}H]Ketanserin (specific activity, 61.5 Ci/mmol, New England Nuclear) was used to measure $5HT_2$ receptors, following the method previously reported (14). Briefly, individual frontal cortices were homogenized as described before and the final resuspension was made in ice-cold 50 mM Tris buffer (pH 7.7 at 25°). Saturation studies were carried out using six concentrations of [^{3}H]ketanserin (0.1–4.0 nM), which were incubated with 4 mg of original wet weight of frontal cortex in 2.5 ml of assay buffer, in the absence or presence of 500 nM cinanserin, which was used to define nonspecific binding to $5HT_2$ receptors. Specific binding represented 75% of total binding at 0.5 nM [^{3}H]ketanserin. The incubation was carried out for 30 min at 37° before the reaction was stopped by filtration over Whatman GF/C glass fiber filters. Filters were washed quickly with three 5-ml portions of ice-cold 50 mM Tris buffer and were counted in 5 ml of Ecolume by scintillation counting of an efficiency of 43%.

Tryptophan 5-hydroxylase activity. The activity of the enzyme tryptophan 5-hydroxylase was measured in rat striatum, following a modification of the fluorimetric method of Gal and Patterson (15). Briefly, rat striata were homogenized in HEPES buffer (50 mM HEPES, 20 mM dithiothreitol, 2 mM $CaCl_2$, 50 μM ferrous ammonium sulfate, and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4) with a motor-driven Teflon pestle and were centrifuged at 12,000 rpm for 10 min. An aliquot of the supernatant was added to the assay buffer (30 mM KH_2PO_4, 6 mM 2-aminoethylisothiouronium bromide, 0.2 mM pargyline, 16 μM ferrous ammonium sulfate) with 40 μg of catalase, 0.2 mM L-tryptophan, and 0.1 mM DL-6-methyl-5,6,7,8-tetrahydropterine. The samples either were incubated at 37° or were kept on ice (tissue blanks). A standard curve was prepared separately using L-5-hydroxytryptophan (0–1100 ng) dissolved in the assay buffer. After an incubation with 0.3 mg of *ortho*-phthaldialdehyde, the formation of L-5-hydroxytryptophan was measured by fluorescence spectroscopy (Perkin-Elmer LS-5 spectrophotometer) with 360–370 nm as excitatory wavelength and 470–505 nm as emission wavelength.

Data analysis. The weighted, nonlinear, least squares, iterative curve-fitting program LIGAND was used for the analysis of saturation and competition experiments (11). Saturation experiments were analyzed either by fitting the "specific" binding (defined as the difference between total binding and "nonspecific" binding delineated by 10 μM alprenolol) or by fitting the total binding, with the nonspecific binding being allowed to float and fitted by the program as a linear function of ^{3}H-ligand concentration. All saturation and competition studies were initially analyzed with a one-site model (i.e., one saturable binding site); the data were then analyzed according to a two-site model (i.e., two distinct saturable binding sites of different affinities) and the results of this curve fitting was statistically compared with a one-site model by an *F* test. The two-site model was accepted if the fit was significantly better ($p < 0.05$) with respect to the one-site analysis. LIGAND also allows for the simultaneous analysis of multiple competition curves for the same ligands, which provides more information for the accurate calculation of binding parameters.

An ANOVA test was used in the statistical analysis of the experiments and comparison between any two groups was made by the Dunnett *t* test. When comparisons were made between assays run in parallel or between different assay conditions (nonspecific binding defined by 10 μM alprenolol or by LIGAND), comparisons were also made by paired Student *t* test.

Drugs. The following drugs were donated: ketanserin tartrate (Janssen Pharmaceutica, Beerse, Belgium), ICI 89,406 (Imperial Chemical Industries, Wilmington, DE), DMI (Merrel Dow Pharmaceuticals Inc., Cincinnati, OH), and cinanserin HCl (Squibb & Sons Inc., Princeton, NJ).

The following compounds were purchased: *l*-alprenolol and PCPA HCl, (Sigma, St. Louis, MO) and CGS-12066B (Research Biochemicals Incorporated, Natick, MA).

Results

Time Course of β-Adrenergic Receptor Down-Regulation following DMI Treatment

Rats were treated with a single daily injection of either saline or DMI for 1, 2, 4, or 7 days and were killed 24 hr after the last injection. The data from the saline-treated rats were pooled, because they were not statistically different from each other, for comparisons with the DMI-treated rats. Utilizing the common procedure of defining nonspecific binding of [^{3}H]DHA with the β-adrenergic receptor antagonist alprenolol, it can be seen in Fig. 1 that a significant decrease (−19.4%) in the B_{max} of [^{3}H]DHA binding to rat cerebral cortex membranes did not occur until after a 7-day DMI treatment. However, as we show in the accompanying paper, [^{3}H]DHA also labels another binding site and alprenolol inhibits [^{3}H]DHA binding to this site (10). If the saturation data for [^{3}H]DHA binding were analyzed using the nonlinear, least squares, iterative curve-fitting procedure LIGAND, allowing the computer program to define nonspecific binding as a linear function of ^{3}H-ligand concentration, a significantly lower B_{max} was obtained in control animals, more closely representing the true β-adrenergic receptor number. With the nonspecific binding of [^{3}H]DHA defined by LIGAND in this way, it can be seen that the specific β-adrenergic receptor binding of [^{3}H]DHA was decreased significantly 24 hr after a single injection of DMI (−23%). Indeed, although the reduction following a 7-day treatment with DMI was somewhat greater (−37%), this decrease was not significantly greater than the decrease seen after a single injection. All of these changes in B_{max} occurred without significant changes in K_d values.

