A BIOLOGICAL ACTIVITY OF THE A-CHAIN OF INSULIN AND THE INACTIVITY OF A SYNTHETIC ANALOGUE CONTAINING AN INTACT INTRACHAIN DISULFIDE BRIDGE

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Received November 6, 1967

The biological activity of the separated chains of insulin has been investigated frequently, but the question whether these chains have insulin-like activity remains unanswered, because the peptide chains are often contaminated either with intact insulin or with each other (Langdon, 1960; Fisher and Zachariah, 1960; Meek and Bolinger, 1966; cf. Dixon and Wardlaw, 1961). It is especially important that claims that these chains have a low level of activity be accompanied by independent evidence that they are not contaminated to a degree that could explain this low biological activity. Volfin and coworkers (1964) demonstrated insulin-like activity of a synthetic A-chain preparation on the isolated rat diaphragm, but the surprisingly high activity which they found needs to be confirmed.

We have investigated the biological activity of the A-chain, because the interpretation of such a study is simplified by better methods of purifying the separated natural chains (Marglin and Merrifield, 1967) and by an isolated-cell assay (Rodbell, 1964; Gliemann, 1965). We have also studied a synthetic A-chain analogue which has an intact Cys_{6-11}**

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**Abbreviations: Cys, cystine; ASH, A-chain-tetrathiol; $ASSO_3$, A-chain-tetra-S-sulfonate; BSH, B-chain-dithiol; $BSSO_3$, B-chain-di-S-sulfonate; [Ala^7 Ala^{20}]-bovine-insulin-A-chain, a wholly synthetic analogue of the bovine A-chain wherein the half-cystines normally occupying positions 7 and 20 are replaced with alanines (cf. Biochemistry (1967). 6:362).

disulfide bridge, in order to find out whether this disulfide bond modified the biological activity of the reduced A-chain.

The results indicate that the fully reduced natural bovine A-chain (ASH) has a potency of approximately 1/30000 that of intact insulin on the evolution of $^{14}CO_2$ from U-^{14}C-glucose. However, ASH does not have truly insulin-like activity, since there is no concomitant stimulation of incorporation of ^{14}C into fatty acids or glyceride-glycerol by the isolated cells. The synthetic analogue with the intact disulfide bridge is devoid of any measurable activity on glucose oxidation or triglyceride synthesis.

Materials and methods

Natural bovine ASH was prepared by reducing highly purified $ASSO_3$ (Marglin and Merrifield, 1967) with excess 2-mercaptoethanol by the method of Du et al. (1965). The mercaptoethanol was removed by extracting the precipitated ASH with ethyl acetate four times (Katsoyannis and Tometsko, 1966). The ASH was kept in deaearated water at pH 5.0 for the several-minute interval between its preparation and the determination of its biological activity, and the suspension was then adjusted to pH 7.4. The resulting solution was then diluted in the incubation buffer.

[Ala^{7}Ala^{20}]bovine-insulin-A-chain (the analogue with the intact intra-chain disulfide bridge) was synthesized by the solid phase method, using procedures previously reported for a synthesis of normal A-chain (Marglin and Merrifield, 1966). After protecting groups were removed with sodium in liquid ammonia, the bis-dithiol form was oxidized to the disulfide with potassium ferricyanide (Hope et al., 1962). Under the conditions used, uptake of a stoichiometric amount of potassium ferricyanide indicated that all sulfhydryl groups present were converted to disulfide. The peptide was then purified by ion-exchange chromatography and gel filtration. The purified peptide was homogeneous and monomeric as judged by amino acid analysis, thin-layer chromatography, and gel filtration on Sephadex G-25. Details of this synthesis will be published elsewhere.

Biological activity was determined by a modification of the isolated adipose cell methods of Rodbell (1964) and Gliemann (1965). U-^{14}C-glucose was used in all experiments. All peptides were tested in amounts ranging from 0.1 to 100 μg/ml.

Oxytocic assays on the rat uterus were done by Dr. John. M. Stewart, Rockefeller University.

