Diabetologia (1998) 41: 287–291

Diabetologia
© Springer-Verlag 1998

Differential effects of proinsulin C-peptide fragments on Na^+, K^+-ATPase activity of renal tubule segments

Y. Ohtomo[1], T. Bergman[2], B.-L. Johansson[3], H. Jörnvall[2], J. Wahren[3]

[1] Department of Woman and Child Health, Pediatric Unit, St. Göran's Children's Hospital, Stockholm, Sweden
[2] Department of Medical Biochemistry and Biophysics, Karolinska Institute, Stockholm, Sweden
[3] Department of Surgical Sciences, Section of Clinical Physiology, Karolinska Hospital, Stockholm, Sweden

Summary Proinsulin C-peptide has been shown to stimulate the activity of Na^+K^+ ATPase of rat renal tubule segments. Thirty-six peptides and amino acids, corresponding to parts of the intact rat C-peptide and suitable controls were screened for capacity to stimulate Na^+, K^+-ATPase in an attempt to determine potential active sites in the C-peptide molecule. The carboxy-terminal tetra and penta peptides were found to elicit 92–103 % of the intact molecule's activity, and the remaining segment, des-(27–31) C-peptide, did not possess stimulatory activity. Peptides from the middle C-peptide segment, however, centering around a GGPEAG sequence, stimulated Na^+, K^+-ATPase activity (36–80 % of the intact molecule's effect) but this effect was not balanced by corresponding inactivity of other parts. Furthermore, it was paralleled by activity of a non-native dipeptide D-form. It is concluded that the latter effect and that of the middle segment may represent complex interactions other than the apparently specific effects of the C-terminal segment. [Diabetologia (1998) 41: 287–291]

Keywords C-peptide, active sites, fragments, renal tubule segments, Na^+, K^+-ATPase activity.

Since the discovery of insulin biosynthesis in 1967 [1, 2] it has generally been held that C-peptide, the connecting segment of proinsulin, does not possess biological activity. Possible metabolic effects of C-peptide have been looked for in animals and humans but with negative results [3–5]. The C-peptide structure of different species varies considerably in both chain length and amino acid sequence [6], a fact that together with the negative experimental results has been taken to support the view that C-peptide does not exert biological effects. However, several studies during the last 5 years have raised doubts concerning this view. Replacement on a short-term basis (1–3 h) of physiological C-peptide levels in insulin-dependent diabetic patients, who are deficient in C-peptide, has been shown to be accompanied by decreased glomerular hyperfiltration [7], augmented whole body [7] and skeletal muscle [8] glucose utilization, increased blood flow to skeletal muscle during physical exercise [9] and improved autonomic nerve function in insulin-dependent patients with signs of autonomic neuropathy [10]. Prolonged C-peptide administration (1–3 months) results in improvements in renal function, as evidenced by diminished albumin excretion [11, 12] and amelioration of autonomic nerve dysfunction in patients with autonomic neuropathy [12]. In vitro studies have confirmed that C-peptide stimulates glucose transport in skeletal muscle and that this effect occurs via pathways not including the insulin receptor [13]. Moreover, in vitro and in vivo studies suggest that C-peptide may be involved in the feedback inhibition of insulin secretion [14, 15].

The different C-peptide effects may in part be explained by the peptide's ability to stimulate Na^+, K^+-ATPase [16]. Thus, C-peptide has been shown to stimulate Na^+, K^+-ATPase of renal tubule segments, probably by activating a receptor coupled to a pertussis toxin-sensitive G-protein with subsequent activation of Ca^{2+}-dependent intracellular signalling path-

Received: 31 July 1997 and in revised form: 30 October 1997

Corresponding author: Professor J. Wahren, Section of Clinical Physiology, Karolinska Hospital, S-17176 Stockholm, Sweden

ways. The stimulatory effect of C-peptide was markedly enhanced in the presence of neuropeptide Y [16]. The above findings are in agreement with previous observations indicating the presence of C-peptide receptors in cultured beta cells [17].

