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Modification of the Rat Adipocyte A_1 Adenosine Receptor-Adenylate Cyclase System during Chronic Exposure to an A_1 Adenosine Receptor Agonist: Alterations in the Quantity of $G_{s\alpha}$ and $G_{i\alpha}$ Are Not Associated with Changes in Their mRNAs

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SUMMARY

The A_1-adenosine receptor (A_1AR) adenylate cyclase system in rat adipocytes undergoes heterologous desensitization following chronic *in vivo* exposure to an A_1AR agonist (+)-N^6-(R-phenylisopropyl)adenosine [*J. Biol. Chem.* **262**:841–847 (1987)]. This desensitization involves an absolute increase in adenylate cyclase activity and a refractoriness to receptor ligands that are inhibitory to adenylate cyclase. In this study, receptor changes were characterized using an A_1AR antagonist radioligand, [^{3}H]8-[4-[[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropyl xanthine. Saturation binding studies demonstrated a 47% decrease in total A_1AR density without a change in K_D. Agonist competition studies revealed a decreased percentage of receptors, from 55% to 35%, in the high affinity state following desensitization. An increase in $G_{s\alpha}$ of 49% was found by Western blotting using specific $G_{s\alpha}$ antibodies. Further, an antibody that recognizes $G_{i\alpha1}$ and $G_{i\alpha2}$ was used to quantitate these subtypes of $G_{i\alpha}$ and both were decreased by 59% following desensitization. However, when an antibody that recognizes $G_{i\alpha3}$ was used, no change in $G_{i\alpha3}$ was found, demonstrating, in this case, differential regulation of $G_{i\alpha}$ subtypes. The mechanisms responsible for changes in $G_{s\alpha}$ and $G_{i\alpha}$ were studied by measuring the levels of their mRNAs from normal and desensitized adipocytes. Using either labeled cDNAs ($G_{i\alpha2}$, $G_{i\alpha3}$) or oligonucleotides ($G_{s\alpha}$, $G_{i\alpha1}$), Northern analysis demonstrated that mRNAs for $G_{s\alpha}$ and all three isoforms of $G_{i\alpha}$ are present in adipocytes but that there are no changes in the levels of any of these transcripts following desensitization. These data suggest that desensitization of the A_1AR-adenylate cyclase system involves a down-regulation of A_1ARs and an additional loss of A_1AR agonist high affinity sites. Further, an increase in $G_{s\alpha}$, a decrease in $G_{i\alpha1}$ and $G_{i\alpha2}$, and no change in $G_{i\alpha3}$ were found. The regulation of $G_{s\alpha}$ and the subtypes of $G_{i\alpha}$ in this system does not occur by altering the levels of their respective transcripts.

For several decades it has been known that adenosine can modulate important physiological responses (1). It has only been in the past 10 years, however, that the biochemical mediators of these responses, namely AR and related signal transduction proteins, have become amenable to study. In this time, two types of AR, A_1 and A_2, have been identified which are distinguished by their relative affinities for a group of agonists (1, 2). Further, it is clear that these receptors can be coupled to either inhibition (A_1AR) or stimulation (A_2AR) of adenylate cyclase via the inhibitory G protein G_i or the stimulatory G protein, G_s, respectively (2, 3). The availability of high affinity A_1 agonist radioligands and photoaffinity probes has allowed investigators to identify A_1AR in brain, heart, testis, and fat in a variety of species (2, 3).

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The phenomenon of desensitization or tachyphylaxis is known to occur following the chronic activation of receptors coupled in a stimulatory fashion to the enzyme adenylate cyclase (4). Much less is known concerning the phenomenon of desensitization in inhibitory receptor systems. We and others have begun to probe the mechanisms involved in the desensitization of the A_1AR-adenylate cyclase system. Hoffman and colleagues (5) showed a diminished ability of A_1AR agonists to inhibit lipolysis in rats that had been treated with a 6-day infusion of the A_1AR agonist (*R*)-PIA. Further, they showed a marked increase in basal and isoproterenol-stimulated cAMP accumulation in adipocytes isolated from treated animals. Parsons and Stiles (6), using a similar rat model, characterized the desensitized adipocyte A_1AR-adenylate cyclase system. They showed that there was a 2-fold increase in the level of adenylate cyclase activity when measured under basal or isoproterenol- or forskolin-stimulated conditions. Further, a 40–60% decrease in the ability of A_1 adenosine agonists to inhibit adenylate cyclase activity was shown. This was also true for prostaglan-

din-mediated adenylate cyclase inhibition. This type of change in adenylate cyclase, where chronic exposure to one type of receptor agonist causes diminished responses to stimulation of other receptor types, is called heterologous desensitization and may occur via one of several mechanisms (4). Accompanying these functional changes was a 32% decrease in the number of A_1AR agonist binding sites, as well as a 37% decrease in the α subunit of G_i ($G_{i\alpha}$) and a 44% increase in the α subunit of G_s ($G_{s\alpha}$), as defined by toxin-mediated [^{32}P]ADP-ribosylation. Clearly, the changes in G proteins could account for the differences in adenylate cyclase activity found between membranes from control and treated animals. Studies of A_1AR desensitization have been hindered, until recently, by the lack of an A_1AR antagonist radioligand of sufficiently high affinity and low nonspecific binding to evaluate total receptor number and receptor-G protein coupling. Furthermore, the mechanisms whereby chronic A_1AR agonist exposure changes the levels of $G_{i\alpha}$ and $G_{s\alpha}$ remain obscure. In this study, we further characterize A_1AR in this model using an A_1AR antagonist radioligand, [^{3}H]XAC (7), and compare it with known models of receptor desensitization. Furthermore, we have measured $G_{s\alpha}$ and $G_{i\alpha}$ protein levels by Western blotting and quantitated specific mRNA levels for several G_α species, in an effort to begin clarifying the mechanism whereby G_α levels are regulated in this model of heterologous desensitization.

Experimental Procedures

Materials. Animals and supplies were purchased as described earlier (6) and as noted below. [^{3}H]XAC (specific activity ~130 Ci/mmol), ^{125}I-Protein A (2–10 μCi/μg), and [α^{32}P]ATP were from Dupont-New England Nuclear. Deoxycytidine 5′-[α^{32}P]triphosphate and [γ-^{32}P] ATP were from Amersham. Chemicals for RNA studies were from the following suppliers. Agarose (type II, EEO medium), salmon sperm DNA, Ficoll (type 400), MOPS, SDS, Tris base, guanidinium isothiocyanate, sodium pyrophosphate, ethidium bromide, and formamide were from Sigma and were molecular biology grade. Glycerol and formaldehyde were from Mallinckrodt. EDTA, sodium citrate, sodium acetate, and 2-mercaptoethanol were from Fluka. Oligo(dT)-cellulose and tRNA were from Bethesda Research Laboratories and cesium chloride was from Bio-Rad.

