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Mechanism of Lipolytic Action of a New *Alpha*-2 Adrenergic Antagonist of the Piperazinopyrimidine Family: RP 55 462[1]

JEAN SÉBASTIEN SAULNIER-BLACHE, MOHAMMED TAOUIS, MICHÈLE DAUZATS, ANDRÉ UZAN, MICHEL BERLAN and MAX LAFONTAN

Institut National de la Santé et de la Recherche Médicale, Unite 317, Institut de Physiologie, Université Paul Sabatier (J.S.S-B, M.T., M.D., M.B., M.L.), rue François Magendie, 31400 Toulouse, France and Rhône-Poulenc Santé (U.A.), Centre de Recherches, Quai du Moulin de Cage, Gennevilliers, France

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ABSTRACT

The *in vivo* lipid mobilizing effect of *alpha*-2 adrenergic antagonist has been demonstrated previously. This has attracted attention to the putative interest of such compounds in a lipid-mobilizing strategy. RP 55462 [6-chloro-4-(isopropylamino)-5-(methyl)-2 piperazinopyrimidine], a piperazinopyrimidine derivative, has already been shown to exert *alpha*-2 adrenergic antagonist actions on fat cell function *in vitro*. Moreover, RP 55462 exhibits a direct *in vitro* lipolytic action which is independent of its *alpha*-2-blocking potency. When administered i.v. RP 55462 is also able to induce an increment in plasma nonesterified levels in dogs. The mechanism of action of RP 55462 was studied and the nature of its lipomobilizing effect was explored. RP 55642-dependent lipolysis was not affected by *beta* adrenergic blockers on rat fat cells and RP 55462 had no direct effect on adenylyl-cyclase activity on fat cell membranes. Moreover, RP 55462 did not compete with [^{3}H]phenyl isopropyl adenosine binding (A1-adenosine receptor agonist) on fat cell membranes. In fact, RP 55462 inhibited, in a dose-dependent manner, the cyclic AMP (cAMP)-dependent phosphodiesterase (PDE) activity in rat adipose tissue. Several derivatives with the piperazinopyrimidine structure also inhibited cAMP-dependent PDE activity and exerted lipolytic effects. A short structure-activity study was performed with various derivatives. In dogs, by contrast with yohimbine, the *in vivo* lipid mobilizing effect of RP 55462 was not abolished by pretreatment with propranolol, and lasted longer. It is concluded that the *in vivo* lipolytic activity of RP 55462 is connected with its ability to inhibit cAMP-dependent PDE activity; a property of several piperazinopyrimidine derivatives. The lipid mobilizing effect induced *in vivo* by RP 55462 results from a combination of its *alpha*-2 adrenergic antagonist properties and its direct lipolytic action mediated by cAMP-dependent PDE inhibiting effects.

Recent investigations in our laboratory have demonstrated the lipid mobilizing effect of an *alpha*-2 adrenergic antagonist (yohimbine) which suggested a putative interest of such compounds in lipid mobilizing strategies. Oral administration of yohimbine to fasting human subjects promotes an increase in plasma NEFA and glycerol levels. This action can be prevented by a meal or by the administration of a *beta*-antagonist like propranolol before yohimbine intake (Galitzky *et al.*, 1988; Taouis *et al.*, 1988). This effect is attributable to: 1) an increase in synaptic norepinephrine (due to an increment of the orthosympathetic tone through central and/or peripheral effects) with a resultant rise in *beta* adrenergic-dependent lipolysis and 2) a blockade of the antilipolytic post junctional *alpha*-2 adrenoceptors of the adipocyte.

In the course of our search for new *alpha*-2 adrenergic antagonists, we studied a new agent, RP 55462 [6-chloro-4-(isopropylamino)-5-(methyl)-2 piperazino- pyrimidine] which represents a new chemical family of *alpha*-2 adrenergic antagonists (Gueremy *et al.*, 1986). We first confirmed the *alpha*-2 adrenergic antagonist properties of RP 55462 on white fat cells by functional (inhibition of the *alpha*-2 adrenergic-dependent antilipolysis) and binding studies (competition with [^{3}H]yohimbine binding on *alpha*-2 adrenoceptors on human fat cell membranes). We also demonstrated that RP 55462 was able to promote an increment of the plasma NEFA levels after i.v. injection in dogs, an effect which could be attributable to its *alpha*-2-blocking potencies. However, we noticed that RP 55462 was able to activate lipolysis *in vivo* on isolated fat cells from different species. This effect, which had never been described with other *alpha*-2 adrenergic antagonists, like yohimbine, idazoxan and phentolamine, is apparently linked to a specific

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ABBREVIATIONS: NEFA, nonesterified acids; cAMP, cyclic AMP; PDE, phosphodiesterase; KRBA, Krebs-Ringer bicarbonate albumin buffer; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid; PIA, phenyl isopropyl adenosine; ADA, adenosine deaminase; IBMX, isobutyl methyl xanthine.

mechanism of action which is independent from the *alpha*-2-blocking properties of the agent (Saulnier-Blache *et al.*, 1989).