Rat C-peptide constitutes a 31-residue segment of proinsulin. It is not known which part(s) of the molecule possess biological activity. Consequently, the present study was undertaken to examine potential active sites of the C-peptide molecule by studying the stimulatory effect of different C-peptide fragments on the Na^+, K^+-ATPase activity of rat proximal tubule segments.

Materials and methods

Animals. Male Sprague-Dawley rats (ALAB, Sollentuna, Sweden), aged 40–45 days and weighing 150–200 g, were used.

Preparation of proximal convoluted tubule segments. Tubular segments were obtained from collagenase-perfused rat kidneys [18]. Tubules were stored on ice until dissection was completed, maximally for 30 min.

Incubation of tubules with peptides and/or drugs. The tubule segments were incubated for 30 min at room temperature in 1 μl microdissection solution alone (control tubules) or in 1 μl microdissection solution containing one of the C-peptide fragments. The ratio of cell volume to buffer volume was approximately 1:1000.

Determination of Na^+,K^+-ATPase activity. The preincubation period was stopped by reduction of the temperature of the tubular segments to 4 °C. The segments were then made permeable by hypotonic shock, freezing and thawing, to allow labelled ATP to enter the cells and to change the cellular sodium concentration [18]. The ^{32}P-phosphate liberated by hydrolysis of ATP was separated by filtration through a Millipore filter (Millipore Corp, Bedford, MA, USA) after absorption of the unhydrolysed nucleotide on activated charcoal and radioactivity was determined in a liquid scintillation counter.

In each study, total ATPase activity and ouabain-insensitive ATPase activity were measured on 6 to 10 different tubule segments. This procedure was repeated on tubules from at least three animals. Na^+,K^+-ATPase activity (pmol $^{32}P_i$ hydrolysed per mm tubule per h) was then calculated as the difference between the mean value for total ATPase and the ouabain-insensitive ATPase activity, and expressed either as absolute values or as percentage above the corresponding control measurement.

Peptides. Synthetic rat C-peptide (1 and 2), randomly scrambled C-peptide 1, C-peptide fragments containing 2–26 amino acid residues, and control peptides were obtained from Ferring AB (Malmö, Sweden), Sigma Chemical Company (St Louis, MI, USA) or synthesized as described [19]. The scrambled C-peptide had the same composition as the native peptide but with the residues in random order. The purity of all peptides, established by HPLC or capillary electrophoresis [20], was greater than 99 %.

Preparation of purified Na^+,K^+-ATPase. Purified Na^+,K^+-ATPase from proximal convoluted tubular cells was prepared for control studies after selective removal of membrane components by sodium dodecyl-sulphate [21]. The enzyme preparation was incubated with C-peptide or different fragments, and the Na^+,K^+-ATPase activity was subsequently determined. The enzyme (2 μg) was added to 80 μl of the microdissection solution [18] and the peptides were added to a concentration of $5 \cdot 10^{-7}$ mol/l. Na^+,K^+-ATPase activity was assayed immediately as described above.

Statistical analysis. Values are given as mean ± SEM. Standard statistical methods were employed using Student's *t*-test.

Results

Rat C-peptide 1 stimulated Na^+,K^+-ATPase in renal tubular segments as described previously [16]. The effect of C-peptide 2 tended to be slightly greater than that of C-peptide 1 but the difference did not attain statistical significance. The combined results with the C-peptide fragments indicate the presence of a distinct active site in the C-terminal region of the molecule (Fig. 1). In addition, fragments from the middle segment exerted a significant stimulatory effect but the nature of this effect was less distinctive than that of the C-terminal fragments, as discussed below.

The C-terminal tetra- and penta-peptides possess 92 and 103 %, respectively, of the intact molecule's activity. Consistent with this finding, the remaining des(27–31) C-peptide showed no stimulatory effect (Fig. 1). The human C-terminal pentapeptide (EGSLQ), in which two of five residues are identical to those of the rat peptide fragment (EVARQ), also elicited stimulation of Na^+,K^+-ATPase, corresponding to 75 % of the intact molecule's activity (rat C-peptide 1, Table 1).

