Identification of a Ca^{2+}/calmodulin-binding domain within the carboxyl-terminus of the angiotensin II (AT_{1A}) receptor

Walter G. Thomas*, Luisa Pipolo, Hongwei Qian

Molecular Endocrinology Laboratory, Baker Medical Research Institute, P.O. Box 6492, St. Kilda Road Central, Melbourne, 8008, Australia

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Abstract To identify regulators of the type 1A angiotensin II receptor (AT_{1A}), we investigated the interaction of cellular proteins with a fusion protein containing the rat AT_{1A} receptor carboxyl-terminus. An ~20 kDa cytoplasmic protein interacted with the fusion protein in a Ca^{2+}-dependent manner and was identified as calmodulin. A control peptide with high affinity for Ca^{2+}/calmodulin and a peptide corresponding to a membrane proximal portion of the AT_{1A} receptor carboxyl-terminus with analogy to known calmodulin-binding sequences were synthesised and tested for calmodulin-binding. Using in vitro binding assays combined with gel shift analysis, we demonstrated the formation of complexes between calmodulin and both peptides, which were Ca^{2+}-dependent and of 1:1 stoichiometry. Affinity gels produced from these peptides also purified calmodulin from cell extracts. These results suggest a novel feedback regulation of the AT_{1A} receptor by Ca^{2+}/calmodulin and identify the membrane proximal region of the carboxyl-terminus as a focal point for interactions important for AT_{1A} receptor function.

Key words: Calmodulin-binding protein; Receptor; Angiotensin; AT_{1A} receptor

1. Introduction

Seven transmembrane-spanning receptors that couple to heterotrimeric guanyl nucleotide-binding proteins (G-proteins) represent a large superfamily of cell surface receptors. These receptors convert a diverse array of sensory and hormonal stimuli into intracellular signals that mediate cellular responses. The general paradigm of G-protein coupled receptor (GPCR) action is that stimulated receptors undergo a conformational change that exposes cytoplasmic domains in the receptor, which bind to and activate G-proteins. The termination of GPCR signalling is mediated by receptor phosphorylation and the recruitment to the receptor of accessory proteins termed arrestins, which prevent further G-protein coupling and direct receptors to clathrin-coated pits for internalisation (reviewed in [1–4]).

Recent reports indicate that proteins other than heterotrimeric G-proteins and arrestins can associate with GPCRs and regulate their function. For example, the Na^+/H^+ exchanger regulatory factor interacts with the β_2-adrenergic receptor through a PDZ motif in the carboxyl-terminus of the receptor [5], endothelial nitric-oxide synthase interacts with the carboxyl-terminus of bradykinin B2 and angiotensin AT_{1A} receptors [6], the small non-heterotrimeric G-proteins ARF and RhoA associate with M3 muscarinic and angiotensin AT_1 receptors [7] and the tyrosine phosphatase SHP-1 couples to the sst2 somatostatin receptor [8]. Presumably, such associations increase the repertoire of GPCR actions.

The type 1 (AT_1) angiotensin receptor is a GPCR and mediates the important cardiovascular actions of the peptide hormone, angiotensin II (AngII), including vasoconstriction and blood pressure regulation, fluid and electrolyte homeostasis, neuromodulation and cell growth [9]. Two closely related AT_1 receptor subtypes (AT_{1A} and AT_{1B}) exist in rodents and stimulation of AT_1 receptors by AngII results in $G\alpha_{q/11}$ mediated signalling [10], including phospholipase C-β1-dependent production of diacylglycerol and inositol(1,4,5)trisphosphate (IP_3) with the resultant activation of protein kinase C and release of calcium from intracellular stores. The actions of raised intracellular calcium are mediated through small acidic calcium-binding proteins, typified by calmodulin (CaM), and AngII stimulation of AT_1 receptors activates Ca^{2+}/CaM-dependent kinases and pathways [11–13]. The processes that terminate AT_1 receptor signalling are also poorly understood, but may involve the regulatory carboxyl-terminal region, which is targeted for phosphorylation, directs receptor internalisation and may recruit modulatory proteins to the activated receptor [14].

