ENHANCED SPECIFIC INSULIN BINDING AND INSULIN ACTION WITH C-TERMINAL B-CHAIN PENTAPEPTIDE DERIVED FROM INSULIN

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1. Introduction

At increased concentrations compared to insulin, desoctapeptide insulin (DOP) and desasparagine desalanine insulin (DAA), resolved free of traces of insulin contamination, are inherently full insulin agonists in fat and muscle tissue [1]. These degraded insulins also act through the insulin receptor [1]. These findings indicate that if there is a discrete 'active center' in the insulin molecule, it would appear to reside in the DOP core. In terms of insulin action, the relative importance of the ligand versus that of the insulin receptor and post-receptor events will not be considered here.

Since the degraded insulins have such a markedly decreased capacity to bind to the insulin receptor, studies on role of the C-terminal portion of the B-chain of the insulin molecule become of considerable interest. Studies of a human insulin variant with leucine substituted for phenylalanine at positions 24 (and 25) also demonstrate a markedly decreased insulin activity and decreased binding indicating the importance of the C-terminal portion of the B-chain [2]. We found that the pentapeptide Arg–Gly–Phe–Phe–Tyr comprising residues B22–26 of insulin was biologically inactive alone but weakly potentiative of the actions of suboptimal concentrations of insulin or DOP [1]. Now we report that at $\geqslant 10^{-4}$ M, the peptide alone is inactive, but markedly enhances both the biological activity of suboptimal concentrations of insulin (or DOP) and the specific binding of labelled insulin to its receptor. These effects require a peptide containing arginine. The enhanced binding is not due to decreased insulin degradation, but constitutes a true enhancement of the binding process itself.

2. Methods and materials

Adipocytes were prepared from the epididymal tissue of 120–180 g rats (Wistar strain fed ad libitum). Adipocytes were either incubated in media for measurement of various metabolic or enzymatic parameters or incubated in ^{125}I-labelled insulin for insulin binding. $^{14}CO_2$ production was measured as nmol D-[1-^{14}C]glucose to $^{14}CO_2$. 10^6 cells^{-1}. h^{-1} at 37°C as in [3]. For measurement of glycogen synthase activity, extracts were prepared [3] and glycogen synthase activity assayed as in [4]. Binding of labelled insulin was done as in [5].

^{125}I-Labelled insulin was obtained through the courtesy of Dr George Vandenhoff from the University of Virginia Diabetes Research and Training Center. Binding assays were conducted in the absence and presence of unlabelled insulin at 60 μg/ml final conc.

UDP-glucose, glucose 6-P, bovine serum albumin (fraction V) and rabbit liver glycogen were obtained from Sigma Biochemicals. The glycogen was purified before use by passing a 5% solution over a mixed-bed ion-exchange resin (Amberlite MB-3) as in [6]. Collagenase (*Clostridium histolyticum*) was obtained from Worthington Biochemicals. Bacitracin was obtained from Pfizer. β-Alanylpentapeptide amide was prepared as in [7]. Crystalline single-peak beef insulin (lot ID6-04-94-195) containing 0.49% proinsulin and <0.003% glucagon, was obtained from Eli Lilly through the courtesy of Dr W. W. Bromer. [1-^{14}C]Glucose was obtained from New England Nuclear. DOP resolved free of trace insulin contamination was prepared as in [1].

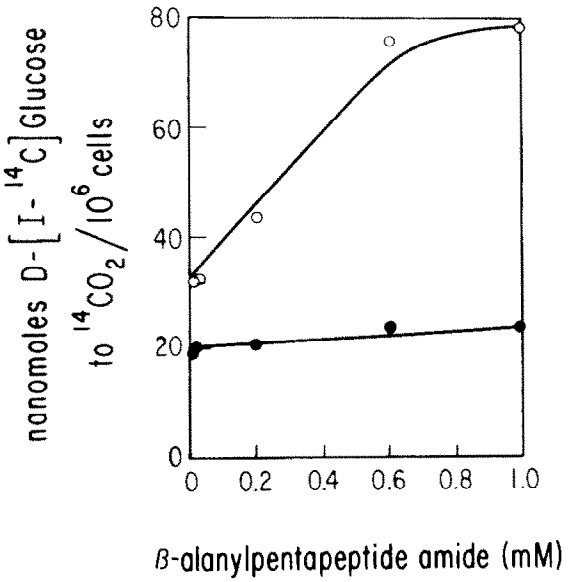

Fig.1. Effect of varying concentrations of the β-alanylpentapeptide amide on $^{14}CO_2$ production from glucose in the absence and presence of insulin. Cells were isolated and washed in Krebs-Ringer phosphate buffer (pH 7.4) containing 30 mg bovine serum albumin ml. [1-^{14}C]Glucose conversion to $^{14}CO_2$ was measured after incubation of cells at 37°C for 1 h in a medium containing 0.2 mM D-[1-^{14}C]glucose with the indicated concentrations of the β-alanylpentapeptide amide in the absence (•) and presence (○) of insulin (10^{-10} M).

