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Evidence for *Alpha* Adrenergic Activation of Phosphorylase and Inactivation of Glycogen Synthase in Rat Adipocytes

Effects of *Alpha* and *Beta* Adrenergic Agonists and Antagonists on Glycogen Synthase and Phosphorylase

JOHN C. LAWRENCE, JR.,[1] AND JOSEPH LARNER

University of Virginia School of Medicine, Department of Pharmacology, Charlottesville, Virginia 22903

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SUMMARY

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The effects of *alpha* and *beta* adrenergic agonists and antagonists on the percentages of phosphorylase *a* and glycogen synthase I activities were investigated in isolated rat adipocytes. (−)-Epinephrine, (−)-norepinephrine, and (−)-isoproterenol increased the concentration of adenosine cyclic 3′,5′-monophosphate (cAMP) and the percentage of phosphorylase *a* activity in a dose-dependent manner. Isoproterenol was about 10 times more potent than epinephrine or norepinephrine with respect to these increases as well as to decreases in the percentage of glycogen synthase I activity. Although all three agents decreased synthase I activity to the same extent, the maximal effects of epinephrine and norepinephrine on phosphorylase *a* activity were approximately 25% greater than the maximal effect of isoproterenol. In the presence of the *alpha* adrenergic antagonists phentolamine, phenoxybenzamine, and dihydroergotamine, the maximal effect of norepinephrine on increasing the percentage of phosphorylase *a* was reduced to that of isoproterenol. All three *alpha* adrenergic antagonists potentiated the ability of norepinephrine to increase the concentration of cAMP. The maximal effect of (−)-phenylephrine on phosphorylase *a* activity was less than the maximal effect of isoproterenol. When cells were incubated with isoproterenol plus phenylephrine, phosphorylase *a* activity was increased to levels observed with epinephrine or norepinephrine. Methoxamine also increased phosphorylase *a* activity, and the effects of isoproterenol and methoxamine on phosphorylase were additive. Incubation of cells with 5 mM dibutyryl cAMP decreased synthase I activity and increased phosphorylase *a* activity to the level observed with isoproterenol. When dibutyryl cAMP was added to cells together with isoproterenol or epinephrine, no further increase in phosphorylase *a* activity over that obtained with the catecholamines alone was observed. However, when cells were incubated with phenylephrine plus dibutyryl cAMP, the percentage of phosphorylase *a* activity was increased to that observed with epinephrine. (−)-Propranolol completely blocked the rise in cAMP observed following incubation of cells with

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[1] Predoctoral Fellow in the Department of Pharmacology, University of Virginia School of Medicine.

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epinephrine, norepinephrine, or phenylephrine; yet an increase in phosphorylase *a* activity and a decrease in synthase I activity were still observed. Incubation of cells with 20 μM propranolol together with 1 μM phenoxybenzamine did not reverse the decrease in synthase I activity produced by 200 milliunits/ml of adrenocorticotrophic hormone, but completely abolished the effect of 200 μM phenylephrine. Incubation of cells with methoxamine (1–10 μM) decreased glycogen synthase I activity. Phentolamine (20 μM) completely blocked this effect of methoxamine, as well as the ability of methoxamine to increase phosphorylase *a* activity.

INTRODUCTION

Recent studies have provided evidence that the catecholamine stimulation of gluconeogenesis and glycogenolysis in rat liver involves mechanisms independent of changes in cAMP[2] (1–7). It now appears that the primary means by which catecholamines activate rat hepatocyte phosphorylase and inactivate glycogen synthase is through *alpha* adrenergic receptor activation (6). Several reports describe effects of catecholamines on rat adipocyte glycogen synthase and phosphorylase (8–12), but the data are not complete enough to characterize the adrenergic receptors responsible for these effects. In this report we present results demonstrating that the rat adipocyte has both *alpha* and *beta* adrenergic receptors and that the activation of either of these receptors leads to an increase in phosphorylase *a* activity and a decrease in glycogen synthase I activity.

MATERIALS AND METHODS

Fat cells were prepared from epididymal adipose tissue of 120–180-g Wistar rats fed ad libitum (13). All incubations of adipocytes were performed using plastic bottles or tubes and a medium composed of Krebs-Ringer-phosphate buffer (128 mM NaCl, 1.4 mM $CaCl_2$, 1.4 mM $MgSO_4$, 5.2 mM KCl, and 10 mM Na_2HPO_4) and 30 mg/ml of bovine serum albumin (fraction V, Sigma, lot 16C-0027). The medium was prepared daily and adjusted to pH 7.4 with NaOH after addition of albumin. Fat pads were cut into small pieces, and 1–3 g of tissue were incubated in 3–8 ml of medium and 1 mg/ml of crude collagenase (*Clostridium histolyticum*, Worthington, lot 45J112X) for 1 hr at 37°. The cells were filtered through two layers of cheesecloth, washed twice with 10 ml of medium, and resuspended in 10 ml of medium per gram of original tissue. Cells were counted in a hemocytometer, and 4–8 × 10^5 cells/ml were used.

Cells were incubated at 37° in 5 ml of medium with additions (20 μl) as indicated. A 1-ml sample of cells was removed for cAMP determination. The remaining cells (in 4 ml) were centrifuged for 15 sec in a clinical centrifuge; after the medium had been removed, the incubation was terminated by adding 0.6 ml of cold buffer (100 mM KF and 10 mM EDTA, pH 7.0). The cells were then homogenized in a chilled glass homogenizer at 0° (10 strokes with a Teflon pestle driven at 1000 rpm). After centrifugation of the homogenates at 10,000 × *g* for 15 min, the supernatants were collected and assayed for glycogen synthase and phosphorylase activities.

The incubation of the 1-ml cell sample was terminated at the same time as the above 4-ml incubation sample by adding 1 ml of 100 mg/ml trichloroacetic acid. The tubes were vigorously agitated for 30 sec, using a Vortex-Genie mechanical mixer, then centrifuged at 10,000 × *g* for 10 min. The supernatants were removed and extracted four times with 3 volumes of water-saturated ether. After a 5-fold dilution, samples (0.5 ml) were acetylated and the cAMP content of the original cell sample was determined by radioimmunoassay using the procedures described by Harper and Brooker (14).

Glycogen synthase was assayed by the incorporation of radioactivity from UDP-[*U*-^{14}C]glucose into glycogen essentially as described by Thomas *et al.* (15). Phosphorylase activity was assayed in the direc-

[2] The abbreviations used are: cAMP, adenosine cyclic 3′,5′-monophosphate; ACTH, adrenocorticotrophic hormone; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

tion of glycogen synthesis from [*U*-^{14}C]glucose-1-P as described by Gilboe *et al.* (16). The experimental conditions used in these assays were those previously described (12). Glycogen synthase I activity is expressed as a percentage of the total synthase activity (assayed in the presence of 7.2 mM glucose-6-P). None of the agents used in this study altered total synthase activity. Phosphorylase *a* activity is expressed as a percentage of the total phosphorylase activity (measured in the presence of 2.0 mM AMP).[3] The activation of phosphorylase was associated with an increase in the total activity similar to that previously observed in rat adipocytes (10) and in a purified preparation of swine phosphorylase (17).

