CMLS, Cell. Mol. Life Sci. 61 (2004) 2782–2790
1420-682X/04/212782-09
DOI 10.1007/s00018-004-4258-x

CMLS Cellular and Molecular Life Sciences

Research Article

C-peptide stimulates Na^+,K^+-ATPase via activation of ERK1/2 MAP kinases in human renal tubular cells

Z. Zhong[a, b], O. Kotova[b], A. Davidescu[a], I. Ehrén[c], K. Ekberg[a], H. Jörnvall[d], J. Wahren[a], and A. V. Chibalin[b, *]

[a] Section of Clinical Physiology, Department of Surgical Sciences, Karolinska Institutet, 17177 Stockholm (Sweden)
[b] Section of Integrative Physiology, Department of Surgical Sciences, Karolinska Institutet, von Eulers väg 4, 4 tr, 17177 Stockholm (Sweden), Fax +46 8 335436, e-mail: alexander.Chibalin@kirurgi.ki.se
[c] Section of Urology, Department of Surgical Sciences, Karolinska Institutet, 17177 Stockholm (Sweden)
[d] Department of Medical Biochemistry and Biophysics, Karolinska Institutet, 17177 Stockholm (Sweden)

Received 15 June 2004; received after revision 14 September 2004; accepted 14 September 2004

Abstract. Proinsulin-connecting peptide (C-peptide) exerts physiological effects partially via stimulation of Na^+,K^+-ATPase. We determined the molecular mechanism by which C-peptide stimulates Na^+,K^+-ATPase in primary human renal tubular cells (HRTCs). Incubation of the cells with 5 nM human C-peptide at 37 °C for 10 min stimulated $^{86}Rb^+$ uptake by 40% ($p<0.01$). The carboxy-terminal pentapeptide was found to elicit 57% of the activity of the intact molecule. In parallel with ouabain-sensitive $^{86}Rb^+$ uptake, C-peptide increased α subunit phosphorylation and basolateral membrane (BLM) abundance of the Na^+,K^+-ATPase α_1 and β_1 subunits. The increase in BLM abundance of the Na^+,K^+-ATPase α_1 and β_1 subunits was accompanied by depletion of α_1 and β_1 subunits from the endosomal compartments. C-peptide action on Na^+,K^+-ATPase was ERK1/2-dependent in HRTCs. C-peptide-stimulated Na^+,K^+-ATPase activation, phosphorylation of α_1-subunit and translocation of α_1 and β_1 subunits to the BLM were abolished by a MEK1/2 inhibitor (20 μM PD98059). C-peptide stimulation of $^{86}Rb^+$ uptake was also abolished by preincubation of HRTCs with an inhibitor of PKC (1 μM GF109203X). C-peptide stimulated phosphorylation of human Na^+,K^+-ATPase α subunit on Thr-Pro amino acid motifs, which form specific ERK substrates. In conclusion, C-peptide stimulates sodium pump activity via ERK1/2-induced phosphorylation of Thr residues on the α subunit of Na^+,K^+-ATPase.

Key words: Na pump; sodium pump; C-peptide; kidney; MAP kinase; PKC.

Introduction

C-peptide, the connecting segment of proinsulin, is secreted by pancreatic β cells into the circulation together with insulin in equimolar quantities. One role of C-peptide is to participate in the proper folding of proinsulin by facilitating correct disulfide bond formation between the A and B chains. In patients with type 1 diabetes and in animal models of the disease, administration of C-peptide in physiological concentrations results in improvements of diabetes-induced functional and structural changes of peripheral nerves [1–3]. C-peptide in replacement doses prevents diabetes-induced deficits in nerve fiber regeneration [4], protects against glucose-induced apoptosis of nerve cells, and stimulates cellular proliferation [5, 6]. Moreover, C-peptide in replacement doses corrects glomerular hyperfiltration characteristic of the early stages of diabetic nephropathy, reduces urinary excretion of albumin and prevents the development of glomerular

* Corresponding author.

hypertrophy in type 1 diabetes [1, 7, 8]. C-peptide, given in replacement doses to type 1 patients, augments skeletal muscle and myocardial blood flow and increases the rate of contraction and stroke volume of the left ventricle [9–11].

The molecular mechanisms by which C-peptide exerts its effects are now beginning to emerge. A series of studies during the past decade have demonstrated that C-peptide binds to a G-protein-coupled membrane binding site on a number of different cell types [12], thereby triggering Ca^{2+}-dependent intracellular signaling pathways [13], including protein kinase C (PKC) isoforms and the mitogen-activated protein (MAP) kinase cascade [14, 15]. Some data indicate that C-peptide may exert insulinomimetic effects via interaction with the insulin signaling pathways at the level of the insulin receptor or downstream of it [15]. Acute stimulation with C-peptide leads to subsequent activation of both Na^+,K^+-ATPase and endothelial nitric oxide synthase (eNOS) [16–18]. C-peptide stimulates Na^+,K^+-ATPase activity [17] via PKCα activation in rat medullary thick ascending limb cells [19]. Activation of the Na pump and eNOS are of particular clinical interest, as they are reported to be deficient in diabetes [20–23].