In addition, non-terminal segments centering on the mid-third portion exhibited 35–80 % of the stimulatory activity of the entire molecule, while longer or slightly shorter fragments showed a similar stimulatory capacity (Fig. 1). However, the glycine di- and tripeptide sequences of the mid-portion fragment stimulated Na^+,K^+-ATPase activity only slightly, and the non-native di-, tri-, tetra- or pentapeptides with ala-

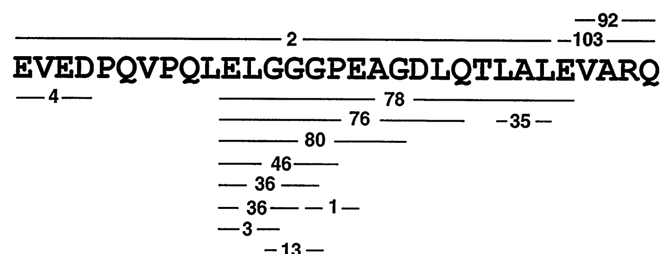

Fig. 1. Stimulation of Na^+,K^+-ATPase activity by C-peptide fragments. The amino acid sequence of rat C-peptide 1 is indicated. Numbers denote the percentage of the entire molecule's stimulating activity that is retained by the fragments. All peptides were tested at $5 \cdot 10^{-7}$ mol/l

Table 1. Summary of the effects of intact C-peptide, C-peptide fragments, constituent amino acids and controls on Na^+, K^+-ATPase activity of renal tubule cells

Peptides and amino acids, $5 \cdot 10^{-7}$ mol/l	Na^+, K^+-ATPase activity	
	% above basal	% of C-peptide 1
Rat C-peptide 1	44.9 ± 1.8	100
Rat C-peptide 2	51.4 ± 2.5	114
Randomly scrambled C-peptide 1	0.9 ± 1.3	2
G	0.1 ± 2.8	0
L	0.6 ± 0.8	1
ELG	1.2 ± 2.0	3
ELGG	16.3 ± 1.6	36
ELGGG	16.1 ± 0.6	36
ELGGGP	20.5 ± 0.8	46
ELGGGPEAG	36.1 ± 0.9	80
ELGGGPEAGDLQ	33.9 ± 3.9	76
ELGGGPEAGDLQTLALE	34.8 ± 7.7	78
EVED	1.7 ± 2.3	4
LAL	15.8 ± 2.2	35
GPE	0.6 ± 2.7	1
EVARQ	46.3 ± 1.1	103
VARQ	41.3 ± 3.6	92
EGSLQ	33.6 ± 2.9	75
Des-(27–31) C-peptide 1	1.0 ± 2.8	2
GG	7.0 ± 2.8	16
GGG	5.9 ± 3.8	13
AA	–1.8 ± 1.6	0
AAA	0.3 ± 3.2	1
AAAA	0.1 ± 1.5	0
AAAAA	0.7 ± 2.6	1
L-GL	4.1 ± 1.9	9
D-GL	–1.2 ± 1.1	0
L-LG	0.5 ± 1.2	1
D-LG	32.3 ± 4.0	72
D, L-LG	28.3 ± 0.9	63
L-LG + D-LG (1 : 1)	28.4 ± 0.4	63
AcG	2.6 ± 2.1	6
AcLG	0.6 ± 3.3	1
AcQ	1.2 ± 1.4	3
AcE	2.7 ± 3.2	6
SF	2.9 ± 3.5	6

Means ± SEM are given. Each data point represents the average value of 18–30 measurements on 6–10 tubule segments from three different rats

nine had no measurable effect (Table 1). In addition, the dipeptide L-LG had little or no stimulatory capacity. In spite of this, D-LG stimulated Na^+,K^+-ATPase to 72 % of the effect of the intact molecule (Table 1). D,L-LG showed 63 % activity, as did the mixture of L-LG and D-LG (Table 1). AcLG showed no detectable effect. Likewise, individual amino acids (G, L) or the acetylated derivatives, AcG, AcE and AcQ were almost without effect. Overall this activity from fragments of the mid-third segment shows three distinctions from that of the C-terminal fragment: 1) the mid-third fragment activities are not balanced by lack of activity of the des-mid fragments; 2) the mid-third fragments appear to be also active with non-native configurations, like D-peptides; and 3) they are less active than the C-terminal segments, even though the differences are not great.