Statistically significant differences in the results were determined using Dunnett's test (18) for comparing multiple treatments with a control, or Tukey's method (19) for comparing differences between treatment groups. Differences at the $p < 0.05$ level were considered significant.

(−)-Epinephrine bitartrate (−)-isoproterenol bitartrate, (−)-norepinephrine bitartrate, (−)-phenylephrine HCl, dihydroergotamine tartrate, N^6,$O^{2'}$-dibutyryl cAMP, ACTH (porcine, 85 units/mg), and rabbit liver glycogen were obtained from Sigma. The glycogen was purified before use by passing a 5% solution over a mixed-bed ion-exchange resin (Amberlite MB-3) as described by Larner *et al.* (20). (−)-Propranolol was a gift from Ayerst Laboratories. Phentolamine HCl was obtained from Ciba-Geigy; phenyoxybenzamine HCl, from Smith Kline & French; and methoxamine, from Burroughs Wellcome. UDP-[*U*-^{14}C]glucose and α-D-[*U*-^{14}C]glucose-1-P were purchased from New England Nuclear.

RESULTS

Khoo *et al.* (10) previously demonstrated that activation of phosphorylase and inactivation of glycogen synthase by epinephrine in rat adipocytes corresponded with increases in cAMP. These observations supported the concept that cAMP mediates the effects of epinephrine on glycogen synthase and phosphorylase. To investigate this relationship further, studies were carried out with three *beta* adrenergic agonists. Figure 1A shows the concentrations of cAMP resulting from a 4.5-min incubation of fat cells with increasing concentrations of (−)-isoproterenol, (−)-epineph-

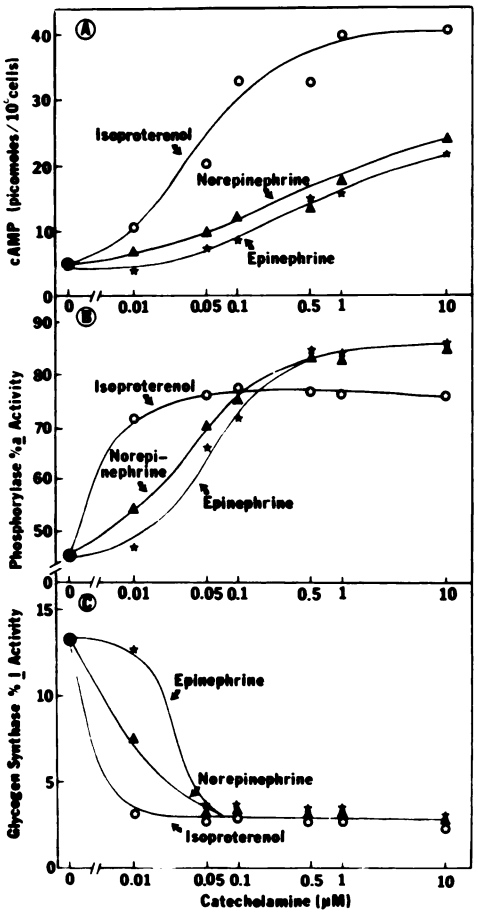

FIG. 1. *Dependence of adipocyte glycogen synthase, phosphorylase, and cAMP on concentrations of adrenergic agonists*

Fat cells were incubated for 4 min at 37° before the indicated concentrations of (−)-isoproterenol, (−)-norepinephrine, or (−)-epinephrine were added. After 4.5 min, the incubations were terminated and glycogen synthase, phosphorylase, and cAMP were assayed as described under MATERIALS AND METHODS.

[3] The percentage of phosphorylase *a* activity is equivalent to 100 times the activity ratio (−AMP:+AMP) used by others (10). Use of the term phosphorylase *a* is not meant to imply a similarity to forms of the enzyme from other tissues.

TABLE 1

Failure of gel filtration to alter percentage of phosphorylase a or glycogen synthase I activity of extracts from cells incubated with norepinephrine and isoproterenol

Adipocytes were incubated in 10 ml of medium without additions or with 10 μM (−)-norepinephrine or 10 μM (−)-isoproterenol for 4.5 min at 37°. Extracts containing glycogen synthase and phosphorylase were prepared after the incubations were terminated by adding 1.2 ml of buffer composed of 100 mM KF and 10 mM EDTA (pH 7.0). Extracts were kept on ice, and AMP (1 mM) was added as indicated. Samples (0.4 ml) of each were filtered through columns (1.1 × 6.5 cm) containing Sephadex G-25 that had been equilibrated with the same buffer. Glycogen synthase and phosphorylase activities in the extracts, as well as the activities eluted in the void volume of the columns, were assayed as described under MATERIALS AND METHODS.

Addition to cells	Addition to extracts	Glycogen synthase I activity		Phosphorylase *a* activity		Total phosphorylase activity recovered from column
		Extract kept on ice	Extract passed through column	Extract kept on ice	Extract passed through column	
		%		%		%
None	None	14.8	13.6	39.5	36.5	77
None	AMP	—[a]	—[a]	71.5	38.9	81
Norepinephrine	None	3.9	3.3	80.1	78.4	84
Isoproterenol	None	3.7	4.1	64.7	65.3	79

[a] Not measured.

rine, and (−)-norepinephrine. In keeping with the findings of Butcher and Sutherland (21) in adipose tissue homogenates, isoproterenol was more potent with respect to increasing cAMP than either norepinephrine or epinephrine, with the latter two agents being approximately equal in potency. As shown in Fig. 1B and C, this order of potency was also maintained for the activation of phosphorylase and the inactivation of glycogen synthase. While higher concentrations of all three agonists equally decreased glycogen synthase I activity, the maximal activation of phosphorylase observed with isoproterenol was less than that observed with either epinephrine or norepinephrine. The difference in maximal effects was not explained by cAMP, because the increase in the cyclic nucleotide observed with isoproterenol (10 μM) was twice that seen with epinephrine (10 μM) or norepinephrine (10 μM).

