Abstract

In this study, we present data that support the presence of two distinct calmodulin binding sites within the angiotensin II receptor (AT1A), at juxtamembrane regions of the N-terminus of the third intracellular loop (i3, amino acids 214–231) and carboxyl tail of the receptor (ct, 302–317). We used bioluminescence resonance energy transfer assays to document interactions of calmodulin with the AT1A holo-receptor and GST-fusion protein pull-downs to demonstrate that i3 and ct interact with calmodulin in a Ca2+-dependent fashion. The former is a 1–12 motif and the latter belongs to 1-5-10 calmodulin binding motif. The apparent Kd of calmodulin for i3 is 177.0±9.1 nM, and for ct is 79.4±7.9 nM as assessed by dansyl-calmodulin fluorescence. Replacement of the tryptophan (W219) for alanine in i3, and phenylalanine (F309 or F313) for alanine in ct reduced their binding affinities for calmodulin, as predicted by computer docking simulations. Exogenously applied calmodulin attenuated interactions between G protein βγ subunits and i3 and ct, somewhat more so for ct than i3. Mutations W219A, F309A, and F313A did not alter Gβγ binding, but reduced the ability of calmodulin to compete with Gβγ, suggesting that calmodulin and Gβγ have overlapping, but not identical, binding requirements for i3 and ct. Calmodulin interference with the Gβγ binding to i3 and ct regions of the AT1A receptor strongly suggests that calmodulin plays critical roles in regulating Gβγ-dependent signaling of the receptor.

Introduction

Angiotensin II (Ang II) plays crucial roles in the regulation of cardiovascular functions, all of which rely on signal propagation elicited by Ang II binding to G protein-coupled receptors (GPCRs). The most important Ang II receptor appears to be the AT1 receptor, which signals primarily through Gq/11 family G proteins, and which couples to Ca2+ mobilization, proliferation and/or hypertrophic signals in most cell types [1]. Despite intensive interest in the signal transduction pathways of the AT1 receptor, our understanding of many aspects of its signaling mechanisms is incomplete. Although most signals appear to be mediated through G proteins, other proteins can bind to and regulate AT1 receptor signal transduction [2].

Like other GPCRs, the heptahelical structure of the AT1 receptor consists of seven transmembrane α-helical barrels connected by three extracellular loops and three intracellular loops. The amino terminus is oriented extracellularly, whereas the carboxyl terminal tail faces the cytoplasm. The carboxyl terminal tail and the intracellular loops contain regulatory sites for G protein coupling, phosphorylation and protein-protein interactions. G proteins are thought to mediate signals through direct interaction of their α and/or βγ subunits with the intracellular domains of the GPCRs, generally at the juxtamembrane regions of the third intracellular loops (i3) and carboxyl terminus [3], [4], [5], [6], [7], [8].

There is a growing awareness that GPCRs signal by forming multimolecular complexes that include G proteins and many other proteins [9], [10], [11], [12], [13], [14]. In that regard, proteins other than G proteins have been shown to directly interact with the intracellular domains of GPCRs, primarily with the i3 loop and carboxyl tail. Binding of these so-called RIPS (receptor interacting proteins) can regulate receptor function, trafficking and signaling, either through, or independent of G proteins [2], [15], [16], [17]. For example, the β2 adrenergic receptor complexes with the Ca2+ channel Cav1.2, ensuring specificity and efficiency of signal propagation [9]. Thus, RIPS could be regulators for GPCRs [18], [19], [20], [21], could regulate signal propagation between GPCRs and their downstream binding partners [22], [23], and could possibly mediate signal propagation independent of G protein activation [10], [24]. Exploring RIPS and their binding sites in GPCRs could yield new clues for understanding signal propagation mechanisms of the GPCRs and developing therapeutic methods targeting their interactions.

Several putative RIPS for the 55 amino acid carboxyl terminal tail of the AT1A receptor have been identified in recent years [10], [25], [26], [27]. In contrast, few putative RIPS have been reported yet for the relatively short (24 amino acids) i3 loop of the AT1A
One RIP that has drawn increasing interest of late is calmodulin (CaM), which is a small (~17 kDa) acidic protein consisting of 148 residues. CaM has four EF-hand motifs with a Ca²⁺ binding site in each of the EF-hands. Binding of four Ca²⁺ ions to the EF hands elicits conformational changes that expose hydrophobic residues on the surface of CaM, enabling CaM to interact with its various target peptides. CaM recognition sequence motifs in proteins are highly variable, suggesting that features other than the primary sequence are critical for CaM binding. A typical CaM binding region is often characterized as a ~20 amino acid a-helix with critical hydrophobic residues clustered on the opposite side of the helix from clusters of basic amino acid residues [28], [29].

