MOLECULAR PHARMACOLOGY, 15, 588-597

In Vitro and *In Vivo* Kinetic Analysis of the Interaction of a Norbornyl Derivative of Propranolol with β-Adrenergic Receptors of Brain and C6 Glioma Cells; an Irreversible or Slowly Reversible Ligand

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(Received July 31, 1978)
(Accepted November 27, 1978)

SUMMARY

LUCAS, MARGUERITE, HOMBURGER, VINCENT, DOLPHIN, ANNETTE & BOCKAERT, JÖEL (1979) *In vitro* and *in vivo* kinetic analysis of the interaction of a norbornyl derivative of propranolol with β-adrenergic receptors of brain and C6 glioma cells. An irreversible or slowly reversible ligand. *Mol. Pharmacol.*, **15**, 588-597.

When injected into rats (30 mg/kg, i.p.), a norbornyl derivative of propranolol, 1-(2-exo-bicyclo[2,2,1] Hept-2-ylphenox)-3-[(1-methyl-ethylamino]-2-propanol (FM 24) blocked by 85% the cerebral cortex β-adrenergic receptors measured on membranes prepared five hours after the treatment. The preparation of membranes included extensive washing and a 75 min incubation period at 37°. Injection of propranolol at the same dose did not modify the number of β-adrenergic receptors in these membranes. When membranes were prepared 11 hours after a single injection of FM 24, 50% of β-adrenergic receptors were still blocked; more than 24 hours were necessary for complete recovery. A detailed analysis of the FM 24 interaction with β-adrenergic receptors was performed on β-adrenergic receptors of C6 glioma cells. FM 24 behaves as a competitive antagonist of the [^{3}H]-dihydroalprenolol binding and of the isoproterenol sensitive adenylate cyclase (K_I = 50 μM) when measured during a short period after starting the reaction. When measured after different incubation periods, FM 24 blocked the β-adrenergic receptors in a mixed competitive and non-competitive manner. The non-competitive inhibition was time and concentration dependent. The time course of this non-competitive inhibition was delayed by the presence of the β-adrenergic agonist isoproterenol. When membranes were washed, only the non-competitive inhibition of both [^{3}H]-dihydroalprenolol binding and β-adrenergic sensitive adenylate cyclase remained. It is proposed that the FM 24 formed a reversible complex with β-adrenergic receptors which is slowly transformed to a more stable complex. An analysis of the dissociation of this more stable complex showed two components, one which dissociated within one hour and the other which did not dissociate even after 5 hours of incubation.

This work was supported by L.R.A. 219 from the Centre National de la Recherche Scientifique (CNRS) and by the Institut National de la Recherche Médicale (INSERM) C.L.R. N° 76.4.04.26 and ATP N° 58-78-90.

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INTRODUCTION

Three classes of ligand have been used to study the characteristics of hormonal or neurotransmitter receptors sites. The first class is composed of ligands which bind with and dissociate from their receptors rapidly, and includes competitive agonists

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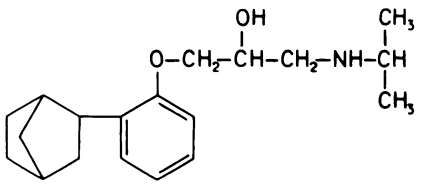
and antagonists. The second class is composed of irreversible ligands which bind covalently to receptors. Examples of this class are photoaffinity labels (1–3) and compounds interacting with reactive groups such as sulphydryl groups (4–7). Such ligands may be useful for purifying (5) or studying the turnover of the receptor molecules. However they are generally very unstable, and because of their high reactivity they are also involved in non-specific interactions (7). They are of uncertain use for *in vivo* blockade of the sites. Members of the third class bind tightly to their receptors and dissociate very slowly. Indeed, the snake venom neurotoxins, which are the best example of this class, have been considered for a long time to bind irreversibly to acetylcholine receptors (8, 9), although detailed analysis has now shown that a very slow dissociation does in fact occur (10–12). These toxins have been used extensively for purification (11, 13), synaptic localization (14, 15) and investigation of turnover (15, 16) of the acetylcholine receptor. No such slow reversible ligand does exist for β-adrenergic receptors. The β-adrenergic antagonist which has the slowest dissociation constant is the hydroxybenzylpindolol. However, it has been observed that most of the hydroxybenzylpindolol binding is reversed in less than three hours, excluding the use of this ligand to perform experiments similar to these conducted with neurotoxins (17).

In this report, we present evidence that norbornyl derivative of propranolol, FM 24[2] (Fig. 1), binds to the β-adrenergic receptor both *in vitro* and *in vivo* with characteristics comparable to those of the snake venom neurotoxins.