Animal treatment. Male rats (275–350 g) were weighted and underwent subcutaneous implantation of a miniosmotic pump (Alza Corp., Palo Alto, CA) which delivered 75 nmol/kg/hr (*R*)-PIA. After a 2-, 4-, or 6-day period, treated and sham operated control animals were sacrificed and adipocyte membranes were prepared from epididymal fat pads as previously described (6). Treated animals experienced weight gain similar to that of controls. This is the same model used previously by us (6) and Hoffman *et al.* (5).

Membrane preparation. This was carried out exactly as described previously (6) with the following exceptions. After the first centrifugation, membranes for adenylate cyclase measurements were resuspended in 75 mM Tris (pH 7.4 at 30°), 12.5 mM $MgCl_2$, 2.5 mM dithiothreitol, 200 mM NaCl, 6 units/ml ADA at a protein concentration of ~2.0 mg/ml. For binding, membranes were resuspended in 5.0 mM Tris (pH 7.4 at 37°), 1 mM $MgCl_2$, 0.1 mM EDTA, 6 units/ml ADA, incubated at 37° for 15 min, recentrifuged, and resuspended to ~0.8 mg/ml protein in the same buffer. Total membrane protein yield from isolated adipocytes was approximately 1.0 mg/fat pat, with no significant difference between fat pads from control and treated animals. Membranes were used immediately, without freeze-thaw. Protein determinations were by the method of Bradford (8), using bovine serum albumin as the standard.

Radioligand binding. Saturation binding was carried out as follows. The final reaction volume of 250 μl contained 150 μl of membranes (~120 μg of protein), 50 μl of [^{3}H]XAC diluted to give final concentrations ranging from 0.25 to 5.0 nM of radioligand, and 50 μl of H_2O or 5 $\times$ 10^{-5} M (*R*)-PIA to define total and nonspecific binding, respectively, at each concentration of radioligand.

Competitive binding assays included 150 μl of membranes, 50 μl of [^{3}H]XAC (final concentration ~1.5 nM), and 50 μl of a dilution of (*R*)-PIA to give a range of concentrations from 10^{-10} to 3 $\times$ 10^{-5} M (*R*)-PIA. All incubations were then carried out for 1 hr at 37° and were terminated by rapid filtration onto No. 32 filters (Schleicher & Schuell), which had been presoaked for 1 hr in 0.3% polyethylenimine. Filters were washed with 3 $\times$ 3 ml of ice-cold membrane buffer containing 0.05% CHAPS and then counted using a general purpose liquid scintillation cocktail. All binding experiments were performed with duplicate determinations at each point. The conditions used in these binding experiments were found to minimize nonspecific and maximize specific binding in adipocyte membranes and have been reported elsewhere (9).

Resulting binding data were analyzed with the assistance of a computer using the previously described (10, 11) ALLFIT and SCATFIT programs, which employ general nonlinear, least squares, curve-fitting routines with statistical analysis methods. Analysis of saturation curve data was performed by determining K_D and B_{max} for each animal and then comparing control and treated groups using Student's test. Analysis of competition curves was carried out by normalizing data from all experiments and then subjecting the mean data points for each concentration of competing ligand to computerized curve fitting.

Adenylate cyclase assays were performed exactly as described (6), and cAMP isolation was according to the method of Salomon *et al.* (12). Where indicated, values are expressed as mean ± standard error. Student's test was used for statistical evaluation.

Western blotting. Western blotting was performed by first resolving samples (60–100 μg of protein/lane) on 10% SDS-polyacrylamide gels (stock 30% acrylamide solutions contained 0.4% bis-acrylamide instead of the usual 0.8% to improve resolution of $G_{i\alpha}$ subtypes) (13). Proteins were then transferred to nitrocellulose filters (0.2 μm; Schleicher & Schuell) using a Novoblot apparatus (Pharmacia) at 4 mA/cm^2 for 6–7 hr. The filter was blocked by a 1-hr incubation with 3% gelatin in a buffer of 10 mM Tris (pH 7.5) and 500 mM NaCl. Antibody incubation was performed at a 1/500 dilution in the same buffer, except that 1% gelatin was used. After an incubation of 12–14 hr at 25°, the filter was washed in the same buffer without antibody three times and then incubated with the same buffer and 10^7 cpm of ^{125}I-protein A for 1 hr. The filter was washed with three changes of 50 mM Tris (pH 7.5) and 0.05% Tween 20 (Sigma). Autoradiography was carried out using Kodak X-OMAT AR-5 film and single Cronex Lightening Plus intensifier screens for a period of 18 to 72 hr at −80°. Films were developed using an Aplhatek AX-350 automated developer. The ^{125}I-Protein A signal was detected either by cutting and counting bands or by scanning densitometry (Bio-Rad Model 620 Videodensitometer). Scanning densitometry data are presented in arbitrary units. Control experiments were performed to determine the relationship between the quantity of protein assayed and the signal that was detected. The amount of ^{125}I-Protein A detected was linear with the amount of membrane protein added (data not shown). This ensured that comparisons between normal and desensitized membranes were valid. For $G_{i\alpha1}$ and $G_{i\alpha2}$ blots, which were detected using the same antibody, aggregate values were obtained and expressed as $G_{i\alpha1+2}$, because separate quantitation of the bands was not possible.

RNA preparation. RNA was prepared using the guanidinium/cesium chloride method (14), except that 25 mM sodium citrate was used and sarkosyl was omitted. Total tissue or isolated adipocyte RNA was used as indicated in the text. The average total RNA yield/fat pad was 206 μg of mRNA from both control and treated animals. The total RNA yield for adipocytes isolated from one fat pad was approximately 95 μg, again with no difference between adipocytes from control and treated animals. Messenger RNA was isolated from total RNA by the oligo(dT)-cellulose method (15). Purity and concentration of RNA were

estimated by UV spectrophotometry and samples were stored at −80° in a buffer of 10 mM Tris, 1 mM EDTA (pH 8.0).

RNA electrophoresis and transfer. RNA samples (10–15 μg/lane) were added to 2–4 volumes of RNA sample buffer for a total volume of 25 μl. RNA sample buffer consisted of 160 μl of dye buffer (5.0 ml of glycerol, 5 ml of 10× TBE, 25 mg of bromphenol blue, and 25 mg of xylene cyanol), 720 μl of deionized formamide, 160 μl of 10× MOPS buffer, 260 μl of formaldehyde (37%), and 200 μl H_2O. RNA was denatured by heating this mixture to 90° for 10 min and then resolved by electrophoresis through 1% agarose gels containing 0.2 M formaldehyde, 1× MOPS buffer, and 0.2 μg/ml ethidium bromide. Electrophoresis buffer consisted of 1× MOPS buffer and 0.2 μg/ml ethidium bromide. Agarose gels were photographed under long-wave UV light illumination before transfer. Ribosomal RNA of 18 S and 28 S visualized by ethidium bromide staining, served as internal controls. RNA was then transferred to nylon sheets (Zeta-probe; Bio-rad) using 10× SSC as the transfer buffer and these were then baked for 2 hr at 80° in a vacuum oven.

