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Interaction with calmodulin is important for the secretion of thimet oligopeptidase following stimulation

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Abstract

Thimet oligopeptidase (EC 3.4.24.15; EP24.15) was originally described as a neuropeptide-metabolising enzyme that is highly expressed in the brain, kidneys and neuroendocrine tissue. EP24.15 lacks a typical signal-peptide sequence for entry into the secretory pathway and is secreted by cells via an unconventional and unknown mechanism. Here, we identify a novel calcium-dependent interaction between EP24.15 and calmodulin (CaM) that is important for the stimulated, but not constitutive, secretion of EP24.15. We demonstrate that in vitro, EP24.15 and CaM physically interact only in the presence of Ca²⁺ with an estimated K_d of 0.52 μM. Confocal microscopy confirmed that EP24.15 co-localises with CaM in the cytosol of resting HEK293 cells. This co-localisation markedly increases when cells are treated with either the calcium ionophore A23187 or the protein kinase A activator forskolin. Overexpression of CaM in HEK293 cells is sufficient to greatly increase the A23187-stimulated secretion of EP24.15, which can be inhibited by the CaM inhibitor calmidazolium. The specific inhibition of PKA with KT5720 reduced the A23187-stimulated secretion of EP24.15 and inhibited the synergistic effects of forskolin with A23187. Treatment with calmidazolium and KT5720 nearly abolished the stimulatory effects of A23187 on EP24.15 secretion. Together, these data suggest that the interaction between EP24.15 and calmodulin is regulated within cells and is important for the stimulated secretion of EP24.15 from HEK293 cells.

Keywords

calmodulin; protein kinase A; thimet oligopeptidase; 14-3-3ε; unconventional secretion; protein-protein interaction

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Introduction

Protein and neuropeptide secretion are crucial biological events that provide extracellular access to molecules involved in cell signalling. In the conventional rough endoplasmic reticulum-Golgi apparatus (ER-Golgi) secretory pathway, there is dynamic interplay between cell organelles and their constituents. The initial step of this pathway is the co-translational translocation of the protein into the lumen of the rough endoplasmic reticulum, and the pathway culminates with the exocytosis of secretory vesicles containing the molecules. The vast majority of secreted proteins contain a hydrophobic signal-peptide sequence that targets the protein to the secretory pathway [1,2]. However, several proteins that lack a signal-peptide sequence are also secreted, and this secretion is mediated through the so-called “alternative” or “unconventional” secretory mechanism [3]. The various stages involved in protein secretion by this latter mechanism remain to be identified, and multiple pathways for unconventional protein secretion may exist [3-5].

Several proteins that do not contain signal peptides are secreted without undergoing early entry into the ER-Golgi secretory pathway, including neurolysin [6,7], interleukin -1 β [8,9], HIV-tat, Leishmania hydrophilic acylated surface protein B [10-12], the chromatin-binding proteins HMGB1 and En2, galectin-1, galectin-3 [13,14], thioredoxin, basic fibroblast growth factor 1 and 2 [3], and the GRASP proteins [15]. Thimet oligopeptidase (EC3.4.24.15; EP24.15) also lacks a signal-peptide sequence for entry into the secretory pathway but has been shown to be secreted from neuroendocrine tissues [16-18] as well as from distinct cell lineages. However, little is known regarding the mechanism and major molecular components of the unconventional secretory pathway.

The secretion of EP24.15 from AtT20 cells, a mouse pituitary tumour cell line, can be stimulated by A23187 and corticotrophin-releasing hormone and blocked by brefeldin A and nocodazole. However, EP24.15 is not present in the secretory vesicles of AtT20 cells [5]. Subcellularly, EP24.15 has been shown to extensively co-localise with syntaxin-6, an integral trans-Golgi network protein, in the perinuclear region of AtT20 cells [19]. EP24.15 was also found to associate with small vesicular organelles distributed throughout the cell body, and some, but not all, of these organelles were also positive for adrenocorticotrophic hormone [19,20]. Moreover, ultrastructural experiments using electron microscopy have demonstrated that EP24.15 is strongly associated with the cytoplasmic face of the membranes of neurosecretory elements in the rat brain, including the endoplasmic reticulum, Golgi cisternae, tubulovesicular organelles, synaptic vesicles and endosomes [21]. Taken together, these data strongly suggest that EP24.15 secretion occurs by an unconventional pathway, but requires components of the classic secretory pathway [4,22].

Many specialised processes (e.g., cell signalling, synapse formation and maintenance, neurotransmitter and hormone release, axonal transport and nerve cell targeting) are tightly regulated by protein-protein interactions. The interaction of EP24.15 with the scaffold protein 14-3-3 ϵ was previously described to facilitate the secretion of EP24.15 from human embryonic kidney 293 (HEK293) cells stimulated with forskolin [4]. The interaction of EP24.15 and 14-3-3 ϵ dramatically increases when EP24.15 is phosphorylated on Ser⁶⁴⁴ by protein kinase A (PKA). Furthermore, EP24.15 secretion induced by the calcium ionophore A23187 can be stimulated *in vivo* by overexpressing 14-3-3 ϵ or by treating HEK293 cells with the PKA activator forskolin [4,23]. However, the molecular mechanisms involved in the alternative secretion of EP24.15 are not well characterised, and additional proteins that participate in unconventional secretory pathway used by EP24.15 remain to be discovered.

In the present study, we demonstrate that EP24.15 and calmodulin (CaM) interact both *in vitro* and *in vivo* and that this interaction is important for the unconventional secretion of

EP24.15 following stimulation. Our results suggest that in vitro, EP24.15 interacts with CaM only in the presence of calcium with an estimated K_d of 0.52 μM . The overexpression of CaM significantly increases the stimulated secretion of EP24.15 in HEK293 cells, and the co-localisation of CaM and EP24.15 increases when these cells are treated with either A23187 (1 or 10 μM) or forskolin (10 μM). These data suggest a novel interaction between CaM and EP24.15 that has physiological implications for the unconventional secretion of EP24.15 following stimulation.

