Cyclic AMP impairs the rapid effect of insulin to enhance cell-surface insulin-binding capacity in rat adipocytes

Jan W. ERIKSSON,* Peter LÖNNROTH and Ulf SMITH

Department of Medicine II, University of Gothenburg, Sahlgren's Hospital, S-413 45 Gothenburg, Sweden

The aim of this study was to characterize further the interaction between cyclic AMP (cAMP) and insulin binding and action. Rat adipocytes were preincubated at 37 °C for 20 min, and, after energy depletion with KCN, cell-surface ¹²⁵I-insulin binding was measured. As recently reported [Eriksson, Lönnroth & Smith (1992) *Diabetes* 41, 707–714], preincubation with insulin rapidly increased the number of cell-surface insulin binding sites up to ~5-fold through recruitment within the plasma membrane. This was completely abolished by the presence of 4 mM-*N*⁶-monobutyryl cAMP (a non-hydrolysable cAMP analogue) or 1 μM-isoprenaline, without any apparent change in receptor internalization. Insulin-stimulated receptor tyrosine kinase activity was attenuated by the cAMP analogue only if the exposure of the adipocytes was prolonged to 60 min. The cellular sensitivity to insulin, assessed as 3-*O*-methylglucose uptake, was markedly decreased by the cAMP analogue, and this could be attributed to the impaired cell-surface binding. However, evidence for post-receptor interactions between cAMP and insulin was also found: an impairment of maximal insulin-stimulated 3-*O*-methylglucose transport and a delay in the rate of activation of the glucose transport system by insulin. In conclusion, these data demonstrate that β-adrenergic stimulation and elevated cAMP levels markedly impair the ability of insulin to enhance cell-surface insulin-binding capacity. This novel interaction may be an important mechanism for the cellular insensitivity to insulin produced by cAMP.

INTRODUCTION

β-Adrenergic stimulation rapidly produces insulin resistance *in vivo* [1–3]. This effect can also be elicited *in vitro* by catecholamines or by direct exposure to various cyclic AMP (cAMP) analogues, as shown in studies with adipocytes, skeletal-muscle strips and cardiac myocytes [4–12]. Previous work has indicated that this is partly due to decreased insulin binding, which leads to a decreased sensitivity to insulin [8–12]. However, there is also evidence for post-binding perturbations elicited by β-adrenergic stimulation and elevated cAMP levels, which may impair cellular responsiveness as well as sensitivity to insulin. Such effects of cAMP have been demonstrated with both insulin-stimulated glucose transport and translocation of receptors for insulin-like growth factor II [12–14]. The post-binding interaction between cAMP and insulin has in part been attributed to a decrease in the insulin-receptor tyrosine kinase activity [15–17], although one previous study was unable to show an effect of isoprenaline on this enzyme [13]. Moreover, previous data have indicated a decreased intrinsic activity [18,19] and an increased phosphorylation of glucose transporters [20,21] in conjunction with isoprenaline treatment. In a recent study, cAMP treatment decreased mRNA for the insulin-responsive glucose transporter (GLUT 4), suggesting a transcriptional gene repression [22].

We have recently demonstrated a novel effect of insulin to up-regulate markedly and rapidly the number of cell-surface insulin-binding sites in rat adipocytes, probably occurring through a changed receptor conformation [23]. To elucidate further the rapid interactions between cAMP and insulin at the receptor level, rat adipocytes were treated for 20 min with insulin in the presence or absence of isoprenaline or *N*⁶-monobutyryl cAMP (*N*⁶-mbcAMP), a cAMP analogue which is not hydrolysed by the cAMP phosphodiesterase [24]. To stop receptor internalization and recycling, the cells were energy-depleted with KCN and, after extensive washing, ¹²⁵I-insulin was added to allow as-

essment of the cell surface binding [23,25–28]. Furthermore, insulin-stimulated 3-*O*-methylglucose transport and insulin-receptor tyrosine kinase activity were measured.

