PHOTOCATALYZED LABELING OF ADIPOCYTE PLASMA MEMBRANES WITH 8-AZIDOADENOSINE

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SUMMARY: In an effort to investigate adenosine binding components on plasma membranes of intact adipocytes, a photoreactive derivative of adenosine, (2-^{3}H)-8-azidoadenosine, has been synthesized. Photolysis of the adenosine derivative in the presence of intact adipocytes results in the incorporation of radioactivity into several protein components of the plasma membrane. In the absence of light, 8-azidoadenosine specifically inhibits the net uptake of adenosine by intact adipocytes. In addition, adenosine, other nucleosides and nucleoside transport inhibitors affect the net uptake of 8-azidoadenosine. These studies suggest that one or more of the labeled proteins may be functionally involved in the adenosine transport system.

INTRODUCTION

Adenosine interacts with the plasma membranes of a variety of cells. The transport of this nucleoside has been shown to be effected by a facilitated diffusion mechanism (1,2). In addition, there appear to be other functional interaction sites associated with the surface membrane (3,4). The identification of specific functional components of plasma membranes has been the subject of a myriad of studies. The technique of photoaffinity labeling has been utilized to specifically label several functional binding sites on plasma membranes, including those for acetylcholine (5), glucagon (6), epidermal growth factor (7), concanavalin A (8), glucose (9), adenosine triphosphate (10), cyclic 3',5'-adenosine

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monophosphate (11), local anesthetics (12) and anions (13). In this study we have utilized this technique with an azide derivative of adenosine in an attempt to characterize the adenosine binding components of adipocyte plasma membranes and their functional role in the adenosine transport system.

MATERIALS AND METHODS

Nucleosides were obtained from Sigma Chemical Co.; (2-^{3}H) adenosine was obtained from New England Nuclear; (8-^{3}H)adenosine, 3-O-(^{3}H)methylglucose and (4,5-^{3}H)leucine were obtained from ICN Pharmaceuticals. Dipyridamole was a gift from Boehringer Ingelheim, Ltd., Elmsford, New York, 10523. All other chemicals were of reagent grade.

(2-^{3}H)-8-Bromoadenosine was synthesized essentially according to the procedure of Ikehara and Uesugi (14). This product was converted to (2-^{3}H)-8-azidoadenosine by a modification of the method of Holmes and Robbins (15). All procedures were performed in the absence of bright light. Most of the radioactivity (97%) ran as a single spot and co-chromatographed with cold 8-azidoadenosine (R_f of 0.79) on silica gel plates developed in chloroform/methanol/water (65/25/4). Irradiation of the radioactive derivative resulted in photo-products which also co-chromatographed with those obtained from the cold nucleoside. The synthesis of (2-^{3}H)-8-azidoadenosine from (2-^{3}H)adenosine afforded an overall yield of 40%.

Intact adipocytes were prepared by the method of Rodbell (16) from the epididymal fat pads of male Sprague-Dawley rats (160-200gm). Fat cell number was determined by counting in a hemocytometer. Plasma membranes were prepared by the procedures of McKeel and Jarett (17) as modified by Carter et al. (18).

The photolysis apparatus consisted of a General Electric medium pressure 400 watt mercury arc lamp located approximately 4 cm from a 100 ml Kontes (pyrex) water-jacketed vessel. The reaction tube containing intact adipocytes in Krebs-Ringer bicarbonate buffer (pH 7.4) containing 1% bovine serum albumin was flushed with 95% O_2/5% CO_2 and capped prior to irradiation. Cells were photolyzed for 10 min at 37°C with stirring. Following photolysis, the cells were washed, homogenized, and the plasma membranes isolated on a linear sucrose graident.

Isolated plasma membranes were subjected to sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis according to the procedure of Gurd et al. (19). Gels were stained for protein with Coomassie brilliant blue and for carbohydrate with the periodic acid-Schiff reagent, according to Fairbanks et al. (20). Molecular weight determinations were derived from parallel calibrated gels utilizing ribonuclease, β-lactoglobulin, chymotrysinogen A, aldolase, ovalbumin, bovine serum albumin, transferrin and phosphorylase a as standard marker proteins. Protein was determined by the method of Lowry et al. (21) as modified by Hartree (22), using bovine serum albumin as a standard. The distribution of

