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CYCLIC AMP MODULATES INSULIN BINDING AND INDUCES POST-RECEPTOR INSULIN RESISTANCE OF GLUCOSE TRANSPORT IN ISOLATED RAT ADIPOCYTES

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SUMMARY: The effect of cAMP on insulin binding and insulin stimulation of glucose transport was investigated in isolated rat adipocytes. Preincubation for 30 min in medium containing 16 mmol/l glucose and either db-cAMP or bromo-cAMP in concentrations of 10^{-4}-10^{-3} M inhibited high affinity binding of insulin by 15 to 30 % and glucose transport by 30 to 50 %. Preincubation with IBMX (10^{-4}-10^{-3} M) reduced insulin binding by 25 % and glucose transport by 70 %. Closer analysis of these data indicated that preincubation with these compounds caused not only a decrease in insulin binding but also a post-receptor resistance. High intracellular cyclic AMP-levels seem therefore to induce insulin resistance at both receptor and post-receptor levels.

Whereas an effect of insulin on intracellular cAMP levels is well documented (1,2), a modulatory action of cAMP on insulin stimulation of D-glucose transport is still controversial. There is on the one hand evidence for an inhibitory effect of high cAMP levels on insulin-stimulated glucose transport which has been obtained either using the indirect method of 1-[^{14}C] glucose oxidation (3,4) or assaying glucose transport in the presence of high activities of adenosine deaminase (5). On the other hand, other workers found that isoproterenol stimulated glucose transport in rat (6) and human adipocytes (7). D-glucose transport was inhibited by a phosphodiesterase inhibitor in human adipocytes (7). Schoenle et al. (8) working with different cAMP modulating agents on hypophysectomized rats in which glucose transport is maximally stimulated, concluded that cAMP does not modulate insulin stimulated glucose transport.

Since we showed earlier (9) that preincubation of fat cells with catecholamines decreases high affinity binding and

Abbreviations: cAMP, adenosine cyclic 3',5' monophosphate; IBMX, 3-isobutyl-1-methylxanthine; db-cAMP, N^6, O^2-dibutyryl-cAMP, bromo-cAMP, 8-bromoadenosine 3':5'-cyclic monophosphate

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insulin stimulation of glucose transport, the effect of cAMP on these parameters was reassessed.

MATERIALS AND METHODS

Fat cells were prepared according to (10) from male Sprague-Dawley rats fed ad libitum (180-220 g body weight). Krebs-Ringer-Hepes buffer (pH 7.4, 37°C) containing 2.5 g/dl crystalline bovine serum albumin was used for all incubations. In one set of experiments high intracellular levels of cAMP were obtained in the fat cells by preincubation for 30 min with IBMX (10^{-4}-10^{-3} M) from IGA-Chemie, Steinheim, FRG. In the other experiments, the high intracellular cAMP levels were simulated by the addition of db-cAMP or bromo-cAMP (10^{-4}-10^{-3} M). The incubations were carried out in the presence of 16 mmol/l D-glucose since the intracellular levels of ATP are then stable even following addition of catecholamines (unpublished observations).

3-O-Methylglucose transport: 3-O-methylglucose transport was measured by the method of Whitesell and Gliemann (11) as described (12). Aliquots (100 µl) of the concentrated cell-suspension (5 x 10^6 cells/ml) were drawn together with 200 µl 3-O-methylglucose (final concentration 0.5 mmol/l) and a tracer of 0.1 µCi 3-O-methyl-D-[^{14}C]-glucose (Radiochemical Centre, Amersham) into a mixing pipette (Gilson medical electronics, France). After 4 seconds, the uptake was stopped by diluting the cells in 5 ml NaCl (0.9 g/dl) which contained phloretin (1 mmol/l). Cells and medium were separated by centrifugation through silicone oil (1000 x g for 30 sec). The cell layer was removed by a pipette, added to scintillation fluid and the radioactivity was measured. The amount of 3-O-methylglucose in extracellular fluid or taken up by diffusion was determined in samples which contained 1 mmol/l phloretin throughout. All other uptake values were corrected by this value.

