

# The antilipolytic effect of insulin does not require adenylate cyclase or phosphodiesterase action

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Insulin antagonized the lipolytic actions of epinephrine in rat epididymal adipocytes when the phosphodiesterase inhibitor, Ro 20-1724, was present. Adipocytes were depleted of functional cAMP by inhibiting adenylate cyclase with *N*<sup>6</sup>-phenylisopropyladenosine in the presence of adenosine deaminase such that Ro 20-1724 no longer stimulated lipolysis. The cAMP analogs 8-thioisopropyl-cAMP or 8-thiomethyl-cAMP, which are resistant to phosphodiesterase hydrolysis, were subsequently added to bypass adenylate cyclase and phosphodiesterase action. Under these conditions, insulin antagonized the lipolytic effects of these analogs, even in the presence of Ro 20-1724.

*Lipolysis      Rat adipocyte      Insulin      Phosphodiesterase      Adenylate cyclase      Cyclic AMP*

## 1. INTRODUCTION

It is generally accepted that one of the principal functions of insulin is to regulate fat mobilization by inhibiting the rate of lipolysis. Early studies demonstrated an insulin-mediated decrease of cAMP levels in adipose tissue stimulated with epinephrine [1]. Subsequently, both insulin inhibition of adenylate cyclase [2,3] and insulin stimulation of cAMP phosphodiesterase [4–7] have been reported. This led to the hypothesis that the antilipolytic effect of insulin in adipose tissue could be fully accounted for by a decrease in cAMP concentrations as a result of phosphodiesterase activation and/or adenylate cyclase inhibition [8–11]. However, several groups have challenged this mechanism based on the observation that insulin could inhibit lipolysis in the absence of any measurable changes in cAMP concentrations [12–16]. In each case the evidence supporting these conclusions derive from cAMP measurements. These measurements can be misleading since they

are extremely dependent on the time and conditions of the incubation [11,16].

This paper presents a new approach not dependent on cAMP measurements to address the controversy surrounding the requirement of cAMP decreases for the antilipolytic effects of insulin. By incubating adipocytes depleted of functional cAMP with phosphodiesterase resistant cAMP analogs, we present conditions where the antilipolytic effect of insulin is independent of both phosphodiesterase and adenylate cyclase action and therefore cAMP concentrations.

## 2. MATERIALS AND METHODS

Insulin was a gift from Dr Ron Chance of Eli Lilly Co. and Ro 20-1724 was a gift from Hoffmann La Roche. 8-Thioisopropyl-cAMP and 8-thiobenzyl-cAMP were purchased from ICN and *N*<sup>6</sup>-phenylisopropyladenosine (PIA) was from Boehringer Mannheim. All other reagents were obtained from Sigma.

Adipocytes were prepared as in [17] from epididymal fat pads of rats fed ad libitum. Approx. 50 mg cells in 1.0 ml Krebs-Henseleit buffer

**Abbreviation:** Ro 20-1724, d-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidione

containing 1.2 mM  $\text{CaCl}_2$ , 2% bovine serum albumin, and 280 mU/ml adenosine deaminase were incubated for 2 h at 37°C in plastic, 20-ml scintillation vials under an atmosphere of 95%/5%  $\text{O}_2:\text{CO}_2$  yielding linear rates of glycerol release. The concentration of  $(\text{NH}_4)_2\text{SO}_4$  added with adenosine deaminase had no effect on glycerol release. Glycerol was measured in cell extracts deproteinized with perchloric acid and neutralized with KOH according to [18].

### 3. RESULTS

#### 3.1. Effects of Ro 20-1724 on insulin antilipolytic effect

To examine the role of phosphodiesterase activation in the antilipolytic response of insulin, the effect of the phosphodiesterase inhibitor Ro 20-1724 on the insulin antagonism of epinephrine-stimulated lipolysis in adipocytes was determined. The ability of insulin to inhibit epinephrine-stimulated glycerol release was unchanged by a maximally effective concentration of Ro 20-1724 (table 1).

