G<sub>i</sub> Down-regulation as a Mechanism for Heterologous Desensitization in Adipocytes*

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Prolonged incubation of rat adipocytes with (-)N<sup>6</sup>-phenylisopropyl adenosine (PIA) (an A<sub>1</sub> adenosine receptor agonist) leads to down-regulation of each of the three subtypes of G<sub>i</sub> (Green, A., Johnson, J. L., and Milligan, G. (1990) J. Biol. Chem. 265, 5206-5210). To determine whether other inhibitors of adenylcyclase would have similar actions, we incubated adipocytes in primary culture with PIA, prostaglandin E<sub>1</sub> (PGE<sub>1</sub>), or nicotinic acid. After various times cells were homogenized, and crude membrane fractions were analyzed on Western blots using antipeptide antisera to α<sub>2</sub> and β-subunits of G-proteins (SG1 (which binds to α<sub>1</sub> and α<sub>2</sub>), I3B (which binds to α<sub>3</sub>), BN2 (binds to β-subunits) and CS1 (recognizes forms of α<sub>2</sub>)). PIA and PGE<sub>1</sub> caused approximately 90% down-regulation of α<sub>1</sub> and α<sub>3</sub>, and about 50% loss of α<sub>2</sub> and β-subunits. In contrast, nicotinic acid at concentrations up to 1 mM had no effect on levels of any of these G<sub>i</sub> subtypes. None of the compounds altered levels of either a 43- or 47-kDa form of α<sub>2</sub>. PIA caused about a 50% decrease in binding of [3H]DPCPX (an A<sub>1</sub> adenosine receptor antagonist), indicating adenosine receptor down-regulation; however, neither PGE<sub>1</sub> nor nicotinic acid treatment altered [3H]DPCPX binding. None of the treatments affected the activity of adenylcyclase when measured in the presence of 100 μM forskolin and 10 mM Mn<sup>2+</sup>, indicating that the catalytic subunit of adenylcyclase is not altered.

To determine whether G<sub>i</sub> down-regulation results in heterologous desensitization, we incubated adipocytes with maximally effective concentrations of PIA (300 nM), PGE<sub>1</sub> (3 μM), or nicotinic acid (1 mM) for 4 days. The cells were then washed and incubated for an additional 30 min with various concentrations of these compounds to determine their ability to inhibit lipolysis. PIA caused a (marked) decrease in the sensitivity of the cells to both PIA and PGE<sub>1</sub>, thus indicating heterologous desensitization. Similarly, PGE<sub>1</sub> decreased the sensitivity of the cells to both PGE<sub>1</sub> and PIA, again demonstrating heterologous desensitization. In contrast, prolonged incubation with nicotinic acid decreased the sensitivity of the cells to nicotinic acid but had no effect on the sensitivity of the cells to PIA.

Adenylylcyclase in membranes from PGE<sub>1</sub>-treated cells showed decreased sensitivity to inhibition by PIA. In contrast, adenylylcyclase showed normal sensitivity to PIA in membranes from nicotinic acid-treated cells. Together with the finding that PGE<sub>1</sub> has no effect on either A<sub>1</sub> adenosine receptors nor on the catalytic subunit of adenylylcyclase, these findings suggest that heterologous desensitization of lipolysis is at least partly due to down-regulation of the G-protein(s) responsible for inhibition of adenylylcyclase.

It is well established that exposure of a cell to an agonist can cause desensitization, so that a second exposure to the agonist is less effective than the first. This phenomenon has been studied extensively, and various mechanisms have been described. First, relatively short term exposure to a ligand (minutes) can induce a change in the receptor. For example, β-adrenergic receptors can become phosphorylated by a specific enzyme (β-adrenergic receptor kinase) after exposure to an agonist (1, 2). Second, after more prolonged exposure to an agonist, the number of receptors on the cell surface can decrease, by a process termed down-regulation. Down-regulation is generally considered to occur in two steps. First, fairly rapid sequestration of receptors from the cell surface through endocytosis. This is followed by a slower loss of total cellular receptors involving intracellular degradation, although at least a portion are often recycled back to the cell surface (3, 4). In addition to increased degradation of receptors, recent studies have suggested that alterations in receptor mRNA turnover may also play a role (5, 6).

