Desensitization of Adenosine Receptor–mediated Inhibition of Lipolysis

The Mechanism Involves the Development of Enhanced Cyclic Adenosine Monophosphate Accumulation in Tolerant Adipocytes

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Abstract

Adipocytes contain adenosine receptors, termed A_1 receptors, which inhibit lipolysis by decreasing adenylate cyclase activity. The inhibition of lipolysis by adenosine agonists in vivo acutely suppresses the plasma concentrations of free fatty acids (FFA) and triglycerides. We have found that infusions of the adenosine receptor agonist phenylisopropyladenosine (PIA) initially decreases plasma FFA concentrations; however, with prolonged exposure (6 d), rats become very tolerant to the effects of the drug. Adipocytes isolated from epididymal fat pads from PIA-infused rats have altered lipolytic responses. When lipolysis is stimulated with a relatively high concentration of isoproterenol (10^{-7} M), PIA does not inhibit lipolysis in adipocytes from the infused animals. However, PIA inhibits isoproterenol-stimulated cyclic AMP (cAMP) accumulation in adipocytes from the infused rats although with decreased sensitivity compared with controls. The explanation for the impaired antilipolytic effect appears to be due to the fact that isoproterenol-stimulated cAMP accumulation is markedly increased in cells from infused rats. Indeed, basal lipolysis and lipolysis stimulated with lower concentrations of isoproterenol (10^{-9}, 10^{-8} M) are effectively inhibited by PIA. cAMP accumulation is greatly increased in adipocytes from infused rats when stimulated by isoproterenol, ACTH, and forskolin. The results have some striking analogies to changes induced in nerve cells by prolonged exposure to narcotics. These data suggest that tolerance to PIA develops in adipocytes as a consequence of enhanced cAMP accumulation.

Introduction

Prolonged exposure of tissues to a variety of hormones or drugs often leads to a blunted response when the tissue is subsequently exposed to the agonist. This phenomenon has been termed desensitization, tolerance, or tachyphylaxis. A great deal has been learned over the past decade about the mechanisms by which receptor systems that activate adenylate cyclase may desensitize (1, 2). For example, uncoupling and down-regulation of beta adrenergic receptors frequently have been found to be associated with the desensitization of adenylate cyclase activation that occurs after prolonged exposure to catecholamines.

Much less is known about desensitization of receptors which function by inhibiting adenylate cyclase. Muscarinic cholinergic, opiate, and alpha$_2$ adrenergic receptors are examples of receptors that inhibit adenylate cyclase in a variety of cell types. The fall in the intracellular accumulation of cyclic AMP (cAMP)[1] that occurs when these receptors are activated may explain many of the physiological effects of these receptors. Desensitization of responses mediated by these inhibitory receptors may occur after prolonged exposure to agonists (3–5).

Adipocytes have been found to contain adenosine receptors which inhibit hormone-stimulated lipolysis (6–8). These adenosine receptors, termed A_1 receptors, function by inhibiting adenylate cyclase (9, 10), and are very efficacious in inhibiting catecholamine-stimulated lipolysis in adipocytes. Indeed, it has previously been shown that administration of adenosine-receptor analogs leads to a marked suppression of plasma free fatty acids (FFA) presumably reflecting inhibition of lipolysis in vivo (11–13). We have recently found that single injections of the adenosine receptor agonist phenylisopropyladenosine (PIA) acutely suppress both FFA and triglycerides in rats (Hoffman, B. B., E. Dall'Aglio, C. Hollenbeck, H. Chang, and G. Reaven, manuscript submitted for publication).

The present studies examine the effects of prolonged infusions of PIA. We found that rats became tolerant to the metabolic effects of the drug over time. Experiments in adipocytes isolated from these chronically infused rats revealed an unexpected mechanism for the desensitization.

Methods

Chemicals were obtained from the following sources: Collagenase, Worthington Biochemicals, Freehold, NJ; (−) isoproterenol, ACTH, and adenosine deaminase, Sigma Chemical Co., St. Louis, MO; (−) N^6-phenylisopropyladenosine, Boehringer Mannheim Diagnostics, Inc., Houston, TX; [^{3}H]N^6-phenylisopropyladenosine, Amersham Corp., Arlington Heights, IL. All other chemicals were obtained from standard commercial sources.

