Characterization of $[^3\text{H}]$Adenosine Binding to Fat Cell Membranes*

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$[^3\text{H}]$Adenosine binding to fat cell plasma membrane preparations was examined by a vacuum filtration technique. Appreciable binding of adenosine to fat cell membranes could only be demonstrated in the presence of the adenosine deaminase inhibitors, erythro-9-(2-hydroxy-3-nonyl)adenine or deoxycoformycin. The binding of adenosine to these membranes was rapid, reaching near equilibrium within 10 min at 37°C, and was reversible. Bound adenosine dissociated very rapidly following a 100-fold dilution at 0°C or 22°C. Only 7% of equilibrium-bound adenosine remained at 2 min following 100-fold dilution at 22°C. Scatchard plots of equilibrium binding data were nonlinear, suggesting at least two populations of adenosine binding sites possessing different affinities for adenosine. The apparent dissociation constants, $K_d$, and maximum binding capacities, $B_{max}$, were 9.5 x 10^-6 M and 25 pmol/mg of protein and 9.5 x 10^-4 M and 1700 pmol/mg of protein for the high and low affinity adenosine binding sites, respectively.

The high affinity sites display maximum binding at pH 7.5, above pH 7.5, or below pH 6.5 adenosine binding to the fat cell membranes declines sharply. The divalent cations calcium or magnesium, at concentrations greater than 1 mM, inhibit the binding of adenosine to the high affinity sites of the fat cell membranes. Adenosine binding is reduced by prior exposure of the membranes to trypsin, chymotrypsin, or neuraminidase or by thermal denaturation. Purine derivatives compete with adenosine for binding to the membrane site in the following potency order: adenosine > ATP > ADP > cyclic AMP = AMP = inosine. Inhibition studies of high affinity adenosine binding performed with these purine derivatives indicate this binding is to multiple components of differing specificity and not to a single, homogeneous class of binding sites. Theophylline, but not dipyridamole or $p$-nitrobenzylthioguanosine, is a potent inhibitor of adenosine binding to the membranes. One micromolar theophylline inhibits adenosine binding 14%. Highly purified fat cell plasma membranes possess the major portion of adenosine binding sites identified with the crude fat cell plasma membranes. The highly purified fraction likewise displayed the two populations of adenosine binding sites identified in the crude preparation. Adenosine (100 μM) did not affect the specific binding of $[^3\text{H}]$dihydralprenalin to fat cell plasma membranes. Neither 10 μM $[^3\text{H}]$isoproterenol nor 10 μM $[^3\text{H}]$alprenolol affected the ability of fat cell membranes to bind $[^3\text{H}]$adenosine. This study demonstrates that fat cell plasma membranes possess sites which bind $[^3\text{H}]$adenosine with high affinity. The relationship between these adenosine binding sites and the influence of adenosine on cyclic AMP levels in intact fat cells and on adenylyl cyclase activity is discussed.

Dole (1) first demonstrated the ability of adenosine to inhibit epinephrine-stimulated free fatty acid release from fat pads. This observation has since been amply confirmed in adipose tissue (2, 3) and isolated fat cell suspensions (4, 5). Additionally, it has been shown that adenosine and adenosine-containing compounds such as 5′-AMP, ADP, ATP, and NAD possess a capacity to inhibit glucagon- (6), ACTH- (7), and norepinephrine-stimulated (3, 7) lipolysis in adipose tissue. Schwabe et al. (8) demonstrated that adenosine is continuously released to the medium by fat cell suspensions. Sattin and Rall (9) first reported the ability of adenosine to elevate adenosine 3′:5′-monophosphate (cyclic AMP) levels in brain slices. Shimizu and associates (10, 11) confirmed this observation. Subsequent reports have shown adenosine stimulation of cyclic AMP levels in platelets (12–14), isolated bone cells (15), and cultured cell lines (16–21). Adenosine stimulation of cyclic AMP levels in several clones of mouse neuroblastoma cells required, however, the presence of RO-20-1724, a cyclic AMP phosphodiesterase inhibitor (18). Adenosine also stimulates adenylyl cyclase activity in cell membrane preparations (22–27), although some of these preparations display a biphasic stimulation and inhibition of the cyclase by adenosine (22, 24, 25). In contrast, epinephrine- and glucagon-stimulated cyclic AMP accumulation are inhibited by adenosine (22, 26, 28). The abbreviations used are: ACTH, adrenocorticotrophic hormone; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; $K_d$, equilibrium dissociation constant; $B_{max}$, maximum binding capacity; NBTG, $p$-nitrobenzylthioguanosine.
sine in isolated hepatocytes (28). Hepatic adenylate cyclase is likewise inhibited by adenosine (29-31). Micromolar concentra-
tions of adenosine potently inhibit the rise in cyclic AMP
accumulation stimulated by lipolytic hormones in intact rat
fat cells (4, 32, 33). Higher concentrations of adenosine also
inhibit the activation of fat cell "ghost" adenylate cyclase by
hormones (4).

