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CELLULAR CYCLIC AMP LEVELS MODULATE INSULIN SENSITIVITY AND RESPONSIVENESS - EVIDENCE AGAINST A SIGNIFICANT ROLE OF G_i IN INSULIN SIGNAL TRANSDUCTION

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Summary: Treating rats with pertussis toxin (PTX) both elevated the adipocyte cAMP levels and impaired sensitivity and responsiveness to the antilipolytic effect of insulin in the presence of different ß-adrenergic agonists. However, in the presence of a fixed medium concentration of the degradable cAMP analogue, 8-bromo-cAMP, the effect of insulin was similar in PTX- and control cells. Elevating the cAMP levels in control cells either through different concentrations of the cAMP analogue or addition of adenosine deaminase impaired both insulin sensitivity and responsiveness to a similar extent as that seen in PTX-treated cells. The antilipolytic effect of insulin was exerted through the activation of the cGMP-inhibitable phosphodiesterase (cGI-PDE) as it was dose-dependently impaired by the specific cGI-PDE inhibitor OPC 3911.
The results show the importance of the cellular cAMP levels in modulating insulin sensitivity and action. G_i plays a minor role, if any, for the signal transduction of the antilipolytic effect of insulin.

Receptor phosphorylation and tyrosine kinase activation play an important role in insulin signal transduction [1]. In addition, recent studies have suggested that the inhibitory GTP-binding protein (G_i) may play a role. For instance, insulin-resistant cells from animals made diabetic with streptozotocin show an impaired response to G_i activation and also have a low G_i protein content [2]. Several recent reports have shown that pertussis toxin (PTX) treatment leads to an impairment of insulin sensitivity in different cells [3-5]. Furthermore, insulin attenuates ADP-ribosylation by PTX *in vitro* [6] and G_i and related proteins can be phosphorylated *in vitro* by insulin receptor kinase(s) [7-9] but not in intact cells [6, 10]. Insulin responsiveness is also decreased in cells exposed to PTX. This includes maximally insulin-stimulated glucose transport [11] and translocation of IGF II receptors [12]. In addition, adenosine and other agents activating G_i improve the responsiveness to insulin on these pathways in normal cells [13].

Taken together, there is much indirect evidence to support a role for G_i in insulin signal transduction and responsiveness. However, G_i also modulates adenylyl cyclase activity and, thus, cAMP formation. cAMP exerts a marked in-

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sulin-antagonistic effect both at the receptor [11, 14] and postreceptor [13, 15] levels. Thus, in order to attribute an altered insulin effect following PTX-treatment to an impaired G_i-linked signal transduction pathway, it is critical to exclude the effect of concomitant alterations in cAMP levels.

The present studies were designed to address this issue by studying the antilipolytic effect of insulin. The data clearly suggests that the most important effect of PTX is to elevate the cAMP levels to such an extent that both insulin sensitivity and responsiveness become impaired.

MATERIALS AND METHODS

Collagenase , (-)-isoprenaline, 8-bromo-cAMP, and bovine serum albumin (BSA) were from Sigma Chemical Co. (St Louis, MO, USA), [-^{32}P] ATP (3000 Ci/mmol) was from Du Pont (Boston, Mass., USA). Prenalterol was a gift from Astra-Hässle (Mölndal, Sweden) and OPC 3911 was donated by Dr. Per Belfrage, University of Lund, Sweden. Adenosine deaminase (ADA) was from Boehringer Mannheim (Mannheim, Germany), medium 199 from Statens Bakteriologiska Laboratorium (Stockholm, Sweden) and pertussis toxin from List Biological Laboratories, (Campbell, CA., USA).

Male Sprague Dawley rats (150 - 200 g), fed ad libitum, were sacrifized and the epididymal fat pads excised and minced. Fat cells were isolated in medium 199 with 0.8 mg/ml collagenase and 4% BSA (w/v) [16]. Where indicated, the rats were injected with either 3 μg pertussis toxin (PTX) or 0.9% NaCl (control cells) 64-72 h before sacrifize. Under the conditions used, subsequent ADP-ribosylation in vitro of a 43 KDa protein, consistent with G_i, is completely inhibited (16). This was also confirmed in random experiments by the lack of inhibition of lipolysis by adenosine, a G_i agonist.