Probe labeling. DNA probes for $G_{i\alpha 3}$ and $G_{i\alpha 2}$ were derived from an HL60 cDNA library and a U937 cDNA library, respectively. JD43 ($G_{i\alpha 2}$) is a 1078-base pair *Bgl*II/*Eco*RI fragment and JDHL9 ($G_{i\alpha 3}$) is a 966-base pair *Bgl*II/*Eco*RI fragment, as previously described (16, 17). These human cDNAs shared approximately 92% homology with corresponding rat cDNAs (16–20). These were then labeled, using a Random Primed DNA labeling kit (Boehringer Mannheim) and [α^{32}P]dCTP (~3000 Ci/mmol), to a specific activity of 1–3 × 10^7 cpm/μg. Oligonucleotide probes for rat $G_{s\alpha}$ (complementary to bases encoding amino acids 379–394) and $G_{i\alpha 1}$ (complementary to bases encoding amino acids 118–133) (19) were end-labeled using T_4 polynucleotide kinase (Bethesda Research Laboratories), as described (15).

Hybridization and washing of RNA blots. RNA filters were prepared for hybridization by soaking for 2 hr at 42° in 50% formamide, 2× Denhardt's solution, 5× SSC, 1% SDS, and 200 μg/ml salmon sperm DNA. Hybridization to cDNA probes were carried out overnight at 42° with the same buffer and 5–10 × 10^6 cpm of each probe. Oligonucleotide probes were used with the same buffers, except that 0.05% sodium pyrophosphate and 0.1 mg/ml tRNA were included. Filters probes with cDNAs were washed in 2× SSC, 0.1% SDS, for 15 min at room temperature, then 0.5× SSC, 1% SDS, for 15 min at room temperature, and then twice in 0.1× SSC, 0.1% SDS, at 55° for 30 min. Filters probed with oligonucleotides were washed twice with 3.3× SSC, 0.01% sodium pyrophosphate, 0.1% SDS, at room temperature for 20 min, then for 30 min in the same buffer at 55°, and finally with 2× SSC, 0.1% SDS, at 55° for 30 min. Autoradiography was carried out as described above.

As a control, poly(A)$^+$ mRNA was prepared from total RNA using standard oligo(dT)-cellulose chromatography, as described (15). Poly(A)$^+$ and poly(A)$^-$ fractions were then resolved on formaldehyde/agarose gels, transferred to nylon filters, and hybridized to either cDNA or oligonucleotide probes, as described above. In each case, it was demonstrated that the transcript detected in the total RNA was only seen in the poly(A)$^+$ fraction and not in the poly (A)$^-$ fraction. Dilutional Northern analysis was carried out by diluting total RNA with 10 mM Tris, 1 mM EDTA, pH 8.0, and then adding the same volume of RNA sample buffer to each sample with further processing as described above. Specific transcripts were quantitated using scanning densitometry of the hybridized filter autoradiographs. Equal quantities of total RNA were loaded onto each lane, as assessed by UV spectrophotometry, by ethidium bromide staining, and by probing with a α-tubulin cDNA.

Results

Time Course of Adenylate Cyclase Changes

The time course of adenylate cyclase changes was investigated by assaying adipocyte membranes at 2 and 4 as well as 6 days of (*R*)-PIA treatment. Table 1 shows these data. Of note, the changes in basal, forskolin-stimulated, and (*R*)-PIA-inhibited activities did not occur simultaneously but rather sequentially. There was no significant difference in any of the activities measured at day 2 when compared with control. At 4 days, there was a significant difference in basal activity while differences in forskolin-stimulated activity in this series (five experiments) were not statistically significant. However, in a larger series (14 experiments) looking only at the day 4 time point, forskolin-stimulated adenylate cyclase activity was significantly different ($p < 0.02$) in adipocytes from treated (294 ± 28 pmol/mg/min) when compared with control (224 ± 15 pmol/mg/min) animals. Notably, in all studies, (*R*)-PIA inhibition of forskolin-stimulated adenylate cyclase activity was not changed at days 2 or 4. As Table 1 shows, only 6 full days of (*R*)-PIA treatment results in loss of (*R*)-PIA inhibition, which falls dramatically by 73%.

A_1AR Quantitation

Although previous experiments using an agonist radioligand have shown a reduction in the number of high affinity agonist binding sites in adipocyte membranes after 6 days of treatment (6), it was necessary to utilize an antagonist radioligand to determine the actual A_1AR density. Representative saturation binding data comparing [^{3}H]XAC binding in adipocyte membranes from animals at 6 days of (*R*)-PIA exposure and controls are shown in Fig. 1. This time point was chosen because it was the only one where significant changes in the ability of (*R*)-PIA to inhibit adenylate cyclase were found. There was a 47% decrease in the total A_1AR number (from 1.39 ± 0.19 pmol/mg of protein to 0.74 ± 0.13 pmol/mg; eight experiments; $p < 0.02$) in desensitized membranes when compared with control, but no significant change in K_D (control, 1.39 ± 0.37 nM; treated, 0.72 ± 0.11 nM; eight experiments; $p > 0.1$).

With the availability of a high affinity antagonist radioligand, agonist competition analysis is now feasible. This was performed on seven pairs of control and treated animals both in the presence and absence of guanyl nucleotides. Fig. 2 shows binding in adipocyte membranes from animals treated for 6 days and from controls. The agonist competition curve in membranes from treated animals is steeper and shifted to the right when compared with control (curves were normalized for graphic representation). Each curve is best described by a model demonstrating two agonist affinity states of high (K_H) and low (K_L) affinity. Although K_H and K_L are not significantly different between the curves, there is a significant decrease, from 55 to 35%, in the percentage of receptors occupying the high affinity state ($\%R_H$) in desensitized membranes when compared with control (Table 2). In the presence of guanyl nucleotides, both treated and control curves were steepened and shifted to the right, as seen in the *inset* of Fig. 2. There was no significant difference in K_H, K_L, or $\%R_H$ between these curves when computer modeling was performed (data not shown).

Western Blotting of G proteins

Western blots were first performed using adipocyte membranes from day 6 treated animals and controls. The availability of specific antibodies that detect $G_{s\alpha}$[1] $G_{i\alpha 1+2}$ (13), and $G_{i\alpha 3}$[1] permits quantitation of these proteins in normal and desensitized membranes using the Western blot technique. This technique circumvents many of the difficulties and limitations

[1]P. Goldsmith, C. Unson, and A. Spiegel, manuscript in preparation.