Fain and associates (4, 33, 34) have demonstrated that
adenosine inhibition of norepinephrine-stimulated cyclic AMP
accumulation in rat fat cells is rapid, enhanced by the presence
of inhibitors of adenosine deaminase, readily reversed by
adding purified adenosine deaminase to the medium, and
antagonized by methylxanthines. Adenosine covalently linked
to stachyose, although too large to penetrate the cell mem-
brane, has recently been shown to be nearly equipotent as free
adenosine in inhibiting cyclic AMP accumulation by both
chicken and rat fat cells (34). These studies suggest 1) that
adenosine may regulate the intracellular accumulation of
cyclic AMP via binding to a site on the plasma membrane,
and 2) that methylxanthines may exert their antagonism of
cyclic AMP action through competition with adenosine for this
binding site.

This paper reports the results of studies designed to probe
adenosine interactions with the fat cell membrane by utilizing
tritiated adenosine and a direct binding approach. A prelimi-
nary report of this study has been presented (35).

MATERIALS AND METHODS

Female, 200- to 250-g Sprague-Dawley rats (Charles River CD
strain) were fed laboratory chow ad libitum. The (-)-
\(^{3}H\)hydralazine (specific activity 32.6 Ci/mmol) and
\(^{3}H\)adenosine, labeled at positions 2 and 8, (specific activity, 30 to
50 Ci/mmol) were obtained from New England Nuclear, Boston,
MA. The purity of the tritiated adenosine was greater than 97% as
determined by the supplier and thin layer chromatography in this
laboratory. The following compounds were generously provided by
the sources indicated: cryptidine-9-(2-hydroxy-3-nonyl)adenine, Dr.
Donald Namm, The Wellcome Research Laboratories, Research
Adenosine, Dr. Harold Stork, Boehringer-Mannheim, West Ger-
many; 2',5'-dideoxyadenosine, Drug Research and Development,
Sigma or Boehringer-Mannheim. New York, NY. 50 Ci/mmol) were obtained from New England Nuclear, Boston.

To assay or frozen and maintained at -20°C until assayed. No loss of
adenosine binding was noted in fat cell membranes stored in this
fashion for up to 2 weeks. Highly purified fat cell plasma membranes
were prepared according to the method of McKeel and Jarett (30)
with the following modifications. The pellet (P-3) was resuspended
and layered on 25 ml of 35% sucrose, 1 mM EDTA, and 5
mM Tris-HCl, pH 7.4, and centrifuged at 24,000 rpm for 90 min in a
Beckman model L ultracentrifuge fitted with a SW 25.1 rotor. The
pellet of plasma membranes at the sucrose/sample interface
was collected, diluted with 4 volumes of 0.25 M sucrose, 1 mM EDTA,
and 10 mM Tris-HCl, pH 7.4, and centrifuged at 16,000 x g for 15
min. The pellet was resuspended in 10 mM Tris-HCl, pH 7.4, 5 mM
MgCl\(_2\) buffer. The amount of membrane protein was determined by
method of Lowry et al. (39) using crystalline bovine serum albumin
as a standard.

Binding assays were routinely performed in 10 mM Tris-HCl, pH
7.4, 5 mM MgCl\(_2\) buffer at a final volume of 0.2 ml containing 200 to
400 \(\mu\)g of membrane protein, 10 to 100 nM \(^{3}H\)adenosine and an
adenosine deaminase inhibitor. Incubations of the radiolabeled,
competitors, and membranes were initiated by the addition of
membranes into the incubation vessel. The incubation vessels (Falcon,
17 x 100 mm, No. 2017 tubes) were incubated at the indicated
temperature with constant shaking for up to 60 min. To assess
binding in the presence of membranes, the membranes were
incubated in all experiments under identical conditions as those with membranes. Assay tubes were routinely in
tuplicate for both tubes containing fat cell membranes and those
containing just buffer in place of the membranes. At the end of an
incubation period, 8 ml of ice cold incubation buffer was added to
each tube to terminate the reaction. The contents of the tubes were
rapidly filtered by vacuum through a single Whatman GF/C filter
(24 mm diameter) or Millipore HA filter (24 mm, 0.45 \(\mu\)m). The filter
was then washed with an additional 8 ml aliquot of this same buffer
and the radioactivity was measured by liquid scintillation. The
membranes were incubated in all experiments under identical
conditions utilizing 30 mM radioligand, binding of \(^{3}H\)adenosine
in the tubes containing membranes represented 1000 to 1200 cpm,
while binding in the absence of membranes represented 100 to
200 cpm (\(^{3}H\)Adenosine binding).

Binding of \(^{3}H\)adenosine by the membranes was calculated by
subtracting the no-membrane control value from the value obtained
in the presence of membranes. Binding data were then converted to
units of femtomoles bound per mg of membrane protein.

The nature of the radioactivity bound by the fat cell membranes
was determined as follows. Fat cell membranes (460 \(\mu\)g) were
incubated with 100 nM \(^{3}H\)adenosine for 20 min at 4°C under
standard assay conditions. Following this period, the mixture
was filtered and washed on a Whatman GF/C filter. Control incubations
were performed as above omitting the fat cell membranes. Filters from
these three identical incubations were then immersed in 3
ml of ice cold 1 N HClO\(_4\). The perchloric acid extracts were passed
over columns of Norit/Celite, washed with water, and the adenine
and derivatives eluted with 50% aqueous pyridine according to Hepp
et al. (41). The eluant of the pyridine wash was collected, lyophilized,
and the adenine and derivatives rechromatographed on a modified
cellulose plate using polyethyleneimine-impregnated plates of cellulose
MN 300 (Brinkmann CELL 300 PEI) (0.1 mm). The developing solvent
was 1-butanol, glacial acetic acid and water (59:25:25) or 1-butanol,
formic acid, and water (77:13:10).