We have previously determined that the ligand [^{3}H]CGP-12177 is a more specific ligand for β-adrenergic receptors. Similar B_{max} values were obtained (10) whether nonspecific binding was defined by alprenolol or by LIGAND. Fig. 2 demonstrates that the B_{max} of β-adrenergic receptors labeled by [^{3}H]CGP-12177 was decreased in rat cerebral cortex 24 hr after a single injection of DMI. In the case of this ligand, a time course in the reduction of β-adrenergic receptors following DMI treatment for up to 7 days was apparent, with the decrease at 7 days (−34.1%) being significantly greater than the decrease seen at 1 (−9.3%) or 2 days (−19.6%) of treatment. However, a single acute injection of DMI, with the animal being sacrificed 2 hr later, showed no decrease in β-adrenergic receptor number (saline = 5.40 ± 0.10 versus acute DMI = 5.36 ± 0.16 fmol/mg of tissue). There was no change in the affinity of [^{3}H]CGP-12177 for β-adrenergic receptors after any of the DMI treatments. The same pattern of β-adrenergic receptor down-regulation after DMI treatment was observed in rat hippocampal membranes (Fig. 2). However, no significant change in β-

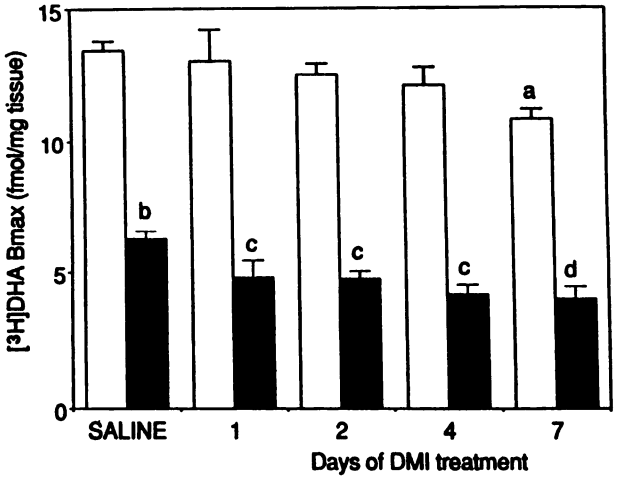

Fig. 1. Time course of the effect of DMI treatment on [^{3}H]DHA binding to rat cerebral cortex membranes. Saturation curves of [^{3}H]DHA (the concentration range was 0.2 to 8.0 nM) were analyzed by LIGAND, defining its nonspecific binding either with 10 μM alprenolol (□) or having the 'nonspecific' binding determined by the computer program as a linear function of ^{3}H-ligand concentration (■). The B_{max} (fmol/mg of tissue) for control, alprenolol-defined, [^{3}H]DHA binding was 13.43 ± 0.37. The control density of [^{3}H]DHA binding (6.30 ± 0.26 fmol/mg of tissue) was significantly lower ($p < 0.001$) when nonspecific binding was defined by LIGAND. No significant changes in K_d values were detected following DMI treatments; in saline-treated animals, the K_d of [^{3}H]DHA was 1.81 ± 0.08 nM when nonspecific binding was defined by 10 μM alprenolol, which was significantly greater than the K_d of 0.74 ± 0.06 nM obtained when the nonspecific binding was determined by LIGAND. $^a p < 0.01$ versus saline-treated rats with nonspecific binding defined by 10 μM alprenolol (Dunnett *t* test). $^b p < 0.001$ versus saline-treated rats when nonspecific binding was determined by 10 μM alprenolol (paired *t* test). $^c p < 0.05$ versus saline-treated rats with nonspecific binding defined by LIGAND (Dunnett *t* test). $^d p < 0.01$ versus saline-treated rats with nonspecific binding defined by LIGAND (Dunnett *t* test).

