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Protein Kinase C recognition sites in the cytoplasmic domain of Endothelin Converting Enzyme-1c

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ABSTRACT

Endothelin Converting Enzyme-1 (ECE-1) is essential for the production of the potent vasoconstrictor Endothelin-1 (ET-1). The activation of Protein Kinase C (PKC) by phorbol 12-myristate 13-acetate (PMA) can increase ECE-1 phosphorylation, which in turn promotes ECE-1c trafficking to the cell surface where ET-1 production occurs. This study has identified the specific residues in the N-terminal cytoplasmic tail of ECE-1c isoform that are phosphorylated upon the activation of PKC. Levels of phosphorylation are expressed as a % phosphorylation in untreated CHO-K1 cells. We transfected CHO-K1 cells with wild type and mutant forms of ECE-1c (Ala^{4}-ECE-1c, Ala^{35}ECE-1c and $Ala^{4/35}$ECE-1c) to confirm the involvement of Thr^{4} and Ser^{35} residues in PMA induced phosphorylation of ECE-1c. Phosphorylation of wild type ECE-1c increased in response to PMA treatment (150 ± 13%, unpaired *t*-test, $P < 0.05$, significantly different compared to untreated control). The two single mutants and one combined mutant significantly reduced the PMA induced phosphorylation (103–117 ± 6–13%; unpaired *t*-test; $n = 8$; $P < 0.05$ significantly different compared to untreated control). The mutations had no effect on the basal ECE-1c phosphorylation. In addition, they had no effect on the catalytic activity as evidenced by the similar rate of substrate cleavage compared to wild type. This study is the first to confirm the residues phosphorylated upon the activation of PKC by PMA. The results complete a gap in our understanding of the mechanism(s) behind PKC induced trafficking of ECE-1.

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1. Introduction

Endothelin-1 (ET-1) is the one of the most potent vasoconstrictors known thus far [1]. It is produced by the cleavage of its precursor Big Endothelin-1 (BigET-1) which is a 38 amino acid peptide with no biological activity [2]. BigET is cleaved between Trp21 and Val22 by the highly specific metalloprotease known as Endothelin Converting Enzyme-1 (ECE-1) to produce the 21 amino acid bioactive peptide ET-1 [2]. Therefore the expression and localisation of ECE-1 is the rate limiting step in the production of ET-1. ECE-1 (and therefore ET-1) has been implicated in the pathogenesis of a range of other diseases including cancer [3], cardiovascular [4–6] and Alzheimer's disease [7–9].

ECE-1 is a type II integral membrane bound protein with a large extracellular C-terminal domain, a short N-terminal domain and a single transmembrane region. The catalytic activity is confined to the C-terminal domain. Four isoforms (ECE-1a, b, c and d) of ECE-1 have been cloned and all are encoded by a single gene but under the control of different promoters [10,11]. The specific localisation of ECE-1 can affect its access to the BigET-1 substrate and hence ET-1 production. The only differences amongst the isoforms exists in the intracellular N-terminal region and these differences are attributed to the differential sub cellular trafficking of the ECE-1 isoforms [12]. Therefore phosphorylation can indirectly regulate ECE-1 activity in terms of ET-1 production [13,14]. The sequence of ECE-1 contains several sites for possible post-translational modifications including as many as 10 glycosylation sites [15], and palmitoylation [16] at a conserved cysteine residue close to the transmembrane domain. Phosphorylation of ECE-1 is thus far the most widely studied post translational modification [12,14,17].

Phosphorylation of ECE-1 can be described as either constitutive or stimulated. Previous work has shown that ECE-1 is constitutively phosphorylated at residues Ser^{18} and Ser^{20} (ECE-1c numbering) by Protein Kinase C (PKC) [17]. These residues are absent in ECE-1a and as such this isoform is therefore not constitutively phosphorylated [17]. The stimulated phosphorylation of ECE-1 is mediated by PKC as shown in previous studies conducted

Abbreviations: PMA, phorbol 12-myristate 13-acetate; CHO cells, Chinese Hamster Ovary cells; ECE-1, Endothelin Converting Enzyme-1; BigET-1, Big Endothelin-1; ATCC, American Type Culture Collection.

