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Interactions Between Adenosine and α_1-Adrenergic Agonists in Regulation of Respiration in Hamster Brown Adipocytes

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SUMMARY

Respiration in brown adipocytes can be increased by β-adrenergic receptor agonists or by α_1-adrenergic receptor agonists (phenylephrine and norepinephrine). Previous studies have shown that β receptor-stimulated respiration is inhibited by adenosine and that enzymatic removal of adenosine produced by fat cells under normal incubation conditions enhances the respiratory response to β receptor activation. The present experiments were performed to determine the effect of adenosine on the respiratory response elicited by agonists of α_1 receptors. The α-adrenergic agonists phenylephrine and norepinephrine (in the presence of the β-adrenergic antagonist propranolol) stimulated respiration and the respiratory response to each agent was enhanced when endogenous adenosine was removed with adenosine deaminase. Addition of hydrolysis-resistant analogues of adenosine inhibited phenylephrine-stimulated respiration, and, since N^6-phenylisopropyladenosine was more effective than was 5′-*N*-ethylcarboxamidoadenosine, we conclude that an A1 receptor is involved. In contrast, the P site agonist 2′,5′-dideoxyadenosine did not inhibit phenylephrine-stimulated respiration but did cause some inhibition of isoproterenol-stimulated respiration. These results suggest that adenosine, acting via A1 receptors, modulates α_1-adrenergic effects on thermogenesis in brown fat cells, an action that is analogous to its inhibition of β-adrenergic receptor-stimulated thermogenesis.

Adenosine has been found to be an important regulator of metabolism in white fat cells (1). Among the more prominent actions of the nucleoside on these cells is its ability to attenuate adenylate cyclase (1, 2) and thereby inhibit a variety of metabolic activities, such as triglyceride hydrolysis, which are dependent upon cellular levels of cyclic AMP (3–5). Moreover, the nucleoside is formed within fat cells (5, 6) and released into the extracellular environment. The regulatory role of this endogenous adenosine has been investigated in studies using adenosine deaminase or methyl xanthines which block adenosine-receptor interactions. Results from such investigations (5, 7–10) have found that either enzymatic removal of adenosine or blockade of its receptors with methyl xanthines promotes cyclic AMP formation and activates cyclic AMP-dependent processes in white fat cells. These and related findings have led to the view that adipocyte adenylate cyclase is under a tonic inhibition exerted by endogenously formed adenosine (8–10).

While most studies have addressed adenosine regulation of cell responses dependent upon cyclic AMP, adenosine is also capable of influencing cell function through mechanisms apparently unrelated to cyclic AMP. Many of these other actions of adenosine are insulin-like and include activation of a low K_m phosphodiesterase (11), stimulation of glucose metabolism (12–14), and activation of mitochondrial pyruvate dehydrogenase (14). Adenosine is also capable of potentiating insulin actions on white fat cells (12).

The actions of adenosine on brown adipose tissue have been investigated in much less detail than the actions on white fat cells. Brown adipose tissue serves a different physiologic function than does white adipose tissue; the physiologic function of this tissue is metabolic heat production, and this activity is stimulated by catecholamines (15). Thermogenesis in brown fat cells results from the presence in the mitochondria of a specific regulated proton conductance pathway (16). Increases in the proton permeability loosens the coupling between oxidative phosphorylation and electron transport and mitochondrial respiration and heat production increase (16). β-Adrenergic agonists stimulate thermogenesis in brown adipocytes, and this response is generally thought to result from activation of adenylate cyclase and a cyclic AMP-dependent activation of triglyceride hydrolysis (15–17). Respiration increases as a consequence of the interaction of fatty acids with the mitochondrial proton leak pathway. Not surprisingly, β-adrenergic stimulated respiration is antagonized by stable adenosine analogues and

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ABBREVIATIONS: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DDA, 2′,5′-dideoxyadenosine; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; NECA, 5′-*N*-ethylcarboxamidoadenosine; N_i, the guanyl nucleotide-binding protein mediating inhibitory regulation of adenylate cyclase; PIA, *N*-phenylisopropyladenosine; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

enhanced by the enzymatic removal of endogenous adenosine (18–20), and these effects of the nucleoside are secondary to attenuation of adenylate cyclase activity (18, 21).

Brown fat cells possess α_1-adrenergic receptors, and their selective activation is also capable of stimulating respiration (17, 22–25). The α-adrenergic response is obtained with phenylephrine (in the presence of propranolol), is not associated with activation of adenylate cyclase (22, 23), but is associated with a breakdown of phosphoinositides (25) and accumulation of inositol phosphates (26) and with increased calcium efflux from the cells (27). The mechanisms whereby the presumed elevation of intracellular calcium is translated to an increase in respiration are not known. In a previous study (28), we reported that the respiratory response of isolated brown fat cells to selective α_1 receptor activation with phenylephrine could be partially inhibited by 2-chloroadenosine. In the present communication, we have investigated this phenomenon in greater detail. We now report that brown adipocytes secrete sufficient endogenous adenosine to severely limit the extent of α_1-adrenergic-stimulated respiration and that removal of endogenous adenosine greatly augments the respiratory response to selective α_1 receptor activation. It is also shown that adenosine exerts its control over α_1 receptor-stimulated respiration via an A1-type adenosine receptor and that its inhibitory effect is blocked by pertussis toxin.