Lipolysis was studied as previously described [16] in the absence or presence of 1 U/ml adenosine deaminase (ADA) and the indicated concentrations of the various additions. Glycerol release to the incubation medium was measured radiometrically [17] and taken as an index of lipolysis. In a few experiments, glycerol was analysed enzymatically with the glycerokinase method [18]. These methods were found to be highly correlated ($r = 0.98$, $n = 63$).

Cell and medium cAMP was measured as described previously [16] after 4 min incubation at 37°C in the absence of a PDE inhibitor.

RESULTS AND DISCUSSION

The antilipolytic effect of insulin was completely abolished by 10 μM OPC 3911, a specific inhibitor of the cGMP-inhibitable low K_m phosphodiesterase (cGI-PDE) [19, 20] when lipolysis was stimulated with either maximal or submaximal concentrations of 8-bromo-cAMP or isoprenaline (Fig. 1). Furthermore, increasing concentrations of OPC 3911 gradually impaired the responsiveness to the antilipolytic effect of insulin when lipolysis was stimulated by 10^{-6}M isoprenaline (data not shown). Thus, stimulation of this particular enzyme is essential for the antilipolytic effect of insulin as also concluded in a recent report [21]. Previous studies with different cAMP analogues have also indicated that insulin exerts its effect by activating PDE [22, 23] although the isoform was unknown.

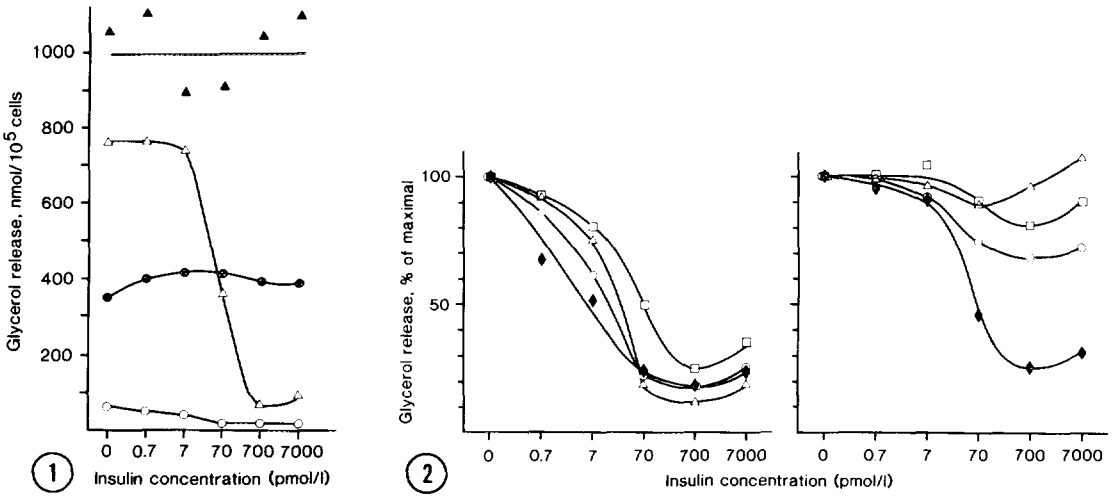

Fig. 1. **The effect of the specific cGI-PDE inhibitor, OPC 3911, on the antilipolytic effect of insulin.**
Isolated cells were incubated with the indicated concentrations of insulin in the absence (○, △) or presence (●, ▲) of 10 μM-OPC 3911. Further additions were 5 nM isoprenaline (○, ●) or 5 mM 8-bromo-cAMP (△, ▲). To convert pmol/l of insulin to μU/ml, divide by 7.0.

