MOLECULAR PHARMACOLOGY, 19:228–235

Adenosine Receptors in Fat Cells

Identification by (−)-N^6-[^{3}H]Phenylisopropyladenosine Binding

THOMAS TROST[1] AND ULRICH SCHWABE[2]

Institut für Pharmakologie und Toxikologie, Medizinische Hochschule Hannover, 3000 Hannover 61, and Institut für Pharmakologie und Toxikologie, Universität Bonn, 5300 Bonn 1, Federal Republic of Germany

Received September 8, 1980; Accepted November 4, 1980

SUMMARY

TROST, T., AND U. SCHWABE. Adenosine receptors in fat cells: identification by (−)-N^6-[^{3}H]phenylisopropyladenosine binding. *Mol. Pharmacol.* 19:228–235 (1981).

The binding of (−)-N^6-[^{3}H]phenylisopropyladenosine to its receptor sites was investigated in plasma membranes of rat fat cells. The binding was rapid, reversible, stereospecific, saturable, and dependent on protein concentration, pH, and temperature. Pretreatment of fat cell membranes with adenosine deaminase resulted in a 3 to 4-fold increase in specific binding. The specific binding was of high affinity with an equilibrium dissociation constant (K_D) of 6.1 nM and was saturable with 1.9 pmoles of (−)-N^6-[^{3}H]phenylisopropyladenosine per milligram of protein. Rate constants of association and dissociation were $k_1 = 1.95 \times 10^7$ M^{-1} min^{-1} and $k_2 = 0.040$ min^{-1}, respectively. In competition experiments the (−)-isomer of N^6-phenylisopropyladenosine was 11-fold more potent than the (+)-isomer in competing for the binding sites. Specific binding was most effectively displaced by the following purine-modified adenosine derivatives which have been classified as *R* site adenosine agonists: (−)-N^6-phenylisopropyladenosine monophosphate, N^6-isobutyladenosine, N^6-cyclohexyladenosine, 2-chloroadenosine, 2-fluoroadenosine, N^6-phenyladenosine, and N^6-benzyladenosine, with IC_{50} values ranging from 17 to 139 nM. The ribose-modified adenosine derivatives 3′-deoxyadenosine, 2′,5′-dideoxyadenosine, and 2′-deoxyadenosine, which have been classified as *P* site adenosine agonists, showed IC_{50} values ranging from 9.7 to 78 μM. Similar IC_{50} values were obtained by several 5′-phosphorylated adenosine derivatives. Pharmacologically inactive compounds such as inosine, hypoxanthine, and adenine did not substantially inhibit binding at concentrations up to 100 μM. The methylxanthines isobutylmethylxanthine (IC_{50} 55 μM), theophylline (IC_{50} 61 μM), and caffeine (IC_{50} 150 μM), which have been classified as antagonists for *R* site adenosine receptors, competed for the binding sites of (−)-N^6-[^{3}H]phenylisopropyladenosine in a manner which parallels their known pharmacological activity in fat cells. Specific binding was not displaced by other phosphodiesterase inhibitors (papaverine or Ro 20-1724), and not by dipyridamole or nicotinic acid. Our results indicate that rat fat cells have adenosine receptors which are labeled by (−)-N^6-[^{3}H]phenylisopropyladenosine. On the basis of the order of affinity of agonists and antagonists they appear identical with the *R* site adenosine receptors which mediate the inhibitory effects of adenosine on cyclic AMP formation and lipolysis in fat cells.

INTRODUCTION

Adenosine plays a significant role as an endogenous modulator of cyclic AMP accumulation and lipolysis in isolated fat cells (1–4). The effects of adenosine have been assumed to be mediated by an interaction with an extracellular receptor in fat cells (5). This assumption was further supported by the fact that adenosine inhibited hormone-stimulated adenylate cyclase activity of fat cell membrane preparations (1, 6–8). Studies with several adenosine analogues have shown that the inhibitory effects of adenosine in fat cells are mediated by processes occurring at two different sites which have been classified as ribose site (*R* site) and purine site (*P* site) receptors (9, 10). The *R* site is presumably located on the external surface of the cell membrane and has strict structural requirements in the ribose moiety of adenosine, whereas the *P* site is intimately associated

This study was supported by the Deutsche Forschungsgemeinschaft.

[1] Institut für Pharmakologie und Toxikologie, Medizinische Hochschule Hannover.

[2] Institut für Pharmakologie und Toxikologie, Universität Bonn.

0026-895X/81/020228-08$02.00/0

with the adenylate cyclase catalytic subunit located intracellularly and is inhibited by adenosine analogues with an intact purine moiety (10). The *R* site-mediated inhibition of cyclic AMP formation in fat cell membrane preparations by adenosine and N^6-substituted analogues such as N^6-phenylisopropyladenosine is dependent on the presence of inhibitory concentrations of GTP (11). Methylxanthines antagonize the inhibitory effects of adenosine and its purine-modified analogues at the *R* site receptor in fat cells (1–3, 5), and this inhibition again is GTP-dependent (11).

Attempts have been made to identify adenosine receptors in fat cells by binding studies using [^{3}H]adenosine (12). The binding of [^{3}H]adenosine was rapid and reversible and consisted of a high-affinity component (K_D = 10 μM) and a low-affinity component (K_D = 950 μM). Other binding characteristics differed considerably from what might have been predicted for physiological adenosine receptors. N^6-Phenylisopropyladenosine failed to compete with adenosine binding; adenine, which is devoid of adenosine-like activity in intact fat cells, had the highest affinity, and adenosine antagonists, such as theophylline and isobutylmethylxanthine, produced less than 50% inhibition of [^{3}H]adenosine binding at concentrations up to 100 μM (12).

Because of success in using (−)-N^6-[^{3}H]phenylisopropyladenosine to identify adenosine receptors in rat brain (13), we used this compound to characterize the postulated adenosine receptors in rat fat cell membranes.