The chromatographic procedure using polyethyleneimine-cellulose
plate and the 1-butanol-acetic acid resulted in very discrete spots
and reproducible separations except for that of labeled adenosine
derivatives eluted with 50% aqueous pyridine according to Hepp
et al. (41). The eluant of the pyridine wash was collected, lyophilized,
and 300 (Brinkmann CELL 300 PEI) (0.1 mm). The developing solvent
was 1-butanol, glacial acetic acid and water (59:25:25) or 1-butanol,
formic acid, and water (77:13:10).

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and reproducible separations except for that of labeled adenosine
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et al. (41). The eluant of the pyridine wash was collected, lyophilized,
Adenosine Binding to Fat Cell Plasma Membranes

Initial experiments utilizing a rapid filtration assay failed to demonstrate any appreciable binding of \(^{3}H\)adenosine to crude plasma membranes prepared from rat fat cells. As shown in Table I, adenosine binding to fat cell membranes could only be demonstrated when an inhibitor of adenosine deaminase was present in the incubation medium. Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), a potent adenosine deaminase inhibitor, at 1 to 10 \(\mu\)M increased the amount of adenosine bound by these membranes. Deoxycoformycin, another potent inhibitor of adenosine deaminase (42), similarly provided the condition necessary to observe \(^{3}H\)adenosine binding to the membranes (Table I). Since the amount of adenosine deaminase activity of these membrane preparations varied, binding studies were routinely performed in the presence of 100 \(\mu\)M EHNA.

The binding of \(^{3}H\)adenosine at 10 nM radioligand increased linearly with the protein concentration of the fat cell membranes utilized in the assay from 60 to 450 \(\mu\)g of membrane protein/tube (Fig. 1A). A 350- to 400-\(\mu\)g aliquot of membrane protein was routinely used in the assay of adenosine binding to the fat cell membranes.

The kinetics of adenosine binding to fat cell membranes at 30 nM adenosine was rapid; more than 90% of the equilibrium binding value was obtained within 60 s (Fig. 1B). Increasing the temperature at which the incubation was performed to 37°C reduced the equilibrium adenosine binding capacity of the membranes by 30%. This reduction in the adenosine binding may be the result of increased metabolism of some critical component of the system at this temperature or perhaps the result of simple heat denaturation of the particulate membranes. Exposing the fat cell membranes alone to 37°C for 15 min reduced the ability of these membranes to subsequently bind \(^{3}H\)adenosine following a 20-min incubation at 4°C by 30 to 40% (data not shown). To avoid this complication, binding assays were routinely performed in an ice bath (0-4°C) or at 22°C in a shaking water bath.

Saturating studies were next conducted. The results of these studies are shown in Fig. 2. Adenosine binding by fat cell membranes was concentration-dependent but could not be saturated at concentrations of adenosine as high as 1 mM. Analysis of this binding data by the method of Scatchard (43), as shown in the inset of Fig. 2, produced a nonlinear plot. A simple interpretation of these data suggests the existence of two populations of binding sites with differing affinities for adenosine. The higher affinity sites display an apparent dissociation constant \(K_d\) of 9.5 \(\pm\) 3 \(\times\) 10\(^{-6}\) M (\(n = 3\)) and a maximum binding capacity \(B_m\) of 28 \pm 6 pmol of adenosine bound/mg of membrane protein. The lower affinity adenosine binding sites display an apparent \(K_d\) of 9.5 \(\pm\) 2 \(\times\) 10\(^{-4}\) M and a \(B_m\) of 1700 \pm 300 pmol of adenosine bound/mg of membrane protein. The low binding affinity of this population explains the inability of 1 mM adenosine to saturate these sites and suggests these sites have little physiological relevance.

The identity of the radioactivity bound by the fat cell membranes was determined. Under the standard assay conditions using 100 nM \(^{3}H\)adenosine, at least 80% of the bound radioactivity was identified as adenosine (Table II). Using the two solvent systems, no radioactivity in the form of inosine or adenine was detected by thin layer chromatography. It was of interest to determine more precisely the subcellular localization of the adenosine binding components of fat cells. Fat cells were isolated and divided into two equal portions. Crude fat cell plasma membranes (similar to those used in the above studies) were prepared from one of these

### Table I

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (\mu)M</th>
<th>Adenosine concentration (nM)</th>
<th>(^{3}H) Adenosine bound mg/mg protein</th>
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<tr>
<td>Experiment A</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Erythro-9-(2-hydroxy-3-nonyl)adenine</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>10</td>
<td>49</td>
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<td></td>
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<td>10</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>10</td>
<td>79</td>
</tr>
<tr>
<td>Deoxycoformycin</td>
<td>1</td>
<td>30</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>30</td>
<td>179</td>
</tr>
<tr>
<td>Deoxycoformycin</td>
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<td>30</td>
<td>210</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>30</td>
<td>205</td>
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</tbody>
</table>