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by us [18,19] and others [7,20,21] where phosphorylation of ECE-1 is increased following the activation of PKC by phorbhol esters such as phorbol 12-myristate 13-acetate (PMA). This results in the trafficking of ECE-1 to the cell surface where the catalytically active ectodomain can be shed. [22]. Despite the confirmation of the sites of constitutive phosphorylation [17], the sites of PKC mediated (stimulated) phosphorylation are yet to be confirmed. Cytoplasmic tails of ECE-1 isoforms are known to contain potential recognition sites for a number of kinases [14,19]. Although not constitutively phosphorylated, we have previously identified Tyr^4 and Ser^{35} (ECE-1c numbering) as potential sites for recognition by PKC.

The stimulated phosphorylation of ECE-1 is likely to be the mechanism by which ECE-1 expression and localisation is regulated by physiological/pathophysiological factors. This is supported by studies which show that a high glucose environment can increase the expression of the ECE-1c isoform, a process which is sensitive to inhibitors of PKC [20]. Furthermore, high glucose mediated PKC activation results in the trafficking of ECE-1c to the cell surface [20]. Since ECE-1c is considered as a dominant isoform expressed in endothelial cells and hence is a principal regulator of blood pressure [19]. We have used CHO-K1 cells transfected with wild type and mutated forms of ECE-1c, to confirm the potential sites of recognition by PKC in the N-terminal domain of ECE-1c.

2. Material and methods

Ripa buffer in ddH_2O (50 mM TrisHCl, 100 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.1% SDS, 0.5% deoxycholic acid sodium salt, 1% Triton X-100, 1 μg/ml aprotinin, 5 μg/ml leupeptin, 0.7 μg/ml pepstatin (SIGMA)).

Wash buffer 1 in ddH_2O (50 mM Tris–HCl, NaCl, 1% TritonX-100, 0.5% deoxycholic acid sodium salt, 1 μM PMSF, 1 μg/ml aprotinin, 5 μg/ml leupeptin, 0.7 μg/ml pepstatin (SIGMA)).

Wash buffer 2 in ddH_2O (50 mM Tris–HCl, 0.5 M NaCl, 0.1% TritonX-100, 0.05% deoxycholic acid sodium salt);

Wash buffer 3 in ddH_2O (50 mM Tris–HCl, 0.1% TritonX-100, 0.05% deoxycholic acid sodium salt).

2.1. Cell culture

CHO-K1, CHO-P, Cos-7, SWISS 3T3 and HEK 293 cells were obtained from American Type Culture Collection (ATCC). EA.hy926 (Endothelial cell line) were a generous gift from Dr. Edgell (Pathology Department, University of North Carolina, USA). The cells were maintained in media containing 50% α Mem, 40% foetal Calf Serum and 10% DMSO.

2.2. Transfections

For each well of a 12-well plate, 0.6 μg of plasmid DNA was diluted with 60 μl of OptiMem, whilst 4.8 μl of Lipofectamine was diluted with 60 μl of OptiMem. Both diluted solutions were mixed together and allowed to stand at room temperature for 20 min. After incubation, 480 μl OptiMem was added to DNA/Lipofectamine mixture, which was added to each well after washing with 2 ml of OptiMem. After 4–5 h incubation, cells were grown in complete media for a further 24–36 h.

2.3. Generation of a stable CHO-K1 cell line expressing ECE-1c

Cells were seeded into culture plates and transfected when 70% confluent. Transfection was conducted using lipofectamine transfection reagent and 1 μg of DNA (pcDNA3) encoding the ECE-1c sequence and previously linearised using the *Pvu* II restriction site. The next day, cells were trypsinized and split 10^4 fold into fresh culture plates containing the selective agent G_{418} (1 mg/ml, Gibco, USA). Media was changed twice a week until colonies of 100–200 cells were visible. Cells from these colonies were seeded onto 6 well plates and maintained in G_{418} containing α Mem for a further two weeks.

2.4. Generation of mutants

Single and double mutants of potential PKC phosphorylation sites were generated by substituting Thr^4 and/or Ser^{35} with alanine. The various mutations were introduced into the wild type ECE-1c expression vector using PCR-based site-directed mutagenesis (QuickChange, Stratagene). Oligonucleotides used for mutagenesis were (5′–3′):

– sense 1, GCCACCATGATGTCGGMCTACAAGCGGGCCACG;
– antisense 1, CGTGGCCC-GCTTGTAGKCCGACATCATGGTGGC;
– sense 2, CAGGTGAACTTCCAC-GMCCCGCGGAGTGGCCAGAGG;
– antisense 2, CCTCTGGCCACTCCGCG-GGKCGTGGAAGTTCACCTG.