In vivo studies. Male sprague-Dawley rats (350–400 g) were fed standard laboratory chow ad lib. and maintained on a 12-h light–dark (06.00 h/18.00 h) cycle. Rats were fasted overnight before blood was obtained on the next day. Rats were injected or implanted subcutaneously with osmotic minipumps (Alza Corp., Palo Alto, CA) at 10.00 h. PIA was dissolved in 2.5% dimethyl sulfoxide in normal saline; control rats were injected subcutaneously with vehicle alone or implanted with minipumps containing vehicle as indicated. Blood was obtained from the tail for the measurement of FFA (14) and triglycerides (TG) by kit (Reagents Applications, Inc., San Diego, CA).

In vitro studies with isolated fat cells. Fat cells were prepared from epididymal fat pads according to the method of Rodbell (15) as previously described (16). Aliquots of diluted cells were fixed in a solution of 2%

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1. *Abbreviations used in this paper:* A_1, adenosine; cAMP, cyclic AMP; EC_{50}, effective concentration; FFA, free fatty acids; PIA, phenylisopropyladenosine; TG, triglycerides.

osmium tetra oxide in collidine buffer and counted in a Coulter counter (Coulter Electronics, Inc., Hialeah, FL) to determine cell number.

To measure lipolysis, fat cells were placed in plastic vials (1×10^5 cells/ml) in Krebs (in mM: NaCl, 121; KCl, 4.9; KH_2PO4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25.5)-4% albumin-2.5 mM glucose buffer, pH 7.4. Drugs were added to the cells and incubated at 37°C for 1 h in an atmosphere of O_2 (95%), CO_2 (5%). The rate of glycerol production is linear during this period. At the end of the incubation, an aliquot (0.2 ml) of infranatant was removed from each mixture for measurement of lipolysis. The rate of lipolysis is expressed as glycerol release; glycerol was measured by the enzymatic method described by Wieland (17).

To measure cAMP accumulation, we preincubated isolated fat cells for 30 min and then added the drugs. The reaction was then stopped in 10 min by the addition of 1 ml of 12% trichloroacetic acid. cAMP accumulation achieved a steady state value by 10 min. Samples were then frozen at −70°C and stored until assay. After thawing, samples were centrifuged (1,240 *g*, 20 min, 4°C), and the supernatant was extracted six times with water-saturated ethyl ether. cAMP was then measured by radioimmunoassay (18).

Measurement of adenosine receptors. Isolated adipocytes were prepared as described above. Adenosine receptors were measured with [^{3}H]phenylisopropyl adenosine in membranes prepared from these cells as previously described (16). Data from saturation curves were analyzed using a nonlinear least squares fitting program run on a HP 9816 computer (Hewlett-Packard Co., Palo Alto, CA). Results are expressed as fmol/mg protein; protein was measured by the method of Lowry (19) using bovine serum albumin as standard.

Results

The ability of PIA (0.150 μmol/kg subcutaneously) to decrease plasma FFA and TG concentration in overnight fasted rats is illustrated in Fig. 1 *A* and *B*. Repetitive injections of PIA decreased FFA from ~0.68 μmol/ml to <0.10 μmol/ml. The FFA remained well suppressed for the 9-h duration of the experiment. Vehicle injections had no effect on the concentration of FFA (Fig. 1 *A*). Plasma TG concentrations were also measured in these rats (Fig. 1 *B*). PIA caused a suppression of TG from 115 to 50 mg/dl within 1 h of the first PIA injection. The degree of suppression of TG by PIA remained relatively stable during the next 8 h even though the concentration of FFA was persistently decreased.