#### 3.2. Depletion of functional cAMP

PIA, an inhibitor of adipocyte adenylate cyclase [19], was used to inhibit cAMP production in these cells. Authors in [20] have demonstrated that  $10^{-7}$  M PIA, in the presence of adenosine

Table 1

The effect of insulin on lipolysis stimulated by epinephrine and Ro 20-1724

Effector	Glycerol release ( $\mu\text{mol/g}$ per h)	
	– Insulin	+ Insulin
No addition	$1.21 \pm 0.15$	$0.85 \pm 0.09$
Epinephrine	$7.42 \pm 0.31$	$2.01 \pm 0.13$
Epinephrine + Ro 20-1724	$11.1 \pm 0.68$	$2.43 \pm 0.14$

Results are expressed as the means  $\pm$  SE of duplicates from 3 different cell preparations. Concentrations of effectors were: insulin,  $10^{-10}$  M; epinephrine,  $5 \times 10^{-7}$  M; Ro 20-1724,  $10^{-4}$  M. Adipocytes were incubated for 2 h at 37°C in medium containing 280 mU/ml adenosine deaminase, as described in section 2

Table 2

Depletion of functional cAMP in adipocytes

Effector	Glycerol release ( $\mu\text{mol/g}$ per h)
Control	$1.16 \pm 0.09$
Ro 20-1724	$2.61 \pm 0.10$
PIA	$0.23 \pm 0.02$
PIA + Ro 20-1724	$0.30 \pm 0.02$

Results are expressed as the means  $\pm$  SE of duplicates from 4 different cell preparations. The concentration of PIA was  $10^{-7}$  M and Ro 20-1724  $10^{-4}$  M. All other conditions were identical to those in fig.1

deaminase to reduce extracellular adenosine accumulation, lowers intracellular cAMP levels to a point where adipocytes can be considered depleted of functional cAMP. In agreement with these authors, we observed that PIA markedly decreased basal lipolysis (table 2). Evidence that PIA effectively inhibited cAMP production was obtained by using the specific phosphodiesterase inhibitor Ro 20-1724 which, unlike the methylxanthines, is not an adenosine antagonist [21,22]. In the presence of PIA and adenosine deaminase, the stimulation of glycerol release by Ro 20-1724 was negligible compared to the large effect of Ro 20-1724 in the absence of PIA ([20]; table 2). In adipocytes incubated with PIA, exogenously added cAMP analogs were still effective as lipolytic agents indicating that PIA did not interfere with the actions of cAMP at steps distal to cAMP generation (fig.1).

#### 3.3. Insulin effect on 8-thiomethyl-cAMP and 8-thioisopropyl-cAMP

To delineate the role of adenylate cyclase inhibition in the antilipolytic actions of insulin, adipocytes were incubated with cAMP analogs which would bypass adenylate cyclase action. Insulin antagonized the lipolytic effects of both 8-thiomethyl-cAMP and 8-thioisopropyl-cAMP (fig.1), two analogs that permeate the plasma membrane [23]. Since these experiments were performed under conditions where the adipocytes were functionally depleted of intracellular cAMP, we conclude that insulin does not require adenylate cyclase inhibition for its antilipolytic response.