To identify modulators of AT_1 receptor function, the association of cellular proteins with the AT_{1A} receptor carboxyl-terminus was examined. We report a previously unrecognised interaction between the Ca^{2+}-sensing molecule, CaM, and the carboxyl-terminus of the AT_{1A} receptor, which was dependent upon the membrane proximal region of the receptor tail. This region is a positively charged amphipathic α-helix and as such appears to be an archetypal Ca^{2+}/CaM-binding domain. We propose a novel feedback regulation of the AT_{1A} receptor by Ca^{2+}/CaM that may represent a common mechanism for regulating GPCRs.

2. Materials and methods

2.1. Production of a maltose-binding protein (MBP)-AT_{1A} fusion protein

A MBP fusion protein, containing amino acids 302–359 of the AT_{1A} receptor carboxyl-terminus (MBP-AT_{1A}CT), was made by PCR from the pRc/CMV/AT_{1A} template [15] using sense (5′-CTGCCTGAACCCT**GAA**TTCTACGGCTTTC-3′, bold letters indicate mutations) and antisense (5′-GCCAGTGTGCT**GTCGA**CCT-GTCACTCCAC-3′, bold letters indicate mutations) primers, incorpo-

*Corresponding author. Fax: (61) (3) 9521-1362.
E-mail: walter.thomas@baker.edu.au

Abbreviations: AT_1, AT_2, AT_{1A}, AT_{1B}, types and subtypes of angiotensin II receptors; AngII, angiotensin II; G-protein, guanyl nucleotide-binding protein; GPCR, G-protein-coupled receptor; IP_3, inositol(1,4,5)trisphosphate; CaM, calmodulin; HBSS, Hank's buffered salt solution; CHO-K1, Chinese hamster ovary cells; MBP, maltose-binding proteins; MBP-AT_{1A}CT, MBP fusion protein containing the AT_{1A} carboxyl-terminus

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rating unique restriction sites (5′ *Eco*RI and 3′ *Sal*I, underlined). The 216 bp PCR product was digested with *Eco*RI and *Sal*I and ligated into these sites of the pMal-c2 vector (New England Biolabs). To generate a construct that produces MBP only, the pMal-c2 vector was restriction-digested with *Eco*RI and *Xba*I, the overhangs were filled-in using Klenow and the plasmid was re-ligated. This generated an in-frame stop codon (TAG) in the polylinker region. These constructs were confirmed by sequencing.

Plasmid DNA for MBP-AT_{1A}CT and MBP was transformed into the TB-1 strain of *Escherichia coli* and cultures at A_{600} of 0.4–0.5 were induced with IPTG (0.1 mM, 3 h) to stimulate fusion protein production. Bacterial lysates were incubated with amylose resin (New England Biolabs) and the specifically bound fusion proteins eluted with 100 mM maltose. Fusion proteins were >90% pure as judged by SDS-PAGE. Affinity columns were made by applying 1 mg of purified fusion protein to 1 ml of amylose beads and incubating for 30 min at room temperature followed by extensive washing in Hank's buffered salt solution (HBSS).

2.2. Interaction of ^{35}S-labelled cellular proteins with the MBP-AT_{1A}CT fusion protein

Chinese hamster ovary cells (CHO-K1) cells were maintained in α-MEM containing foetal bovine serum (10%) plus antibiotics. At 70% confluence, a 100 mm plate of cells was washed once with 10 ml of DMEM devoid of L-methionine and L-cysteine and the cells were starved in 10 ml of this media for 2 h. Cells were then incubated in 4 ml of media supplemented with 400 μCi/ml Tran^{35}S-Label (∼70% [^{35}S]-L-methionine, ∼15% [^{35}S]-L-cysteine, ICN pharmaceuticals) for 16 h. The cells were washed three times with 10 ml of ice-cold HBSS and lysed in 2 ml of a 50 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 1 μg/ml aprotinin and 1 μg/ml pepstatin. The plates were rocked for 30 min at 4°C and cell extracts clarified by centrifugation (14 000×*g* for 15 min). Typically, 80–90% of [^{35}S] label was incorporated into protein as revealed by precipitation with trichloroacetic acid.

Fusion proteins immobilised on amylose resin (∼50 μg protein on 50 μl of resin) were washed twice with 1 ml of assay buffer (100 mM Tris-HCl, pH 7.5, 200 mM NaCl) and then resuspended in 60 μl of assay buffer containing either 2 mM $CaCl_2$ or 4 mM EGTA. 40 μl of [^{35}S]-labelled CHO-K1 cell extract was added and the suspension was mixed and incubated for 30 min at 22°C. Following pelleting and removal of the supernatant, the affinity beads were washed four times with 500 μl of assay buffer containing either 4 mM EGTA or 2 mM $CaCl_2$. The final pellets were resuspended in 50 μl of SDS-PAGE sample buffer, heated at 95°C for 5 min and resolved by 12% SDS-PAGE. Gels were fixed and dried and apposed to a Fuji type BAS-IIIs phosphoimaging plate for 1–3 days. The imaging plate was read in a FUJIX Bio-imager Analyser BAS1000 to visualise the radioactive proteins.