Na^+,K^+-ATPase is a ubiquitously expressed plasma membrane cation pump, essential for maintenance of intracellular and extracellular sodium and potassium concentrations, cell volume, osmotic balance and electrochemical gradients [24, 25]. The regulation of Na^+,K^+-ATPase can be achieved via multiple mechanisms, including changes in intrinsic activity, subcellular distribution and cellular abundance [26–28]. The catalytic subunit of the Na^+,K^+-ATPase is a substrate for protein kinases [25, 26, 28], and pump phosphorylation is an important molecular mechanism for the short-term control of its activity in response to hormonal stimulation [26].

Na^+,K^+-ATPase in renal proximal tubular cells is a key target of a number of hormones, where the enzyme plays an essential role in Na^+ handling [26]. The mechanisms for regulation of Na^+,K^+-ATPase activity have been thought to be similar in humans and rodents. However, species differences [28, 29] in the structure of Na^+,K^+-ATPase must be considered, especially with regard to the role of PKC. PKC-mediated phosphorylation of Ser23 of the Na^+,K^+-ATPase α subunit plays an important role in regulation of Na^+,K^+-ATPase activity and membrane trafficking in rodent tissue [30–32]. PKC has been implicated in insulin-induced stimulation of Na^+,K^+-ATPase [27, 33, 34]. However, the human α subunit lacks Ser23, highlighting important species differences in the regulation of the pump. In vitro phosphorylation of GST fusion proteins, containing the N terminus of the Na^+,K^+-ATPase α subunit [29], or in vitro phosphorylation of purified human kidney Na^+,K^+-ATPase [35] indicate that the human α_1 subunit is a poor substrate for PKC.

C-peptide stimulates ERK1/2 via PKC-dependent signaling pathways [14, 36]. Moreover, MAP kinases have been implicated in the regulation of Na^+,K^+-ATPase. Activation of the ERK1/2 signaling pathway leads to an increase in synthesis of Na^+,K^+-ATPase subunits [37, 38] and short-term stimulation of Na^+,K^+-ATPase activity [38]. Consequently, in this study, we examined physiological effects of C-peptide on Na^+,K^+-ATPase ion-transporting activity in human renal tubular cells in order to clarify the roles of PKC and MAP kinases.

Materials and methods

Antibodies and reagents. Rabbit polyclonal anti-NK1 antibodies were kind gifts of Dr. E. Feraille. Specific anti-α_1 subunit monoclonal antibodies were obtained from Dr. M. Caplan (Yale University, New Haven, Conn.); rabbit polyclonal anti-β_1 and β_2 subunit antibodies were from Dr. P. Martin-Vasallo (University La Laguna, Tenerife, Spain). Mouse antibodies raised against a phospho-Thr-Pro motif were from Cell Signaling (Beverly, Mass.). Kinase inhibitors PD98059 and GF109203X were from Calbiochem (La Jolla, Calif.). Pertussis toxin (PTX) was from Sigma (St Louis, Mo.). Cell culture media and reagents were obtained from Invitrogen (Lidingö, Sweden). Dimethylsulfoxide from Calbiochem was used as a solvent for protein kinase inhibitors. All other reagents were of analytical grade. Human recombinant C-peptide was obtained from Schwarz Pharma (Monheim, Germany). Scrambled C-peptide (the same amino acid residues as in C-peptide but in random order) and C-terminal pentapeptide (EGSLQ) were from Sigma Genosys (Cambridge, UK).

Cell culture. Human renal tubular cells (HRTCs) were cultured from the unaffected outer cortex of renal tissue obtained from non-diabetic patients undergoing elective nephrectomy for renal cell carcinoma. Tissue collection was undertaken with the informed consent of the subject and approval by the Karolinska Institutet ethics committee. The cells were cultured in RPMI 1640 (Life Technologies, Paisley, UK) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 5 mM glucose, 10 mM HEPES, bensylpenicillin (100 U/ml) and streptomycin (100 µg/ml) and passaged at near confluence by trypsinization. Growing cells exhibited epithelial morphology with a central nucleus, a granular cytoplasm and cobblestone appearance on light microscopy. Cells from the second and third passages were used for experiments.

Cell incubation. For activity assays, cells were serum starved overnight, washed in PBS and preincubated for 30 min in PBS with either 0.2% DMSO or 20 µM PD98059 (MEK1 inhibitor), 1 µM or 10 µM GF109203X

(PKC inhibitor) and 100 ng/mL PTX (G-protein inhibitor). After pre-exposure to DMSO/inhibitors, cells were stimulated with C-peptide (5 nM) for 10 min. After treatment, cells were washed twice with ice-cold PBS, and harvested by scraping cells into ice-cold lysis buffer A (20 mM Tris pH 8.0, 135 mM NaCl, 1 mM $MgCl_2$, 2.7 mM KCl, 10 mM $Na_4P_2O_7$, 0.5 mM Na_3VO_4, 10 mM NaF, 1 µM okadaic acid, 1% Triton X-100, 10% v/v glycerol, 0.2 mM PMSF, 10 µg/ml leupeptin, and 10 µg/ml aprotinin). Homogenates were rotated for 60 min at 4 °C and subjected to centrifugation (20,000 g for 10 min at 4 °C). Protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.). Lysates were kept at –80 °C before subsequent Western blot analysis or immunoprecipitation with appropriate antibodies.