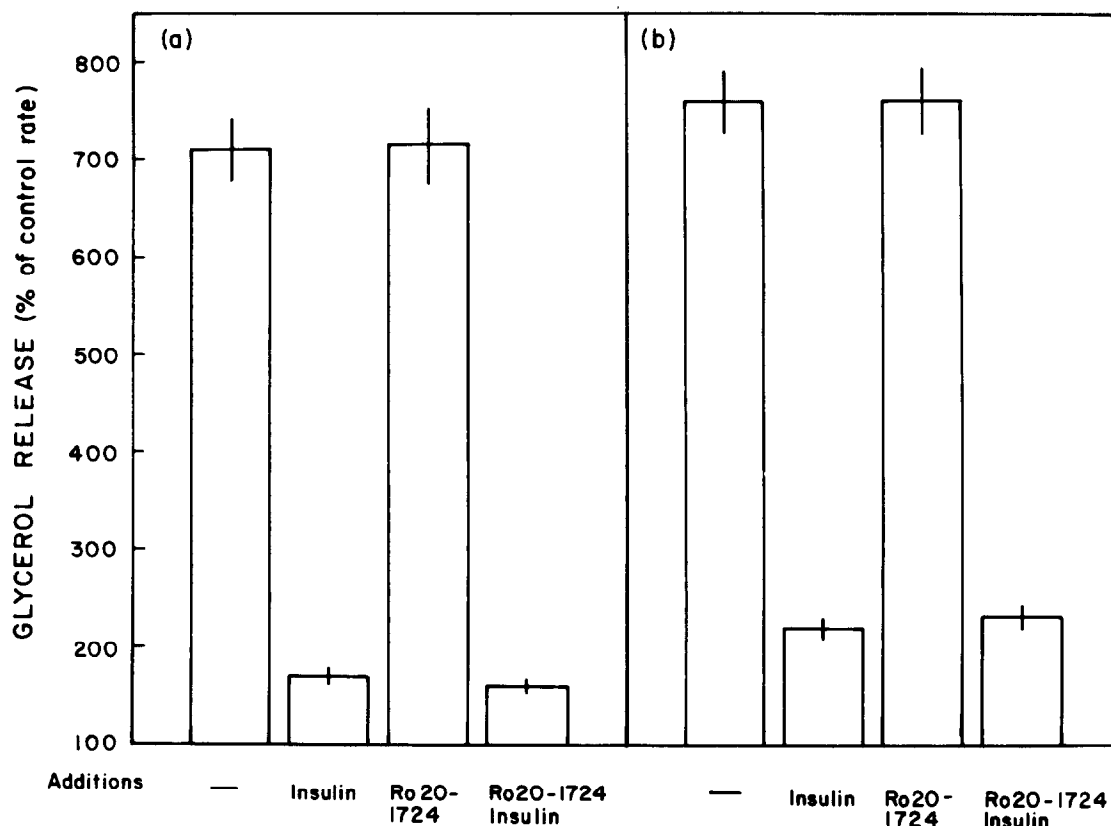


Fig.1. The effect of insulin and Ro 20-1724 on lipolysis stimulated by (a) 8-thiomethyl-cAMP and (b) 8-thioisopropyl-cAMP. Adipocytes were incubated in the presence of  $10^{-7}$  M PIA and 280 mU/ml adenosine deaminase as described in section 2 with either (a) 8-thiomethyl-cAMP ( $2.5 \times 10^{-4}$  M) or (b) 8-thioisopropyl-cAMP ( $2.5 \times 10^{-4}$  M). Other effector concentrations were as in table 1. Results are expressed as the means  $\pm$  SE of duplicates from 4 different cell preparations. The control rate of glycerol release was  $1.11 \pm 0.06$   $\mu$ mol/g per h.

The analogs chosen, 8-thiomethyl-cAMP and 8-thioisopropyl-cAMP, also have the feature of being resistant to hydrolysis by phosphodiesterase [25,26]. This was supported by the observation in vivo that phosphodiesterase inhibition by Ro 20-1724 had no effect on the rate of glycerol release (fig.1). The lipolytic effect of these two analogs cannot be due to phosphodiesterase inhibition since, in these cells depleted of functional cAMP, phosphodiesterase inhibition alone did not stimulate lipolysis. Under these conditions, insulin antagonized the effects of either 8-thiomethyl-cAMP or 8-thioisopropyl-cAMP even in the presence of Ro 20-1724 (fig.1). Similar results were also obtained using another phosphodiesterase-resistant analog, 8-thiobenzyl-cAMP (not shown).

These data clearly indicate that under these conditions the antilipolytic response of insulin is independent of any effect on either phosphodiesterase or adenylate cyclase.

#### 4. DISCUSSION

The antilipolytic effect of insulin has often been correlated with a decrease in cAMP concentrations in adipose tissue [1,8–11,24]. Although this is not an invariant finding [12–16], some have proposed that this decrease in cAMP levels fully explains the antilipolytic actions of this hormone [8–11]. Both sides of this controversy have used cAMP measurements as the basis for their conclusions. These measurements can be misleading since they

are extremely dependent on experimental conditions. Since the spike rise in the concentration of cAMP is the important parameter for activation of the cAMP-dependent protein kinase and therefore lipolysis, time points for cAMP measurements must be chosen carefully. The possibility that cAMP may be sequestered or compartmentalized further complicates these measurements [27,28]. Differences in cell density, the pre-incubation conditions of adipocytes, and insulin concentrations have all been shown to influence the relationship between the antilipolytic effect of insulin and intracellular cAMP levels [11,16]. Furthermore, in many studies the accumulation of adenosine, an agent that lowers cAMP levels, has not been controlled. In light of these problems, an alternate approach was undertaken to determine the requirement of cAMP changes for the inhibition of lipolysis by insulin.