Stimulation of phosphorylase *b* in the cellular extracts by endogenous AMP or other effectors was considered as an explanation for the greater maximal effect of norepinephrine on phosphorylase. However, separation of small molecules from the enzymes by gel filtration using columns of Sephadex G-25 did not alter the effects of norepinephrine (10 μM) or isoproterenol (10 μM) on either glycogen synthase or phosphorylase (Table 1). The apparent increase in phosphorylase *a* activity produced by adding AMP (1 mM) to an extract from control cells was abolished by the column treatment, demonstrating the effectiveness of the separation. In other experiments, the effects on phosphorylase produced by incubating cells with 10 μM norepinephrine or 10 μM isoproterenol were not altered by a 5-fold dilution of the extracts.[4] These results indicate that the changes in both glycogen synthase and phosphorylase activities due to norepinephrine and isoproterenol reflect stable modifications of the enzymes.

The time courses of phosphorylase activation and glycogen synthase inactivation by 10 μM norepinephrine and 10 μM isoproterenol are shown in Fig. 2B and C. The maximal effects of these agents on both enzymes were observed after a 4.5-min incubation. The percentage of phosphorylase *a* activity was again greater with norepinephrine than with isoproterenol, even though isoproterenol produced

[4] J. C. Lawrence, Jr., and J. Larner, unpublished observations.

higher concentrations of cAMP (Fig. 2A). The difference in the effects of the two catecholamines on phosphorylase was more pronounced after 1 min, but was observed at all incubation intervals tested. Also shown in Fig. 2 are the increase in phosphorylase *a* activity and decrease in synthase I activity produced by 200 μM (−)-phenylephrine, a comparatively pure *alpha* adrenergic agonist.

Incubation of fat cells for 9.5 min with increasing concentrations of (−)-propranolol alone did not detectably alter the concentration of cAMP (Fig. 3A) or the percentages of phosphorylase *a* (Fig. 3B) or glycogen synthase I activities (Fig. 3C). Propranolol (20 μM) completely blocked the increase in cAMP produced by epinephrine or norepinephrine, yet did not abolish the increase in phosphorylase *a* activity observed with these agents. A

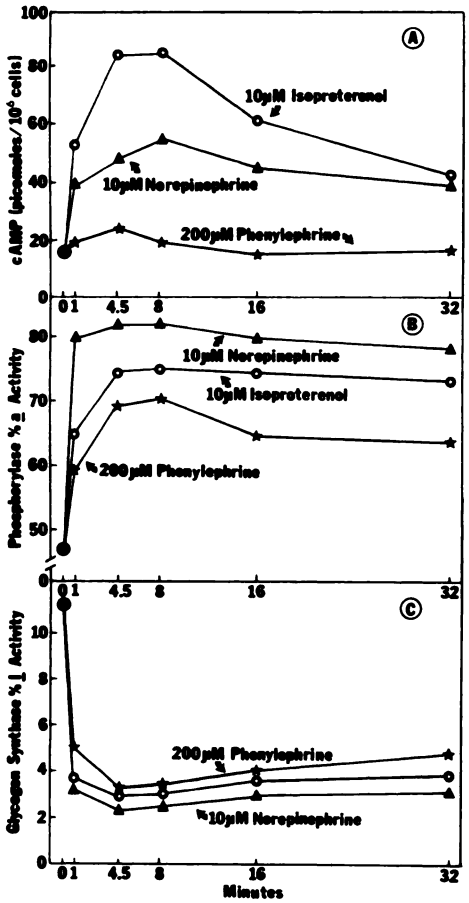

FIG. 2. *Dependence of adipocyte phosphorylase a activity, glycogen synthase I activity, and cAMP on time of incubation with isoproterenol, norepinephrine, and phenylephrine*

Fat cells were incubated at 37° for 32 min, then centrifuged and homogenized. (−)-Isoproterenol (10 μM), (−)-norepinephrine (10 μM), or (−)-phenylephrine (200 μM) was added after the appropriate period of this incubation to obtain the times of exposure indicated below. Enzyme activities and cAMP levels were determined as described under MATERIALS AND METHODS.

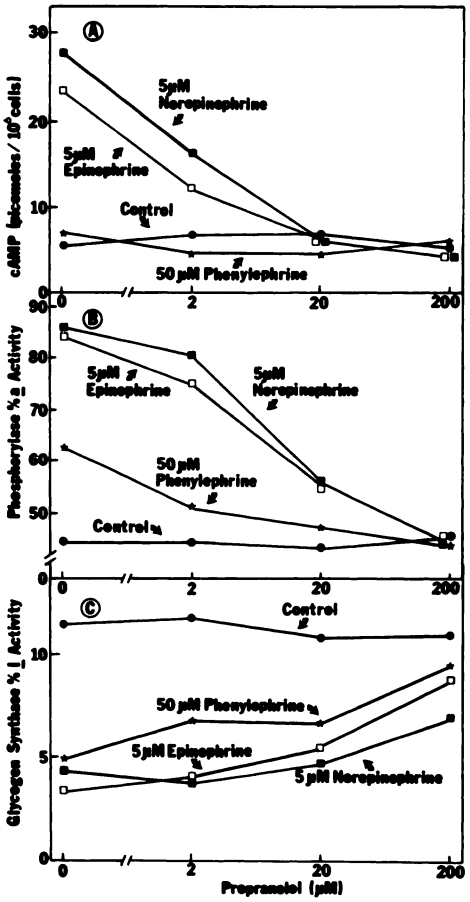

FIG. 3. *Effects of increasing concentrations of propranolol on activation of phosphorylase and inactivation of glycogen synthase by adrenergic agonists*

Fat cells were incubated with the indicated concentrations of (−)-propranolol at 37°. After 4 min, 5 μM (−)-norepinephrine, 5 μM (−)-epinephrine, or 50 μM (−)-phenylephrine was added. The incubations were terminated after 4.5 min, and glycogen synthase, phosphorylase, and cAMP were assayed as described under MATERIALS AND METHODS.

decrease in synthase I activity was observed with epinephrine and norepinephrine even in the presence of 200 μM propranolol (Fig. 3C). However, the effects of the catecholamines on phosphorylase were abolished by 200 μM propranolol. The effects of this concentration of propranolol may not result entirely from *beta* adrenergic receptor blockade. As discussed by Fain (22), propranolol at concentrations above 20 μM has nonspecific effects on fat cells, some of which may be related to local anesthetic properties of the antagonist. Such nonspecific actions may partially contribute to the effects of propranolol at the concentrations used in this study. In the other experiments presented, the concentration of propranolol used was limited to 20 μM to minimize nonspecific effects of the antagonist.

Phenylephrine (50 μM) increased the percentage of phosphorylase *a* activity (Fig. 3B) and decreased the percentage of glycogen synthase I activity (Fig. 3C), even in the presence of 2 μM propranolol. These results suggested that *alpha* adrenergic receptor activation might be responsible in part for the effects of the mixed-action adrenergic agonists epinephrine and norepinephrine.