Like many GPCRs, CaM participates in Ca²⁺ signaling; CaM can bind to and modulate the functions of enzymes, ion channels and receptors involved in a variety of cellular processes such as muscle contraction, cell cycle progression and cytoskeletal organization [30], [31], [32], [33]. Recently, CaM has been shown to directly interact with a handful of GPCRs, and to modify their functions. These include metabotropic glutamate receptor [34], [35], [36], [37], [38], [39], µ-opioid receptor [40], angiotensin II AT₁A receptor [41], D₂ dopamin receptor [23], [28], [29].

Materials and Methods

Materials

GST expression vector pGEX-4T-1 and Glutathione-Sepharose 4B beads were purchased from Amersham Biosciences (Piscataway, NJ). The E. coli BL21 gold strain was purchased from Stratagene (La Jolla, CA). The yellow fluorescent protein fusion protein expression vector eYFP-N1 and the Renilla luciferase (RLuc) protein expression vector eYFP-N1 and the Renilla luciferase (RLuc) protein expression vector were purchased from Clontech (Mountain View, CA). CaM and G protein Gβ subunits were purchased from Upstate Biotechnology (Charlottesville, VA). Dansyl chloride, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7), and antibiotic-antimycotic solution were purchased from Invitrogen-GIBCO (Carlsbad, CA). Electrophoresis supplies and lipofectamine 2000 were purchased from Invitation (Carlsbad, CA). CaM and G protein Gβ subunits were purified from bovine brain, and protease inhibitor cocktail set I, from Clontech (Mountain View, CA). CaM and G protein Gβ subunits were synthesized by Invitrogen. CaM-induced downstream of GST, GST-TSYTLIWKALKKAYEIQKPRNDIDFRKL. Forward and reverse primers were GGA TGC ACC AGC TAT ACC CT1 TT1 ATT and GAA TTC AAT TAT TAT CCT AAA GAT GTC with pCDNA3.1-AT1A receptor as template. GST-AT3(213-234) were generated by introducing a stop codon into the vector of GST-AT3(213-234) after the codon for 234R. Forward and reverse primers for generation of the stop codon were CAA AAG AAC AAA CCA AGA TAA GAT GAC AAT TAT AT1 A receptor, and to establish their functional significance. The purpose of the current study was to identify and characterize CaM binding domain(s) in the AT1A receptor, and to establish their functional significance. The work described in this manuscript supports the existence of two recent studies. In that regard, one group used peptides and fusion proteins to identify a CaM binding site between residues 305–327 in the juxtamembrane region of the carboxyl terminal tail of the AT1A receptor [41], although the functional significance of this interaction was not studied. The purpose of the current study was to identify and characterize CaM binding domain(s) in the AT1A receptor, and to establish their functional significance. The work described in this manuscript supports the existence of two distinct CaM binding sites located in amino terminal juxtamembrane regions of the i3 loop and carboxyl tail of the AT1A receptor. Moreover, the work supports a role for CaM as a regulator for signal propagation at the interface between AT1A receptor and G protein Gβγ subunits.

Oligonucleotide primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA), and verified either by PAGE or HPLC depending upon the length of the primers.

Cell Culture

Human Embryonic Kidney 293 (HEK293) cells from ATCC (Manassas, VA) were fed on a medium containing F-12 nutrient mixture, 1% antibiotic-antimycotic solution, and 10% fetal bovine serum. The cells were cultured at 37°C in a humidified incubator with 95% air and 5% CO₂.
GST-fusion Protein Purification and Pulldown Assays

GST-fusion proteins were expressed in E. coli BL21 strain cells, and purified by affinity chromatography using immobilized glutathione sepharose 4B beads. Proteins were eluted by reduced glutathione in 50 mM Tris-HCl buffer (pH 8.0), and then concentrated with Centricon filters with 3 kDa MW cut-off. The eluted proteins were either used immediately or stored at −80°C for future use. For GST-fusion protein pull-down assays, fusion proteins were incubated with either cell lysates or purified protein(s) at 4°C for 3 hours, following which glutathione-sepharose 4B beads were added and incubation continued at 4°C for 1 hour. The beads were then recovered by brief centrifugation, followed by 4 washes with the corresponding incubation buffer. The fusion proteins and the proteins to which they were bound were then separated by SDS-PAGE, and identified by staining with Coomassie blue or by immunoblot.

Measurements of Dansyl-CaM Fluorescence

Dansyl-CaM was synthesized according to a standard method [45], [46], [47], [48]. 300 μl of CaM purified from bovine brain (2 mg/ml in a buffer containing 20 mM NH4HCO3, pH 7.4 and NIH. The F309A, Y312A, and F313A mutant structures were refined using 3000 steps of energy minimization using xplor-NIH [51]. The CaM in PDB 1 cdm and liq5 crystal structures of the bovine CaM-CaMKII peptide complex (PDB code 1iq5 [50]). The CaM in PDB 1 cdm were added through homology modeling based on 1iq5, the coordinates of the 10 missing residues (74–83) of the CaM in 1 cdm were added through homology modeling based on 1iq5, and the resulting structure was further refined by 2000 steps of energy minimization using xplor-NIH [51].