Results

In a yeast two-hybrid screen to identify proteins that interacted with EP24.15, we found the CaM could interact with EP24.15 (data not shown). To confirm these results and further investigate the possible functional relevance of this interaction, we cloned the full-length cDNA encoding the rat CaM and expressed the CaM protein in *E. coli* using the pGEX-4T2 plasmid system (Fig. 1A). CaM was expressed as a fusion protein with GST (GST-CaM; Fig. 1A), purified, covalently immobilised onto a glutathione-sepharose column, as previously described, and incubated with EP24.15 (10 μg) either in the absence (Fig. 1B, lane 3) or in the presence of increasing Ca^{2+} concentrations (Fig. 1B, lanes 4–8). After extensive washing to remove non-specifically bound proteins, the CaM-associated protein complexes were eluted by boiling the resin in SDS sample buffer. A western blot of these complexes demonstrated that EP24.15 physically interacts with CaM (Fig. 1B). In vitro, even the lowest Ca^{2+} concentration tested (0.12 μM) was sufficient for the interaction between EP24.15 and CaM, although these proteins do not associate in the absence of calcium. Covalently immobilised GST alone was used as a negative control, and even in the presence of 1200 μM Ca^{2+} , EP24.15 did not interact with the control GST (Fig. 1B, lane 2).

The Ca^{2+} -dependent interaction between EP24.15 and CaM was also analysed using a surface plasmon resonance-based biosensor (Biacore T100; GE Health Care), which allows for the real-time detection and monitoring of molecular binding events [24,25]. A sensogram from a representative experiment is shown in Fig. 2. CaM was covalently immobilised on a CM5 chip, and association and dissociation slopes were obtained with the addition of an increasing concentration of EP24.15. The association slopes increase proportionally to the EP24.15 concentration from 0.1–5.0 μM . The association between EP24.15 and CaM occurs rapidly (within seconds), while the dissociation occurs more slowly (Fig. 2). Under these experimental conditions, the interaction between EP24.15 and CaM was estimated to have a K_d of 0.52 μM .

To investigate the possible in vivo interaction between EP24.15 and CaM, double-labelling immunocytochemical experiments using confocal microscopy were conducted in HEK293 cells (Fig. 3). HEK293 cells were transiently transfected with a modified pCMV plasmid that was empty (negative control) or encoded EP24.15, CaM and/or 14-3-3 ϵ . The overexpression of EP24.15, CaM or 14-3-3 ϵ in these cells was confirmed by western blot (data not shown). EP24.15 and CaM were distributed throughout the cells, as analysed by confocal microscopy (Fig. 3). However, the discrete co-localisation of these two proteins could be observed after superimposing the individual localisation patterns (Fig. 3M and 3O). Because we had previously shown that 14-3-3 ϵ affects the unconventional secretion of EP24.15 [4], we investigated the effect of the overexpression of 14-3-3 ϵ with both EP24.15 and CaM on the localisation patterns. Following the overexpression of 14-3-3 ϵ , the co-localization of EP24.15 and CaM was more pronounced (Fig. 3Q). Treatment with the protein kinase A (PKA) activator forskolin, caused an incremental change in the cellular co-localisation of EP24.15 and CaM (Fig. 3N and 3P), which is more evident in cells overexpressing EP24.15, CaM and 14-3-3 ϵ (Fig. 3R). The Control experiments in which the primary antisera were omitted or pre-absorbed showed no specific staining (data not shown).

Therefore, these findings suggest that the EP24.15 and CaM interaction can be regulated in vivo by PKA activation, particularly in cells that also express 14-3-3 ϵ .

CaM is involved in many cellular functions, including cell signalling and exocytosis [26]. Therefore, we were interested in the importance of CaM for the secretion of EP24.15 in HEK293 cells. We found that the EP24.15 activity and protein expression were not altered in cells overexpressing CaM or 14-3-3 ϵ (data not shown). Moreover, the constitutive secretion of EP24.15 was not affected in HEK293 cells overexpressing i) CaM, ii) EP24.15, iii) CaM and EP24.15, iv) CaM and 14-3-3 or v) CaM, EP24.15 and 14-3-3 (data not shown). In contrast, CaM overexpression alone enhanced the A23187-stimulated secretion of EP24.15 from HEK293 cells (Fig. 4A). Co-treatment of these cells with both A23187 and the PKA activator forskolin produced a synergistic effect on EP24.15 secretion (Fig. 4A). As expected, EP24.15 overexpression in HEK293 cells was sufficient to cause a proportional increase of in EP24.15 secretion upon A23187 stimulation, which was also potentiated by forskolin (Fig. 4A). However, the overexpression of both CaM and EP24.15 in these cells produced no additional increase in the secretion of EP24.15 following stimulation (Fig. 4), suggesting that the stimulated secretion of EP24.15 could be regulated by additional proteins. As mentioned above, 14-3-3 ϵ has previously been shown to facilitate the secretion of EP24.15 [4]. Indeed, A23187-stimulated EP24.15 secretion was higher in HEK293 cells overexpressing CaM, EP24.15 and 14-3-3 (Fig. 4). However, forskolin showed no further synergistic effects on the A23187-stimulated secretion of EP24.15, suggesting additional limiting steps in this unconventional secretory pathway. The synergistic effects of forskolin and A23187 on EP24.15 secretion were completely blocked by the specific PKA inhibitor KT5720, while the stimulatory effect of A23187 was only partially inhibited by this compound (Fig. 4A).