MATERIALS AND METHODS

(−)-N^6-Phenylisopropyladenosine was tritiated by the Radiochemical Centre, Amersham, England, by an exchange procedure using tritium gas and was purified by paper chromatography. The tritiated product was supplied as a solution in water and ethanol (7:3) and was stored at 2°. The specific activity of (−)-N^6-[^{3}H]phenylisopropyladenosine was 26.0 Ci/mmole. Other compounds used in this study were bovine serum albumin (Serva Feinbiochemica, Heidelberg, Federal Republic of Germany), treated with charcoal according to the method of Chen (14); crude bacterial collagenase (Worthington Biochemical Corporation, Freehold, N. J.); Ficoll 400 (Pharmacia Fine Chemicals, Uppsala, Sweden); adenosine, cyclic AMP, 5′-AMP, ADP, ATP, inosine, adenine, *S*-adenosylhomocysteine, hypoxanthine, and adenosine deaminase from calf intestine (200 units/mg) (Boehringer Mannheim, Mannheim, Federal Republic of Germany); 1,N^6-ethenoadenosine, 2-chloroadenosine, 7-deazaadenosine (tubercidin), and 2′-deoxyadenosine (Sigma Chemical Company, St. Louis, Mo.); 2′,5′-dideoxyadenosine and 3′-deoxyadenosine (cordycepin) (P-L Biochemicals, Milwaukee, Wisc.); dipyridamole (Dr. Karl Thomae GmbH, Biberach, Federal Republic of Germany); nicotinic acid, theophylline, caffeine, and papaverine (Merck AG, Darmstadt, Federal Republic of Germany); 3-isobutyl-1-methylxanthine (Aldrich Chemical Company-Europe, Düsseldorf, Federal Republic of Germany); (±)-propranolol (Rheinpharma, 6831 Plankstadt, Federal Republic of Germany); and (−)-noradrenaline HCl (Fluka AG, Buchs, Switzerland). 4-(3-butoxy-4-methoxybenzyl)-2-Imidazolidinone (Ro 20-1724) was kindly provided by Dr. W. E. Scott (Hoffmann La Roche, Nutley, N. J.); N^6-mono-substituted adenosine derivatives by Dr. K. Stegmeier (Boehringer Mannheim, Mannheim, Federal Republic of Germany); eritadenine by Dr. Takeyama (Tanabe Seiyaku, Saitama, Japan). 3′-*O*-Methyldenosine, 2-fluoroadenosine, and deoxycoformycin were a gift of Dr. John Daly (Bethesda, Md.).

Preparation of fat cell plasma membranes. Isolated fat cells were prepared according to the method of Rodbell (15). The procedure for preparation of plasma membranes was essentially that described by McKeel and Jarett (16). After the last centrifugation the membranes were resuspended in 5–7 ml of incubation buffer (50 mM Tris-HCl, pH 7.4, and 1 mM $MgCl_2$) to a final protein concentration of 80–120 μg/100 μl and incubated with adenosine deaminase (1 μg/ml) for 10 min at 30° in all experiments except where indicated. For measurement of (−)-N^6-[^{3}H]phenylisopropyladenosine binding, the membranes were used either immediately after preparation or after storage at −18° for up to 1 week without essential loss of specific binding. The protein concentration was determined by the method of Lowry *et al.* (17), using bovine serum albumin as standard.

Binding assay. The measurement of (−)-N^6-[^{3}H]phenylisopropyladenosine binding to fat cell membranes was performed by a vacuum filtration technique as previously described for rat brain membranes (13). Briefly stated, aliquots of fat cell membranes (80–120 μg/100 μl) were incubated at 37° with 10 nM (−)-N^6-[^{3}H]phenylisopropyladenosine in a total volume of 350 μl of incubation buffer (50 mM Tris-HCl, pH 7.4, and 1 mM $MgCl_2$). Following incubation, 300 μl were removed and rapidly filtered through a Whatman GF/B glass fiber filter. The filters were washed with two 5-ml portions of ice-cold incubation buffer and counted after 6-hr storage at 4° following the addition of 10 ml of Triton-based scintillation fluid (Quickszint 402, Zinsser Analytic GmbH, Frankfurt, Federal Republic of Germany). In each experiment, nonspecific binding was determined by measurement of the amount of radioactivity retained on filters when incubations were performed in the presence of 100 μM (−)-N^6-phenylisopropyladenosine. The nonspecific binding, including counter background, nonspecific adsorption to filters, and nonspecific adsorption to protein, was approximately 30% of the total counts bound in the absence of adenosine deaminase and approximately 10% in the presence of adenosine deaminase. (−)-N^6-[^{3}H]-Phenylisopropyladenosine nonspecifically adsorbed to the filters was less than 0.2% of the total counts filtered and was not altered by high concentrations of unlabeled (−)-N^6-phenylisopropyladenosine.

RESULTS

The binding of (−)-N^6-[^{3}H]phenylisopropyladenosine to fat cell membranes was dependent on the pretreatment of the membranes with adenosine deaminase (1 μg/ml) during the membrane preparation. In adenosine deaminase-treated membranes, specific binding was 3 to 4-fold higher than that in untreated membranes, whereas nonspecific binding was not changed (Table 1). The effect of adenosine deaminase treatment is probably due to removal of endogenous adenosine still present in the

TABLE 1

Effect of adenosine deaminase treatment of rat fat cell membranes on (−)-N^6-[^{3}H]phenylisopropyladenosine binding

Rat fat cell membranes, which had been treated or not with adenosine deaminase (1 μg/ml) during the membrane preparation procedure, were incubated for 15 min with 10 nM (−)-N^6-[^{3}H]phenylisopropyladenosine and binding was determined as described under Materials and Methods. Values for nonspecific binding of the radioligand were 0.147 ± 0.025 pmole/mg of protein in control membranes and 0.152 ± 0.014 pmole/mg of protein in membranes treated with adenosine deaminase. Values are means ± standard error of the mean of three separate experiments.

Addition	Specific binding of (−)-N^6-[^{3}H]phenylisopropyladenosine	
	Control	Adenosine deaminase
	pmoles/mg protein	
None	0.259 ± 0.044	0.966 ± 0.138
Deoxycoformycin		
1 nM	0.239 ± 0.038	0.959 ± 0.126
10 nM	0.239 ± 0.035	0.555 ± 0.106
100 nM	0.232 ± 0.030	0.488 ± 0.086
1000 nM	0.227 ± 0.041	0.489 ± 0.082

repeatedly washed membrane preparation. However, N^6-phenylisopropyladenosine is resistant to deamination by the deaminase (13, 18) and as a possible competitive inhibitor may bind to the enzyme, which then may increase the amount of radioligand binding. The soluble adenosine deaminase did not bind (−)-N^6-[^{3}H]phenylisopropyladenosine under our experimental conditions. We have further considered the possibility that traces of adenosine deaminase become associated with the membranes and thus may be the reason for an additional binding. As a control we have used the adenosine deaminase inhibitor deoxycoformycin to assess the enzyme as an additional binding site for the radioligand, although this experimental approach primarily will be a control for the effectiveness of the added adenosine deaminase to remove the endogenous adenosine. In fact, deoxycoformycin markedly reduced the specific binding of (−)-N^6-[^{3}H]phenylisopropyladenosine in the presence of added deaminase, but, even at the highest concentrations of the deaminase inhibitor, binding was still 2-fold higher than that obtained in the absence of adenosine deaminase (Table 1). Deoxycoformycin had no substantial effect on radioligand binding in the absence of adenosine deaminase.