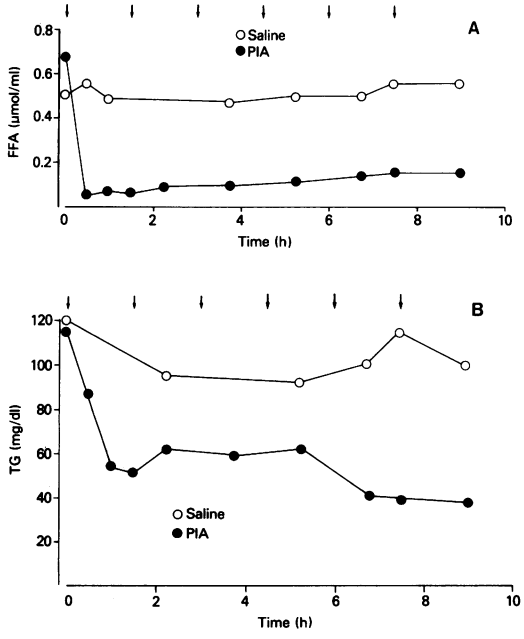

Figure 1. Suppression of FFA and TG by multiple injections of PIA. Fasted rats were injected repetitively with PIA (0.150 μmol/kg) (closed symbols) or vehicle (open symbols) as indicated by the arrows. Blood was sampled at various times during the course of the experiment. In (*A*) the values of FFA are illustrated whereas in (*B*) TG concentrations are shown. The results represent the means of two experiments that agreed within 10%.

In view of the major effects of repeated injections of PIA on both FFA and TG concentrations, we were interested in extending these results into longer term studies. Consequently, osmotic minipumps were implanted subcutaneously in rats; the putative rate of infusion of PIA (0.150 μmol/kg per h) was chosen so that it would be similar to that used in the intermittent injection experiments described above (0.150 μmol/kg per 1.5 h). Controls were implanted with osmotic minipumps containing vehicle alone (day 1). FFA and TG were first measured in these rats 24 h after the implantation of the minipumps (day 2). The rats in both groups were fasted overnight. As indicated in Fig. 2 *A*, the concentration of FFA in the vehicle infused rats was 0.92±0.12 μmol/ml, which was significantly higher than the fasting concentration of FFA in the PIA-infused group, 0.45±0.05 μmol/ml ($P < 0.001$). However, the partial suppression in the concentration of FFA 24 h after insertion of the minipump was not as great as we had seen with repetitive, acute injections (Fig. 1 *A*).

In an attempt to explain this result, we gave both groups of rats a subcutaneous injection of PIA (0.150 μmol/kg) exactly as described above. The animals receiving vehicle infusions had the expected marked fall in FFA. However, in the animals receiving the PIA infusion, the supplemental, acute injection of PIA did not further suppress FFA (Fig. 2 *A*). This result suggests

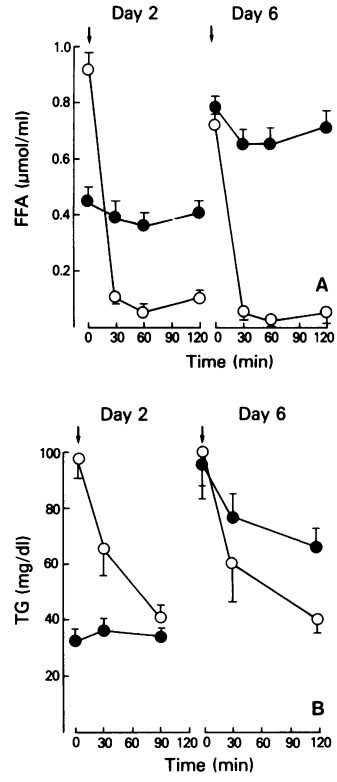

Figure 2. Effect of infusion of PIA on (*A*) FFA and (*B*) TG. Rats were implanted with osmotic minipumps that infused either PIA (0.150 μmol/kg per h) or vehicle. 24 h after the start of the infusion (day 2), fasting FFA and TG were measured in both groups; then, animals in each group received a subcutaneous injection of (−)PIA (0.150 μmol/kg), and FFA and TG were measured at the indicated times after the injection. A similar protocol was followed in the same rats on day 6 after implantation of the minipumps. The arrows indicate an injection of PIA as described. The results represent the mean±SEM of five experiments in vehicle-infused and 11 experiments in PIA-infused rats. ○, control; ●, PIA-infused.

that the rats had become partially tolerant to PIA within 24 h of the onset of the continuous infusion. The lack of further response to the acute injection of PIA suggests that the partial effect of PIA was not due to inadequate plasma concentrations of the drug in the infused rats.