EXPERIMENTAL

Materials

Pig monocomponent insulin and mono-[¹²⁵I-Tyr-A14]-insulin (sp. radioactivity 200–300 μCi/μg) were purchased from Novo (Copenhagen, Denmark). 3-*O*-[¹⁴C]Methyl-D-glucose (sp. radioactivity 59 μCi/μmol) was from Amersham International (Amersham, Bucks., U.K.), and [γ-³²P]ATP (sp. radioactivity 3 Ci/μmol) from DuPont (Geneva, Switzerland). Collagenase, BSA (fraction V), wheat-germ agglutinin, Glu⁸⁰Tyr²⁰, *N*⁶-monobutyryl cAMP (*N*⁶-mbcAMP), isoprenaline, trypsin and proteinase inhibitors were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Adenosine deaminase (ADA) was obtained from Boehringer Mannheim (Mannheim, Germany) and medium 199 from Statens Bakteriologiska Laboratorium (Stockholm, Sweden).

Cell preparation and incubation conditions

Male Sprague-Dawley rats, fed *ad libitum* and weighing 160–200 g, were stunned and decapitated. The epididymal fatpads were immediately excised and minced. Fat-cells were isolated in medium 199 with 1 mg of collagenase/ml and 40 mg of BSA/ml. The cells were then filtered through a nylon mesh and washed four times with fresh medium. The preincubations were performed with the indicated agents (lipocrit 5–10%) at 37 °C for 20 min in the presence of 1 unit of ADA/ml and 30 mM-Hepes. The buffering capacity of the incubation medium was sufficient to maintain the pH within the range 7.30–7.50 even in the presence of lipolytic agents. After the preincubations and unless otherwise specified, the cells were energy-depleted with 2 mM-KCN for 5 min before the binding assays as previously described [23].

Abbreviations used: cAMP, cyclic AMP; *N*⁶-mbcAMP, *N*⁶-monobutyryl cAMP.

* To whom correspondence should be addressed.

¹²⁵I-insulin binding to intact cells

To remove bound and free insulin and other agents from the preincubation medium that might directly interfere with binding, the cells were washed four times in medium 199 with 10 mg of BSA/ml and 2 mM-KCN [23]. Portions of cells and medium were transferred to 16 °C, and 0.2 ng of ¹²⁵I-insulin/ml and 0–3.5 µg of unlabelled insulin/ml were added. After 2 h, when steady state was reached, specific ¹²⁵I-insulin binding was measured as cell-associated radioactivity remaining after subtracting non-specific binding (in the presence of 3.5 µg of unlabelled insulin/ml).

¹²⁵I-insulin binding to the total and intracellular receptor pools

For assessment of binding to the entire pool of cellular receptors, the cells were solubilized essentially as described by Marshall *et al.* [29] after preincubation and washing. They were treated with 0.4 % Triton X-100 for 60 min at 4 °C in the presence of the proteinase inhibitors leupeptin (100 µM), pepstatin (70 µM), phenylmethanesulphonyl fluoride (500 µM) and aprotinin (10 µg/ml). Receptors were precipitated with poly(ethylene glycol) with IgG as a carrier. The samples were vortex-mixed, centrifuged at 11 000 g for 5 min, and the pellets were carefully washed three times and resuspended in medium 199 with 40 mg of BSA/ml and proteinase inhibitors. Binding of ¹²⁵I-insulin (0.2 ng/ml) in the presence or absence of 3.5 µg of unlabelled insulin/ml (non-specific binding) was performed for 18 h at 4 °C. The receptors were again precipitated, washed three times, and receptor-bound radioactivity was determined (non-specific binding subtracted).

Binding to intracellular receptors was assessed by treating cells with trypsin (1 mg/ml) at 16 °C for 10 min, followed by trypsin inhibitor (1 mg/ml) for 5 min, and then solubilization and ¹²⁵I-insulin binding as described above. This methodology was used to evaluate receptor internalization after preincubation with insulin or other agents [23,29].