2.3. Preparation of peptide affinity media

A peptide corresponding to the putative CaM-binding domain of the AT_{1A} receptor (CLGKKFKKYFLQLLKYIPPK-amide, referred to as 1ACT, for AT_{1A} carboxyl-terminus peptide) and the model CaM-binding peptide (CLKLKKLLKLLKKLLKLG-amide, referred to as CBCP, for CaM-binding control peptide) were synthesised (Chiron mimotopes) with a amino-terminal cysteine to allow immobilisation on an iodoacetate-activated gel (SulfoLink Coupling gel, Pierce). 5 mg of each peptide was dissolved in 1 ml of 50 mM Tris-HCl, 5 mM EDTA, pH 8.5 and mixed with 2 ml Sulfolink Coupling gel for 45 min at room temperature. Unbound peptide was removed by extensive washing with 50 mM Tris-HCl, 5 mM EDTA, pH 8.5 and uncoupled iodoacetate groups in the gel were inactivated with a 50 mM cysteine wash. Finally, the column was washed with 1 M NaCl, equilibrated with 50 mM Tris-HCl, pH 7.5 and stored as a 50% suspension.

The method for identifying proteins that interact with the peptide affinity beads was the same as that described above for the AT_{1A} carboxyl-terminus fusion protein. Some SDS-PAGE gels were also Western-blotted to a PVDF membrane (Polyscreen, NEN) and probed with a monoclonal antibody to CaM (Upstate Biotechnology) and visualised using Renaissance ECL (NEN). As a control, a sample (1 ng) of pure bovine CaM (Sigma) was run in parallel to confirm the immunodetection process.

2.4. In vitro binding assay and gel shift analysis

Gel shift analysis of complexes formed between CaM and 1ACT or CBCP, using urea-polyacrylamide gel electrophoresis, was performed as described by Erickson-Viitanen and Degrado [16]. Reactions (30 μl) containing 300 pmol (∼5 μg) of pure CaM and increasing amounts (0–3000 pmol) of the peptides 1ACT or CBCP, in 100 mM Tris-HCl, pH 7.5, 4 M urea and either 0.1 mM $CaCl_2$ or 1 mM EGTA, were incubated at 22°C for 30 min. 15 μl of a 50% glycerol/0.1% bromphenol blue solution was added to each reaction and the complexes resolved in 12.5% acrylamide gels containing 4 M urea and either 1 mM $CaCl_2$ or 1 mM EGTA. The gels were fixed and stained with Coomassie blue.

3. Results and discussion

The carboxyl-terminus of the AT_1 receptor is important for receptor function. It interacts with and activates G-proteins [17,18] and other signalling molecules [19,20], indicating a contribution to receptor activation, while the identification of phosphorylation sites [21,22] and internalisation motifs [23,24] suggests a key role in receptor desensitisation. To identify possible regulators of AT_{1A} receptor function, we investigated the interaction of [^{35}S]-labelled cellular proteins with a purified MBP fusion protein containing the entire receptor carboxyl-terminus. As shown in Fig. 1, in the presence Ca^{2+}, we observed the interaction of a small (∼20 kDa) cytoplasmic protein with the MBP-AT_{1A}CT fusion protein coupled to amylose resin. This interaction was not observed with the MBP protein alone (not shown) or when Ca^{2+} was chelated with excess EGTA (Fig. 1). Based on its size (∼20 kDa) and the Ca^{2+}-dependence of the interaction, we wondered whether this protein was CaM or a similar member of the small acidic EF-hand containing family of proteins that mediate the effects of intracellular Ca^{2+}. Given that one of the primary cellular responses to activation of the AT_1 receptor by AngII is the elevation of intracellular Ca^{2+} levels and the activation of Ca^{2+}/CaM-dependent pathways [11–13], we wanted to determine if this interacting protein was CaM and to identify the site of interaction within the AT_{1A} carboxyl-terminus.