Both set of primers contained wobble nucleotides (M = A or C, K = T or G) in order to produce either Ala or Asp. To help for screening of positive clones, the first site removed a *Sal*I site whereas a silent *Sac*II was generated by the second set of primers. The PCR product was *Dpn*I digested to remove parental DNA for 1 h at 37 °C. The nicked vector DNA incorporating the mutations was then transformed into DH5α *Escherichia coli*. Plasmid-bearing colonies (grown on Luria broth ampicillin plates) were screened for the relevant removed cut site or silent restriction site. Positive clones for each mutant were confirmed by sequence analysis (Micromon, Monash University, Australia).

CHO-K1 cells were then transfected and protein amounts quantified by Western analysis before the phosphorylation analysis of ECE-1c and its PKC site knockouts.

2.5. Phosphorylation studies

When transfected CHOK-1 cells were 70% confluent they were serum starved for 16 h. Following incubation with $[^{32}P]$ Pi (80 μCi/well) in phosphate free medium (400 μl/well) for 2 h, cells were stimulated by PMA (2 μM) for 10 min at 37 °C. After stimulation cells were washed on ice twice with cold HBSS (1 ml/well). Membranes were solubilised with 0.3 ml/well of Ripa buffer. The lysates were harvested, centrifuged (14000 g for 30 min) and the supernatant pre-cleared by the addition of Protein A-agarose (10 μl) and BSA (6%, 10 μl) at 4 °C for at least 1 h. Pre-cleared lysates were then incubated overnight (4 °C) with 20 μl of Protein A-agarose beads and 2 μl of serum containing ECE-1 antibodies. The beads were washed, twice in each of wash buffers 1 & 2 twice followed by once in wash buffer 3. The immuno-precipitates were finally resuspended in 55 μl of SDS sample buffer, heated at 100 °C for 5 min and resolved by 8% SDS–PAGE. Gels were fixed, dried and exposed overnight against a Fuji-type BAS-IIIs PhosphorImaging plate. The plates were subsequently read in a FUJIX Bio-imaging Analyser BAS 1000 and the data analysed using MacBAS version 1.0.

2.6. ECE-1 activity assays

ECE-1 activity was measured based on the ability of the CHO-K1 cell lysates to cleave $BigET_{18-34}$ (DIIWVNTPEHVVPYGLG, Auspep, Vic, Australia) a truncated version of the natural substrate. $BigET_{18-34}$ (5 μg; dissolved in 10% DMSO and 90% TBS) was incubated in the presence of cell lysate. The reaction was stopped at time = 0 and 3 h by mixing an aliquot of the reaction mixture with

four times the volume of 1% TFA in methanol. The samples were dried (Speed-Vac, Savant) and stored in −20 °C until analysed by HPLC and LC/MS for evidence of ECE-1 mediated substrate cleavage.

2.7. High performance liquid chromatography (HPLC)

Each sample, diluted to 250 µl in solvent A, was injected onto a compressed 8 × 10 cm C18 Nova-pack reversed phase column. Solvent A was 0.08% TFA and solvent B was 70% acetonitrile (ACN) and 0.08% TFA. Solvents were filtered (0.45 µm) and degassed under vacuum prior use. Separation was achieved using a linear gradient of 3–100% B over 30 min at a flow rate of 1 ml/min. Absorbance of emerging peaks was measured at 214 nm using a Waters HPLC system (510 pumps, gradient controller 680 injector U6K and a model 441 UV absorbance detector). The peptides corresponding to the emerging peaks were collected manually and identities determined by MALDI-ToF as described previously [22].

2.8. SDS–PAGE and Western blot analysis

Membrane proteins were resolved on a 8% SDS–PAGE under reducing conditions, and transferred onto a PVDF membrane. The transfer was conducted at a constant current of 400 mA for 1.5 h at 4 °C. Non specific binding was blocked by incubation with 5% skim milk at room temperature for 45 min. ECE-1c antiserum was raised in house (against ECE-1c Ile^{379}–Pro^{577}) using previously published methods [18]. Membranes were incubated with ECE-1 antiserum (1:5000 dilution) followed by appropriate secondary antibodies. Immunoreactive bands were detected using ECL chemiluminescence reagent.