TABLE 1

Time course of adenylate cyclase changes during (*R*)-PIA treatment

Animals were treated with (*R*)-PIA for the indicated times and sacrificed and adipocyte membranes were prepared as described in the text. The data given below are for basal, forskolin (10^{-5} M)-stimulated, and (*R*)-PIA (10^{-6} M)-mediated inhibition of forskolin-stimulated adenylate cyclase activity. Data from five experiments, each consisting of one animal at each time point, are shown.

	Day 0 (control)	Day 2	Day 4	Day 6
Basal (pmol of cAMP/mg/min)	12.8 ± 1.1	22.1 ± 5.2[a]	65.8 ± 14.3[b]	77.1 ± 22.3[b]
Forskolin (pmol of cAMP/mg/min)	212 ± 14	242 ± 14[a]	319 ± 46[a]	420 ± 14[b]
(*R*)-PIA (% inhibition of forskolin-stimulated activity)	44 ± 4	44 ± 7[a]	35 ± 2[a]	12 ± 3[b]

[a] Not significantly different compared with control.
[b] $p < 0.05$ when compared with control.

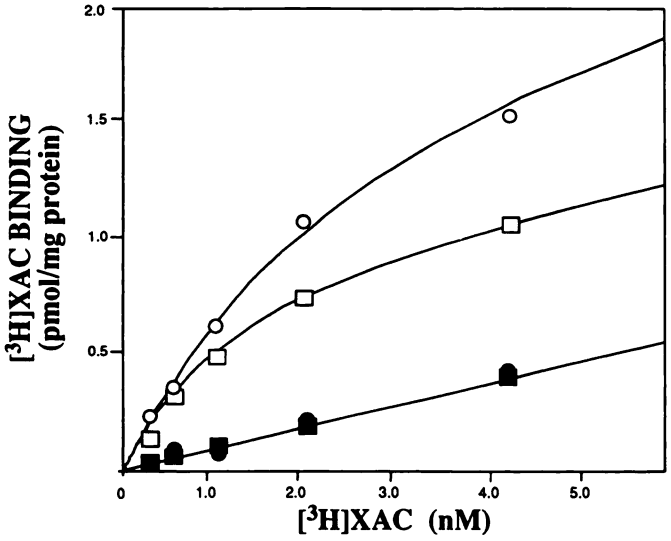

Fig. 1. [^{3}H]XAC saturation binding in adipocyte membranes from (*R*)-PIA-treated and control rats. Adipocyte membranes from 6-day treated rats and concurrent controls were prepared as described in Experimental Procedures. [^{3}H]XAC was added to the concentrations given on the *abscissa*. Nonspecific binding was defined by the addition of 10^{-5} M (*R*)-PIA. After incubation at 37° for 60 min., samples were washed on filters and counted as described. The *open* and *filled circles* represent total and nonspecific binding in control membranes, respectively. The *open* and *filled squares* represent total and nonspecific binding in adipocyte membranes from treated animals. The data shown here are the means of duplicates from an experiment that is representative of the seven performed. Composite data from all seven experiments are found in Results. Curves were modeled with the aid of a computer (SCATFIT), as described (9, 10).

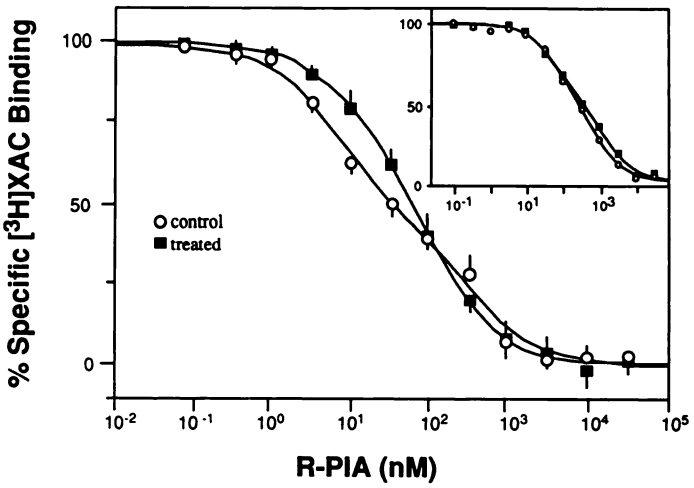

Fig. 2. Competitive binding of (*R*)-PIA in adipocyte membranes from treated and control rats. Binding was carried out using 1.5 nmol of [^{3}H]XAC and the indicated concentration of (*R*)-PIA. Data from control (○) and day 6 treated animals (■) are included. The data shown are the mean values of normalized data from seven experiments. The *error bars* show the standard error for each point. The curves were generated by a computer modelling program (SCATFIT, ALLFIT) and their analysis is found in Table 2. *Inset*, competitive binding in the presence of 10^{-5} M 5′-guanylylimidodiphosphate is shown. These data are the mean values of normalized curves from seven experiments. The *axes* and *symbols* are the same as those in the larger figure.

TABLE 2

Computer analysis of competitive A_1AR binding in adipocyte membranes from control and six-day desensitized animals

K_H, high affinity dissociation constant; K_L, low affinity dissociation constant. These data are derived from seven separate competitive binding experiments.

	Control	Treated	
K_H (nM)	3.5 ± 0.8	5.9 ± 2.7	NS[a]
K_L (nM)	175 ± 52	69 ± 27	NS
$\%R_H$	55 ± 5	35 ± 8	$p < 0.002$

[a] NS, not significant.

implicit in the toxin labeling experiments previously reported (6). For example, pertussis toxin labels not only the three subtypes of $G_{i\alpha}$ but also $G_{o\alpha}$, which may exist in 10-fold excess over $G_{i\alpha}$ in membranes (21). Quantitation of $G_{s\alpha}$ was performed using a polyclonal antibody (RM/1) raised against a synthetic peptide representing the carboxyterminal decapeptide of $G_{s\alpha_3}$ as deduced from its cDNA.[1] Western blotting with this $G_{s\alpha}$-specific antibody (Fig. 3A) confirmed previous data (6) by showing a 49 ± 10% increase in $G_{s\alpha}$ (12 experiments; $p < 0.001$) in membranes from animals treated for 6 days when compared with controls.

When an antibody (AS/7) (13) that recognizes $G_{i\alpha 1+2}$ was used (Fig. 3B), a 59 ± 5% reduction (eight experiments; $p < 0.001$) was found in $G_{i\alpha 1+2}$ in membranes from animals undergoing 6 days of treatment, when compared with controls. This decrease is greater than that found previously by pertussis toxin labeling (6), which showed a 37% decrease in desensitized membranes. It is also noteworthy, as seen in Fig. 3C, that the doublet seen corresponding to $G_{i\alpha 1}$ at 41 kDa and $G_{i\alpha 2}$ at 40 kDa is reduced uniformly, suggesting that both subtypes of $G_{i\alpha}$ are decreased in desensitized membranes.