When these same rats were studied 4 d later (day 6), the basal concentration of FFA in the PIA-infused animals was indistinguishable from that seen in the vehicle-infused controls. Indeed, an acute injection of PIA had essentially no effect on the FFA in the drug-infused rats (Fig. 2 *A*). This result suggests that these rats had become increasingly tolerant to PIA with time.

Similar results were obtained with the measurement of plasma TG in these rats: TGs were suppressed on day 2 but were indistinguishable from controls on day 6 (Fig. 2 *B*).

To determine whether the desensitization to PIA-mediated suppression of FFA reflected direct or indirect changes in adipocyte responsiveness to the adenosine analog, isolated adipocytes were prepared from rats made tolerant to PIA with a 6-d infusion of the drug and from vehicle-infused controls. Fig. 3 illustrates dose-response curves of isoproterenol-stimulated lipolysis in adipocytes from controls and PIA-infused rats. These experiments were conducted in the presence of adenosine deaminase so that the potentially differential sensitivity of the two groups to endogenously related adenosine would not be a confounding variable. As indicated in Fig. 3, basal lipolysis was greatly enhanced in adipocytes from infused rats, whereas the maximal responses were similar in the two groups. PIA was then used to inhibit lipolysis stimulated with various concentrations of isoproterenol (Fig. 4). PIA (10^{-7} M) effectively inhibited basal lipolysis and lipolysis activated with 10^{-9} M and 10^{-8} M isoproterenol in adipocytes from PIA infused rats. However, PIA was ineffective in inhibiting lipolysis when the adipocytes were activated with 10^{-7} M isoproterenol. Consequently, PIA's ability to inhibit lipolysis in cells from the infused rats was related to the concentration of isoproterenol used to stimulate lipolysis. PIA effectively inhibited lipolysis in the control adipocytes that had been stimulated with up to 10^{-7} M isoproterenol. These results suggested the possibility that there was an altered response to isoproterenol in the cells from the PIA-infused rats rather than an inherent unresponsiveness to PIA.

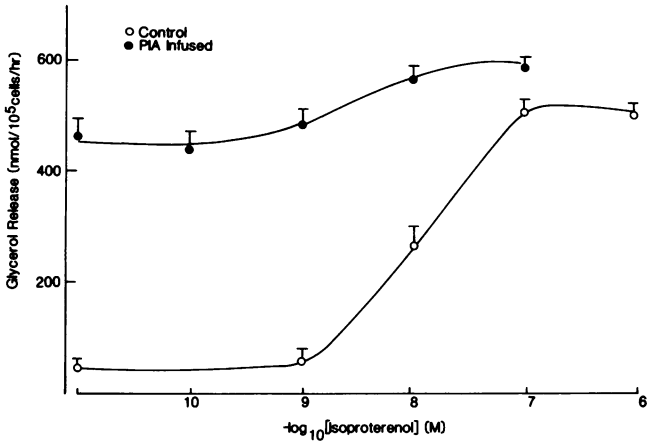

Figure 3. Isoproterenol-stimulated lipolysis in adipocytes from control and PIA-infused rats. In the presence of adenosine deaminase, isoproterenol-stimulated glycerol release was measured in the two groups. The results are the means±SEM of six experiments.

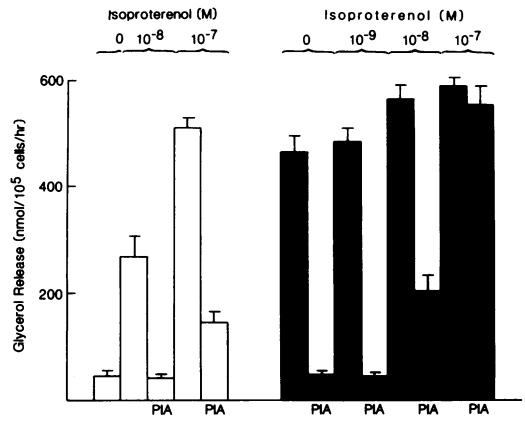

Figure 4. Ability of PIA (10^{-7} M) to inhibit lipolysis stimulated by various concentrations of isoproterenol. Adipocytes from control (clear bars) and PIA-infused rats (solid bars) were stimulated with various concentrations of isoproterenol in the presence and absence of added PIA (10^{-7} M). Adenosine deaminase was present in all assay tubes. These experiments represent the means±SEM of 3–7 separate studies.