Materials and Methods

Preparation of brown adipocytes. Male hamsters weighing between 60 and 80 g were purchased from Charles River, Lakeview, NJ, and housed in a climate-regulated vivarium for at least 1 week before use. The animals were anesthetized by intraperitoneal injection of sodium pentobarbital, and the interscapsular and cervical brown fat depots were excised. The white adipose and connective tissues were removed and the brown fat was minced with scissors for 5 min. Adipocytes were prepared from the minced tissue by digestion with crude bacterial collagenase (2.0 mg/ml) for 12 min in the presence of 0.33 mg/ml soybean trypsin inhibitor. The buffer used was (in mM): 115 NaCl, 1.0 $CaCl_2$, 15 $NaHCO_3$, 4.6 KCl, 1.4 $MgSO_4$, and 30 HEPES adjusted to pH 7.4 after addition of 40 mg/ml bovine serum albumin. Following collagenase treatment, the digested tissue was filtered through nylon mesh and the adipocytes were washed with buffer of identical composition. An aliquot of the final cell suspension was stained with acridine orange and cell number was determined using a hemocytometer.

Measurement of respiration, lipolysis, and inositol phosphates. Oxygen consumption was measured polarigraphically at 37° using a YSI model 53 oxygen meter equipped with a YSI model 5331 oxygen probe (22). Approximately 50,000 cells were present in the respiratory chambers which contained 3.0 ml of buffer. In some experiments, an Instech (Instech Laboratories, Horsham, PA) model 600B oxygen chamber system fitted with a YSI model 125/05 electrode was used. The volume contained by this chamber was 0.60 ml and approximately 15,000 cells were used. The electrical output from the electrodes was visualized on Kipp and Zonen chart recorders. Glucose, fructose, and pyruvate (10 mM, 10 mM, 1.0 mM) were present in all media to optimize the respiratory responses (22) and were also present for those studies measuring lipolysis and accumulation of inositol phosphates. Lipolysis was measured as glycerol production during an incubation lasting for 30 min. The glycerol content of the media was measured as described (5). For measurement of inositol phosphates, phospholipids were labeled during a cell incubation with [^{3}H]inositol (30 μCi/ml) for 2–3 hr. Cell suspensions were then reincubated with norepinephrine. The incubations were terminated by addition of cold trichloroacetic acid, and inositol phosphates were separated by anion exchange chromatography as described (29).

Respiration experiments were conducted as follows. The cells were introduced into the chamber in buffer containing glucose, fructose, and pyruvate. Basal oxygen consumption was then measured for 3–5 min at which time phenylephrine, isoproterenol, norepinephrine, adenosine deaminase, or stable analogues of adenosine were injected using a Hamilton syringe. Phenylephrine was used at a concentration of 10 μM, which has been found to be maximally effective at promoting respiration and turnover of phospholipids (23). PIA and NECA were dissolved in ethanol and further diluted in saline. Injections of an equivalent volume of diluent were never observed to affect respiration. Adenosine deaminase, as a suspension in $(NH_4)_2SO_4$, was diluted into saline on the day of the experiment and used without purification. Unless noted otherwise, propranolol was present at 1.0 μM to block the β-adrenergic activity of the phenylephrine. The "effective stimulation times" presented are estimates of the lag (hysteresis) in the time course of phenylephrine action and were calculated as the time from addition of the phenylephrine to the respiratory chamber to the intersection of lines extrapolated from basal and maximally stimulated respiration.

Membrane preparation and toxin labeling. Brown adipocyte membranes were prepared (18, 21) from the interscapular and cervical depots following disruption in a Polytron homogenizer (Brinkman Instruments) using three pulses, each lasting for 10 sec, at a setting of 5.5 and five passes of the crude homogenate in a Potter-Elvehjem homogenizer equipped with a Teflon pestle. The homogenization medium was Tris-EDTA (25 mM; 1.0 mM), pH 7.4 with 250 mM sucrose. The membranes were stored at −70° in Tris-EDTA at a protein concentration of 1.0 mg/ml until use. The membranes (150 μg of protein) were treated with pertussis toxin (1.0 μg) for 30 min in 0.10 ml of a solution consisting of 25 mM Tris (pH, 7.4), 10 mM thymidine, 10 mM dithiothreitol, 1.0 mM ATP, 15 μM NAD, 1 mg/ml digitonin, 1 mg/ml ovalbumin, 0.10 mM GTP, and 6 μCi [^{32}P]NAD. The pertussis toxin was preactivated by incubation with 25 mM dithiothreitol for 10 min at 37°. The ribosylation reaction was terminated by addition of 1 ml of a solution of 25 mM Tris, 1.0 mM EDTA, and 75 mM NAD. The membranes were collected by centrifugation at 15,000 ×g for 30 min and solubilized in 0.050 ml of Tris-SDS prior to analysis by SDS-polyacrylamine gel electrophoresis. Autoradiography was generally for 4 days in the presence of an enhancing screen.

