

Increased sensitivity of fat cell adenylate cyclase to stimulatory agonists during fasting is not related to impaired inhibitory coupling system

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In rat adipocytes, inhibition of the forskolin-stimulated cyclic AMP response by nicotinic acid and *N*⁶-phenylisopropyladenosine was unaltered by a 72 h fasting. Under assay conditions favouring inhibition, basal and forskolin-stimulated adenylate cyclase responses to inhibition by GTP and nicotinic acid were also unimpaired by fasting. Under the same conditions, however, low GTP concentrations elicited a clear activatory effect in membranes from fasted but not from fed rats. Fasting failed to alter the incorporation of [³²P]ADP ribose into the α -subunit of *N*_i and the attenuation of nicotinic acid inhibitory action that are both induced by pertussis toxin. These results, demonstrating unimpaired inhibitory control of adenylate cyclase in starved rat adipocytes, suggest that the permissive effect of fasting on the action of stimulatory receptor agonists in fat cells reflects a specific increase in the activity of the adenylate cyclase stimulatory coupling system.

Adenylate cyclase Starvation N_i protein Nicotinic acid Hormonal sensitivity (Rat adipocyte)

1. INTRODUCTION

Hormonal regulation of lipolysis in adipose tissue is a cyclic AMP-dependent process in which the hormonal signal is brought about by receptor-mediated modulation of adenylate cyclase, the enzyme catalysing cyclic AMP biosynthesis. Within the last few years, it has become clear that adenylate cyclase catalytic activity (C) is under the dual control of stimulating (lipolytic) and inhibiting (antilipolytic) agents [1]. Although converging on the same enzyme, the receptor-mediated signals initiated by these agents are transduced by two different trimeric GTP-dependent regulatory complexes called *N*_s and *N*_i, respectively. Both stimulation and inhibition of C require Mg²⁺ and GTP and cause dissociation of the N proteins into α - and β -subunits. The β -

subunit, the role of which is to sequester and inactivate the α -subunits, is common to *N*_s and *N*_i, while the α -subunits of these proteins are clearly different. α_s , the α -subunit of *N*_s, is a 45 kDa protein which, when bound to GTP, interacts with C and stimulates the enzyme. α_i , the α -subunit of *N*_i, is a 41 kDa protein which, when bound to GTP, is released from *N*_i together with the β -subunit which in turn associates with α_s -GTP and thereby antagonises stimulation of C [1–10].

It is a well established fact that fasting increases the blood free fatty acid level. In vitro studies have revealed that the sensitivity of rat adipose tissue to lipolytic agents such as the catecholamines is increased by starvation [11,12] and it has been suggested that a better coupling of the β -adrenoceptors to adenylate cyclase could be responsible for this alteration [12]. In [13], we have provided direct evidence in favour of such a mechanism by showing that a 72 h fasting increases the sensitivity of the fat cell β -

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adrenoceptor-coupled adenylate cyclase system to guanine nucleotides. However, as adenylate cyclase is dually regulated by GTP [3,4] and since the above experiments were performed in the presence of sufficient amounts of adenosine to attenuate adenylate cyclase stimulation, these studies do not provide any information on whether the tighter stimulatory coupling found in starvation is due to increased function of the stimulatory transducing process or to decreased activity of the inhibitory coupling system. In this respect, it is noteworthy, that a reduced sensitivity of catecholamine-stimulated lipolysis to the antilipolytic effect of adenosine has been recently reported in adipocytes from starved rats [14]. These observations and the lack of information on the modulation of the inhibitory GTP binding regulatory protein N_i by fasting have prompted us to carry out the present investigations.

2. MATERIALS AND METHODS

The origins of the following reagents were: PMSF, leupeptin, benzamidine, trypsin inhibitor, ATP, phosphoenolpyruvate, IBMX, NAD^+ and nicotinic acid from Sigma; pyruvate kinase, GTP, Guopp(NH)p, adenosine deaminase and N^6 -phenylisopropyladenosine (N_6 -PIA) from Boehringer Mannheim; forskolin and bovine serum albumin (fraction V fatty acid poor) from Calbiochem; cyclic [3H]AMP (spec. act. 25 Ci/mmol) and cyclic AMP binding protein from Amersham, collagenase (CLS, spec. act. 148 U/mg) from Worthington, [α - ^{32}P]NAD $^+$ (spec. act. 37.5 Ci/mmol) from NEN and purified pertussis toxin from List Laboratories.