The time course of the decrease in $G_{i\alpha 1+2}$ is seen in Fig. 4A. Animals were examined after 0 (control), 2, 4, and 6 days of (*R*)-PIA infusion. The decline was gradual, so that at each time point there was less $G_{i\alpha 1+2}$ than at the one before, with the maximum decrease being found at day 6. The time course of the increase in $G_{s\alpha}$ is seen in Fig. 4C. This species is increased in membranes from treated animals at the first time point

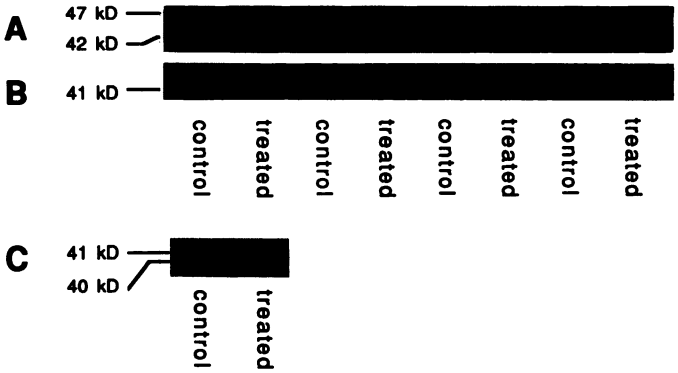

Fig. 3. Western blots of adipocyte membranes from control and desensitized animals. Adipocyte membranes from control animals and animals treated for 6 days with (*R*)-PIA were subjected to electrophoresis on 10% SDS-polyacrylamide gels. Equal amounts of protein were loaded in each lane. Proteins were then transferred to nitrocellulose filters and subjected to Western blot analysis as described in Experimental Procedures. A, A resultant autoradiogram when a polyclonal antibody specific for $G_{s\alpha}$ (RM/1) was used. This antibody identifies two bands that exactly comigrate with cholera toxin substrates in adipocyte membranes. B, Results when the same membranes where run on gels, transferred, and then probed with polyclonal antibodies specific for $G_{i\alpha1+2}$ (AS/7). C, An enlargement in which resolution of $G_{i\alpha1}$ and $G_{i\alpha2}$ can be discerned.

examined, day 2, and reaches its maximum increase at day 4, with no further change at day 6.

In contrast to the changes seen in $G_{i\alpha1}$ and $G_{i\alpha2}$, $G_{i\alpha3}$ did not change at any point during the 6-day course of (*R*)-PIA treatment. This is seen in Fig. 4B, which shows a Western blot in which an antibody (EC/2)[1] directed against the terminal decapeptide of $G_{i\alpha3}$ was used. At 2, 4, and 6 days of treatment, no significant change was found in this subtype when compared with control.

Northern Analysis

To elucidate the mechanism whereby G_α subunit levels are modulated in desensitized membranes following treatment with an A_1AR agonist, we assessed G_α mRNAs by Northern analysis. In order to ensure that tissue used for RNA preparation was desensitized, we utilized the following approach: pairs of control and treated animals following a 6-day infusion of (*R*)-PIA were killed and one epididymal fat pad from each was immediately processed with RNA extraction buffer while the other was used for membrane preparation and subsequent adenylate cyclase assays. Tissue from animals whose adipocyte membrane preparation demonstrated heterologus desensitization, as determined by a diminished capacity of (*R*)-PIA to inhibit adenylate cyclase activity and an enhanced effect of stimulatory agents, were further processed for Northern analysis. This approach was necessary because ~10% of animals from this series did not demonstrate behavior typical of desensitization. In nearly all cases, this could be attributed to malfunction of the miniosmotic pump or to concurrent illness of the animal.

$G_{s\alpha}$ blots. An oligonucleotide (48-mer) was synthesized based on sequence from the distal third of the $G_{s\alpha}$ (bases encoding amino acids 379–394) and was used as a probe (19). Fig. 5 shows a representative experiment. The $G_{s\alpha}$ mRNA migrates at 1.8 kilobases, in agreement with previously published data for this transcript (20). When seven pairs of control and treated animals are compared and the amount of RNA loaded into each lane was accounted for by measurement of α-tubulin and ethidium bromide staining, there was no significant difference in the

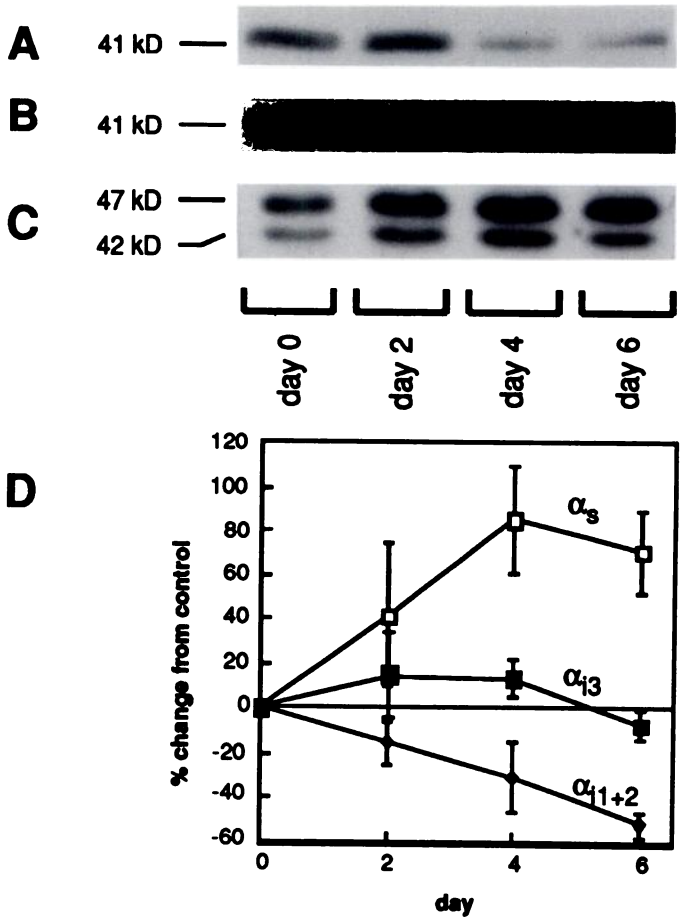

Fig. 4. Western blot analysis of the changes in G_α subunits in adipocytes over time during desensitization. Animals were killed after 0 (control), 2, 4, and 6 days of exposure to (*R*)-PIA and adipocyte membranes were prepared. Each experiment (five in total) consisted of one rat at each of the time points indicated and antibodies against $G_{i\alpha1+2}$ (AS/7), $G_{i\alpha3}$ (EC/2), and $G_{s\alpha}$ (RM/1) were used for Western analysis, as described in the text and in Fig. 3. The figure shows autoradiographs of resultant blots (A, $G_{i\alpha1+2}$; B, $G_{i\alpha3}$; C, $G_{s\alpha}$) from one of four such experiments performed. The graph (D) shows mean data ± standard errors from all four experiments where Western blots were analyzed either by scanning densitometry of autoradiograms or by cutting and counting of radiolabeled bands.

amount of $G_{s\alpha}$ mRNA found in adipose tissue when quantitated by scanning densitometry (control, 3.29 ± 0.29; treated, 2.92 ± 0.23; $p = 0.49$). This contrasts with a significant increase in the quantity and function of $G_{s\alpha}$ protein found in adipocytes from animals treated in the same manner.