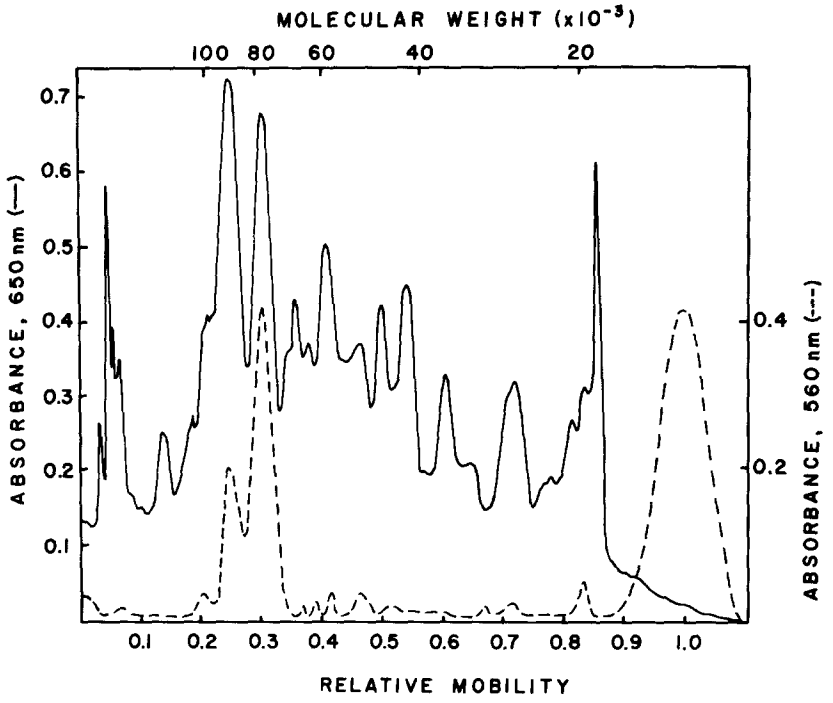

Fig. 1. Densitometer scans of sodium dodecyl sulfate-10% polyacrylamide gels of adipocyte plasma membranes (180 µg) stained for protein with Coomassie Blue (————) and glycoprotein with the periodic acid-Schiff reagent (------). Bromphenol blue was used as the tracking dye.

tritium in the gel was determined by digestion and counting of 1mm gel slices in a scintillation cocktail containing 25% Triton X-100 and 0.4% Butyl-PBD fluoroalloy in toluene.

Uptake studies were performed with duplicate samples containing 2-4 x 10^5 cells/ml at 37° C for 2 min with stirring in Krebs-Ringer bicarbonate buffer (pH 7.4) containing 1% bovine serum albumin in a total volume of 0.71 ml. Reactions were terminated by the rapid oil centrifugation technique of Gammeltoft and Gliemann (23). Packed cells which floated through the dinonyl phthalate were transferred to vials containing scintillation cocktail and counted.

RESULTS AND DISCUSSION

Plasma membranes of intact adipocytes were isolated and analyzed by electrophoresis on sodium dodecyl sulfate-10% polyacrylamide gels. Gels were stained for protein with Coomassie Blue and for glycoprotein with the periodic acid-Schiff reagent. This procedure indi-

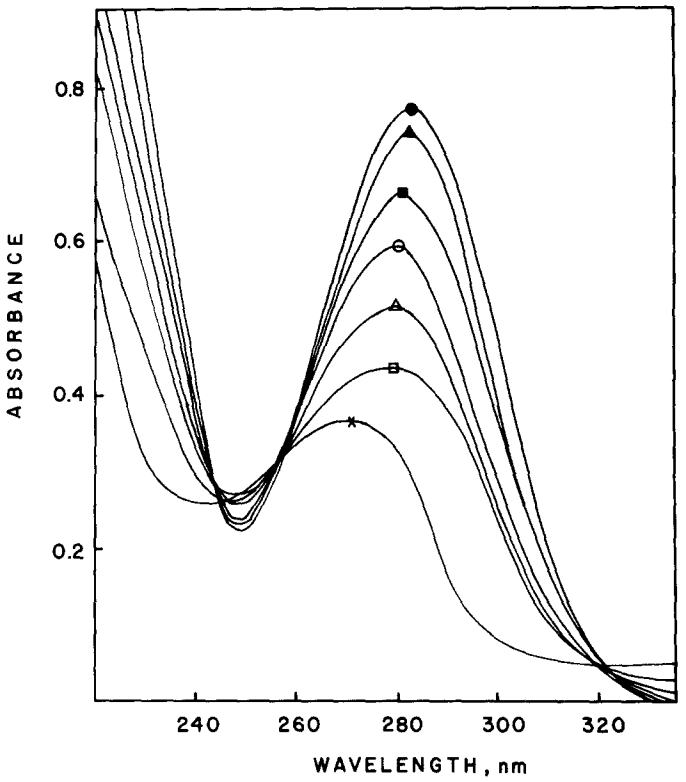

Fig. 2. Absorption spectra of 8-azidoadenosine irradiated in Krebs-Ringer phosphate buffer, pH 7.4, with light greater than 300 nm at 37° C for varying time periods. (●), unirradiated; (▲), 10 sec; (■), 20 sec; (○), 40 sec; (△), 60 sec; (□), 2 min; and (×), 10 min after irradiation.

cates the presence of more than 25 protein components with apparent molecular weights ranging from 18,000 to 180,000. Staining with the Schiff reagent reveals the presence of two major glycoproteins with apparent molecular weights of 78,000 and 94,000, several minor glycoproteins and substantial glycolipid. A densitometer scan of these gels is shown in Fig. 1. Analysis of the appropriate enzymatic markers as previously described (24) showed the membrane preparation to be 85% pure.