The phenomena described above can readily explain homologous desensitization, where exposure of a cell to a hormone results in subsequent insensitivity to that hormone. Another commonly reported phenomenon is known as heterologous desensitization, in which treatment of a cell with a hormone subsequently can make the cell less sensitive to another hormone that works through a different, distinct receptor. Heterologous desensitization has been described for many signaling systems, including receptors coupled to adenylylcyclase (7-9) and to phospholipase C (10, 11). Mechanisms of heterologous desensitization, however, are relatively poorly understood.

We reported that A<sub>1</sub> adenosine receptors can be down-regulated in rat adipocytes following prolonged incubation
with an agonist, namely PIA\(^1\) (12). The \(\alpha_1\) adenosine receptor is coupled to inhibition of adenylyl cyclase through one or more of a group of GTP-dependent regulatory proteins (G-proteins), termed \(G_i\), of which three subtypes have been identified, termed \(G_i1, G_i2,\) and \(G_i3\).

By pertussis toxin labeling we found that, in addition to adenosine receptor down-regulation, chronic exposure of adipocytes to PIA causes selective loss of \(G_i\) from the cells (12). More recently, we have used a series of specific antisera to demonstrate that PIA causes a marked (approx 90%) loss of \(G_i1\) and \(G_i3\), with a more modest (50%) loss of \(G_i2\) (13). When PIA is washed away, \(G_i\) down-regulation is reversible, suggesting that it is a real regulatory phenomenon rather than a simple toxic effect. Furthermore, Stiles and co-workers (14, 15) have reported similar findings after chronic infusion of PIA into rats \(in vivo\).

More recent studies suggest that G-protein down-regulation is a common phenomenon resulting from chronic exposure to agonists. Thus, down-regulation of \(G_i2\) was observed following prolonged treatment of hamster smooth muscle DDT1 MF-2 cells with PIA (16). \(G_i1\) is down-regulated by chronic treatment of rat spinal cord dorsal root ganglion cocultures with opiates (17), and \(G_i3\) down-regulation has been observed following treatment of NG108-15 cells with PGE\(_1\) (18). Since a number of agonists can presumably couple through a single G-protein, it is clear that this phenomenon of G-protein down-regulation could account for heterologous desensitization.

In the current studies, we have evaluated effects of other inhibitors of adenylyl cyclase: first, to determine whether G-protein down-regulation is specific to the adenosine receptor system in adipocytes or may be a more general regulatory phenomenon, and second, to determine whether G-protein down-regulation is a mechanism for heterologous desensitization. We have used prostaglandin \(E_1\) and nicotinic acid, both of which are potent inhibitors of adenylyl cyclase and lipolysis in fat cells (19).

### MATERIALS AND METHODS AND RESULTS

The effects of prolonged treatment of adipocytes with PIA, \(PGE_1\), and nicotinic acid on G-proteins are summarized in Table I. PIA and \(PGE_1\) caused about a 90–95% loss of \(\alpha_1\) and \(\alpha_3\) and about 50% loss of \(\alpha_2\) and \(\beta\)-subunits. In contrast, nicotinic acid did not affect levels of any of the G-protein subunits. These findings demonstrate, first, that \(G_i\) down-regulation is not unique to the \(\alpha_1\) adenosine receptor. Second, the findings demonstrate that down-regulation of \(\alpha_1, \alpha_2,\) and \(\alpha_3\) is not secondary to chronic inhibition of lipolysis, because nicotinic acid was used at a concentration (1 mM) that was equally effective as an inhibitor of lipolysis as either of the other agents (Fig. 1). Furthermore, experiments using a wide range of concentrations of nicotinic acid (10 \(\mu\)M to 1 mM) failed to demonstrate an effect of chronic exposure to nicotinic acid on levels of any of the \(G_i\) subtypes (not shown). Finally, to ensure that the lack of effect of nicotinic acid is not due to breakdown of the compound, cells were incubated for up to 4 days with 1 mM nicotinic acid. Media was then assayed for nicotinic acid based on its ability to inhibit lipolysis in freshly isolated cells, which revealed that nicotinic acid levels did not alter appreciably over the 4-day incubation period (data not shown). The stability of nicotinic acid was also confirmed by analyzing the media for nicotinic acid on thin layer chromatography (see "Materials and Methods"), which demonstrated that the nicotinic acid was intact after the 4-day incubations.