Measurement of ouabain-sensitive $^{86}Rb^+$ uptake. The transport activity of Na^+,K^+-ATPase was measured by ouabain-sensitive $^{86}Rb^+$ uptake under conditions of initial rate, as described elsewhere [39]. The cells were grown on 100-mm dishes (Costar, Cambridge, Mass), serum starved overnight and preincubated in serum-free RPMI 1640 without or with ouabain (1 mM) and kinase inhibitors for 30 min at 37 °C. Thereafter, cells were incubated in the absence or presence of C-peptide (10 min) and/or inhibitors. The transport activity of Na^+,K^+-ATPase was determined after the addition of 50 µl of medium containing tracer amounts of $^{86}RbCl$ (100 nCi/sample; Amersham, Little Chalfont, UK) for 5 min. Incubation was stopped by cooling on ice and dishes were washed three times with an ice-cold solution containing 150 mM choline chloride, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 2 mM $BaCl_2$, and 5 mM HEPES, pH 7.4. Cells were lysed in 750 µl lysis buffer A, and the radioactivity was measured by liquid scintillation. Protein content was determined in parallel by using the bicinchoninic acid assay (Pierce). Ouabain-sensitive $^{86}Rb^+$ uptake was calculated as the difference between the mean values measured in triplicate samples incubated without or with 0.2 mM ouabain and was expressed as a percentage of the control. Basal ouabain-sensitive $^{86}Rb^+$ uptake was 16.3±2.4 pmol Rb^+ per microgram of protein per minute.

Metabolic labeling of HRTCs with $^{32}P_i$. $^{32}P_i$ metabolic labeling was performed as described elsewhere [34] to investigate in vivo phosphorylation of α subunits of Na^+,K^+-ATPase. Cells growing on 100-mm dishes, were serum starved overnight and incubated for 3 h at 37 ° C in serum-free RPMI 1640 containing $^{32}P_i$ (1 mCi/ml). C-peptide and/or inhibitors were added during the last 20 or 50 min of incubation time, as described above. Incubation was terminated by cooling on ice. Cells were lysed in buffer A and α subunits were immunoprecipitated with polyclonal anti-NK1 rabbit antibodies. The bead pellets were mixed with Laemmli buffer (60 µl; 62.5 mM Tris-HCl, 2% SDS, 10% glycerol, and 10 mM DTT), separated by SDS-PAGE, and transferred to polyvinylidenedifluoride (PVDF) membranes (Millipore, Bradford, Mass). Phosphoproteins were analyzed using the Bio-Imaging Analyzer BAS-1800II (Fuji, Tokyo, Japan), and quantification was performed using the Image Gauge software, version 3.4 (Fuji). In each experiment, the amount of radioactivity incorporated into the α subunit was corrected for the amount of protein detected by Western blot, and the quantitative data were shown as percent of basal.

Immunoprecipitation. The immunoprecipitation of Na^+, K^+-ATPase α subunit was performed as described previously [34]. HRTCs were lysed in 0.5 ml ice-cold lysis buffer A. Insoluble material was removed by centrifugation (12,000 g for 10 min at 4 °C). Aliquots of supernatant (300 µg protein) were immunoprecipitated overnight at 4 °C with 50 µl of polyclonal anti-NK1 rabbit antibodies. Immunoprecipitates were collected on protein A-Sepharose (Pharmacia Biotech, Uppsala, Sweden). Beads were washed four times in lysis buffer A, twice in 0.1 M Tris (pH 8.0) and 0.5 M LiCl, once in 10 mM Tris (pH 7.6), 0.15 M NaCl, and 1 mM EDTA; and once in 20 mM HEPES, 5 mM $MgCl_2$, and 1 mM DTT. Pellets were resuspended in Laemmli sample buffer.

Phosphoamino acid analysis. The phosphorylated α subunits were immunoprecipitated, resolved by SDS-PAGE, transferred to PVDF membranes and the ^{32}P-labeled Na^+, K^+-ATPase α subunits were identified on the membrane by a Bio-Imaging Analyzer BAS-1800II and excised. Thereafter, the phosphorylated α subunits were hydrolyzed in 6 M HCl and analyzed by two-dimensional high-voltage electrophoresis on cellulose thin-layer plates. Phosphoamino acid analysis was performed essentially as described by Boyle et al. [40]. Phosphoamino acids, on thin-layer electrophoresis plates, were analyzed using the Bio-Imaging Analyzer BAS-1800II.