Chemicals. Unless stated otherwise, chemicals and materials were purchased from standard sources. The bovine serum albumin (fraction V) and PIA were from Boehringer-Mannheim; collagenase, type I, was from Cooper Biomedical; NECA, adenosine deaminase (type VIII; 247 units/mg), soybean trypsin inhibitor, phenylephrine, norepinephrine, and isoproterenol were from Sigma; forskolin was from Calbiochem-Behring; DDA was from P&L Biochemicals; propranolol was from Ayerst; pertussis toxin was from List Biologicals; EHNA was from Burroughs-Wellcome; and [^{3}H]inositol was from New England Nuclear. Prazosin was a gift from Pfizer.

Results

A previous study (28) found that 2-chloroadenosine could inhibit respiration in isolated brown adipocytes stimulated with phenylephrine in the presence of propranolol. Since brown adipocytes release adenosine into the extracellular environment (18), we wished to determine how the respiratory response to α_1 receptor activation would be affected by incubation conditions that prevented the accumulation of adenosine. The stimulation of brown adipocytes with a maximally effective concentration of phenylephrine under conditions which permitted the accumulation of endogenous adenosine produced a less than 2-fold increase in oxygen consumption. When adenosine accumulation was prevented by addition of adenosine deaminase, the respiratory response was dramatically enhanced becoming

nearly 3-fold greater than basal (Fig. 1). By itself, however, adenosine deaminase had no significant effect on oxygen consumption, an observation reported previously by us (18) and others (19, 20). Results from other studies have found that adenosine deaminase potentiates the lipolytic and respiratory responses of white and brown adipocytes to β-adrenergic agonists by decreasing the EC_{50} values while not altering the maximum responses (3, 5, 7, 20). In contrast, the sensitivity of brown adipocytes to phenylephrine, as indicated by the concentration needed to produce a half-maximal activation of respiration, was decreased only slightly by adenosine deaminase (EC_{50} values: control, 0.90 ± 0.07 M; adenosine deaminase, 1.0 g/ml, 0.75 ± 0.06 M). It appears, therefore, that adenosine deaminase potentiation of phenylephrine-stimulated respiration results more from an increase in the maximum respiratory response than from a change in cell sensitivity.

The respiratory response to α receptor activation appears to have a detectable lag period before a steady rate of stimulated oxygen uptake is achieved. When adenosine deaminase was present, however, the respiratory response to phenylephrine appeared to develop more rapidly. The lag period in phenylephrine action was estimated, as described in Materials and Methods, and found to decrease from a value of 1.11 ± 0.18 min when adenosine deaminase was not present to a value of 0.68 ± 0.06 min when adenosine deaminase was added.

Because brown adipocyte respiration can be increased by activation of either β- or α_1-adrenergic receptors, and since phenylephrine has the potential of interacting with β-adrenergic receptors, we considered the possibility that potentiation of phenylephrine-stimulated respiration with adenosine deaminase could have resulted from magnification of any β-adrenergic activity of this agent which persisted in the presence of 1.0 μM propranolol. Several types of experiments were conducted in order to test this possibility. In the experiment depicted in Fig. 2, we determined the effects of adenosine deaminase on phenylephrine-stimulated respiration in the presence of higher concentrations of propranolol. Phenylephrine-stimulated respiration was decreased by 1 μM propranolol, but increasing the concentration of the blocker to 10 μM produced only a small further decrease. Adenosine deaminase potentiated phenylephrine-stimulated respiration, and the degree of potentiation was similar at 1.0 μM and 10.0 μM propranolol (Fig. 2, *top panel*).

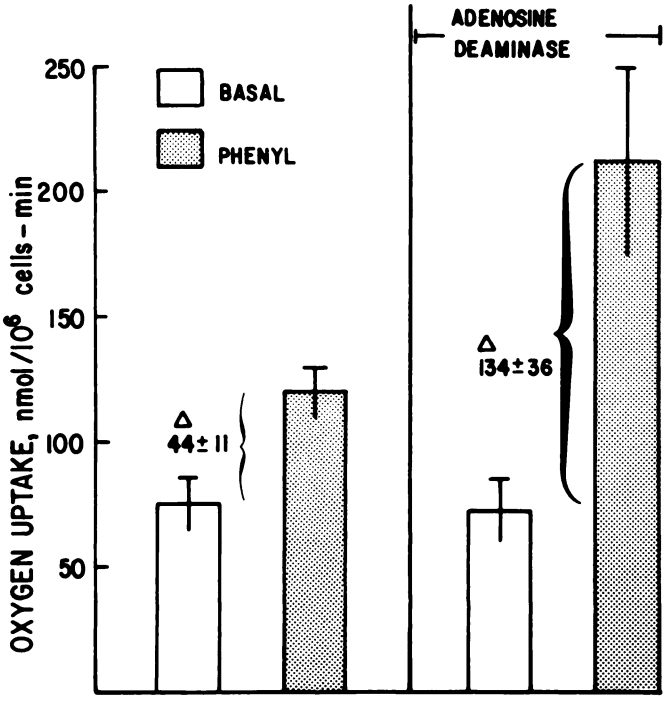

Fig. 1. Potentiation of phenylephrine-stimulated respiration by adenosine deaminase. Brown adipocytes were prepared as described and introduced into the respiratory chamber in 3.0 ml of buffer containing 10 mM glucose, 10 mM fructose, 1.0 mM pyruvate, and 1.0 μM propranolol. Adenosine deaminase was present at a final concentration of 1 μg/ml. Phenylephrine was added to a final concentration of 10 μM. The results are the means and standard errors of five individual experiments. Each experiment had two to five replicate determinations for each point.