The specific CaM inhibitor calmidazolium partially blocked the A23187-stimulated secretion of EP24.15 (Fig. 4B). Interestingly, treatment with both KT5720 and calmidazolium essentially abolished the secretion of EP24.15 following stimulation (Fig. 4B). Importantly, the cell viability was greater than 99% by both Trypan blue exclusion and MTT in all experiments, indicating that the EP24.15 secretion measured in the above experiments was not due to non-specific cell leakage (data not shown). Taken together, these data further suggest that the stimulated secretion of EP24.15 is a cellular event regulated in part by the CaM pathway.

Next, we used a confocal microscopy to measure the increase in the intracellular cytosolic calcium concentration ($[Ca^{2+}]_i$) in HEK293 cells treated with A23187 (Fig. 5A and 5B). Although this technique has limitations (the maximum detectable calcium concentration is below the maximum level that can be reached within the cell), we observed an increase in the $[Ca^{2+}]_i$ proportional to the A23187 concentration (Fig. 5A and 5B). In parallel, the secretion of EP24.15 following stimulation was measured at distinct A23187 concentrations (Fig. 5C). These data indicate that HEK293 cells secrete EP24.15 only when the $[Ca^{2+}]_i$ is above 4 μ M (Fig. 5C). In addition, we performed double-labelling immunocytochemical experiments for confocal microscopy to analyse the effect of the increased $[Ca^{2+}]_i$ on the co-localisation of EP24.15 and CaM (Fig. 6). The HEK293 cells were transiently transfected with the modified pCMV plasmid vector expressing EP24.15, CaM and/or 14-3-3 ϵ (or an empty control) and were treated with various concentrations of A23187. Interestingly, the intracellular co-localisation of EP24.15 and CaM increased when the cells were treated with 1 μ M A23187 (Fig. 6O and 6Q), conditions that were not sufficient to induce EP24.15 secretion (Fig. 5C). A large increase in EP24.15 and CaM co-localisation occurred in cells overexpressing these two proteins that were treated with 10 μ M of A23187 (Fig. 6P), with or without the additional overexpression of 14-3-3 ϵ (Fig. 6R). Therefore, the co-localisation of

EP24.15 and CaM seems to be controlled by intracellular calcium concentrations that are not sufficient to induce EP24.15 secretion.

Additionally, we have investigated putative CaM binding sites [27] on EP24.15. The EP24.15 sequence was subjected to the CaM target database algorithm, and only the highest propensity for interaction with CaM was considered. As seen in Fig. 7A, there were two regions of the highest predicted binding to CaM, amino acids 141-152 and 261-271 separated by approximately 110 residues. When these two distinctly separated regions (by primary sequence) were depicted on the X-Ray crystallographic structure of EP24.15, they comprised adjacent alpha-helices on the surface of the enzyme quite distant from the active site of the enzyme (Fig. 7B). The catalytic zinc, depicting the core of the active site, is rather distant from the CaM binding region (~26Å from the centroid comprising amino acids 261-271 and ~30Å from the centroid composed of amino acids 141-152; Fig. 7).

Discussion

Here we have characterised a novel functional interaction between thimet oligopeptidase (EC3.4.24.15; EP24.15) and calmodulin (CaM), which enhances the secretion of EP24.15 following stimulation. Therefore, the interaction with CaM is likely to be of physiological significance, contributing to the release of EP24.15 into the extracellular environment where this enzyme functions in the metabolism of pathophysiologically significant neuropeptides [reviewed in [16,17,28-35].

EP24.15 does not contain a signal peptide for regular entry in the secretory pathway or a hydrophobic sequence to directly anchor it to the cell membrane [36]. However, previous reports have suggested that the secretion of EP24.15 uses the rough endoplasmic reticulum-Golgi apparatus (ER-Golgi) pathway, as the stimulated secretion of EP24.15 is inhibited by both brefeldin A and nocodazole [5]. Consistent with this hypothesis, immuno-electron microscopy demonstrated that EP24.15 is associated with the cytosolic face of the ER, Golgi and plasma membrane [24]. EP24.15 has also been shown to be present in lipid rafts, dynamically arranged membrane regions known to facilitate protein-lipid and protein-protein interactions [37], in cultured neurons [38,39], and these lipid rafts could contribute to EP24.15 secretion. In a previous study, we characterised the interaction between EP24.15 and the scaffold protein 14-3-3 ϵ , the regulation of this interaction by protein kinase A (PKA) and the importance of this interaction for the secretion of EP24.15 following stimulation [4]. A high-throughput yeast two-hybrid screen performed in our laboratory suggested that EP24.15 might also interact with CaM. Here, we have further investigated this potential interaction between EP24.15 and CaM. The *in vitro* physical interaction between EP24.15 and CaM occurred only in the presence of calcium, suggesting that this binding might be dynamically regulated. We observed this interaction in real time using surface plasmon resonance and estimated the K_d of this interaction to be 0.52 μ M. The interaction between EP24.15 and CaM is characterised by a fast association and a slow dissociation, which could be physiologically relevant for cell signalling that involves an intracellular calcium increase. The co-localisation of EP24.15 and CaM was also observed in the cytoplasm of HEK293 cells, particularly in cells overexpressing these two proteins and 14-3-3 ϵ . While the co-localisation between EP24.15/CaM is less evident in cells not overexpressing EP24.15 and CaM, we believe that this is related to the relatively low expression level of these proteins in HEK293 cells. However, the estimated K_d for the interaction between EP24.15 and CaM is consistent with the hypothesis that these molecules interact physiologically. Furthermore, the intracellular co-localisation of EP24.15 and CaM is strongly induced following treatment of HEK293 cells with A23187, and to a lesser extent with forskolin, further suggesting the functional significance of this association.