Results

$^{14}CO_2$ evolution from ^{14}C-glucose: From the linear portion of a dose-response curve made with standard insulin, ASH from natural insulin was estimated to have approximately 1/30000 the activity of insulin on a weight basis. This estimate is based on the average of several experiments, and the degree of CO_2 evolution was found to increase with increasing doses of the peptide. Responses equivalent to 100 μU of insulin could be obtained when sufficient ASH was used. In contrast, [Ala^{7}Ala^{20}]bovine-insulin-A-chain had no measurable effect on the cells' basal $^{14}CO_2$ evolution over a wide dose range. However, slight inhibition of $^{14}CO_2$ evolution by this analogue was noted in the presence of insulin, but only with very large doses (Table 1). This point is under further investigation.

Conversion of ^{14}C-glucose to fatty acids and glyceride-glycerol: The results are recorded in Table 1. Despite the easily-demonstrated ability of ASH to increase $^{14}CO_2$ production from U-^{14}C-glucose, it did not measurably enhance incorporation of ^{14}C into fatty acids or glyceride-glycerol. [Ala^{7}Ala^{20}]bovine-insulin-A-chain also did not promote additional synthesis of triglycerides. Neither peptide inhibited triglyceride synthesis stimulated by insulin.

Oxytocic activity: [Ala^{7}Ala^{20}]bovine-insulin-A-chain had no oxytocic activity in the rat uterus assay, even when it was tested in concentrations of 10 μg/ml.

Discussion

Insulin acts on the fat cell to increase synthesis and storage of

TABLE 1. COMPARISON OF PARAMETERS OF INSULIN-LIKE ACTIVITY ON ISOLATED FAT CELLS. Results are expressed in c. p. m. ± standard deviation.

Test substance	$^{14}CO_2$ evolution	^{14}C in total fatty acids	^{14}C in glyceride-glycerol
Cells alone	2911 ± 148	965 ± 34	3355 ± 56
Insulin 5 μU	3328 ± 134	1231 ± 174	3445 ± 191
Insulin 10 μU	5085 ± 626	2831 ± 191	4026 ± 136
Insulin 100 μU	6439 ± 203	3848 ± 78	4566 ± 126
ASH (nat.) 10 γ	4711 ± 608	828 ± 53	3510 ± 27
[Ala^7Ala^{20}]-A-chain, 100 γ	3089 ± 363	1077 ± 8	3439 ± 127
[Ala^7Ala^{20}]-A-chain, 100 γ, + insulin, 100 μU	5909 ± 17	3654 ± 186	4536 ± 162
Blank	3009 ± 288	861 ± 11	3232 ± 119

The fat cells were prepared by the method of Gliemann and were used within 30 min. The suspensions of the freshly reduced peptide or of the disulfide form of [Ala^7Ala^{20}]-A-chain were adjusted to pH 7.4 and the solutions added to the cell suspension just prior to assay. The volume of the incubation mixture was 1.0 ml. U-^{14}C-glucose was used instead of 1-^{14}C-glucose. The duration of the incubation was 30 min. A water blank was treated with mercaptoethanol, extracted with ethyl acetate, and put into the assay medium in a manner identical to the peptide chains.

TABLE 2. SUPPORTING EVIDENCE THAT THE BIOLOGICAL ACTIVITY OF ASH IS NOT DUE TO INSULIN OR TO BSH. Results in c. p. m. ± standard deviation.

Test substance	$^{14}CO_2$ evolution
Cells alone	2196 ± 220
Insulin 100 μU	4409 ± 50
$ASSO_3$ 10 γ	2310 ± 37
$ASSO_3$ 10 γ + insulin 100 μU	4297 ± 75
$BSSO_3$ 10 γ	2397 ± 334
$BSSO_3$ 10 γ + insulin 100 μU	5015 ± 75
ASH 1.0 γ	2274 ± 9
BSH 1.0 γ	2463 ± 0
ASH 1.0 γ + BSH 1.0 γ	2424 ± 132
ASH 1000 γ	6491 ± 168
ASH 1000 γ + BSH 1.0 γ	6950 ± 18
Blank	2043 ± 71