$G_{i\alpha}$ blots. Recent reports have established the existence of at least three different mRNAs ($G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$) (13, 16–20) encoding what had previously been thought to be only one $G_{i\alpha}$ polypeptide. Unlike the multiple forms of $G_{s\alpha}$, it appears that the subtypes of $G_{i\alpha}$ originate from different genes (18, 20) rather than alternative splicing of a single gene. The functional significance of these subtypes is unclear, however; it has been suggested by Birnbaumer and colleagues (22) that $G_{i\alpha3}$ couples muscarinic receptors to a K^+ channel in atrial myocytes. Because it is not clear which of the forms of $G_{i\alpha}$ is responsible for inhibition of adenylate cyclase, probes for all three were used in Northern analysis.

The first interesting finding was that adipocytes contain transcripts for all three forms of $G_{i\alpha}$ (Fig. 6). Fig. 6, B and C, shows representative blots using random prime-labeled cDNA

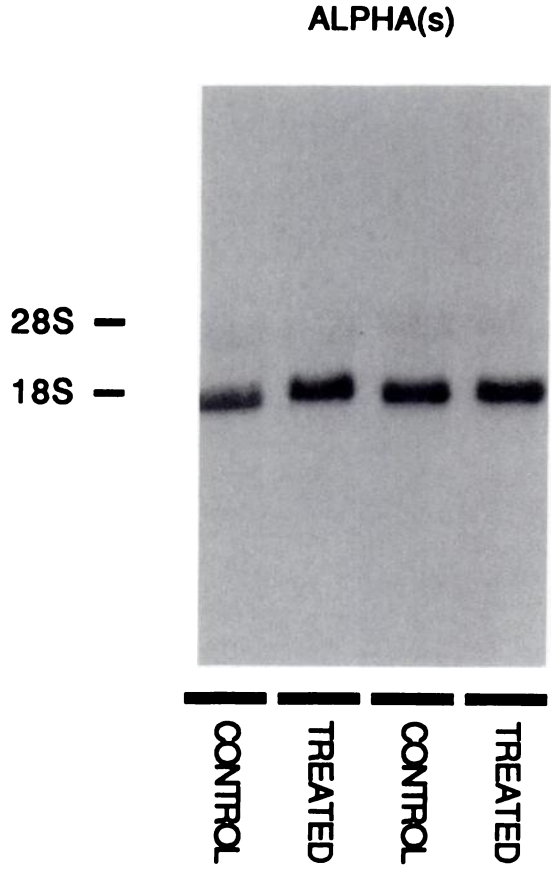

Fig. 5. Northern blot of total RNA from normal and desensitized adipose tissue using a probe for $G_{s\alpha}$ mRNA. Total RNA (~10 μg) from fat pads of two pairs of control and 6-day (*R*)-PIA-treated animals were resolved on agarose gels, transferred to nylon filters, and probed with a radiolabeled oligonucleotide complementary to a 48-base pair sequence of the 3′ region of $G_{s\alpha}$ mRNA. Migration of the 18 S and 28 S ribosomal RNA is shown. These two pairs of data are representative of seven that were analyzed. Scanning densitometry of all seven pairs of data (see Results) showed no significant difference in the amount of this transcript in fat pads from treated and control animals.

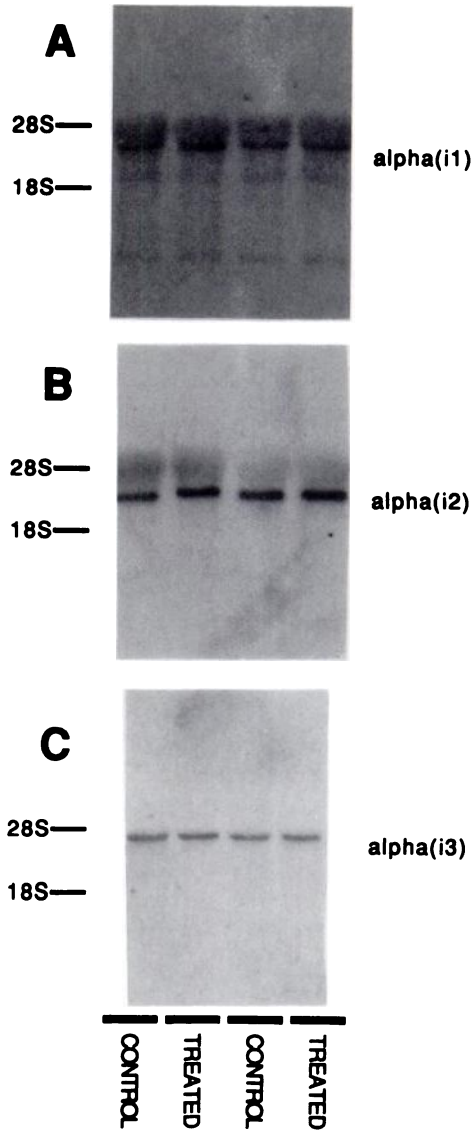

Fig. 6. Analysis of $G_{i\alpha}$ transcripts. The same RNA filter seen in Fig. 5 was stripped and then underwent hybridization with probes specific for $G_{i\alpha 1}$, $G\alpha 2$, and $G_{i\alpha 3}$. A, The resultant autoradiogram when a 48-mer oligonucleotide specific for $G_{i\alpha 1}$ was hybridized to the filter. B and C, Autoradiograms when cDNAs complementary to mRNAs for $G_{i\alpha 2}$ and $G_{i\alpha 3}$, respectively, were used. Scanning densitometry data from seven pairs of adipocyte RNA samples from control and 6-day treated animals (see Results) showed no significant difference in the quantity of the mRNAs encoding any of the three $G_{i\alpha}$ proteins.

probes for $G_{i\alpha 2}$ and $G_{i\alpha 3}$, respectively. Transcripts migrate at 2.6 kilobases for $G_{i\alpha 2}$ and 3.6 kilobases for $G_{i\alpha 3}$. When the amount of RNA loaded into each lane was accounted for, there was no difference between treated and control in the relative amount of mRNA, as assessed by scanning densitometry, for either $G_{i\alpha 2}$ (control, 2.38 ± 0.43; treated, 2.84 ± 0.48; $p = 0.57$) or $G_{i\alpha 3}$ (control, 0.73 ± .05; treated, 0.98 ± 0.20; $p = 0.37$). A synthetic oligonucleotide (48-mer) spanning a unique sequence of $G_{i\alpha 1}$ (bases encoding amino acids 118–133) was used lastly (Fig. 6A). A 3.4-kilobase transcript was delineated and no difference was seen in the quantity of transcript for $G_{i\alpha 1}$ in adipocytes from treated or control animals (control, 0.76 ± 0.09; treated, 0.67 ± 0.04; $p = 0.45$). It should be pointed out that unique and specific probes for $G_{i\alpha 1}$ and $G_{i\alpha 3}$ mRNAs were used to establish that, although these transcripts have similar (but not identical) sizes, they are distinct messages. Therefore, the data seen in Fig. 5, A and C, are not an artifact of cross-hybridization.