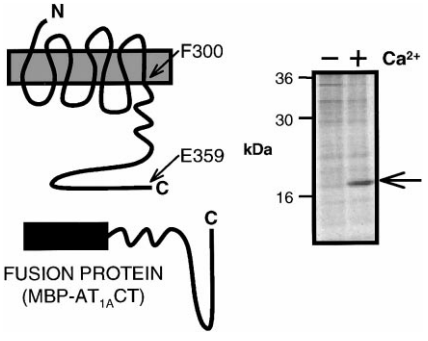

Fig. 1. Ca^{2+}-dependent interaction of proteins with the rat AT_{1A} carboxyl-terminus. Shown (top left) is a schematic representation of the 359 amino acid, seven transmembrane-spanning AT_{1A} receptor. A maltose-binding fusion protein (MBP-AT_{1A}CT) was constructed that contained the entire AT_{1A} receptor carboxyl-terminus (from Phe^{300} to Glu^{359}). This fusion protein was purified and immobilised on amylose resin for use as an affinity matrix to identify proteins that interact with the AT_{1A} receptor tail. In the presence of Ca^{2+} (+), but not EGTA (−), a small ∼20 kDa [^{35}S]-labelled protein (arrow) was found to associate with the fusion protein. This experiment was repeated three times with similar results.

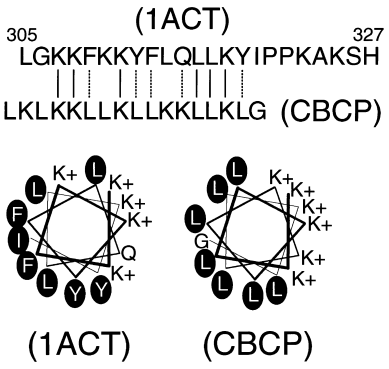

Fig. 2. The proximal AT_{1A} carboxyl-terminus is a positively charged amphipathic helix. Shown are an alignment of residues 305–327 of the rat AT_{1A} carboxyl-terminus and a leucine/lysine repeating peptide that forms a perfect amphipathic helix with nM affinity for calmodulin [28]. Identical and similar residues are indicated by lines and dashed lines, respectively. Shown below are the helical wheel representations for the AT_{1A} receptor carboxyl-terminus peptide (termed 1ACT) and the CBCP (termed CBCP). Note, the similarity in charge and amphipathicity of the receptor sequence and the control peptide.

Ca^{2+}-binding to CaM leads to a conformation change that allows specific binding to sequences within target proteins, including enzymes, channels and receptors [25]. Ca^{2+}/CaM-binding motifs in target proteins commonly involve a preponderance of basically charged residues often within regions predicted to form amphipathic α-helices [25,26]. Using computer modelling, we predicted [24] that the 54 amino acid cytoplasmic carboxyl-terminus of the AT_{1A} receptor starts with Leu^{305} and that the region from Lys^{307} to Ile^{320} has a high probability of forming a positively-charged amphipathic α-helix. This prediction was subsequently confirmed by Franzoni et al. [27] using synthetic peptides corresponding to this region

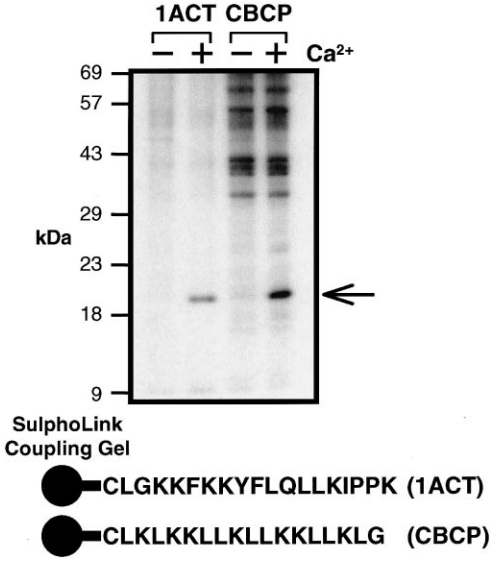

Fig. 3. Ca^{2+}-dependent interaction of proteins with 1ACT and CBCP peptide affinity gels. The 1ACT and CBCP peptides were covalently attached through an introduced N-terminal cysteine to SulphoLink coupling gel. The resulting affinity gels were used to identify interacting [^{35}S]-labelled proteins. In the presence of Ca^{2+} (+), but not EGTA (−), an ∼20 kDa [^{35}S]-labelled protein (arrow) was found to associate with both the 1ACT and CBCP affinity gels. This experiment was performed four times with similar results.