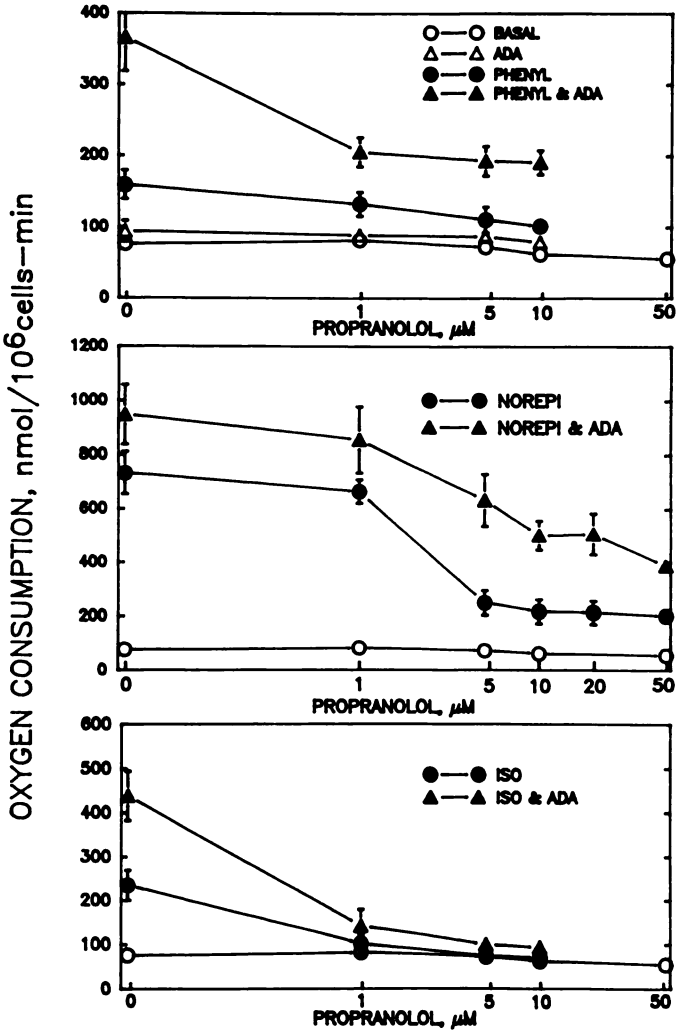

Fig. 2. Effect of propranolol on phenylephrine-, norepinephrine-, and isoproterenol-stimulated respiration. Brown adipocytes were prepared as described and introduced into the respiratory chamber in buffer containing 10 mM glucose, 10 mM fructose, and 1.0 mM pyruvate and the indicated concentration of propranolol. Respiration was stimulated by administration of either phenylephrine (*top panel*) (10 μM), norepinephrine (*middle panel*) (0.50 μM), or isoproterenol (*bottom panel*) (5 nM). Adenosine deaminase (*ADA*) was present where indicated at a final concentration of 1 μg/ml. Unstimulated respiration (basal) and respiration measured after addition of adenosine deaminase are shown in the *top panel* but only basal lipolysis is represented in the *middle* and *lower panels*. The results are the means and standard errors of 10 experiments using phenylephrine and isoproterenol and 5 experiments using norepinephrine; each experiment had two to five replicate determinations for each point.

In contrast, when isoproterenol was used to stimulate respiration to an extent comparable to that observed with phenylephrine, addition of 1.0 μM propranolol caused almost a complete inhibition of the respiratory response (Fig. 2, *bottom panel*).

Also shown in Fig. 2 (*middle panel*) are data obtained using norepinephrine as the α-adrenergic ligand. The concentration of norepinephrine used in this study (0.50 μM) was selected because it was found to be the lowest concentration capable of activating α_1-adrenergic receptors, as judged from its ability to promote accumulation of inositol phosphates (Table 1). Norepinephrine-stimulated respiration was partially, but not completely, inhibited by addition of propranolol. Because norepinephrine is a more potent β-adrenergic agonist than is phenylephrine, the concentration of propranolol was increased to 50 μM, and approximately 25% of norepinephrine-stimulated respiration persisted in the presence of this high concentration of propranolol. Addition of adenosine deaminase enhanced norepinephrine-stimulated respiration, and this potentiation was evident even in the presence of 50 μM propranolol. In experiments conducted in parallel, the ability of norepinephrine to stimulate lipolysis was measured to assess the completeness of propranolol blockade of norepinephrine's β-adrenergic activity. In contrast to respiration, norepinephrine activation of lipolysis was completely blocked by propranolol (Table 2). The portion of norepinephrine-stimulated respiration not blocked by propranolol has been shown to result from the activation of α-adrenergic pathways (17, 22, 23) and, as was found when phenylephrine was used, this α-adrenergic component is enhanced when endogenous adenosine is removed.

We (22) and others (17, 23) have reported that the respiratory response to phenylephrine was blocked by the selective α_1-adrenergic receptor blocking drug prazosin. The data presented in Table 3 show that prazosin blockade of phenylephrine-stimulated respiration is still evident when adenosine deaminase is present, providing additional support for the participation of α receptors in this response. Since unpurified preparations of adenosine deaminase were used in these studies, experiments were performed to provide assurance that the observed effects were due to removal of adenosine and not to another constituent of the enzyme preparation. Introduction of $(NH_4)_2SO_4$ into the incubation media to produce a concentration identical to that obtained when adenosine deaminase was used had no effect on phenylephrine (Table 3)- or isoproterenol (not presented)-stimulated respiration. The enhancement of phenylephrine-stimulated respiration with adenosine deaminase was not observed when the inhibitor of adenosine deaminase, EHNA, was added to the respiratory chambers before adenosine deaminase (Table 3).