The present study is focused on the clarification of the effects of RP 55462. This compound seems to be a good candidate for use in antiobesity therapy through lipomobilization with a combination of *alpha*-2 adrenolytic action and direct lipolytic activity. The study was conducted in order to determine the *in vitro* lipolytic mechanism of action of RP 55462 and to delineate the contribution of a direct (not *alpha*-2 adrenergic dependent) lipolytic action in the lipid mobilizing effect of RP 55462 *in vivo*.

A large part of the lipolysis studies were carried out on the fat cells of the rat in order to exclude possible interactions of RP 55462 with *alpha*-2 adrenoceptors in as much as rat fat cells are characterized by a small number of functional *alpha*-2 adrenoceptors (Carpéné *et al.*, 1983) and a weak *alpha*-2 adrenergic responsiveness (Rebourcet *et al.*, 1988).

We demonstrated that the *in vitro* lipolytic activity of RP 55462 is mediated by inhibition of cAMP-dependent PDE activity. Moreover, the *in vivo* lipid mobilizing effect of RP 55462 is the result of a combination of its *alpha*-2 adrenergic properties and its direct lipolytic action.

Methods

Animals. Male Sprague-Dawley rats weighing between 250 and 350 g and male golden hamsters (*Mesocricetus auratus*) weighing between 100 and 145 g, were housed at 20–22°C and were fed *ad libitum* before use. They were sacrificed by decapitation after an overnight fast. Epididymal and perirenal adipose tissue were removed immediately and used for biological assays.

Fat cell preparation. Isolated adipocytes were obtained from adipose tissue according to Rodbell's method (1964) with minor modifications. The tissue was cut into small pieces and incubated for 30 to 40 min at 37°C under vigorous agitation in KRBA, pH 7.5, with 35 mg/ml of bovine serum albumin and 6 mM glucose (KRBA buffer) containing 1.6 mg/ml of collagenase. After incubation the cells were filtered through a silk screen (250 μm mesh) and washed twice with the KRBA buffer in order to eliminate the stroma-vascular fraction and the collagenase.

Lipolysis measurements. Isolated adipocytes (20–30 mg of total lipid) were dispersed in 1 ml of KRBA buffer containing 10 μl of various concentrations of RP 55462 or the other agonists tested. After 90 min of incubation under gentle agitation at 37°C the reaction was stopped in ice and an (200 μl) aliquot of the incubation medium was taken to determine the glycerol released by the cells according to Wieland's method (1957). The lipid content of the incubation vials was determined gravimetrically after extraction according to the method of Dole and Meinertz (1960). Results are expressed in moles of glycerol released per 100 mg of total cell lipid.

Preparation of adipocyte membranes. Crude membranes were obtained after hypotonic lysis of the adipocytes. The lysing medium was composed of 2.5 mM $MgCl_2$, 1 mM $KHCO_3$, 2 mM Tris-HCl, 100 μM EGTA (pH 7.5; 35 mOsmol) and the following protease inhibitors: leupeptin (1 μg/ml), benzamidine (0.1 mM) and 100 μM phenylmethyl sulfonyl fluoride. For adenylyl-cyclase assays the lysing medium was complemented with 0.2 mM ATP. The lysis of adipocyte suspensions was performed at 20–22°C in order to minimize trapping of membranes in the coalescing fat cake. Fat cell ghosts were obtained by centrifugation (40,000 × *g*, 10 min at 15°C). The pellet was washed and resuspended in 1 ml of lysing medium and frozen immediately at −80°C until use in binding studies or adenylyl-cyclase assays.