Interestingly, dynamic calcium signalling in HEK293 cells is, in part, mediated by the ability of calcium to regulate other second messengers, including cyclic AMP (cAMP) [40]. This suggests that an increase in the intracellular calcium concentration will also activate adenylate cyclase, increasing the cAMP level and activating PKA [41]. PKA has been implicated in many cellular processes, including exocytosis [42], modulation of other protein kinases, regulation of the intracellular calcium concentration and the regulation of transcription [43]. Furthermore, EP24.15 has been shown to be phosphorylated by PKA on Ser⁶⁶⁴, which in turn regulates its interaction with the scaffold protein 14-3-3 ϵ and facilitates the secretion of EP24.15 [4,23]. CaM has also been previously shown to efficiently interact with 14-3-3 ϵ in the presence of calcium [44]. Our experiments have shown that the co-localisation of EP24.15 and CaM and the A23187-stimulated secretion of EP24.15 is greater in HEK293 cells overexpressing 14-3-3 ϵ together with EP24.15 and CaM. The overexpression of CaM in HEK293 cells resulted in a significant increase in the A23187-stimulated secretion of EP24.15, but had no effect on its constitutive secretion. The addition of forskolin had a synergistic effect on the A23187-stimulated secretion of EP24.15 that was completely abolished by the specific PKA inhibitor KT5720, and KT5720 alone partially blocked the stimulatory effects of A23187 on EP24.15 secretion. The inhibition of CaM by calmidazolium significantly decreased the A23187-stimulated EP24.15 secretion. Furthermore, the inhibition of both CaM and PKA practically abolished the secretion of EP24.15 following A23187 treatment. These data indicate that PKA and CaM act synergistically to promote the secretion of EP24.15 following stimulation. One possible interpretation for the above results is that PKA activation directly and indirectly (through 14-3-3 ϵ) promotes the binding of EP24.15 and CaM to positively regulate the unconventional secretion of the enzyme.

CaM is a small, acidic protein of approximately 148 amino acids (16.7 kDa) that exists in at least two different configurations: i) apocalmodulin, which lacks calcium, and ii) calcium-CaM, which can bind up to four calcium ions and undergo post-translational modifications such as phosphorylation, acetylation, methylation and proteolytic cleavage that modulate its action [45]. Upon binding calcium, CaM undergoes large and dramatic conformational changes. Though the crystal structure of calcium-loaded CaM exhibits a bilobed shape with a bridging alpha helix, when calcium is bound, the protein is rather flexible [27,46,47] and can increase its affinity for potential target proteins. Thus, CaM can form a compact globular conformation by altering the bending of the central helix to straddle the target helix. The predictive algorithm for CaM target binding is based solely on properties of individual amino acids in the primary sequence, and is coupled with helix propensity and hydrophobicity [48]. This scheme fits very well in the biological context of the sequences depicted as the strongest binding region for EP24.15 (Fig. 7). Though distinct with respect to the linear sequence, two regions separated by more than 100 amino acids, these regions in the atomic resolution structure of the enzyme reside next to each other on the surface of the protein and relatively far from the interior active site of the enzyme to provide a modulatory platform for potential regulation of EP24.15.

Moreover, CaM is a ubiquitously expressed protein that can bind to and regulate a multitude of different protein targets and affect many different cellular functions, including vesicle recycling and exocytosis [26]. CaM mediates processes such as inflammation, metabolism, apoptosis, muscle contraction, intracellular movement, short-term and long-term memory, nerve growth and immune response [49]. Calcium-CaM is a positive regulator of exocytosis in neuroendocrine cells [50,51], where it activates the opening of fusion pores at the plasma membrane [52-54] and the fusion of secretion granules [55].

Interestingly, we found that even under conditions in which EP24.15 is not secreted, EP24.15 and CaM co-localise in the cell (Fig. 6). This co-localisation may have additional

physiological significance such as regulating the spatial localisation of EP24.15 within the cell. One exciting possibility is that a small increase in the intracellular calcium concentration could contribute to the recruitment of EP24.15 to active signal transduction locations, thereby regulating the metabolism of intracellular peptides. However, upon larger increases in the intracellular calcium concentration, the interaction with CaM contributes to the increase in the secretion of EP24.15, which would affect both intracellular and extracellular peptide metabolism. Recent studies have identified a number of intracellular peptides that are likely endogenous substrates and products of EP24.15 [56]. Besides, the overexpression of EP24.15 alters both angiotensin II and isoproterenol signal transduction in HEK293 cells, suggesting a physiological function for the intracellular substrates/products of EP24.15 [57]. This suggests that the regulation of the intracellular activity of EP24.15 could be an important step in control on signal transduction pathways.

In conclusion, our results suggest that the interaction with CaM may play an important role in the secretion of EP24.15 following stimulation. The interaction between EP24.15 and CaM was shown to be regulated both by the intracellular calcium concentration and PKA activation, which are frequently observed during signal transduction. We suggest that CaM might facilitate EP24.15 secretion by spatially positioning the enzyme closer to specific regions of the plasma membrane to allow its secretion upon further elevation of intracellular calcium concentration.

Experimental Procedures

Reagents

Bovine serum albumin (BSA), forskolin, KT5720, calmidazolium, A23187 and protease inhibitors were purchased from Sigma-Aldrich (St. Louis, MO, USA). Salts, β -mercaptoethanol and Triton X-100 were purchased from AMRESCO (Solon, OH, USA) and/or Sigma-Aldrich. The quenched-fluorescence substrate (QFS) and JA2 inhibitor were generous gifts from Dr. A. Ian Smith (Baker Medical Research Institute, Australia).