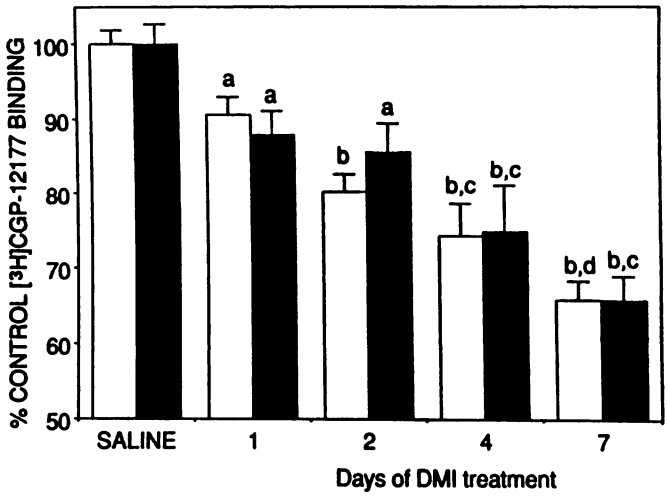

Fig. 2. Time course of the effect of DMI treatment on [^{3}H]CGP-12177 binding to rat cerebral cortex and hippocampus membranes. [^{3}H]CGP-12177 saturation curves for cerebral cortex (□) and hippocampus (■) membranes were analyzed by LIGAND with nonspecific binding defined by 10 μM alprenolol. The control B_{max} value (fmol/mg of tissue) in cerebral cortex (mean ± SE of at least six separate determinations) was 5.40 ± 0.10. No significant changes in K_d values from those in the saline-treated rats (0.13 ± 0.01 nM) were observed following DMI treatments. In rat hippocampal membranes, the percentages (mean ± SE of five or six separate determinations) of [^{3}H]CGP-12177 binding with respect to saline-treated rats (B_{max} = 2.55 ± 0.10 fmol/mg of tissue) were: DMI, 1-day = 88.1 ± 3.1%^{a}; DMI, 2-day = 85.7 ± 3.8%^{a}; DMI, 4-day = 75.0 ± 6.1%^{b,c}; and DMI, 7-day = 65.9 ± 3.1%^{b,c}. $^a p < 0.05$ versus saline-treated rats (Dunnett *t* test). $^b p < 0.01$ versus saline-treated rats (Dunnett *t* test). $^c p < 0.05$ versus DMI 1-day-treated rats (Dunnett *t* test). $^d p < 0.01$ versus DMI 1-day- and $p < 0.05$ versus DMI 2-day-treated rats (Dunnett *t* test).

adrenergic receptors was found after a 7-day treatment with the antidepressant in striatal membranes from the same rats (saline = 7.10 ± 0.37 versus DMI = 6.33 ± 0.30 fmol/mg of tissue).

[^{3}H]CGP-12177 has similar affinities for the β_1- and the β_2-adrenergic receptor subtypes (16). In order to investigate the effect of DMI treatment on β_1- and β_2-adrenergic receptor subtypes individually, competition curves of the semiselective β-adrenergic receptor antagonist ICI 89406 (17) for [^{3}H]CGP-12177 binding to cortical membranes were analyzed by the computer program LIGAND into two sites, the higher affinity site representing ICI 89406 binding to β_1-adrenergic receptors (K_i = 1.12 ± 0.10 nM) and the lower affinity site representing ICI 89406 binding to β_2-adrenergic receptors (K_i = 126.7 ± 11.7 nM). Rats were treated for 7 days with DMI and, as shown in Fig. 3, a significant 29% decrease in β_1-adrenergic receptors occurred, with no significant change in β_2-adrenergic receptors.

Investigation of the Role of Serotonin in the Down-Regulation of β-Adrenergic Receptors by DMI

Influence of 5HT$_2$ receptor blockade on the DMI-induced β-adrenergic receptor down-regulation. In order to investigate whether 5HT$_2$ receptors could be involved in the down-regulation of β-adrenergic receptors, rats were concurrently treated with DMI and the 5HT$_2$ receptor antagonist ketanserin for a 7-day period. Ketanserin was either administered by intraperitoneal injection, at the same time as DMI was administered, or it was released continuously by implanted osmotic minipumps (see Materials and Methods for details). As can be seen in Table 1, treatment with ketanserin alone (either by intraperitoneal injection or by minipump) did not affect the density of β-adrenergic receptors measured by [^{3}H]CGP-12177. The resolution of ICI 89406 competition curves

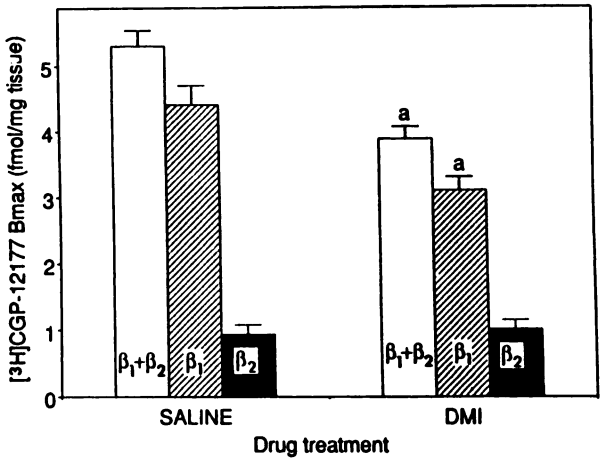

Fig. 3. Effect of 7-day DMI treatment on β-adrenergic receptor subtypes in rat cerebral cortex membranes. The density of total β-adrenergic receptors (□) was determined by saturation experiments of [^{3}H]CGP-12177 binding with nonspecific binding defined by 10 μM alprenolol and analyzed by LIGAND. The resolution of β-adrenergic receptors into β_1- (▨) and β_2- (■) adrenergic receptor subtypes was obtained from analyzing the competition curves of the semiselective β-adrenergic receptor subtype antagonist ICI 89406 for 0.2 nM [^{3}H]CGP-12177 binding. The competition curves were analyzed by LIGAND using the K_d of [^{3}H]CGP-12177 for the total β-adrenergic receptor population obtained from saturation experiments. The competition curves were best fitted to a two-site model, with the K_i for the higher affinity site, representing β_1-adrenergic receptors, being 1.12 ± 0.10 nM and the K_i for the lower affinity site, representing β_2-adrenergic receptors, being 126.7 ± 11.7 nM. $^a p < 0.01$ versus saline-treated rats (Dunnett t test).