**FIG. 1.** Binding of \(^{3}H\)adenosine to fat cell plasma membranes. Effects of concentration of membrane protein, time, and temperature. A, fat cell plasma membranes (60 to 450 \(\mu\)g of protein) were incubated with 10 nM \(^{3}H\)adenosine in a final volume of 0.2 ml for 10 min at 0°C in the standard assay buffer (10 mM Tris-HCl, pH 7.4, 5 mM MgCl\(_2\), 0.1 mM EHNA). Data points are the mean values of triplicate determinations of a representative experiment.
portions. Highly purified fat cell plasma membranes were prepared from the other portion according to the method of McKeel and Jarett (38). Adenosine binding was then assayed in both this highly purified fraction and the standard crude fat cell plasma membrane preparation. The results of these studies are shown in Table III. Nearly 70% of the total adenosine binding capacity of the crude membrane fraction measured at 0.1 and 1 μM adenosine was retained in the highly purified plasma membranes. A somewhat higher percentage (78%) of adenosine binding measured at 100 μM was recovered in the highly purified fraction. The highly purified fat cell plasma membranes also display two populations of adenosine binding sites with affinities similar to those displayed in Fig. 2 (data not shown). These data demonstrate that adenosine binding components can be identified and are perhaps localized in the plasma membrane of the fat cell.

Dissociation of adenosine bound by fat cell membranes at equilibrium with 30 nm adenosine was examined following a 100-fold dilution (Fig. 3). Dissociation was very rapid when performed at 22°C or 0°C. More than 50% of the bound adenosine dissociated within 50 s following a 100-fold dilution at either temperature. Data obtained from the vacuum filtration assay thus probably underestimate actual binding by approximately 10% since some dissociation is possible during the period of filtration and washing (<5 s). This same technique has been used with fat cell membranes to characterize several other rapidly dissociating radioligands (40, 44). The nonlinearity of the semilogarithmic plot of adenosine dissociation probably reflects dissociation from the two populations of binding sites.

The pH dependence of adenosine binding to fat cell membranes was also examined. Adenosine binding was maximum when performed at pH 7.5, declining sharply at pH regions above 7.5 or below 6.5 (data not shown). Binding assays were therefore, routinely carried out at pH 7.4.

Concentrations of either calcium or magnesium below 200 μM did not appreciably affect the capacity of the fat cell membranes to bind adenosine (data not shown). Concentrations of either divalent cation greater than 1 mM inhibited adenosine binding. 50% inhibition being attained at 15 mM calcium or 30 mM magnesium. Fat cell membranes washed twice with 1 mM ethylenediaminetetraacetic acid (EDTA) and resuspended in 10 μM EDTA demonstrated no reduction in their capacity to bind adenosine (data not shown).

### Table III

| Binding of [3H]adenosine to crude and highly purified plasma membranes prepared from isolated rat fat cells |
|-----------------|-----------------|-----------------|-----------------|
| Plasma membrane type | Total protein yield | [3H]adenosine binding capacity assayed at a final adenosine concentration of: |
|                  | mg | 0.1 μM | 1.0 μM | 100 μM |
| Crude plasma membranes | 22.1 | 8.7 ± 0.8 | 70.3 ± 6.6 | 4020.8 ± 221 |
| Highly purified plasma membranes | 14.4 | 5.9 ± 0.7 | 48.5 ± 3.2 | 3156.0 ± 225 |
| % of adenosine binding recovered in the highly purified plasma membranes | 68% | 69% | 78% |

### Table II

Adenosine binding to fat cell membranes: identification of bound radioactivity

Fat cell membranes (640 μg of membrane protein) were incubated with 100 nM [3H]adenosine and varying amounts of unlabeled adenosine to yield a final adenosine concentration ranging from 0.1 μM to 1.0 mM. Incubations were performed in 10 mM Tris-HCl, pH 7.4, 5 mM MgCl2 containing 0.1 mM EHNA at 4°C for 50 min. Binding was assayed by vacuum filtration on a Whatman GF/C filter. The inset to this figure is a transformation of the data according to the method of Scatchard (43). Bound adenosine (B/F) is expressed in picomoles/mg of membrane protein. Data points are the mean values of triplicate determinations of a representative experiment. B/F, ratio of bound to free.

### Table I

Adenosine Binding to Fat Cell Plasma Membranes 3117

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Bound radioactivity, cpm ( % of label recovered)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-butanol/H2O/glacial acetic acid (50:25:25)</td>
<td>ATP, ADP</td>
</tr>
<tr>
<td>4 (1%)</td>
<td>60 (14%)</td>
</tr>
</tbody>
</table>

* Additional thin layer chromatography performed with 1-butanol:H2O:formic acid (77:10:13) as the developing solvent demonstrated that nearly 100% of the label chromatographed as adenosine with no label detected in the position of adenine.*
Adenosine Binding to Fat Cell Plasma Membranes

Fig. 2. Dissociation of \[^{3}H\]adenosine from fat cell plasma membranes. Fat cell plasma membranes (2.1 mg) were incubated with 30 nM \[^{3}H\]adenosine for 20 min at indicated temperatures in a final volume of 0.5 ml of assay medium. At the end of this incubation period, adenosine binding was assayed in a 0.1-ml aliquot by vacuum filtration on Whatman GF/C filters. Nonspecific binding was determined in the absence of membranes and this value subtracted as background. The incubation mixture was then diluted more than 100-fold with assay medium of corresponding temperature and adenosine binding assayed in 10-ml aliquots at the indicated times. The data represent the mean value of triplicate determinations of a representative experiment.