In another experiment, we tested the effect of the adenylate cyclase inhibitor DDA on respiration (Table 4). If phenylephrine-stimulated respiration results from β-adrenergic receptor-mediated activation of adenylate cyclase, then DDA should cause some inhibition of the response to phenylephrine. Exposure of adipocytes to DDA caused some inhibition of isoproterenol-stimulated respiration but failed to inhibit respiration stimulated with phenylephrine; actually, DDA produced a slight, but not statistically significant, increase in phenylephrine-stimulated respiration. In another experiment, we measured the rates of lipolysis in brown fat cells incubated with adenosine deaminase, seeking some indication of adenylate cyclase activation by phenylephrine (Table 5). However, evidence for phenylephrine stimulation of lipolysis was not obtained. Since one action of forskolin is to augment receptor-mediated stimulation of adenylate cyclases (30), we looked for evidence of phenylephrine stimulation of lipolysis in cells incubated with submaximal concentrations of forskolin. The

TABLE 1

Accumulation of inositol phosphates in norepinephrine-stimulated brown adipocytes

Brown adipocytes were preincubated for 2–3 hr with 30 μCi/ml [^{3}H]inositol at 37° in media containing glucose (10 mM), fructose (10 mM), and pyruvate (1.0 mM). Following preincubation, the adipocytes were diluted with fresh media to produce a cell density of 800,000–1,500,000 cells/ml, and propranolol was added to a final concentration of 10 μM; immediately thereafter, norepinephrine was added to yield the final concentrations indicated. The adipocytes were then reincubated at 37° for 1 min at which time the incubation was terminated by addition of ice-cold trichloroacetic acid to a final concentration of 15%. Inositol phosphates (inositol phosphate, IP; inositol diphosphate, IP_2; inositol triphosphate, IP_3) were measured following ether extraction of the trichloroacetic acid by anion exchange chromatography as described. Each data point presented is the mean and standard error of four individual experiments.

[Norepinephrine]	Inositol phosphate accumulation		
	IP	IP_2	IP_3
μM	*cpm/10^6 cells*		
0	862 ± 89	229 ± 31	168 ± 21
0.10	819 ± 111	206 ± 30	167 ± 11
0.50	979 ± 72	271 ± 18[a]	233 ± 30[a]
1.0	1019 ± 131[a]	404 ± 39[a]	483 ± 41[a]
5.0	1159 ± 97[a]	493 ± 52[a]	527 ± 53[a]
10.0	1116 ± 142[a]	529 ± 58[a]	497 ± 49[a]

[a] Significantly greater than control, $p < 0.05$ by paired Student's t test.

TABLE 2

Blockade of norepinephrine-stimulated lipolysis by propranolol

Brown adipocytes were incubated for 30 min in media containing glucose (10 mM), fructose (10 mM), and pyruvate (1.0 mM). Norepinephrine was present at 0.50 μM and adenosine deaminase at 1.0 μg/ml. Unstimulated lipolysis was measured to 308 ± 41 nmol of glycerol/10^6 cells-30 min in the absence of adenosine deaminase and 282 ± 48 nmol glycerol/10^6 cells-30 min in the presence of 1 μg/ml adenosine deaminase. Each value is the mean ± standard error of four individual experiments.

[Propranolol]	Glycerol production	
	Norepinephrine	Norepinephrine + adenosine deaminase
μM	*nmol/10^6 cells-30 min*	
0	944 ± 111	1016 ± 130
1.0	713 ± 89	799 ± 98
5.0	596 ± 87	632 ± 96
10.0	391 ± 45	418 ± 54
50.0	282 ± 22	271 ± 30

TABLE 3

Effects of ammonium sulfate, prazosin, and EHNA on phenylephrine stimulation of brown adipocyte respiration

Brown adipocytes were placed in the respiratory chamber in 3.0 ml of buffer containing 10 mM glucose, 10 mM fructose, 1 mM pyruvate, and 1.0 μM propranolol. Phenylephrine was present where indicated at a final concentration of 10 μM. Each value is the mean ± standard error of five individual experiments.

Additions	Oxygen consumption	
	Control	Phenylephrine
	nmol/10^6 cells-min	
None	78.6 ± 7.5	113.8 ± 15
NH_4SO_4, 0.32 mM	66.0 ± 69	115.0 ± 8.3
Adenosine deaminase 1 μg/ml		266.0 ± 31
Adenosine deaminase + Prazosin, 1.0 μM	ND[a]	69.8 ± 12
Adenosine deaminase + EHNA, 50 μM	78.4 ± 7.7	112.0 ± 7.3

[a] ND, not determined.