Radioligand binding studies. Hamster fat cell A1-adenosine receptors were identified with [^{3}H]PIA. Thawed crude membranes were washed once in 50 mM Tris-HCl and 1 mM EDTA, pH 7.5, and then in 50 mM Tris HCl and 10 mM $MgCl_2$, pH 7.5, and finally resuspended in this medium containing 1 μg/ml of ADA. Incubation buffer consisted in 100 μl of aqueous radioligand solution (10 nM) and 100 μl of membrane suspension (100–200 μg of protein) made up to a final volume of 400 μl with 50 mM Tris HCl, 10 mM $MgCl_2$ and 1 μg/ml of ADA, pH 7.5. RP 55462 or other competing agents were added to the assay mixture at various concentrations ranging from 10^{-6} to 10^{-3} M. Incubations were carried out at 37°C in a water bath for 30 min under constant shaking. At the end of the incubation, the mixture was diluted with 4 ml of ice-cold buffer and vacuum filtered immediately through Whatman GF/C glass fiber filters faced on a Millipore manifold. The filters were washed twice with 10 ml of ice-cold buffer and counted in 4 ml of scintillation medium in a Packard scintillation spectrometer at an efficiency of 45%. Residual binding was expressed as percentage of total [^{3}H]PIA binding (100% binding was defined in the absence of competitors). Protein content was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Determination of the cAMP-dependent phosphodiesterase activity. The cAMP-PDE activity was assayed on white adipose tissue homogenates, according to the method described by De Mazancourt and Giudicelli (1984). Epididymal adipose tissue was removed from rats, washed twice in 75 mM Tris and 10 mM $MgCl_2$, pH 7.4, buffer. The tissue was homogenized in a Potter apparatus in 10 ml of buffer and centrifuged 5 min at 3000 rpm at 4°C. The cAMP-PDE activity was determined in the liquid phase; 60 to 90 μg of protein were incubated at 37°C for 10 min in a final volume of 250 μl containing 0.62 μM cAMP, 2.4 nM [^{3}H]cAMP, 5 mM $MgCl_2$, 0.04% bovine serum albumin and 30 mM Tris-HCl, pH 7.4. A warm denaturized homogenate (2 min at 90°C) was used as blank. The reaction was stopped by addition of 200 μl of 0.24 M $Ba(OH)_2$ and 200 μl of 0.24 M $ZnSO_4$. After centrifugation for 10 min at 5000 × *g*, 200 μl of supernatant were counted in 4 ml of scintillation medium in a Packard scintillation spectrometer. All measurements were run in triplicate. After removing the blank value, cAMP-PDE activity was expressed in picomoles of the cAMP transformed per milligram of protein per minute. RP 55462 and other compounds were tested as inhibitors of basal cAMP-PDE activity.

Adenylate-cyclase assays. Adenylate-cyclase assays were performed in duplicate in a final volume of 50 μl consisting of 10 μl of adipocyte membrane suspension (20–30 μg of protein), 20 μl of buffer or pharmacological agents at a suitable concentration and 20 μl of concentrated incubation mixture containing the following agents to reach a final composition: 40 mM Tris-HCl, 2 mM Mg Cl_2, 0.1 to 10 μM GTP, 100 μM EGTA, 0.5 mM IBMX, 0.5 mM cAMP, 20 μM ATP, 0.5 μCi of [α^{32}P]ATP, 5 mM creatine phosphate, 0.2 mg/ml of creatine kinase, 0.2 mg/ml of bovine serum albumin and 2 μg/ml of ADA at pH 7.4. The reaction was initiated with 10 μl of thawed membranes washed once and then resuspended in 5 mM Tris-HCl and 0.5 mM $MgCl_2$; pH 7.5, containing antiprotease compounds: leupeptin, 5 μl/ml; pepstatin, 2 μg/ml; soybean trypsin inhibitor, 10 μg/ml; and 0.05 mM L-1-chloro-3- < 4-tosylamido > 4-phenyl-2-butanon. After a 20-min incubation at 30°C the reaction was stopped by addition of 100 μl of a solution containing 2% lauryl sulfate, 40 mM ATP and 1.4 cAMP as well as trace amounts of [α-^{3}H]cAMP (7–8000 counts/min). [^{32}P]cAMP was isolated by Dowex 50WX8 and neutral alumina chromatography as described previously (Salomon *et al.*, 1974) with a 75 to 80 yield, and measured for radioactivity with a Packard liquid scintillation spectrometer.

***In vivo* studies.** Male Beagle dogs (15–20 kg) were used for the *in vivo* studies.A bolus injection of propranolol, RP 55462 or yohimbine were made at time 10 min into the jugular vein, with the help of a catheter, in alert dogs. Blood samples were collected in heparinized tubes and centrifuged immediately. Plasma was collected and frozen at −20°C until utilization. Plasma NEFA levels were determined by a titrimetric method, after extraction, using the technique of Dole and Meinertz (1960).

Chemicals. RP 55462 and its analogs defined in table 3 were kindly given by Rhône-Poulenc Santé (Gennevilliers, France), (−)-N6-R-(G-[^{3}H])phenylisopropyl adenosine and (2,8-[^{3}H]) adenosine 3',5'-cyclic phosphate were obtained from Amersham International (Amersham,

England)., Propranolol and ICI-118551 were obtained from ICI Pharma (Alderly Park, England). Betaxolol was obtained from Synthelabo (Paris, France). Yohimbine hydrochloride, bovine serum albumin (Fraction V), IBMX, caffeine, forskolin, sodium fluoride, theophylline and (−)-epinephrine bitartrate were purchased from Sigma Chemical Co. (St. Louis, MO). Collagenase (from *Clostridium histolyticum* at 0.4 U/mg), ADA, (−)-PIA and enzymes for glycerol assays came from Boehringer Mannheim (Mannheim, FRG). All other chemicals and organic solvents were of reagent grade.