FM 24

FIG. 1. *Structural formula of FM 24*

MATERIALS AND METHODS

Adenylate cyclase assay and (–) [^{3}H]-dihydroalprenolol binding in C6 glioma cells. Culture of C6 glioma cells, particulate fraction preparation, [^{3}H]-DHA binding and adenylate cyclase activity measurements were performed as previously described (18). [^{3}H]-DHA binding and adenylate cyclase activities were determined in strictly the same conditions. Briefly, particulate fractions of C6 glioma cells (20–25 μg protein) were incubated at 30° in a total volume of 50 μl containing 100 mM TRIS-HCl pH 8, 5 mM $MgSO_4$, 1 mM cyclic AMP, 0.2 mM ATP, 0.2 mg/ml creatine kinase, 20 mM phosphocreatine and 1 mM EDTA. For adenylate cyclase assays tracer amounts of [α^{32}P]-ATP and [^{3}H]-cyclic AMP were added together when indicated. For binding measurements [^{3}H]-DHA was added at the beginning of incubation. The incubation was terminated by addition of 1 ml of cold (4°) 50 mM TRIS-HCl (pH 8) containing 20 mM $MgCl_2$, and the samples were filtered through GF/C Whatman filters. The filters were then washed and the bound radioactivity determined by scintillation counting. Specific binding was defined as the difference between the amount of [^{3}H]-DHA bound in the absence of (total binding) and in the presence of (nonspecific binding) 10 μM unlabeled alprenolol.

[^{3}H]-DHA binding in rat cerebral cortex. These binding experiments were conducted essentially as previously described (19).

Cerebral cortex from rats (male Sprague Dawley, 200–300 g) were dissected free from the external capsule at 4° and homogenized (0.05 g wet weight/ml) in 10% sucrose. The homogenate was centrifuged at 1000 × *g* for 10 minutes and the supernatant was then centrifuged at 47,000 × *g* for 30 minutes. The pellet was washed 5 times with a 15 min incubation period at 37° between each wash. The final pellet was resuspended in 20 volumes (of initial fresh weight) of TRIS-HCl (50 mM, pH 8 containing EDTA 5 mM, ascorbic acid 0.1% and

[2] The abbreviations used are: FM 24: 1 (2-exo-bicyclo[2,21]Hept-2-ylphenoxy-3-[(1-methylethyl)-amino]-2-propanol,hydrochloride; [^{3}H]-DHA: (–)-[^{3}H]-dihydroalprenolol.

pargylline 1 μM) and filtered through a silk screen. Aliquots (usually 200 μl) were incubated for 30 min at 23° with [^{3}H]-DHA in a total volume of 240 μl (protein concentration 1-2 mg/ml). Nonspecific binding was determined in the presence of (−)-alprenolol (1 μM). At the end of the incubation period the samples were diluted with 1 ml ice-cold TRIS-HCl, 50 mM, pH 7.8, filtered through a glass fiber filter (GF/B, Whatman) and washed with 5 × 4 ml of the same medium.

Chemicals. (−)-[^{3}H]-dihydroalprenolol, [^{3}H]-cyclic AMP and [α^{32}P]-ATP were purchased from the New England Nuclear Corp.; (−)-isoproterenol, (−) noradrenaline and (±) propranolol were purchased from Sigma Chemical Company; FM 24 was synthetized by Pharmindustrie of the Pharmuka Group.

RESULTS

Apparent irreversible blockade of β-adrenergic receptors of rat brain after in vivo injection of FM 24. Kinetic of reappearance. FM 24, propranolol (30 mg/kg) or saline were administered i.p. to rats. After 5 hr, the cerebral cortex membranes were prepared, washed five times with a 15 min incubation at 37° between each wash and the characteristics of [^{3}H]-DHA binding were investigated. Figure 2 shows that the propranolol administration did not alter either the affinity or the total number of binding sites. On the contrary, FM 24 at the same dose markedly reduced the total number of β-adrenergic receptors (by 86%). Their apparent affinity was not highly modified. However, the low specific binding capacity after FM 24 treatment did not allow an accurate determination. After all the washes and the 75 min incubation period at 37° the total number of binding sites for control membranes was 141 fmoles/mg protein compared to 169 ± 8 fmoles/mg protein (N = 12) when membranes were prepared in normal conditions.