Binding studies: After preincubation of fat cells (4.5-5.5 x 105 cells/ml) with IBMX, db-cAMP or bromo-cAMP in the concentrations given above, 5 or 10 µU/ml Mono-A14-[^{125}I]-insulin (specific activity 250 µCi/µg, NOVO industrie, Bagsvaerd, Denmark) were added alone or together with unlabeled insulin in concentrations between 15 and 1000 µU/ml. After 20 min, 400 µl aliquots were transferred into polyethylene centrifuge tubes of a high speed centrifuge and separation of cells from medium was performed by centrifugation through dinonylphtalate (13). The tubes were cut at the oil layer and radioactivity of the cell layer was measured. Non-specific binding was assessed by addition of an excess of unlabeled insulin (1 U/ml) to the labeled insulin.

For statistical evaluations the 2-tailed student's t-test for paired observations was used.

RESULTS

Preincubation of fat cells with IBMX (10^{-3} M) for 30 min inhibited insulin stimulation of glucose transport by 70.5 % ± 1.7 (Fig. 1A) at all insulin concentrations. A smaller but significant inhibition was obtained following

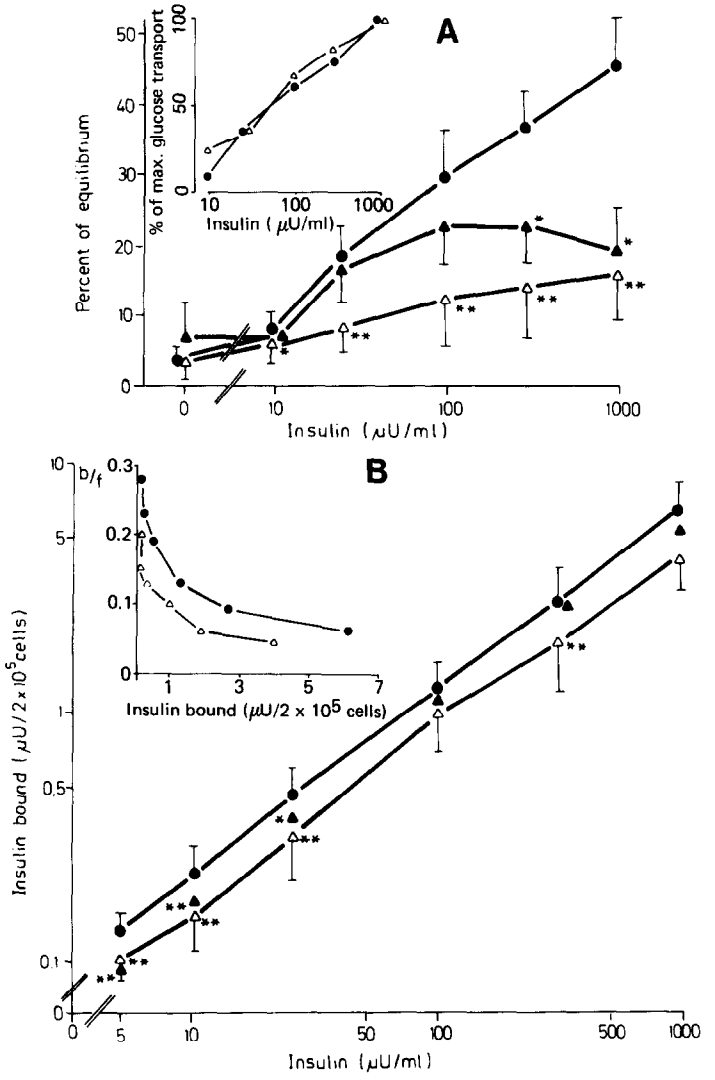

Figure 1 Effect of preincubation with IBMX on insulin-stimulation of D-glucose transport and insulin binding
A. Dose response curve of insulin on 3-O-methylglucose transport. Cells were preincubated (30 min, 37°C) in absence (●-●) or presence of 10^{-4} M (▲-▲) or 10^{-3} M (Δ-Δ) IBMX, and subsequently insulin in the concentrations given on the abscissa was added. D-glucose transport was measured after 20 min as described in Materials and Methods. Transport values are expressed as fraction of the equilibrium space filled in 4 seconds. The values represent the mean ± SEM of 8 experiments independently performed in duplicate; *indicates $p < 0.05$ and ** $p < 0.01$ as evaluated by the two-tailed t-test for paired observations. In the insert, the values obtained with control cells (●-●) and cells preincubated with IBMX (10^{-3} M) (Δ-Δ) are expressed as fraction of the maximal insulin effect.
B. Binding of A14-[^{125}I]-insulin to cells treated as in A. Binding of A14-[^{125}I]-insulin was determined after 20 min as described in Materials and Methods. Values represent the mean + SEM of 6 independently performed experiments measured in duplicate. Significance is indicated as in A. In the insert the Scatchard plot analysis of the values obtained with control cells (●-●) and cells preincubated with IBMX (10^{-3} M, Δ-Δ) is shown.