Results

In vitro Studies

Comparison of the lipolytic activity of RP 55462 with other lipolytic agents. In order to determine the lipolytic efficiency of RP 55462 on rat fat cells, dose-effect curves were compared with those of different lipolytic agents like epinephrine, forskolin, IBMX and theophylline, the mechanism of action of which was well known. All the agents tested initiated a dose-dependent increase of the lipolytic activity with a decrease in the glycerol production at the highest doses (5×10^{-4} M) for some agents: RP 55462, IBMX and theophylline (fig. 1). The EC_{50} values and maximal effect values were determined for each lipolytic agent (table 1). The maximal lipolytic effect of RP 55462 reached 70% of that induced by 0.1 μM isoproterenol. It was lower than the lipolytic action of epinephrine and forskolin but quite similar that of IBMX, theophylline and caffeine. The sensitivity of RP 55462 (EC_{50} value) was lower than that of epinephrine or forskolin but quite equivalent to those of IBMX, theophylline and caffeine. All the comparative experiments

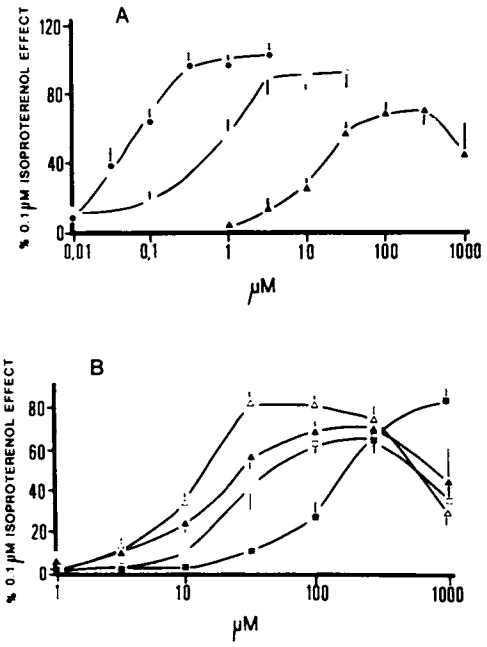


Fig. 1. Comparison of the lipolytic activity of RP 55462 and of different lipolytic agents on isolated rat fat cells. Isolated adipocytes were incubated in KRBA buffer with increasing concentrations of RP 55462 (A and B)(▲) (−)-epinephrine (A,●), forskolin (A,○), IBMX (B,△) and theophylline (B,□) and caffeine (B,■). Glycerol release was measured as described under "Materials and Methods." The lipolytic activity was expressed as a percentage of the effect initiated by 0.1 μM isoproterenol (6.23 ± 0.62 μmol of glycerol per 100 mg of lipid) after subtraction of the basal activity (0.29 ± 0.02 μmol of glycerol per 100 mg of lipid). Each point represents the mean ± S.E. of four separate experiments performed in duplicate.

TABLE 1

Sensitivity (EC_{50} values) and maximal effects of RP 55462 and different lipolytic agents on rat fat cells

EC_{50} values and maximal stimulating effects were calculated from the individual dose-effect curves depicted in Fig. 1. Results are mean ± S.E. of 4 separate experiments.

Compounds	EC_{50}	Maximal Effect
	μM	μmol glycerol/ 100 mg lipid
Epinephrine	0.075 ± 0.004	6.45 ± 0.96
Forskolin	0.44 ± 0.06	5.81 ± 0.99
IBMX	11.2 ± 1.5	5.11 ± 0.47
RP 55462	16.8 ± 1.7	4.35 ± 0.40
Theophylline	39.8 ± 3.2	4.14 ± 0.43
Caffeine	189.3 ± 16.8	4.94 ± 0.24

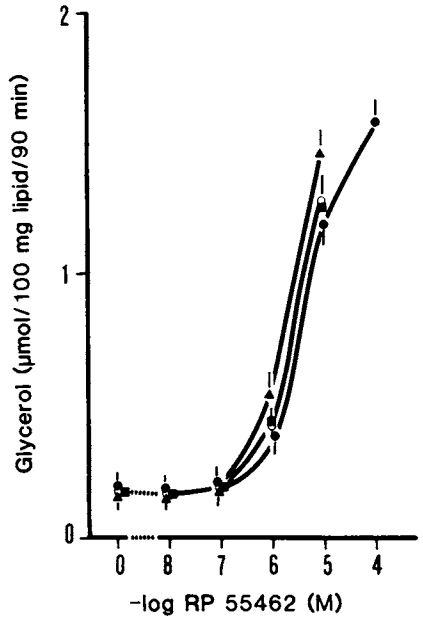


Fig. 2. Influence of three *beta* adrenergic antagonists on the lipolytic activity of RP 55462 in rat fat cells. Isolated adipocytes were incubated in KRBA buffer containing 2 μg/ml of ADA with increasing concentrations of RP 55462 in the absence (●) or presence of 10^{-6} M propranolol (▲) 10^{-4} M betaxolol (■) or 10^{-5} M ICI 118551 (○). Glycerol release was determined as described under "Materials and Methods." Each point is the mean ± S.E. of four separate experiments performed in duplicate.

described here were performed without removing adenosine from the incubation medium as some agents are known to act through an antagonization of A1 adenosine receptor sites.