These results favor the assumption that adenosine deaminase is not an important site of (−)-N^6-[^{3}H]phenylisopropyladenosine binding, since deoxycoformycin only partially inhibited the effect of added adenosine deaminase. Deoxycoformycin itself obviously does not bind to adenosine sites on the fat cell membranes. Although final proof under these circumstances is not possible, all available evidence supports the interpretation that the effects of adenosine deaminase are due to removal of endogenous adenosine. Therefore, in all experiments, adenosine deaminase-treated membranes were used with the exception of those experiments in which adenosine deaminase-degradable compounds were studied.

Specifically bound (−)-N^6-[^{3}H]phenylisopropyladenosine was linear with protein concentrations in the range of 25–200 μg (data not shown) and usually represented 90% of the total binding (Table 1). Specific binding was furthermore dependent on pH, showing a broad optimum over the pH range 5.0–7.4 and a marked decline at pH 4.5 and 8.0. The analysis of the temperature dependence revealed a sharp peak of specific binding at 37°, whereas nonspecific binding was not substantially altered over the entire temperature range tested (Fig. 1).

With increasing concentrations of (−)-N^6-[^{3}H]phenylisopropyladenosine, specific binding was saturable (Fig. 2). Scatchard analysis of these binding data as is shown in the *inset* to Fig. 2 yields a linear plot suggesting a single class of binding sites. From these data an apparent dissociation constant (K_d) of 6.1 nM and a maximal number of binding sites (B_{max}) of 1.89 pmoles of radioligand bound per milligram of membrane protein were calculated.

When (−)-N^6-[^{3}H]phenylisopropyladenosine was incubated with fat cell membranes in the presence of increasing concentrations of unlabeled (−)-N^6-phenylisopropyladenosine, 50% inhibition of binding was obtained at a concentration of 13.0 nM (−)-N^6-phenylisopropyladenosine (Fig. 3). As is shown in the *inset* of Fig. 3, Scatchard analysis of the data yields a linear plot suggesting the existence of a single class of binding sites. The apparent dissociation constant (K_D) calculated from these data was 4.1 nM and the maximal number of binding sites (B_{max}) was 1.5 pmoles/mg of protein. These values are in excellent agreement with those obtained from the radioligand saturation studies described above (see Fig. 2).

Figure 4 shows the time course of (−)-N^6-[^{3}H]phenylisopropyladenosine binding to fat cell membranes. The

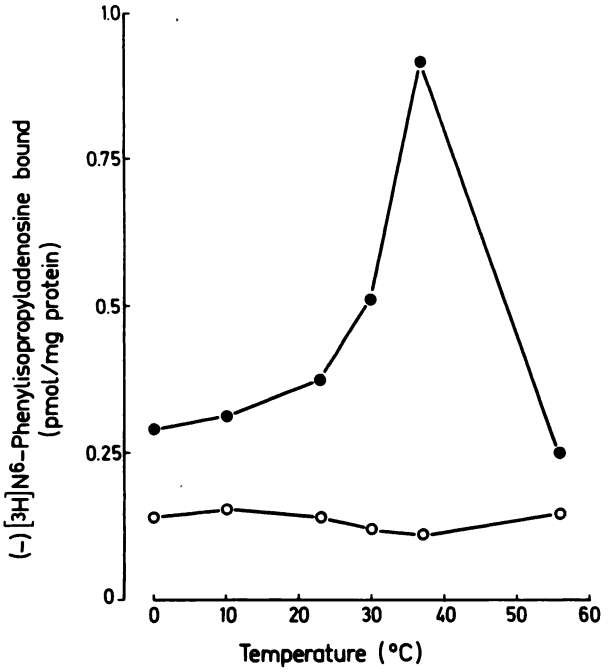

FIG. 1. *Temperature dependence of (−)-N^6-[^{3}H]phenylisopropyladenosine binding to rat fat cell membranes*

Rat fat cell membranes were incubated at various temperatures for 15 min under standard assay conditions in the absence (●) or the presence (○) of 100 μM (−)-N^6-phenylisopropyladenosine. Each value is the mean of two separate experiments.

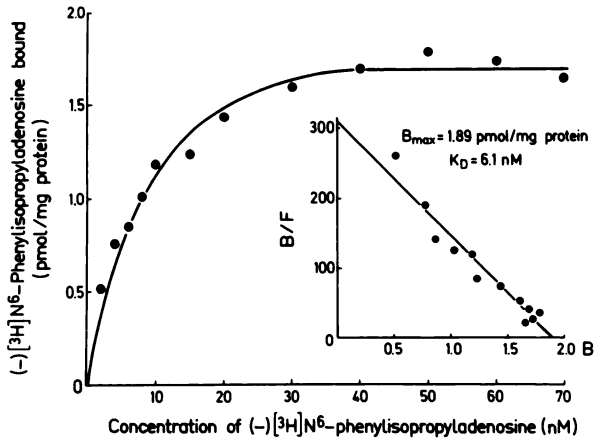

FIG. 2. *Saturation of (−)-N^6-[^{3}H]phenylisopropyladenosine binding to rat fat cell membranes*

Specific binding to rat fat cell membranes was determined as described under Materials and Methods. Each value is the mean of two or three separate experiments. *Inset:* Scatchard plot of the same data. The line was calculated by linear regression analysis (r = 0.968) of the data for specific (−)-N^6-[^{3}H]phenylisopropyladenosine binding, yielding values for K_D = 6.1 nM and B_{max} = 1.89 pmoles/mg of protein. *B*, (−)-N^6-[^{3}H]phenylisopropyladenosine bound (picomoles per milligram of protein); *F*, concentration of free (−)-N^6-[^{3}H]phenylisopropyladenosine (micromolar).

specific binding occurred rapidly, being 50% complete after approximately 3 min. Equilibrium was reached at approximately 10 min. The observed forward rate constant (k_{ob}) can be calculated from the slope of the line in the *inset* to Fig. 4 and is 0.235 min^{-1}. The second-order rate constant can be obtained from $k_1 = k_{ob} - k_2/[(-)\text{-}N^6\text{-}[^3\text{H}]\text{phenylisopropyladenosine}] = 1.95 \times 10^7$ M^{-1} min^{-1}, where k_2 is the independently determined rate constant of the dissociation of the radioligand from its binding sites (see Fig. 5). Nonspecific binding measured in the presence of 100 μM (−)-N^6-phenylisopropyladenosine reached a maximum of 0.140 ± 0.009 pmole/mg of protein (mean ± standard error of the mean; n = 3) after only 1 min of incubation and than slowly decreased during 30 min to 50% of the maximal value obtained at 1 min.