If insulin were to act through decreases of cAMP concentrations, this could be accomplished through either adenylate cyclase inhibition or phosphodiesterase stimulation (it has been established that insulin does not affect the partitioning of cAMP across the plasma membranes [16,28]). Although early reports of inhibition of adenylate cyclase by insulin exist [2,3], confirmation of these reports has been difficult. To study the role of adenylate cyclase inhibition, adipocytes were depleted of functional cAMP using the adenylate cyclase inhibitor PIA as in [20]. PIA effectively inhibited the generation of cAMP since in the presence of PIA the effects of the phosphodiesterase inhibitor Ro 20-1724 were negligible. Under these conditions which approximate an adenylate cyclase-deficient cell, insulin antagonized the lipolytic effects of exogenously added cAMP analogs which bypass adenylate cyclase. This clearly demonstrates that adenylate cyclase inhibition is not required for the antilipolytic effect of insulin.

Phosphodiesterase activation by insulin has been consistently observed in a number of insulin-sensitive tissues including adipose tissue (see [29] and references therein), however, the role of this effect has been disputed (review, [30]). The phosphodiesterase inhibitor Ro 20-1724 did not affect the insulin antagonism of lipolysis stimulated by epinephrine ([24]; table 1) suggesting that phosphodiesterase activation is not required. Some investigators have had difficulty in obtaining this

result [1,13] because the responses to insulin were examined on maximally stimulated rates of lipolysis where insulin is ineffective because of the high intracellular cAMP concentrations [31].

To test the hypothesis that phosphodiesterase activation is not required for insulin inhibition of lipolysis, 8-thiomethyl-cAMP and 8-thioisopropyl-cAMP, two phosphodiesterase-resistant analogs [25,26], were used. Evidence that these analogs were not hydrolysed *in vivo* was obtained by assessing the effects of phosphodiesterase inhibition on lipolysis stimulated by these analogs in adipocytes depleted of functional cAMP. The best controlled studies have indicated that at submaximally stimulated rates of lipolysis, these rates reflect intracellular cAMP concentrations well [10,11,16,23,32-34]. Therefore, the observation that phosphodiesterase inhibition had no effect on the rate of lipolysis stimulated by either analogs is further confirmation that these analogs are not appreciably hydrolysed in the cell. In adipocytes depleted of endogenous cAMP, insulin antagonized the lipolytic effects of these analogs even in the presence of the phosphodiesterase inhibitor Ro 20-1724. This clearly represents conditions where the antilipolytic effect of insulin does not involve adenylate cyclase or phosphodiesterase action and hence cAMP changes.

The dissociation of the antagonistic effects of insulin from changes in hormonally stimulated levels of cAMP agrees with similar observations in liver [35]. A potential site of insulin action under these conditions is the direct inhibition of the cAMP-dependent protein kinase as has been observed in liver [35,36] and muscle [37,38]. Lipolysis is regulated through the phosphorylation of a hormonally-sensitive lipase by cAMP-dependent protein kinase [39]. Insulin decreases the phosphorylation of the stimulated lipase [40] which may be accomplished through either an inhibition of protein kinase activity or an increase of protein phosphatase activity. A postulated inhibition of cAMP-dependent protein kinase could also account for an increase in protein phosphatase activity since in adipose tissue insulin has been shown to decrease the phosphorylation and activity of a protein phosphatase inhibitor which is controlled by cAMP-dependent protein kinase [41]. Further studies will be necessary to determine the validity of these mechanisms.

## NOTE ADDED IN PROOF

Mooney et al. [42] recently reported that insulin antagonized lipolysis stimulate by exogenous cAMP in digitonin permeabilized rat adipocytes, further supporting the hypothesis that insulin action does not require adenylate cyclase inhibition.

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