Incubation of cells with increasing phentolamine concentrations decreased the percentage of phosphorylase *a* activity observed with 100 μM norepinephrine to the level observed with 0.05 or 10 μM isoproterenol (Fig. 4). The concentrations of norepinephrine selected were 10 times greater than those of isoproterenol because we wanted to use concentrations of these agonists that had produced approximately the same increases in cAMP, and isoproterenol was about 10 times more potent with respect to increasing cAMP than was norepinephrine (Fig. 1). None of the concentrations of phentolamine tested detectably altered control percentages of glycogen synthase I or phosphorylase *a* activities. However, as shown in Fig. 4A, phentolamine potentiated the increase in cAMP due to 0.5 and 100 μM norepinephrine, but not that due to 0.05 μM isoproterenol. A similar finding with epinephrine and phentolamine in human fat cells was reported by Robison *et al.* (23). In some experiments we observed an increase in cAMP due to phentolamine in the presence of higher concentrations of isoproterenol (Fig. 4A), which might be due to blockade of *alpha* adrenergic effects. In this regard, Hittelman *et al.* (24) previously reported that isoproterenol showed some *alpha* adrenergic activity in hamster white fat cells.

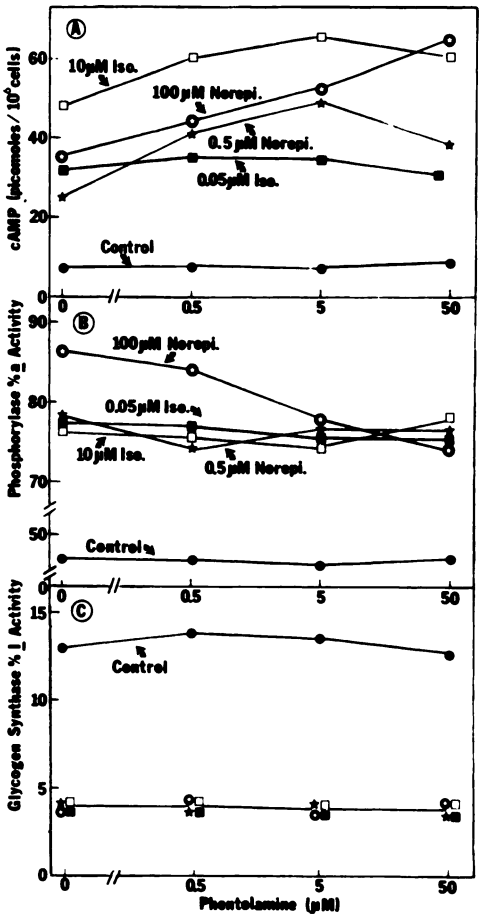

FIG. 4. *Effects of phentolamine on activation of phosphorylase and increases in cAMP produced by isoproterenol and norepinephrine*

Adipocytes were incubated with the concentrations of phentolamine indicated for 4 min at 37°. (−)-Norepinephrine or (−)-isoproterenol was added, and after 4.5 min enzyme activities and cAMP were assayed as described under MATERIALS AND METHODS.

To investigate further the effects of propranolol and phentolamine on the activation of phosphorylase and inactivation of

TABLE 2

Effects of phentolamine and propranolol on glycogen synthase I, phosphorylase a, and concentrations of cAMP in adipocytes incubated with ACTH and increasing concentrations of norepinephrine

Cells were incubated for 4 min at 37° with or without 20 μM (−)-propranolol or 20 μM phentolamine before the indicated concentrations of (−)-norepinephrine and ACTH were added. After 4.5 min, the incubations were terminated and glycogen synthase, phosphorylase, and cAMP were assayed as described under MATERIALS AND METHODS. The results presented are the mean values ± standard errors from four experiments performed on different days.

Additions	Glycogen synthase I activity			Phosphorylase *a* activity			cAMP		
	No antagonist	Propranolol	Phentolamine	No antagonist	Propranolol	Phentolamine	No antagonist	Propranolol	Phentolamine
	%	%	%	%	%	%	*pmoles/10⁶ cells*		
None	10.6 ± 1.0	11.0 ± 1.1	10.7 ± 0.9	47.0 ± 2.3	47.3 ± 3.0	47.4 ± 2.2	7.6 ± 0.4	7.2 ± 0.3	6.4 ± 0.3[a]
ACTH, 200 milliunits/ml	3.4 ± 0.3	3.0 ± 0.1	3.4 ± 0.5	76.1 ± 1.6	74.5 ± 1.9	75.5 ± 2.4	31.8 ± 5.1	34.3 ± 2.7	34.6 ± 3.3
Norepinephrine, 1 μM	3.7 ± 1.1	8.2 ± 0.6[b]	3.5 ± 0.2	81.7 ± 1.9	51.4 ± 2.6[a]	76.5 ± 2.8	25.3 ± 2.9	7.3 ± 0.3[a]	36.4 ± 5.7
Norepinephrine, 4 μM	3.2 ± 0.4	5.2 ± 0.3[c]	3.5 ± 0.4	84.7 ± 1.1[d]	59.2 ± 4.4[c]	75.9 ± 3.0[e]	26.2 ± 3.3	7.7 ± 0.3[a]	37.4 ± 5.1[e]
Norepinephrine, 10 μM	3.2 ± 0.1	3.6 ± 0.1[c]	3.3 ± 0.1	83.2 ± 2.6[d]	69.1 ± 3.9[c]	76.5 ± 2.7[e]	30.3 ± 2.7	8.8 ± 0.3[a]	43.6 ± 3.7[e]

[a] Not significantly different from results obtained without adrenergic antagonists or other additions.

[b] $p < 0.05$ vs. results obtained without adrenergic antagonists or other additions.

[c] $p < 0.01$ vs. results obtained without adrenergic antagonists or other additions.

[d] $p < 0.05$ vs. results obtained with ACTH alone.

[e] $p < 0.05$ vs. results obtained without adrenergic antagonists.

glycogen synthase by norepinephrine, the experiments presented in Table 2 were performed. ACTH was used as a control, since this hormone presumably does not act through *alpha* or *beta* adrenergic receptors. Consistent with this reasoning, neither 20 μM propranolol nor 20 μM phentolamine altered the concentration of cAMP or the percentages of phosphorylase *a* or glycogen synthase I activities produced by incubating cells with 200 milliunits/ml of ACTH. The increase in cAMP observed with ACTH was approximately the same as that produced by incubating cells with norepinephrine (1–10 μM), but the percentage of phosphorylase *a* activity was greater with the catecholamine. Incubation of fat cells with phentolamine increased the concentrations of cAMP observed with norephinephrine, but decreased the percentage of phosphorylase *a* activity to that observed with ACTH. Propranolol abolished the rise in cAMP produced by 4 μM norepinephrine, but an increase in the percentage of phosphorylase *a* activity and a decrease in glycogen synthase I were still observed.