Figure 5 shows the dissociation of (−)-N^6-[^{3}H]phenylisopropyladenosine from its binding sites as determined by adding an excess of the unlabeled compound to an equilibrated mixture of the radioligand and fat cell membranes. The dissociation was relatively slow but constant over the entire time measured, yielding a rate constant k_2 of 0.040 min^{-1} (*inset*, Fig. 5). Within 15 min after the addition of 100 μM (−)-N^6-phenylisopropyladenosine, 47% of the radioligand specifically bound to the membranes was dissociated from its binding sites.

The ratio k_2:k_1 of the rate constants was 2.1 nM and provides an independent estimate of the equilibrium dissociation constant K_D for the interaction of the radioligand with its binding sites in fat cell plasma membranes. This value is in good agreement with the apparent K_D values calculated from the saturation and competition experiments (6.1 and 4.1 nM) illustrated in Figs. 2 and 3.

The binding of (−)-N^6-[^{3}H]phenylisopropyladenosine to fat cell plasma membranes was stereospecific, as was demonstrated by a lower IC_{50} value for the (−)-isomer (13 nM) as compared with the (+)-isomer (IC_{50} = 142 nM) (Fig. 6; Table 2). Thus, an 11-fold higher concentration of the (+)-isomer was required to cause half-maximal inhibition of binding.

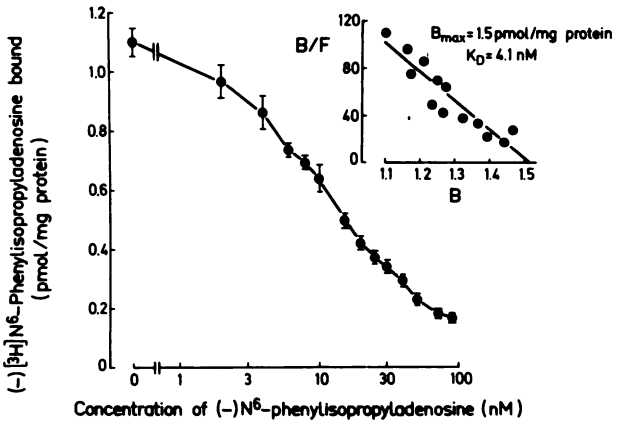

FIG. 3. *Displacement of (−)-N^6-[^{3}H]phenylisopropyladenosine (10 nM) from rat fat cell membranes by unlabeled (−)-N^6-phenylisopropyladenosine*

Rat fat cell membranes were incubated with increasing concentrations of unlabeled (−)-N^6-phenylisopropyladenosine and specific binding was determined as described under Materials and Methods. Mean values ± standard error of the mean of three or four separate experiments are shown. *Inset:* Scatchard plot of the same data. The line was calculated by linear regression analysis (r = 0.916) of the data for specific (−)-N^6-[^{3}H]phenylisopropyladenosine binding, yielding values for K_D = 4.1 nM and B_{max} = 1.51 pmoles/mg of protein. *B*, (−)-N^6-[^{3}H]phenylisopropyladenosine bound (picomoles per milligram of protein); *F*, concentration of free (−)-N^6-[^{3}H]phenylisopropyladenosine (micromolar).

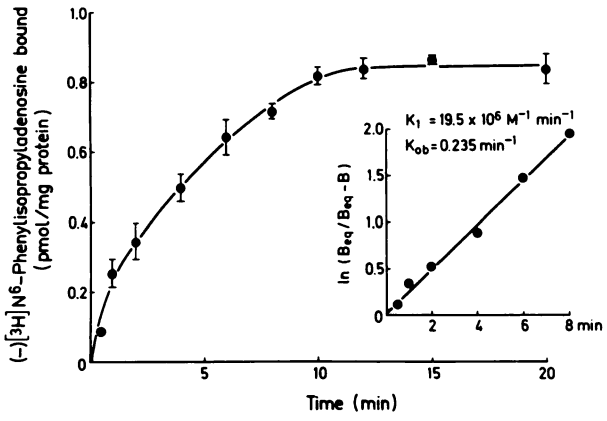

FIG. 4. *Time course of specific binding of (−)-N^6-[^{3}H]phenylisopropyladenosine to rat fat cell membranes*

At times indicated, 300-μl aliquots were removed from the incubation medium and specific binding of (−)-N^6-[^{3}H]phenylisopropyladenosine was determined as described under Materials and Methods. Values are the means ± standard error of the mean of three separate experiments. *Inset:* pseudo-first order plot of (−)-N^6-[^{3}H]phenylisopropyladenosine binding. The slope of the line, determined by linear regression analysis (r = 0.997), provides an estimate of the observed rate constant (k_{ob}). B_{eq}, amount of (−)-N^6-[^{3}H]phenylisopropyladenosine bound at equilibrium; *B*, amount bound at each time; k_1, second order rate constant for the association, calculated from the equation $k_1 = (k_{ob} - k_2)/[(-)\text{-}N^6\text{-}[^3\text{H}]\text{phenylisopropyladenosine}]$; k_2, rate constant for the dissociation of the receptor-ligand complex (see Fig. 5).