The time-course of the evolution of the total number of binding sites accessible to [^{3}H]-DHA after a single injection of FM 24 (30 mg/kg i.p.) was analyzed by Scatchard analysis. The $t_{1/2}$ of reappearance was about

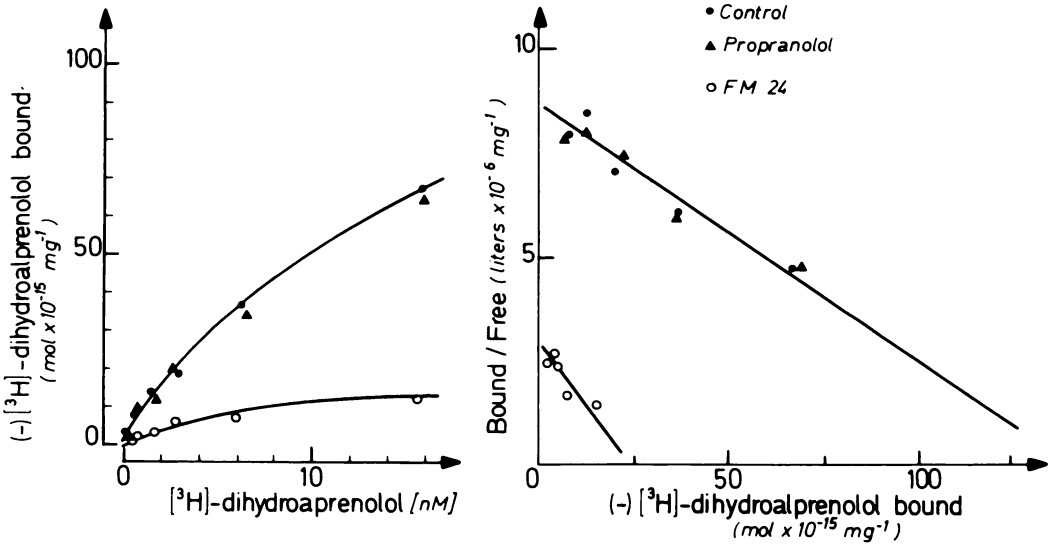


FIG. 2. *Effect of in vivo injection of FM 24 and propranolol on the number of β-adrenergic receptors present in rat cerebral cortex membranes*

(±) propranolol, FM 24 (30 mg/kg) or saline were administered i.p. into rats. Five hours later the membranes were prepared as described under methods and assayed for [^{3}H]-DHA bindings. For the highest [^{3}H]-DHA concentration used, the non-specific binding represented 59%, 48% and 87% of the total binding in the membranes prepared from control, propranolol and FM 24 treated animals, respectively. The non-specific binding decreased linearly with [^{3}H]-DHA decreasing concentrations.

11 hours. More than 24 hours were necessary for complete recovery (Fig. 3).

Rapid competitive and slow non-competitive inhibition of the β-adrenergic sensitive adenylate cyclase and [^{3}H]-DHA binding by FM 24 determined in vitro on C6 glioma cell membranes. Figure 4 (part A) shows that when all compounds necessary to measure the adenylate cyclase activities plus isoproterenol and propranolol or FM 24 were present at the beginning of incubation, and the reaction was measured for 5 min, propranolol and FM 24 behaved as competitive inhibitors of the isoproterenol-stimulated adenylate cyclase (K_I apparent = 9.6 nM and 52 nM, respectively). In the experiment shown in Fig. 4 (Part B), the membranes were incubated in the absence or the presence of either propranolol or FM 24 (.5 μM) in standard medium except that ATP (plus a tracer amount of [α^{32}P]-ATP) and isoproterenol were added after 15 min. In these conditions, propranolol still behaved as a competitive inhibitor (K_I app. = 8 nM) whereas FM 24 blocked the β-adrenergic stimulation in a mixed competitive (shift to the right of the dose-response curve; K_I app. 20 nM) and a non-competitive manner (decrease in the maximal velocity of the reaction). These results suggest that the competitive inhibition by FM 24 is not dependent on the incubation time whereas the non-competitive blockade of the β-adrenergic-sensitive adenylate cyclase is clearly time dependent. Experiments conducted with 1 μM and 10 μM FM 24 gave qualitatively the same results (data not shown). FM 24 or propranolol did not significantly modify the basal (Fig. 4) or NaF-stimulated (data not shown) adenylate cyclase activities.

When membranes were pretreated with FM 24 (50 nM) for 12 min at 30°, and washed 5 times, FM 24 did not alter the apparent affinity of adenylate cyclase of β-adrenergic stimulation but still reduced the maximal stimulation without modifying the affinity of the remaining sites (Fig. 5).