In an effort to determine if the adipocytes from the PIA-infused rats indeed did respond differently to isoproterenol than did the control cells, we compared the ability of isoproterenol to activate cAMP accumulation in the two groups. These studies were conducted in the presence of adenosine deaminase so that they would be comparable to the lipolysis experiments illustrated in Fig. 4. Fig. 5 illustrates the dose-response curves of isoproterenol-stimulated cAMP accumulation in isolated adipocytes from the two groups of rats. These data indicate that isoproterenol causes substantially greater cAMP accumulation in cells isolated from PIA-infused rats.

In a further effort to determine the mechanism for PIA's apparent inability to inhibit lipolysis in adipocytes stimulated with 10^{-7} M isoproterenol, we also measured A_1 receptors and their ability to inhibit cAMP accumulation. A_1 receptors were measured in membranes prepared from isolated adipocytes with [^{3}H]PIA. There was a modest decrease in the number of A_1 receptors in adipocyte membranes from PIA-infused animals (779±104 fmol/mg protein) compared with controls (1,098±113 fmol/mg protein), $P < 0.03$, $n = 8$. There was no change in the dissociation constant (K_d) of [^{3}H]PIA, which was 2.3±0.6 nM in controls and 3.1±1.0 nM in the PIA treated group, $P < 0.26$.

Since A_1 receptors in adipocytes are thought to inhibit lipolysis by suppressing intracellular cAMP accumulation, we ex-

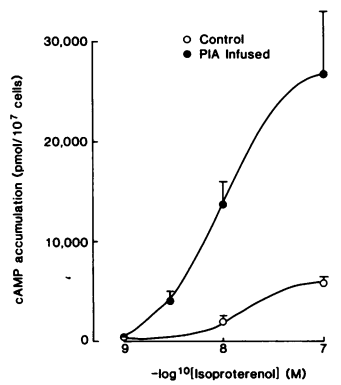

Figure 5. Isoproterenol-stimulated cAMP accumulation in adipocytes isolated from control and PIA-infused rats. Dose-response curves were obtained in the presence of adenosine deaminase (1 U/ml) to remove endogenously released adenosine. The results are the mean±SEM of four separate experiments.

amined this response in isolated adipocytes from control and PIA-infused rats. The ability of PIA to suppress isoproterenol-stimulated cAMP accumulation is shown in Fig. 6. Two important observations follow from these data. First, the adipocytes from the PIA-infused rats are less sensitive to PIA. PIA's effective concentration, 50% (EC_{50}) is $8.6\pm4.9 \times 10^{-12}$ M in controls and $6.8\pm2.5 \times 10^{-10}$ M in adipocytes from the infused rats, $P < 0.02$. Secondly, note that the maximal response to PIA, when expressed as percentage inhibition of cAMP accumulation, is very similar in the two groups (Fig. 6). Consequently, in adipocytes from PIA infused rats, high concentrations of PIA markedly suppressed isoproterenol (10^{-7} M)-stimulated cAMP accumulation without inhibiting lipolysis. These results suggested that the apparent inability of PIA to inhibit lipolysis in adipocytes from the infused rats may have been due to the markedly enhanced cAMP response that occurred when these cells were stimulated with isoproterenol.

We next asked whether the enhanced cAMP accumulation in response to isoproterenol was specific for beta adrenergic receptors or if other stimulators of adenylate cyclase might also cause exaggerated responses in the adipocyte from PIA-infused rats. Several stimulators of cAMP accumulation with different mechanisms of action were evaluated in adipocytes from controls and PIA-infused rats (Table I). ACTH, acting at a specific cell-surface receptor, was similar to the beta adrenergic agonist isoproterenol in that a markedly greater cAMP response was found in adipocytes from PIA-infused rats. Forskolin, which more directly activates adenylate cyclase independently of cell surface receptors, also caused a much greater response in the adipocytes for the infused rats. Isolated adipocytes spontaneously release adenosine, which interacts with the A_1 receptors to inhibit cAMP accumulation (6, 7). Consequently, we examined the effects of isoproterenol and forskolin in the presence of adenosine deaminase, an enzyme which metabolizes adenosine to inosine. Much greater stimulation of cAMP accumulation also occurred in the presence of adenosine deaminase in the adipocytes from PIA-infused rats, indicating that the differences between the two groups were not merely due to enhanced inhibition of adenylate cyclase by endogenously released adenosine in the control cells.