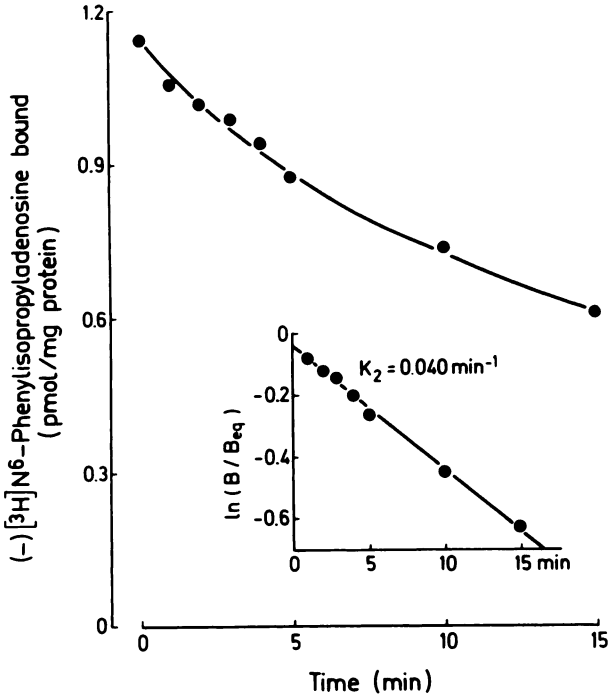

FIG. 5. *Dissociation of specific (−)-N^6-[^{3}H]phenylisopropyladenosine binding*

Rat fat cell membranes were equilibrated with (−)-N^6-[^{3}H]phenylisopropyladenosine (10 nM) for 15 min, after which a large excess of unlabeled (−)-N^6-phenylisopropyladenosine (100 μM) was rapidly added to the mixture at time zero. Alquots of 300 μl were sampled at the indicated times and specific binding was determined as described under Materials and Methods. Each value is the mean of three separate experiments. *Inset:* first-order plot of the dissociation of the radioligand from the binding sites. The slope of the line, determined by linear regression analysis (r = 0.998), provides an estimate of the dissociation rate constant k_2. B, The amount of (−)-N^6-[^{3}H]phenylisopropyladenosine bound at each time after the addition of unlabeled compound; B_{eq}, the amount at equilibrium at time zero.

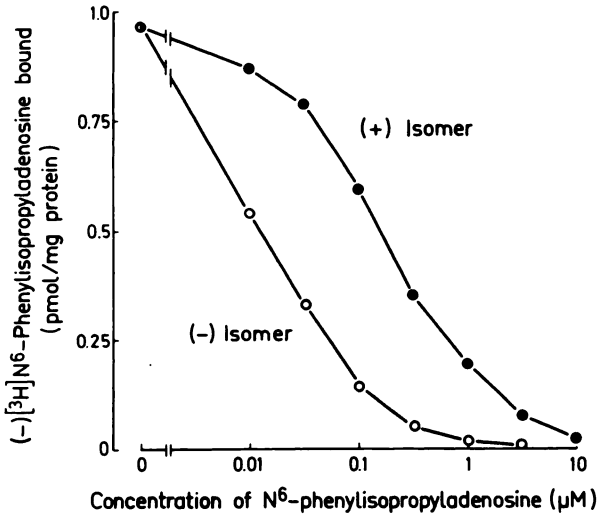

FIG. 6. *Inhibition of specific (−)-N^6-[^{3}H]phenylisopropyladenosine binding to rat fat cell membranes by the (−)- and (+)-stereoisomers of N^6-phenylisopropyladenosine*

Rat fat cell membranes were incubated for 15 min under standard assay conditions as described under Materials and Methods. Each value is the mean of three separate experiments.

Several methylxanthines which are known as adenosine antagonists and phosphodiesterase inhibitors have been tested for their ability to compete with (−)-N^6-[^{3}H]-phenylisopropyladenosine binding. The order of effectiveness was isobutylmethylxanthine > theophylline > caffeine, and the IC_{50} values ranged from 55 to 150 μM (Fig. 7; Table 2). Other phosphodiesterase inhibitors, such as papaverine, dipyridamole, and Ro 20-1724, had no effect at concentrations up to 100 μM (Table 2).

Moreover, several other substances were tested for their effects on (−)-N^6-[^{3}H]phenylisopropyladenosine binding (Table 2). Several N^6-monosubstituted adenosine derivatives as well as 2-chloroadenosine and 2-fluoroadenosine, which have been classified as R site effectors of adenosine action (10), were highly effective in reducing specific binding of the radioligand. Since it yielded an IC_{50} value of 0.017 μM, (−)-N^6-phenylisopropyladenosine monophosphate was, next to (−)-N^6-phenylisopropyladenosine, the most effective adenosine derivative, whereas N^6-benzyladenosine (IC_{50} = 0.139 μM) required

TABLE 2

IC_{50} values for inhibition of (−)-N^6-[^{3}H]phenylisopropyladenosine binding to rat fat cell membranes

IC_{50} values were determined by using 10 nM of the radioligand and at least four concentrations of the displacing compound in two to six separate experiments. Values given in parentheses indicate the percentage inhibition obtained by 100 μM of the added compound if inhibition was less than 50%.

Compound	IC_{50}
	μM
(−)-N^6-[^{3}H]Phenylisopropyladenosine	0.013
(−)-N^6-[^{3}H]Phenylisopropyladenosine monophosphate	0.017
N^6-Isobutyladenosine	0.018
N^6-Cyclohexyladenosine	0.019
N^6-n-Propyladenosine	0.019
2-Chloroadenosine	0.023
2-Fluoroadenosine	0.023
N^6-Phenyladenosine	0.024
N^6-O-Xylyladenosine	0.088
N^6-Benzyladenosine	0.139
(+)-N^6-Phenylisopropyladenosine	0.142
7-Deazaadenosine	25.7
2′,5′-Dideoxyadenosine	31.4
Cyclic AMP	34.0
S-Adenosylhomocysteine	34.5
5′-ATP	44.7
Isobutylmethylxanthine	55.4
5′-ADP	56.3
Theophylline	61.4
5′-AMP	64.4
Caffeine	150.3
1,N^6-Ethenoadenosine	>100(31%)
Inosine	>100(23%)
3′-O-Methyladenosine	>100(16%)
Dipyridamole	>100(11%)
Papaverine	>100(7%)
Eritadenine	>100(3%)
Propranolol	>100(1%)
Hypoxanthine	>100(0%)
Adenine	>100(0%)
Noradrenaline	>100(0%)
Nicotinic acid	>100(0%)
Ro 20-1724	>100(0%)