FM 24 and propranolol (100 nM) blocked competitively the interaction of [^{3}H]-DHA with β-adrenergic receptors when the binding assay was performed during a 5 min incubation period (K_D = 50 nM and 25 nM, respectively) (Fig. 6A). On the contrary,

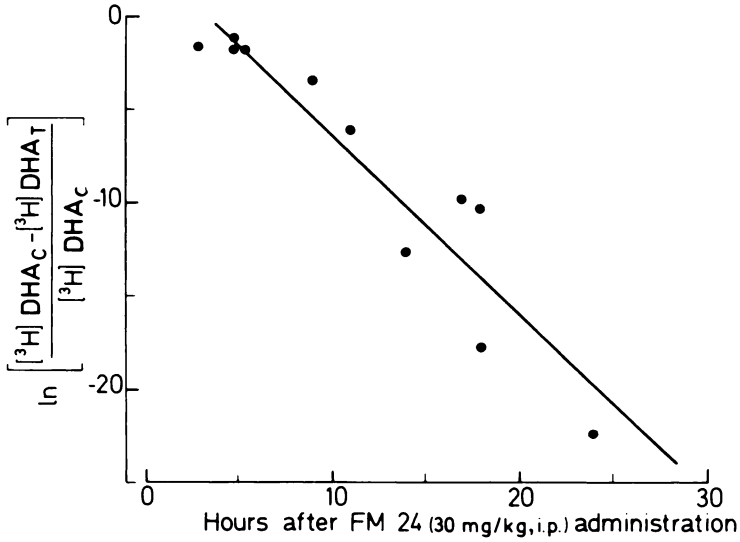


FIG. 3. *Time course of the reappearance of β-adrenergic receptors in rat cerebral cortex membranes after injection of FM 24*

Rats were injected i.p. with FM 24 (30 mg/kg) or saline (controls). After different periods of time, pairs of treated and control rats were killed and the specific binding of [^{3}H]-DHA was performed on cerebral cortical membranes as described under METHODS. The concentration of β-adrenergic receptors remaining was estimated by Scatchard plot analysis for each time period. [^{3}H]-DHA$_C$ = total number of specific binding sites in membranes of control rats; ^{3}H -DHA$_T$ = total number of specific binding sites in membranes of treated animals.

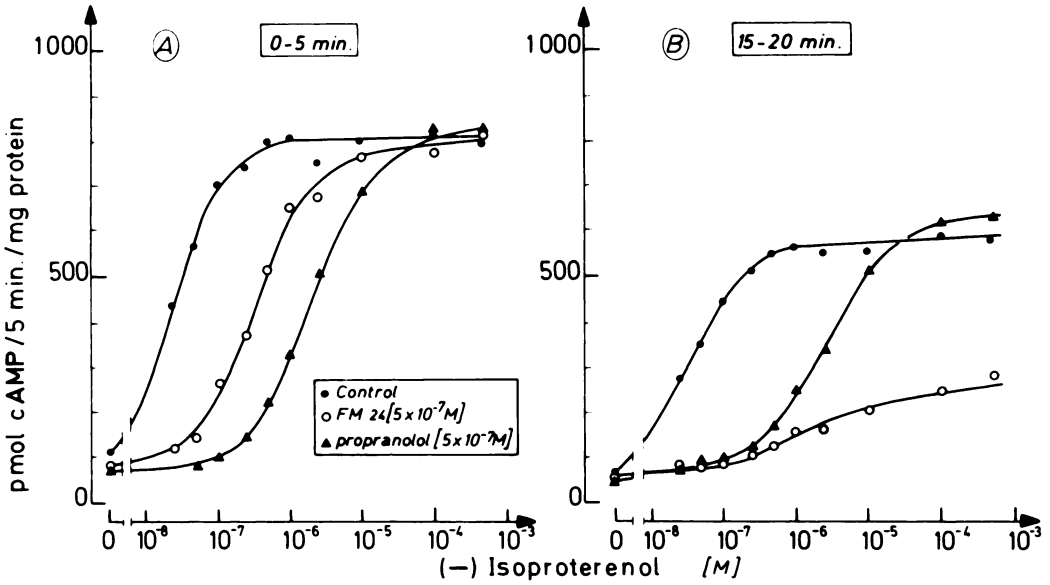


FIG. 4. *Effects of FM 24 and propranolol on β-adrenergic sensitive adenylate cyclase of C6 glioma cell membranes*

A. The effects of these antagonists were tested without preincubation. All drugs and adenylate cyclase assay components were present during the 5 min assay. B. The effects of these antagonists were tested after 15 min preincubation period. Membranes were incubated with FM 24 or propranolol with all the adenylate cyclase assay components except for isoproterenol and ATP (plus a tracer amount of [α^{32}P]-ATP) which were added together in 5 μl, after a 15 min preincubation period. The adenylate cyclase assay reaction was then allowed to proceed for 5 min. The amount of protein was 47 μg in 50 μl.

when membranes were pretreated during 12 min with 50 nM, 0.1 μM or 500 nM FM 24, washed extensively and assayed for [^{3}H]-DHA binding, it is clear that FM 24 reduced in a concentration-dependent manner the number of β-adrenergic receptor sites accessible to the labeled ligand (Fig. 6B).