TABLE 4

Effect of DDA on phenylephrine- and isoproterenol-stimulated respiration

Brown adipocytes were placed in the respiratory chamber in 3.0 ml of buffer containing 10 mM glucose, 10 mM fructose, 1 mM pyruvate, and 1 μg/ml adenosine deaminase. Propranolol was present at 1 μM when phenylephrine was used. Basal respiration was measured for 2–4 min at which time phenylephrine or isoproterenol was added. Approximately 5 min later, DDA was injected into the respiratory chambers at the indicated concentrations. Each value is the mean ± standard error of five individual experiments.

	Oxygen consumption
	nmol/10⁶ cells-min
Basal	122 ± 23
DDA, 100 μM	111 ± 12
Phenylephrine, 10 μM	305 ± 39
Phenylephrine, 10 μM + DDA, 50 μM	392 ± 78
Phenylephrine, 10 μM + DDA, 100 μM	445 ± 83
Isoproterenol, 5 nM	518 ± 71
Isoproterenol, 5 nM + DDA, 50 μM	403 ± 62[a]

[a] Significantly less than value obtained with isoproterenol, $p < 0.05$ by paired Student's *t* test.

TABLE 5

Phenylephrine does not stimulate lipolysis in the presence of adenosine deaminase

Brown adipocytes were incubated for 30 min in media containing glucose (10 mM), fructose (10 mM), pyruvate (1 mM), propranolol (1.0 μM), and adenosine deaminase (1 μg/ml). Phenylephrine, forskolin, and isoproterenol were present at the indicated concentrations. Each value is the mean ± standard error of eight individual experiments.

	Glycerol production
	nmol/10⁶ cells-30 min
Basal	281 ± 22
Phenylephrine, 10 μM	293 ± 17
Forskolin, 0.50 μM	509 ± 64
Forskolin + phenylephrine	559 ± 79
Isoproterenol, 20 nM	777 ± 66

results of that study also showed no indication of phenylephrine activation of lipolysis.

The objective of our next experiment was to characterize the receptor mediating adenosine inhibition of phenylephrine-stimulated respiration as being either an A1 or an A2 site. Purine receptors inhibitory to adenylate cyclase, termed A1 sites, are characterized pharmacologically by N^6 derivatives of adenosine such as PIA being more potent than are N^5 derivatives such as NECA (2), and circumstantial evidence for the presence of such a receptor on brown adipocytes has been presented (18, 20). The data in Fig. 3 show that, whereas PIA and NECA both inhibited phenylephrine-stimulated respiration, PIA was considerably more effective than was NECA, suggesting that adenosine A1 receptors serve to mediate this response. In this study, adenosine deaminase was present to exclude any contribution from the endogenous adenosine.

Our last experiment sought to reexamine the effects of pertussis toxin on adenosine inhibition of phenylephrine-stimulated respiration. Our previous study (28) found adenosine inhibition of phenylephrine-stimulated respiration to persist following treatment of the adipocytes with pertussis toxin. However, membranes prepared from cells exposed to the toxin still showed labeling of a 41-kDa peptide upon incubation with pertussis toxin and [^{32}P]NAD (21). It follows that these proteins available for pertussis toxin-catalyzed labeling could have mediated adenosine inhibition of phenylephrine-stimulated

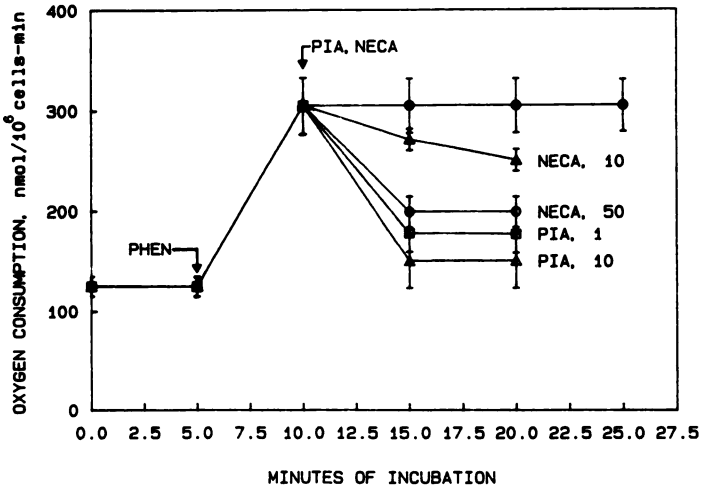

Fig. 3. Inhibition of phenylephrine-stimulated respiration by analogues of adenosine. Brown adipocyte respiration was stimulated with phenylephrine (10 μM) in the presence of propranolol (1.0 μM) and adenosine deaminase (1.0 μg/ml). PIA or NECA was then added to yield the final concentrations, in nM, indicated by the numbers to the *right* of each line. Each data point is the mean and standard error of five individual experiments.