preincubation with 10^{-4} M IBMX. This indicated that the effect was concentration-dependent. When transport data were expressed as percentages of the maximal response, no shift of the dose response curve was seen (Fig. 1A, insert), which means that the reduction of the insulin effect was mainly due to a decrease of the responsiveness (14) of the glucose transport to insulin. Insulin binding (Fig. 1B) was reduced by 25 % ± 4 following preincubation with 10^{-3} M IBMX at all insulin concentrations. A parallel decrease of insulin binding in the Scatchard plot (Fig. 1B, insert) indicated that this was due to a reduction in the number of insulin receptors. With 10^{-4} M IBMX, insulin binding was decreased by 23 % ± 4 and only at insulin concentrations below 100 μU/ml.

In order to analyze whether the inhibition of insulin binding and stimulation of glucose transport described above was indeed due to an increase in intracellular cAMP levels, fat cells were preincubated with the membrane-permeable cAMP analogue db-cAMP. We found that db-cAMP had similar effects on glucose transport and insulin binding as IBMX and catecholamines; glucose transport was reduced by 52 % ± 6 by cAMP (10^{-3} M) at insulin concentrations above 10 μU/ml, and as shown in Fig. 2A, insert, this was also mainly due to a decrease of responsiveness. Insulin binding was also significantly reduced by 28 % ± 3 at concentrations between 5 and 100 μU/ml of insulin (Fig. 2B), and here, too, Scatchard plot analysis of insulin binding showed that this involved mainly a reduction of high affinity binding (Fig. 2B, insert). Preincubation with 10^{-4} M db-cAMP had the same effects but they were less marked. Similar observations were made when fat cells were preincubated with bromo-cAMP (10^{-4}-10^{-3} M). At 10^{-3} M, bromo-cAMP decreased insulin stimulation (10-100 μU/ml) of glucose transport by 33 % ± 1 (n=4) and high affinity binding by 17 % ± 3 (n=4) (data not shown).

DISCUSSION

We reported earlier (9) that preincubation with catecholamines (norepinephrine 10^{-9}-10^{-7} M), isoproterenol 10^{-7}-10^{-5} M) induces insulin resistance in fat cells in vitro and in the present study we could show that preincubation with IBMX, db-cAMP or bromo-cAMP has a similar effect. A preincuba-