Determination of the *in vitro* lipolytic mechanism of action of RP 55462. Amongst the possible mechanisms of action of RP 55462, infraction with *beta* adrenoceptors or A1-adenosine receptor sites and direct impact on adenylate cyclase were tested.

Interaction with *beta* adrenoceptors. The influence of three *beta*-blockers (propranolol, betaxolol and ICI-118551) was tested on the lipolytic dose-effect curve of RP 55462 on rat fat cells. Lipolytic assays were performed with 2 μg/ml ADA in the incubation buffer. Removal of adenosine promoted an increment of basal lipolysis and greater sensitivity (shift to the left of the dose-response curve of RP 55462). As shown in figure 2, the dose-effect curve of RP 55462 was not modified either by higher concentrations of propranolol (mixed *beta*-1-*beta*-2 antagonist), betaxolol (*beta*-1-antagonist) or ICI 118551 (*beta*-2-

antagonist). Apparently, RP 55462 does not interact with rat fat cell *beta* adrenoceptors.

Adenylyl-cyclase activity. The effect of increasing concentrations of RP 55462 on adenylyl-cyclase activity of adipocyte membranes was compared with the stimulation obtained with agents known to activate this enzyme: isoproterenol, forskolin and NaF. Whatever the concentration of RP 55462 used, (0.1 μM to 1 mM) basal adenylyl-cyclase activity (21 ± 4 pmol of cAMP/mg of protein per 1 min) was never increased by this agent whereas isoproterenol (10 μM), forskolin (0.1 μM) and NaF (20 mM) induced an increase over basal activity of 138, 97 and 533%, respectively. The absence of any direct interaction of RP 55462 with adenylyl-cyclase is demonstrated.

Interaction with A1-adenosine receptors. The effect of RP 55462 on [^{3}H]PIA (A1-adenosine agonist) binding was tested on hamster adipocyte membranes in as much as we had a good practice of [^{3}H]PIA binding on the membranes and we obtained binding parameters which were more accurate than in the rat. [^{3}H]PIA bound to hamster fat cell membranes with higher affinity and capacity K_d= 1.1 ± 0.1 nM; maximal binding = 440 ± 40 fmol/mg of protein). Competition of [^{3}H]PIA binding with RP 55462 was compared with the competition obtained with PIA, theophylline and IBMX.

As shown in figure 3, theophylline and IBMX inhibited [^{3}H] PIA binding in a dose-dependent manner whereas RP 55462 had no significant inhibitory effect. The inhibition of [^{3}H]PIA binding by higher PIA concentrations was considered as defining the nonspecific binding of the radioligand. The results suggested that RP 55462 does not have any interaction with A1-adenosine receptor sites.

Inhibition of cAMP-dependent PDE activity. The effect of increasing concentrations of RP 55462 were tested on the basal cAMP-PDE activity of rat epididymal adipose tissue homogenates.

As shown in figure 4, RP 55462 inhibited, in a dose-dependent manner, the basal cAMP-PDE activity. A significant effect was obtained with 1 μM RP 55462 leading to 10% inhibition of the basal activity. The maximal effect was observed with 1000 μM leading to 95% inhibition of the basal activity; 50% of the maximal inhibition (IC_{50}) was obtained at 40 μM RP 55462.

A parallel was shown to exist between the lipolytic activity of 50 μM RP 55462, IBMX, theophylline and caffeine and the

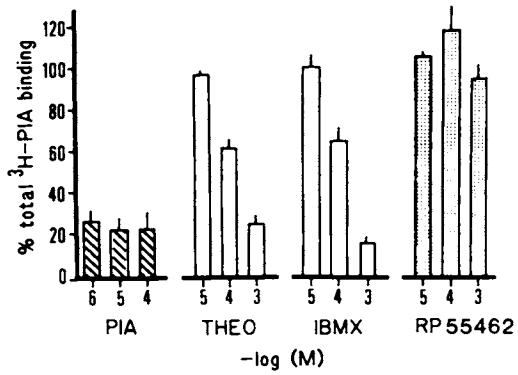


Fig. 3. Competition of [^{3}H]PIA binding on hamster fat cell membranes with RP 55462, PIA, IBMX and theophylline (THEO). The membrane suspension was incubated with 10 nM [^{3}H]PIA in the absence (100% total binding) or in the presence of increasing concentrations of PIA, IBMX and THEO. The residual binding was expressed as a percentage of the total [^{3}H]PIA binding. Each column represents the mean ± S.E. of four separate experiments.