The experimental conditions were the same as those in the legend to Table 1. BSH was made from $BSSO_3$ in the same manner as ASH from $ASSO_3$ (see text). $ASSO_3$ was contaminated with less than 0.05% $BSSO_3$ as determined by mouse convulsion assay (Marglin and Merrifield, 1967). Both ASH and BSH are the natural chains. In the experiment in which ASH was 1000 γ and BSH 1.0 γ (a molar ratio of ASH : BSH = 1390 : 1), efficient chain recombination might occur to form insulin (cf. Du et al., 1965; Katsoyannis and Tometsko, 1966) because of the limiting amount of BSH present. This would produce a maximum of 1.7 γ insulin. 100 μU insulin = 0.0045 γ. From this information, it can be calculated that the amount of insulin formed from a small amount of BSH in 10 γ ASH would be too small to account for the increased CO_2 evolution.

fat from carbohydrate, and any determination of insulin-like activity should include some measurement of this. It is possible that many non-hormonal thiols will promote increased glucose oxidation, as has already been noted in the case of reduced glutathione by Renold and co-workers (1960). The synthesis of fat in adipose tissue may be uniquely dependent on insulin. Although in our study the reduced A-chain had a small but measurable effect on $^{14}CO_2$ evolution from U-^{14}C-glucose in the isolated fat cell, it did not really have insulin-like activity (cf. Mirsky, 1965). This observation also applies to the study of Meek and Bolinger (1966), in which only $^{14}CO_2$ evolution from 1-^{14}C-glucose was reported.

Because natural ASH had no measurable effect on ^{14}C incorporation into fatty acids or glyceride-glycerol, its ability to promote CO_2 evolution from glucose could not have been due to insulin or to resynthesis of insulin in the incubation medium by its combination with B-chain. The absence of insulin in our ASH preparation is demonstrated by assay of $ASSO_3$, which totally failed to increase $^{14}CO_2$ evolution. This indicated that the degree of contamination of ASH with insulin was less than 1/100000 (Table 2). That this increased CO_2 evolution is not due to B-chain contaminating our natural ASH is also indicated by the observation that reduced B-chain (BSH), when it was deliberately added to the A-chain incubation medium, failed to increase significantly the CO_2 evolution caused by ASH (Table 2, cf. Meek and Bolinger, 1966). We also found that synthetic ASH affected these parameters of insulin-like activity in a manner identical to natural ASH. It is unlikely that ASH merely potentiated or spared endogenous insulin associated with these cells, because of the failure of ASH to stimulate fatty acid synthesis. Thus the increased evolution of CO_2 in the presence of ASH must be due to ASH alone.

The insulin A-chain has a 20-membered ring similar to that in oxytocin, and oxytocin has insulin-like activity in adipose tissue (Mirsky and Perisutti, 1961). [Ala^{7}Ala^{20}]bovine-insulin-A-chain resembles the normal

A-chain in structure even more than does oxytocin, and it therefore might be expected to have insulin-like activity. However, our analogue with the intrachain disulfide bridge was inactive in the fat cell system where natural and synthetic ASH increased CO_2 production from glucose. It also had no oxytocic activity in the rat uterus assay. These results agree with Mirsky's generalization (1962, 1965) that oxytocic activity and glucose oxidation by adipose tissue preparations are parallel in the oxytocin series. They also support the implication from studies with vasopressin and oxytocin analogues that disulfide bonds of peptides with such 20-membered rings may not be of prime importance in their biological activity (Schwartz et al., 1964; Jošt and Rudinger, 1967). Further experiments with insulin would be desirable before this suggestion is extended to that hormone. Our experiments suggest that the stimulation of glucose oxidation by the A-chain is not enhanced by an intrachain disulfide bridge. They further suggest that the lack of complete insulin-like activity in ASH is not due to the absence of an intrachain disulfide bridge.

Acknowledgements: The interest and consideration given this work by Drs. R. B. Merrifield and M. A. Rizack are gratefully acknowledged. This study was aided in part by National Science Foundation Grant GB-6035.

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