The possibility that the C-peptide or its fragments might exert a direct stimulation of Na^+,K^+-ATPase as distinct from a membrane-mediated intracellular activation was examined. A purified preparation of Na^+,K^+-ATPase was incubated with C-peptide 1, EVARQ, ELGGGPEAG, D-LG and L-LG. No measurable activation was found with any of these peptides, demonstrating that the effect is not likely to be exerted on the ATPase directly, but indirectly and presumably via a receptor.

Discussion

We measured the extent of stimulation of Na^+,K^+-ATPase in renal tubular segments, a well-established model for the study of Na^+,K^+-ATPase activity [18]. Measurements were made after preincubation with different C-peptide derivatives, including the intact rat C-peptides 1 and 2, and a scrambled C-peptide 1. In addition, free and acetylated amino acids were studied. Half-maximal activation of Na^+, K^+-ATPase by $5 \cdot 10^{-7}$ mol/l C-peptide has been observed previously [16]. Consequently, this concentration was used for all peptides and amino acids in the present study even though it represents a pharmacological rather than physiological concentration. The results, given in Table 1 and summarized in Figure 1, present a consistent picture by showing full activity of the rat C-peptides 1 and 2, and complete absence of effect of the scrambled C-peptide 1. These results with the full-size peptides confirm and extend those obtained previously with the intact C-peptide [16] and establish the reproducibility of the enzyme measurements. Thus established, two novel effects become apparent. One is that two segments of the C-peptide appear to be of special functional importance, the C-terminal segment and a mid-third segment. The other is that these segments differ in activity with only the C-terminal segment being capable of consistently replacing the entire molecule.

Thus, regarding the C-terminal segment, almost the entire C-peptide effect was reproduced by 4–5-residue C-terminal segments, which elicited 92–103 % of the effect of the intact C-peptide (Table 1). Consistent with this finding is the observation that the remainder of the molecule, the des-(27–31) C-peptide, was almost inactive (Table 1). This finding is of particular interest since des-(27–31) C-peptide is secreted from rat pancreatic beta-cells and may account for as much as 10 % of the rat beta cell secretory granules [22]. As indicated in Table 1, the human C-terminal pentapeptide (EVARQ) also exhibits an effect. Although different, the human peptide has two residues, positioned at both terminals, that are identical with the rat form. Considering these sets of peptides, it appears as if the major part of functional importance in the C-peptide stimulation of Na^+,K^+-

ATPase [16] is the C-terminal segment. The present finding of a considerable activity in the C-terminal segment of the molecule, although novel regarding the C-peptide, resembles that noticed with many other biologically active peptides. Thus, peptides frequently have defined functional sites, corresponding to small segments which interact with receptors or other proteins. Specifically, both gastrin and CCK show active sites involving C-terminal pentapeptides [23, 24].

A more complex finding is that a mid-segment of the C-peptide also elicits considerable effects (Table 1, Fig. 1). In this case, however, the interpretation is not as straightforward; the activity is present in several segments from the middle part of the C-peptide and it is not balanced by corresponding inactive segments. In addition, non-natural, D-amino acid containing peptides are also active. The prerequisite for this activity appears to be at least one (if D-peptide) or two (if L-peptide) of the central tri-glycine residues. However, the activity has three further abnormalities. One is that this activity, although quite extensive, is never as pronounced as that from the C-terminal segment (Fig. 1). Another is that the activity is absent with the whole des-(27–31) C-peptide (Table 1). Thus, it appears as if parts of the central segment only in special combinations give effects in our enzyme assay, apparently with an optimum for a less than 10-residue segment containing the central Gly-sequence, but decreasing in effect when longer portions from either the N- or C-terminal regions are included (Fig. 1). This is not typical of single-receptor interactions and is therefore concluded to reflect a complex pattern of several or indirect effects. Finally, in tests of this middle segment with dipeptides and free amino acids, corresponding to the constituents of this region, we found that the free amino acids have essentially no effect on the enzyme system, the tripeptide Gly-Gly-Gly possesses only a limited effect, and that the D-form of the dipeptide Leu-Gly (D-LG) has a considerable effect, while the L-form (L-LG) is inactive (Table 1). This differential dipeptide effect is reproducible and observed with the peptides prepared in different ways. Thus, the D,L-mixture, both when prepared from the separately synthesized D and L dipeptides, and from the racemic mixture synthesized as such, gave identical and lower values than the D-form (Table 1). In conclusion, it appears as if a central portion of the C-peptide has a considerable but not complete effect and that the effect is noticeable both in native forms and in non-native D-isomers of C-peptide segments. The D-form activity is reminiscent of that recently reported in another study [25].