3. Results

When $^{14}CO_2$ production from labelled glucose was measured as shown in fig.1, the β-alanylpentapeptide amide containing B22 arginine of the B-chain was inactive alone over 10^{-4}–10^{3} M. However in the presence of a submaximal concentration of insulin, at 10^{-4} M it stimulated $^{14}CO_2$ production from glucose 2-fold and at 10^{-3} M, 4-fold. Experiments with glycogen synthase activation were performed next. Fig.2 demonstrates that in the presence of a submaximal concentration of insulin, the pentapeptide significantly stimulated glycogen synthase activation, an intracellularly mediated event [8]. Again the peptide alone was inactive. To determine whether the peptide was acting in the presence of suboptimal insulin concentrations by decreasing insulin degradation, experiments were performed in the presence of bacitracin which itself is known to suppress insulin degradation. Fig.3 demonstrates that, in the presence of a submaximal concentration of insulin, the peptide stimu-

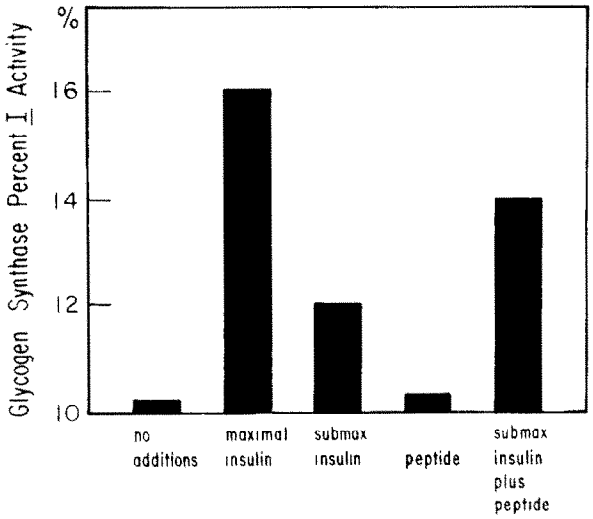

Fig.2. Stimulation of glycogen synthase activation by the β-alanylpentapeptide amide in the presence of insulin. 1 ml of cells (5×10^6) was incubated in the absence of glucose for 15 min at 37°C with or without 0.6 mM β-alanylpentapeptide amide in the absence and presence of insulin (0.4×10^{10} M), then centrifuged for 15 s in a clinical centrifuge. After the medium had been aspirated, the incubation was terminated by adding 0.5 ml cold buffer (100 mM KF and 10 mM EDTA, pH 7.0) to the cells, followed by homogenization at 0°C. After centrifugation at 10 000 × *g* for 15 min, the supernatants were used for enzyme assay. The results (expressed as % I activity of glycogen synthase) are mean values from 3 independent expt. Maximal insulin value presented was obtained from incubation of cells with 10^{-8} M insulin.

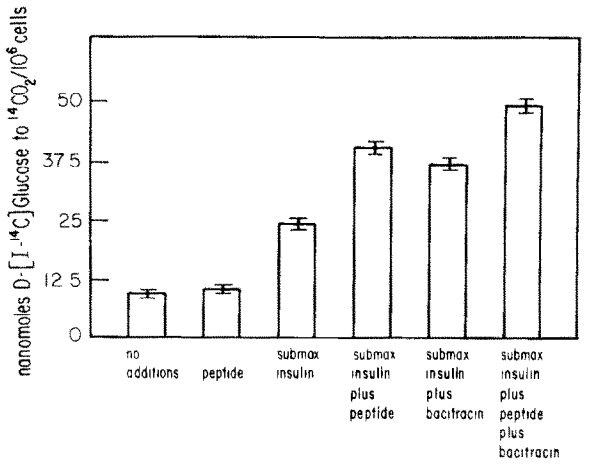

Fig.3. Effect of the β-alanylpentapeptide amide on $^{14}CO_2$ production from [1-^{14}C]glucose in the absence and presence of bacitracin. The incubations were conducted with or without 0.6 mM β-alanylpentapeptide amide and/or 1 mM bacitracin in the absence or presence of insulin (10^{-10} M). Other conditions were as in the legend to fig.1. Error bars represent ± 1 SD.

lated $^{14}CO_2$ production from labelled glucose in the absence and in the presence of 1 mM bacitracin, which eliminates degradation of insulin [9]. We have confirmed this action of bacitracin by submitting reaction mixtures to Sephadex G-50 molecular sieving, which separates degraded from unaltered and aggregated insulin. With bacitracin present, no detectable insulin degradation occurred in the absence or presence of β-alanyl pentapeptide amide. This was determined by measuring the sizes of the 3 peaks present: peak 1, aggregated insulin; peak 2, native insulin; peak 3, degraded insulin. In the absence of bacitracin insulin degradation was clearly observed. The presence of bacitracin itself also enhanced the effect of submaximal insulin, probably by protecting the hormone from degradation.