Time and concentration dependence of the non-competitive inhibition of the β-adrenergic-sensitive adenylate cyclase by FM 24. In the experiment presented in Fig. 7, membranes were incubated for different periods of time with various concentrations of FM 24. Isoproterenol (100 μM) was then added together with ATP (plus a tracer amount of [α^{32}P]-ATP) and the adenylate cyclase reaction was measured for 5 min. The isoproterenol concentration was high enough to obtain maximal stimulation of the adenylate cyclase even in presence of 10 μM FM 24 when the reaction was measured during the first 5 min of incubation. Thus, this experiment monitored the evolution of the maximal adenylate cyclase stimulation. It is clear that this non-competitive blockade by FM 24 was time and concentration dependent. The IC 50 for this inhibition varied as a function of time from 2 μM to 25 nM. The time course of inhibition was prolonged when the β-adrenergic receptors sites were protected by isoproterenol (100 μM) (Fig. 8).

Reversibility of FM 24-induced non-competitive inhibition of the β-adrenergic stimulated adenylate cyclase. FM 24 (500 nM) was incubated for 20 min at 30° with C6 glioma cell membranes. Following this, isoproterenol was added at a concentration (10^{-4} M) sufficient to occupy in less than 1 minute (18) all the free β-adrenergic receptors and which are liberated from FM 24 during the course of the experiment (Fig. 9).

A control was performed without addition of isoproterenol (Fig. 9). After different periods of time aliquots were removed and the adenylate cyclase was tested for its ability to respond to a high concentration

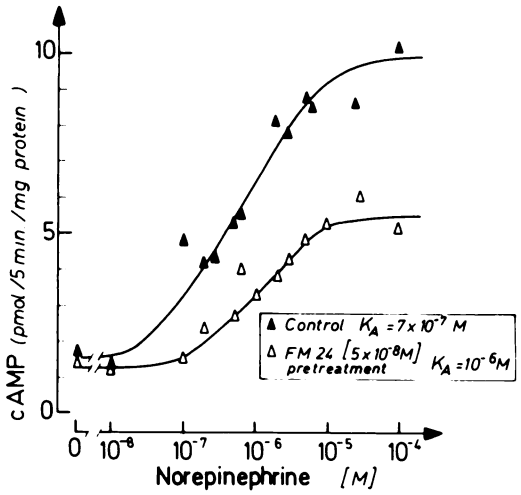


FIG. 5. *β-Adrenergic sensitive adenylate cyclase in membranes pretreated with FM 24*

Membranes (2.4 mg/ml) were preincubated for 12 min in a standard incubation medium in the presence or absence of 50 nM FM 24. The reaction between FM 24 and β-adrenergic receptors was stopped by the addition of propranolol 10^{-5} M. The membranes were then washed five times in Tris-HCl 25 mM, pH 8, $MgCl_2$ 5 mM. The adenylate cyclase assay was then performed between 8 and 13 min as described under METHODS.

of isoproterenol. Thus, in this experiment again, the non-competitive inhibition of β-adrenergic sensitive adenylate cyclase by FM 24 was followed; this inhibition was of 68% after 20 min preincubation period and increased slightly for the next 5 hours.

On the contrary, when isoproterenol was added after the 20 min preincubation period, two components in the non-competitive inhibition were revealed, one which was reversible during a period of 30 min to 1 hour and another which was irreversible or which slightly increased during the following 4 hours (representing 30% of blockade, Fig. 9). Even after 5 hours of incubation, there was enough isoproterenol in the incubation medium to activate maximally the adenylate cyclase, i.e., the same adenylate cyclase activities were found when the samples which had received isoproterenol for the whole period were then tested either with or without further addition of isoproterenol. Other appropriate controls are described in the legend of Fig. 9.