Fig. 2. **Dose-response curves for the inhibitory effect of insulin on agonist-stimulated lipolysis in adipose cells from control (left) and pertussis toxin-treated (right) rats.**
Isolated cells were incubated with the indicated concentrations of insulin in the presence of prenalterol 100 μM (♦) or isoprenaline 5 nM (○), 10 nM (△) or 1 μM (□). Data are the means of 3-4 experiments.

However, there have also been other reports that the antilipolytic effect of insulin was mediated through a reduced beta-adrenergic binding [24], inhibited adenylyl cyclase activity [25] or impaired cAMP-binding to the cAMP-dependent protein kinase (cAMP PrK) [26-28]. The importance of these latter effects is conjectural since the finding that, in the presence of OPC 3911, insulin had no effect on lipolysis stimulated by either the degradable cAMP analogue, 8-bromo-cAMP, or isoprenaline (Fig. 1) clearly shows that cGI-PDE activation is the key mechanism for the antilipolytic effect.

In control cells, insulin virtually completely inhibited lipolysis stimulated by both submaximal and maximal concentrations of the partial ß-adrenergic agonist, prenalterol, or the full agonist isoprenaline (Fig. 2 - left). However, the insulin sensitivity was gradually impaired by increasing concentrations of isoprenaline (EC_{50} for insulin ~ 8 vs ~ 34 pM at 5 x 10^{-9} M and 10^{-6} M, respectively). Cells from PTX-treated animals were generally less sensitive to insulin (EC_{50} at 10^{-4} M prenalterol ~ 4 vs ~ 40 pM in control and PTX cells, respectively). A further impairment in insulin sensitivity was seen in the presence of increasing concentrations of isoprenaline but EC_{50} could not be adequately measured due to the low insulin response (Fig. 2 - right). A normal ability of

Table I. Total cell and medium cAMP levels in fat cells from control and pertussis toxin-treated animals

Addition to cells	cAMP conc. (p mol/10^5 cells)	
Isoprenaline (nmol/)	Control	PTX
-	0.3	1.6
5	0	1.6
50	1.0	4.6
100	1.2	113.8
500	1.3	85.6
1000	1.6	111.5

Isolated fat cells were incubated with the indicated agents for 4 min. Total cell and medium cAMP levels were then analysed. The data are from one representative experiment.

insulin to almost completely inhibit lipolysis, as in control cells (Fig. 2 - left), was only seen in PTX cells in the presence of the weak ß-agonist prenalterol (Fig. 2 - right).

This marked reduction in sensitivity and responsiveness to the antilipolytic effect of insulin in cells from PTX-treated rats could be due to; 1) high cAMP levels exceeding the capacity of the cGI-PDE in combination with a cAMP-mediated impairment of the insulin signal or; 2) a loss of an insulin signal transduction pathway that is mediated through the inhibitory GTP-binding protein.

Total cell and medium cAMP levels after 4 min. stimulation (Table 1) were markedly elevated in cells from PTX- treated animals at all isoprenaline concentrations tested. Even basal, non-stimulated levels were higher in PTX-treated cells but this difference could not be adequately quantified due to the relative insensitivity of the assay and the absence of a PDE inhibitor in the medium. In control cells, removal of adenosine by ADA reduced both insulin sensitivity and responsiveness in the presence of isoprenaline to a similar extent as that seen in cells from PTX-treated animals (data not shown). Also, increasing concentrations of 8-bromo-cAMP in control cells gradually reduced insulin sensitivity and, at high concentrations, responsiveness to insulin (Fig. 3). A supramaximal concentration of 8-bromo-cAMP (50 mM) completely abolished the antilipolytic effect of insulin (Fig. 3).