The effects of various enzymatic and temperature pretreatments on the ability of fat cell membranes to bind adenosine are shown in Table IV. Pretreatment of the membranes with 0.1 mg/ml of trypsin or chymotrypsin for 3 min at 37°C reduced the adenosine binding capacity 40 to 70%. Ovomucoid trypsin inhibitor at 0.16 mg/ml partially reversed this trypsin effect. The inhibitor alone slightly enhanced the binding of adenosine to the membrane, perhaps by inhibiting endogenous protease activity of the membrane preparation. Neuraminidase pretreatment also reduced the adenosine binding capacity of the fat cell membranes, while hyaluronidase was without effect. Exposing the membranes to 50°C or 100°C for 1 min reduced adenosine binding to fat cell membranes approximately 40 and 70%, respectively. These observations suggest binding of adenosine by the fat cell membranes is thermally labile and requires the integrity of a protein component(s) of the membrane and perhaps key neuraminic acid residues.

To test the specificity of the high affinity adenosine binding to fat cell membranes, the binding of \[^{3}H\]adenosine at a concentration of 30 nM was assayed in the presence of various purine derivatives. As shown in Table V, \(N^\circ\)-(phenylisopropyl)adenosine was ineffective as a competitor with adenosine for binding to the membranes, whereas the 9',5'-dideoxyadenosine analog was very potent. The order of effectiveness of the other purine derivative inhibitors was adenine > ATP = ADP > AMP = cyclic AMP = inosine. Increasing the concentration of 5'-AMP from 10 to 100 \(\mu\)M resulted in no significant increase in inhibition. Similarly, increasing the adenosine concentration from 1 to 100 \(\mu\)M only slightly increased the inhibition of \[^{3}H\]adenosine binding to the membranes. These data strongly suggest that the high affinity adenosine binding is not to a single, homogeneous class of binding sites, but more likely represents binding to multiple classes of high affinity sites of differing specificities. GTP had no effect (data not shown).

\(p\)-Nitrobenzylthioguanosine (NBTG) and dipyridamole are potent inhibitors of adenosine uptake in a variety of systems (14, 45-47). Ebert and Schwabe (5) reported that 20 \(\mu\)M dipyridamole almost completely blocked adenosine uptake in rat fat cells. Rosenblit and Levy (48) similarly reported that 30 \(\mu\)M dipyridamole inhibited adenosine uptake in rat fat cells 74%. As shown in Table VI, both NBTG and dipyridamole were poor inhibitors of adenosine binding to fat cell membranes, NBTG, at 100 \(\mu\)M inhibited adenosine binding 27% while dipyridamole, at 100 \(\mu\)M, inhibited adenosine binding only 8%.

The observed antagonism of adenosine by theophylline (33, 34) prompted our examination of the effect of theophylline on the binding of adenosine by the fat cell membranes (Table VI). Theophylline inhibited adenosine binding in a dose-dependent fashion. Binding by fat cell membranes at 30 nM adenosine was inhibited 8% by 0.1 \(\mu\)M theophylline and 23% by 10 \(\mu\)M theophylline. By comparison, 1-methyl-3-isobutyl xanthine was very ineffective as a competitor of adenosine binding to the fat cell membranes.

Carlson (49) originally reported that nicotinic acid, but not nicotinamide, was an effective inhibitor of lipolysis in rat adipose tissue. Allen and Clark (50) reported that 100 \(\mu\)M nicotinic acid inhibited basal, epinephrine-, adrenocorticotropic hormone-, and fluoride-stimulated adenylate cyclase activity of fat cell homogenates. Pereira and Holland (3) reported, in addition, that nicotinamide-adenine dinucleotide

| TABLE IV |

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Binding of [^{3}H]adenosine to fat cell plasma membranes: effect of various enzymatic and temperature pretreatments on subsequent binding of [^{3}H]adenosine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[^{3}H]adenosine bound</td>
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<tr>
<td></td>
<td>fmol/mg protein</td>
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<tr>
<td>None</td>
<td>124</td>
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<tr>
<td>Trypsin (0.1 mg/ml)</td>
<td>73</td>
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<tr>
<td>Chymotrypsin (0.1 mg/ml)</td>
<td>65</td>
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<tr>
<td>Neuraminidase (0.1 mg/ml)</td>
<td>66</td>
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<tr>
<td>Hyaluronidase (0.1 mg/ml)</td>
<td>124</td>
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<tr>
<td>Heating at 50°C for 1 min</td>
<td>56</td>
</tr>
<tr>
<td>Heating at 100°C for 1 min</td>
<td>26</td>
</tr>
<tr>
<td>Incubation at 4°C, no treatment</td>
<td>158</td>
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<tr>
<td>None</td>
<td>79</td>
</tr>
<tr>
<td>Ovomucoid trypsin inhibitor (0.16 mg/ml)</td>
<td>82</td>
</tr>
<tr>
<td>Trypsin (0.1 mg/ml)</td>
<td>22</td>
</tr>
<tr>
<td>Both</td>
<td>67</td>
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</tbody>
</table>
Adenosine Binding to Fat Cell Plasma Membranes

Effects of various purine derivatives on binding of [3H]adenosine to fat cell plasma membranes

