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Characterization of Rat White Fat Cell α_{1B}-Adrenoceptors

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SUMMARY

In isolated rat white adipocytes, epinephrine (in the presence of 10 μM propranolol) increased the uptake of [^{32}P]P_i into phosphatidylinositol in a dose-dependent fashion. When the cells were pretreated with the irreversible antagonist chlorethylclonidine, this α_1-adrenergic effect was markedly diminished. The effect of epinephrine was dose-dependently antagonized by selective α_1-adrenergic antagonists, with the potency order prazosin > 5-methylurapidil ≥ WB4101. Binding studies using crude membrane preparations were performed with the ligands [^{3}H]bunazosin and ^{125}I-HEAT. Both ligands bound to membrane sites with high affinity (K_d values of 0.75 ± 0.20 nM for [^{3}H]bunazosin and 125 ± 20 pM for ^{125}I-HEAT), in a rapid, reversible, and saturable (B_{max}, 9–12 fmol/mg of protein) fashion, and with the expected pharmacological characteristics for α_1-adrenoceptors. Binding displacement studies with these ligands indicated a potency order of prazosin > 5-methylurapidil ≥ WB4101. Northern blot analysis using receptor subtype-specific gene probes showed that adipocyte mRNA hybridized with the α_{1B}-adrenergic probe. All these data suggest that the α_1-adrenoceptors of rat white adipocytes belong to the α_{1B} subtype.

Catecholamines are among the main modulators of adipocyte metabolism (1). Three basic types of adrenoceptors are present in these cells, i.e., α_1-, α_2-, and β-adrenoceptors (1). The effects of catecholamines on cAMP accumulation are mediated through regulation of adenylate cyclase activity, which is activated by β-adrenoceptors and inhibited by α_2-adrenoceptors. Activation of α_1-adrenoceptors is associated with phosphoinositide turnover and calcium mobilization (1, 2).

Activation of α_1-adrenoceptors in white adipocytes increases the uptake of [^{32}P]P_i into PI (3–6). This increased PI labeling seems to be secondary to the initial breakdown of phosphatidylinositol-4,5-bisphosphate to inositol-1,4,5-trisphosphate and diacylglycerol, by a phosphoinositidase (7). Other α_1-adrenergic actions have been observed in adipocytes and include activation of glycogen phosphorylase (8, 9), inactivation of glycogen synthase (5, 8, 9), and stimulation of $^{42}K^+$ efflux (10); these actions seem to be due to elevated cytosol [Ca^{2+}].

Heterogeneity of α_1-adrenoceptors has been demonstrated by pharmacological and molecular biological techniques. The existence of two subtypes of α_1-adrenoceptors, α_{1A} and α_{1B}, was initially suggested by pharmacological data (11); these receptors have now been cloned and expressed (12, 13). A new subtype, the α_{1C}-adrenergic receptor, not previously evidenced by pharmacological criteria, has also now been cloned and expressed (14).

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In the present study, we examined the subtype of α_1-adrenoceptor present in isolated rat adipocytes, using a metabolic response (PI labeling), radioligand binding techniques, and Northern blot analysis.

Materials and Methods

l-Epinephrine, *dl*-propranolol, and prazosin were obtained from Sigma Chemical Co. (St. Louis, MO). Chlorethylclonidine, 5-methylurapidil, and WB4101 were obtained from Research Biochemicals Inc. (Natick, MA). Collagenase was from Worthington (Freehold, NJ). [^{32}P]P_i (carrier-free) and ^{125}I-HEAT (2200 Ci/mmol) were from New England Nuclear (Boston, MA). [^{3}H]Bunazosin (54 Ci/mmol) was generously provided by Eizai Co., Ltd. (Japan). The α_1-adrenoceptor gene probes were generously provided by Drs. Lomasney, Cotecchia, Caron, and Lefkowitz (Duke University).

Male Wistar rats (300–350 g), fed *ad libitum*, were used. Rats were sacrificed by decapitation, and the epididymal fat pads were removed. Fat cells were isolated by the method of Rodbell (15). The methodology to study PI labeling in fat cells has been reported in detail elsewhere (3). In brief, adipocytes were incubated in Krebs-Ringer Tris buffer supplemented with 6% albumin and 10 μCi/ml radioactive phosphate, for 60 min. Phospholipids were extracted with chloroform/methanol (2:1), followed by silicic acid adsorption. Separation of phospholipids was performed using one-dimensional thin layer chromatography. Incorporation of label was determined directly, by counting silica gel scrapings of individual phospholipids. The incorporation of label and the phospholipid composition were identical to those reported previously (3).