Discussion

PIA acutely suppresses both plasma FFA and TG concentrations in rats. However, with continuous infusion, partial tolerance to the drug is evident within 24 h and is even more evident at 6 d. We have found that adipocytes isolated from rats that become tolerant to PIA after a chronic infusion of the drug have altered responses to PIA in vitro. However, the impaired ability of PIA to inhibit lipolysis in isolated adipocytes appears to be mainly due to a marked accentuation in cAMP accumulation in response to isoproterenol rather than an absolute ineffectiveness of the A_1 receptors.

Desensitization of PIA-mediated suppression of plasma FFAs was noted after a prolonged infusion of the drug. We did not attempt to develop an assay for PIA in blood; however, it appears likely that the drug was present during the course of the infusion because the rats receiving PIA infusions did not respond to acute injections of the compound. This suggests that maximally effective concentrations of PIA were already present and that a further increment in drug concentration was without additional effect. Consequently, the data suggest that the adipose tissue in these rats was truly tolerant to PIA. Hypothetically, it is possible that an agonist compound such as PIA could be metabolized to a form which fortuitously acts as an antagonist at the same receptor. However, there is little reason to speculate that PIA's action was being blocked in vivo by a receptor antagonist since washed adipocytes in vitro were less sensitive to the drug. After a 6-d infusion of PIA, FFA did not fall in response to a large injection of PIA. This suggests that the stimulation of lipolysis in these overnight fasted rats is quite marked, analogous to the in vitro situation where PIA did not inhibit lipolysis in adipocytes from tolerant rats that had been stimulated with a high concentration of isoproterenol (10^{-7} M). Consequently, it may be that counter-regulatory mechanisms, such as enhanced secretion of lipolytic hormones, might contribute to the tolerance to PIA observed in intact rats.

A component of the blunted response to PIA in adipocytes isolated from PIA-infused rats might be due to the modest down-regulation in the number of A_1 receptors in these cells. However, there was a marked loss in sensitivity to PIA-mediated inhibition of cAMP accumulation; this 80-fold increase in PIA's EC_{50} may only partly be due to the receptor loss. It is possible that some of the remaining receptors do not couple effectively to the in-

Table I. Stimulation of cAMP Accumulation in Adipocytes from Control and PIA-infused Rats

	cAMP accumulation		
	Controls	PIA-infused	*P*
	pmol/10⁷ cells	*pmol/10⁷ cells*	
Basal	172±13 (12)	206±18 (15)	NS
Isoproterenol (10^{-7} M)	294±32 (7)	3,971±990 (10)	$P < 0.01$
ACTH (3 U/ml)	286±47 (4)	3,590±1,168 (4)	$P < 0.05$
Forskolin (10^{-5} M)	681±97 (4)	2,729±531 (5)	$P < 0.02$
In the presence of adenosine deaminase (1 U/ml)			
Basal	295±15 (4)	404±28 (4)	$P < 0.02$
Isoproterenol (10^{-7} M)	6,134±524 (9)	23,254±1,346 (12)	$P < 0.001$
Forskolin (10^{-5} M)	2,663±624 (4)	27,516±3,654 (3)	$P < 0.001$

Adipocytes were isolated from the epididymal fat pads of vehicle-infused (controls) and PIA-infused rats 6 d after implanting osmotic minipumps. Stimulation and measurement of cAMP accumulation were performed as indicated in Methods. The numbers in parentheses refer to the number of separate experiments expressed as mean±SEM.