A solution of 8-azidoadenosine (5×10^{-5} M) in Krebs-Ringer phosphate buffer (pH 7.4) was irradiated with light greater than

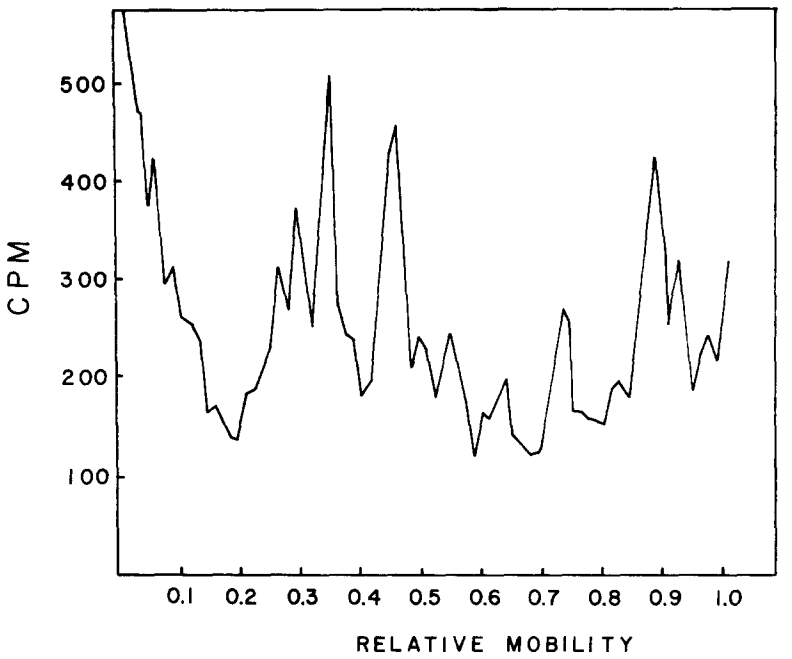

Fig. 3. Sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis of isolated plasma membranes (180 µg) isolated from intact adipocytes (6×10^6 cells) which have been photolyzed in the presence of (2-^{3}H)-8-azidoadenosine (500 µCi; 8.0 µM; 12.3 Ci/mmol).

300 nm for varying periods of time. Analysis of the ultraviolet spectrum suggested that complete photolysis was effected in 10 min, as shown in Fig. 2. A 10 min irradiation period was thus used for the adipocyte labeling studies. An analysis of the Coomassie Blue staining patterns of sodium dodecyl sulfate-polyacrylamide gels of isolated plasma membranes obtained from irradiated and unirradiated adipocytes suggested that the irradiation procedure had no effect on the membrane protein components.

Fig. 3 illustrates a representative sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of isolated plasma membranes obtained following irradiation of (2-^{3}H)-8-azidoadenosine (500 µCi, 8.0 µM, 12.3 Ci/mmole) in the presence of intact adipocytes. Photolysis results in the labeling of several membrane

components. The covalent incorporation of radioactivity is dependent upon photolysis of (2-^{3}H)-8-azidoadenosine, since incubation of unirradiated reagent with isolated plasma membranes does not result in the labeling of any membrane proteins. Furthermore, photolysis must be performed in the presence of plasma membranes since the mixing of preirradiated (2-^{3}H)-8-azidoadenosine with plasma membranes also does not result in the labeling of membrane proteins.

Several potential adenosine binding components exist in the plasma membrane. In addition to a transport system for this nucleoside (1,2), adenosine appears to interact with other membrane associated receptors and/or enzymes. A number of enzymes including adenosine deaminase, phosphoribosyltransferase, and nucleoside phosphorylase have been found to copurify with the plasma membrane fraction of some cell types (3). Adenosine has been shown to be a potent inhibitor of phosphodiesterase (25) and adenylate cyclase (26,27) of adipocytes. A binding site on the exterior surface of the adipocyte has been suggested to explain the facilitating effect of adenosine on the action of insulin (4).