For the binding studies, a crude membrane preparation was obtained from the isolated adipocytes (4). [^{3}H]Bunazosin binding was measured

ABBREVIATIONS: PI, phosphatidylinositol; SSC, sodium chloride/sodium citrate; kb, kilobases; ^{125}I-HEAT, 2{β-(4-hydroxy-3-^{125}I-iodophenyl)ethylaminomethyl}tetralone.

as described by Kobatake *et al.* (16), with some minor modifications. In brief, 1 mg of membrane protein was incubated with [^{3}H]bunazosin (0.05–2.5 nM in saturation experiments or 1.25 nM in binding displacement experiments), in 50 mM Tris, 10 mM $MgCl_2$, pH 7.5, for 20 min at 25°, in a total volume of 0.5 ml. The incubation was terminated by the addition of 10 ml of ice-cold buffer; the suspension was filtered through GF/C filters, which were then washed three times with 10 ml of the same buffer and counted in a scintillation counter with an efficiency of 40%. Nonspecific binding was evaluated in the presence of 1 μM prazosin; specific binding usually represented 60% of total binding at the K_d. Binding of ^{125}I-HEAT was assayed as described by Lomasney *et al.* (12), with some modifications. Membranes (300 μg of protein) were incubated with ^{125}I-HEAT (10–300 pM in saturation experiments and 100 pM in binding competition experiments), in 1 ml of 50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.2% albumin, pH 7.4, for 60 min at 25°. The incubation was terminated by the addition of 10 ml of ice-cold buffer, and the mixture was filtered through GF/C filters, which were washed three times with 10 ml of the same buffer and counted in a γ counter. Nonspecific binding was determined in the presence of 1 μM prazosin; specific binding represented ≈40% of total binding at the K_d. The K_i values were calculated according to the method of Cheng and Prusoff (17).

Total RNA was extracted with phenol and precipitated with ethanol, as described (18). To perform the Northern blot analysis, 100 μg of total RNA were subjected to electrophoresis in a 1.5% agarose gel with 17% formaldehyde; electrophoresis and electrotransfer were performed using standard conditions, and the filters were hybridized under high stringency conditions (18), with 50% formamide. After hybridization, the membranes were washed three times with 2× SSC (150 mM sodium chloride, 15 mM sodium citrate, pH 7.4), 0.1% sodium dodecyl sulfate, for 5 min at room temperature, twice with 0.1× SSC, 0.1% SDS, at 50° for 30 min, and twice with 0.1× SSC at room temperature for 5 min.

Gene-specific probes were labeled by nick translation, to a specific activity of $>10^8$ cpm/μg. In all experiments, 1 μg of radioactive probe was used for the hybridization. After the washes, the filters were autoradiographed for 7–10 days at −70°.

Hybridization probes were as follows: the α_{1A}-adrenoceptor probe was the 0.85-kb *Bam*HI fragment (12); the α_{1B}-adrenoceptor probe was the 0.81-kb *Eco*RI-*Bam*HI fragment of the hamster α_1-adrenoceptor cDNA (13) (subcloned in SP65 at the *Eco*RI site); and the α_{1C}-adrenoceptor probe was the 1.02-kb *Bgl*II-*Hind*III fragment (14).

Results

In agreement with our previous results (3, 5, 6), epinephrine in the presence of 10 μM propranolol stimulated PI labeling in a dose-dependent fashion, with an EC_{50} of ≈1 μM (Fig. 1, *left*). Pretreatment of the cells with the alkylating antagonist chlorethylclonidine (100 μM for 15 min) markedly diminished the α_1-adrenergic effect, from a 3.5-fold increase in labeling to a stimulation of only 60–80% over basal (Fig. 1, *left*); the pretreatment with chlorethylclonidine was without effect on PI labeling by itself (data not shown), and it was receptor specific, as evidenced by the fact that the stimulation of PI labeling induced by 0.1 μM oxytocin was not altered (545 ± 75 and 505 ± 40% of basal labeling in control and chlorethylclonidine-treated cells, respectively; means ± standard errors of three experiments, in duplicate, in each case).

We next examined the effect of reversible α_1-adrenergic-selective antagonists. The effect of 10 μM epinephrine (in the presence of 10 μM propranolol) was dose-dependently inhibited by these antagonists, with the order of potency prazosin > 5-methylurapidil ≥ WB4101 (Fig. 2, *right*); the K_i values are presented in Table 1.