Crude fat cell plasma membranes (350 μg) were incubated with 30 nM tritiated adenosine, with or without the indicated concentration of agent, in 0.2 ml of standard assay medium. The incubation was carried out at 4°C for 20 min. Adenosine binding was assayed by vacuum filtration on Whatman GF/C filters. Data are expressed as the mean values ± S.E. for n separate experiments.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration (μM)</th>
<th>% Inhibition</th>
<th>n</th>
</tr>
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<tbody>
<tr>
<td>Adenosine</td>
<td>1</td>
<td>6.1 ± 2.9</td>
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<tr>
<td></td>
<td>10</td>
<td>21.9 ± 3.5</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>49.5 ± 4.8</td>
<td>9</td>
</tr>
<tr>
<td>N'-(Phenylisopropyl)adenosine</td>
<td>1</td>
<td>1.4 ± 1.0</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.3 ± 3.0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2.0 ± 1.4</td>
<td>6</td>
</tr>
<tr>
<td>2',5'-Dideoxyadenosine</td>
<td>1</td>
<td>10.3 ± 5.4</td>
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</tr>
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<td>100</td>
<td>53.8 ± 4.1</td>
<td>4</td>
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<tr>
<td>AMP</td>
<td>1</td>
<td>2.8 ± 1.4</td>
<td>8</td>
</tr>
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<td></td>
<td>10</td>
<td>15.5 ± 5.8</td>
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<td>100</td>
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<td>Cyclic AMP</td>
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</tr>
<tr>
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<td>10</td>
<td>5.5 ± 5.5</td>
<td>4</td>
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<tr>
<td></td>
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<td>17.4 ± 7.8</td>
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<tr>
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<td>6.1 ± 3.2</td>
<td>6</td>
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<tr>
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<td>17.1 ± 2.8</td>
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<tr>
<td></td>
<td>100</td>
<td>43.1 ± 5.1</td>
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<tr>
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<tr>
<td></td>
<td>100</td>
<td>18.5 ± 4.4</td>
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(NAD) inhibited epinephrine-stimulated lipolysis of rat adipose tissue. As shown in Table VI, NAD inhibited the binding of adenosine to the fat cell membranes, whereas nicotinic acid was an ineffective competitor of adenosine binding, as was nicotinamide (data not shown).

The effects of several of these compounds on the low affinity adenosine binding were probed using 1 mM [3H]adenosine to test the specificity of the low affinity binding component (Table VII). 2',5'-Dideoxyadenosine, N'-(phenylisopropyl)adenosine, and theophylline at 10 μM failed to appreciably inhibit adenosine binding to the low affinity sites. A 100-μM concentration of adenosine reduced binding by approximately 20%. However, adenosine inhibition of the high affinity sites probably accounts for most of this inhibition.

We examined the possibility that adenosine may be inhibiting hormone-stimulated cyclic AMP accumulation via modification of the hormone/receptor interaction. β-Adrenergic hormone binding to fat cell membranes, as probed with (−)-[3H]dihydralprenol (37, 51), was not significantly altered by 100 μM adenosine (data not shown). Likewise, neither 10 μM (−)-alprenolol nor 10 μM (−)-isoprotenerol affected [3H]adenosine binding to the fat cell membranes (data not shown).

DISCUSSION

Adenosine regulates adenylate cyclase activity and intracellular cyclic AMP levels in a wide spectrum of tissues and cell types. Specific plasma membrane receptors for adenosine have been postulated as the site of adenosine action in brain slices (9, 11, 52), platelets (14, 22), cultured human astrocytoma and glioma cells (19, 24), isolated bone cells (15), mouse neuroblastoma cells (23), transformed human lung fibroblasts (21), and Leydig tumor cells (27), although studies aimed at direct identification of these sites have not been reported. Plasma membrane adenosine receptors were proposed by Davies (7) as
the site of adenosine action in adipose tissue. A similar proposal was advanced by other groups on the basis of studies performed with isolated fat cells (5, 34). The present study is, to the best of our knowledge, the first report characterizing \( ^1 \)H-adenosine binding to a plasma membrane preparation. The plasma membrane fractions prepared from isolated white fat cells exhibit a capacity to bind adenosine. The binding of adenosine at nanomolar concentrations to these membranes can be assayed by the vacuum filtration technique herein described.

The kinetics of adenosine binding to fat cell plasma membranes was rapid, reaching 50% of the equilibrium level within 1 min at 0°C, 22°C, and 37°C. Adenosine inhibition of norepinephrine-stimulated cyclic AMP accumulation in fat cells is very rapid; if added 1 min after norepinephrine, adenosine reduced cyclic AMP accumulation during the next minute (33). Adenosine has also been shown to act rapidly in cultured neuroblastoma cells (18), isolated bone cells (15), and platelets (14).

Addition of adenosine deaminase to incubated fat cells increased basal cyclic AMP accumulation and potentiated the norepinephrine-stimulated increase in cyclic AMP (53, 54). Near maximal effects of adenosine deaminase on norepinephrine-stimulated cyclic AMP accumulation in fat cells were achieved at approximately 20 s of incubation (33). Inhibitors of adenosine deaminase were required in the present study to detect appreciable adenosine binding to the fat cell plasma membranes. These inhibitors also potentiate adenosine inhibition of norepinephrine-stimulated cyclic AMP accumulation in fat cells (33, 34). A similar observation was reported in lymphocytes, where adenosine deaminase reverses and EHNA potentiates the stimulation of cyclic AMP levels by adenosine (20).