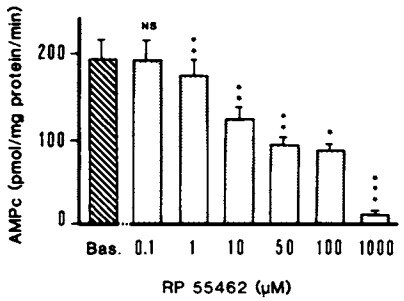


Fig. 4. Influence of RP 55462 on the basal (Bas) cAMP-PDE activity of rat epididymal adipose tissue. Tissue homogenates were incubated with increasing concentrations of RP 55462 and the residual Bas cAMP-PDE activity was measured as described under "Materials and Methods." Each column represents the mean ± S.E. of three separate experiments performed in triplicate. A Student's paired *t* test was used to estimate the significance of inhibition with regard to the Bas activity. ★P < .05; ★★P < .02; ★★★P < .01.

TABLE 2

Comparison between the lipolytic effects and the inhibition of cAMP-PDE activity of RP 55462, IBMX, theophylline and caffeine

The same dose (50 μM) of each agent was used to determine the impact on the lipolytic activity in isolated fat cells incubated in KRBA buffer as described under "Materials and Methods" and the potencies to inhibit the cAMP-PDE activity in epididymal rat adipose tissue homogenates. Results are the mean of four separate experiments performed in triplicate. The weakest lipolytic agents (theophylline and caffeine) had the lowest inhibiting effect on cAMP-PDE activity.

	Lipolytic Activity (% of 0.1 μM isoprenaline activity)	Inhibition of cAMP-PDE (% inhibition of basal cAMP-PDE activity)
IBMX	82 ± 3	72 ± 2
RP 55462	56 ± 3	51 ± 3
Theophylline	41 ± 10*	21 ± 1*
Caffeine	10 ± 5*	16 ± 1*

* P < .05; as compared with IBMX-Student's paired *t* test.

capacity of the same concentration of these agents to inhibit the cAMP-PDE basal activity (table 2). The same order of potency was obtained with the two biological effects: IBMX > RP 55462 > theophylline > caffeine.

Comparative study of differently substituted piperazinopyrimidine derivatives. A comparative study of the lipolytic effects and inhibiting action on the cAMP-PDE activity of differently substituted piperazinopyrimidine derivatives was performed. This short study clearly revealed that substitution of the R1 group on piperazine nucleus by a methyl residue leads to agents which are inactive toward lipolysis and cAMP-PDE activity whatever the chemical nature of the R4, R3 and R2 moieties of the molecule (table 3).

Moreover, when the group R1 was an hydrogen atom, changes in the chemical structure of substitutants R3 and R4 did not noticeably modify the cAMP-PDE inhibition potential or lipolytic activity of the different derivatives.

In spite of the limited number of compounds available for this study, it is clear that there is a strict association between the lipolytic activity of some piperazinopyrimidine derivatives and their ability to inhibit cAMP-dependent PDE-activity.

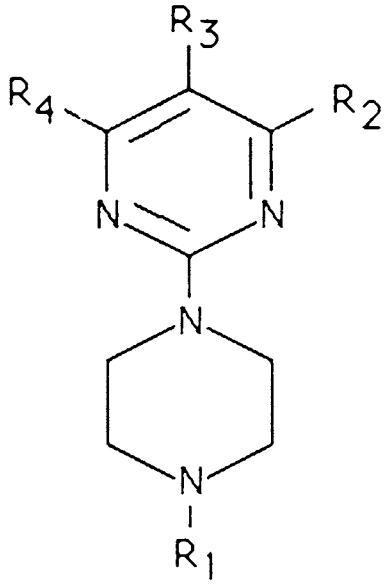
In Vivo Studies

The influence of a preinjection (i.v.) of propranolol (*beta* adrenergic antagonist) on the lipomobilizing effect promoted by an i.v. injection of RP 55462 was compared with the influ-

TABLE 3

Relation between the chemical structure, the cAMP-PDE inhibition capacity and the lipolytic activation of the same dose (50 μM) of piperazinopyrimidine derivatives

The inhibition of cAMP-PDE activity (n = 3) for each compound and the lipolytic activity (n = 6) are rounded off to the nearest percentage. Lipolytic efficacy (expressed as percentage of maximal lipolytic effect initiated by 0.1 μM isoproterenol) was defined from lipolysis studies performed on intact fat cells incubated in KRBA buffer as described under "Materials and Methods."