Insulin binding was determined as a marker for the plasma membrane, since previous studies have demonstrated that at least 95% of cellular insulin receptors are located on the cell surface in adipocytes (3, 36). None of the treatments altered insulin binding to the membranes, indicating that the recovery of plasma membranes was similar in the various groups of cells (Table I). Similarly, the activity of adenylyl cyclase, maximally stimulated with a combination of forskolin and Mn\(^{2+}\) ions, was not altered by any of the treatments. Furthermore, Coomassie Blue-stained gels run on membranes following the various treatments revealed an essentially identical pattern of visible bands (not shown). Together, these findings strongly suggest that the recovery of plasma membranes was very similar following the different treatments.

Time-course experiments, using a maximally effective concentration of \(PGE_1\) (3 \(\mu\)M), demonstrated that down-regulation was detectable by 1 day and maximal by about 3 days (Fig. 6). This time course is similar to that observed in the presence of a maximally effective concentration of PIA (13).

Fig. 8 demonstrates the effect of chronic treatment of adipocytes with PIA, \(PGE_1\), and nicotinic acid on the subsequent sensitivity of lipolysis to PIA and \(PGE_1\). Cells were incubated for 4 days with the compounds at concentrations determined to be maximally effective for \(G_i\) down-regulation (i.e. 300 nM PIA or 3 \(\mu\)M \(PGE_1\)), or with 1 mM nicotinic acid. The cells were then washed and incubated with various concentrations of PIA (Fig. 8A) or \(PGE_1\) (Fig. 8B), and the rate of glycerol release was determined over a 30-min incubation. As we reported previously (12), prolonged treatment of adipocytes with PIA decreased the subsequent sensitivity of the cells to PIA (Fig. 8A). Prolonged treatment with \(PGE_1\) also resulted in decreased sensitivity to PIA (Fig. 8B). More interestingly, the \(PGE_1\)-treated cells also showed decreased sensitivity to PIA (Fig. 8A), and similarly, the PIA-treated cells showed reduced sensitivity to \(PGE_1\) (Fig. 8B). These findings demonstrate that PIA and \(PGE_1\) induce both homol-

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1. The abbreviations used are: PIA, (-)N\(^6\)-phenylisopropyl adenosine; \(PGE_1\), prostaglandin \(E_1\); \(G_i\), G-protein, any member of a family of GTP-dependent regulatory proteins; \(\alpha_1, \alpha_2, \alpha_3\), the \(\alpha\)-subunits of \(G_i1, G_i2,\) and \(G_i3; \alpha_2\)-subunit of \(G_i2; SDP-PAGE, sodium dodecyl sulophenylacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine.

2. Portions of this paper (including "Materials and Methods," part of "Results," and Figs. 1–5 and 7) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
Blots. Subunits of Gi, Gi2, and Gi3 were visualized using antisera as indicated. Membranes were isolated from the cells and the G, PIA and PGE1, I3B. The incubated with the indicated concentrations of PIA separate experiments. While low concentrations of PGE1 inhibit lipolysis, at higher concentrations the inhibition is partially reversed. Although these findings suggest that down-regulation of Gi could be involved in the mechanism of heterologous desensitization. In contrast, nicotinic acid did not induce Gi down-regulation, since nicotinic acid, which does not cause Gi down-regulation, causes only homologous desensitization.