Incubation of cells with phenoxybenzamine (1 μM) or dihydroergotamine (1 μM) did not alter the control percentage of phosphorylase *a* activity or the percentage of phosphorylase *a* activity observed with 10 μM isoproterenol (Table 3). However, like phentolamine, these antagonists potentiated the increase in cAMP produced by norepinephrine. Also, incubation of fat cells with either phenoxybenzamine or dihydroergotamine reduced the percentage of phosphorylase *a* activity observed with norepinephrine to that observed with isoproterenol.

Incubation of cells with 5 mM dibutyryl cAMP increased the percentage of phosphorylase *a* activity to that observed with isoproterenol (Table 4). In these experiments, cells were also incubated with 200 μM phenylephrine, 10 μM epinephrine, and 10 μM isoproterenol, which, when added alone, produced their respective maximal effects on the percentages of glycogen synthase I and phosphorylase *a* activities (Table 5 and Fig. 1). All three agents, as well as dibutyryl cAMP, decreased glycogen synthase I activity to the same extent. When cells were incubated with epinephrine or isoproterenol, together with dibutyryl cAMP, the percentage of phosphoryalse *a* activity was no different from that observed following incubation of cells with the catecholamines alone. However, when cells were incubated with phenylephrine plus dibutyryl cAMP, the percentage of phosphorylase *a* activity was increased to that observed with epinephrine.

Table 5 summarizes the results obtained from experiments in which increasing concentrations of phenylephrine were added to cells either together with 10 μM isoproterenol or following a 4-min incubation

TABLE 3

Effects of phenoxybenzamine and dihydroergotamine on activation of phosphorylase and increase in cAMP produced by isoproterenol and norepinephrine

Fat cells were incubated for 4 min with or without 1 μM phenoxybenzamine or 1 μM dihydroergotamine before 10 μM (−)-norepinephrine or 10 μM (−)-isoproterenol was added. After 4.5 min, the incubations were terminated and phosphorylase *a* activity and cAMP were measured as described in MATERIALS AND METHODS. The cAMP concentration in cells incubated without additions was 15.1 ± 1.8 pmoles/10^6 cells. The results presented are the mean values ± standard errors of three experiments performed on different days.

Alpha adrenergic antagonist	Phosphorylase *a* activity			Increase in cAMP		
	No addition	Norepinephrine	Isoproterenol	No addition	Norepinephrine	Isoproterenol
	%	%	%	%	%	%
None	47.5 ± 1.3	80.4 ± 1.0	72.8 ± 1.0[a]	0	271 ± 15	435 ± 41
Phenoxybenzamine	47.2 ± 1.7	74.0 ± 1.5[a]	71.0 ± 0.2	8 ± 17	425 ± 21	465 ± 31
Dihydroergotamine	47.1 ± 1.5	73.5 ± 0.8[a]	74.2 ± 1.5	32 ± 11	457 ± 24	452 ± 40

[a] $p < 0.05$ vs. results obtained with norepinephrine alone.

TABLE 4

Effect of dibutyryl cAMP on activation of phosphorylase and inactivation of glycogen synthase by alpha and beta adrenergic agonists

Fat cells were incubated at 37° with or without 5 mM dibutyryl cAMP for 10 min before the addition of 200 μM (−)-phenylephrine, 10 μM (−)-isoproterenol, or 10 μM (−)-epinephrine. After 4.5 min, glycogen synthase and phosphorylase were assayed as described under MATERIALS AND METHODS. The results represent the mean values ± standard errors from four experiments performed on different days.

Additions	Glycogen synthase I activity		Phosphorylase *a* activity	
	Control	Dibutyryl cAMP	Control	Dibutyryl cAMP
	%	%	%	%
None	11.3 ± 0.6	3.1 ± 0.1	42.7 ± 0.5	74.1 ± 1.2
Phenylephrine	3.3 ± 0.1	2.9 ± 0.2	65.1 ± 1.3[a]	81.2 ± 1.6[a]
Isoproterenol	2.7 ± 0.1	3.1 ± 0.2	70.8 ± 1.0	73.8 ± 2.0
Epinephrine	2.7 ± 0.1	2.9 ± 0.2	81.4 ± 1.1[a]	82.6 ± 1.7

[a] $p < 0.05$ vs. results obtained with isoproterenol or dibutyryl cAMP alone.

with 20 μM propranolol. Incubation of cells with phenylephrine (0.02–2 mM) resulted in a small increase in cAMP that was blocked by propranolol. Propranolol partially reversed but did not abolish a decrease in glycogen synthase I activity or an increase in phosphorylase *a* activity produced by incubation of cells with phenylephrine. When phenylephrine was added to cells together with isoproterenol, no further decrease in synthase I activity was observed, although the percentage of phosphorylase *a* activity was increased over that produced by isoproterenol alone. A depression of the rise in cAMP produced by isoproterenol was observed with phenylephrine. We have noted in other experiments that this effect of phenylephrine could be partially reversed by phentolamine.[4]

If the ability of phenylephrine to increase phosphorylase *a* activity over the increase produced by isoproterenol was due to the *alpha* adrenergic agonist properties of phenylephrine, this effect should be abolished by an *alpha* adrenergic antagonist. Incubation of cells with 20 μM phentolamine (Fig. 5) was without effect on basal phosphorylase *a* activity or the increase in phosphorylase *a* activity produced by 10 μM isoproterenol; however, phentolamine reduced the effect of 200 μM phenylephrine on increasing phosphorylase *a* activity. When cells were incubated with isoproterenol plus phenylephrine in the presence of phentolamine, no further increase in the percentage of phosphorylase *a* activity over that produced by isoproterenol was observed. In other experiments, replacement of phentolamine with 1 μM phenoxybenzamine produced the same results.[4]

Further evidence that *alpha* adrenergic receptor activation can lead to an increase in the percentage of phosphorylase *a* activity was provided by the observation that 10 μM methoxamine, an agent thought to activate only *alpha* adrenergic receptors (25), increased phosphorylase *a* activity (Fig. 6). In other experiments, the maximal activation of effect of methoxamine on phosphorylase was observed at 10 μM.[4] The extent of phosphorylase activation was approximately the same as that produced by 200 μM phenylephrine in the presence of 20 μM propranolol (Table 5). As also shown in Fig. 6, the effects of methoxamine and 10 μM isoproterenol on activation of phosphorylase activity were additive.