Additional evidence suggesting adenosine exerts its effects via a surface receptor is based on experiments utilizing an adenosine derivative which is too large to penetrate cell membranes (55). Adenosine covalently linked to a water-soluble oligosaccharide, stachyoside, with a molecular weight greater than 1500, has been shown to virtually equipotent to free adenosine in inhibiting cyclic AMP accumulation by rat fat cells (34). Olson et al. (55) first demonstrated the ability of this novel adenosine compound to exert an adenosine-like, dose-dependent coronary vasodilation following intracoronary infusion into dogs.

Dipyridamole is a potent inhibitor of the uptake of \( ^1 \)H-adenosine into rat fat cells (5, 48). Ebert and Schwarz (5) observed that dipyridamole (10 \( \mu \)M) potentiated the ability of adenosine to inhibit lipolysis in fat cells rather than reduce it, although it almost completely blocked the uptake of adenosine into the cells. These investigators concluded that the plasma membrane was probably the site of this action of adenosine in the fat cell. Dipyridamole (40 to 100 \( \mu \)M) likewise failed to block the reduction by adenosine of norepinephrine-stimulated cyclic AMP accumulation (53). Neither dipyridamole nor NBTG, another potent inhibitor of adenosine uptake (14, 46), were effective inhibitors of adenosine binding to fat cell membranes.

The binding of \( ^1 \)H-adenosine to fat cell plasma membranes was inhibited by ATP, ADP, and 5'-AMP. Dole (1) originally reported that addition of ATP, 5'-AMP, or adenosine (at 0.8 to 4 \( \mu \)M) to incubated adipose tissue inhibited the lipolytic action of epinephrine. Kappeler (6) demonstrated that adenosine, 5'-AMP, ADP, and ATP inhibited glucagon-stimulated lipolysis in adipose tissue; half-maximal inhibition by adenosine was obtained between 10 and 100 \( \mu \)M. Pereira and Holland (3) reported a 44% inhibition of norepinephrine-stimulated lipolysis in adipose tissue by 1 \( \mu \)M adenosine and a similar level of inhibition by 1 to 10 \( \mu \)M ATP, ADP, or 5'-AMP. Adenosine (1 \( \mu \)M) produced an 80% inhibition of norepinephrine-stimulated cyclic AMP accumulation in the presence of theophylline (53). Dilute fat cell suspensions were more sensitive to inhibition of norepinephrine-stimulated cyclic AMP accumulation by adenosine and by adenosine-containing compounds (8). In this system, 0.1 \( \mu \)M adenosine almost completely inhibited the cyclic AMP accumulation due to norepinephrine and adenosine-containing compounds displayed the following order of potency: adenosine > 5'-AMP > ADP > ATP (8). How much of the inhibition of both \( ^1 \)H-adenosine binding and hormone-stimulated lipolysis and cyclic AMP accumulation by these adenosine-containing compounds is due to adenosine released via their metabolism has not been established.

Adenosine was a very potent inhibitor of adenosine binding to fat cell membranes (Table V). From a structural standpoint, this observation is certainly not unexpected. Dole (1) reported early that unlike adenosine, adenosine enhanced epinephrine-stimulated lipolysis in adipose tissue. Davies (7) subsequently reported a 71% stimulation of norepinephrine-induced lipolysis in adipose tissue by 0.4 \( \mu \)M adenosine. In fat cells, significant elevation of theophylline-induced lipolysis was demonstrated with 1 \( \mu \)M adenosine (5). In contrast to the situation in fat cells, cyclic AMP levels of cultured mouse neuroblastoma cells were stimulated by adenosine and inhibited by adenosine (18). 2-Chloroadenosine stimulation of adenylate cyclase in mouse neuroblastoma preparations was actually antagonized by adenosine (25). Likewise, bone cell adenylate cyclase is stimulated by adenosine and inhibited by adenosine (25). Adenosine at both 18 and 73 \( \mu \)M, however, had no effect on norepinephrine-stimulated adenylate cyclase activity of fat cell ghosts (4).

Fain et al. (4, 56) reported that 5 to 10 \( \mu \)M 2',5'-dideoxyadenosine inhibited norepinephrine-stimulated cyclic AMP accumulation in fat cells approximately 50%. Fifty percent inhibition of norepinephrine-stimulated adenylate cyclase activity in fat cell ghosts was obtained with 5 \( \mu \)M 2',5'-dideoxyadenosine (4). This adenosine analog was a potent inhibitor of \( ^1 \)H-adenosine binding in fat cell plasma membranes (Table V). 2',5'-Dideoxyadenosine has also been shown to be a potent inhibitor of both glucagon-stimulated adenylate cyclase activity in rat liver membranes (28, 31) and glucagon- and epinephrine-stimulated cyclic AMP accumulation in intact hepatocytes (29).