The findings described above suggest that Gi down-regulation is central to the mechanism of heterologous desensitization in this system. However, it is also possible that changes at the level of the receptors occur. To investigate this question, adipocytes were incubated with various compounds for 4 days as before, and then A1 adenosine receptors were measured using the antagonist radioligand [3H]DPCPX (32). PIA caused about 58% loss of binding (Table I), consistent with adenosine receptor down-regulation as we have reported (12). In contrast, PGE1 did not affect [3H]DPCPX binding. These experiments were performed with [3H]DPCPX at a concentration of 0.2 nM, which is close to the reported Kd of A1 adenosine receptors for this antagonist (32). Therefore changes in the receptor, whether number or affinity, would have been detected by this approach. This demonstrates that PGE1-induced heterologous desensitization is not due to an alteration at the level of the adenosine receptor.

Since the antilipolytic activity of the compounds described is thought to be due primarily to inhibition of adenylylcyclase (19), we determined the ability of PIA to inhibit adenylylcyclase in membranes from cells treated with PIA, PGE1, or nicotinic acid (Fig. 10). The effect of PIA was determined in the presence of 10 μM isoprenaline, because preliminary experiments revealed that in the absence of a stimulator, adenylylcyclase activity was very low. In membranes from control cells, PIA produced a maximal inhibition of about 50%, with a half-maximally effective concentration of about 1 nM (Fig. 10A). Adenylylcyclase in membranes from both PIA-treated and PGE1-treated cells was much less sensitive to inhibition by PIA, as revealed by a rightward shift in the dose-response curve. This demonstrates heterologous desen-

In Fig. 9, adipocytes were incubated with PIA or nicotinic acid for 4 days as before and then washed, and the effect of nicotinic acid on lipolysis was determined over a 30-min period. Nicotinic acid-treated cells showed a markedly decreased sensitivity to nicotinic acid. In addition, the PIA-treated cells showed a markedly decreased sensitivity to nicotinic acid. Therefore, nicotinic acid can induce homologous desensitization, even though it does not induce heterologous desensitization. The decreased sensitivity of PIA-treated cells again demonstrates heterologous desensitization. From these findings it is evident that the compounds that cause Gi down-regulation, i.e. PIA and PGE1, will induce both homologous and heterologous desensitization. In contrast, however, nicotinic acid, which does not cause Gi down-regulation, causes only homologous desensitization.

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sensitivity at the level of adenylylcyclase. In contrast, nicotinic acid had no effect on the sensitivity of cyclase to inhibition by PIA. Membranes from both PIA-treated and PGE1-treated cells also showed decreased sensitivity to PGE1-inhibition of adenylylcyclase (Fig. 10B), which is again indicative of both homologous and heterologous desensitization. Again, nicotinic acid treatment did not affect the sensitivity of adenylylcyclase to PGE1. However, all three treatments did decrease the sensitivity of adenylylcyclase to nicotinic acid (Fig. 10C). This is again consistent with heterologous desensitization induced by PIA and PGE1, while nicotinic acid induces only homologous desensitization, presumably because it does not down-regulate Gi. Interestingly, PIA and PGE1 caused similar changes in the sensitivity of cyclase to inhibition by PGE1 and PGEa, while PIA had the greater effect on intact cells, measuring lipolysis (compare Figs. 8 and 10). This observation suggests that PIA may have other effects distal to those identified here (see "Discussion").

**DISCUSSION**

We reported previously that the three subtypes of Gi can be down-regulated to various degrees by PIA, an A1 adenosine receptor agonist, in isolated adipocytes. The experiments presented in the current report were designed to answer two questions. First, is the phenomenon of Gi down-regulation unique to the adenosine receptor? The findings clearly demonstrate that, like PIA, PGE1 can down-regulate each form of Gi in adipocytes, with a time course similar to that which we reported for PIA-induced down-regulation. Furthermore, the pattern was the same for the two agents, i.e. a1 and a3 were down-regulated by approximately 90%, whereas a2 was decreased by only about 50%. Thus it is clear that Gi down-regulation is not uniquely an effect of A1 adenosine receptor-coupled agonists. Gi down-regulation is also not produced by all receptor agonists coupled to inhibition of adenylylcyclase, because nicotinic acid did not cause down-regulation. However, this latter observation is difficult to interpret, since the mechanism of action of nicotinic acid is poorly understood.