Because changes in $G_{s\alpha}$ and $G_{i\alpha}$ proteins were seen at early time points (*vide supra*), Northern analysis was performed using total RNA from fat pads from control and treated animals with probes for $G_{s\alpha}$ and $G_{i\alpha 2}$. These results (data not shown) agreed with those obtained for 6 days of treatment, i.e., there was no difference in the quantity of either transcript. Thus, in treated animals, at times when changes in G_{α} subunits are significantly less ($G_{i\alpha 1+2}$) or greater ($G_{s\alpha}$) than control, there is no change in the levels of their respective transcripts.

As a control, total RNA was prepared from isolated adipocytes from control and treated animals and subjected to the same analysis described above for the total fat pad, to exclude the possibility that RNA from nonadipocyte cells was masking changes in RNA from the adipocytes themselves. RNA yields from adipocytes isolated using the standard collagenase digestion (6) were a minimum of ~50% compared with the whole fat pad. Further, there was no difference in the levels of any of the $G_{i\alpha}$ or $G_{s\alpha}$ transcripts in adipocyte RNA preparations from

control and treated animals. In order to ensure that the results of Northern analysis were not an artifact caused by an excess of RNA loaded onto the gels, dilutions of RNA samples were loaded onto agarose gels and transferred to nylon filters. RNA from normal and desensitized adipocytes demonstrated equivalent labeling over a 20-fold range of loaded RNA when probes for $G_{s\alpha}$ and $G_{i\alpha 2}$ were used. The amount of labeling was linear with the amount of RNA loaded onto the gels (data not shown). These controls confirm the conclusion that there is no difference in $G_{s\alpha}$ and $G_{i\alpha}$ mRNA levels from control and desensitized adipocytes.

Discussion

Perhaps the most interesting and novel event involved in the process of desensitization in the A_1AR-adenylate cyclase system is the time-dependent regulation of the quantity and function of G protein α-subunits and, thus, the activity of adenylate cyclase. Following a 2-day treatment with (*R*)-PIA, at a time when there are trends toward an increase of $G_{s\alpha}$ and a decrease of $G_{i\alpha 1+2}$, there are no significant changes in basal or forskolin-stimulated adenylate cyclase activity or in the ability of (*R*)-PIA to inhibit forskolin-stimulated activity. By day 4 of treatment when there are substantial changes in $G_{s\alpha}$ and $G_{i\alpha 1+2}$, only basal and forskolin-stimulated adenylate cyclase activities are enhanced. It is not until day 6, when the largest decreases in $G_{i\alpha 1+2}$ (59%) are observed, that significant loss in the ability of (*R*)-PIA to inhibit adenylate cyclase occurs. It appears that the stimulatory G protein components of adenylate cyclase respond more rapidly than the inhibitory G proteins and that substantial alterations in the quantities of α-subunits must occur before an effect on adenylate cyclase activity is evident. These data are consistent with results found in a model of pertussis toxin-treated hepatocytes by Ui and colleagues (23). They found that greater than 50% of $G_{i\alpha}$ had to be ADP-ribosylated before a significant loss of receptor-mediated inhibition of adenylate cyclase activity could be observed. We cannot exclude the possibility that a change in A_1AR from day 4 (not measured) to day 6 (see below) also has an effect in the precipitous loss of A_1AR-mediated inhibition of adenylate cyclase.

The availability of a high affinity A_1AR antagonist radioligand has allowed us to characterize receptor changes associated with heterologous desensitization of the rat adipocyte A_1AR. The changes found following 6 days of (*R*)-PIA treatment include a reduction of the total number of receptors as well as an uncoupling of the receptor from its G protein, indicated by a reduction in the percentage of receptors in the high affinity state. These data are consistent with our previous observations but extend the findings markedly.

Using Western analysis, with antibodies specific for the various subtypes of $G_{i\alpha}$, we have been able to document that there is differential regulation of these subtypes. While $G_{i\alpha 1}$ and $G_{i\alpha 2}$ are clearly decreased, $G_{i\alpha 3}$ is unperturbed at all time points observed. One possible explanation for the lack of change of $G_{i\alpha 3}$ is that the A_1AR is not coupled to $G_{i\alpha 3}$ but this hypothesis cannot be pursued until specific functions are assigned for the $G_{i\alpha}$ subtypes in this system. It is possible, however, to drawn some inferences about the coupling of $G_{i\alpha}$ subtypes with effector mechanisms. This system typifies a heterologous type of desensitization, i.e., multiple receptors that are inhibitory to adenylate cyclase have diminished effect following exposure to an agonist of one receptor type. This refractoriness could possibly be caused by the reduction in the function or quantity of a component that is common to the mechanism utilized by all inhibitory receptors, namely the $G_{i\alpha}$ protein. Because $G_{i\alpha 1}$ and $G_{i\alpha 2}$ are decreased whereas $G_{i\alpha 3}$ is unchanged, it is possible that in this system either $G_{i\alpha 1}$ and/or $G_{i\alpha 2}$ mediates the inhibition of adenylate cyclase, whereas $G_{i\alpha 3}$ may not.

Although data are presented that show concurrent changes in A_1AR and G proteins during prolonged A_1 adenosine agonist changes, we are not able to prove a causal relationship between chronic receptor occupancy and the alterations described. Because this is an *in vivo* model, complex alterations in the neuroendocrine axis of the animals cannot be excluded as an explanation. However, there are several reasons why we believe the alterations are a consequence of chronic receptor activation. These include the fact that changes seen are similar to those described in other models of desensitization (4). Furthermore, and more importantly, in a study where isolated adipocytes were incubated *in vitro* with (*R*)-PIA over several days, Green (24) demonstrated a decrease in A_1ARs by agonist binding and decreased $G_{i\alpha}$ by pertussis toxin labeling (24), suggesting that the changes are due to a mechanism involving only the adipocyte itself.

We also addressed the issue of mechanisms whereby G proteins are regulated during desensitization of the A_1AR-adenylate cyclase system. The availability of nucleotide sequence data and of cDNAs for the α-subunits of G_i and G_s provides the opportunity to determine whether the changes observed in α-subunits are due to changes in the levels of their respective mRNAs. We selected probes that, in total, should hybridize to mRNAs for all known forms of the G_α subunit mRNAs.