Because an increase in cellular cAMP appears to be sufficient to inactivate glycogen synthase maximally, the abilities of *alpha* adrenergic antagonists to oppose the effects of norepinephrine or epinephrine are probably obscured by *beta* adrenergic receptor activation. Phenylephrine has some activity as a *beta* adrenergic agonist in fat cells, although much less than epinephrine and norepinephrine (26). This is indicated in Table 5, where incubation of cells with phenylephrine resulted in a small increase in cAMP that was blocked by propranolol. Therefore both an *alpha*

TABLE 5

Effects of phenylephrine on glycogen synthase I, phosphorylase a, and concentrations of cAMP in adipocytes incubated with propranolol and isoproterenol

Cells were incubated for 4 min at 37° with or without 20 μM (−)-propranolol before the indicated concentrations of (−)-phenylephrine or (−)-phenylephrine plus 10 μM (−)-isoproterenol were added. After 4.5 min the incubations were terminated, and glycogen synthase and phosphorylase were assayed as described under MATERIALS AND METHODS. The cAMP concentration in cells incubated without additions was 9.1 ± 1.0 pmoles/10^6 cells. The results presented are the mean values ± standard errors of three experiments performed on different days.

Phenylephrine	Glycogen synthase I activity			Phosphorylase *a* activity			Increase in cAMP		
	No addition	Propranolol	Isoproterenol	No addition	Propranolol	Isoproterenol	No addition	Propranolol	Isoproterenol
mM	%	%	%	%	%	%	%	%	%
0	10.0 ± 1.6	10.6 ± 1.1	2.7 ± 0.3	40.7 ± 1.7	41.5 ± 1.3	74.9 ± 2.2	0	−6 ± 10	394 ± 98
0.02	4.0 ± 0.3	6.5 ± 0.5[a]	3.1 ± 0.1	56.8 ± 3.5	44.0 ± 2.5[b]	78.2 ± 1.5	13 ± 13	10 ± 24	309 ± 70
0.2	3.8 ± 0.4	6.1 ± 0.7[a]	3.0 ± 0.1	64.7 ± 2.3	47.0 ± 2.3[c]	83.8 ± 0.8[d]	26 ± 9	1 ± 15	325 ± 51
2	3.2 ± 0.2	5.9 ± 0.8[a]	3.0 ± 0.1	65.5 ± 2.2	48.0 ± 3.2[a]	83.6 ± 2.2[d]	42 ± 4	9 ± 13	253 ± 55

[a] $p < 0.01$ vs. results obtained without phenylephrine or other additions.

[b] Not significantly different from results obtained without phenylephrine or other additions.

[c] $p < 0.05$ vs. results obtained without phenylephrine or other additions.

[d] $p < 0.05$ vs. results obtained with isoproterenol alone.

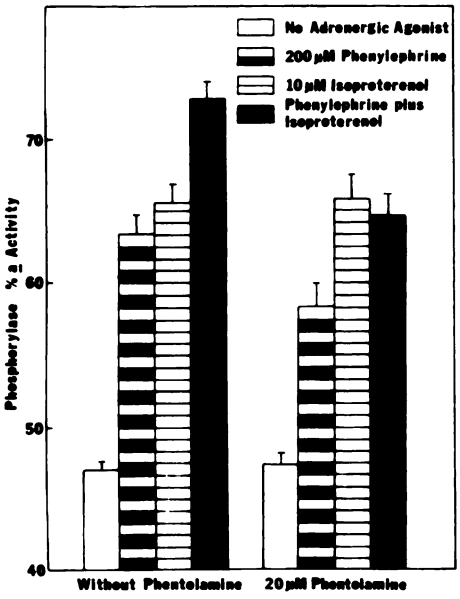

FIG. 5. *Effects of phentolamine on activation of phosphorylase by phenylephrine and isoproterenol*

Adipocytes were incubated with or without 20 μM phentolamine for 4 min at 37°. (−)-Phenylephrine, (−)-isoproterenol, or both were added as indicated and incubated with the cells for 1.5 min before the incubations were terminated. The results represent the mean values + standard errors from four experiments performed on different days.

and a *beta* adrenergic antagonist should be necessary to abolish the effects of phenylephrine on glycogen synthase. The effects of 20 μM propranolol and 1 μM phenoxybenzamine on the inactivation of glycogen synthase by 200 μM phenylephrine were investigated (Fig. 7). Again, 200 milliunits/ml of ACTH were used as a control for possible nonspecific effects of the two adrenergic antagonists. Propranolol only partially reversed the decrease in synthase I activity due to phenylephrine, presumably by blocking the *beta* adrenergic receptor-mediated rise in cAMP. Phenoxybenzamine altered neither the control nor the percentages of synthase I activity produced by incubating cells with phenylephrine. When both antagonists were added together, the effect of phenylephrine was abolished, but no difference in the effect of ACTH was observed.

Adipocytes were incubated with increasing concentrations of methoxamine to investigate further the *alpha* adrenergic receptor-mediated decrease in glycogen synthase I activity (Fig. 8). The maximal effect of methoxamine on decreasing glycogen synthase I activity (approximately 50%) was observed at a concentration of 10 μM. The same decrease in glycogen synthase I activity was observed with 200 μM phenylephrine in the presence of 20 μM propranolol (Fig. 7).

One interpretation of these results is that both *alpha* and *beta* adrenergic receptor-mediated processes contribute to the abilities of phenylephrine (as well as those of epinephrine and norepinephrine) to inactivate glycogen synthase and to activate phosphorylase. Blockade of the *beta* adrenergic receptor by propranolol leaves only the *alpha* adrenergic receptor-mediated effects of phenylephrine (Table 5). In contrast, incubation of cells with methoxamine results in inactivation of glycogen synthase and activation of phosphorylase

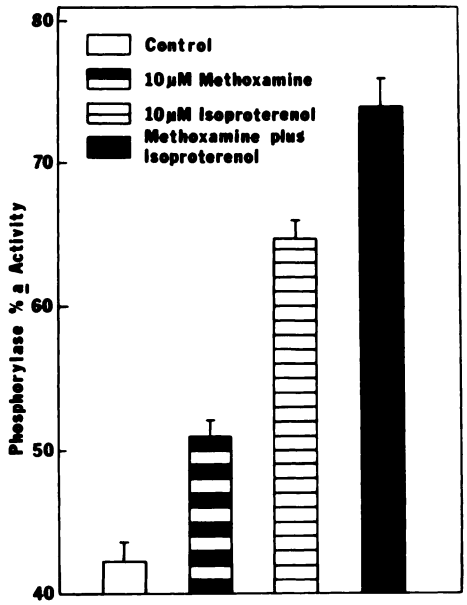

FIG. 6. *Activation of phosphorylase by methoxamine in the absence and presence of isoproterenol*

Adipocytes were incubated for 4 min at 37° before 10 μM methoxamine and 10 μM (−)-isoproterenol were added as indicated. Incubations with the adrenergic agonists were terminated after 1.5 min, and phosphorylase was assayed. The results represent the mean values + standard errors from four experiments performed on different days.