TABLE 1

Density of β-adrenergic receptors, measured by [^{3}H]CGP-12177, in rat cerebral cortex membranes following chronic treatment (7 days) with DMI: effect of concomitant ketanserin administration either by intraperitoneal injection (IP) or by osmotic minipump implantation (MP)

The density of total β-adrenergic receptors (β_1 + β_2) was determined by saturation experiments of [^{3}H]CGP-12177 binding with nonspecific binding defined by 10 μM alprenolol analyzed by LIGAND. No significant change was detected in K_d values (saline = 0.14 ± 0.03 nM). The resolution of β-adrenergic receptors into β_1- and β_2-adrenergic receptors was obtained from competition curves of the semiselective β-adrenergic receptor subtype antagonist ICI 89406 for 0.2 nM [^{3}H]CGP-12177 binding. The complete competition curves were analyzed by the computer program LIGAND for two sites using the K_d of [^{3}H]CGP-12177 for β-adrenergic receptors obtained from saturation experiments. Nonspecific binding was defined by LIGAND.

Treatment	Density		
	β_1 + β_2	β_1	β_2
	fmol/mg of tissue		
Saline	5.32 ± 0.23	4.41 ± 0.28	0.94 ± 0.14
DMI	3.88 ± 0.19[a]	3.11 ± 0.21[a]	1.01 ± 0.14
Ketanserin (IP)	5.55 ± 0.20	4.86 ± 0.32	1.07 ± 0.15
DMI + ketanserin (IP)	3.98 ± 0.13[b]	3.11 ± 0.16[b]	0.99 ± 0.16
Ketanserin (MP)	5.59 ± 0.17	4.64 ± 0.30	1.00 ± 0.09
DMI + ketanserin (MP)	3.83 ± 0.15[c]	2.54 ± 0.12[c]	1.02 ± 0.14

[a] $p < 0.01$ versus saline-treated animals (Dunnett t test).
[b] $p < 0.01$ versus saline- or ketanserin (IP)-treated animals (Dunnett t test).
[c] $p < 0.01$ versus saline- or ketanserin (MP)-treated animals (Dunnett t test).

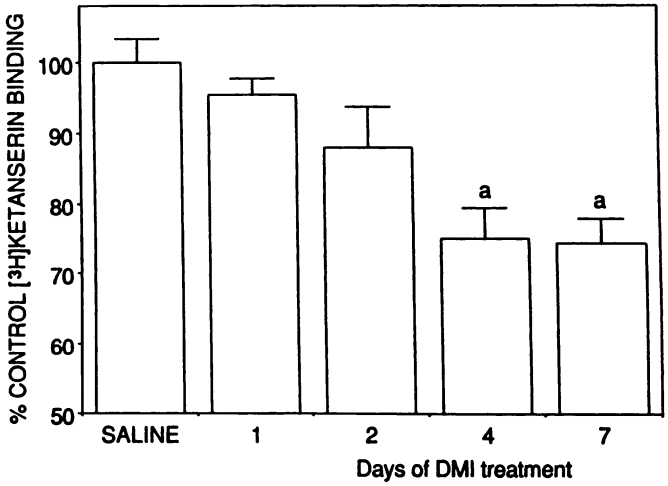

Fig. 4. Time course of DMI treatment on 5HT$_2$ receptor binding in rat frontal cortex. The density of 5HT$_2$ receptors was determined by saturation experiments with [^{3}H]ketanserin. The control B_{max} was 21.07 ± 0.94 fmol/mg of tissue. No significant change in K_d values was detected with the different treatments (K_d in saline-treated rats was 0.53 ± 0.06 nM). $^a p < 0.01$ versus saline-treated rats (Dunnett t test).

for [^{3}H]CGP-12177 binding indicated that neither β-adrenergic receptor subtype was altered by ketanserin treatment. The treatment of rats with ketanserin did not prevent the down-regulation of β-adrenergic receptors elicited by DMI. Although the combined treatment with ketanserin (via minipump) and DMI produced a somewhat larger decrease in β-adrenergic receptors, this effect was not statistically significant ($p > 0.05$).

Prolonged treatment with DMI has also been reported to down-regulate the number of 5HT$_2$ receptors in rat cerebral cortex (18). We investigated the time course of this modification and the effect of a concomitant administration of ketanserin on the adaptive changes of 5HT$_2$ receptors following DMI administration. Fig. 4 shows that a significant reduction in the number of 5HT$_2$ receptors measured with [^{3}H]ketanserin was evident after 4 days of treatment with DMI (−25%). This

TABLE 2

Density of $5HT_2$ receptors in rat frontal cortex after DMI treatment: effect of concomitant ketanserin administration either by intraperitoneal injection (IP) or by osmotic minipump implantation (MP)

Rats were sacrificed 24 hr after the last injection of DMI. The B_{max} of $5HT_2$ receptors was determined by saturation experiments with [^{3}H]ketanserin, with nonspecific binding defined by 500 nM cinanserin. No differences in K_d values (0.54 ± 0.05 nM) were detected among the experimental groups.