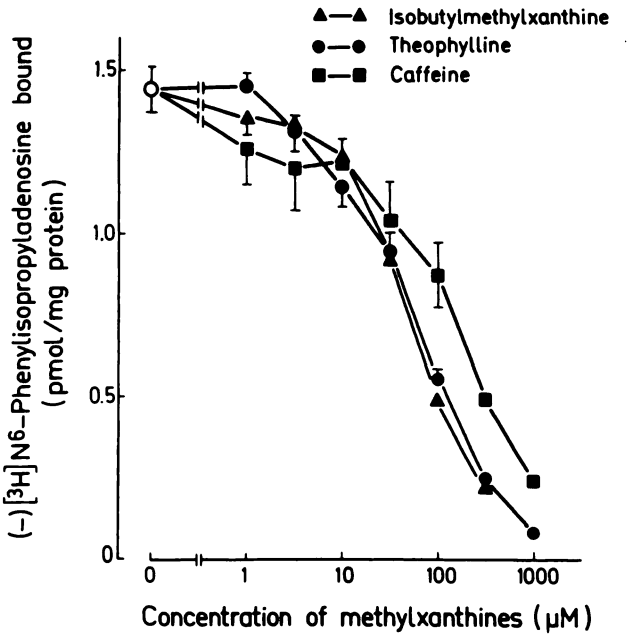

FIG. 7. *Inhibition of specific (−)-N^6-[^{3}H]phenylisopropyladenosine binding to rat fat cell membranes by methylxanthines*

Rat fat cell membranes were incubated for 15 min under standard assay conditions as described under Materials and Methods. Values are means ± standard error of the mean of three separate experiments.

approximately 10-fold higher concentrations for 50% inhibition of binding. Several other N^6-substituted adenosine derivatives, as well as 2-chloroadenosine and 2-fluoroadenosine, showed IC_{50} values in the nanomolar concentration range. All other derivatives tested were at least 3 orders of magnitude less active than (−)-N^6-phenylisopropyladenosine in reducing the binding of the radioligand. 2′,5′-Dideoxyadenosine, which has been classified as a *P* site effector of adenosine action (10), was approximately 2000 times less effective than (−)-N^6-phenylisopropyladenosine. Eritadenine, which can be classified as a pure *P* site agonist in fat cells (19), had no effect on binding. Other adenosine analogues which are neither *R* site nor *P* site effectors (10) (such as 7-deazaadenosine, 1,N^6-ethenoadenosine, or 3′-*O*-methyladenosine) were at least 2000 times less effective than (−)-N^6-phenylisopropyladenosine. The phosphorylated adenosine derivatives cyclic AMP, ATP, ADP, and 5′-AMP required concentrations between 34 and 64 μM for 50% inhibition of binding. A similar IC_{50} value was obtained for *S*-adenosylhomocysteine. The adenosine metabolites inosine, hypoxanthine, and adenine, which are only weak inhibitors of cyclic AMP accumulation and lipolysis in intact cells (5, 20), did not substantially compete with (−)-N^6-[^{3}H]phenyl- isopropyladenosine binding. The antilipolytic agent nicotinic acid, which markedly reduces cyclic AMP levels in fat cells (21), was ineffective as an inhibitor of binding. Propranolol and noradrenaline had no effect on binding of the radioligand.

Finally, adenosine and some adenosine analogues which are deaminated by adenosine deaminase were studied in the absence of this enzyme. A 50% inhibition of specific (−)-N^6-[^{3}H]phenylisopropyladenosine binding occurred at a concentration of 0.11 μM (−)-N^6-phenylisopropyladenosine (Table 3), a concentration being 8 times higher than that in the presence of the enzyme (Table 2). Adenosine (IC_{50} = 0.65 μM) was approximately 6 times less effective than (−)-N^6-phenylisopropyladenosine in reducing binding of the radioligand, whereas 3′-deoxyadenosine and 2′-deoxyadenosine were approximately 2–3 orders of magnitude less effective than (−)-N^6-phenylisopropyladenosine (Table 3).

DISCUSSION

The results of the present study demonstrate in a rat fat cell membrane preparation the presence of binding sites for (−)-N^6-[^{3}H]phenylisopropyladenosine which display the characteristics of *R* site adenosine receptors. N^6-Phenylisopropyladenosine has been classified as an *R* site adenosine agonist in several adenosine-sensitive cellular systems (10, 22). In the fat cell, N^6-phenylisopropyladenosine is a very potent adenosine derivative with respect to inhibition of adenylate cyclase, cyclic AMP accumulation, and lipolysis (1, 5, 8, 11, 18, 23). N^6-Phenylisopropyladenosine shares these properties with several other N^6-substituted adenosine derivatives, all of which are resistant to degradation by adenosine deaminase (9, 18). Furthermore, no phosphorylation of (−)-N^6-[^{3}H]-phenylisopropyladenosine occurred in another binding study with this radioligand (13). In contrast to adenosine, which is a substrate of many different enzymes, (−)-N^6-[^{3}H]phenylisopropyladenosine is thus far a relatively stable ligand for labeling of adenosine receptors.

Binding of the radioligand to fat cell plasma membranes was saturable, rapid, reversible, and dependent on pH, temperature, and the presence of adenosine deaminase. The Scatchard plots from the saturation and competition experiments were linear, suggesting a single class of binding sites. The K_D values, calculated independently from the saturation and competition curves and from the kinetic data, were in the same order of magnitude—ranging from 2 to 6 nM. These values are in good agreement with the nanomolar concentrations of N^6-phenylisopropyladenosine necessary to inhibit hormone-stimulated cyclic AMP accumulation and lipolysis in intact rat fat cells (4, 5, 8, 23) and adenylate cyclase in rat fat cell membranes (11).

Concerning the specificity of binding, there was an excellent correlation between binding and the pharmacological activity of several adenosine derivatives in the

TABLE 3

IC_{50} values for the inhibition of (−)-N^6-[^{3}H]phenylisopropyladenosine binding to rat fat cell membranes in the absence of adenosine deaminase

The IC_{50} values were determined by using 10 nM of the radioligand and five or six concentrations of each compound in two or three separate experiments.

Compound	IC_{50}
	μM
(−)-N^6-Phenylisopropyladenosine	0.11
Adenosine	0.65
3′-Deoxyadenosine	9.7
2′-Deoxyadenosine	78.5

fat cell system. Binding proved to be highly specific. The (−)-isomer of N^6-phenylisopropyladenosine was 11 times more effective than the (+)-isomer in competing with the radioligand for the binding sites. A stereospecific effect of N^6-phenylisopropyladenosine in fat cells has previously been described (18, 24).