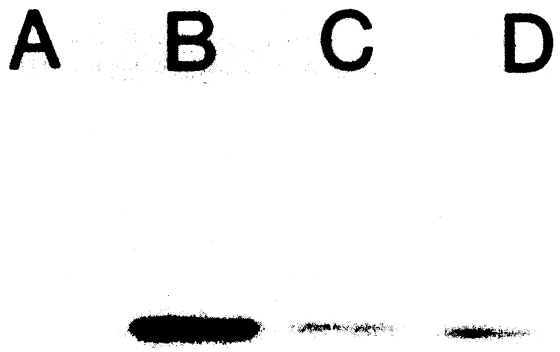

Fig. 4. Autoradiogram of an SDS-polyacrylamide gel of membrane proteins following pertussis toxin-induced ribosylation of brown adipose membranes from untreated and pertussis toxin-treated hamsters. Membranes (150 μg) were incubated with [^{32}P]NAD and preactivated pertussis toxin as described. Lane *A*, membranes from hamsters given 10 μg of pertussis toxin. Lane *B*, membranes from untreated hamsters; lane *C*, membranes from hamsters given 5.0 μg of pertussis toxin; lane *D*, membranes from hamsters given 1.0 μg of pertussis toxin.

respiration. In the present study, adipocytes were harvested from hamsters treated (intraperitoneal injection) with 1.0, 5.0, or 10.0 μg of pertussis toxin and sacrificed 3 days later. Plasma membranes were prepared for estimation of the status of N_i by measurement of *in vitro* ribosylation with [^{32}P]NAD (Fig. 4). Incubating membranes with pertussis toxin caused the incorporation of radiolabel into a single protein band having an apparent molecular weight of 40,000–42,000. Membranes prepared from hamsters given 10 μg of pertussis toxin showed virtually no protein labeling by pertussis toxin, suggesting that this treatment regimen had effectively modified all of the N_i

molecules present in the cell membrane. In contrast, membranes from hamsters treated with 1 or 5 μg of pertussis toxin still displayed a considerable amount of labeling of the protein substrate.

Adipocytes were isolated and stimulated with either isoproterenol or phenylephrine (Table 6). The ability of adenosine deaminase to enhance the respiratory responses to isoproterenol or phenylephrine was used as evidence for the presence of an adenosine inhibition of stimulated respiration. Both isoproterenol- and phenylephrine-stimulated respiration is increased by adenosine deaminase, and this phenomenon was observed in cells from hamsters given 1.0 μg of pertussis toxin. When cells from animals given 5.0 g of pertussis toxin were used, a different picture emerged. The respiratory response to isoproterenol was greater than that measured in cells from untreated animals and from animals given 1.0 μg of pertussis toxin and was not potentiated by adenosine deaminase. The absence of adenosine deaminase potentiation of isoproterenol-stimulated respiration indicated that adenosine regulation of adenylate cyclase had been blocked by treatment with pertussis toxin. In contrast, when the respiratory stimulus was provided by phenylephrine, adenosine deaminase still enhanced the response, indicating that adenosine regulation of phenylephrine-stimulated respiration was present. When cells from hamsters given 10 μg of pertussis toxin were studied, however, a large response to phenylephrine was observed, and this response was no longer potentiated by adenosine deaminase.

Discussion

There is no doubt that one important action of adenosine on both white and brown adipocytes is to modify cellular responses to activation of β-adrenergic receptors and that this function is subserved by an inhibitory action on the adenylate cyclase complex. Adipocytes, however, can also be stimulated through mechanisms initiated by α_1-adrenergic receptors and, in many instances, these responses are qualitatively similar to those observed following β receptor activation [e.g., activation of glycogen phosphorylase (31, 32) and increased brown adipocyte thermogenesis (17, 22–24)]. The salient observation reported in the present communication is that one such response to selective α_1-adrenergic receptor activation, namely, increased thermogenesis in brown adipocytes, is modulated by adenosine in a fashion analogous to its inhibition of β-adrenergic-stimulated thermogenesis in these cells. Since PIA was a more potent inhibitor than NECA, it would appear that the receptor mediating this response can be characterized as being A1, the same designation given to adenosine receptors that inhibit adenylate cyclase (2).

TABLE 6

Effect of pertussis toxin on adenosine deaminase potentiation of isoproterenol- or phenylephrine-stimulated respiration

Brown adipocytes from hamsters treated with varying doses of pertussis toxin were prepared as described and introduced into the respiratory chamber in 3.0 ml of buffer containing 10 mM glucose, 10 mM fructose, and 1.0 mM pyruvate, and 1.0 μM propranolol. Adenosine deaminase (ADA) was present where indicated at a final concentration of 1 μg/ml. Phenylephrine was added to a final concentration of 10 μM and isoproterenol to a final concentration of 5 nM. Basal (unstimulated) respiration was 122 ± 17 nmol/O_2/10^6 cells-min. Each value is the mean ± standard error of five individual experiments.

Pertussis toxin	Oxygen consumption			
	Phenylephrine		Isoproterenol	
	−	+ ADA	−	+ ADA
μg/hamster	nmol/10^6 cells-min			
0	171 ± 19	285 ± 31	222 ± 41	421 ± 52
1	168 ± 21	309 ± 19	241 ± 29	405 ± 39
5	198 ± 27	322 ± 44	395 ± 53	441 ± 43
10	302 ± 44	327 ± 31		