DISCUSSION

FM 24 has been recently described as a long lasting β-adrenergic blocking agent (20). When this compound is administered *in vivo*, its duration of action on isoproterenol-induced tachycardia, norepinephrine-induced lipolysis (20) blockade of isoproterenol-activated adenylate cyclase in heart was far longer than that of propranolol (21). Some evidence has been provided suggesting that this compound is not metabolized into an active metabolite but rather that it is tightly bound to the β-adrenergic receptor of heart (21). Since this compound could be of particular interest in biochemical and pharmacological studies of β-adrenergic receptors, we have extensively investigated the kinetics of its action both *in vivo* and *in vitro*. When injected into rats, FM 24 but not propranolol (30 mg/kg, i.p.) blocked by 85 ± 2% (n = 3) the brain β-adrenergic receptors after extensive washing of the membranes and their *in vitro* incubation for a period of 75 min at 37° (15 min incubation between each wash). Even after a further 4 hours *in vitro* incubation of the membranes at 37°, there was no significant reversion of the blockade (78.3 ± 3.4% blocked, n = 4). These observations could be interpreted as an irreversible or very slowly reversible effect of FM 24 on β-adrenergic receptor sites. A decision between these two possibilities is however, difficult, as has been previously found for the blockade of acetylcholine receptor by snake venom neurotoxin (11). The rate of dissociation of FM 24 from β-adrenergic receptors is very slow *in vitro* (see above) but also *in vivo* since after a single injection of FM 24 (30 mg/kg) 24 hours are necessary for a total recovery of the β-adrenergic receptors (Fig. 3). Furthermore, this recovery is likely to be a combination of two phenomena: the dissociation of FM 24 from the β-adrenergic receptors and the synthesis of new receptors. A more complete study using labeled FM 24 is obviously needed to quantify the relative importance of these two components. In order to provide more detailed analysis of the interaction of FM 24 with β-adrenergic receptors, we decided to use the β-adrenergic receptor coupled with adenylate cyclase in C6 glioma cells

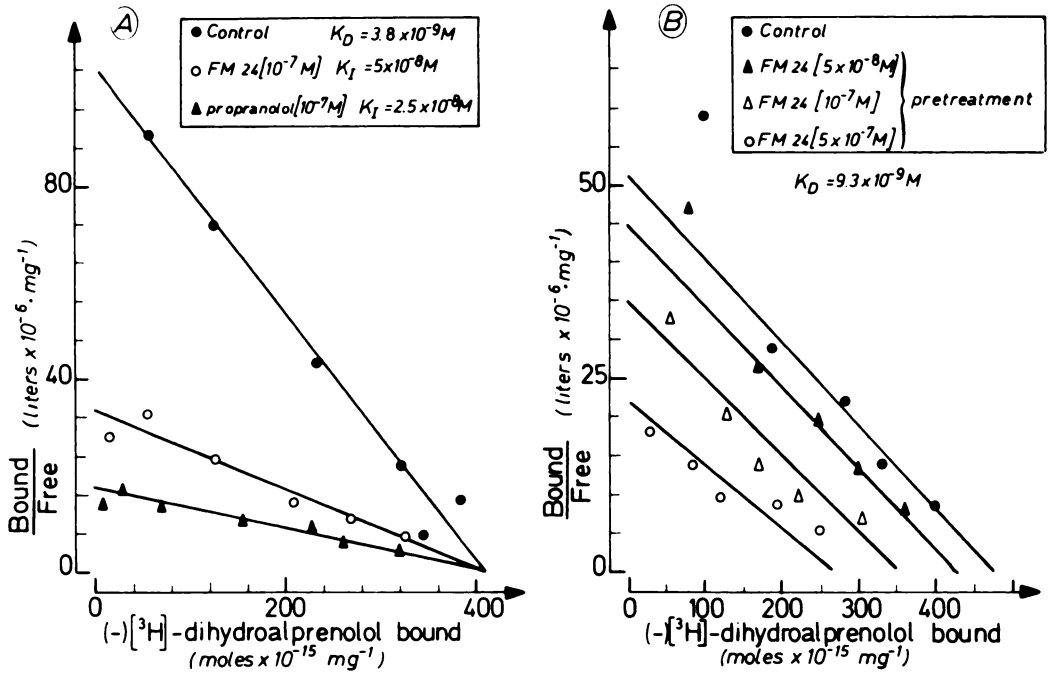


FIG. 6. *Effects of FM 24 on [³H]-DHA binding in C6 glioma cell membranes*

A. The effects of FM 24 and propranolol were tested without preincubation. [³H]-DHA binding was measured directly after 5 min of incubation without washing the membranes (24 μg protein in 50 μl). B. The effects of FM 24 were tested after a 12 min preincubation period. Membranes (2.4 mg/ml) were preincubated for 12 min in a standard binding assay medium (except that [³H]-DHA was not present) in the presence of different concentrations of FM 24. The reaction between FM 24 and β-adrenergic receptors was stopped by the addition of propranolol (10 μM). The membranes were washed five times in Tris-HCl 25 mM, pH 8, $MgCl_2$, 5 mM. They were then assayed for [³H]-DHA binding as described under METHODS.