Preparation of basolateral membrane and endosomes from HRTCs. The cells were preincubated for 30 min either with 0.2% DMSO or 20 µM PD98059. After preexposure to inhibitors, cells were incubated with C-peptide (5 nM) and scrambled C-peptide (5 nM) for 15 min. After treatment, cells were washed twice with ice-cold PBS, and harvested by scraping into 500 µl ice-cold buffer [12 mM HEPES, 300 mM mannitol, pH 7.6 (Tris 1 M), 0.5 mM Na_3VO_4, 0.2 mM PMSF, 5 µg/ml leupeptin, 5 µg/ml aprotinin, and 1 µM microcystin]. Cells were homogenized by Pellet Pestle twice for 1 min, and passage through an insulin syringe with a 21G needle 10–12 times into an Eppendorf tube. Homogenates were precleaned by centrifugation at 2,500 g for 15 min. Supernatants were collected. Pellets were resuspended in 500 µl ho-

mogenization buffer, and spun down at 2,500 g for 15 min. Supernatants were collected and transferred to the tubes, containing the first 500 µl, and centrifuged at 20,000 g for 20 min, +4 °C on a TLA 100.2 rotor. Basolateral membranes (BLMs) were further purified [41], using a Percoll gradient. The yellow layer of the pellet was resuspended again in the supernatant (carefully removed from the brown pellet containing mitochondria and cell ghosts) and centrifuged at 48,000 g for 30 min. The supernatant was discarded, and the pellet was resuspended in 1 ml buffer (300 mM mannitol and 12 mM HEPES, pH 7.6, adjusted with Tris) by gentle pipetting. To form a Percoll gradient, 0.19 g undiluted Percoll (Pharmacia Biotech) was added to a 1-ml suspension (0.8–1.0 mg of protein). The suspension was gently mixed and centrifuged at 48,000 g for 30 min, and the ring of BLMs, light endosomal fraction (the top one-third of the tube), and pellets were collected and frozen at –20 °C. Protein content was determined in parallel using the bicinchoninic acid assay (Pierce) and samples, containing BLMs and light endosomes (10 µg protein) were resolved on SDS-PAGE.

Western blot analysis. Aliquots of cell lysates (30 µg protein), fractions of BLMs and light endosomes (10 µg protein), or immunoprecipitates were resuspended in Laemmli sample buffer. Proteins were then separated by SDS/PAGE, transferred to PVDF membranes, blocked with 7.5% non-fat milk, washed with TBST (10 mM Tris HCl, 100 mM NaCl, 0.02% Tween 20) and incubated with appropriate antibodies overnight at 4 °C. Membranes were washed with TBST and incubated with an appropriate secondary antibody. Proteins were visualized by enhanced chemiluminescence and quantified by densitometry.