Drug treatment	B_{max}
	fmol/mg of tissue
Saline	21.07 ± 0.94
DMI	15.67 ± 0.74[a]
Ketanserin (IP)	15.50 ± 0.66[a]
DMI + ketanserin (IP)	13.75 ± 0.83[a]
Ketanserin (MP)	15.81 ± 0.80[a]
DMI + ketanserin (MP)	13.19 ± 0.82[a]

[a] $p < 0.001$ versus saline-treated rats (Dunnett t test).

reduction was not significantly different from that observed after the 7-day treatment with the antidepressant. As has been reported previously (19), Table 2 indicates that ketanserin itself, either following intraperitoneal injection or minipump implantation, was able to down-regulate the number of $5HT_2$ receptors. However, this effect was not additive with the DMI-induced down-regulation; although the rats treated with the two drugs demonstrated a larger reduction in [^{3}H]ketanserin B_{max}, this effect was not significantly greater than that produced by DMI or ketanserin administered alone.

Effect of serotonin depletion on DMI-induced down-regulation of β-adrenergic receptors. Rats received a prior treatment with PCPA (350 mg/kg daily, intraperitoneally) for 3 days before the treatment with DMI for 8 days was begun. During the DMI treatment, PCPA administration was maintained by injection of 125 mg/kg every other day. The rats were sacrificed 24 hr after the last DMI injection. Fig. 5 shows that the PCPA treatment reduced striatal tryptophan hydroxylase activity by more than 90%. The co-treatment with DMI and PCPA produced a similar decrease in tryptophan hydroxylase activity, whereas DMI alone did not change the activity of the enzyme.

β-Adrenergic receptors were quantified using either [^{3}H]DHA or [^{3}H]CGP-12177. When binding was measured using

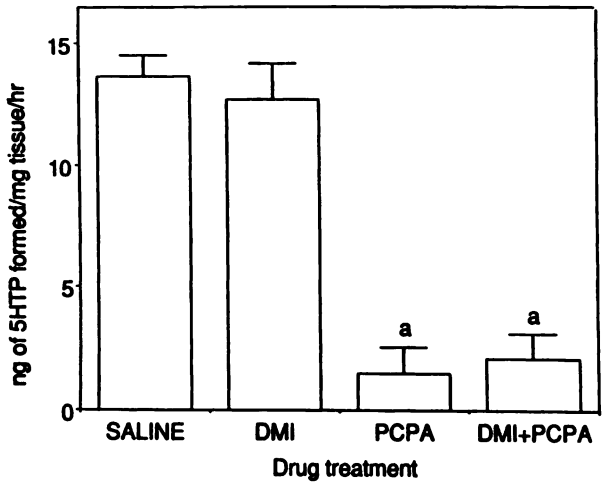

Fig. 5. Rat striatal tryptophan hydroxylase activity following DMI and/or PCPA treatment. The values are the mean ± standard error of six separate determinations. [a] $p < 0.001$ versus saline-treated rats (Dunnett t test).

[^{3}H]DHA in the typical manner, with nonspecific binding defined by 10 μM alprenolol, it can be seen in Table 3 that the DMI treatment alone significantly reduced "specific" binding by 14%. PCPA treatment alone significantly increased binding by 15% and the co-treatment with DMI and PCPA produced binding levels that were not significantly different from those in the saline-treated animals. However, if [^{3}H]DHA binding to β-adrenergic receptors was defined by the computer program LIGAND, with nonspecific binding determined as a factor proportional to ^{3}H-ligand concentration, the following observations were made: DMI significantly reduced β-adrenergic receptor binding by 38%; PCPA treatment did not affect β-adrenergic receptor binding; and the co-treatment with DMI and PCPA produced a significant decrease in β-adrenergic receptors of 20%, which was not significantly different from that produced by DMI alone. Similarly, as shown in Fig. 6, when β-adrenergic receptors were measured specifically with

TABLE 3

[^{3}H]DHA binding to rat cerebral cortex membranes after a prolonged treatment with DMI (8 days): effect of serotonin depletion following PCPA injection

Rats were killed 24 hr after the last DMI injection. Nonspecific binding for [^{3}H]DHA (concentration range of 0.2 to 6.0 nM) was either defined by 10 μM alprenolol or defined by the computer program LIGAND. No significant change was detected in K_d values following the drug treatment.

Treatment	B_{max}	
	Nonspecific binding defined by 10 μM alprenolol	Nonspecific binding defined by LIGAND
	fmol/mg of tissue	
Saline	10.78 ± 0.54	5.92 ± 0.50[a]
DMI	9.25 ± 0.43[b]	3.66 ± 0.08[c]
PCPA	12.45 ± 0.50[b]	5.74 ± 0.13
DMI + PCPA	10.98 ± 0.59	4.71 ± 0.33[d]

[a] $p < 0.001$ versus saline-treated group when nonspecific binding was defined by 10 μM alprenolol (paired t test).
[b] $p < 0.05$ versus saline-treated rats (paired t test).
[c] $p < 0.01$ versus saline-treated rats (Dunnett t test).
[d] $p < 0.05$ versus saline- or PCPA-treated rats (Dunnett t test).