Partial purification of insulin receptors

Samples of the cell suspension were treated with 0.5 % Triton X-100 in Hepes buffer (50 mM, pH 7.4), containing 10 mM-MgSO₄ and the proteinase inhibitors described above. After vortex-mixing and freezing at –80 °C for 1 h, the cell suspension was thawed and homogenized. After centrifugation at 17 000 g for 15 min at –4 °C, the triacylglycerol layer was carefully removed and the infranant centrifuged at 100 000 g for 60 min. The supernatant was applied to a wheat-germ agglutinin-agarose column and allowed to recirculate three times. After careful washing, the purified insulin receptors were eluted with 0.3 M-N-acetylglucosamine in Hepes buffer. Insulin binding was determined by adding 0.2 ng of ¹²⁵I-insulin to 100 µl of the eluate. After 18 h at 4 °C the receptors were precipitated with poly(ethylene glycol) together with IgG, and specific binding was measured.

Insulin-receptor tyrosine kinase activity

Triplicate 40 µl samples of the eluate containing the purified insulin receptors were incubated for 15 min at 20 °C with 2 mM-MnCl₂ in the absence or presence of 100 000 µ-units of insulin/ml; 15 µM-ATP was added, and 15 min later Glu⁸⁰Tyr²⁰ (2.5 mg/ml), [³²P]ATP (~ 10⁶ c.p.m.) and unlabelled ATP (final concn. 15 µM) were added to a total volume of 100 µl and the incubations continued for another 15 min. Then 75 µl samples were spotted on Whatman 3 MM papers, washed with 10 % trichloroacetic acid containing 10 mM-Na₄P₂O₇, and then once with 95 % ethanol followed by 95 % acetone. Radioactivity was determined by scintillation counting, and phosphate incorporation into

Glu⁸⁰Tyr²⁰ was expressed per fmol of bound ¹²⁵I-insulin to adjust for differences in receptor yield.

[¹⁴C]3-O-Methylglucose transport

This assay was performed as previously described [30]. After preincubation with the indicated agents, portions of the cell suspension were pulsed with [¹⁴C]3-O-methylglucose (50 µM, 1.0 µCi) without prior energy depletion. Glucose uptake was stopped after 5–10 s with ice-cold phloretin (0.3 mM). Cell-associated radioactivity was measured and extracellular [¹⁴C]3-O-methylglucose subtracted.

Statistical analysis

Statistical significance of differences was tested with Student's two-tailed *t* test for paired data. Results are expressed as means ± S.E.M. unless otherwise indicated.

RESULTS

¹²⁵I-insulin binding to intact cells

Preincubation of adipocytes for 20 min with insulin followed by energy depletion and extensive washing increased cell-surface ¹²⁵I-insulin binding in a concentration-dependent manner (EC₅₀ ~ 20 µ-units/ml; results not shown). At a maximally effective concentration of insulin (1000 µ-units/ml), the increase was 4–5-fold compared with control cells (Fig. 1). Scatchard analysis confirmed that the effect of insulin to enhance binding capacity was due to an increase in the apparent number of binding sites without any change in apparent receptor affinity (Fig. 1, insert). This effect was completely abolished by the presence of 4 mM-N⁶-mbcAMP (Fig. 1) or 1 µM-isoprenaline (binding 33 ± 11 % compared with cells treated with insulin alone; *n* = 3, *P* < 0.05).