Methylxanthines antagonize the effects of adenosine on adenylate cyclase as well as cyclic AMP accumulation in intact fat cells, but they do not appear to antagonize the inhibitory effects of ribose-modified analogues of adenosine, such as 2′,5′-dideoxyadenosine (4, 9, 11). Accordingly, the methylxanthines have been classified as *R* site adenosine antagonists (3, 10, 11). The concentrations of methylxanthines which are effective in competing for (−)-N^6-[^{3}H]phenylisopropyladenosine binding are in the range of pharmacologically active concentrations of these agents in antagonizing the inhibitory effect of adenosine and N^6-substituted adenosine derivatives. The IC_{50} values obtained for inhibition of radioligand binding for theophylline (61 mM) and caffeine (150 μM) agree well with those of a previous report in their half-maximally effective concentrations (38 and 193 μM, respectively) in antagonizing the inhibitory effect of N^6-methyladenosine on adenylate cyclase activity in fat cell membranes (11), whereas isobutylmethylxanthine (IC_{50} = 55 μM) was more potent in the adenylate cyclase system (IC_{50} = 9 μM). A similar order of the effectiveness has been demonstrated for activation of lipolysis in intact rat fat cells by theophylline and caffeine, whereas isobutylmethylxanthine was 15 times more potent as a lipolytic agent than was theophylline (25). Other phosphodiesterase inhibitors such as papaverine and Ro 20-1724, which were inactive as adenosine antagonists in the adenylate cyclase preparation (11), likewise did not affect (−)-N^6-[^{3}H]phenylisopropyladenosine binding.

The N^6-monosubstituted adenosine derivatives as well as 2-chloroadenosine or 2-fluoroadenosine were potent inhibitors of radioligand binding, with IC_{50} values ranging from 17 to 139 nM. Similar IC_{50} values for these compounds have been obtained from inhibition of noradrenaline-induced cyclic AMP accumulation in isolated rat fat cells [ranging from 0.6 to 112 nM (9)] or from inhibition of isoproterenol-stimulated adenylate cyclase activity in rat fat cell membranes [ranging from 5 to 20 nM (11)]. It is worth mentioning that N^6-*O*-xylyladenosine and N^6-benzyladenosine, which were less potent as inhibitors of cyclic AMP accumulation in fat cells (9), were similarly less potent among the N^6-substituted analogues investigated in the present study.

All other compounds tested were at least 3 orders of magnitude less active than (−)-N^6-phenylisopropyladenosine in reducing the binding of the radioligand. 2′,5′-Dideoxyadenosine, which has been classified as a *P* site compound (10), was approximately 2000 times less active than (−)-N^6-phenylisopropyladenosine in displacing the radioligand from its binding sites. Moreover, the IC_{50} value of this compound obtained in the present study (IC_{50} = 31.4 μM) agrees well with that obtained from inhibition of cyclic AMP levels in isolated rat fat cells (IC_{50} = 10 μM) (9). Eritadenine, which has been found to elicit only *P* effects in fat cells (19), had no substantial effect on binding. Other adenosine analogues which are neither *R* site nor *P* site effectors, such as 7-deazaadenosine, 1,N^6-ethenoadenosine, or 3′-*O*-methyladenosine (11), were at least 2000 times less active than (−)-N^6-phenylisopropyladenosine. The phosphorylated adenosine derivatives cyclic AMP, ATP, ADP, and 5′-AMP were 2600–5000 times less active than (−)-N^6-phenylisopropyladenosine as inhibitors of binding. The greater effectiveness of these nucleotides on cyclic AMP accumulation in fat cells (20) than on binding in this study may be due to degradation of the nucleotides to adenosine during incubation with the isolated fat cells. *S*-Adenosylhomocysteine, a substrate of the *S*-adenosylhomocysteine hydrolase, which has recently been identified as a soluble adenosine binding protein (26), was 3 orders of magnitude less active than (−)-N^6-phenylisopropyladenosine in competing for the radioligand; therefore, it seems unlikely that this enzyme substantially contributed to the observed binding of (−)-N^6-[^{3}H]phenylisopropyladenosine in our binding experiments. Breakdown products of adenosine metabolism, such as inosine, hypoxanthine, or adenine, which were only poor inhibitors of cyclic AMP accumulation or lipolysis in fat cells (5, 20), did not substantially compete with the binding of the radioligand. Interestingly, nicotinic acid was completely ineffective as an inhibitor of binding. The antilipolytic effect of this compound, which inhibits cyclic AMP accumulation in fat cells (21), obviously is not mediated by an interaction with adenosine receptors. As to be expected, dipyridamole, an inhibitor of adenosine uptake in isolated fat cells (5), as well as propranolol and noradrenaline had no effect on binding.

The binding of some adenosine deaminase-sensitive compounds such as adenosine, 3′-deoxyadenosine, and 2′-deoxyadenosine was tested in an adenosine deaminase-free assay. In the absence of this enzyme, (−)-N^6-phenylisopropyladenosine itself was approximately 10 times less active in competing for the radioligand than it was in the presence of adenosine deaminase. The adenosine generated during the preparation or incubation of the membranes in the absence of adenosine deaminase is probably responsible for a reduced effect of (−)-N^6-phenylisopropyladenosine on binding. Similarly, adenosine (IC_{50} = 650 nM) inhibited radioligand binding 10- to 100-fold less effectively than cyclic AMP accumulation in isolated fat cells (IC_{50} = 8 nM) (9) or adenylate cyclase activity of fat cell membranes (K_i = 27 nM) (27). Nevertheless, 3′-deoxyadenosine and 2′-deoxyadenosine had less effect on the binding of (−)-N^6-[^{3}H]phenylisopropyladenosine than did adenosine and thus showed an order of effectiveness similar to that in a previous study on the inhibition of cyclic AMP accumulation in rat fat cells (4).

In conclusion, this report demonstrates that (−)-N^6-[^{3}H]phenylisopropyladenosine binding techniques first developed for the study of adenosine receptors in brain (13) are more generally applicable to other adenosine-sensitive systems. The close correlation between the results obtained in these binding experiments and in previous adenylate cyclase studies provides a further tool for future studies on the molecular mechanisms of adenosine-mediated inhibition of hormone and drug-induced stimulation of adenylate cyclase in fat cells.

ACKNOWLEDGMENTS

The excellent technical assistance of Miss Helga Hannemann and Mrs. Anna-Carmen Bauer is gratefully acknowledged.