In agreement with the data of Kobatake *et al.* (16), we observed that [^{3}H]bunazosin bound to adipocyte membranes in a rapid, reversible, and stereospecific manner, with the pharmacological characteristics of α_1-adrenoceptors (data not shown). Saturation experiments and their Scatchard analysis indicated the presence of a single class of sites for this ligand, with a K_d of 0.75 ± 0.20 nM and a B_{max} of 12 ± 3 fmol/mg of protein (means ± standard errors of three experiments, in

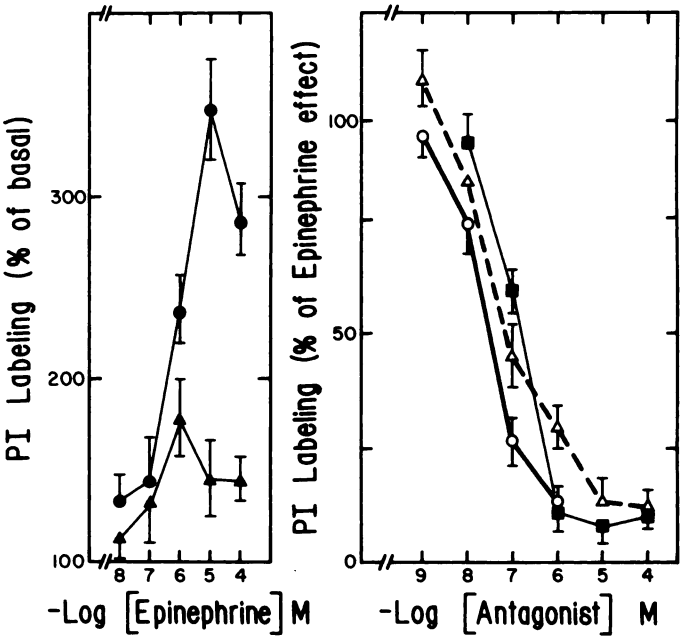

Fig. 1. Effect of α_1-adrenergic agents on PI labeling in isolated rat adipocytes. *Left*, adipocytes were preincubated without (●) or with 100 μM chlorethylclonidine (▲) for 15 min, washed, and incubated with 10 μM propranolol and different concentrations of epinephrine. *Right*, cells were incubated with 10 μM epinephrine (plus 10 μM propranolol) and different concentrations of prazosin (○), 5-methylurapidil (△), or WB4101 (■). Results are expressed either as percentage of basal, which was 1934 ± 301 cpm/10^6 cells (*left*) or as percentage of the stimulation induced by 10 μM epinephrine (*right*). The means are plotted, and *vertical lines* represent the standard error, of five or six experiments, using different cell preparations.

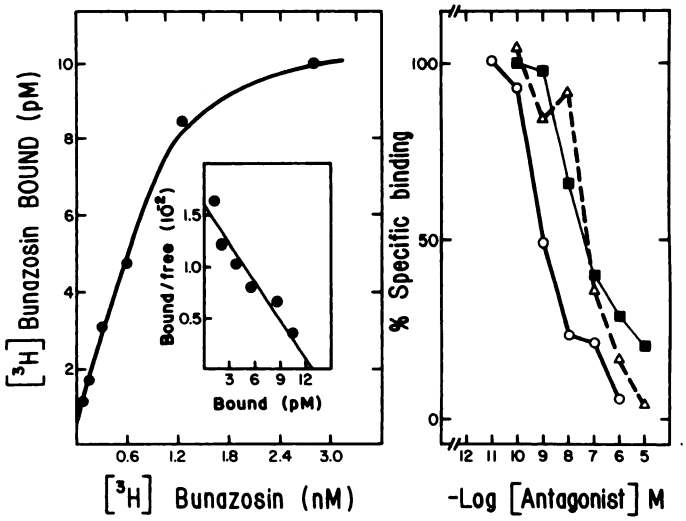

Fig. 2. Saturation and displacement of specific [^{3}H]bunazosin binding. *Left*, specific binding as a function of radioligand concentration (*inset*, Scatchard plot analysis). *Right*, displacement of [^{3}H]bunazosin specific binding by the indicated concentrations of prazosin (○), 5-methylurapidil (△), or WB4101 (■). Experiments representative of three to five, using different membrane preparations, are presented.