Compounds	R1	R2	R3	R4	% Inhibition of cAMP-PDE	Lipolytic Activity
RP 55 462	H	NH—CH—(CH3)2	CH3	Cl	54 ± 3	71 ± 4
RP 58 746	H	NH—CH—(CH3)2	SCH3	Cl	57 ± 2	46 ± 3
RP 58 743	H	NH—CH—(CH3)2	Br	Cl	65 ± 4	85 ± 6
RP 58 741	CH3	NH—CH—(CH3)2	CH3	Cl	12 ± 1	3 ± 1
RP 65 541	CH3	NH—C—(CH3)3	SCH3	Cl	19 ± 2	1 ± 1
RP 65 542	CH3	NH—CH—(CH3)2	SOCH3	Cl	16 ± 1	2 ± 1

ence on the lipomobilizing effect induced by yohimbine. Experiments were carried out on alert dogs. As shown in figure 5, the increase in plasma NEFA levels induced by yohimbine was abolished completely under propranolol treatment whereas the increase in NEFA levels induced by RP 55462 was delayed in time but never abolished completely by propranolol pretreatment; a noticeable lipomobilizing action is still preserved after the administration of the *beta*-blocking agent.

Discussion

The *in vivo* lipid mobilizing action of *alpha*-2 adrenergic antagonists has already been demonstrated (Galitzky *et al.*, 1988; Taouis *et al.*, 1988). We described previously that RP 55462, representing a new family of *alpha*-2 adrenergic antagonists, in addition to its *alpha*-2 adrenolytic potencies, was also able to activate the lipolysis of isolated fat cells from different species. This lipolytic activity was independent of its *alpha*-2 adrenolytic properties (Saulnier-Blache *et al.*, 1989). The aim of the present study was to determine the *in vivo* and *in vitro* lipolytic mechanism of action of RP 55462. It is demonstrated that this mechanism is associated to an inhibition of adipose tissue cAMP-PDE activity. Moreover, the lipomobilizing effect promoted by RP 55462 administration results from both impacts *i.e.* its direct action on cAMP-PDE and its *alpha*-2 antagonist effects.

The *in vitro* lipolytic activity of RP 55462 was not modified by *beta* receptor blockade (propranolol, betaxolol or ICI 118551) (fig. 2). So the lipolytic activity of the drug is not mediated by the stimulation of a *beta* adrenergic receptor.

RP 55462 was not able to directly stimulate adenylyl-cyclase activity on fat cell membranes, dismissing any direct or indirect interaction with the enzyme as is the case with some compounds (forskoline and NaF) which are able to stimulate lipolysis by direct activation of the adenylyl-cyclase complex without interaction with specific receptors.

The lipolytic dose effect-curve obtained with RP 55462, compared with that of different lipolytic agents (fig. 1) revealed that the maximal effect as well as the sensitivity of RP 55462 were quite equivalent to those of the alkylated xanthins (IBMX, theophylline and caffeine). Under our working conditions, when rat fat cells were incubated in a buffer without ADA to remove adenosine released in the incubation buffer, the lipolytic action of the alkylated xanthins is attributable to the inhibition of cAMP-PDE activity and to the blockade of the inhibitory A1 - adenosine receptor. Because RP 55462 behaved like the xanthins in terms of efficiency, a possible action on both mechanisms was explored. Competition binding studies with [^{3}H]PIA, an agonist specific for the A1-adenosine receptor on fat cell membranes (fig. 3) revealed that RP 55462 was unable to interact with this receptor unlike IBMX or theophylline.

In fact, RP 55462 inhibited, in a dose-dependent manner, the cAMP-PDE activity of rat adipose tissue as found with IBMX, theophylline and caffeine (fig. 4). The cAMP-PDE activity measured corresponds to the total population of cAMP-PDE contained in the rat adipose tissue, without discrimination

of the different isoforms. From our results, we cannot conclude to a specific inhibition of the low K_m cAMP-PDE or the particulate cAMP-PDE (endoplasmic reticulum-associated) implicated in the regulation of lipolysis and specifically inhibited by cilostamide (Elks *et al.*, 1985, Degerman *et al.*, 1987). The good correlation existing between the lipolytic activity and the capacity of IBMX, RP 55462, theophylline and caffeine to inhibit the cAMP-PDE activity (table 3) allowed us to conclude that the lipolytic activity of RP 55462 was certainly mediated by the inhibition of cAMP-PDE activity of the fat cell. This action easily explains the enhancement of the isoproterenol- or synacthen-induced lipolysis (through limitation of cAMP degradation) reported previously (Saulnier-Blache *et al.*, 1989). The connection between the lipolytic action and the inhibition of cAMP-PDE activity is also confirmed by the comparative study performed with various piperazinopyrimidine derivatives (table 3). The agents exerting a lipolytic effect also inhibited cAMP-PDE activity whereas those inactive on lipolysis did not affect noticeably the activity of cAMP-PDE.