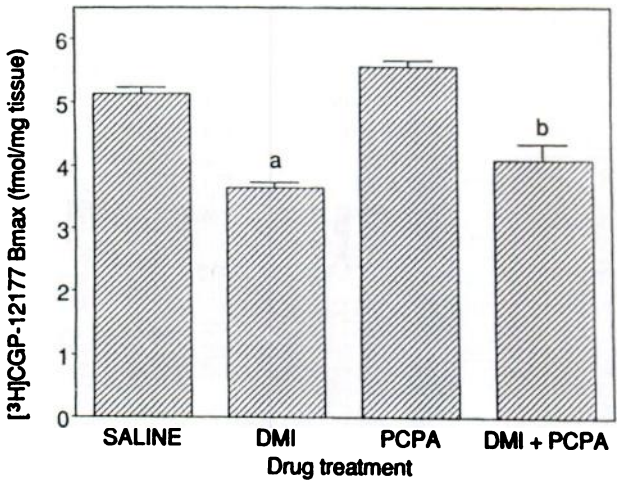

Fig. 6. Effect of serotonin depletion following PCPA injection of [^{3}H]CGP-12177 binding to rat cerebral cortex membranes after a chronic treatment with DMI (8 days). [^{3}H]CGP-12177 saturation curves were analyzed by LIGAND with nonspecific binding defined by 10 μM alprenolol. The measured B_{max} values (fmol/mg of tissue) were: saline = 5.14 ± 0.10; DMI = 3.64 ± 0.10[a]; PCPA = 5.58 ± 0.10; and DMI + PCPA = 4.08 ± 0.27[b]. No changes in K_d values of [^{3}H]CGP-12177 were detected (saline = 0.099 ± 0.001 nM). [a] $p < 0.01$ versus saline-treated rats (Dunnett t test). [b] $p < 0.01$ versus saline or PCPA-treated rats (Dunnett t test).

[^{3}H]CGP-12177, with nonspecific binding defined by 10 μM alprenolol, DMI treatment produced a 29% reduction in β-adrenergic receptor B_{max} and PCPA did not significantly alter β-adrenergic receptor binding, whereas the co-treatment with PCPA and DMI produced a significant 21% decrease in β-adrenergic receptor B_{max}, which was not significantly different from that elicited by DMI treatment alone. None of the treatments altered the affinity of the ^{3}H-ligands for their binding sites.

Discussion

β-Adrenergic receptors were one of the first types of central nervous system neurotransmitter receptor to be studied with radioligand binding techniques (20). [^{3}H]DHA was used as the radioligand for their initial characterization and has remained the most popular ligand for the study of these receptors. Numerous reports have demonstrated that the chronic treatment of rats with antidepressants leads to the down-regulation of β-adrenergic receptors as labeled by [^{3}H]DHA, as well as the reduction in the ability of β-adrenergic agonists to stimulate adenylate cyclase activity (1–4). However, differences have been noticed in the time course for these two events (3, 4, 12, 13). Similarly, manipulations of serotonergic systems have been reported to block the ability of antidepressants to down-regulate β-adrenergic receptor binding (5–9), although in some reports, paradoxically, the reduced sensitivity of adenylate cyclase to stimulation by β-adrenergic agonists was unaffected (8, 9).

Our recent studies have demonstrated that [^{3}H]DHA labels an additional site besides β-adrenergic receptors and that the separation of these two sites cannot be adequately obtained with the drugs currently used to define [^{3}H]DHA nonspecific binding (10). The additional [^{3}H]DHA site has some of the pharmacological characteristics of a serotonergic binding site and may be identified with the $5HT_{1B}$ serotonin receptor subtype (10). A possible explanation for the differential effects of serotonergic manipulations on "β-adrenergic receptors" labeled by [^{3}H]DHA and β-adrenergic stimulation of adenylate cyclase by DMI is thus suggested, because such manipulations may modulate the serotonergic and β-adrenergic receptor components of [^{3}H]DHA binding differently.

We have demonstrated that it is possible to use [^{3}H]DHA to label β-adrenergic receptors selectively, if the binding data are analyzed with the computer program LIGAND and the nonspecific binding is defined as a factor proportional to ^{3}H-ligand concentration (10). We also further demonstrated that another β-adrenergic receptor ligand, [^{3}H]CGP-12177, does not suffer from the additional labeling of the serotonergic site and that it can be used to selectively measure β-adrenergic receptors, whether its nonspecific binding is defined by alprenolol or by LIGAND.

One of the major premises of current psychopharmacology is that the β-adrenergic receptor down-regulation is in some way related to the therapeutic mechanism of action of antidepressants, because both responses require that antidepressants be given for a number of days before the response is apparent (1–4). Indeed, previous studies investigating the down-regulation of β-adrenergic binding, using [^{3}H]DHA as a radioligand, have demonstrated that at least 1 week of treatment (in general) is required to down-regulate the "β-adrenergic receptor" binding sites. Indeed, in the present studies, if [^{3}H]DHA binding is characterized in the typical manner, with nonspecific binding defined by 10 μM alprenolol, we also found that a significant decrease in its binding was not apparent until after 7 days of DMI treatment (Fig. 1). However, if the specific binding of [^{3}H]DHA to only β-adrenergic receptors was measured (with the LIGAND program defining the nonspecific binding), a significant decrease in β-adrenergic receptors was apparent 24 hr after a single injection of DMI (Fig. 1). The decrease in β-adrenergic receptors required time to occur, because the effect was not apparent 2 hr after the DMI injection. Furthermore, using the specific β-adrenergic receptor radioligand [^{3}H]CGP-12177, we were also able to demonstrate a significant decrease in β-adrenergic receptors 24 h after a single injection of DMI (Fig. 2). Using [^{3}H]DHA as ligand, the decrease seen after a 7-day versus a 1-day treatment with DMI was not significantly greater. However, using [^{3}H]CGP-12177 as radioligand, it was apparent that the decrease at 7 days was greater than the decrease seen at earlier time points. A very early study (21) using another β-adrenergic receptor ligand, [^{125}I]iodohydroxybenzylpindolol, also reported a significant decrease of β-adrenergic receptors after a single DMI injection, which became maximal after seven daily injections.