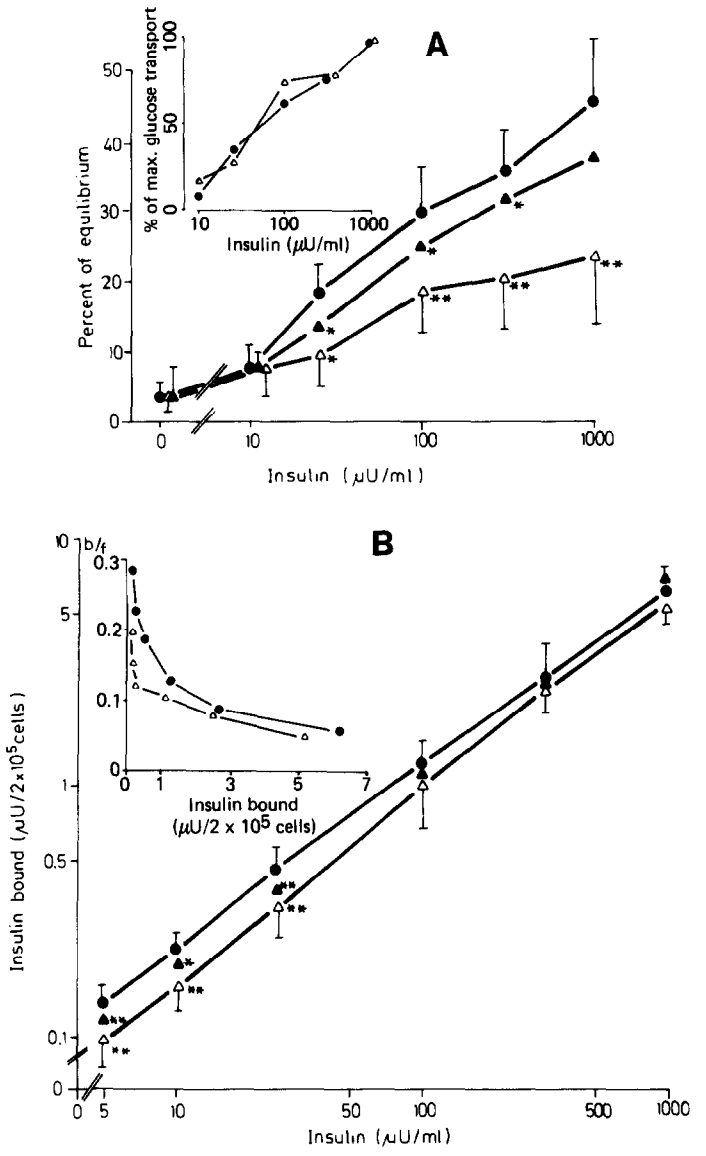

Figure 2 Effect of preincubation with db-cAMP on insulin stimulation of D-glucose transport and insulin binding.
A. Dose response curve of insulin on 3-O-methylglucose transport. Cells were preincubated (30 min, 37°C) in absence (●-●) or presence of 10^{-4} M (▲-▲) or 10^{-3} M (Δ-Δ) db-cAMP and subsequently insulin in the concentrations given on the abscissa was added. D-glucose transport was measured after 20 min as described in Material and Methods. Transport values are expressed as fraction of the equilibrium space filled in 4 seconds. The values represent the mean ± SEM of 12 independently performed experiments each measured in duplicate; *indicates $p < 0.05$ and ** $p < 0.01$ as evaluated by the two-tailed student's t-test for paired observations. In the insert, the values obtained with control cells (●-●) and cells preincubated with db-cAMP (10^{-3} M, Δ-Δ) are expressed as fraction of the maximal insulin effect.
B. Binding of A14-[^{125}I]-insulin to cells treated as in A. Binding of A14-[^{125}I]-insulin was determined after 20 min as

tion period of 30 min produced consistent results with these compounds. This indicates that preexposure of fat cells to increased intracellular cAMP levels significantly inhibits insulin binding and insulin-dependent glucose transport. As pointed out in the introduction, contradictory results have been reported on the modulatory role of cAMP on glucose transport, but this is probably due to the fact that different experimental protocols had been used. For example, it has been shown that catecholamines have opposite effects on insulin induced glucose transport depending on whether the cells are preincubated with catecholamines for about 30 min (9) or whether catecholamines are added simultaneously with insulin (6,7). Moreover, some of the experimental protocols used were not suitable for the study of the influence of cAMP on insulin signal transmission. This applies to the experiments where high cAMP levels were induced after complete stimulation of glucose transport by insulin so that it was rather the influence of cAMP on the termination of glucose transport which was then investigated (5,8,15).
The results reported in (3,4) support the view that cAMP has a modulatory action on glucose transport but they were obtained using an indirect method of glucose transport determination. Moreover, 10 mM NaF was used in one of these studies (3) and NaF itself inhibits glucose transport (unpublished observation) and interferes with the phosphorylation reactions on the insulin receptor (16).

The effects of cAMP on insulin binding has to our knowledge not yet been investigated. A recent report, however, has shown that cAMP potentiates the down regulation of epidermal growth factor receptor (17). In our experiments, catecholamines, IBMX, db-cAMP and bromo-cAMP all decreased high affinity binding of insulin within the physiological concentration range of insulin.