\( N^\circ - (\text{Phenylisopropyl}) \text{adenosine}, which cannot be deaminated by adenosine deaminase, was shown by Fain (53) to be the most potent adenosine analog tested with respect to inhibition of cyclic AMP accumulation in intact fat cells. Cyclic AMP accumulation and lipolysis due to 0.1 \( \mu \)M norepinephrine in the presence of adenosine deaminase was almost completely blocked by 0.01 \( \mu \)M \( N^\circ - (\text{Phenylisopropyl}) \text{adenosine} (56). However, this adenosine analog, even at 73 \( \mu \)M, was virtually without effect on adenylate cyclase activity of fat cell ghosts (4). \( N^\circ - (\text{Phenylisopropyl}) \text{adenosine} failed to inhibit adenosine binding to the fat cell membranes (Table VI).}

Why \( N^\circ - (\text{Phenylisopropyl}) \text{adenosine}, the most potent inhibitor of norepinephrine-stimulated lipolysis and cyclic AMP accumulation in the intact cells, is so ineffective as an inhibitor of both adenylate cyclase and adenosine binding remains obscure. Perhaps the protocols used to prepare the fat cell ghosts and membranes alter, in a critical fashion, the character of the site increasing its affinity for 2',5'-dideoxyadenosine.
while simultaneously reducing its affinity for \( N^\prime \)-(phenylisopropyl)adenosine. Blume and Foster (23) reported the selective loss of 2-chloroadenosine-stimulatable adenylyl cyclase activity of neuroblastoma cells following fractionation, although basal cyclase activity did not change. Peté et al. (26) similarly noted that purified membrane fractions prepared from neuroblastoma cells displayed a marked reduction in adenylyl cyclase responsiveness to the stimulatory action of adenosine. Alternatively, \( N^\prime \)-(phenylisopropyl)adenosine and 2,5'-dideoxyadenosine could exert their influence on cyclic AMP accumulation in fat cells via separate distinct sites (56).

Another common observation concerning adenosine action and mammalian cell cyclic AMP metabolism is that regardless of whether adenosine stimulates (9, 12, 14, 18, 19) or inhibits (33, 34) the accumulation of cyclic AMP, methylxanthines appear to antagonize its action in these systems. In rat fat cells, theophylline inhibits uptake of tritiated adenosine and opposes the action of adenosine (5, 48, 56). The present studies demonstrate that theophylline inhibits adenosine binding to fat cell membranes. Significant inhibition of adenosine binding was detected with 0.1 \( \mu M \) theophylline, a concentration of theophylline too low to inhibit cyclic AMP phosphodiesterase (57).

Fain et al. (56) found 1-methyl-3-isobutyl xanthine to be more potent than theophylline in inhibiting adenosine uptake in the intact fat cell. The present study shows that theophylline inhibits adenosine binding to fat cell membranes. In contrast, 1-methyl-3-isobutyl xanthine failed to inhibit adenosine binding even at 100 \( \mu M \). These data suggest that inhibitors of adenosine uptake of intact fat cells, such as dipryridamole and methylxanthines, are generally poor inhibitors of adenosine binding. Although theophylline does inhibit \([H] \)-adenosine binding, this effect is probably not related to the effect of theophylline on adenosine uptake. Unlike adenosine uptake, inhibition of adenosine binding by theophylline appears to be specific for this methylxanthine. McKenzie et al. (58) recently similarly reported that the ability of adenosine to relax intestinal smooth muscle is antagonized by theophylline but not 1-methyl-3-isobutyl xanthine.

The present study demonstrates that highly purified plasma membranes prepared from fat cells do possess adenosine binding sites. This fat cell plasma membrane preparation was previously shown to be enriched in both adenylyl cyclase activity (38) and \( \beta \)-adrenergic receptors (37). Since adenosine antagonizes the action of catecholamines on lipolysis and cyclic AMP accumulation in intact fat cells, it was of interest to explore the possibility that adenosine might inhibit or modify catecholamine binding to its putative \( \beta \)-adrenergic receptor. Adenosine possessed no significant ability to alter catecholamine interaction with its receptor as probed with (\( \beta \))-\([H] \)-dihydroalprenolol. \( \beta \)-Adrenergic agonists and antagonists had no effect on \([H] \)-adenosine binding to the fat cell membranes.

It is interesting to speculate on the function of these adenosine binding components identified in the fat cell plasma membrane preparations. The inability of dipryridamole to effectively compete for these binding sites with adenosine would argue against these sites being involved in adenosine uptake. The required presence of EHNA or deoxycoformycin to demonstrate adenosine binding would similarly rule out adenosine deaminase. Several characteristics of the binding sites suggest these sites may be involved in the regulation of adenylyl cyclase activity. Adenosine has been shown to be an inhibitor of fat cell cyclic AMP phosphodiesterase activity (4, 7). The action of adenosine in fat cells is to inhibit, rather than potentiate, cyclic AMP accumulation arguing against this enzyme being the binding site. In addition, 1-methyl-3-isobutyl xanthine, one of the most potent phosphodiesterase inhibitors, failed to compete with adenosine for these sites.

Perhaps some of these adenosine binding components are regulatory sites on the adenylate cyclase complex which modulate the activity of this enzyme. This scheme is only speculative, however, and the precise identity and function of these adenosine binding components remains to be established. Rosenblit and Levy (48) have recently approached this same problem by using a photoactive derivative of adenosine, 8-azido2-H-adenosine. Photolysis of this derivative with intact fat cells led to its incorporation into several protein components of the membrane (48). However, the specificity of this labeling technique was not addressed in this report (48).

**REFERENCES**

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