To study further the mechanism of action of the peptide, we determined its effect on specific binding of labelled insulin to its receptor. Fig.4 demonstrates that the pentapeptide increased the specific binding of ^{125}I-labelled insulin to adipocyte receptors as a function of increasing concentration of the peptide. Furthermore, the concentration dependence of enhanced insulin binding is the same as that of enhanced insulin bioactivity. The B-chain C-terminal hetapeptide, Gly–Phe–Phe–Tyr–Pro–Lys derived from the C-terminal of B-chain, but without B22 Arg, showed no effect even in the presence of submaximal concentrations of insulin (not shown). Similar experiments with suboptimal concentrations of DOP in place of insulin demonstrated analogous results to those with insulin (not shown).

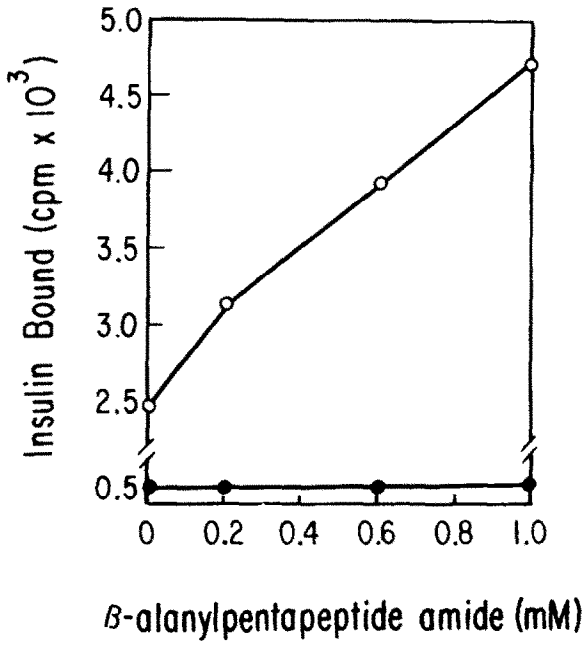

Fig.4. Effect of the β-alanylpentapeptide amide on ^{125}I-labelled insulin binding to adipoytes. Cells (5×10^5) in 0.5 ml Krebs-Ringer phosphate buffer (pH 7.4) containing 3% bovine serum albumin were incubated at 25°C for 30 min with ^{125}I-labelled insulin (0.7×10^{-10} M spec. act. 1.2 Ci/μmol) in the presence of varying concentrations of the β-alanylpentapeptide amide in the absence of (○) and presence of (●) cold native insulin (60 μg/ml). Aliquots (300 μl) of the cell suspension were transferred to 400 μl polyethylene tubes containing 90 μl dinonly phthalate and rapidly centrifuged for 1 min in a Beckman microfuge (10 000 × g). After centrifugation, the tubes were cut through the oil layer and the cells transferred to counting vials and counted.

4. Discussion

Here, we have shown that β-alanylpentapeptide amide containing B22 Arg was inactive alone, but at 10^{-4} M it potentiated the actions of insulin, including activation of $^{14}CO_2$ production from glucose and glycogen synthase activation. Even in the presence of bacitracin, under which conditions insulin degradation was essentially completely suppressed, the peptide still stimulated insulin action, indicating that the peptide acts through a mechanism different from the protection of insulin from degradation. The concentrations of peptide required for the stimulation of insulin action correspond to those required for the increased binding. The close correlation between the biological potency and the effect on insulin binding strongly suggests that the stimulation of insulin action by the peptide may be directly related to increased binding of insulin to the insulin receptor.

In [10,11] evidence was presented that the peptides derived from the C-terminal of the B-chain of insulin containing arginine were active alone. The insulin-like activity of the peptide alone reported in [10,11], however, was more notable when the experiments were performed in whole animals or with tissues rather than with cells. For example, the activity of the peptide was much lower with adipocytes compared with diaphragm, despite the fact that adipocytes are well known to be one of the cells most sensitive to insulin [3]. Their results can be more reasonably interpreted therefore, if we assume that the insulin-like activity of the peptide is not due to an inherent activity of the peptide per se, but due to potentiation

by the peptide of the action of endogenous insulin or degraded insulin present in the animals or tissues used. Taken together with our data demonstrating the DOP is fully active in terms of maximal response, it was concluded that DOP contains the 'active center' of the insulin molecular if indeed there is a single 'active center' and the C-terminal peptide of the B-chain is not the 'active center' alone, but potentiates insulin action. Studies are underway detailing the kinetics of the effects of the peptide on the association and dissociation rate processes in insulin binding and will be reported subsequently. These peptides should now prove valuable as probes in studying insulin receptor function in insulin action.

Acknowledgements

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