2.1. Preparation of crude adipocyte membranes

Male Wistar rats (140–160 g) maintained under constant temperature (22°C) and lighting conditions (8 a.m.–6 p.m.) were fed ad libitum or fasted (72 h) before being killed by decapitation. Epididymal fat pads from 10 rats were pooled and isolated fat cells were prepared according to Rodbell [15] with slight modifications (collagenase concentration, 0.5 mg/ml; time of digestion, 30 min). Crude membranes were prepared as in [13] except that the medium used for the cell disruption contained the following protease inhibitors: PMSF (100 μ M), benzamidine (15 μ g/

ml), leupeptin (5 μ g/ml) and trypsin inhibitor (5 μ g/ml). The final pellet was resuspended in 1 mM $KHCO_3$ resulting in a suspension containing 1–2 mg protein/ml which was immediately used for the adenylate cyclase assays. Protein was determined by the method of Lowry et al. [16].

2.2. Cyclic AMP production by isolated fat cells

Fat cells ($1-2 \times 10^5$) were incubated for 30 min at 37°C in 1 ml Earle's medium-20 mM Hepes containing 2% albumin, 1 mM IBMX (or 0.5 mM Ro-201724), 50 mU/ml adenosine deaminase and the different effectors tested. Reactions were stopped by adding 5 mM EDTA followed by 5 min immersion in a boiling water bath. Cyclic AMP was extracted and determined as in [17].

2.3. Assays of adenylate cyclase

Adenylate cyclase activity was assayed according to a modification of the method of Birnbaumer et al. [18]. Incubations were performed at 25°C in a final volume of 0.1 ml containing 25 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$, 0.1 M NaCl, 1 μ M GTP, 0.4 mM ATP, 1 mM IBMX, 50 mU/ml adenosine deaminase, 1 mM EGTA, 10 mM phosphoenolpyruvate and 25 μ g/ml pyruvate kinase. Reactions were initiated by the addition of 20–30 μ g crude membrane protein and stopped after 20 min by the addition of ice-cold buffer (4 mM EDTA-50 mM Tris-HCl, pH 7.4) followed by 2 min immersion in a boiling water bath. Cyclic AMP was extracted and determined as described above.

2.4. Pertussis toxin-catalysed labeling of membrane proteins with [α - ^{32}P]NAD $^+$

Pertussis toxin (IAP) was preactivated with 20 mM DTT for 10 min at 30°C. Membranes (usually 100 μ g protein) were incubated for 20 min at 30°C in a final volume of 0.1 ml containing 100 mM Tris (pH 8.0), 5 mM $MgCl_2$, 10 mM thymidine, 1 mM ATP, 0.1 mM GTP and the indicated concentrations of [α - ^{32}P]NAD $^+$ and pertussis toxin. Following the labeling reaction, the membranes were washed, solubilized and subjected to electrophoresis on 12.5% polyacrylamide gel in the presence of 0.1% SDS [19]. Autoradiography was then conducted by exposure of the electropherograms to Kodak X-Omat films for 2–3 days at $-80^\circ C$. Bands of radioactivity identified by autoradiography were excised from

the dried gels, dissolved in 0.4 ml of 30% H_2O_2 by heating for 90 min at 75°C and counted.

3. RESULTS

3.1. Effects of 72 h fasting on the inhibition of cyclic AMP production in intact adipocytes

In adipocytes, as in many other cells, both nicotinic acid and the adenosine analog N^6 -PIA inhibit the increase in cyclic AMP production induced by forskolin [20–22], a diterpene which stimulates adenylate cyclase through a receptor-independent mechanism [23]. To investigate the influence of fasting on this inhibitory process, we have compared the effects of increasing concentrations of nicotinic acid and N^6 -PIA on the cyclic AMP response to $10\text{ }\mu\text{M}$ forskolin in fat cells from fasted and fed rats. As shown in fig.1, the magnitudes of forskolin stimulation were similar

in both experimental groups and fasting did not alter the sensitivity (concentrations eliciting half-maximal inhibition = IC_{50} values) and the maximal response of cyclic AMP to the inhibitory effect of nicotinic acid and N^6 -PIA.

3.2. Effect of 72 h fasting on adenylate cyclase inhibitory responses

The dose-related inhibition of forskolin-stimulated adenylate cyclase activity by nicotinic acid is shown in fig.2. As can be seen, forskolin-stimulated adenylate cyclase activity was unchanged by fasting and the inhibitory curves found in fasted and fed rats were strictly superimposable. The same picture was also observed in separate experiments (not shown) where inhibition of isoproterenol ($1\text{ }\mu\text{M}$)-stimulated adenylate cyclase was studied as a function of N^6 -PIA concentrations.