Using Northern analysis, we have determined that there is no change in the quantity of mRNA encoding any of the α-subunits ($G_{s\alpha}$, $G_{i\alpha 1}$, $G_{i\alpha 2}$ and $G_{i\alpha 3}$) at any time point during desensitization (Fig. 5 and 6). Thus, at times when there are dramatic increases in $G_{s\alpha}$ and decreases in $G_{i\alpha 1}$ and $G_{i\alpha 2}$, there are no alterations in their mRNAs. The fact that $G_{s\alpha}$ and $G_{\alpha 1+2}$ are regulated in opposite directions and no changes are seen in either transcript even by dilution methods suggests that the regulation in this system is not brought about by changes in transcript levels and that the desensitization process involves modulation of G protein α-subunits by a posttranscriptional mechanism. There is, of course, precedent for posttranscriptional control of protein levels. Regulation has been found to occur during translational events, by means of increased efficiency of translation or by changes in RNA stability (25, 26), and during posttranslational events (26). One or more of these mechanisms may be relevant to the G_α subunits in A_1AR desensitization.

This study extends our previous investigation into the mechanisms of desensitization of inhibitory receptor adenylate cyclase systems. It is now clear that chronic activation of the A_1AR by agonists can result in receptor down-regulation, receptor-G protein uncoupling, and modulation of α-subunit quantity. The data in this study suggest that G protein α-subunit levels may be posttranscriptionally regulated. Further work will be required to elucidate the mechanism(s) by which G protein α-subunit levels are modulated.

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References

1. Daly, J. W., Y. Kuroda, J. W. Phyllis, H. Shimiza, and M. Ui. *Physiology and Pharmacology of Adenosine Derivatives*. Raven Press, New York (1983).
2. Stiles, G. L. Adenosine receptors: structure, function and regulation. *Trends Pharmacol. Sci.* **87:**486–490 (1986).
3. Daly, J. W. Adenosine receptors. *Adv. cyclic Nucleotide Protein Phosphorylation Res.* **19:**29–46 (1985).
4. Sibley, D. R., and R. J. Lefkowitz. Molecular mechanisms of receptor desensitization using the *beta*-adrenergic receptor-coupled adenylate cyclase system as a model. *Nature (Lond.)* **317:**124–129 (1985).
5. Hoffman, B. B., H. Chang, E. Dalli Aglio, and G. M. Reaven. Desensitization of adenosine receptor-mediated inhibition of lipolysis: the mechanism involves the development of enhanced cyclic adenosine monophosphate accumulation in tolerant adipocytes. J. Clin. Invest. **78:**185–190 (1986).
6. Parsons, W. J., and G. L. Stiles. Heterologous desensitization of the inhibitory A_1 adenosine receptor-adenylate cyclase system in rat adipocytes. *J. Biol. Chem.* **262:**841–847 (1987).
7. Jacobson, K. A., D. Ukena, K. L. Kirk, and J. W. Daly. A [^{3}H]xanthine amine congener of 1,3-dipropyl-8-phenylxanthine: an antagonist for adenosine receptors. *Proc. Natl. Acad. Sci. USA* **83:**4089–4093 (1986).
8. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.* **72:**248–254 (1976).
9. Bumgarner, J. R., V. Ramkumar, and G. L. Stiles. Altered thyroid status regulates the adipocyte A_1 adenosine receptor-adenylate cyclase system. *Life Sci.* **44:**1705–1712 (1989).
10. De Lean, A., P. J. Munson, and D. Rodbard. Simultaneous analysis of families of sigmoidal curves: applications to bioassay, radioligand assay and physiological dose-response curves. *Am. J. Physiol.* **235:**E97-E102 (1978).
11. DeLean, A., A. A. Hancock, and R. J. Lefkowitz. Validation and statistical analysis of a computer modeling method for quantitative analysis of radioligand binding data for mixtures of pharmacologic receptor subtypes. *Mol. Pharmacol.* **21:**5–16 (1982).
12. Salomon, Y., C. Londos, and M. Rodbell. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58:**541–548 (1974).
13. Goldsmith, P., K. Rossiter, A. Carter, W. Monds, C. G. Unson, R. Vinitsky, and A. M. Spiegel. Identification of the GTP-binding protein encoded by G_{i3} complementary DNA. *J. Biol. Chem.* **263:**6476–6479 (1988).
14. Maniatis, T., E. F. Fritsch, and J. Sambrook. *Molecular cloning*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).
15. Davis, G. D., M. D. Dibner, and J. F. Batte. *Basic Methods in Molecular Biology*. Elsiever Science Publishing Company, New York (1986).
16. Didsbury, J. R., Y.-S. Ho, and R. Snyderman. Human G_i protein *alpha*-subunit: deduction of amino acid structure from cloned cDNA structure. *FEBS Lett.* **211:**160–164 (1987).
17. Didsbury, J. R., and R. Snyderman. Molecular cloning of a new human G protein: evidence for two G_i *alpha*-like protein families. FEBS Lett. **219:**259–263 (1987).
18. Jones, D. T., and R. R. Reed. Molecular cloning of five GTP-binding protein cDNA species from rat olfactory neuroepithelium. *J. Biol. Chem.* **262:**14241–14249 (1987).
19. Brann, M. R., R. M. Collins, and A. Spiegel. Localization of mRNAs encoding the α-subunits of signal-transducing G-proteins within rat brain and among peripheral tissues. FEBS Lett. **222:**191–198 (1987).
20. Itoh, H., R. Joyama, T. Kozasa, T. Sukamoto, M. Matsuoka, and Y. Kaziro. Presence of three distinct molecular species of G_i protein α-subunit. *J. Biol. Chem.* **263:**6656–6664 (1988).
21. Steinweis, P. C., and J. Robishaw. Isolation of two proteins with high affinity for guanine nucleotides in membranes from bovine brain. *J. Biol. Chem.* **259:**13806–13813 (1984).
22. Codina, J., J. Olate, J. Abramowitz, R. Mattera, R. G. Cook, and L. Birnbaumer. a_i-3 cDNA encodes the α-subunit of G_k, the stimulatory G protein of receptor-regulated K^+ channels. *J. Biol. Chem.* **263:**6746–6750 (1988).
23. Okajima, F., Y. Takumitsw, Y. Kondo, and M. Ui. P_2-purinergic receptors are coupled to two signal transduction systems leading to inhibition of cAMP generation and to production of inositol trisphosphate in rat hepatocytes. *J. Biol. Chem.* **262:**13483–13490 (1987).
24. Green, A. Adenosine receptor down-regulation and insulin resistance following prolonged incubation of adipocytes with an A_1 adenosine receptor agonist. *J. Biol. Chem.* **262:**15702–15707 (1987).
25. Jacobs-Lorena, M., and H. M. Fried. Translational regulation of ribosomal protein regulation, in *Translational Regulation of Gene Expression* (J. Ilan, ed.), Chap. 4. Plenum Press, New York (1987).
26. Matthews, M. B. *Translational Control*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1985).

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