2.9. Statistical analysis

For all comparisons, data were analysed by student's *t*-test. All data are presented as Mean ± SEM and a value of $P < 0.05$ was considered to be significantly different.

3. Results

3.1. Phosphorylation site knock out and ECE-1c expression in CHO-K1 cells

We have previously identified Tyr^4 and Ser^{35} (ECE-1c numbering) as potential PKC recognition sites in the N-terminal domain of ECE-1c [19]. Here we conducted site directed mutagenesis to determine their role in the stimulated phosphorylation of ECE-1c and its activity. The following three mutants were generated: single knock-out mutant Ala^4-ECE-1c and Ala^{35}-ECE-1c, and a double knock-out mutant Ala$^{4/35}$-ECE-1c. Western blot analysis of the CHO-K1 lysates indicated that these mutations had no effect on protein expression compared to wild type (Fig. 1). Furthermore, stimulation by PMA failed to have any significant effect on the expression of ECE-1c mutants compared to wild type.

3.2. Phosphorylation site knock out and ECE-1c phosphorylation by PKC in CHO-K1 cells

The single mutation Ala^4 and the combined mutation Ala$^{4/35}$ had no significant effect on the basal level of ECE-1c phosphorylation. However, the single mutation Ala^{35} increased the basal level of ECE-1c phosphorylation ($^{\dagger}P < 0.02$ significant compared to untreated wild type ECE-1c; $n = 8$; Fig. 2). Stimulation by PMA increased the phosphorylation of wild type ECE-1c as shown previously ($^{*}P < 0.007$ significantly different compared to no treatment; $n = 8$; Fig. 2). However PMA treatment failed to significantly increase the phosphorylation of both single mutants (Ala^4 and Ala^{35}) and the combined mutant Ala$^{4/35}$ ($^{**}P < 0.05$ significantly different compared to PMA treated wild type ECE-1c; unpaired *t*-test; $n = 8$; Fig. 2).

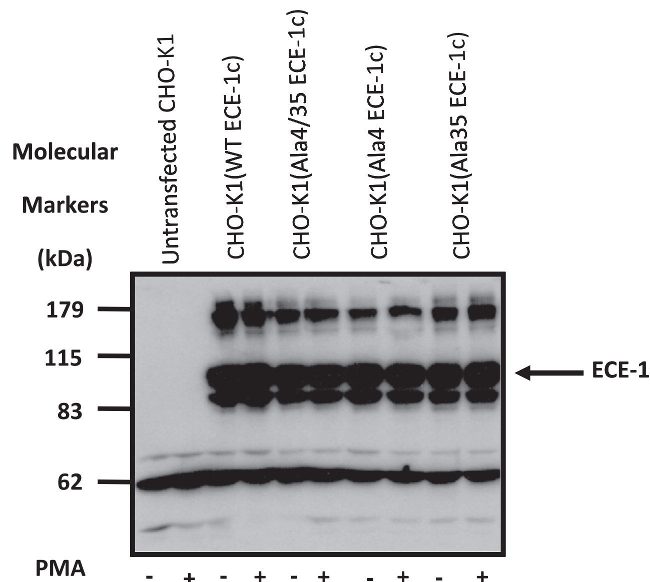

Fig. 1. The effect of mutations in the N-terminal cytoplasmic domain on ECE-1c expression. SDS–PAGE (8%) and Western blot analysis of CHO-K1 cell lysates transfected with either wild type (WT) or mutant forms of ECE-1. 48 h after transfection cells were treated with PMA for 10 min prior to lysis. 20 µl of lysate was analysed on a 8% SDS–PAGE gel. ECE-1c is indicated by an arrow.

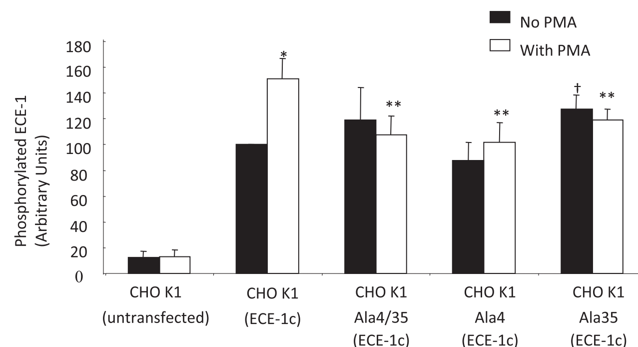

Fig. 2. Effect of mutating putative ECE-1c phosphorylation sites on PKC mediated phosphorylation of ECE-1c. Results are expressed as a percentage change compared to unstimulated control. *Significantly different compared to wild type with no treatment; **Significantly different compared to wild type with PMA treatment; † Significantly different compared to wild type with no PMA treatment; $n = 8$, unpaired *t*-test.