CaM-ATct(302–317) modeling. The peptide CaMKII was manually mutated to ATct(302–317) in the molecular modeling software InsightII (Accelrys, Burlington, MA) based on the 1-50 motif alignment. This was accomplished by keeping the backbone structure intact while replacing the side-chains. The complex then was refined using 3000 steps of energy minimization using xplor-NIH. The F309A, Y312A, and F313A mutant structures were modeled based on the refined CaM-ATct structure through similar procedures.

CaM-ATT3(214–231) modeling. The peptide cCaMKKp was manually mutated to ATT3(214–231) in InsightII. This was done assuming an α-helical structure for ATT3(214–231). The first key motif residues in the two peptides (residue “1” in 1–16 and 1–12 motifs) were aligned, i.e., the first Leu in cCaMKKp was aligned to the first Leu in ATT3(214–231). Then, a protein docking procedure was conducted which minimizes the above CaMAT3(214–231) complex energy using a flexible protein-peptide docking algorithm [32]. The docking method used a Monte-Carlo annealing simulation and considered various movements including rigid peptide translation, rigid peptide rotation, peptide torsion on side-chain and backbone, and protein side-chain torsion. By keeping the protein backbone fixed, we considered both the flexibility of peptide and protein side-chain, in the docking simulation. 100 independent Monte-Carlo docking simulations were conducted from different random seeds. All of the obtained conformations were ranked using the same energy function used in the docking algorithm. The energy function includes atomic pairwise interaction energy between protein and peptide and atomic solvation contribution. The conformation with the lowest energy was chosen and further refined using 3000 steps of energy minimization in xplor-NIH. The Y213A, L217A, and W219A mutant structures were modeled based on refined CaMAT3(214–231) structures in similar ways to those with the CaM-ATct (302–317) mutations. Evaluation of binding affinities: The binding affinities between CaM and the peptides were evaluated using the same energy function used in the docking program. Because the mutated residues were buried in the interface and the complex structures did not experience significant changes, the contribution of solvation energies was excluded. The final binding energy included the protein-ligand atomic pairwise interactions, which were described by a distance-dependent function (Equation).

\[ E_{\text{pl-contact}} = A_{ij} \left( R_{ij} - B_{ij} \right)^{C_{ij}} + D_{ij} |A_{ij}| \]

\[ 1.0 \leq |R_{ij}| \leq 1.0 \leq C_{ij} \leq 0.2 |D_{ij}| \leq 1.0 \]

Rij is the actual distance between a pair of protein-ligand atoms i and j, Aij is the force constant related to the atom pairs, Bij is a typical interaction distance between atoms of i and j, the exponent Cij determines the interaction’s distance dependence, and Dij corresponds to a basic packing background [52]. All units in the equation are in Å. All possible pair-wise contacts between protein atoms and peptide atoms within a contact cutoff of 15.0 Å were calculated, and the binding affinity was calculated as the sum of all of those interactions.

Bioluminescence Resonance Energy Transfer (BRET)

BRET measures the transfer of energy between a donor luminescent source and an acceptor fluorophore. Our luminescent donor source was luciferase, whereas the acceptor fluorophore was yellow fluorescent protein, (YFP). When luciferase degrades its substrate coelenterazine, energy is released in the appropriate wavelength to excite YFP. The Forster energy transfer from luciferase to YFP only occurs when the donor and acceptor are <100 Å [53]. This means that the emission of light from YFP is a function of close physical proximity of the luciferase to the YFP. We used this method to demonstrate cellular interaction (close physical proximity) between the AT1A receptor and CaM. In order to perform the BRET assays, we constructed expression vectors of AT1A receptor-eYFP and RLuc-CaM (or CaM-RLuc), and co-transfected HEK293 cells with the two vectors. The AT1A receptor-eYFP expression vector was constructed by insertion of the AT1A receptor without stop codon into BamH I and BamHI sites at the N-terminus of eYFP and in frame with the eYFP in the eYFPN1 vector. Expression vectors of CaM-RLuc and RLuc-CaM were kindly provided by Dr. Justin Turner (Medical University of South Carolina), in which CaM was ligated in-frame with Renilla luciferase either at its N-terminus (CaM-RLuc) or C-terminus (RLuc-CaM) [43], [45].