It has previously been reported that the time course of the decrease in β-adrenergic receptors in the hippocampus was slower than that in the cortex, suggesting that the hippocampus may be a more primary site for the antidepressant action of DMI (22). However, in our study we found that the hippocampus also showed a significant decrease in β-adrenergic receptors 24 hr after a single DMI injection and that the pattern of modifications observed in rat hippocampal membranes resembled those observed in rat cerebral cortex (Fig. 2). As reported previously (4), no change was detected in β-adrenergic receptors after a 7-day treatment with DMI in striatal membranes from the same rats. We found that the effects of DMI occurred solely at the β_1- and not the β_2-adrenergic receptor subtype (Fig. 3), as previously reported (23).

Two studies were undertaken to investigate the role of serotonergic mechanisms in the action of DMI on β-adrenergic receptors. Because DMI is also a potent inhibitor of 5HT reuptake and because its chronic administration has also been reported to down-regulate $5HT_2$ receptors in rat brain (18), in one experiment ketanserin, a $5HT_2$ receptor antagonist, was co-administered with DMI to investigate whether the blockade of the effects of endogenous 5HT at $5HT_2$ receptors would modulate the ability of DMI to down-regulate β-adrenergic receptors. Irrespective of whether ketanserin was administered by intraperitoneal injection or was continuously infused by an implanted osmotic minipump, ketanserin had no influence on the β-adrenergic receptor down-regulation by DMI.

We confirmed the down-regulation of $5HT_2$ receptors elicited by chronic DMI treatment (18) and found that the time course for this effect was somewhat slower than for the down-regulation of β-adrenergic receptors, becoming significant only at day 4. The chronic administration of ketanserin significantly down-regulated $5HT_2$ receptors, as has been reported previously (19). Interestingly, the effect of ketanserin was not additive with the effect of DMI, suggesting that possibly both effects are mediated by a similar mechanism. However, it is also possible that there is a 'ceiling' effect on the ability of $5HT_2$ receptors to down-regulate and that, although ketanserin and DMI may work by different mechanisms, only the same maximal down-

regulation can occur. Dose-response studies are needed to resolve this question.

It has been reported previously that serotonin depletion, following the administration of the tryptophan hydroxylase inhibitor PCPA (5, 6) or lesion of serotonergic forebrain systems with the neurotoxin 5,7-dihydroxytryptamine (7, 8), is able to block the ability of DMI to down-regulate β-adrenergic receptors. Moreover PCPA treatment (6) and 5,7-dihydroxytryptamine lesion (8, 9, 24) alone have been shown to increase the B_{max} of [^{3}H]DHA. Indeed, if the β-adrenergic receptor binding of [^{3}H]DHA was defined in the typical way using 10 μM alprenolol, we replicated these observations and found that "β-adrenergic receptor" binding was not decreased following 8 days of DMI treatment if tryptophan hydroxylase had been inhibited by more than 90% by PCPA treatment and that PCPA treatment alone significantly increased [^{3}H]DHA binding. However, if the selective binding of [^{3}H]DHA to β-adrenergic receptors was resolved by the computer program LIGAND or if β-adrenergic receptors were investigated with the selective ligand [^{3}H]CGP-12177, with nonspecific binding defined by 10 μM alprenolol, we found that PCPA, which alone did not change the B_{max} of β-adrenergic receptors, was not able to block the ability of DMI to down-regulate β-adrenergic receptor binding. When either [^{3}H]CGP-12177 or [^{3}H]DHA (with nonspecific binding defined by LIGAND) was used as radioligand, the data suggest that there may have been a tendency for the PCPA treatment to reduce the effect of DMI, although the decrease of β-adrenergic receptors following DMI under the influence of PCPA was not statistically significantly different from that after the DMI treatment alone. However, this observation may not indicate a role of serotonin in this effect, because PCPA has also been shown to reduce, although to a lesser extent, norepinephrine levels (25), which may have influenced the effect of DMI.

The fact that PCPA treatment increased [^{3}H]DHA binding when nonspecific binding was defined by 10 μM alprenolol, whereas the "true" β-adrenergic receptors (see above) were not changed, suggests that PCPA treatment increased the serotonergic component of [^{3}H]DHA binding. Indeed, if one subtracts the true β-adrenergic receptor binding levels of [^{3}H]DHA (defined by LIGAND) from the [^{3}H]DHA binding defined by 10 μM alprenolol, one can derive apparent values for the serotonergic component of [^{3}H]DHA binding. If this is done, it is apparent that DMI itself also increases the binding to the serotonergic component (+15%). This observation would explain why a longer time course may be required for the effect of DMI on the β-adrenergic receptor binding of [^{3}H]DHA (defined in the typical manner) to become apparent, because DMI is simultaneously increasing the serotonergic component as it is decreasing the β-adrenergic receptor component of [^{3}H]DHA binding.