As the stimulation of protein phosphorylation by cAMP dependent kinases is one of the main effects of cAMP, the inhibition of insulin binding observed here could be due to the effect of cAMP on insulin-receptor phosphorylation. According to recent work on the molecular level, insulin receptor phosphory-

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(Fig. 2 Cont'd.)
described in Material and Methods. The values represent the mean ± SEM of 7 independently performed experiments measured in duplicate. Significance is indicated as in A. In the insert the Scatchard plot analysis of values obtained with control cells (●-●) and cells preincubated with cAMP (10^{-3} M, Δ-Δ), is shown.

lation is not cAMP dependent (18), but more recently it has has been found (19) that besides phosphotyrosine, phosphoserine is formed on the ß-subunit of the insulin receptor. A selective modulation of serine phosphorylation by cAMP could lead to the decrease of high affinity binding observed in our experiments and could have been obscured in the overall measurements of phosphorylation by the predominant, cAMP independent tyrosine phosphorylation.

Since high intracellular levels of cAMP decrease both insulin binding and insulin-dependent glucose transport, the question arises as to whether the resistance of glucose transport to insulin is due to the simultaneously induced reduction in insulin binding. If this should be the case, we should observe a decrease in sensitivity of glucose transport to insulin in our experiments. IBMX, db-cAMP and bromo-cAMP, however, caused mainly a decrease in responsiveness of glucose transport to insulin so that an additional effect of high cAMP levels on the post-receptor signal transmission has to be assumed. In conclusion, high intracellular cAMP levels seem to induce insulin resistance both at a receptor and post-receptor level in fat cells in vitro. Since preincubation with catecholamines have the same effects in fat cells in vitro (9), it seems possible that the catecholamine-induced insulin resistance in vivo could be due to high intracellular cAMP levels.

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REFERENCES

1. Renner, R., Kemmler, W., Hepp, K.D. (1974)Eur.J.Biochem. 49, 129-141
2. Kono, T., Barham, F.W. (1974)J.Biol.Chem. 248, 7417-26
3. Hollenberg, M.D., Cuatrecasas, P. (1975)Fed.Proc. 34, 1556-1563
4. Taylor, W.M., Mak, L.M., Halperin, M.L. (1976)Proc.Natl. Acad.Sci.(USA) 73, 4359-4363
5. Green, A. (1983)FEBS Letters 152, 261-264
6. Ludvigsen, C., Jarett, L., McDonald, J.M. (1980)Endocrinology 106, 786-790
7. Kashiwagi, J., Foley, J.E. (1982)Biochem.Biophys.Res.Comm. 107, 1151-1157
8. Zapf, J., Waldvogel, M., Schoenle, E., Froesch, E.R. (1981) FEBS Letters 135, 199-202
9. Kemmler, W., Häring, H.U., Baumgarten, M., Kirsch,D. (1982) Diabetologia 23, 179
10. Rodbell, M. (1964)J. Biol.Chem. 239, 375-380
11. Whitesell, R.R., Gliemann, J. (1979)J.Biol.Chem. 254, 5276-5283

12.Häring, H.U., Biermann, E., Kemmler, W.(1981)Am.J.Physiol. 240, E556-565
13.Gliemann, J., Osterhind, K., Veniten, J.(1972)Biochem. Biophys.Acta 286, 1-9
14.Kahn, C.R.(1978)Metabolism 27, 1893-1902
15.Häring, H.U., Baumgarten, M., Kemmler, W.(1981)Diabetologia 21,279
16. Machicao, F., Urumow, T., Wieland, O.H.(1982)FEBS Letters 149, 96-100
17.Leof, E.B., Olashaw, N.E., Pledger, W.J., O'Keefe, E.J. (1982)Biochem.Biophys.Res.Comm. 109, 83-91
18.Häring, H.U., Kasuga, M., Kahn, C.R.(1982)Biochem.Biophys. Res.Comm. 108, 1538-1545
19.Kasuga, M., Zick, Y., Blith, D.L., Karlson, F.A., Häring, H.U., Kahn, C.R.(1982)J.Biol.Chem. 257, 9891-9894