membranes. When FM 24 and isoproterenol were added together and for a short period of time, FM 24 interacted reversibly with β-adrenergic receptors with an apparent K_I of 50 nM as shown by its competitive behavior on the adenylate cyclase activation (shift to the right and parallelism of the dose-activation curves, maximal activation) (Fig. 4A). Even after a 15 min incubation of FM 24 at a concentration high enough to saturate all the β-adrenergic receptors, a subsequent addition of various concentrations of isoproterenol gave a dose activation curve which presents a shift to the right (compared to the control) allowing the calculation of a K_I app. (20 nM) similar to the K_I app. measured during the 0–5 min experiment (Fig. 4B). This indicates that on the β-adrenergic receptors which were not blocked non-competitively by FM 24 there was still a competition between FM 24 and isoproterenol. For clarity we designate this reversible complex as $RL_1 : R + L \rightleftarrows RL_1$.

In addition to the formation of this complex, a more stable complex RL_2 was formed, the amount of which was time and FM 24-concentration dependent (Fig. 7). The reversibility of RL_2 was so slow that it gave noncompetitive inhibition of the isoproterenol-sensitive adenylate cyclase (Fig. 4B; Fig. 5). At least part of FM 24 involved in this complex was not removed by washing (Figs. 5 and 6B). On the contrary, after washing, the complex RL_1 was completely dissociated since the FM 24 blockade of the isoproterenol-sensitive adenylate cyclase and [³H]-DHA binding on washed membranes was only non-competitive (Figs. 5 and 6B). The rate of formation of RL_2 was decreased by the occupation of the β-adrenergic receptor by isoproterenol (Fig. 8).

However, as expected if the RL_2 complex is slowly reversible, the concentration of RL_2 reached the same level in the presence or absence of isoproterenol (Fig. 8). We have attempted to measure the dissociation of RL_2 in a long term adenylate cyclase experiment (Fig. 9). The results indicated that in

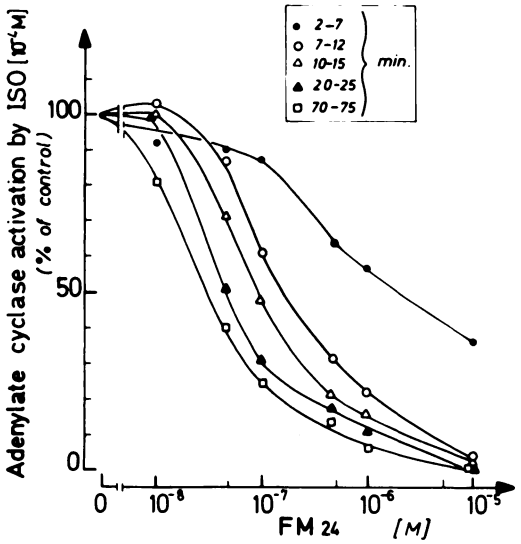


FIG. 7. *Time and concentration dependence of the non-competitive inhibition of the β-adrenergic sensitive adenylate cyclase by FM 24*

Membranes were incubated with different concentrations of FM 24 and all the adenylate cyclase assay components except for isoproterenol (10^{-4} M final) and ATP (plus a tracer amount of [α^{32}P]-ATP which were added together (in 5 μl) after different preincubation periods (as indicated on the figure). The reaction was allowed to proceed for 5 min. Note that two other series were run in the same experiment. 1. A series which did not receive FM 24 in the preincubation and isoproterenol in the incubation period. The basal adenylate cyclase activity decreased linearly from 54 to 24 pmoles/5 min/mg protein when tested between 2–7 min and 70–75 min, respectively. 2. A series which did not receive FM 24 in the preincubation period but which received isoproterenol (100 μM final) in the incubation period. The β-adrenergic sensitive adenylate cyclase activity decreased linearly from 467 to 200 pmoles/5 min/mg protein when tested between 2–7 min and 70–75 min respectively. The amount of protein was 63 μg in 50 μl. The 100% stimulation of adenylate cyclase refers, for each incubation period, to the ratio of activities determined in series 2 (maximally simulated) versus series 1 (basal). The residual stimulation obtained in the presence of FM 24 was expressed as a percent of that control stimulation.