¹²⁵I-insulin binding to total and intracellular receptor pools

Preincubation of adipocytes with insulin (1000 µ-units/ml), N⁶-mbcAMP (4 mM) or the combination of these agents did not significantly change insulin binding to the solubilized total cellular receptor pool (101 ± 14, 108 ± 7 and 92 ± 9 % of binding to receptors from control cells respectively; *n* = 6). The effects of insulin and N⁶-mbcAMP on receptor internalization was assessed by treating the cells with trypsin before solubilization. Preincubating the cells with insulin (1000 µ-units/ml) for 20 min increased intracellular binding capacity from 36 ± 7 to 57 ± 9 % (*n* = 6, *P* < 0.02) of total binding (no trypsin), reflecting an enhanced receptor internalization [23]. Thus in the intact cell the fraction of the remaining plasma-membrane-associated binding sites available for ligand binding is apparently increased by insulin stimulation (see the Discussion section). The intracellular proportion of receptors was not significantly altered by addition of 4 mM-N⁶-mbcAMP (37 ± 6 % alone and 70 ± 6 % when combined with insulin treatment).

Tyrosine kinase activity of partially purified insulin receptors

Pretreating the intact cells with insulin (1000 µ-units/ml) at 37 °C for 20 min enhanced the tyrosine kinase activity of subsequently wheat-germ-agglutinin-purified receptors by 3–4-fold compared with untreated cells (Table 1). The insulin-stimulated activity was unchanged by the presence of N⁶-mbcAMP during the preincubations, whereas N⁶-mbcAMP alone produced a modest activation by 50–100 % (Table 1). When insulin (100 000 µ-units/ml) was added directly to the purified receptors to achieve maximal intrinsic tyrosine kinase activity, this was virtually identical whether the cells had been pretreated or not with insulin and/or cAMP (~ 10–15-fold above non-stimulated

Fig. 1. Effects of insulin and *N*⁶-mbcAMP on ¹²⁵I-insulin binding to intact cells

The adipocytes were preincubated at 37 °C for 20 min in the presence of 1000 μ-units of insulin/ml (Ins; ■ in insert), 4 mM-*N*⁶-mbcAMP, the combination of these agents (▲ in insert) or without additions (● in insert). Then 2 mM-KCN was added and the cells were extensively washed. ¹²⁵I-insulin (0.2 ng/ml) and unlabelled insulin (0–3.5 μg/ml) were added and insulin binding was determined as described in the Experimental section. Data are means ± S.E.M. of six separate experiments, and *P* values refer to differences compared with untreated cells (not significant if no indication). Scatchard curves are from one representative experiment repeated twice (insert).

Table 1. Tyrosine kinase activity of partially purified insulin receptors

Activity is expressed as pmol of phosphate incorporated in Glu⁸⁰-Tyr²⁰ per fmol of bound ¹²⁵I-insulin. ^a*P* < 0.001, ^b*P* < 0.02, ^c*P* < 0.01 for comparisons with control cells (no addition).

Addition to cells	Insulin added to receptors (10 ⁵ μ-units/ml)...		Activity	
			– (<i>n</i> = 12)	+ (<i>n</i> = 9)
None			0.28 ± 0.05	3.15 ± 0.46
Insulin (1000 μ-units/ml)			1.00 ± 0.17 ^a	3.24 ± 0.53
<i>N</i> ⁶ -mbcAMP (4 mM)			0.48 ± 0.06 ^b	3.47 ± 0.51
Insulin + <i>N</i> ⁶ -mbcAMP			0.93 ± 0.20 ^c	3.39 ± 0.65

activity of receptors from control cells; Table 1). When preincubation of cells with *N*⁶-mbcAMP was prolonged to 60 min, a small decrease (~30%; *n* = 3, *P* < 0.05) of the insulin-stimulated tyrosine kinase activity was demonstrated. However, this inhibitory effect of the cAMP analogue was not consistently found after 30 or 40 min preincubation (results not shown).