We first stably transfected HEK293 cells with the AT1A receptor-eYFP vector by using lipofectamine 2000 solution

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PLOS ONE | www.plosone.org 3 June 2013 | Volume 8 | Issue 6 | e65266
and carboxyl terminal tail of the AT1A receptor, in a Ca²⁺-downs demonstrated that CaM associated with both the i3 loop Coomassie blue staining (Figure 1A). GST fusion protein pull-
proteins were separated by SDS-PAGE and visualized by purified using gluthathione-sepharose 4B beads. The fusion side (IPTG), following which the GST-fusion proteins was induced by isopropyl-
GST-fusion proteins was induced by isopropyl-β-D-thiogalactoside (IPTG), following which the GST-fusion proteins were purified using glutathione-sepharose 4B beads. The fusion proteins were separated by SDS-PAGE and visualized by Coomassie blue staining (Figure 1A). GST fusion protein pull-downs demonstrated that CaM associated with both the i3 loop and carboxyl terminal tail of the AT1A receptor, in a Ca²⁺-dependent manner. Further, the interactions occurred not only with CaM from rat brain lysates (upper panel, Figure 1B) but also with pure bovine brain CaM (lower panel, Figure 1B), indicating that the interactions between the fusion proteins and CaM are direct.

Interaction of CaM with the AT1A Receptor in HEK293 Cells
To test whether the interaction between the AT1A holo-receptor and CaM also occurs in living cells, we employed a BRET assay. When HEK293 cells were co-transfected with expression vectors of both the AT1A receptor fused with yellow fluorescent protein (AT1A receptor-eYFP), and CaM fused with luciferase either at its C-terminus (RLuc-CaM) or N-terminus (CaM-RLuc), the BRET ratio was significantly increased in cells co-transfected with YFP-AT1A receptor and RLuc-CaM, and YFP-AT1A receptor and CaM-RLuc, as compared to cells transfected with a control (RLuc). The BRET signals induced by YFP-AT1A receptor+CaM-RLuc (26-fold) and YFP-AT1A receptor+RLuc-CaM (0.75-fold) were markedly higher than that induced by co-transfection of RLuc-CaM and lacking RLuc-CaM or CaM-RLuc. These results provide further support for CaM-receptor interactions in that functional CaM is important for the BRET signal to occur. Interestingly, treatment of the co-transfectants with 100 nM Ang II at 2, 4 and 6 minutes did not alter the magnitude of the BRET ratio when compared with those without Ang II treatment (Figure 2). This suggests that the association between CaM and the AT1A receptor is

Statistical Analysis
Results were expressed as means ± S.E. ANOVA and student t-tests were used for the statistical analyses of the data.

Results

Interactions of CaM with the i3 Loop or Carboxyl Tail of the AT1A Receptor
As an initial approach to identify interaction between CaM and the AT1A receptor, we constructed expression vectors encoding GST fused to the i3 loop, GSTATi3(213–242), or to the carboxyl terminal tail, GST-ATct(297–359) of the AT1A receptor. The vectors were introduced into E. coli (BL21 strain). Expression of GST-fusion proteins was induced by isopropyl-β-D-thiogalactoside (IPTG), following which the GST-fusion proteins were purified using glutathione-sepharose 4B beads. The fusion proteins were separated by SDS-PAGE and visualized by Coomassie blue staining (Figure 1A). GST fusion protein pull-downs demonstrated that CaM associated with both the i3 loop and carboxyl terminal tail of the AT1A receptor, in a Ca²⁺-dependent manner. Further, the interactions occurred not only with CaM from rat brain lysates (upper panel, Figure 1B) but also with pure bovine brain CaM (lower panel, Figure 1B), indicating that the interactions between the fusion proteins and CaM are direct.

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constitutive. If this is true, a complex containing AT1A receptor and CaM could be pulled out from the cells.

Potential CaM Binding Sites in the i3 Loop and Carboxyl Terminal Tail of the AT1A Receptor

In order to identify potential CaM binding sites in the AT1A receptor, we searched the Calmodulin Target Database at the Ontario Cancer Institute (http://calcium.uhnres.utoronto.ca/ctdb/flash.htm). The database evaluates amino acid sequences for the presence of characteristics associated with known CaM binding sites. Using this algorithm, two putative CaM binding sites were identified in the N-terminal juxtamembrane region of the i3 loop spanning amino acids from 210 to 234, and in the juxtamembrane area of the carboxyl tail spanning amino acids from 325 to 359. Accordingly, we constructed four GST fusion proteins containing the N- or C-terminal portions of both the i3 loop and the carboxyl tail (Figure 3A). These were utilized to document potential interactions with CaM by GST-fusion protein pull-down assays. Results of those experiments showed that the N-terminal sequences in the i3 loop ATi3N (213–234) and the carboxyl tail ATctN (297–324) interact with purified CaM. In contrast, the C-terminal sequences in the i3 loop ATi3C (235–242), and in the carboxyl tail ATctC (325–359), had no interactions with CaM (Figure 3B). The experimental data agree with the theoretical predictions from the CaM Target Database in that the predicted CaM binding domains associate with CaM, whereas nearby sequences do not.