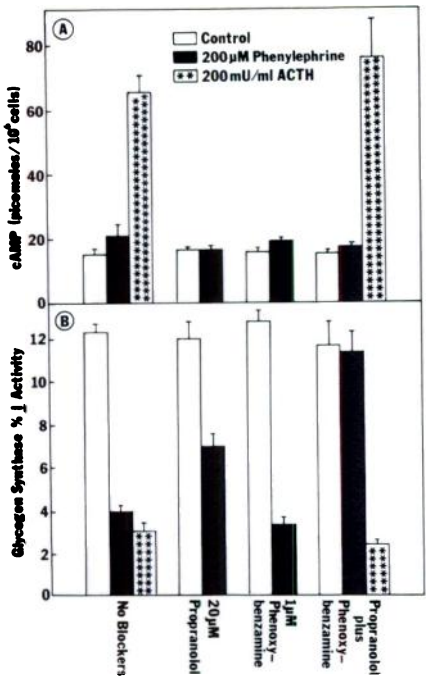

FIG. 7. *Blockade of inactivation of glycogen synthase by phenylephrine with propranolol and phenoxybenzamine*

Fat cells were incubated for 4 min with 20 μM (−)-propranolol and 1 μM phenoxybenzamine at 37°. Following a 4.5-min incubation with 200 μM (−)-phenylephrine or 200 milliunits/ml of ACTH, glycogen synthase activity and cAMP were measured as described under MATERIALS AND METHODS. The results presented represent the mean values + standard errors of four experiments performed on different days.

through only an *alpha* adrenergic receptor-mediated mechanism. If this interpretation is correct, an *alpha* adrenergic antagonist should completely block the effects of methoxamine. As shown in Fig. 9, 20 μM phentolamine abolished both the inactivation of glycogen synthase and the activation of phosphorylase produced by incubating cells with 10 μM methoxamine.

DISCUSSION

Using human adipocytes, Burns and Langley (27) demonstrated that phentolamine potentiated the ability of norepinephrine to increase lipolysis but did not potentiate the effect of isoproterenol. These authors suggested that both *alpha* and *beta* adrenergic receptors were present in human fat cells and acted in a divergent manner in mediating the effects of epinephrine on lipolysis. Robison *et al.* (23) later showed that these effects on lipolysis were reflected by changes in cAMP. It was proposed that *beta* adrenergic receptor activation increased cAMP and lipolysis in the human adipocyte, and that *alpha* adrenergic receptor activation served to oppose these increases. This hypothesis would explain why phentolamine potentiated the increase in cAMP produced by the mixed-action adrenergic agonist epinephrine, but not the relatively pure *beta* adrenergic agonist isoproterenol. Hittelman *et al.* (24) studied the effects of various agents on the concentrations of cAMP in hamster fat cells and obtained results supporting the above proposal. Yet, because of unsuccessful attempts to demonstrate inhibition of cAMP accumulation by *alpha* adrenergic agonists in rat adipo-

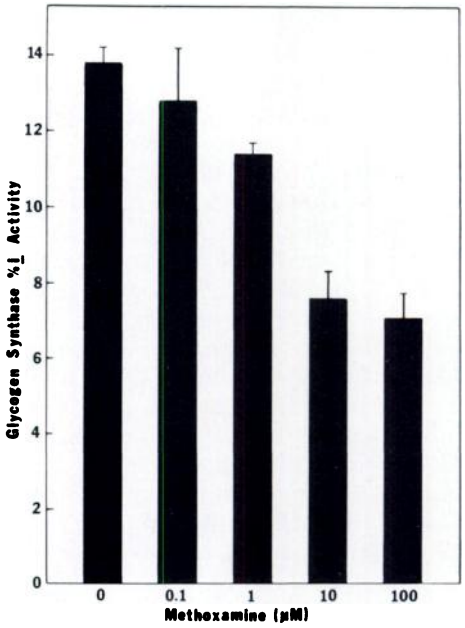

FIG. 8. *Dependence of adipocyte glycogen synthase I activity on concentration of methoxamine*

Fat cells were incubated for 4 min at 37° before the indicated concentrations of methoxamine were added. After 1.5 min the incubations were terminated and glycogen synthase was assayed. The results represent the mean values + standard errors from three experiments performed on different days.

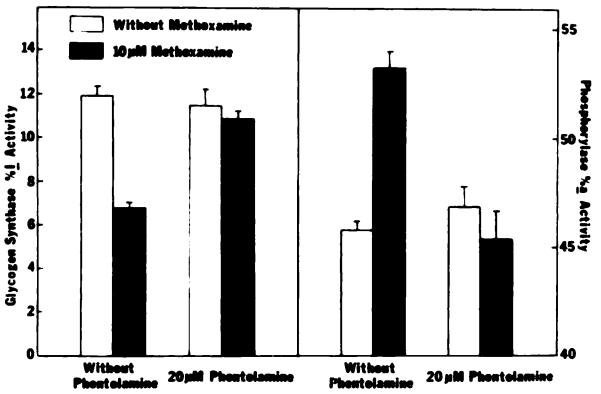

FIG. 9. *Blockade by phentolamine of effects of methoxamine on adipocyte glycogen synthase and phosphorylase*

Adipocytes were incubated at 37° in the presence and absence of 20 μM phentolamine for 4 min, then for 1.5 min with or without 10 μM methoxamine, before glycogen synthase and phosphorylase were assayed. The results represent the mean values + standard errors from five experiments.

cytes (27, 28), these cells were believed to be devoid of this *alpha* adrenergic receptor-mediated function.

In the present study, the ability of phentolamine to potentiate the increase in cAMP produced by norepinephrine is clearly evident (Fig. 4 and Table 2). Furthermore, similar potentiating effects were produced by two other *alpha* adrenergic antagonists, phenoxybenzamine and dihydroergotamine (Table 3). Fain (22) has pointed out that because of many nonspecific effects of *alpha* adrenergic antagonists on adipose tissue, the potentiation of the effects of epinephrine by these drugs does not necessarily result from a specific effect on *alpha* adrenergic receptors. For example, Ward and Fain (29) found that dihydroergotamine (20–100 μM) was an inhibitor of adipocyte phosphodiesterase. A decrease in phosphodiesterase activity could result in potentiation of the increases in cAMP by agents like norepinephrine that activate adenylate cyclase. It seems unlikely that this mechanism could account for the results in Table 3, since 20 times less dihydroergotamine was required to observe the potentiation of the effects of norephinephrine on the concentrations of cAMP than was required to inhibit phosphodiesterase (29). Furthermore, no effect of the antagonist was observed on the increase in cAMP produced by isoproterenol. A similar argument can be used for the specificity of the other *alpha* adrenergic antagonists used in the present study. For example, phenoxybenzamine did not potentiate the increase in cAMP produced by isoproterenol (Table 3), and phentolamine did not alter the concentrations of cAMP increased by ACTH (Table 2). Thus the results of this report provide strong evidence for the occurrence of *alpha* adrenergic receptor-mediated inhibition of cAMP accumulation in rat adipocytes.