REFERENCES

1. Schwabe, U., R. Ebert, and H. C. Erbler. Adenosine release from fat cells: effect on cyclic AMP levels and hormone actions *Adv. Cyclic Nucleotide Res.* **5**:569–584 (1975).
2. Fredholm, B. B. Local regulation of lipolysis in adipose tissue by fatty acids, prostaglandins and adenosine. *Med. Biol.* **56**:249–261 (1978).
3. Fain, J. N., and C. C. Malbon. Regulation of adenylate cyclase by adenosine. *Mol. Cell. Biochem.* **25**:143–169 (1979).
4. Trost, T., and K. Stock. Adenosine effects in adipose tissue: effect of adenosine on the cyclic adenosine monophosphate system and lipolysis of fat cells, in *Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides* (H. P. Baer and G. I. Drummond, eds.). Raven Press, New York, 379–393 (1979).
5. Ebert, R., and U. Schwabe. Studies on the antilipolytic effect of adenosine and related compounds in isolated fat cells. *Naunyn-Schmiedebergs Arch. Pharmacol.* **278**:247–259 (1973).
6. Fain, J. N., R. H. Pointer, and W. F. Ward. Effects of adenosine nucleosides on adenylate cyclase, phosphodiesterase, cyclic adenosine monophosphate accumulation, and lipolysis in fat cells. *J. Biol. Chem.* **247**:6866–6872 (1972).
7. McKenzie, S. G., and H. P. Bär. On the mechanism of adenylate cyclase inhibition by adenosine. *Can. J. Physiol. Pharmacol.* **51**:190–196 (1973).
8. Fain, J. N. Inhibition of adenosine cyclic 3′,5′-monophosphate accumulation in fat cells by adenosine, N^6-(phenylisopropyl)adenosine, and related compounds. *Mol. Pharmacol.* **9**:595–604 (1973).
9. Trost, T., and K. Stock. Effects of adenosine derivatives on cAMP accumulation and lipolysis in rat adipocytes and on adenylate cyclase in adipocyte plasma membranes. *Naunyn-Schmiedebergs Arch. Pharmacol.* **229**:33–40 (1977).
10. Londos, C., and J. Wolff. Two distinct adenosine-sensitive sites on adenylate cyclase. *Proc. Natl. Acad. Sci. U. S. A.* **74**:5482–5486 (1977).
11. Londos, C., D. M. F. Cooper, W. Schlegel, and M. Rodbell. Adenosine analogs inhibit adipocyte adenylate cyclase by a GTP-dependent process: basis for actions of adenosine and methylxanthines on cyclic AMP production and lipolysis. *Proc. Natl. Acad. Sci. U. S. A.* **75**:5362–5366 (1978).
12. Malbon, C. C., R. C. Hert, and J. N. Fain. Characterization of [^{3}H]adenosine binding to fat cell membranes. *J. Biol. Chem.* **253**:3114–3122 (1978).
13. Schwabe, U., and T. Trost. Characterization of adenosine receptors in rat brain by (−)[^{3}H]N^6-phenylisopropyladenosine. *Naunyn-Schmiedebergs Arch. Pharmacol.* **313**:179–187 (1980).
14. Chen, R. F. Removal of fatty acids from serum albumin by charcoal treatment. *J. Biol. Chem.* **242**:173–181 (1967).
15. Rodbell, M. Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis *J. Biol. Chem.* **239**:375–380 (1964).
16. McKeel, D. W., and L. Jarett. Preparation and characterization of a plasma membrane fraction from isolated fat cells. *J. Cell. Biol.* **44**:417–432 (1970).
17. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
18. Westermann, E., and K. Stock. Inhibitors of lipolysis: potency and mode of action of α- and β-adrenolytics, methoxamine derivatives, prostaglandin E_1 and phenylisopropyl adenosine, in *Adipose Tissue Regulation and Metabolic Functions* (B. Jean-Renaud and D. Hepp, eds.) Georg Thieme Verlag, Stuttgart, 47–54 (1970).
19. Söchtig, E., and T. Trost. Eritadenine: a new specific effector of adenosine P-site receptors in plasma membranes of rat fat cells. *Pharmacology (Basel)*, in press (1981).
20. Schwabe, U., R. Ebert, and H. C. Erbler. Adenosine release from isolated fat cells and its significance for the effects of hormones on cyclic 3′,5′-AMP levels and lipolysis. *Naunyn-Schmiedebergs Arch. Pharmakol.* **276**:133–148 (1973).
21. Butcher, R. W., C. E. Baird, and E. W. Sutherland. Effects of lipolytic and antilipolytic substances on adenosine 3′,5′-monophosphate levels in isolated fat cells. *J. Biol. Chem.* **243**:1705–1712 (1968).
22. Londos, C., D. M. F. Cooper, and J. Wolff. Subclasses of external adenosine receptors. *Proc. Natl. Acad. Sci. U. S. A.* **77**:2551–2554 (1980).
23. Stock, K., and M. Prilop. Dissociation of catecholamine-induced formation of adenosine 3′,5′-monophosphate and release of glycerol in fat cells by prostaglandin E_1, E_2 and N^6-phenylisopropyladenosine. *Naunyn-Schmiedebergs Arch. Pharmacol.* **282**:15–31 (1974).
24. Prilop, B. Untersuchungen zur Hemmbarkeit des durch Adenosindesaminase in isolierten Adeipocyten ausgelösten Anstiegs von cyclischem Adenosin-3′-5′-Monophosphat und der Lipolyse durch Prostaglandin E_1 und N^6-Phenylisopropyladenosin. Doctoral thesis, Medizinische Hochschule Hannover (1976).
25. Beavo, J. A., N. L. Rogers, O. B. Crofford, J. G. Hardman, E. W. Sutherland, and E. V. Newman. Effects of xanthine derivatives on lipolysis and on adenosine 3′,5′-monophosphate phosphodiesterase activity. *Mol. Pharmacol.* **6**:597–603 (1970).
26. Hershfield, M. S., and N. M. Kredich. S-Adenosylhomocysteine hydrolase as an adenosine-binding protein: a target for adenosine toxicity. *Science (Wash. D. C.)* **202**:757–760 (1978).
27. Cooper, D. M. F., and C. Londos. Evaluation of the effects of adenosine on hepatic and adipocyte adenylate cyclase under conditions where adenosine is not generated endogenously. *J. Cyclic Nucleotide Res.* **5**:289–302 (1979).

Send reprint requests to: Dr. Ulrich Schwabe, Institut für Pharmakologie und Toxikologie, Universität Bonn, Reuterstrasse 2 b, 5300 Bonn 1, Federal Republic of Germany.