3.3. Phosphorylation site knock out and ECE-1c activity

Membrane preparations of CHO-K1 cells transfected with wild type ECE-1c or Ala$^{4/35}$ECE-1c were incubated with BigET$_{18-34}$. The analysis of the reaction mixtures at $t = 0$ min showed only the presence of the substrate BigET$_{18-34}$. The N and C-terminal products of ECE-1 mediated cleavage, DIIW (m/z 545) and VNTPEHVV-PYGLG (m/z 1380) respectively were identified by mass spectrometry in each reaction mixture at $t = 3$ h (Fig. 3). Peak area corresponding to BigET$_{18-34}$ reduced by 88% of initial after 3 h, when incubated with either wild type or Ala$^{4/35}$ECE-1c transfected lysates.

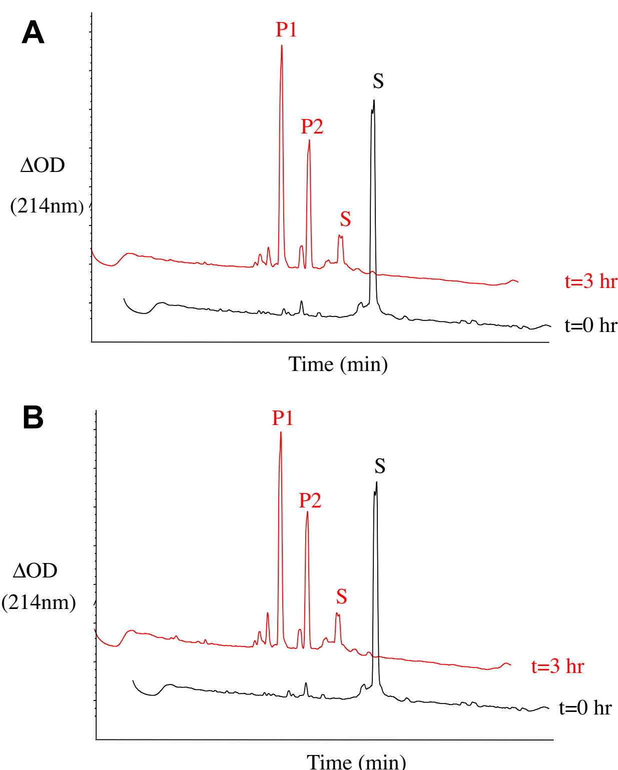

Fig. 3. Reversed-phase HPLC analysis of ECE substrate degradation. Cells were transfected with (A) wild-type ECE-1c or (B) double mutant ($Ala^{4/35}$) ECE-1c. Peak P1 represents the C-terminal product (VNTPEHVVPYGLG; 1380 Da) after cleavage by ECE-1c, peak P2 the N-terminal product (DIIW; 545 Da) and peak S represents the intact peptide (DIIW VNTPEHVVPYGLG; 1907 Da).

4. Discussion

Previous studies suggest that all four isoforms of human ECE-1 (1a, 1b, 1c and 1d) are endogenously expressed in primary human endothelial cells [23]. These isoforms only differ in their short N-terminal cytoplasmic domain, while the transmembrane and extracellular domains remain identical [10,24,25]. Site directed mutagenesis studies have shown that Ser^{18} and Ser^{20} (according to ECE-1c numbering) form conserved phosphorylation sites [17]. Ser^{18} and Ser^{20} are absent in ECE-1a which is therefore not constitutively phosphorylated [17]. Substitution of these residues with alanine creating both single and double knock out mutants resulted in a significant drop in the level of basal ECE-1c phosphorylation [17].