This finding does demonstrate that the loss of Gi is not secondary to chronic inhibition of lipolysis, since nicotinic acid was just as effective at inhibiting lipolysis as was either PIA or PGE1 at the concentrations used. Inhibition of lipolysis by nicotinic acid is pertussis toxin-sensitive (37, 38), and furthermore nicotinic acid inhibition of adenylylcyclase is GTP-dependent (39). Therefore nicotinic acid works through a G-protein-coupled receptor, although the identity of the receptor to which it binds is unknown. Consequently, it is not clear why nicotinic acid failed to down-regulate Gi, while both PIA and PGE1 did. One possibility is that the cells have very few "nicotinic acid receptors." There may be enough nicotinic acid receptors to inhibit lipolysis, but an insufficient number to cause detectable down-regulation of G-proteins. However, this is little more than speculation.

Relatively few receptors are known to couple to inhibition of adenylylcyclase in adipocytes. In addition to A1 adenosine receptors and prostaglandin receptors, a2-adrenergic receptors are thought to be important in inhibition of adenylylcyclase and lipolysis in man. Most investigators have been unable to demonstrate a2-receptors on rat adipocytes, although there is a report that they are indeed present (40). Unfortunately we could not demonstrate an effect of a highly selective a2-agonist (UK 14304) on either lipolysis or relative levels of Gi, (data not shown). Therefore, at present we cannot determine whether a2-receptor activation would lead to down-regulation of Gi.

The second question these studies were designed to address is whether Gi down-regulation could form a basis for heterologous desensitization. This type of desensitization, in which exposure to one agonist results in resistance to another agonist that works through a distinct receptor, has been commonly observed, but the mechanism is not clear. G-proteins can couple different classes of receptor to a single effector. For example, glucagon, ACTH, and β-adrenergic receptors can all activate adenylylcyclase, and all couple through Gi. Conversely as described above, several different receptor types can couple to inhibition of adenylylcyclase through one or more of the subtypes of Gi. Therefore, a change at the level of a G-protein could clearly decrease cellular sensitivity to a range of agonists, providing a mechanism for heterologous desensitization. A number of studies have indicated that a functional change in Gi can account for heterologous desensitization of agonists coupled to activation of adenylylcyclase (8, 41). The findings reported here demonstrate that prolonged exposure of adipocytes to either PIA or PGE1, agonists that inhibit adenylylcyclase, causes actual loss of immunologically detectable Gi from the cells. Furthermore, PIA and PGE1 induce heterologous desensitization of lipolysis. In contrast nicotinic acid, another potent inhibitor of adenylylcyclase and lipolysis, failed to induce Gi down-regulation and resulted in only homologous desensitization. Thus it appears that in this system, Gi down-regulation is required for heterologous but not homologous desensitization.

Clearly, heterologous desensitization could be caused by changes at the level of the receptor, the G-protein(s), the catalytic subunit of adenylylcyclase, or at some downstream location between adenylylcyclase and lipolysis, such as protein kinase A, triglyceride lipase, or cyclic AMP phosphodiesterase. Changes in phosphodiesterase activity have been suggested as a mechanism for desensitization (42), and indeed Conti and co-workers (43) have reported that follicle-stimulating hormone can induce more than a 100-fold increase in mRNA encoding a high affinity CAMP phosphodiesterase in Sertoli cells (43). However, Hoffman et al. (44) have demonstrated that infusion of PIA in vivo induced desensitization in subsequently isolated adipocytes but did not seem to alter the activity of phosphodiesterase. Together with the finding that heterologous desensitization can be seen at the level of adenylylcyclase in membranes isolated from cells after the
various treatments, this suggests that the most likely mechanism involves either receptor changes, G-protein changes, or alterations in adenylylcyclase. We were unable to evaluate the effect of PIA on PGE₁ receptors. However, it was clear that while PIA caused a decrease in A₁ adenosine receptor binding (as would be expected), PGE₁ did not affect adenosine receptors. The catalytic subunit of adenylylcyclase was measured by incubating membranes with a combination of forskolin and Mn²⁺, which is thought to reflect the activity of the catalytic subunit of adenylylcyclase alone (45–47); none of the treatments affected the catalytic subunit, although they were able to alter the sensitivity of cyclase to inhibition. Since both PIA and PGE₁ did down-regulate the various forms of Gᵢ, it seems very likely that Gᵢ down-regulation is involved in heterologous desensitization in this situation. Furthermore, this is strengthened by the finding that nicotinic acid, which did not down-regulate Gᵢ, induced homologous but not heterologous desensitization. Since multiple mechanisms are clearly involved in desensitization the possibility that other changes occur, such as in phosphodiesterase or triglyceride lipase, cannot be ruled out. This may account for the differences in adenylylcyclase desensitization and desensitization of lipolysis. For example, treatment with PIA causes greater desensitization of lipolysis to PIA than to PGE₁, while the shift is equal at the level of adenylylcyclase. This suggests that chronic PIA treatment causes additional effects distal to cyclase inhibition. Further studies will be required to evaluate this possibility.