The effects of isoproterenol and norepinephrine on increasing phosphorylase *a* activity and cAMP were maximal after 4.5 min (Fig. 2). In agreement with the earlier findings of Butcher *et al.* (30) using rat fat pads, the initial increases in cAMP concentration in the presence of norepinephrine or isoproterenol became smaller after longer periods of incubation. In experiments in which cAMP measurements were made, except those represented by Fig. 2, cells were incubated with adrenergic agonists for 4.5 min because this period was optimal for the detection of increases in cAMP. Nevertheless, the results of experiments in which propranolol was used to block the increases in cAMP due to various *beta* adrenergic agonists should be interpreted with caution. For example, Soderling *et al.* (31) presented evidence that very small increases in cAMP were associated with maximal rates of lipolysis

and increases in protein kinase activity in adipose tissue. It could thus be argued that 4 μM norepinephrine increased phosphorylase *a* and decreased glycogen synthase I activity in the presence of 20 μM propranolol (Table 2) because of very small, nondetectable increases in cAMP. However, additional evidence strongly favors the hypothesis that a cAMP-independent pathway exists for phosphorylase activation. First, the maximal effects on phosphorylase activation of epinephrine and norepinephrine, agents that have both *alpha* and *beta* adrenergic agonist activity, were greater than the maximal effect of isoproterenol, even though the concentrations of cAMP were increased more with isoproterenol (Fig. 1). Second, when phenylephrine and isoproterenol (Table 5), or methoxamine and isoproterenol (Fig. 6), were added together at concentrations that produced their maximal effects when added alone, a further increase in phosphorylase *a* activity was observed. Finally, addition of isoproterenol together with dibutyryl cAMP resulted in a level of phosphorylase *a* activity no different from that obtained with either agent alone (Table 4). However, when phenylephrine and dibutyryl cAMP were added together, an additional increase in phosphorylase *a* activity was observed, analogous to the results obtained with isoproterenol plus phenylephrine (Table 5). These experiments with phenylephrine and methoxamine support an *alpha* adrenergic receptor-mediated mechanism for phosphorylase activation. Additional support is provided by the observations that the *alpha* adrenergic antagonists phentolamine (Fig. 4), phenoxybenzamine, and dihydroergotamine (Table 3) reduced the phosphorylase *a* activity obtained with norepinephrine to the level obtained with isoproterenol. Furthermore, the activation of phosphorylase by methoxamine was abolished by phentolamine (Fig. 9).

Establishing the occurrence of *alpha* adrenergic receptor-mediated inactivation of glycogen synthase was complicated by the ability of *beta* adrenergic receptor activation to decrease glycogen synthase I activity maximally, as shown in experiments in which cells were incubated with isoproterenol (e.g., Fig. 1). That dibutyryl cAMP was also effective in this respect is consistent with the idea that increases in cellular cAMP mediate the effects of *beta* adrenergic agonists. However, when the increase in cAMP due to epinephrine (Fig. 3), phenylephrine (Table 5), or norepinephrine (Table 2) was completely blocked by propranolol, inactivation of glycogen synthase by these *alpha* adrenergic agonists was still observed. Also, the relatively pure *alpha* adrenergic agonist methoxamine inactivated glycogen synthase, and this effect was abolished by the *alpha* adrenergic antagonist phentolamine (Fig. 9). The observation that the effects of 200 μM phenylephrine on glycogen synthase I activity were abolished by 20 μM propranolol and 1 μM phenoxybenzamine only when the two antagonists were added together strongly suggests that both *alpha* and *beta* adrenergic receptor-mediated processes contribute to the inactivation of glycogen synthase.

It seems likely that the effects resulting from *beta* adrenergic receptor stimulation are mediated by increased cAMP-dependent protein kinase(s) activity resulting from elevations in intracellular cAMP. An increase in protein kinase activity should result in decreased glycogen synthase I activity and increased phosphorylase *b* kinase activity, leading to phosphorylase activation.

Recent evidence has implicated calcium in the activation of rat hepatocyte phosphorylase by *alpha* adrenergic agonists (32, 33). Because rat liver phosphorylase *b* kinase has been shown to be dependent on calcium for activity (32, 34), an increase in cellular calcium could result in stimulation of phosphorylase *b* kinase activity and, thereby, in activation of phosphorylase. Khoo (35) has demonstrated that adipose tissue phosphorylase *b* kinase is dependent upon calcium for activity. In preliminary experiments, we found that when cells were incubated in a Ca^{++}-free medium containing 1 mM EGTA, the ability of 4 μM norepinephrine to activate phosphorylase was abolished by 20 μM propranolol, although an effect of norepineph-

rine in the presence of propranolol was still observed when the cells were incubated in the same medium to which 2.4 mM Ca^{++} was added back before the addition of norepinephrine.[4] It is therefore possible that a mechanism similar to that proposed for the *alpha* adrenergic receptor-mediated activation of phosphorylase in rat hepatocytes (32, 33) exists in the rat adipocyte. This mechanism would explain the synergistic effects of *alpha* and *beta* adrenergic receptor-mediated activation of phosphorylase. An additional level of interaction between the two adrenergic receptor-mediated mechanisms might also arise from the phosphorylation of phosphorylase kinase by cAMP-dependent protein kinase. Brostrom *et al.* (36) have shown that the activated form of purified rabbit skeletal muscle phosphorylase kinase has a K_m for Ca^{++} that is about an order of magnitude less than the K_m of the nonactivated kinase. While comparable studies have not been performed using purified adipose tissue phosphorylase kinase, phosphorylation of this enzyme might increase its sensitivity to stimulation by Ca^{++}. This mechanism could explain why the activation of phosphorylase by 1 μM norepinephrine was blocked by propranolol (Table 2), although the *alpha* adrenergic receptor-mediated component of phosphorylase activation (i.e., the difference between the norepinephrine and isoproterenol responses) was observed in the absence of propranolol at this concentration of norepinephrine (Fig. 1). In other words, in the absence of events associated with stimulation of *beta* adrenergic receptors, more *alpha* adrenergic receptor stimulation might be required to elicit the *alpha* response. Experiments will be performed to investigate this idea.

In summary, the prominence of the *beta* adrenergic receptor-mediated mechanism of phosphorylase activation and glycogen synthase inactivation in rat adipocytes distinguishes fat cells from rat hepatocytes (6). The mechanism(s) by which *alpha* adrenergic receptor activation acts together with *beta* adrenergic receptor activation to increase phosphorylase *a* activity and decrease glycogen synthase I activity, while acting to oppose the *beta* adrenergic receptor-mediated rise in cAMP, is currently under investigation in this laboratory.

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