As already mentioned, there is some controversy in the literature on the time course of β-adrenergic receptor down-regulation following the administration of DMI. A number of early studies utilized weeks of DMI treatment, although it is not clear from the papers whether earlier time points were adequately examined (1, 2), because more rapid effects have now been reported by the same laboratories (9). However, a few reports have indicated that DMI can down-regulate β-adrenergic receptors after a 2-day treatment (3, 21, 22), even when [^{3}H]DHA was used as ligand (3, 22). In these two studies, a single and low concentration of [^{3}H]DHA and/or a lower concentration of nonradioactive drug to define nonspecific binding were used. This would tend to more selectively label β-adrenergic receptors and to lessen the confounding increase in the serotonergic component of [^{3}H]DHA binding sites that we have shown is simultaneously produced by DMI. Higher concentrations of [^{3}H]DHA will label more of the serotonergic binding sites, which, as we have shown in the accompanying paper, bind [^{3}H]DHA with a lower affinity.

As we discussed in the accompanying paper, it is impossible to successfully quantify [^{3}H]DHA binding to the non-β-adrenergic receptor binding site. We attempted to characterize the increase in the serotonergic binding component induced by DMI and PCPA by analyzing the competition curves of the semiselective $5HT_{1B}$ agonist CGS-12066B for [^{3}H]DHA binding. In our previous studies, we had demonstrated that this agonist demonstrates two components of inhibition of [^{3}H]DHA binding, with a high affinity component of approximately 10–20% of total 2.0 nM [^{3}H]DHA binding, representing its competition for the serotonergic component of [^{3}H]DHA binding. However, in the present study, the binding of CGS-12066B in control tissue could not be resolved into two sites in every animal. When the competition curves for all six control animals were analyzed simultaneously by the program LIGAND, the binding could be resolved significantly into two components. Under these circumstances, with the experimental data from all rats from each drug treatment analyzed simultaneously, we found that DMI and PCPA increased the serotonergic component of [^{3}H]DHA binding by 118% and 71%, respectively. The combined treatment also increased the serotonergic component of [^{3}H]DHA by a similar percentage (data not shown). However, given all the assumptions necessary for this analysis, such as the approximation of the affinity of [^{3}H]DHA for the serotonergic component (as detailed in Ref. 10) this result needs to be confirmed with a selective ^{3}H-ligand for this site.

The present results suggest that the serotonergic system does not play a major role in the down-regulation of β-adrenergic receptors by DMI. This finding complements the observation that the effects of DMI on β-adrenergic receptor agonist stimulation of adenylate cyclase is also not influenced by serotonin depletion (8, 9). Moreover, these results may provide an alternative interpretation of the recent data from Sulser and co-workers (26). Under the common assay procedure for [^{3}H]DHA binding (nonspecific binding defined by 10 μM propranolol), they have shown that the increase of [^{3}H]DHA B_{max} after the lesion of the serotonergic system with 5,7-dihydroxytryptamine was confined to the β-adrenergic population that displayed low affinity for isoproterenol and that this increase could be blocked by the addition of serotonin to the incubation mixture. In the previous paper, we have shown that isoproterenol competes for [^{3}H]DHA (although not for the specific β-adrenergic receptor ligand [^{3}H]CGP-12177) binding in a biphasic manner but that its low affinity site probably defines the serotonergic binding site labeled by [^{3}H]DHA, rather than a low affinity agonist state of the β-adrenergic receptor (10). This suggests that the increase in [^{3}H]DHA binding after serotonin lesion that was observed by Sulser and co-workers (26) could be due to an increase in the serotonergic binding site labeled by [^{3}H]DHA rather than to a change in a low affinity agonist state of the β-adrenergic receptor. Similar observations of the ability of 5-

HT to interact with [^{3}H]DHA binding sites have been made recently by Dhawan and Kellar (27).

Early studies reported that prolonged treatment with DMI (4–8 weeks) was necessary to desensitize β-adrenergic stimulation of adenylate cyclase activity (1). Similarly, early studies also reported that "weeks" of treatment with DMI were required to down-regulate [^{3}H]DHA-labeled β-adrenergic receptors (2, 4). These observations led to the current hypothesis that β-adrenergic receptor down-regulation is related to the therapeutic efficacy of antidepressants, which also has a slow onset. In recent years, however, the effects of DMI on β-adrenergic receptor stimulation of adenylate cyclase have been seen after only 1 week of treatment by the same laboratories (9), whereas the early work of other laboratories that reported significant desensitization after only 1 (21) or a few days (3) of treatment have not been emphasized. In our study, we find that the down-regulation of β-adrenergic receptors can occur 24 hr after a single injection of DMI. These results suggest that a similarity in time course of action cannot be used to support the hypothesis that the therapeutic action of DMI is related to β-adrenergic receptor down-regulation.

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