An interesting observation is that prolonged treatment with PIA causes predominantly a shift to the right in dose-response curve, with little or no decrease in maximal effect. This is true both at the level of adenylylcyclase and at the level of lipolysis and has been reported previously both by us (12) and by Hoffman et al. (44, 48). Since PIA causes a 50–60% decrease in adenosine receptors, this shift in dose-response curve with little change in maximal inhibition suggests that there are "spare receptors" for adenosine on adipocytes.

Very little is known regarding the mechanism of G-protein down-regulation. Longabaugh et al. (15) have reported that steady state levels of mRNA for the various G-proteins are unaltered in adipose tissue from rats infused chronically with PIA, and we have made similar observations in primary cultured adipocytes.³ While the possibility of altered G-protein turnover cannot be ruled out, it seems likely that G-protein down-regulation is due to increased degradation. Indeed Hadcock et al. (16) have recently reported that PIA increases the rate of α₂ degradation in hamster smooth muscle cells. Therefore, the stoichiometry of receptor and G-protein down-regulation is of interest. The concentration of A₁ adenosine receptors in adipocyte plasma membranes appears to be in the range of 0.5–1.5 pmol/mg protein (14, 32, 49). Our estimates suggest that each form of Gᵢ is present at about 7–10 pmol/mg protein. Treatment of adipocytes with maximal concentrations of PIA results in about 60% down-regulation of A₁ adenosine receptors (12), 90% loss of G₁ and G₃, and 50% loss of G₂. Thus it appears that the stoichiometry of down-regulation is such that at least 10 mol of both G₁ and G₃ and 2 mol of G₂ are lost for each mole of adenosine receptors. One possible explanation for this difference could be that the G-proteins and receptors are compartmentalized, but the receptors are recycled back to the plasma membrane more efficiently than the G-proteins. This is supported to a certain extent by the finding that treatment of adipocytes with isoproterenol causes redistribution of Gᵢ from the plasma membrane to a less dense cellular fraction (50). Further work is clearly required on the mechanism of G-protein down-regulation.

Recent evidence suggests that G₂ is the G-protein responsible for coupling receptors to inhibition of adenylylcyclase. Thus, both in neuroblastoma x glioma hybrid, NG108–15 cells (51) and in platelets (52), antibodies directed against α₂ blocked inhibition of adenylylcyclase, but antibodies directed against other forms of α had no effect. Both of these cell types contain G₂ and G₃; hence, the findings suggest that G₃ is not involved in inhibition of adenylylcyclase. However, neither NG108–15 cells nor platelets appear to express G₁, and so the findings do not rule out a role for G₁ in cyclase inhibition.

Interestingly, both PIA and PGE₁, down-regulated G₂ to a lesser degree than either G₁ or G₃. The significance of the effects of these compounds on G₁ and G₃ is difficult to interpret without more knowledge of the mechanism of G-protein down-regulation. If G-proteins are down-regulated as a direct consequence of receptor activation, the findings might suggest that receptors for these compounds can couple to all three of these G-proteins. Further studies will be required to determine which effector system(s) G₁ and G₃ might couple in adipocytes, and whether these effector systems show even more pronounced resistance after treatment with PIA and PGE₁, than does lipolysis, which is presumably regulated primarily by adenylylcyclase and hence, most likely, G₂.