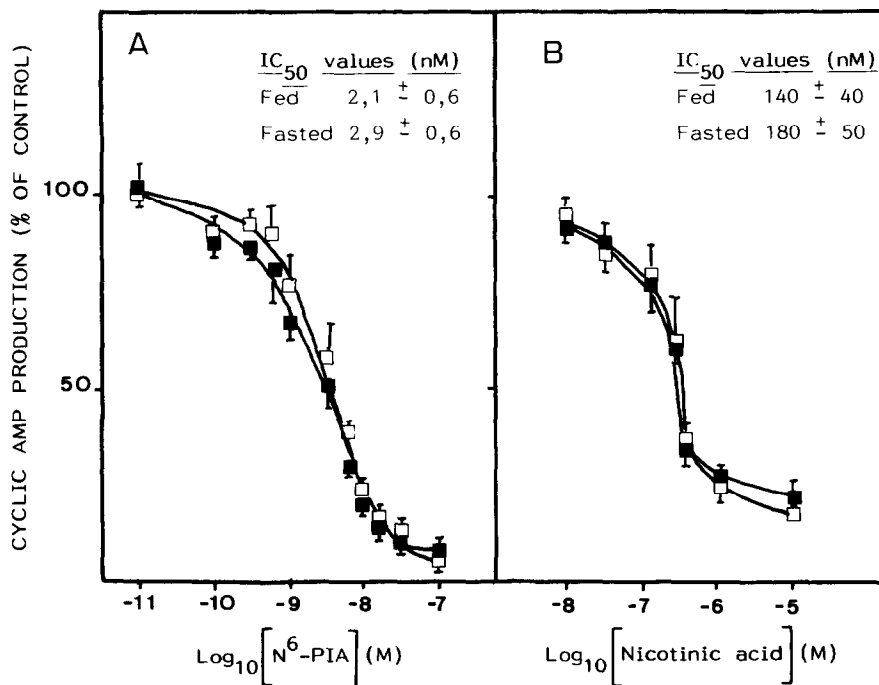


Fig.1. Inhibition of forskolin-stimulated cyclic AMP production by N^6 -PIA (A) and nicotinic acid (B) in rat fat cells: influence of prolonged fasting. Fat cells from fed (■) and fasted (□) rats were incubated at 37°C in buffer containing 2% albumin, 5 mM glucose, 0.5 mM Ro-201724 (A) or 1 mM IBMX (B), 50 mU/ml adenosine deaminase and $10\text{ }\mu\text{M}$ forskolin in the absence (control) or presence of increasing concentrations of N^6 -PIA or nicotinic acid. After 30 min, cyclic AMP was determined. Each point represents the mean $\pm$ SE from 3 (A) and 4 (B) different experiments performed in triplicate. Forskolin-stimulated cyclic AMP productions were (A) 386 ± 93 and 316 ± 116 , and (B) 515 ± 109 and 454 ± 118 pmol/30 min per 10^5 cells in fed and fasted rats, respectively.