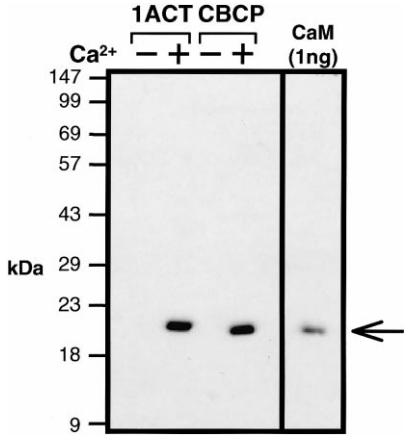

Fig. 4. Western blot analysis identifies the 20 kDa interacting protein as CaM. CHO-K1 cell extracts were purified over the 1ACT and CBCP affinity matrices, separated by SDS-PAGE and Western-blotted with an monoclonal CaM antibody. 1 ng of purified bovine CaM was run as a positive control (arrow). Immunoreactive CaM, with a migration similar to the control CaM, was bound in a Ca^{2+}-specific manner by both peptide affinity gels. This experiment was performed three times with similar results.

examined by circular dichroism and nuclear magnetic resonance. Shown in Fig. 2 is the helical wheel projection of this region, which reveals an amphipathic nature characteristic of a CaM-binding site. For comparison, the helical wheel projection of the model CaM-binding peptide (CBCP, $K_d = \sim 2$ nM for CaM, [28]) and an alignment of the two peptide sequences is shown.

To determine if this basic amphipathic helical region of the AT_{1A} receptor carboxyl-terminus was sufficient to affinity purify the ∼20 kDa Ca^{2+}-dependent protein and could function as a possible CaM-binding site, we coupled the synthetic peptides (1ACT and CBCP) to SulphoLink gel matrix and used these peptide affinity gels to identify interacting [^{35}S]-labelled proteins. As shown in Fig. 3, the CBCP affinity gel purified an ∼20 kDa protein and this association was lost when Ca^{2+} was chelated with EGTA. Similarly, the 1ACT affinity matrix enriched a cytoplasmic protein of ∼20 kDa in a Ca^{2+}-dependent manner. Western blot analysis, using a monoclonal CaM antibody that does not cross-react with other small Ca^{2+}-binding proteins (S100α, S100β, tropinin C and parvalbumin), identified this protein as CaM (Fig. 4).

To determine if the peptide 1ACT (residues 305–327 of the rat AT_{1A} receptor) forms a high affinity complex with Ca^{2+}/CaM, polyacrylamide gel electrophoresis in the presence of 4 M urea was performed. This procedure identifies only high affinity interactions ($K_d < 100$ nM) because 4 M urea dissociates low affinity and non-specific complexes [16]. The stoichiometry of binding was determined by incubating a constant amount of pure CaM (300 pmol) with increasing concentrations (0–3000 pmol) of either 1ACT or the control (CBCP) synthetic peptides. Shown in Fig. 5 are Coomassie blue-stained gels of the gel shift titration experiment. In the presence of Ca^{2+}, the addition of either CBCP or 1ACT resulted in a decreased mobility (upward shift) of CaM indicating the formation of high affinity complexes between CaM and the peptides. The gel shift was complete at equimolar amounts of CaM and peptide (300 pmol) and further addition of peptide did not result in additional bands, which would suggest multivalent complexes. No complexes were formed when Ca^{2+} was

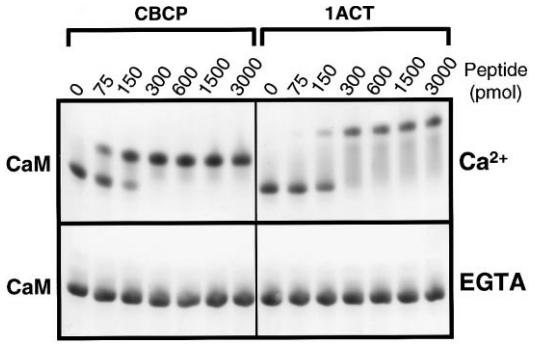

Fig. 5. Complex formation between CaM and the synthetic peptides 1ACT and CBCP in the presence of 4 M urea. Purified CaM (300 pmol per tube) was incubated with increasing amounts (0, 75, 150, 300, 600, 1500 and 3000 pmol) of the peptides 1ACT or CBCP in the presence of 4 M urea and either Ca^{2+} or EGTA. Complexes were resolved by polyacrylamide gel electrophoresis in gels containing 4 M urea and either Ca^{2+} or EGTA to match the samples. The gels were stained with Coomassie blue. Complex formation was observed as a shift in the migration of CaM, in the presence of Ca^{2+}, for both peptides. No CaM shift, at any concentration of added peptide, was observed in presence of EGTA (bottom panels). This experiment was performed four times with similar results.