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³ A. Green and G. Milligan, unpublished observations.
Materials and methods

Deshorpe's modified Eagle medium was purchased from Gibco. Fetal calf serum and horse serum albumin type C (Sigma) were from Amersham Pharmacia Biotech. All other reagents were purchased from Sigma Chemical Co.

Western blotting

Samples were electrophoretically transferred to nitrocellulose membranes by the method of Towbin (1979). The membranes were incubated in 5% milk powder in Tris-buffered saline (TBS) for 1 hour at room temperature. After the blocking step, the membranes were incubated for 2 hours at room temperature with a 1:200 dilution of the primary antibody in TBS containing 1% milk powder. The membranes were then washed three times in TBS and incubated for another 2 hours at room temperature with a 1:1000 dilution of the secondary antibody (anti-rabbit IgG conjugated with horseradish peroxidase) in TBS containing 1% milk powder. After three washes in TBS, the membranes were incubated in a liquid solution containing 0.8% hydrogen peroxide in TBS for 10 min. The membranes were then washed in TBS three times and developed with a "chemiluminescence" reagent (ECL, Amersham) for 4 min. The bands were visualized with autoradiography film and quantified by densitometry using a densitometer (Molecular Dynamics, Sunnyvale, CA). The relative amounts of protein were determined using a standard curve of purified protein.

Western blotting

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RESULTS

Figure 1 shows the effect of PGE1 and PGE2 on the rate of glycogen synthesis in cultured adipocytes. Each data point represents the mean ± S.E.M. for at least 5-10 replicates.

Figure 1. Inhibition of lipolysis in freshly isolated adipocytes. Adipocytes were isolated from overnight fasted rats and incubated with adrenaline (300 nM) or saline (vehicle). The cultures were then incubated for 24 hours in the presence of 100 nM epinephrine and 0.6% (v/v) ethanol. After the incubation period, the cultures were washed with Krebs-Henseleit buffer and incubated for 2 hours in the presence of 100 nM epinephrine and 0.6% (v/v) ethanol. The cultures were then washed with Krebs-Henseleit buffer and incubated for 2 hours in the presence of 100 nM epinephrine and 0.6% (v/v) ethanol.

Figure 2 shows the effect of PGE1 and PGE2 on the rate of glycogen synthesis in cultured adipocytes. Each data point represents the mean ± S.E.M. for at least 5-10 replicates.

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Figure 3 shows the effect of PGE1 and PGE2 on the rate of glycogen synthesis in cultured adipocytes. Each data point represents the mean ± S.E.M. for at least 5-10 replicates.

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Figure 4 shows the effect of PGE1 and PGE2 on the rate of glycogen synthesis in cultured adipocytes. Each data point represents the mean ± S.E.M. for at least 5-10 replicates.

Figure 4. Inhibition of lipolysis in freshly isolated adipocytes. Adipocytes were isolated from overnight fasted rats and incubated with adrenaline (300 nM) or saline (vehicle). The cultures were then incubated for 24 hours in the presence of 100 nM epinephrine and 0.6% (v/v) ethanol. After the incubation period, the cultures were washed with Krebs-Henseleit buffer and incubated for 2 hours in the presence of 100 nM epinephrine and 0.6% (v/v) ethanol. The cultures were then washed with Krebs-Henseleit buffer and incubated for 2 hours in the presence of 100 nM epinephrine and 0.6% (v/v) ethanol.
The effects of the antilipolytic compounds on relative levels of Go subunits, determined by Western blot analysis, are illustrated in Fig. 2. In this experiment, adipocytes were incubated for 4 days with or without each of the antilipolytic agents, then homogenized and membranes were isolated, separated on SDS-PAGE, and blotted as described in the Methods section. Blots containing plasma membranes from control and treated cells were probed with antiseraum to GoL, which binds to the α1 subunit of Go and GoG, (Fig. 2A). As we have previously reported (13), PIA caused a marked decrease in labeling of α1, and a somewhat less pronounced loss of α2. As can be seen, PGE2 had the same effect as PIA on relative levels of α1 and α2. In contrast, nicotinic acid had no effect on the level of either α-subunit. Similarly, PIA and PGE2 both downregulated α2, whereas nicotinic acid had no effect (Fig. 2B).