Taken together, it is clear that at least two effects are observed in the Na^+,K^+-ATPase activation by C-peptide segments. One is a demonstration that the major effect can be obtained by a C-terminal penta/tetra-segment (Fig. 1). This activity is close to fully replacing that of the whole C-peptide and is consistent with the absence of activity in the 26-residue N-terminal remainder of the molecule (Fig. 1). It is concluded that the C-peptide effect, previously observed on Na^+,K^+-ATPase activation may be reproduced by just the C-terminal penta/tetra-peptide, in a manner similar to that of functional segments of bioactive peptides in general [23, 24], most likely reflecting a receptor binding. This finding suggests a possibility of further tests of C-peptide effects by replacements with this short segment only. The other effect(s), involving the central Gly-region, are less well-defined and have inconsistent properties. Thus, longer segments do not give similar effects (including absence of effect of the whole segment 1–26) and a D-dipeptide mimicks much of the activity. The enzyme assay of the Na^+,K^+-ATPase activation is complex, involving whole cell preparations and a membrane-bound enzyme. In addition, the effect of the Gly-peptides and of the D-form may suggest interactions with parts of the segment lacking secondary structure elements. Therefore, this second type of activity is concluded to represent complex interactions not ascribed to specific C-peptide effects. Instead, it resembles the effects recently described for human C-peptide on vascular and neural dysfunction in diabetic rats [25]. The latter finding and the present results agree and suggest that fragments from the midportion, as well as those containing D-amino acids (enantio) C-peptide segments, elicit effects.

In conclusion, using a complex enzyme assay with Na^+,K^+-ATPase activation in cell preparations, we find that much of the bioactivity previously ascribed to the intact proinsulin C-peptide can be ascribed to the C-terminal tetra/penta-peptide fragment, with the remainder of the molecule showing as a negative control, and that a central part exhibits a different bioactivity, possibly representing another type of interaction.

Acknowledgements. This study was supported by grants from the Swedish Medical Research Council (projects no 11201, 3532 and 10832), Skandia Insurance Co. and the Bank of Sweden Tercentenary Foundation.

References

1. Steiner DF, Oyer PE (1967) The biosynthesis of insulin and a probable precursor of insulin by a human islet cell adenoma. Proc Natl Acad Sci USA 57: 473–480
2. Steiner DF, Cunningham D, Spigelman L, Aten B (1967) Insulin biosynthesis: evidence for a precursor. Science 157:697–700
3. Kitabchi AE (1970) The biological and immunological properties of pork and beef insulin, proinsulin, and connecting peptides. J Clin Invest 49:979–987
4. Hagen C, Faber OK, Binder C, Alberti KGMM (1977) Lack of metabolic effects of C-peptide in normal subjects