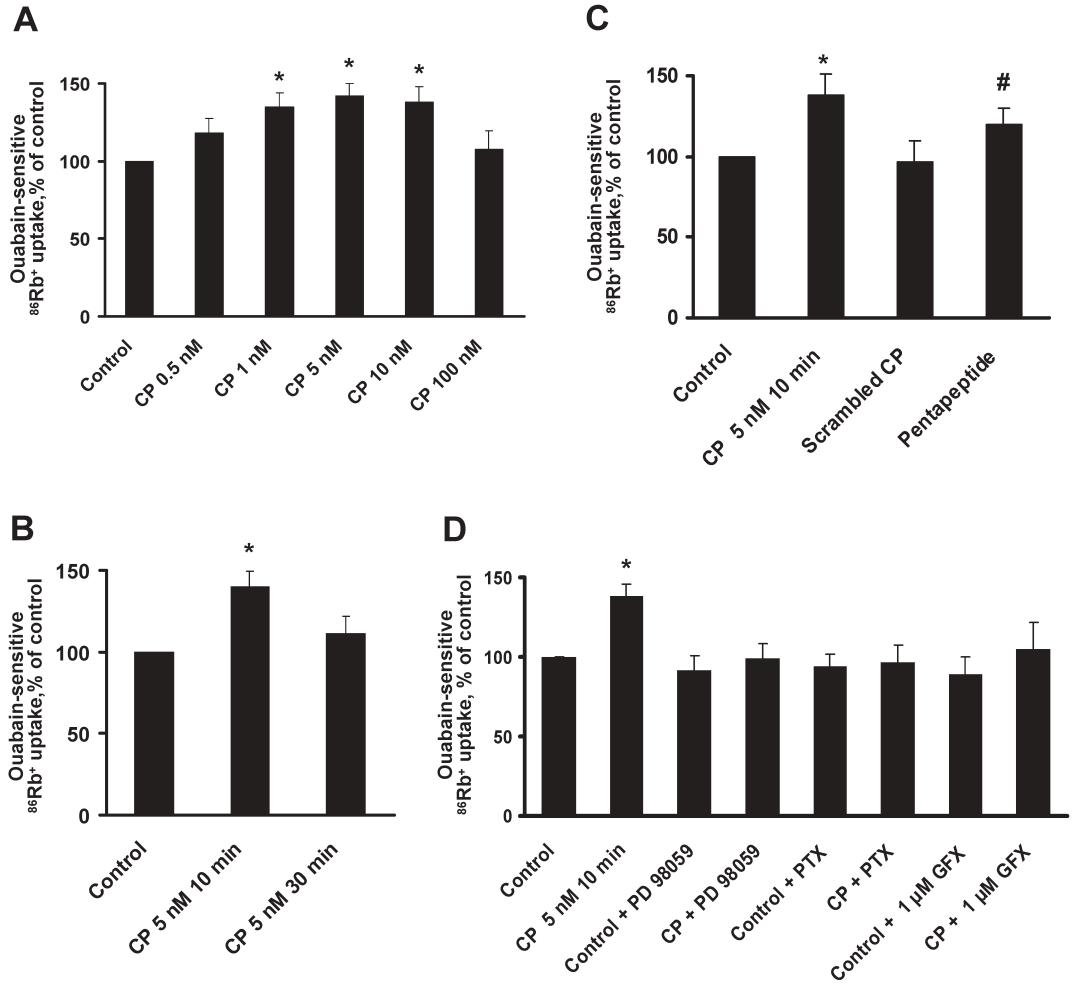

Figure 1. Effect of C-peptide (CP) and inhibitors on ouabain-sensitive $^{86}Rb^+$ uptake in HRTCs. Assays were performed as described in Materials and methods. Results are means ± SE for six independent experiments performed in triplicate. *$p<0.01$ versus control, $^{\#}p<0.05$ versus control. (*A*) Concentration-dependent effect of C-peptide. Cells were incubated with the indicated concentrations of C-peptide for 10 min. *(B)*. Effect of C-peptide on ouabain-sensitive $^{86}Rb^+$ uptake is transient. Cells were incubated with 5 nM C-peptide for 10 and 30 min. (*C*) Cells were incubated with 5 nM C-peptide, C-terminal pentapeptide, and scrambled C-peptide for 10 min. (*D*) Cells were incubated with 5 nM C-peptide for 10 min in the absence or presence of 20 µM PD98059, 1 µM GF109203X, on 100 ng/ml PTX, as indicated.

Statistics. Data are presented as the mean ± SE. Comparisons between groups were performed using Student's t test. For multiple comparisons, one-way ANOVA with Sheffe's correction was used. Significance was established at $p<0.05$.

Results

Effects of C-peptide on ouabain-sensitive $^{86}Rb^+$ uptake. Incubation of HRTCs with 1 nM C-peptide for 10 min significantly stimulated ouabain-sensitive $^{86}Rb^+$ uptake; maximal stimulation (40% above control, $p<0.01$) was achieved with 5 nM of C-peptide. Exposure of cells to 100 nM C-peptide was without effect on ouabain-sensitive $^{86}Rb^+$ uptake (fig. 1A). The effect was transient; at 30 min, the activity had returned to the basal level (fig. 1B). C-terminal pentapeptide elicited a smaller (57% of native peptide), but significant effect, while scrambled C-peptide elicited no effect on the ouabain-sensitive $^{86}Rb^+$ uptake (fig. 1C). The effect of C-peptide was completely inhibited by the presence of either 20 µM of the MEK1/2 inhibitor PD98059, 1 µM of the PKC inhibitor GF109203X, or 100 ng/ml of the G_i protein inhibitor pertussis toxin (fig. 1D). Thus, C-peptide stimulates Na^+,K^+-ATPase activity via PKC-, ERK-, and MAP-kinase dependent pathways.

Phosphorylation of the α subunits of Na^+,K^+-ATPase in HRTC in response to C-peptide. To determine whether C-peptide induces the phosphorylation of Na^+,K^+-ATPase in HRTCs, they were metabolically labeled with $^{32}P_i$ and then incubated with C-peptide for 10 min in the absence or presence of 20 µM PD98059. A specific anti-α subunit antibody was used to immunoprecipitate the pump subunit [34]. Background phosphorylation of the Na^+,K^+-