Full-length CaM cDNA cloning

Total RNA was isolated from rat brain using Trizol according to the manufacturer's instructions (Invitrogen). Subsequently, 3 μ g of total RNA was reverse-transcribed using 200 U of Superscript II reverse transcriptase (Invitrogen) and 10 μ M of a CaM-specific primer (5' CAGCGGCCGCATTTTGCAGTCATCATCTG 3') in a 20 μ l reaction mixture, and following reverse transcription, the RNA was digested with RNase H (Invitrogen). The amplification of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a control to verify the RNA integrity. The CaM-specific cDNA was further amplified by PCR with 0.2 μ M of each primer (sense: 5'-CACTCGAGATGGCTGATCAGCTGACTGAAG-3', anti-sense: 5'-CAGCGGCCGCATTTTGCAGTCATCATCTG-3'), 50 mM $MgCl_2$, 1 $\times$ buffer NH_4^+ , 10 mM of each dNTP, 2 U of Taq polymerase (Biolase) and 100 ng of the template cDNA. The primers were designed based on the gene sequence deposited in GenBank (AF178845.1). The sense primer contained an XhoI site and the anti-sense primer a NotI site (underlined). DNA sequencing was performed as described previously [4] using a multicapillary MegaBACE 1000 DNA sequencer (Amersham Biosciences; Piscataway, NJ, USA), according to protocols supplied with the DYEnamic ET Dye Terminator Cycle Sequencing Kit (Amersham Biosciences).

Cell culture conditions and transient transfections

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μ g/ml) (Invitrogen)

and incubated at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air. The full-length cDNAs encoding rat testis EP24.15, rat brain CaM or 14.3.3 epsilon were subcloned into the pCMV vector (Invitrogen) in frame with the c-myc epitope to generate C terminal fusion proteins. The correct in-frame orientation of all constructs was verified by DNA sequencing (data not shown). The plasmid DNA was purified using a Wizard Plus Kit (Promega, Madison, USA). HEK293 cells were transiently transfected with pCMV plasmids encoding CaM, EP24.15 or 14-3-3 using a Hekfectin Kit (Bio-Rad) according to the manufacturer's protocols. Over 90% of the cells were confirmed to be positively transfected for all constructs (data not shown). HEK293 control cells were similarly transfected with the empty pCMVmyc (mock transfected cells) or pCMV plasmid (mock vector). Protein expression was analysed by western blot as described below, using mock transfected cells as a control.

Western blot

Western blots for EP24.15 were conducted after gel electrophoresis in an 8% SDS polyacrylamide gel and protein transfer to a nitrocellulose membrane at 30 V at 4°C for 18 h. The efficiency of transfer was assessed by staining the nitrocellulose membrane with Ponceau Red, and the membrane was blocked in a buffer containing 25 mM Tris-HCl, pH 7.4, and 125 mM NaCl (TBS) containing 0.1% Tween 20 (TTBS), 5% non-fat dry milk and 1% BSA for 2 h at 21°C. The membrane was incubated with a rabbit anti-EP24.15 primary antibody (1:3000; Proteimax Biotechnology, Cotia, SP, Brazil) for 2 h in 5% fat-free dry-milk diluted in TTBS. After extensive rinsing with TTBS, the membrane was incubated with a peroxidase-labelled anti-rabbit secondary antibody (1:3000 in TTBS; Amersham) for 1 h. Following extensive rinsing with TTBS, the blot was developed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to the manufacturer instructions. The western blot experiments were conducted at least three times, and all experiments produced similar results, confirming the overexpression of EP24.15 after the transient transfection of HEK293 cells with the corresponding pCMV plasmid vector (data not shown). The western blots for CaM or 14-3-3ε were conducted after gel electrophoresis in a 15% SDS-polyacrylamide gel and protein transfer to a nitrocellulose membrane at 30 V at 4°C for 18 h (data not shown). The membranes were fixed with 0.2% glutaraldehyde in potassium phosphate buffer (25 mM KH₂PO₄ + 25 mM K₂HPO₄), washed three times (5 min) in potassium phosphate buffer, incubated in 1 M lysine in 0.1 M NaHCO₃ to re-expose the binding sites and washed three times (5 min) in potassium phosphate buffer. The membranes were blocked in 5% BSA in PBS (pH 7.5) for 4 h at 21°C and incubated with either mouse anti-CaM (Sigma, 1:1000) or mouse anti-14-3-3 (1:500; Santa Cruz) diluted in 1% BSA in PBS for 18 h at 4°C. After four washes (10 min) in wash buffer (0.05% Tween-20 in PBS), the membranes were incubated with a biotinylated anti-mouse secondary antibody (Vector Labs; 1:1000) diluted in wash buffer for 50 min at 21°C, washed four times in wash buffer and incubated with the ABC Reagents (Vector Labs; 50 µL of A reagent and 50 µL of B reagent in 0.1% BSA in PBS) for 40 min. Following an extensive rinsing with wash buffer, the protein bands were developed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to the manufacturer instructions. These experiments were conducted at least three times and all trials produced similar results, confirming the overexpression of both CaM and 14-3-3ε after the transient transfection of HEK293 cells with the respective pCMV plasmid vectors (data not shown).

Confocal microscopy

HEK293 cells were grown on poly-L-lysine (Sigma)-coated coverslips. For some experiments, the cells were stimulated with A23187 (1 or 10 µM) or forskolin (10 µM) for 20 min before immunocytochemistry. After gentle removal of the culture medium, the cells were rinsed once in 0.1 M phosphate buffer (PB) and fixed for 15 min in 4%

paraformaldehyde in PB. After two washes with PB and blocking for 30 min in PB containing 10% normal goat serum, 5% BSA and 0.1% Triton X-100, the cells were incubated at 21°C for 2 h with rabbit anti-EP24.15 (1:4000) and/or mouse anti-CaM (1:100; Sigma-Aldrich) antibodies diluted in PB containing 3% normal goat serum and 0.1% Triton X-100. After three washes with PB, the cells were incubated for 1 h at 21°C with Cy3-conjugated anti-rabbit antibody (1:700; Sigma-Aldrich) and/or an Alexa 488-conjugated anti-mouse antibody (1:700; Sigma-Aldrich) diluted in PB containing 0.1% Triton X-100. Following extensive washing in PB, the coverslips were mounted on glass slides and examined under a Zeiss laser confocal microscope (CLSM 410) equipped with an Axiovert 100 inverted microscope and an argon/krypton laser. Cy3-tagged antibodies were excited at a wavelength of 568 nm, while Alexa 488-labeled molecules were excited at a wavelength of 488 nm. Images were acquired sequentially as single, transcellular optical sections and processed using the Carl Zeiss CLSM software (version 3.1). Final composites were prepared using Adobe Photoshop without modifying the spectral characteristics of the original signal. Control experiments were done using equivalent dilutions of preimmune sera or with immune sera that had been preabsorbed overnight at 4°C with 200 µg/mL or 500 µg/mL of recombinant EP24.15 or CaM, respectively. Specific cell labelling was not observed in any of the control experiments (data not shown).