Dose-response experiments (not shown) demonstrated that the half-maximally effective concentration of PIA for Go downregulation was about 3 μM, and the maximally effective concentration was about 30 μM, as has been previously reported (13). Much higher concentrations of PGE2 were required for half-maximal and maximal downregulation (800 μM and 1 mM, respectively). This apparent low potency is probably due to the higher apparent affinity of the PGE2 receptor for its agonist, suggested by the report of a Kd of 10 nM. (3W)

The experiments illustrated were performed with adipocytes from the incubation medium to determine the efficiency of antilipolytic agents from the presence of PGE2 on various endogenous G-proteins levels (see Methods). However, in practice it was found that adipocytes preincubated for 1 h did not affect the levels of either of the G-protein α-subunits, unlike the ability of PGE2 to induce G-protein downregulation (data not shown). In the study presented here, adipocytes were preincubated for 30 min at a concentration of only about 25,000 cells/ml, and hence it is likely that endogenous adipocytes does not reach sufficiently high concentrations to cause G-protein downregulation.

In Fig. 3, membranes from cells treated with the inhibitors were analyzed with antiserum to α2, which recognizes all forms of α2 subunits. None of the compounds altered the level of either α1 or α2 or 45 kDa bands (see figure legend). The apparent lack of effect on relative levels of Go was confirmed by laser densitometric analysis of the blots (see figure legend).

To establish that the primary culture adipocyte is a good model for studying regulation of sensitivity to inhibition of lipolysis, cells were isolated and maintained in primary culture for up to 4 days (Fig. 7). The effect of various concentrations of PIA was determined on the freshly isolated cells, and after 3 and 4 days in culture. Neither the rate of glycerol release, nor the sensitivity of the cells to inhibition by PIA, varied appreciably over the 4 day period.

Fig. 4. G-protein α-subunits in PIA, PGE2, and nicotinic acid treated cells. Cells were treated as described in the legend to Fig. 2. Western blots were probed with antiserum to α2 to detect α-subunits. Lane 1, control; lane 2, PIA-treated; lane 3, PGE2-treated; lane 4, nicotinic acid-treated. One of three similar blots is illustrated.

Fig. 5. Go in PIA, PGE2, and nicotinic acid treated cells. Cells were treated as described in the legend to Fig. 2. Western blots were probed with antiserum to GoL to detect the α-subunits of Go and GoG, as indicated by the arrows. Gels were loaded as follows: lane 1, control; lane 2, PIA-treated; lane 3, PGE2-treated; lane 4, nicotinic acid-treated. The positions of molecular weight markers, as revealed by Coomassie blue staining, are indicated on the right hand side of the figure.

Fig. 6. Immunological quantitation of Go subtypes. Adipocytes were maintained in primary culture for 4 days with or without each of the antilipolytic agents, then homogenized and membranes were isolated, separated on SDS-PAGE, and blotted as described in the Methods section. Blots containing plasma membranes from control and treated cells were probed with antiserum to GoL, which binds to the α1 subunit of Go and GoG, (Fig. 2A). As we have previously reported (13), PIA caused a marked decrease in labeling of α1, and a somewhat less pronounced loss of α2. As can be seen, PGE2 had the same effect as PIA on relative levels of α1 and α2. In contrast, nicotinic acid had no effect on the level of either α-subunit. Similarly, PIA and PGE2 both downregulated α2, whereas nicotinic acid had no effect (Fig. 2B).

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Fig. 7. Inhibition of lipolysis by PIA after primary culture. Adipocytes were isolated under sterile conditions and established in primary culture. The cells were washed after 2, 3, or 4 day and the rate of lipolysis was determined in the presence of the indicated concentrations of PIA. α1, 1 day; α2, 2 day cells; α1, 4 day cells.