In order to ascertain if 8-azidoadenosine interacts with the adenosine transport system, its effect on the net uptake of adenosine by adipocytes was investigated in the absence of light. Fig. 4 illustrates the effect of increasing concentrations of 8-azidoadenosine on the net uptake of (8-^{3}H)adenosine (1 μM), (4,5-^{3}H)leucine (1 μM) and 3-O-(^{3}H)methylglucose (1 μM). A pronounced inhibitory effect on adenosine uptake is observed at low concentrations of 8-azidoadenosine with a maximum inhibition of 56%. The inhibition appears to be specific since the uptake of leucine and 3-O-methylglucose is uneffected by the presence of 8-azidoadenosine.

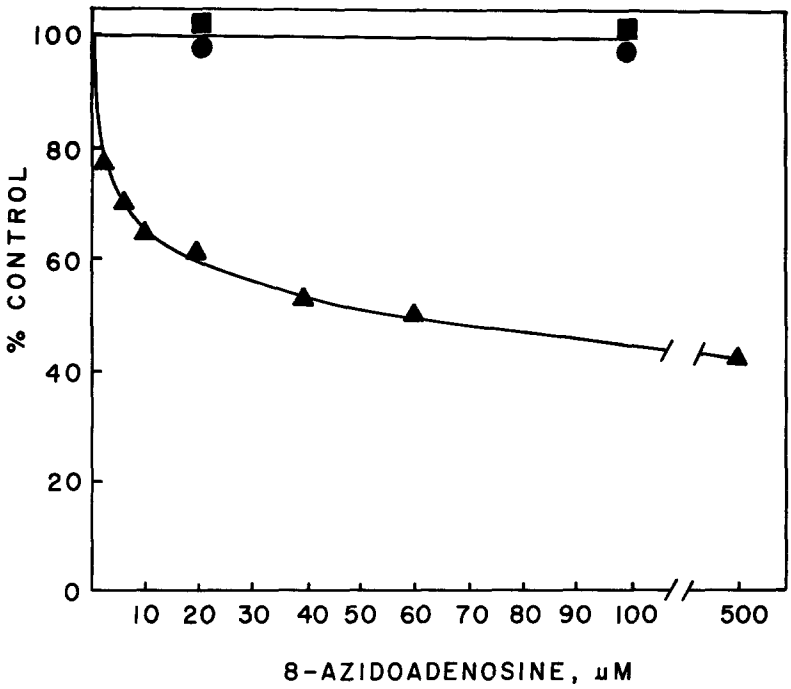

Fig. 4. Effect of 8-azidoadenosine on the net uptake of (8-^{3}H) adenosine (▲), 4,5-(^{3}H)leucine (■) and 3-O-(^{3}H)methylglucose (●). Uptake studies were performed as described in Materials and Methods. 8-Azidoadenosine was incubated with cells at 37°C for 1 min with stirring prior to the addition of the tritiated substrates.

Table I illustrates the effects of a number of nucleosides and nucleoside transport inhibitors on the net uptake of (8-^{3}H) adenosine and (2-^{3}H)-8-azidoadenosine. Of particular significance is the observation that 8-azidoadenosine inhibits the uptake of adenosine and adenosine inhibits the uptake of 8-azidoadenosine, suggesting that the azide analog may utilize the adenosine transport system. A number of chemical agents such as theophylline, dipyridamole and colchicine have been shown to inhibit nucleoside transport (1,2) without affecting intracellular nucleoside metabolism. These compounds were found in this study to inhibit the uptake of both adenosine and 8-azidoadenosine in the adipocyte system.

Table I

EFFECTS OF SELECTED NUCLEOSIDES AND VARIOUS NUCLEOSIDE TRANSPORT INHIBITORS ON THE NET UPTAKE OF (8-^{3}H)ADENOSINE AND (2-^{3}H)-8-AZIDOADENOSINE

Inhibitor	Percent of Control	
	(8-^{3}H)Adenosine (1 μM)	(2-^{3}H)-8-Azidoadenosine (0.5 μM)
Adenosine, 500μM	-	44
Inosine, 500μM	28	62
Guanosine, 500μM	49	76
Uridine, 500μM	36	62
8-Azidoadenosine, 500μM	43	-
Theophylline, 6.25mM	12	29
Dipyridamole, 30μM	26	38
Colchicine, 60μM	73	84

Adipocyte suspensions (2-4 x 10^5 cells) were incubated with the selected inhibitors for 1 min at 37°C with stirring prior to the addition of (8-^{3}H)adenosine or (2-^{3}H)-8-azidoadenosine. The uptake was performed as described in Materials and Methods. Results are expressed as the percent of counts taken up by intact adipocytes to which no inhibitor was added.

The above inhibiton studies of nucleoside uptake suggest the possibility that one or more of the labeled membrane components (Fig. 2) may be functionally involved in the adenosine transport system. Further analysis of the functional specificity of (2-^{3}H)-8-azidoadenosine labeling of intact adipocyte plasma membranes is now in progress.

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