chelated by EGTA, regardless of the amount of peptide added. These data indicate a single, high affinity, Ca^{2+}-dependent binding site for CBCP and 1ACT on CaM.

Our observation of a high affinity CaM-binding site in the proximal region of the AT_{1A} receptor carboxyl-terminus has important implications for receptor function. We propose the following feedback loop between the AT_{1A} receptor and Ca^{2+}/CaM: AngII-stimulated AT_{1A} receptors promote release of Ca^{2+} from intracellular stores, which generates activated Ca^{2+}/CaM complexes. These complexes then feedback to bind the basically charged, amphipathic helical region encompassing residues 305–320 within the AT_{1A} receptor carboxyl-terminus. The proposed CaM feedback is analogous to the feedback pathways reported for other Ca^{2+}-mobilizing receptors and channels (e.g. the inhibition of the *N*-methyl-D-aspartate receptor [29], the CaM regulation of the transient receptor potential-like channel and the negative regulation of phototransduction, which occurs at the level of the rhodopsin receptor [30]).

The role of this putative CaM-AT_{1A} receptor feedback loop is unknown, but the proximal carboxyl-terminus (residues 305–322) has been implicated in a number of important AT_{1A} receptor functions. Foremost, this region is involved in binding and activating heterotrimeric G-proteins [17,18] and point mutations of Tyr^{312}, Phe^{313} and Leu^{314} uncouple the receptor from G-protein-dependent signalling. It is therefore tempting to speculate that once the AngII-stimulated AT_{1A} receptor has activated and released the heterotrimeric G-protein to initiate signalling, the subsequent Ca^{2+} transient results in Ca^{2+}/CaM recruitment to the G-protein-binding site to prevent re-association of G-protein with the receptor. In such a scenario, Ca^{2+}/CaM-binding would be predicted to produce a form of receptor desensitisation. We have also reported that mutations of hydrophobic amino acids at the end of this amphipathic α-helix dramatically reduce AT_{1A} receptor internalisation [24] and Gaborik et al. [31] have linked the region Lys^{307}–Lys^{311} to cell surface expression, suggesting that important determinants of receptor trafficking are associated with this helical region. Interestingly, Ca^{2+}/CaM has been implicated in the general process of receptor endocytosis and, more specifically, in the internalisation of another GPCR, the serotonin receptor [32]. Whether, CaM-binding to the AT_{1A} receptor is involved in receptor internalisation remains to be determined. Finally, the sequence Tyr^{319}–Pro^{322} reportedly binds phospholipase C-γl and a complex of SHP-2 phosphatase/Jak2 kinase [19,20], suggesting a mechanism for the activation of growth factor-like tyrosine kinase signalling pathways by the AT_{1A} receptor [10,33]. Clearly, this region of the carboxyl-terminus is multifaceted, which suggests a complex hierarchy of interactions central to AT_{1A} receptor function.

In conclusion, we have identified, for the first time, the presence of a CaM-binding site within the carboxyl-terminus of the G-protein-coupled AT_{1A} receptor. This high affinity, Ca^{2+}-dependent binding domain is a basic amphipathic α-helix and therefore bears all the hallmarks of a genuine CaM regulatory site. The recent identification of Ca^{2+}/CaM-binding sites within the carboxyl-terminus of another seven transmembrane-spanning GPCR, namely the metabotropic glutamate receptor subtype 5 [34], predicts that Ca^{2+}/CaM-binding to, and modulation of, GPCRs may be a widespread phenomenon. CaM can also regulate the GPCR kinases [35] which phosphorylate these receptors and CaM-dependent protein kinases phosphorylate and modulate the activity of the arrestin proteins [36], which bind to phosphorylated GPCRs and lead to their desensitisation and internalisation. Together, these data identify CaM as a primary regulator of GPCR function.

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