![Figure 2. Interaction of the AT1A receptor with CaM in HEK293 cells.](http://example.com/figure2)

![Figure 3. CaM binding sites in the i3 loop and carboxyl tail of the AT1A receptor.](http://example.com/figure3)

**A**

| GST-ATi3N:    | GST-TSYTLIWKALKKAYEIQNKPR |
|--------------|---------------------------|
| GST-ATi3C:   | GST-NDDIFRII            |
| GST-ATctN:   | GST-LNPLFYGFLGKFKKYFLQLLKYIPPA |
| GST-ATctC:   | GST-KSHSSLSTKMSTLSYRPSDNMSSAKKPA5CFEVE |

**B**

![Recognition of CaM Binding Motifs in the i3 Loop and Carboxyl Tail of the AT1A Receptor](http://example.com/figure3b)
CaM with Ca\(^{2+}\) ions, a conformational change in CaM is induced, and this conformational shift is markedly enhanced by binding of CaM to ATi3(214–231) or ATct (302–317). These data further support the potential interactions of the putative CaM binding regions of the AT1A receptor with CaM.

Effect of Point Mutations on CaM Binding in the i3 Loop and Carboxyl Tail of the AT1A Receptor

If the interactions between the AT1A receptor peptides and CaM are specific, we would expect that mutations of key residues in each of the peptides would reduce the interactions between CaM and the peptides. We modeled three-dimensional complex structures of CaM bound to ATi3(214–231) and to ATct(302–317) in order to predict amino acid changes that could reduce the interactions between the i3 loop or the carboxyl tail of AT1A receptor and CaM (Figure 5A). Based on non-bonded contact analysis on the CaM-peptide complex structures using the HBPLUS program [54], we selected for alanine mutagenesis, six residues within the motifs at predicted contact sites with CaM. The residues chosen for study based on contact site proximity were Y215A, L217A, and W219A located within the i3 loop, and F309A, Y312A, and F313A located within the carboxyl tail. We used the modeled complexes described in Figure 5A to determine the theoretical binding energies of each mutant peptide. The calculated binding energies were higher than wild type CaM-ATi3(214–231) for CaM-ATi3(214–231)W219A (+8%), and lower for CaM-ATi3(214–231)Y215A (-12%) and CaM-ATi3(214–231)L217A (-1%), respectively. The calculated binding energies were found to be higher than wild type CaM-ATct(302–317) for CaM-ATct(302–317)F309A (+7%) and CaM-ATct(302–317)F313A (+10%), and lower for CaM-ATct(302–317)Y312A (-1%). Because higher binding energies correspond to lower affinities, we predicted that GST fusion proteins bearing W219A, F309A and F313A would have reduced efficiencies in pulling down recombinant CaM. In that regard, we performed GST-fusion protein pull-down assays with fixed concentrations of AT1A receptor peptide fusion proteins and CaM to determine relative binding efficiencies. Those studies demonstrated that the relative binding efficiencies for CaM are significantly reduced in the three mutants predicted to have a higher binding energy, GSTATi3N(213–234)W219A (32% of wild type), GST-ATi3N(297–324)F309A (29% of wild type) and GST-ATi3N(297–324)F313A (22% of wild type) (Figure 5B). Indeed, mutants GST-ATi3N(213–234)Y215A, GST-ATi3N(213–234)L217A and GST-ATi3N(297–324)Y312A did not have reduced binding efficiencies (not shown), so those mutants were not further studied.

Titration of Dansyl-CaM Fluorescence Changes with Peptides

We next determined the binding affinities of CaM for peptides ATct(302–317), ATi3(214–231), ATct(302–317)F309A, ATct(302–317)F313A, and ATi3(214–231)W219A by titration of dansyl-CaM fluorescence in the presence of increasing concentrations of the peptides. The apparent dissociation constants (Kd) of CaM for the peptides were 79.4 ± 7.9 nM for ATct(302–317), 177.0 ± 9.1 for ATi3 (214–231), 308.1 ± 11.8 for ATct(302–317)F309A, 388.1 ± 9.4 for ATct(302–317)F313A, and 587.7 ± 13.6 for ATi3(214–231)W219A (Figure 6).
CaM Inhibits the Interaction between G Protein βγ Subunit and the AT1A Receptor