These data suggest that the impaired antilipolytic effect of insulin is due to the high cAMP levels and is consistent with previous findings that the ability of insulin to inhibit lipolysis is lost when the cAMP PrK activity ratio exceeds about 0.6 [29]. This means that insulin-induced activation of cGI-PDE is not sufficient to reduce the cAMP level to such an extent that lipolysis is

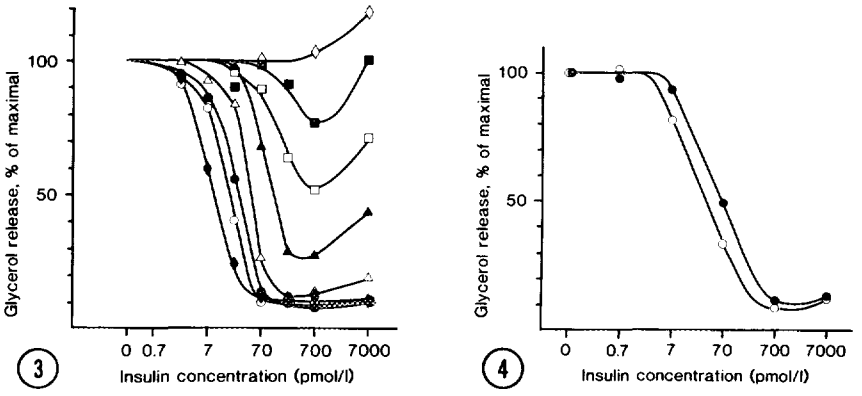

Fig. 3. **Dose-response curves for the inhibitory effect of insulin on lipolysis stimulated by increasing concentrations of 8-bromo-cAMP.**
Isolated cells from control rats were incubated with the indicated concentrations of insulin in the presence of the following concentrations of 8-bromo-cAMP: 1 mM (♦), 2 mM (○), 3mM (●), 5 mM (△), 10 mM (▲), 15 mM (□) 20 mM (■) or 50 mM (◊).

Fig. 4. **Dose-response curves for the inhibitory effect of insulin on 8-bromo-cAMP-stimulated lipolysis in cells from PTX-treated and control animals.**
Isolated cells from control (○) or PTX-treated (●) animals were incubated in the presence of 5 mM 8-bromo-cAMP and the indicated concentrations of insulin. Data are the means of 5 experiments.

inhibited, considering the non-linear relationship between lipolysis and cellular cAMP levels [16].

Elevated cAMP levels also reduce the sensitivity and responsiveness to insulin's stimulatory effect on glucose transport [11, 15] and IGF II receptor translocation [12]. The mechanisms for this probably reside both at the level of the receptor/insulin signal transduction and the effector proteins [11, 14, 30, 31]. In addition, there is evidence for non-cAMP- mediated effects of ß-adrenergic stimulation on the intrinsic activity of the glucose transporting protein [13, 32]. From the present data it is not possible, however, to separate the effect of cAMP on insulin signal transduction from the effect of high substrate concentrations exceeding the V_{max} for PDE.

As shown in Fig. 4, when lipolysis in PTX-treated cells was stimulated by a defined effective concentration of the degradable cAMP analogue, 8-bromo-cAMP, essentially the same sensitivity and responsiveness to insulin was found in PTX-treated as in control cells. The slight difference in insulin sensitivity may be accounted for by higher "basal" cAMP levels in PTX-treated cells. These data clearly show that the cAMP level plays a key role in modulating insulin action suggesting a minor role, if any, of the inhibitory GTP-binding protein in insulin signal transduction, at least when measured as antilipolytic effect of insulin. This is not in agreement with the conclusion of Goren et al. [3] that

PTX abolished the antilipolytic effect of insulin. However, their results may be due to a supramaximal activation and exceedingly high cellular cAMP levels. At supramaximal cAMP levels we also found a marked inhibition of the insulin response both in cells from PTX-treated animals stimulated with isoprenaline and in control cells stimulated with high concentrations of 8-bromo-cAMP (Fig. 3).

In conclusion, the cAMP level is critical for the cellular sensitivity and response to the antilipolytic effect of insulin by impairing the insulin signal transduction mechanisms and/or by constituting the substrate for the cGI-PDE. Consequently, the inherent capacity of cGI-PDE and its ability to become activated by insulin are of profound importance for the final cellular cAMP concentration and, thus, for the antilipolytic effect of insulin.

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