Consideration was given to an artifact which could have been responsible for our findings. The potentiation of phenylephrine-stimulated respiration with adenosine deaminase does not appear to result from an uncovering and magnification of the β-adrenergic activity of this agent. First, adenosine deaminase potentiation of phenylephrine-stimulated respiration was evident in the presence of concentrations of propranolol which completely blocked isoproterenol-stimulated respiration. Second, measurement of lipolysis, an indicator of cell cyclic AMP levels (1, 3, 5, 8, 9), did not provide evidence of phenylephrine stimulation, even when forskolin was present to enhance any adenylate cyclase-stimulatory activity of the α-adrenergic agent (30). Third, the adenylate cyclase inhibitor DDA failed to reduce phenylephrine-stimulated respiration, whereas it did attenuate respiration stimulated by the more potent β-adrenergic agonist isoproterenol. Finally, findings similar to those made with phenylephrine were obtained in experiments using norepinephrine as the α-adrenergic agent. In this case, blockade of the β-adrenergic activity of norepinephrine with a very high concentration (50 μM) of propranolol left a portion of catecholamine-stimulated respiration, attributable to α_1 receptor-activated mechanisms (17, 22, 23), that was enhanced by addition of adenosine deaminase. Taken together, these data are not consistent with the possibility that adenosine regulation of phenylephrine respiration results from a primary action of the nucleoside on adenylate cyclase but, instead, point to a separate action of adenosine on one or more reactions of the α_1 receptor transduction system.

Adenosine inhibition of phenylephrine-stimulated respiration was found to be blocked by pertussis toxin, thereby implicating N_i or another pertussis toxin substrate in this response. This result is not surprising in view of reports of pertussis toxin blockade of adenylate cyclase-unrelated actions of other agents that are capable of inhibiting adenylate cyclases (33, 34). Blockade of adenosine inhibition of phenylephrine-stimulated respiration with pertussis toxin, however, was not evident until virtually all of the N_i molecules present in the cell membrane were modified by the toxin, a finding that explains why treatment of isolated adipocytes with pertussis toxin, which did not allow for complete intoxication of N_i (21), did not block adenosine inhibition of phenylephrine-stimulated respiration (28). Curiously, complete blockade of adenosine inhibition of isoproternol-stimulated respiration was evident following toxin treatments which do not inactivate all of the N_i molecules present in the membrane. If modulation of adenylate cyclase activity with adenosine underlies inhibition of β receptor-stimulated respiration, then our findings would suggest that inhibitory regulation of adenylate cyclase requires more functional N_i molecules than does inhibitory regulation of the α-adrenergic transduction system.

The major question raised by this study concerns the mechanisms whereby adenosine can inhibit phenylephrine action. Adenosine inhibits isoproterenol-stimulated respiration by interfering with receptor activation of adenylate cyclase (18–21), and an attractive possibility is that adenosine exerts a similar

inhibitory affect on the α_1-adrenergic transduction system. Our present data imply that both actions of adenosine are mediated by a common receptor and coupling protein. Since phenylephrine-stimulated respiration is accompanied by a breakdown of phosphoinositides (25, 26), adenosine inhibition of respiration could result from an inhibition of receptor coupling to phosphoinositide metabolism. Support for this possibility comes from studies, reported in abstract form (35), showing that the phenylephrine-stimulated breakdown of phosphatidylinositol 4,5-diphosphate, as measured by the decline in radioactivity associated with this lipid in cells prelabeled with [^{32}P] P_i, is attenuated when adenosine is present. In another study (36), we observed a greater phenylephrine-stimulated phosphoinositide breakdown to occur in cells from hamsters treated with 10 μg of pertussis toxin than in cells from untreated hamsters. Since this treatment regimen with pertussis toxin blocks adenosine inhibition of phenylephrine-stimulated respiration, the greater breakdown of phosphoinositides following pertussis toxin treatment suggests that endogenous adenosine may restrain phenylephrine activation of phospholipase C. The presence of receptor-linked inhibitory regulation of phosphoinositide breakdown has been demonstrated in studies on isolated pituitary cells. In this system dopamine inhibits hormone secretion stimulated by agents that enhance breakdown of phosphoinositides, and this action of dopamine is correlated with a partial inhibition of inositol phosphate accumulation (37). Adenosine, like dopamine, can inhibit pituitary secretion, but its mechanism of action has yet to be explored (38).

Adenosine deaminase potentiation of β and α receptor-initiated respiration is not strictly analogous. The predominant effect of the enzyme on β-adrenergic receptor-stimulated lipolysis and respiration is a reduction in the EC_{50} while the V_{max} of phenylephrine-stimulated respiration is increased by adenosine deaminase. The V_{max} of β receptor-stimulated lipolysis or respiration is not altered by adenosine deaminase because sufficient cyclic AMP can be formed in the presence of adenosine to fully activate the cyclic AMP-dependent protein kinase (9). Finding adenosine deaminase potentiation of maximally effective concentrations of phenylephrine implies that a cellular process linking α receptors with mitochondrial respiration cannot be fully activated in the presence of adenosine. It follows that understanding the apparent change in V_{max} of phenylephrine-stimulated respiration must await elucidation of the cellular-biochemical events which link α_1 receptors to increased respiration.

Recent studies (8, 9) on white adipocytes have emphasized that the basal or quiescent state of these cells results from the presence of endogenous adenosine in the extracellular environment. Mere removal of that adenosine, without addition of a stimulatory ligand, is sufficient for activation of cyclic AMP accumulation and lipolysis. It has been suggested (8, 9) that the considerable variability in results obtained using white cell preparations results from variations in adenosine production among different cell preparations and that obtaining consistent data using adipocyte preparations may require control of extracellular adenosine. Since adenosine is also capable of influencing adipocyte responses to α_1-adrenergic agents, perhaps control of ambient adenosine may be equally important for elimination of some variability present in brown adipocyte preparations.

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