TABLE 1

K_i values for α_1-adrenergic antagonists

Values are the means ± standard errors of six to nine separate experiments.

Antagonist	K_i		
	PI labeling	Binding	
		[^{3}H]Bunazosin	^{125}I HEAT
		nM	
Prazosin	1.25 ± 0.35	0.75 ± 0.25	0.45 ± 0.14
5-Methylurapidil	5.35 ± 2.00	22.75 ± 2.80	14.25 ± 2.85
WB4101	13.50 ± 0.50	23.70 ± 7.00	23.00 ± 7.60

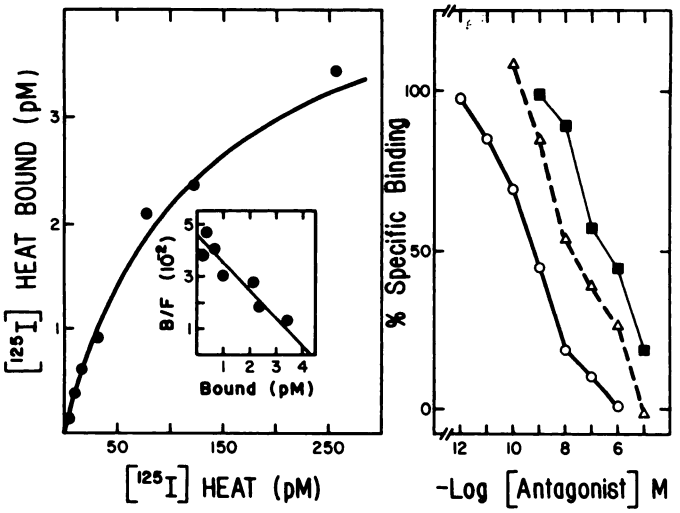

Fig. 3. Saturation and displacement of specific ^{125}I-HEAT binding. *Left*, specific binding as a function of radioligand concentration (*inset*, Scatchard plot analysis). *Right*, displacement of ^{125}I-HEAT specific binding by the indicated concentrations of prazosin (○), 5-methylurapidil (△), or WB4101 (■). Experiments representative of three to five, using different membrane preparations, are presented.

triplicate); a representative experiment is presented in Fig. 2 (*left*). Binding competition experiments showed that the α_1-adrenergic antagonists competed for the [^{3}H]bunazosin binding sites with the order of potency prazosin > 5-methylurapidil ≥ WB4101; a representative experiment is presented in Fig. 2 (*right*), and the K_i values are given in Table 1.

Binding of ^{125}I-HEAT to adipocyte membranes was also rapid and reversible. In saturation experiments, a K_d of 125 ± 20 pM and a B_{max} of 9 ± 2 fmol/mg of protein were determined (means ± standard errors of six experiments, in triplicate); a representative experiment is presented in Fig. 3 (*left*). Agonists competed for the ^{125}I-HEAT binding sites with the following K_i values: *l*-epinephrine, 550 ± 150 nM; *l*-norepinephrine, 970 ± 250 nM; phenylephrine, 1170 ± 90 nM; and *d*-epinephrine, 10000 ± 3000 nM (means ± standard errors of three or four experiments, in triplicate). α_1-Adrenergic antagonists competed for these binding sites with the order of potency prazosin > 5-methylurapidil ≥ WB4101; a representative experiment is presented in Fig. 3 (*right*), and the K_i values are given in Table 1.

Northern blot analysis showed that total adipocyte mRNA hybridized with the α_{1B}-adrenoceptor probe but not with the other subtype-selective probes (Fig. 4). In one experiment, a weak signal was observed using the α_{1A} probe; however, the signal was minor and it was not consistently observed.

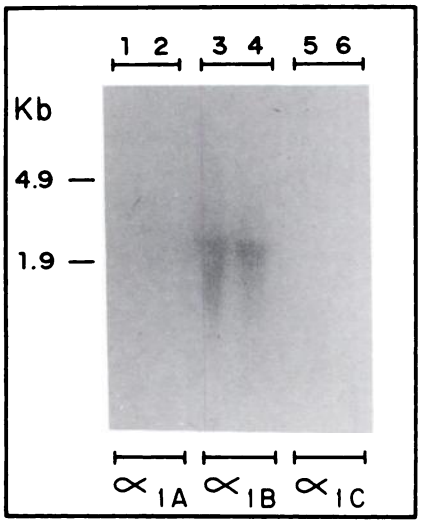

Fig. 4. Northern blot of adipocyte mRNA probed with α_1-adrenoceptor subtype-selective probes. Total RNA (100 μg/lane) was subjected to electrophoresis and electrotransferred. The filter was cut and the membrane fragments were hybridized with the α_{1A}- (*lanes 1* and *2*), α_{1B}- (*lanes 3* and *4*), or α_{1C}-selective (*lanes 5* and *6*) probes. The figure is representative of five experiments, using different cell preparations.