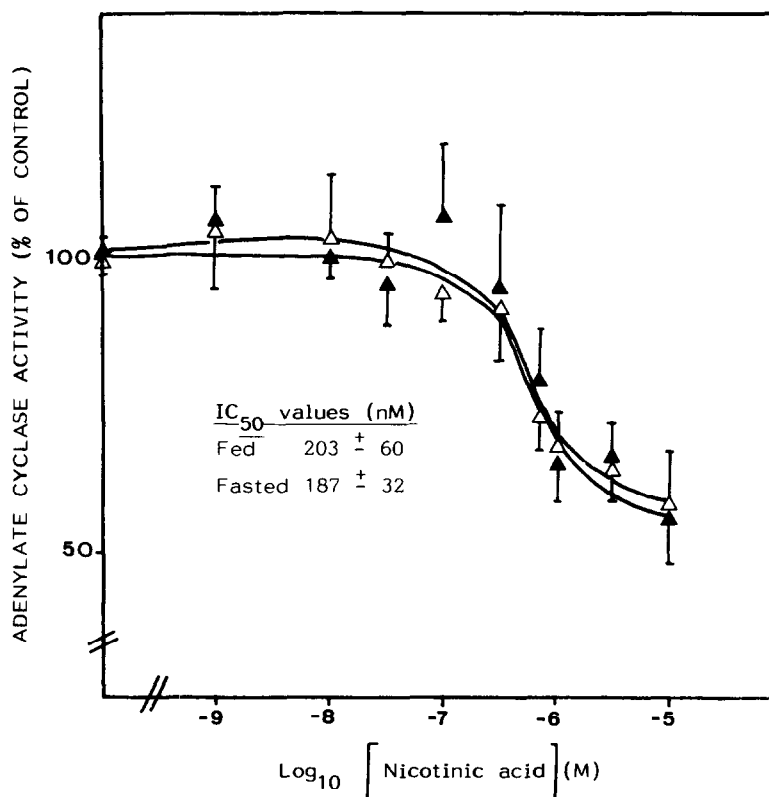


Fig.2. Inhibition of forskolin-stimulated adenylate cyclase activity by nicotinic acid in rat fat cell membranes: influence of prolonged fasting. Fat cell membranes from fed ($\blacktriangle$) and fasted ($\triangle$) rats were incubated with $10 \mu\text{M}$ forskolin in the absence (control) or presence of increasing concentrations of nicotinic acid as described in section 2. Each point represents the mean $\pm$ SE from 3 separate experiments performed in triplicate. Forskolin-stimulated activities were 28900 ± 9000 and 28140 ± 7850 pmol cyclic AMP/20 min per mg protein in fed and fasted rats, respectively.

GTP-dependent inhibition of basal adenylate cyclase activity was also compared in fat cell membranes from fasted and fed rats. In agreement with [4,22], fig.3 shows that GTP concentrations above $0.3 \mu\text{M}$ were required to observe inhibition in both experimental groups. Although maximal inhibition occurred at about $100 \mu\text{M}$ GTP in fat cell membranes from fasted and fed rats, the magnitude of this inhibition (expressed as percentage inhibition of the activity found in the absence of GTP) was 50% less in fasted than in fed rats (28 ± 8 vs $54 \pm 6\%$ in the fed group). It is important to note, however, that the slight activatory phase which we, like others [22], have observed at low GTP concentrations and under the present conditions was much more pronounced in fasted than in fed rats ($+38 \pm 6$ and $+10 \pm 8\%$ stimulations over basal

values, respectively). This difference explains why there was no longer any difference in the GTP-inhibitory curves between fasted and fed rats when the data were normalized with respect to GTP maximally stimulated adenylate cyclase activity (not shown).

3.3. Influence of prolonged fasting on the inhibitory guanine nucleotide regulatory protein

In several cell lines, pertussis toxin (IAP) selectively reduces hormonal- and GTP-mediated adenylate cyclase inhibition by ADP-ribosylating the α_1 -subunit of N_i . This modification also inhibits GTPase activity, presumably by stabilizing the $\alpha_i\beta$ complex [24]. In adipocytes as well, pertussis toxin reduces the ability of various adenylate

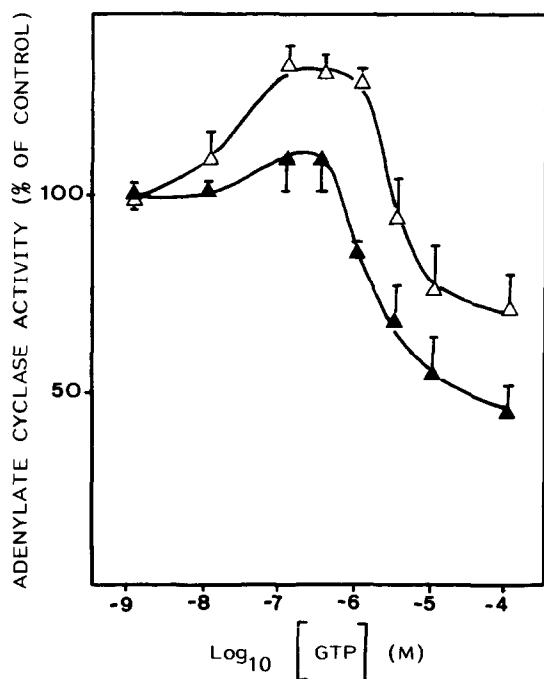


Fig.3. GTP-dependent inhibition of basal adenylate cyclase in rat fat cell membranes: influence of prolonged fasting. Fat cell membranes from fed ($\blacktriangle$) and fasted ($\triangle$) rats were incubated in the absence (control) or presence of increasing concentrations of GTP as described in section 2. Each point represents the mean $\pm$ SE of 3 separate experiments performed in triplicate. Basal adenylate cyclase activities were 2470 ± 580 and 2370 ± 490 pmol cyclic AMP/20 min per mg protein in fed and fasted rats, respectively.