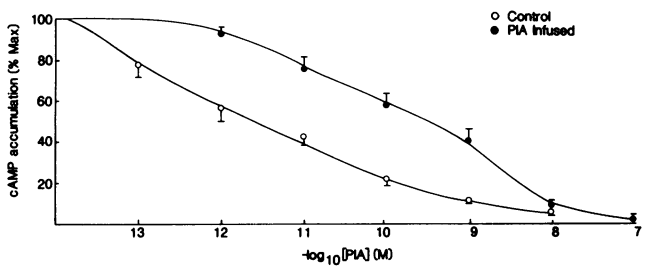

Figure 6. Inhibition of cAMP accumulation by PIA in adipocytes isolated from control and PIA-infused rats. cAMP accumulation was stimulated with 10^{-7} M isoproterenol in the presence of adenosine deaminase and various concentrations of PIA were added. The results are expressed as the percentage of the maximal response found in the absence of added PIA. As indicated in Table I, the absolute cAMP accumulation was greater in the adipocytes from PIA-infused rats than in controls. The results are the mean±SEM of 9 experiments.

hibition of cAMP accumulation. However, it is unlikely that these changes in A_1 receptor number or sensitivity explain the impaired antilipolytic effect of PIA in isolated adipocytes since the drug could maximally inhibit lipolysis in the presence of low concentrations of isoproterenol. Indeed, the evidence suggests that the major explanation for the impaired ability of PIA to inhibit lipolysis was a result of the greatly magnified cAMP response to isoproterenol that occurred in adipocytes isolated from PIA-infused rats. It is likely that the cAMP accumulation in those cells is so much in excess of what is required to maximally activate lipolysis that the PIA-mediated fall in cAMP accumulation is unable to decrease the activation of cAMP-dependent protein kinase. There is evidence that much greater cAMP accumulation can occur in adipocytes than is required to maximally activate protein kinase or lipolysis (20). However, it will be necessary to directly correlate the cAMP concentrations with lipolysis and cAMP-dependent protein kinase activation to confirm this hypothesis.

Nicotinic acid and some heteocyclic carboxylic acid analogs also acutely inhibit lipolysis which leads to suppression of plasma concentrations of FFA and TG (21). Nicotinic acid may inhibit lipolysis by interacting with a specific receptor that inhibits adenylate cyclase activity. For some of these compounds there is evidence that efficacy declines with prolonged use (22). Indeed, Aktories and Jakobs (23) have suggested that tolerance to the antilipolytic effect of 3-carboxy-5-methylpyrazole occurs via desensitization of inhibition of adenylate cyclase in fat cell membranes.

There are a number of analogies between the effects of chronic exposure of adipocytes to PIA and cellular adaptations to narcotics. Nirenberg and colleagues (24) have reported that prolonged exposure to morphine leads to enhanced stimulatory responses in NG108-15 cells. Narcotics are effective inhibitors of adenylate cyclase in these cells. Prolonged activation of opiate receptors (as well as inhibitory muscarinic cholinergic and $alpha_2$ adrenergic receptors) leads to an enhanced ability of prostaglandin E_1 to stimulate cAMP accumulation in these cells (25). When the opiate is removed, basal cAMP is increased—this is a form of "withdrawal." This enhanced cAMP response may account for the tolerance that develops to opiates; cAMP concentrations gradually return to control values even in the continued presence of the opiate agonist (24). We have found that not only do the rats become tolerant to infusions of PIA but also adipocytes in vitro develop rapid rates of lipolysis when PIA is removed. Similar enhanced cAMP responses have been found in pituitary cells and hamster adipocytes after prolonged exposure to drugs or hormones which inhibit cAMP accumulation (23, 26, 27). Consequently, these adaptions occur in neural and nonneural cells and both with narcotics as well as other inhibitors of adenylate cyclase. The underlying basis for this response is unknown. Also, there is some evidence in favor of the possibility that addiction to adenosine in neural tissue can occur (28).

We have found that the enhanced cAMP accumulation in cells from PIA-infused rats occurs with receptor agonists (beta adrenergic and ACTH) as well as forskolin. These data make it unlikely that a receptor alteration is the cause of this phenomenon. Further studies are required to determine the relative importance of possible changes in rates of cAMP production and/or cAMP degradation in the hyperresponsive cells and the detailed mechanism involved.

Our results demonstrate the marked time-dependent attenuation in the effects of PIA with continuous infusion. To what extent this desensitization can be avoided by alternative dosing regimens, such as intermittent exposure to the drug with time for recovery, remains to be determined. However, this model should be an interesting setting in which to study metabolic effects of adenosine agonists and mechanisms of desensitization of inhibitory receptors.

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