Discussion

In the present work, we characterized the α_1-adrenoceptors of isolated rat white adipocytes using different methodological approaches, i.e., a metabolic parameter (PI labeling), binding saturation and displacement studies, and Northern blot analysis. These different approaches gave entirely consistent results.

Although admittedly a secondary response, the α_1-adrenergic stimulation of PI labeling is a large, easy to quantify, reproducible effect in fat cells, and it has been previously characterized pharmacologically (3, 6). The initial event, i.e., the accumulation of inositol-1,4,5-trisphosphate, can also be determined, but usually the adrenergic effect is small, which complicates the pharmacological characterization (19).[1]

It has been difficult to detect α_1-adrenoceptors in white fat cells membranes by direct binding techniques. Initial studies using [^{3}H]prazosin detected a binding site that did not represent α_1-adrenoceptors (20). Only very recently, Kobatake *et al.* (16) succeeded in this task, using [^{3}H]bunazosin, a hydrophilic, highly selective, α_1-adrenergic blocker (21, 22). In the present manuscript, we confirmed their findings and used this ligand to characterize further the subtype of receptor involved. We detected 50% fewer receptors per milligrams of protein than did Kobatake *et al.* (16), which could be due to the strains of rats used (Wistar in our study, compared with Sprague-Dawley). This required the use of very large amounts of membrane protein in the binding assays; fortunately, the nonspecific binding was low. ^{125}I-HEAT was also used in this study, and it was found to be a suitable ligand; it has a much higher specific activity, which allowed us to use less membrane protein in the binding assays. Unfortunately, the nonspecific binding was also higher. In spite of these difficulties, we observed good agreement between binding assays and the metabolic data.

Among the adrenergic agents that have been used for the classification of α_1-adrenoceptors, three have been particularly useful, i.e., chlorethylclonidine (11), WB4101 (23), and 5-meth-

[1] M. E. Torres-Márquez and J. A. García-Sáinz, unpublished observations.

ylurapidil (24). Chlorethylclonidine is an irreversible antagonist that blocks α_{1B}- and α_{1C}- but not α_{1A}-adrenoceptors (11–14). The reversible antagonists 5-methylurapidil and WB4101 have very high affinity for α_{1A}- and α_{1C}-adrenoceptors and lower affinity for receptors of the α_{1B} subtype (12–14, 23, 24). The high sensitivity to chlorethylclonidine of white adipocyte α_1-adrenoceptors rules out the possibility that they belong to the α_{1A} subtype; the relatively low affinities for 5-methylurapidil and WB4101 suggest that these adrenoceptors are of the α_{1B}-subtype. Previously, Morrow and Creese (23) predicted, by analyzing the phentolamine/prazosin potency ratios of our data on epinephrine-stimulated PI labeling, that the adrenoceptors of fat cells belong to the α_{1B} subtype. This is consistent with our present findings. However, we would like to point out that these α_{1B}-adrenoceptors have some differences with respect to receptors in other systems, such as rat hepatocytes. For example, methoxamine, which is only weakly active in liver cells at very high concentrations (25, 26), has good activity in fat cells (3, 5, 8, 9). Obviously, factors such as the type of guanine nucleotide-binding protein(s) involved or the intracellular amplification of the signal participate in the overall response and may explain some of the differences observed.

In our studies, the Northern blot analysis showed hybridization with the α_{1B}-adrenergic probe. Previously, it was observed that adipose tissue mRNA gave some signal with the α_{1A} probe (12, 25). The discrepancy is probably due to the fact that adipose tissue contains, besides adipocytes, several other cell types (vascular, endothelial, etc.). In fact, the presence of some of these cells could explain why we observed a weak α_{1A} signal in one of the fat cell preparations (i.e., cell contamination).

In summary, using biochemical, pharmacological, and molecular biological approaches, our results indicate that rat white adipocyte α_1-adrenoceptors belong to the α_{1B} subtype.

Acknowledgments

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