cyclase inhibitors (adenosine, nicotinic acid, prostaglandins, α_2 -adrenergic amines) to depress cyclic AMP production and lipolysis [8,25]. Two recent reports [22,26] have also shown that exposure of fat cell membranes to both activated pertussis toxin and $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$ results in the radiolabeling of two membranous components, the 41 kDa α_i -subunit of N_i and a 40 kDa protein. Our present results (fig.4) confirm these findings and also indicate that the electrophoretic mobilities of these proteins are unaltered after prolonged fasting. Moreover, calculation of the relative amounts of ^{32}P label incorporated in the 40/41 kDa doublet under the standard incubation conditions described under fig.4 failed to show any significant difference between fasted and fed animal (744 ± 74

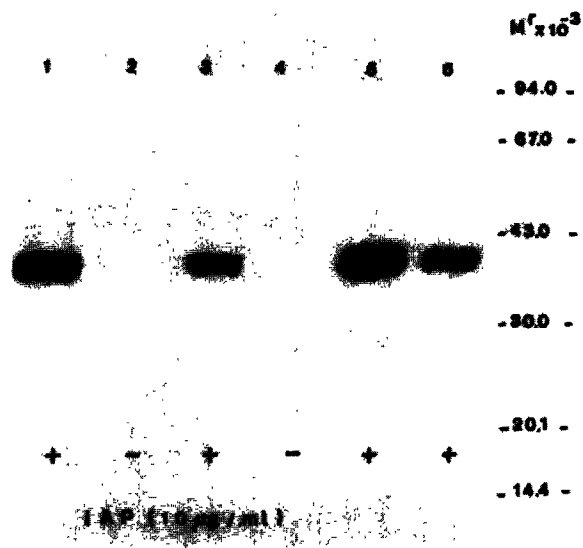


Fig.4. SDS-polyacrylamide gel analysis of pertussis toxin - $[\text{P}^{32}]\text{ADP}$ -ribosylated peptides of adipocyte membranes from fasted and fed rats. Fat cell membranes from fed ($80 \mu\text{g}$, lanes 1-3) and fasted rats ($70 \mu\text{g}$, lanes 4-6) were incubated with 0.5 mM GTP, $5 \mu\text{M}$ $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$ and $50 \mu\text{M}$ NAD^+ in the absence (lanes 2,4) or presence (lanes 1,3,5,6) of pertussis toxin ($10 \mu\text{g}/\text{ml}$). The conditions of incubation, electrophoresis and autoradiography are described in section 2. The data presented are from one experiment representative of two others. The M_r values of the toxin-labeled peptides, 40000 and 41000, were established by comparison with protein standards.

vs $892 \pm 198 \text{ fmol } ^{32}\text{P}/\text{mg}$ protein in membranes from fasted and fed rats, respectively). This was further confirmed by other experiments in which the radiolabeling reaction was investigated as a function of NAD^+ , membrane protein and pertussis toxin concentrations (not shown).

Finally, fat cells from fed and fasted rats were preincubated for 3 h with various concentrations of pertussis toxin and then used to test the ability of pertussis toxin to attenuate the inhibitory action of nicotinic acid on forskolin-stimulated cyclic AMP production. As shown in fig.5, the effect of nicotinic acid was completely abolished by $100 \text{ ng}/\text{ml}$ pertussis toxin in both groups. Moreover, these experiments show that the functional sensitivity of α_i to the toxin (pertussis toxin