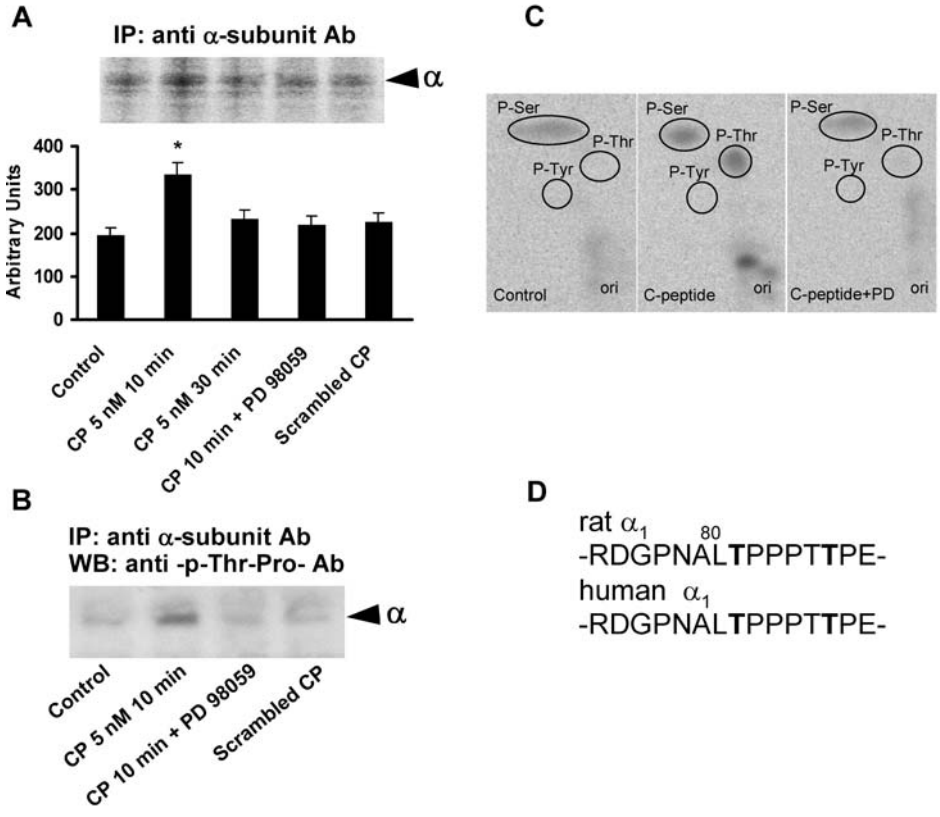

Figure 2. Effect of C-peptide and PD98059 on the phosphorylation of Na^+,K^+-ATPase α subunits in HRTCs. (*A*) Cells were metabolically labeled with $^{32}P_i$ and incubated with 5 nM C-peptide for 10 or 30 min, in the absence or presence of 20 µM PD98059. Cells were lysed, and equal amounts of protein (300 µg) were immunoprecipitated with anti-NK1 antibody. A representative autoradiogram is shown in the upper panel, and quantitative data from four experiments (mean ± SE) are shown in the lower panel. *$p<0.05$ vs control. (*B*) Corresponding Western blot images of immunoprecipitated samples, blotted by anti-phospho-Thr-Pro antibody. (*C*) Representative autoradiograms of phosphoamino acids from immunoprecipitated α subunit from untreated (basal) cells in response to 5 nM C-peptide without or with 20 µM PD98059. The circles represent the positions of unlabeled phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr). The additional spots represent the site of sample application (ori) and non-hydrolyzed peptides. (*D*). Prediction of phosphorylation site for ERK1/2 in the Na^+,K^+-ATPase α subunit. The protein sequences of α_1 subunits of Na^+,K^+-ATPase of human and rat origin were analyzed by the motif-based profile scanning program Scansite 2.0 (http://scansite.mit.edu). Thr81 and Thr86 are the most probable sites for ERK phosphorylation.

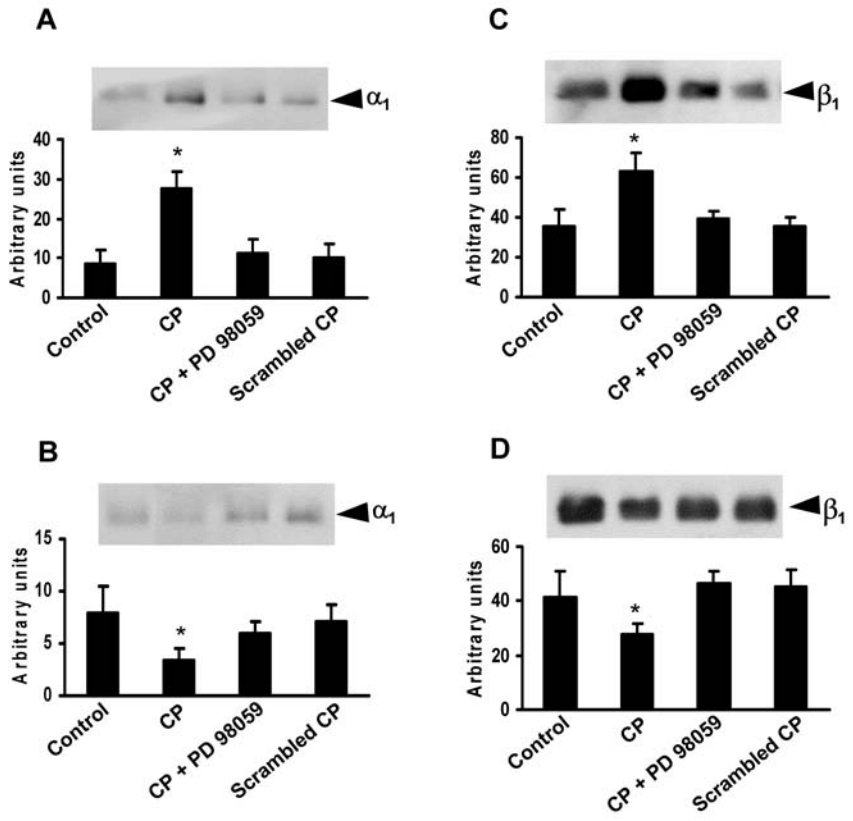

Figure 3. Translocation of Na^+,K^+-ATPase α subunits to the BLM by C-peptide in the absence or presence of PD98059 in HRTCs. Cells were incubated with 5 nM C-peptide in the absence or presence of 20 µM PD98059. BLMs and light endosomes were prepared as described in Material and methods. Na^+,K^+-ATPase α_1 and β_1 subunits were detected by Western blot analysis of the two fractions with isoform-specific antibodies. A representative image is shown in the upper panel of each graph. Quantitative data from six experiments (mean±SE) are shown in the lower panels. *$p<0.05$ vs. control. (*A*) α_1 subunit in BLMs. (*B*) α_1 subunit in light endosomes. (*C*) β_1 subunit in BLMs. (*C*) β_1 subunit in light endosomes.