EP24.15 secretion assay

Both the basal and stimulated secretion of EP24.15 was determined in HEK293 cells by incubating the cells in DMEM containing 0.1% dialysed bovine serum albumin (BSA). Stimulated secretion was induced for 40 min using the calcium ionophore A23187 (10 µM), either in the presence or absence of the PKA activator forskolin (10 µM) that was added to the cells 20 min before the addition of A23187. The PKA inhibitor KT5720 (0.5 µM) or the CaM inhibitor calmidazolium (3 µM) was added to the cells 20 min before the addition of A23187. The secretion of EP24.15 was linear for the first 40 min of stimulation with A23187 (data not shown). After 40 min of stimulation with A23187, the medium was collected, centrifuged for 1 min at 14,000×g to remove cells and debris and immediately used to quantify the extracellular EP24.15 enzymatic activity using the QFS substrate, as described below. Cells that remained attached to the culture dishes were removed, analysed for viability (described below), counted and disrupted by three freeze/thaw cycles. After centrifugation for 10 min at 830×g at 4°C, the supernatant was used to determine the intracellular EP24.15 enzymatic activity. All of the controls and drug treatments were done in triplicate in at least five independent experiments that produced similar results. The data presented are representative of one set of experiments done in triplicate.

EP24.15 enzymatic assay

The enzymatic activity of EP24.15 was determined in triplicate using a continuous assay with a quenched fluorescent substrate (QFS; 7-methoxycoumarin-4-acetyl-P-L-G-P-dK-(2,4-dinitrophenyl), as previously described [58]. To discern peptidolytic activity attributable exclusively to EP24.15, the specific inhibitor N-[1-(R, S)-carboxy-3-phenylpropyl]-Ala-Aib-Tyr-p-aminobenzoate (JA2; 1 µM), was used [59]. Enzymatic determinations were evaluated under linear conditions in which the product formation was directly proportional to the enzyme concentration and obeyed first-order kinetics with less than 10% of the substrate being consumed during the assay. The enzyme assays were done in a final volume of 100 µl containing EP24.15 derived from the cell extracts/media, 10 µM QFS, 1 mM β-mercaptoethanol and TBS (0.025 M Tris HCl, pH 7.4, 0.125 M NaCl).

Protein expression

For binding assays, recombinant EP24.15 and CaM proteins were expressed in *E. coli* (XL1-blue; Stratagene) as glutathione-S-transferase (GST) fusion proteins using the expression

vector pGEX-4T2 (GE Healthcare). The initial protein purification was performed by affinity chromatography using a glutathione-sepharose column (GE Healthcare) with the respective proteins released from the GST-fusion by cleavage with thrombin (100 U; Amersham Biosciences). The intact GST-fusion proteins were eluted from the column with glycine (0.2 M, pH 2.2). The protein was concentrated and further purified by ultrafiltration (Millipore, Bedford, MA, USA) with molecular exclusion limits of <30 kDa and >5 kDa (CaM), >30 kDa and <50 kDa (CaM fused to GST) or >50 kDa (EP24.15), as previously described [60]. The protein concentration was determined with the Bradford assay [61] using BSA as a standard, and protein purity was analysed by Coomassie brilliant blue staining after electrophoresis in a 12% SDS-polyacrylamide gel. All proteins were stored at -80°C until analysed.

In vitro protein-protein binding assays

The CaM-GST fusion protein or GST alone (5 µg) was covalently immobilised on a glutathione-sepharose column (GE HealthCare), incubated with 5 mM of EGTA for 30 min, washed and incubated in buffer containing 150 mM NaCl, 10 mM Tris pH 8.0, 0.3% Triton X-100, 0.1% BSA, 1 mM β-mercaptoethanol and either 0, 0.12, 1.2, 12, 120 or 1200 µM Ca²⁺ for 1 h. Recombinant purified EP24.15 (10 µg) was then incubated with the immobilised GST (control) or GST-CaM resin for 18 h at 4°C. To minimise nonspecific protein association, the resins were extensively washed with the incubation buffer described above. The specifically associated proteins were eluted by boiling the resin in SDS-PAGE sample buffer (100 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol). The association of EP24.15 and CaM was analysed by western blot using the anti-EP24.15 antibody, as described above.

Cell viability

HEK293 cells were rinsed with phosphate-buffered saline (PBS) and treated with 0.4% Trypan blue in PBS for 90 s. Excess dye was removed and the cells were washed twice with PBS before counting. For a positive control (100% permeabilized cells), cells were treated with 50% ethanol for 1 min and then stained as described above. In all of the experiments cellular viability was greater than 98% (data not shown). MTT tests were conducted by adding a 10% vol:vol of a MTT solution (5 mg/mL diluted in PBS) to serum-free DMEM containing 0.1% dialysed bovine serum albumin (420 µL) and incubating the cells at 37°C for 90 min. Subsequently, this medium was discarded and 420 µL isopropyl alcohol/0.04 M HCl was added to each well of a 12-well plate to yield a purple solution. The optical density was measured at 570 nm using a SpectraMax M2 (Molecular Devices, Sunnyvale, CA, USA) [62]. Data were analysed with the GraphPad Prism software package (GraphPad Software, San Diego, CA, USA). Three experiments were done ($n=3$) for each treatment and transfection type.