We wanted to investigate potential roles for CaM interaction with the AT1A receptor. One possibility is that CaM could modulate the coupling of the receptor to G proteins. In that regard, G protein βγ subunits bind to the i3 loop and carboxyl tail of AT1A receptor in a concentration-dependent fashion (Figure 7A). Interestingly, Figure 7B demonstrates that the interactions between Gβγ subunits and the i3 loop or the carboxyl tail of AT1A receptor were pulled down by glutathione-sepharose 4B beads, and subjected to immunoblot with a specific anti-CaM antibody. GST-fusion proteins were visualized in the gels by Coomassie blue staining (the lower gel panel). The summary graph represents relative densities of the ratio of the CaM in the immunoblots and the loaded GST-fusion proteins as determined by Coomassie blue staining. The bars represent mean ± S.E. from 5 independent experiments. * or # stand for P<0.01 as compared with wild type GST-fusion proteins, respectively.

doi:10.1371/journal.pone.0065266.g005

Figure 6. Titration of dansyl-CaM fluorescence with peptides

Figure 6. Titration of dansyl-CaM fluorescence with peptides ATcT(302–317)(1), ATi3(214–231)(2), ATcT(302–317)F309A(3), ATcT(302–317)F313A(4), and ATi3(214–231)W219A(5). Peptides (25–2000 nM) were incubated with 147 nM dansyl-CaM in the presence of 0.1 mM calcium for 1 hour at room temperature. Fluorescence emission of dansyl-CaM was then measured at an emission wavelength of 485 nm. The relative fluorescence intensities F/F0 (the ratio of the total fluorescence intensities and the fluorescence intensity of dansyl-CaM) were plotted against the concentration of the peptides added (curve A), which represents an average of three separate experiments (mean ± S.E.). The curves in B were derived from calculation of the titration data according to a previously described method [58]. The α representing the fractional degree of saturation of dansyl-CaM fluorescence is calculated from formula α = (F−F0)/(F∞−F0) where F∞ is the fluorescence intensity at the saturating level of the peptides added. The reciprocal of the slope gives the apparent dissociation constants (Kd) of CaM for the peptides, which are: 79.4 ± 7.9 nM for ATcT(302–317), 177.0 ± 9.1 nM for ATi3(214–231), 308.1 ± 11.8 nM for ATcT(302–317)F309A, 388.1 ± 9.4 nM for ATcT(302–317)F313A, and 587.7 ± 13.6 nM for ATi3(214–231)W219A (data are expressed as mean ± SE).

doi:10.1371/journal.pone.0065266.g006

Figure 5. Effect of point mutations in ATi3N or ATcTn on their interactions with CaM. A. Modeled structures of CaM-ATi3(214–231) and CaM-ATcT(302–317). The complexes of CaM and CaM binding motif in the i3 loop ATi3(214–231) or the carboxyl terminal tail ATcT(302–317) of the receptor were modeled as described in Experimental Procedures. The target peptides are colored in red. Residues W219 in ATi3(214–231)–SYTLIWKALKKAYEIQKN, and F309 and F313 in ATcT(302–317)–YGFLGKRFKRYFLQQLL are displayed with sticks and are colored in blue. Calcium atoms are shown as orange spheres. The N- and C- termini of CaM are also labeled. Helices and sheets in CaM are colored in green and yellow, respectively. B. Effect of point mutations at ATi3N or ATcTn on their interaction with CaM. 50 pmol of wild type GST-fusion proteins including GST-ATi3N(213–234) and GST-ATcT(297–324), and 50 pmol of mutated GST-fusion proteins including GST-ATi3N(W219A), GST-ATcTn(F309A) and GST-ATcTn(F313A), were incubated with purified bovine brain CaM in a buffer containing 100 mM Tris-HCl (pH 7.5) with 0.1 mM CaCl2. The protein complexes were pulled down by glutathione-sepharose 4B beads, and subjected to immunoblot with a specific anti-CaM antibody. GST-fusion proteins were visualized in the gels by Coomassie blue staining (the lower gel panel). The summary graph represents relative densities of the ratio of the CaM in the immunoblots and the loaded GST-fusion proteins as determined by Coomassie blue staining. The bars represent mean ± S.E. from 5 independent experiments. * or # stand for P<0.01 as compared with wild type GST-fusion proteins, respectively.

doi:10.1371/journal.pone.0065266.g005

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Calmodulin and AT1A Receptor

Tail can be inhibited by CaM. The interaction of 2 pmol of Gbc with 2 pmol of GST-i3 loop was significantly reduced by 2 and 10 pmol of CaM to 77% and 60%, respectively. The interaction of 2 pmol of Gbc with 2 pmol of GST-ct was significantly reduced by 2 and 10 pmol of CaM to 73% and 34%, respectively (Figure 7B).