¹⁴C]3-*O*-Methylglucose transport in adipocytes

Insulin pretreatment of the cells increased the rate of 3-*O*-methylglucose uptake in a concentration-dependent manner. The maximal rate (at 1000 μ-units of insulin/ml) was increased ~10–12-fold compared with non-stimulated cells (4.9 ± 1.1 versus 0.43 ± 0.09 amol/s per cell; *n* = 6, *P* < 0.01). The basal transport rate was modestly increased (0.87 ± 0.18 versus 0.43 ± 0.09 amol/s per cell; *n* = 6, *P* < 0.05), whereas the maximal insulin-stimulated rate was decreased (3.8 ± 0.80 versus

Fig. 2. Time course for the stimulatory effect of insulin on [¹⁴C]3-*O*-methylglucose transport

The cells were preincubated with 1000 μ-units of insulin/ml in the absence (●) or presence (▲) of 1 mM-*N*⁶-mbcAMP at 37 °C for the indicated times. Then [¹⁴C]3-*O*-methylglucose was immediately added and uptake was determined. Data are means ± S.E.M. from four separate experiments and are expressed as percentage of maximal effect on transport rate. The time for half-maximal activation (*t*_{1/2}) was 3.0 ± 0.6 min in control cells and 7.5 ± 1.0 min in *N*⁶-mbcAMP-treated cells (*P* < 0.05).

4.9 ± 1.1 amol/s per cell; *n* = 6, *P* < 0.05) by the presence of 4 mM-*N*⁶-mbcAMP. *N*⁶-mbcAMP also induced an insulin insensitivity, as demonstrated by an ~3-fold increase in EC₅₀ for insulin compared with control cells (46 ± 8 versus 18 ± 2 μunits/ml; *n* = 6, *P* < 0.01). The time course for the onset of activation of glucose transport by insulin was also studied. Fig. 2 shows that when *N*⁶-mbcAMP was present there was a clear delay in the onset of insulin action (*t*_{1/2} ~ 3 min for insulin alone, versus ~7.5 min in combination with *N*⁶-mbcAMP). For comparison, we also assessed the time course for ¹²⁵I-insulin binding to adipocytes at 37 °C without prior energy depletion. However, there were no significant changes in either *t*_{1/2} or time required for steady-state binding in the presence of *N*⁶-mbcAMP as compared with control cells (results not shown).

DISCUSSION

The present data show a marked inhibitory effect of isoprenaline and cAMP on insulin binding. Under the conditions used, this inhibition is of much greater magnitude than that previously found. Earlier studies have shown a 15–50 % decrease in insulin binding to rat adipocytes after exposure to these agents [8–10,12], whereas the present data indicate a decrease by 75–80 % in the presence of high insulin concentrations. The reason for this discrepancy is that the experimental design in this study takes into account the recently reported novel effect of insulin to amplify its binding in insulin-sensitive cells [23]. This effect is abolished by high levels of *N*⁶-mbcAMP or isoprenaline, and is also severely blunted in other states of insulin resistance, such as in cells from obese rats [23] and after amiloride treatment [31].

It is quite conceivable that the effect on binding plays an important role in the regulation of insulin sensitivity, since an enhanced binding capacity is expected to decrease the insulin concentration required to occupy a given number of binding sites. The glucose-transport experiments confirmed previous results, with an impaired sensitivity to the effect of insulin in the presence of *N*⁶-mbcAMP [12]. The decreased insulin sensitivity was quantitatively attributable to the abolished effect of insulin to up-regulate its receptors, with control cells displaying 2–3-fold

greater insulin sensitivity and 2–3-fold increase in insulin binding at around the EC_{50} as compared with N^6 -mbcAMP-treated cells.