and juvenile diabetic patients. Acta Endocrinol 85 [Suppl 209]:29A, (Abstract)
5. Hoogwerf BJ, Bantle JP, Genslen HE et al. (1986) Infusion of synthetic human C-peptide does not affect plasma glucose, serum insulin, or plasma glucagon in healthy subjects. Metabolism 35:122–125
6. Snell CR, Smyth DG (1975) Proinsulin: A proposed three-dimensional structure. J Biol Chem 250:6291–6295
7. Johansson B-L, Sjöberg S, Wahren J (1992) The influence of human C-peptide on renal function and glucose utilization in type 1 (insulin-dependent) diabetic patients. Diabetologia 35:121–128
8. Zierath JR, Galuska D, Johansson B-L, Wallberg-Henriksson H (1991) Effect of human C-peptide on glucose transport in vitro incubated human skeletal muscle. Diabetologia 34:899–901
9. Johansson B-L, Linde B, Wahren J (1992) Effects of C-peptide on blood flow, capillary diffusion capacity and glucose utilization in the exercising forearm of type 1 (insulin-dependent) diabetic patients. Diabetologia 35:1151–1158
10. Johansson B-L, Borg K, Fernqvist-Forbes E, Odergren T, Remahl S, Wahren J (1996) C-peptide improves autonomic nerve function in patients with type 1 diabetes. Diabetologia 39:687–695
11. Johansson B-L, Kernell A, Sjöberg S, Wahren J (1993) Influence of combined C-peptide and insulin administration on renal function and metabolic control in diabetes type 1. J Clin Endocr Metab 77:976–981
12. Johansson B-L, Fernqvist-Forbes E, Wahren J (1995) Effects of C-peptide on nephropathy and neuropathy in IDDM-patients – a clinical study. Diabetologia 38 [Suppl 1] A7 (Abstract)
13. Zierath JR, Handberg A, Tally M, Wallberg-Henriksson H (1996) C-peptide stimulates glucose transport in isolated human skeletal muscle independent of insulin receptor and thyrosine kinase activation. Diabetologia 39:306–313
14. Leclerq-Meyer V, Malaisse WJ, Johansson B-L, Wahren J (1997) Inhibition by C-peptide of insulin release from the isolated perfused rat pancreas. Diabetes & Metabolism 23:149–154
15. Cohen P, Barzilai N, Karnieli E (1995) Suppression of insulin secretion by C-peptide infusions in humans. Isr J Med Sci 31:284–288
16. Ohtomo Y, Aperia A, Sahlgren B, Johansson B-L, Wahren J (1996) C-peptide stimulates rat renal tubular Na^+,K^+-ATPase activity in synergism with neuropeptide Y. Diabetologia 39:199–205
17. Flatt PR, Swanston-Flatt SK, Hampton SM, Bailey CJ and Marks V (1986) Specific binding of the C-peptide of proinsulin to cultured β-cells from a transplantable rat islet cell-tumor. Bioscience Reports 6:193–198
18. Doucet A, Katz AI, Morel F (1979) Determination of Na^+,K^+-ATPase activity in single segments of the mammalian nephron. Am J Physiol 237:F105–F113
19. Bergman T, Jörnvall H, Holmquist B, Vallee BL (1992) A synthetic peptide encompassing the binding site of the second zinc atom (the "structural" zinc) of alcohol dehydrogenase. Eur J Biochem 205:467–470
20. Bergman T, Agerberth B, Jörnvall H (1991) Direct analysis of peptides and amino acids from capillary electrophoresis. FEBS Lett 283:100–103
21. Jorgensen PL (1974) Purification of Na^+,K^+-ATPase. III. Purification from the outer medulla of mammalian kidney after selective removal of membrane components by sodium dodecylsulphate. Biochim Biophys Acta 356:36–52
22. Verchere CB, Paoletta M, Neerman-Arbez M et al. (1996) Des-(27–31)C-peptide. A novel secretory product of the rat pancreatic beta cell produced by truncation of proinsulin connecting peptide in secretory granules. J Biol Chem 271:27475–27481
23. Gregory RA, Tracy HJ (1964) The constitution and properties of two gastrins extracted from hog antral mucosa. Gut 5:103–117
24. Mutt V, Jorpes JE (1968) Structure of porcine cholecystokinin-pancreozymin. Eur J Biochem 6:156–162
25. Ido Y, Vindigni A, Chang K et al. (1997) Prevention of vascular and neural dysfunction in diabetic rats by C-peptide. Science 277:563–566