Intracellular calcium concentration

The measurements of the [Ca²⁺]_i variation in HEK-293T were performed using a LSM 510 confocal microscope (Zeiss, Jena, Germany). One million cells were seeded and allowed to attach to culture dishes (TPP, Germany) 24 h before the measurements. The cells were loaded with 5 µM Fluo-3AM (Invitrogen) for 60 min at 37°C in cell culture medium, pH 7.2 in 0.5% Mg₂SO₄ and 0.1% pluronic acid F-127 and washed three times with culture medium. The fluorophore was excited using a 488 nm argon ion laser, and the emitted light was detected at 515–530 nm using a band-pass filter. Calcium images were collected every second. After recording the basal [Ca²⁺]_i, increasing concentrations of A23187 were added to stimulate calcium responses. At the end of each experiment, 10 µM A23187 was added followed by 50 mM EGTA to establish the maximum (F_{max}) and minimum (F_{min})

fluorescence values. The change in the $[Ca^{2+}]_i$ value was calculated using the following formula: $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$, assuming a 450 nM K_d for Fluo-3AM [63].

Surface plasmon resonance

The surface plasmon resonance analyses were performed using a BIAcore T100 (GE Healthcare). Pure CaM (1000 RU) diluted in phosphate buffer was immobilised on a CM5 chip by amine links in the gold nanoparticles [64] according to the manufacturer's protocols. A constant flow of 10 μ L/min of EP24.15 (0.1, 0.25, 0.5, 1 or 5 μ M) diluted in HBS-EP buffer (10 mM HEPES [pH 7.4], 150 mM NaCl, 3.4 mM EDTA and 0.005% P20 surfactant) containing 5 mM Ca^{2+} or 5 mM EGTA for 3 min was run across the chip. EP24.15 was dissociated in the same buffer for 10 min. The surface was regenerated in 50 mM NaOH for 30 s.

Modeling sites of interaction of calmodulin and EP24.15

To assess where topologically CaM would interact with the EP24.15 target enzyme, we utilized the predictive algorithm for predicting CaM target sites [48]. Utilizing the database of proteins demonstrated to bind to CaM, the algorithm predicts the propensity to form a helix and hydrophobic moment. The value ranges from 0 (no CaM binding) to 9 (very high probability of CaM binding). The results were displayed on the X-ray crystallographic derived structure of EP24.15 as contained in the Protein Databank of the Research Collaboratory for Structural Bioinformatics. PDB ID# 1s4b was used. The view was constructed and rendered with Pymol (Delano Scientific).

Statistical analyses

Results are expressed as the mean $\pm$ SEM as appropriate. Statistical comparisons were performed using ANOVA followed by a Tukey test. A p value less than 0.05 indicated significance.

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Abbreviations

AFU	arbitrary fluorescence units
14-3-3ε	14-3-3 epsilon

BSA	bovine serum albumin
CaM	calmodulin
CMZ	calmidazolium
ER-Golgi	rough endoplasmic reticulum-Golgi apparatus
DMEM	Dulbecco's modified Eagle's medium
EGTA	ethylene glycol tetraacetic acid
EP24.15	thimet oligopeptidase (EC 3.4.24.15)
GST	glutathione-S-transferase
HEK293	human embryonic kidney 293 cells
PBS	phosphate-buffered saline
TBS	Tris-buffered saline
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Figure 1. Recombinant protein expression and *in vitro* protein-protein interaction assays
Panel A: SDS-polyacrylamide gel (12%) stained with Coomassie Brilliant Blue demonstrating the purity of the proteins expressed in *E. coli*. The proteins were purified on a glutathione-sepharose column, and free proteins and GST-fusion proteins were eluted from the column using thrombin or glycine, respectively. The proteins were further purified using size-exclusion centrifugation. **Panel B:** *In vitro* binding experiments were done using GST (5 μ g; lane 2) or GST-CaM (5 μ g; lanes 3–8) immobilized on a glutathione-sepharose matrix. The CaM samples were pre-incubated with 5 mM EGTA. The samples were washed and incubated in increasing concentrations of calcium: 0 μ M (lane 3), 0.12 μ M (lane 4), 1.2 μ M (lane 5), 12 μ M (lane 6), 120 μ M (lane 7) or 1200 μ M (lane 8). The CaM was incubated with EP24.15 (10 μ g) and washed. The associated proteins were eluted by boiling the glutathione-sepharose matrix in SDS-PAGE sample buffer. The eluted proteins were separated by SDS-PAGE, and bound EP24.15 was detected using a western blot with an EP24.15-specific antibody. As a positive control, 0.1 μ g of recombinant EP24.15 was added to the gel (lane 1). The results are representative of three independent experiments.

Figure 2. Interaction between CaM and EP24.15 as measured by surface plasmon resonance CaM was immobilised on a CM5 sensor chip by amine linkage and increasing concentrations of EP24.15 (0.1, 0.25, 0.5, 1 and 5 μM) were applied in a constant flow. Note that EP24.15 rapidly interacted with CaM but dissociation occurred slowly. The K_d between these two proteins was 0.52 μM . The presented results are representative of three independent experiments.