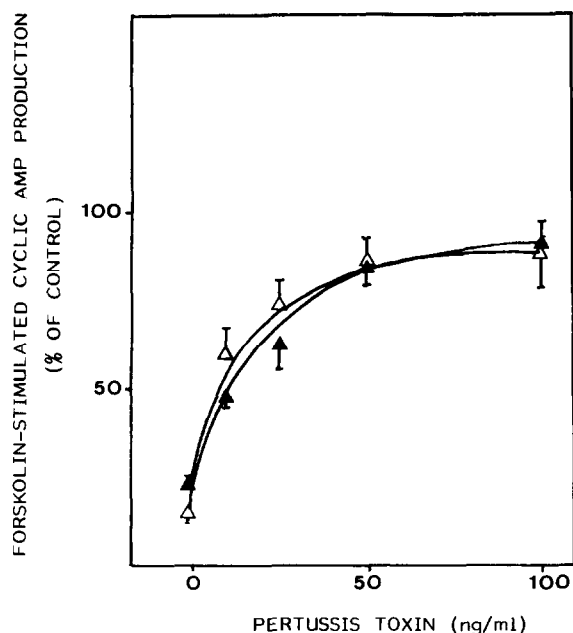


Fig.5. Ability of pertussis toxin to abolish the inhibitory effect of nicotinic acid on forskolin-stimulated cyclic AMP production in fat cells: influence of prolonged fasting. Fat cells from fed ($\blacktriangle$) and fasted ($\triangle$) rats were preincubated for 3 h at 37°C in the absence or presence of increasing concentrations of pertussis toxin in Earle's medium-20 mM Hepes (pH 7.4) supplemented with 4% albumin. Fat cells, washed 3 times with fresh medium, were reincubated in the presence of 10 μ M forskolin, 1 mM IBMX, 50 mU/ml adenosine deaminase and in the absence (control) or presence of 10 μ M nicotinic acid. 30 min later, reactions were stopped and cyclic AMP productions determined. Each point represents the mean $\pm$ SE of 3 separate experiments and is expressed as the percentage of the corresponding control value.

concentration required to reduce by one-half the effect of nicotinic acid) and thereby the ability of α_i to interact with N_s are unchanged by fasting.

4. DISCUSSION

In a recent study, Chohan et al. [14] have reported a decreased responsiveness of lipolysis to the adenosine analog N^6 -PIA in starved rats. As, adenylate cyclase is both positively and negatively regulated, these authors have suggested that decreased adenylate cyclase/lipolysis responsiveness to inhibitory signals could be one of the

mechanisms by which starvation increases the sensitivity of adenylate cyclase and thereby lipolysis to stimulation by β -adrenergic amines [11–13]. This hypothesis was presently tested by investigating the influence of prolonged fasting on the inhibitory responses of stimulated adenylate cyclase and cAMP production to the antilipolytic agents nicotinic acid and N^6 -PIA. To avoid any interference due to the β -adrenoceptor changes induced by fasting in rat fat cells [13], these experiments were performed in the presence of forskolin which stimulates adenylate cyclase through a post-receptor mechanism.

Our results fail to provide any evidence that fasting decreases the sensitivity of adenylate cyclase in membranes and cAMP production in intact adipocytes to the inhibitory drugs, nicotinic acid and N^6 -PIA. Moreover, we were unable to find any significant difference between fasted and fed rats with regard to the sensitivity of basal adenylate cyclase to inhibition by GTP. Finally, experiments performed with pertussis toxin to study selectively the α_i subunit of N_i showed no influence of fasting on both the amount (as judged by [32 P]ADP-ribose incorporation) and the functional activity of α_i (as judged on the ability of pertussis toxin to block cyclic AMP inhibitory responses). From these studies, it thus becomes clear that the fasting-induced increase in fat cell adenylate cyclase responsiveness to β -adrenergic amines [12,13] and guanine nucleotides [13] cannot be accounted for by decreased activity of the inhibitory coupling system but results from a specific increase in the function of the stimulatory one. This is further supported by the finding that, when studied under experimental conditions favoring adenylate cyclase inhibition, low concentrations of GTP induce substantial stimulation of the enzyme in fasted but not in fed rats (fig.3). Studies are currently underway to determine which step(s) of the stimulatory coupling system (amount of N_s , affinity of α_s for GTP and the β -subunit, interaction of α_s with C...) is specifically altered by fasting.

Finally, although the reasons for the discrepancies between our findings (i.e. unimpaired inhibitory response of adenylate cyclase) and those of Chohan et al. [14] (i.e. decreased inhibitory response of lipolysis) remain unclear at present, they could be explained by important differences in the experimental conditions used. In fact,

besides the strains of rats (Wistar vs Sprague-Dawley) and the duration of fasting (72 vs 24 h), the experiments of Chohan et al. [14], contrary to ours, were performed in the absence of any phosphodiesterase inhibitor. As adenosine and N⁶-PIA have recently been reported to stimulate phosphodiesterase in fat cells [27–29], it cannot be excluded that the reduced antilipolytic response to adenosine found by these authors in fasting is related to altered response of this enzyme to adenosine in the starved state.

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