ATPase α subunit was observed after a 3-h incubation of HRTCs in phosphorylation medium. C-peptide (5 nM) increased the phosphorylation of Na^+,K^+-ATPase α subunits 1.7-fold at 10 min; the effect was transient and by 30 min had returned to base line. C-peptide-stimulated Na^+,K^+-ATPase α subunit phosphorylation was inhibited by PD98059 (fig. 2A). This result was confirmed by Western blot analysis with anti-phospho-Thr-Pro-antibody (fig. 2B). α Subunits were immunoprecipitated from cell lysates, resolved by SDS-PAGE and immunoblotted with monoclonal anti-phospho-Thr-Pro antibody. C-peptide induced phosphorylation of α subunits on Thr-Pro motifs. Phosphorylation was abolished in the presence of PD98059. Scrambled C-peptide had no effect on α subunit phosphorylation. To further characterize the phosphoamino acids, the phosphorylated α subunit was excised, hydrolyzed, and analyzed by two-dimensional high-voltage electrophoresis on cellulose thin-layer plates. Under basal conditions, the pump α subunit was phosphorylated on Ser residues. C-peptide stimulation increased phosphorylation of Ser and Thr, but not Tyr residues (fig. 2C). In the presence of PD98059, phosphorylation of Thr residues was abolished, and that of Ser residues was reduced. These data suggest that ERK1/2 MAP kinase is involved in Na^+,K^+-ATPase α subunit phosphorylation, primarily on Thr residues.

C-peptide increases the amount of Na^+,K^+-ATPase in the BLM of HRTCs. To determine the effect of C-peptide on Na^+,K^+-ATPase membrane translocation, surface biotinylation and subsequent streptavidin precipitation were performed. C-peptide stimulation was without effect on Na^+,K^+-ATPase surface abundance on apical membranes of HRTCs (data not shown). These cells, as polarized epithelia, are mainly sensitive to apical cell surface labeling. Therefore, the BLM and light endosome fractions were prepared to further investigate this issue. Western blot analysis of these two fractions demonstrated that C-peptide caused an increase in the amount of α_1 and β_1 subunits of Na^+,K^+-ATPase in BLMs (fig. 3A, C), and corresponding decreases in light endosome fractions (fig. 3B, D). These effects were inhibited by preincubation of cells with 20 µM PD98059. The membrane abundance of another plasma membrane marker, the glucose transporter

GLUT1, and of the β_2 subunit of Na^+,K^+-ATPase was not affected by C-peptide (data not shown). These results indicate that C-peptide specifically activates the Na pump by its translocation from intracellular compartments to basolateral membranes in ERK1/2-dependent manner.

Discussion

Research during the past decade has provided increasing evidence for a physiological role of C-peptide. A number of studies have demonstrated a stimulatory effect of C-peptide on Na^+,K^+-ATPase, an enzyme that plays a crucial role in the maintenance of cellular homeostasis [17, 19]. We evaluated the effect of C-peptide on Na^+,K^+-ATPase in cells of human origin and determined the molecular pathways of C-peptide action. Our results indicate that C-peptide stimulates Na^+,K^+-ATPase activity in HRTCs by activation of a PKC- and MAP kinase-dependent pathway and phosphorylation of the Na^+,K^+-ATPase α subunit on Thr residues by ERK1/2. The stimulation of Na pump activity occurs due to Na^+,K^+-ATPase subunit (α_1 and β_1) translocation to the BLM from a light endosomal fraction.

In HRTCs C-peptide significantly stimulated ouabain-sensitive $^{86}Rb^+$ uptake (~1.4-fold), a measure of cation transport activity of Na^+,K^+-ATPase at 1 nM, with a maximal response obtained during stimulation at 5 nM C-peptide for 10 min. These concentrations are within or close to the physiological range (1–3 nM) of C-peptide in humans. A further increase in incubation time (30 min) or concentration (up to 100 nM) caused only a slight increase in $^{86}Rb^+$ uptake, compared to that of control cells. The C-terminal pentapeptide also significantly stimulated ouabain-sensitive $^{86}Rb^+$ uptake, although the effect was reduced compared to that of the intact C-peptide. These data are in accordance with previous findings [13, 17] showing that C-terminal pentapeptide possesses most of the activity of the entire C-peptide. Scrambled C-peptide was without effect on ouabain-sensitive $^{86}Rb^+$ uptake. The effect of C-peptide was abolished by incubating cells with the MEK1/2 inhibitor PD98059, confirming the involvement of the MAP kinase signaling pathway in C-peptide activation of the Na pump. GF109203X, a PKC inhibitor, used at a concentration that inhibits conventional and novel PKCs [42], also abolished the effect of C-peptide on Na^+,K^+-ATPase. Conventional and novel PKCs play an essential role in C-peptide-dependent activation of the MAP kinase signaling pathway [14]. Our data on ouabain-sensitive $^{86}Rb^+$ uptake thus provide evidence that C-peptide stimulates Na^+,K^+-ATPase activity in HRTC cells by activating a PKC- and MAP kinase-dependent pathway.