In order to confirm that the inhibitory effects of CaM on binding of Gbc subunit to the ATi3 loop and ATct are specific, we performed similar studies using GST fusion proteins that incorporated the same mutations described in Figure 6 (W219A), F309A, and F313A). Figure 7C demonstrates that all three mutant fusion proteins could effectively bind Gbc subunits. The binding for all three mutants was similar in magnitude to the binding to non-mutated ATi3 and ATct fusion proteins (not shown). These data suggest that mutations that decrease CaM binding to the AT1A receptor i3 loop and carboxyl terminal tail have no effect on Gbc binding. Further, CaM had no significant effects on Gbc binding to the three mutant fusion proteins. In the presence of 2 and 10 pmol of CaM, the amount of Gbc binding to GST-ATi3(W219A) was 95%, and 94%, to GST-ATct(F309A) was 93% and 81%, and to GST-ATct(F313A) was 99%, and 83%, respectively (Figure 7C). Thus, these experiments document three key points: (1) Both Gbc and CaM bind to GST-fusion proteins containing each of those two sequences from the AT1A receptor. (2) CaM can impair binding of Gbc to GST-ATi3 and possibly more so to GST-ATct. (3) Binding of CaM to GST-ATi3 and GST-ATct is required for impairment of Gbc binding.

Discussion

What is new about this work is that (1) we have identified and characterized two distinct CaM binding motifs in the AT1A receptor, one each in the amino terminal juxtamembrane regions of the i3 loop and carboxyl terminal tail of the AT1A receptor, and (2) we have demonstrated that CaM impairs the binding of Gbc to GST-fusion proteins containing each of those two sequences from the AT1A receptor. These findings suggest that direct binding of CaM to intracellular regions of the AT1A receptor inhibits Gbc binding, and can modulate its signaling. Thus, CaM binding to the AT1A receptor is functionally significant.

In this manuscript, we have presented multiple lines of evidence supporting the existence of CaM-binding domains within the angiotensin AT1A receptor. (1) The AT1A receptor contains two putative CaM-binding domains as identified by a computer search algorithm and by molecular modeling studies. (2) GST-fusion proteins encompassing the AT1A receptor i3 loop and carboxyl terminal tail efficiently pulled down CaM from rat brain lysates and from solutions of purified bovine CaM, and those interactions were Ca++ dependent. (3) BRET studies demonstrated that CaM and the AT1A holo-receptor are in close proximity (within the Forster radius) when transfected into HEK293 cells, suggesting that the interaction can occur in intact cells. The BRET signal was
functionally significant in that CaM appears to
231). The differences in the affinities of these peptides for CaM
and carboxyl terminus. CaM significantly diminished binding of purified G
bc
determined by GST pull down assays and by dansyl-CaM
loop and carboxyl terminus interact specifically with CaM as
determined by GST pull down assays and by dansyl-CaM
fluorescence spectral measurements. (5) Exogenously applied
CaM significantly diminished binding of purified Gßy subunits
to GST fusion proteins containing the putative CaM binding
regions in the AT1A receptor ß loop and carboxyl terminus. Fusion
proteins containing point mutations of the CaM binding
domains that significantly reduce affinities for CaM had no effect
on binding of purified Gßy subunits to the fusion proteins, but
significantly attenuated the ability of CaM to diminish binding of
purified Gßy subunits. The latter results suggest that CaM binding
to the CaM binding domains is required for CaM to effectively
diminish Gßy subunit interaction with the AT1A receptor ß loop and
carboxyl terminus.

We used a combination of techniques including GST-fusion
protein pull-downs, site-directed mutagenesis, and dansyl-CaM
fluorescence to pinpoint the amino terminal juxtamembrane
regions of the AT1A receptor ß loop and carboxyl terminus of the
receptor as the CaM binding domains. In that regard, truncated
portions of the ß loop (amino acids 213–234) and carboxyl tail
(residues 297–324) were able to efficiently pull-down CaM,
whereas adjacent regions of the receptor were not. Alignment of
the truncated sequences with other well established CaM binding
motifs suggested that the two CaM binding motifs in the AT1A
receptor could be narrowed down to amino acids 214–231 in the
ß loop, and 302–317 in the carboxyl tail. Our work is consistent
with that of Thomas and colleagues, who previously identified a
putative CaM binding domain in a fusion protein from the
juxtamembrane region of the AT1A receptor carboxyl terminus
[41]. Furthermore, the interaction appeared to be of high affinity
in that the peptide induced a CaM mobility shift in a urea gel,
which is generally indicative of an affinity better than 100 nM.
Our work validates and extends their report in a number of
respects. We showed that the AT1A holo-receptor interacts with
CaM by the BRET method. We identified a second CaM
interaction domain in the ß loop of the AT1A receptor, identified
CaM interaction motifs and calculated the affinities of the
interaction of CaM for both sites. We identified three residues in
the AT1A receptor (W219, F309, and F313) that are critical for
efficient coupling of CaM to the receptor, by computer modeling
and mutagenesis. We also showed that CaM binding is necessary
to inhibit binding of G protein ßy dimers to both sites. Moreover,
the binding requirements for ßy and CaM are distinct in that
mutations that reduced the binding of CaM to both regions had
little measurable effect on ßy binding.