Figure 3. Co-localisation of EP24.15 and CaM in HEK293 cells

HEK293 cells were transiently transfected with modified pCMV vectors. Cells in panels A, B, G and H were mock transfected with the empty vector. Cells in panels C, D, I and J were co-transfected with the plasmids expressing EP24.15 and CaM. Cells in panels E, F, K and L were co-transfected the plasmids expressing EP24.15, CaM and 14-3-3ε. The cells in panels B, D, F, H, J and L were treated with 10 μM forskolin. The transfected cells were analysed by confocal microscopy. EP24.15 localization is shown in red (panels A–F), while CaM localization is shown in green (panels G–L). Panels M–R are merged images from A and G (panel M), B and H (panel N), C and I (panel Q), D and J (panel P), E and K (panel Q) or F and L (panel R). Note that forskolin treatment causes the increased co-localisation between EP24.15 and CaM (lower panels N, P and R), which is more evident in cells overexpressing EP24.15, CaM and 14-3-3ε (panel R). These data are representative of three independent experiments.

Figure 4. CaM and the secretion of EP24.15 following stimulation

Panels A and B. The stimulated secretion of EP24.15 was evaluated in HEK293 cells transfected plasmid expressing: i) mock (control cells), ii) CaM, iii) EP24.15, iv) CaM and EP24.15, v) CaM and 14-3-3, or vii) CaM, EP24.15 and 14-3-3. Following transfection, the cells were equilibrated for 1 h in serum-free DMEM medium containing 0.1% dialysed bovine serum albumin. This medium was then replaced with identical fresh medium that was previously equilibrated with CO₂, and the cells were stimulated with 10 μ M A23187. As indicated, the cells were pre-incubated with 10 μ M forskolin, 0.5 μ M KT5720 and/or 3 μ M CMZ for 20 min. These compounds were also present during the A23187 stimulation. The media was collected, and the EP24.15 activity in the media was quantified using the fluorogenic substrate QFS. The secreted EP24.15 activity is expressed here as the percentage of the enzyme secreted based on the total activity measured in the corresponding cell homogenates. A significant increase in A23187-stimulated secretion can be observed in all experimental conditions compared to the mock transfected cells (#, $p < 0.05$). The cells overexpressing CaM, EP24.15 and 14-3-3 and stimulated with A23187 secreted more EP24.15 compared to the other groups (+, $p < 0.05$). Note that the PKA activator forskolin significantly increased EP24.15 secretion (*, $p < 0.001$). The data are expressed as the mean $\pm$ SD of three independent experiments. The data were analysed by ANOVA with a post hoc Tukey test.

Figure 5. Parallel analysis of the free intracellular calcium concentration and EP24.15 secretion in HEK293 cells

Panel A. Representative pseudocolour images of HEK293 cells treated with A23187 (0.01-10 μM) and loaded with the calcium-sensitive fluorophore fluo3-AM, which was used for calcium imaging by confocal microscopy, as indicated. The pseudocolour images show non-stimulated cells and cells stimulated with the indicated concentration of A23187. A23187 (10 μM) and EGTA (50 mM) were added to determine maximum (F_{max}) and minimum (F_{min}) fluorescence intensity, respectively. **Panel B.** The equation $[Ca^{2+}]_i = K_d (F - F_{min}) / (F_{max} - F)$, was used to determine the $[Ca^{2+}]_i$. Note the increase in $[Ca^{2+}]_i$ following the increase in the concentration of A23187. Despite the limitations of the confocal imaging, 10 μM of A23187 was observed to cause a significant increase in the $[Ca^{2+}]_i$, compared to 0.01 and 0.1 μM A23187 (*: p<0.05). **Panel C.** The stimulated secretion of EP24.15 was evaluated in HEK293 cells. The cells were equilibrated for 1 h in serum-free DMEM medium containing 0.1% dialysed bovine serum albumin. This medium was then replaced with identical fresh medium previously equilibrated with CO₂, and the cells were stimulated with 0, 0.01 μM, 0.1 μM, 1 μM, 5 μM, 10 μM or 20 μM A23187 for 40 min. The media was collected, and the EP24.15 activity was quantified using the fluorogenic substrate QFS. Note that under these conditions EP24.15 secretion is not stimulated by 0–5 μM A23187. A significant increase in EP24.15 secretion is only observed when the cells are treated with 10–20 μM of A23187 (*, p<0.001). The data are expressed as mean ± SD of three independent experiments. The data were analysed by ANOVA with a post hoc Tukey test.

Figure 6. Increased intracellular calcium triggers the co-localisation of EP24.15 and CaM
HEK293 cells were transiently transfected with: the empty vector (panels A, B, G and H), there pCMV vectors expressing EP24.15 and CaM (panels C, D, I and J) or pCMV vectors expressing EP24.15, CaM and 14-3-3ε (panels E, F, K and L). The cells were treated with 1 μM A23187 (panels A, C, E, G, I, K) or 10 μM A23187 (panels B, D, F, H, J and L). Following stimulation, the transfected cells were analysed by confocal microscopy. EP24.15 localization is shown in red (panels A–F), while CaM localization is shown in green (panels G–L). Panels M–R are merged images from A and G (panel M), B and H (panel N), C and I (panel O), D and J (panel P), E and K (panel Q) or F and L (panel R). Note that while treatment with 10 μM A23187 induced a very intense co-localization between EP24.15 and CaM in cells overexpressing EP24.15, CaM and 14-3-3 epsilon (panel R), treatment with a concentration of A23187 that did not induce the secretion of EP24.15 lead to the co-localization of EP24.15 and CaM. These data are representative of three independent experiments.

Figure 7.

Prediction of CaM binding propensity and rendering interaction sites on the atomic resolution structure of EP24.15. Panel A. Output from the binding site analysis of the CaM Target Database using EP24.15 as the target sequence (amino acids with the highest propensity are highlighted in red). Panel B. Structural model of the predicted CaM binding sequences based on the coordinates of EP24.15 (PDB ID 1S4B). Red represents the portion of the two adjacent helices of EP24.15 that were predicted to bind CaM (in panel A). The orange ball represents the zinc ion required for catalytic activity of the EP24.15. Rendering was performed using the program Pymol (Delano Scientific).