We further evaluated the phosphorylation of the α subunits of Na^+,K^+-ATPase in response to C-peptide stimulation in HRTCs. These cells were metabolically labeled with $^{32}P_i$, and phosphorylated α subunits were immunoprecipitated with polyclonal anti-α antibodies. Ouabain-sensitive $^{86}Rb^+$ uptake was strongly correlated with maximal effects on α subunit phosphorylation during C-peptide stimulation (5 nM C-peptide for 10 min). The C-peptide effect on $^{86}Rb^+$ uptake and α subunit phosphorylation could be transient due to desensitization of the C-peptide binding site, the signaling pathway, or subsequent activation of protein phosphatases. This response was abolished in the presence of the MEK1/2 inhibitor PD98059, indicating involvement of ERK1/2 in Na^+,K^+-ATPase α subunit phosphorylation.

Using phosphoamino acid analysis, we found that in HRTCs, C-peptide-induced phosphorylation of α subunits occurs on Thr and Ser residues. In the presence of PD98059, phosphorylation of Thr residues was abolished and Ser phosphorylation was decreased. Western blot of immunoprecipitated samples of phosphorylated α subunits with monoclonal antibodies against the phospho-Thr-Pro motif showed increased Thr phosphorylation in response to C-peptide, which was reduced to basal levels by the MEK1/2 inhibitor PD98059. According to a computer-based screening of potential phosphorylation sites of Na^+,K^+-ATPase α subunits, Thr81 in the α_1 subunit exhibits the highest probability for ERK phosphorylation, in comparison with all known ERK phosphorylation sites [35]. HRTCs are cells of proximal tubular origin and almost exclusively express the α_1 subunit of Na^+,K^+-ATPase [43]. Thus, our experimental data are in good agreement with the results from the computer data base screening. The results suggest that C-peptide stimulates Na^+,K^+-ATPase through phosphorylation of its α_1 subunits via an ERK1/2-dependent mechanism, and Thr residues are the main sites of ERK phosphorylation. We have recently shown that in human skeletal muscle cells, insulin stimulates Na^+,K^+-ATPase by phosphorylation of the pump α subunits on the Thr-Pro motif by ERK1/2 and translocation of the Na pump to the cell surface [35]. Collectively, these data indicate that phosphorylation of Na^+,K^+-ATPase α subunits after activation of ERK1/2 MAP kinases may be a universal mechanism of short-term upregulation of Na pump activity.

Regulation of Na pump activity may be achieved by changes in intrinsic activity of the enzyme and/or by changes in its membrane content due to translocation from intracellular compartments. Renal tubular cells are polarized, with ion transport directed from the apical to the basolateral side of the cells. Na^+,K^+-ATPase is mostly expressed in the BLMs [26]. In the initial cell surface biotinylation experiment, the Na^+,K^+-ATPase membrane content was unchanged (data not shown), a finding that most likely reflects experimental conditions, since the biotinylated cell surface label had very limited access to the BLM. Thus, the apical membrane proteins were labeled with higher efficiency. Activity of the Na pump on the BLM of kidney cells is regulated by endocytosis and

exocytosis [30–32, 44]. Thus, we took another approach and isolated BLMs and light endosomes of HRTCs, stimulated by 5 nM C-peptide in the presence and absence of PD98059 and scrambled C-peptide. Both α_1 and β_1 subunit contents were increased significantly upon stimulation of cells with C-peptide and, at the same time, decreased in the endosomal fraction, an effect that was abolished by PD98059. Scrambled C-peptide was without effect on subunit translocation. Thus, C-peptide activates the Na pump by its translocation from endosomes to BLMs in an ERK1/2-dependent manner.

In conclusion, C-peptide stimulates Na^+,K^+-ATPase activity in HRTCs via translocation of α_1 and β_1 subunits to the BLM from an endosomal compartment by a PKC and ERK1/2-dependent mechanism. This activation is linked to ERK1/2-dependent Na^+,K^+-ATPase α subunit phosphorylation on a Thr-Pro motif. Taken together, our findings suggest that ERK1/2 is essential for C-peptide-stimulated Na^+,K^+-ATPase activation. These data support the growing body of evidence for the involvement of ERK1/2 MAPK in Na^+,K^+-ATPase activation in different tissues.

Acknowledgements. We thank Dr. J. R. Zierath and Dr. A. Krook for helpful discussions and critical reading of the manuscript. We especially thank Dr. E. Féraille for the kind gift of NK1 antibody. This work was supported by grants from the Swedish Research Council, the Swedish Heart and Lung Foundation, the Novo-Nordisk Foundation, the Swedish Society of Medicine and Creative Peptides Sweden AB.

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