The mechanisms for the effect of insulin to up-regulate its cell-surface binding sites, and hence also for the impairment produced by β -adrenergic stimulation and elevated cAMP levels, are at present unclear [23]. Obviously, neither insulin nor cAMP affects the total cellular receptor pool, as assessed with solubilized cells. cAMP does not appear to alter receptor internalization or the effect of insulin to increase this process, since the proportion of intracellular binding sites was not significantly changed by N^6 -mbcAMP. The effect of insulin on cell-surface binding is evident despite a concomitant net internalization of receptors, and thus apparently occurs within the receptor pool remaining in the plasma membrane [23]. This has also been confirmed with affinity labelling of ^{125}I -insulin to intact cells, where the increased binding was recovered in subsequently prepared plasma-membrane fractions (0.17 ± 0.02 versus 0.58 ± 0.07 pg of ^{125}I -insulin/ μ g of membrane protein for control and insulin-treated cells respectively; $n = 4$, $P < 0.01$) and specifically at the 135 kDa insulin receptor α -subunit, as shown by SDS/PAGE and autoradiography (J. W. Eriksson, P. Lönnroth & U. Smith, unpublished work). Indirect evidence such as altered interactions between receptors and a polyclonal insulin-receptor antibody or trypsin suggests that the enhanced binding is associated with a conformational change in the receptors or the plasma membrane, leading to the exposure of previously unavailable insulin-binding sites on the cell surface [23].

It is not clear by which mechanism cAMP impairs the effect of insulin to enhance receptor binding, but an altered phosphorylation of the insulin receptor could be involved [15–17,32]. Therefore, insulin-receptor tyrosine kinase activity was assessed after activation by insulin *in situ* in intact cells. However, under the incubation conditions used, the presence of N^6 -mbcAMP did not change the insulin-stimulated tyrosine kinase activity. This suggests that the impairment of insulin action on its binding induced by cAMP is not caused by perturbations of the tyrosine kinase activity. However, the possibility that tyrosine kinase activation is of importance for the conformational change to occur cannot be ruled out, since cAMP may impair some intermediary pathway between those events. Moreover, the receptor up-regulation elicited by insulin is not critical for tyrosine kinase activation. Possibly, 'transphosphorylation' between receptors [33,34] may explain why cAMP-treated cells displayed a normal tyrosine kinase activation despite the impaired insulin binding.

In some previous studies it was found that isoprenaline and cAMP decreased the activation of the tyrosine kinase by insulin in rat adipocytes [15–17]. However, there are also data indicating that, when pH is kept constant, no inhibitory effect on the tyrosine kinase is exerted by isoprenaline [13]. Thus the inhibitory effects reported [15–17] may, in part, be due to a decrease in pH induced by the lipolytic agents used. The time aspects may also be of importance, since in the present work a prolonged exposure to cAMP inhibited the insulin-stimulated tyrosine kinase activity. The mechanism for the effect of N^6 -mbcAMP alone to increase the tyrosine kinase activity modestly (Table 1) is unclear. One speculation may be that the serine phosphorylation of the insulin receptor elicited by cAMP [16,32] plays a role, although this seems not to be consistent with other findings [35,36].

cAMP was demonstrated to delay significantly the onset of insulin action on glucose transport, a perturbation also previously reported in human adipocytes from insulin-resistant subjects, i.e. non-insulin-dependent diabetes mellitus and obesity [37,38]. However, we found no corresponding delay in the time course for insulin binding, suggesting that this kinetic perturbation of glucose transport activation occurs through post-receptor mechanisms.

Such mechanisms are also involved in the decrease in maximally insulin-stimulated 3-*O*-methylglucose transport rate after cAMP treatment, since this cannot be explained by a diminished binding capacity in the face of a large pool of 'spare receptors' for insulin [39,40]. The finding that cAMP can moderately increase 3-*O*-methylglucose transport in the absence of insulin has also recently been reported in a study with human adipocytes [41], but the mechanisms for this are at present unknown.

In conclusion, this study shows that short-term treatment of rat adipocytes with isoprenaline or cAMP markedly impairs the ability of insulin to up-regulate its cell-surface binding sites. This may occur through inhibition of a conformational receptor change elicited by insulin and is not associated with alterations of receptor internalization or insulin-stimulated tyrosine kinase activity. The decreased cell-surface binding after exposure to cAMP is quantitatively related to the impaired sensitivity to insulin action on glucose transport.

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