In conclusion of the *in vitro* studies, a double potency of RP 55462 was revealed: this agent is an *alpha*-2-antagonist (Saulnier Blache *et al.*, 1989) and an inhibitor of cAMP-PDE activity.

In as much as some *alpha*-2-antagonists, when administered *in vivo*, promote lipid-mobilization (Galitzky *et al.*, 1988; Taouis *et al.*, 1988), a comparison of the lipid mobilizing actions of yohimbine and RP 55462 was performed in alert dogs. A lipomobilizing effect of yohimbine (*alpha*-2 adrenergic antagonist) was demonstrated previously in dogs and in human subjects, it was abolished completely by pretreatment with propranolol. These results suggest that the major effect of yohimbine is to increase the sympathetic tone in dogs (Taouis *et al.*, 1988). The activation of the lipolysis is promoted by the release of norepinephrine and can be blocked by a pretreatment with propranolol.

We showed previously a lipid mobilizing effect of RP 55462 (Saulnier-Blache *et al.*, 1989). So, it was interesting to know if the inhibitory effect of RP 55462 on cAMP-PDE, revealed *in vitro*, can be implicated in its lipomobilizing action and can even be dissociated from its *alpha*-2-antagonist-dependent lipomobilizing effect.

Pretreatment of alert dogs with propranolol only suppressed the faster component in the lipid mobilizing effect of RP 55462 (15 and 30 min) without affecting the slower one (at 45 min). On the contrary the lipomobilizing effect of yohimbine was abolished completely (fig. 5) by propranolol pretreatment. These experiments revealed the existence of an *alpha*-2 adrenergic antagonist component in the lipomobilizing action of the RP 55462 which can be blocked by propranolol. This initial effect is followed by another which takes place more slowly and which cannot be blocked by propranolol. The late onset lipomobilizing effect of RP 55462 can probably be attributable to its cAMP-PDE inhibitory action. Infusion of theophylline (a cAMP-PDE inhibitor without *alpha*-2 adrenergic antagonist activity) in dogs, promoted, with a slow onset (45 min) an increase of plasma NEFA levels which cannot be blocked by propranolol (data no shown). Such an action is evoking a similarity with the effects described for RP 55462.

The *in vivo* studies suggested that the lipomobilizing effect of RP 55462, is a combination between an indirect *alpha*-2-blocking action which is abolished by propranolol and a direct lipolytic action due to the inhibition of cAMP-PDE which is resistant to propranolol treatment. To conclude, the piperazinopyrimidine derivative RP 55462 appears to be an original tool possessing a lipid mobilizing activity originating from at least two mechanisms of action. It could also be an experimental tool for *in vitro* studies when cAMP-PDE inhibition is required without any action on A1-adenosine receptor sites in lipolysis studies or adenylate cyclase assays.

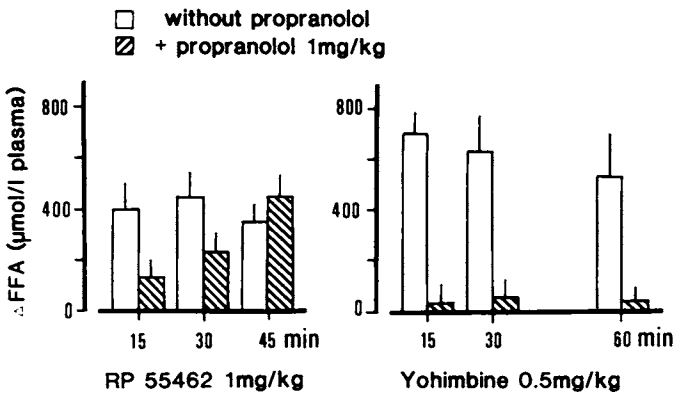


Fig. 5. Influence of propranolol administration on the lipid mobilizing effect of RP 55462 and yohimbine in dogs. Alert dogs were treated (■) or not (□) with 1 mg/kg of propranolol (bolus i.v.) 30 min before injection of 1 mg/kg of RP 55462 (A) or 0.5 mg/kg of yohimbine (B) (bolus i.v.). Blood samples were collected before injection and 15, 30, 45 and 60 min after RP 55462 or yohimbine injection. Plasma NEFA levels were determined as described under "Materials and Methods." Results are expressed as the difference between NEFA levels at time 0 and NEFA levels at time 15, 30, 45 and 60 min. Each point represents the mean ± S.E. of four separate experiments.

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Send reprint requests to: Dr. Max Lafontan, INSERM U-317, Institut de Physiologie, Université Paul Sabatier, Rue François Magendie, 31400, Toulouse, France.