Previous studies have shown the activation of PKC by PMA can increase the total level of ECE-1 phosphorylation [19]. However the specific residues of ECE-1 that are phosphorylated upon the activation of PKC are unknown. In addition, the increased phosphorylation can trigger the trafficking of ECE-1 to the cell surface [18], where it can be shed to produce a soluble and catalytically active counterpart of the membrane bound form [14]. Phosphorylation of multiple residues is widely reported especially in the case of receptor activation. Several serine and tyrosine residues and one threonine residue at the C-terminus are principle phosphorylation sites in the bradykinin B_2 receptor [26,27]. ERK2 is activated by dual phosphorylation [28] and, NEP which is more closely related to ECE-1 was shown to be phosphorylated at both serine and threonine residues [29]. This study used site directed mutagenesis to identify the specific amino acid residues that are phosphorylated upon the activation of PKC by PMA. Thr^4 and Ser^{35} were identified as putative PKC phosphorylation sites within the N-terminal region of ECE-1c [19].

Table 1
Level of ECE-1c phosphorylation (expressed as a% of CHO-K1 without PMA treatment) in different cell types transfected with ECE-1c.

Cell type	−PMA	+PMA	% Change
CHO-K1	100	148	48
CHO P	74	115	41
HEK 296	25	51	26
Cos 7	66	82	16
SWISS 3T3	10	17	7

Studying the mechanism behind the PKC induced phosphorylation of ECE-1c requires a cell type that displays a significant difference between basal and PMA induced phosphorylation of ECE-1c. We therefore transfected several cell types with ECE-1c and the difference between basal and PMA induced phosphorylation was examined. The greatest increase in ECE-1c phosphorylation in response to PMA was observed in CHO-K1 cells (Table 1). These were chosen for further studies on PKC induced ECE-1c phosphorylation. In addition, endothelial cells express all four isoforms of ECE-1 making it difficult to identify which isoforms are effected by phosphorylation without isoform specific antisera.

To eliminate these putative phosphorylation sites (Thr^4 and Ser^{35}), a series of mutants were created by substituting these residues with alanine. All three mutants (Ala^4-ECE-1c, Ala^{35}-ECE-1c and $Ala^{4/35}$-ECE-1c) were characterised by Western blot analysis and were tested for enzyme activity. The mutations had no effect on ECE-1c expression levels compared to wild type, and the stimulation by PMA failed to alter the amount of protein expressed by the cells (Fig. 1). In addition, the enzyme activity was insensitive to the mutations targeting the N-terminus of ECE-1c. This was evidenced by the HPLC analysis of the incubation mixtures containing CHO-K1 lysates and $BigET_{18-34}$ at $t = 0$ and 24 h. The rate of substrate cleavage was identical when incubated with CHO-K1 cells transfected with either wild-type or mutant ECE-1c, and was evidenced by monitoring substrate peak areas. These data indicate that ECE-1c expression and activity are insensitive to mutations targeting the N-terminal cytoplasmic domain of ECE-1c.

Substitution of Thr^4 and Ser^{35} residues with Ala, significantly reduced the PMA induced increase in ECE-1c phosphorylation. This clearly highlights that these residues are phosphorylated upon the activation of PKC by PMA. Furthermore, it confirms previous studies indicating that only residues $Ser^{18/20}$ are constitutively phosphorylated by PKC.

Disease stimuli such as high glucose can increase trafficking of ECE-1c to the cell surface via activation of PKC [20]. The pH of the surrounding cellular environment and therefore the localisation of ECE-1 is a factor determining the specific substrate(s) optimally cleaved by ECE-1 [17]. Small peptide substrates such as bradykinin and substance P have a pH optimum of 5.6–5.8, and BigET has a pH optimum of 7 [13]. Cleavage of BigET is the most widely studied function of ECE-1 and thus PKC induced phosphorylation of ECE-1 can be regarded as a cellular signal which indirectly regulate its activity [14].

This is the first report to identify and confirm the specific amino acid residues in the N-terminal cytoplasmic domain of ECE-1c that are phosphorylated upon the activation of PKC. The results of this study therefore fill a gap in our understanding of the mechanisms behind the trafficking of ECE-1c to the cell surface following the activation of PKC. Furthermore, our results rule out the involvement of MAP kinase and CKII in the constitutive phosphorylation of ECE-1c. This study therefore adds new knowledge to the field of endothelial biology, which has the potential to be used in the therapeutic manipulation of ECE-1 trafficking and hence the production of the potent vasoconstrictor ET-1.

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