We measured the affinities of synthetic peptides corresponding
to those regions by examining the spectral shifts of dansyl-CaM in
the presence of Ca2+ and candidate peptides, assuming that
spectral shifts reflect induction of dramatic conformational
changes in CaM. Indeed, it is known that CaM assumes a
dumbbell-shaped conformation upon loading with Ca2+ [55],[56],
and a compact globular conformation when interacting with
various target peptides [49]. Both putative CaM binding domains
were shown to have relatively high affinities for CaM, 79.4±7.9 nM for AT1(302–317), and 177.0±29.1 for AT1(214–
231). The differences in the affinities of these peptides for CaM
could be functionally significant in that CaM appears to be
somewhat more effective in diminishing Gßy binding to the AT1A
receptor carboxyl terminus than to the ß loop (Figure 7B). These
affinities are similar to those reported for other GPCRs, including
the 5-HT1A (87 nM and 1.70±0.83) and 5-HT2A (63 and 160 nM)
receptors.

CaM is an important regulatory molecule, which functions as
the major calcium-sensor in most cells [30]. CaM has been shown
to regulate many signaling molecules and effectors, including
kinases, phosphatases and other enzymes, ion channels, transcription
factors, receptors and cytoskeletal proteins. CaM has also
been shown to bind to a small number of GPCRs, although the
functional significance of these interactions is only now being
elucidated. CaM binding to D2-dopamine, 5-HT1A, µ-opioid
and group III metabotropic glutamate mGluR7a receptors, regulates
functional coupling of the receptors to pertussis toxin-sensitive
heterotrimeric Gi/o protein ß- subunits [36], [40], [45], [57], [58].
Similarly, CaM binding to the 5-HT2A and V2 vasopressin
receptors attenuates coupling to Go/11 ß-subunits, GTPyS
binding and/or Ca2+ mobilization [42], [43]. CaM binding also
impairs phosphorylation of peptides derived from regulatory
regions of the 5-HT1A, 5-HT2A, and mGluR5 receptors [37],
[43], [45]. Thus, CaM can attenuate both receptor phosphoryl-
ation and propagation of G protein ß-subunit-dependent signals
for a small group of GPCRs.

A role for CaM in regulating Gßy subunit binding to
metabotropic glutamate receptors has previously been proposed.
O’Connor and colleagues showed that CaM can bind to a fusion
protein containing the carboxyl terminus of the metabotropic
glutamate receptor subtype 7 (mGluR7), and that CaM inhibited
ßy binding to the same sequence [34]. The authors suggested that
G protein-mediated signaling was enhanced by CaM through
displacement of Gßy subunits from the carboxyl terminus. El Far
and colleagues confirmed those observations, and described
similar motifs in the carboxyl termini of mGluR4a, mGluR7B,
mGluR8A, and mGluR8B, perhaps indicating that the mutually
exclusive binding of CaM and Gßy to the carboxyl termini of
mGluR is an important signaling mechanism [36]. Up to now, this
effect has only been described for mGluRs, which are class 3
GPCRs [59]. Our work demonstrates that this process is not
limited to mGluRs or class 3 GPCRs, in that it occurs in the
carboxyl terminus of an important class 1 GPCR, the AT receptor
[59]. Additionally, we showed that another intracellular domain
of the receptor (ß loop) also contains a sequence for which CaM and
ßy compete. We further demonstrated that the sequence
requirements for CaM and ßy binding to both sites in the AT1A
receptor overlap, but are not identical. Regardless of whether
CaM prevents binding of ßy, or hastens its displacement, this
process can clearly modulate GPCR signal transduction.

Overall, the present study suggests that CaM may function as a
regulator at interface between AT1A receptor and G proteins by
targeting G protein ßy subunit interacting with the receptor.

Acknowledgments

We thank Dr. John R. Raymond for his editorial review of the manuscript.
We thank Wenle Zhao of the Department of Biostatistics, Bioinformatics &
Epidemiology at MUSC for statistical analysis of the data. We also thank
Dr. Justin Turner (Department of Medicine, MUSC) for providing plasmid
constructs.

Author Contributions

Conceived and designed the experiments: QY. Performed the experiments:
QY RZ. Analyzed the data: QY. Contributed reagents/materials/analysis
tools: QY. Wrote the paper: QY. Structural Modeling and Evaluation of
binding affinity: ZL YQ YX.
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