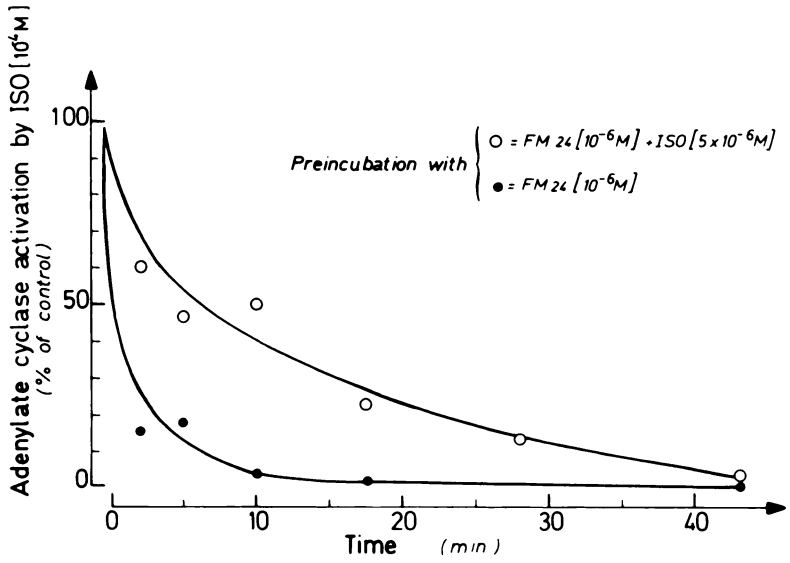


FIG. 8. *Protection by isoproterenol of the non-competitive inhibition of the β-sensitive adenylate cyclase by FM 24*

The experiment is similar to the experiment reported in Fig. 7, for both experimental and control series, except that the preincubation period was performed with or without (5 μM) isoproterenol. The incubation periods were 2 min only. The amount of protein was 20 μg in 50 μl.

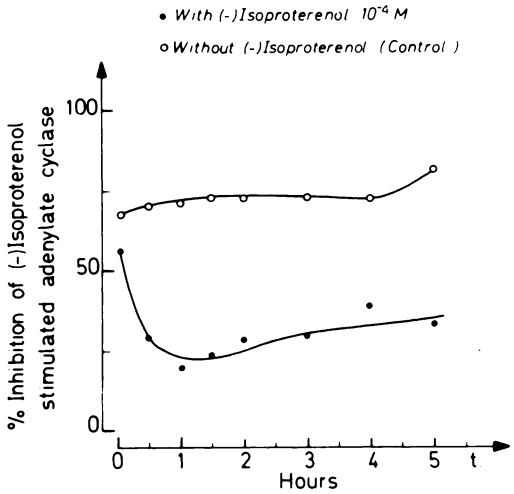


FIG. 9. *Reversibility of the FM 24 induced non-competitive inhibition of β-adrenergic-sensitive adenylate cyclase*

Membranes (1.4 mg protein) were preincubated in 2 ml of standard adenylate cyclase assay medium (except for the absence of ATP and [α^{32}P]-ATP) for 20 min. Four series were performed: series A and B in the absence of FM 24; series C and D in the presence of FM 24, .5 μM. After 20 min of incubation, isoproterenol (100 μM final concentration) was added into series B and D. Following this addition, aliquots of 50 μl were taken after different periods of time from the four series and added to 5 μl containing ATP (final concentration 25 μM; plus a tracer amount of [α^{32}P]-ATP) in the presence or absence of isoproterenol (100 μM final concentration). Reaction was allowed to proceed for five minutes. The percentage inhibition of (−)-isoproterenol stimulated adenylate cyclase was calculated referring to the corresponding series which did not receive FM 24. During the course of the experiment, the adenylate cyclase activities measured in the absence and presence of isoproterenol dropped from 16 to 5 pmoles/5 min/mg protein and 88 to 17 pmoles/5 min/mg protein, respectively. Thus, the stimulation of adenylate cyclase by isoproterenol dropped from 5.5 to 3.4.

the presence of a high concentration of isoproterenol (100 μM) part of RL_2 was reversible in a period of 30 min to 1 hour ($RL_2\alpha$), the remaining part was completely irreversible during a period of 5 hours ($RL_2\beta$). Thus the following model is hypothesized.

$$R + L \leftrightarrows RL_1 \leftrightarrows RL_2\alpha \rightarrow RL_2\beta$$

It is interesting to note that the neurotoxin from Naja Naja interacted with acetylcholine receptors to form mixed complexes having different rate of dissociation (12). Recently Ross et al. (17) have shown that [^{125}I]-iodohydroxybenzylpindolol also formed two different complexes with β-adrenergic receptors, one dissociating with a $t_{1/2}$ of 2 min and the other with a $t_{1/2}$ of 180 min. This slowly reversible complex derives from the rapidly dissociating one in a time-dependent manner. It is possible that the behavior of FM 24 is similar to that of iodohydroxybenzylpindolol, except that FM is also able to form a complex $RL_2\beta$ having a much slower dissociation constant.

ACKNOWLEDGMENTS

We are indebted to Drs. Uzan and Le Fur for donation of FM 24, and interesting discussion, and would like to acknowledge the excellent technical assistance of Michelle Perez and Mina Cherif.

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