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Calcium/calmodulin regulates signaling at the α_{1A} adrenoceptor

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Abstract

Cardiovascular functions are mediated by multiple 7-pass transmembrane receptors whose activation promotes contraction or relaxation of the tissues. The α_1 adrenoceptor type 1A plays important roles in the control of vascular tone and myocardial contractility via Ca^{2+} -dependent actions. Here, using novel FRET-based biosensors, we identified a novel Ca^{2+} -dependent interaction between calmodulin (CaM) and the human α_{1A} adrenoceptor at the juxtamembranous region of its 4th submembrane domain (SMD4_{JM}, a.a. 333-361). SMD4_{JM} houses the known nuclear localization signal of α_{1A} adrenoceptor (NLS, a.a. 334-349). We found that NLS itself also interacts with CaM, but with lower affinity and Ca^{2+} sensitivity, indicating that full interaction between CaM and α_{1A} receptor in this region requires segment a.a. 333-361. Combined K353Q/L356A substitutions in the non-NLS segment of SMD4_{JM} cause a 3.5-fold reduction in the affinity of CaM-SMD4_{JM} interaction. Overexpression of wild-type α_{1A} adrenoceptor in cells enhances phosphorylation of the extracellular signal-regulated kinases 1/2 (ERK1/2) stimulated by A61603, while overexpression of the K353Q/L356A α_{1A} receptor mutant significantly reduces this signal. Norepinephrine stimulates intracellular Ca^{2+} signals that are higher in cells overexpressing wild-type receptor but lower in cells overexpressing the K353Q/L356A receptor compared to non-transfected cells in the same microscopic environments. These data support a novel and important role for Ca^{2+} -dependent CaM interaction at SMD4_{JM} in α_{1A} adrenoceptor-mediated signaling.

Graphical Abstract

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Keywords

α_{1A} adrenoceptor; submembrane domain; calmodulin; calcium; phosphorylation

1. Introduction

Activation of the α_{1A} adrenoceptor triggers vasoconstriction (Minneman, 1988), hypertrophy and proliferation (Gradinaru et al., 2015; Lei et al., 2013). In the heart, the α_{1A} adrenoceptor is more abundant than the α_{1B} or α_{1D} isoform (Jensen et al., 2009), and constitutes ~16% total adrenergic response. However, in failing hearts, α_{1A} receptor accounts for ~39% adrenergic response (Steinfath et al., 1992; Wolff et al., 1998), due to reduced β_1 adrenoceptor expression (Cowley et al., 2015; Woodcock et al., 2008).

The α_{1A} adrenoceptor is a 7-pass transmembrane (7TM) receptor associated with $G\alpha_q\beta\gamma$ heterotrimeric complex. Its activation triggers Ca^{2+} entry, promoting formation of Ca^{2+} -calmodulin (CaM) complexes that modulate numerous CaM-binding proteins. Via other mechanisms, α_{1A} receptor stimulation transactivates epidermal growth factor receptor, activating extracellular signal-regulated kinases (ERK1/2) and survival pathways (Lei et al., 2013; Prenzel et al., 1999). In cardiomyocytes, most α_{1A} adrenoceptor resides on nuclear membrane. A nuclear localization signal (NLS) spans a.a. 334-349 on SMD4 (Wright et al., 2012).

The Ca^{2+} -binding protein calmodulin interacts with ~300 proteins (Shen et al., 2005) yet is not sufficiently expressed for all its targets (Kakiuchi et al., 1982). This shortage generates competition that shapes behaviors of CaM target proteins (Song et al., 2008; Tran, 2005; Tran et al., 2003; Wu and Bers, 2007). Recently, CaM was shown to interact with some 7TM receptors and regulate receptor phosphorylation (Minakami et al., 1997), G-protein association (O'Connor et al., 1999; Turner et al., 2004; Wang et al., 1999), β -arrestin recruitment (Labasque et al., 2008), receptor-mediated signaling (Ehlers et al., 2018; Tran, 2014; Tran et al., 2016). Most binding sites are found on SMD3 (Ehlers et al., 2018; Tran, 2014; Wang et al., 1999; Zhang et al., 2013) or SMD4 (Labasque et al., 2008; Minakami et al., 1997; Thomas et al., 1999; Tran, 2014; Turner et al., 2004), although SMD1 and SMD2 were reported (Ehlers et al., 2018; Tran, 2014).

In the cardiovascular system, actions of CaM at the receptor level are only beginning to be revealed. We recently used a biosensor method to identify CaM-binding sites and found that CaM interactions with some receptors are important for cellular events leading to cardiovascular responses (Ehlers et al., 2018; Tran, 2014; Tran et al., 2016). We found that

interactions between CaM and the angiotensin II type 1 (AT₁) receptor at SMDs 2, 3 and 4 are important for AngII-induced Ca²⁺ signals and ERK1/2 phosphorylation (Ehlers et al., 2018). We observed that CaM binds GPER1 on four SMDs (Tran, 2014). 17 β -estradiol, by activating GPER1, upregulates CaM and inhibits Ca²⁺ efflux, promoting activities of various CaM targets, including GPER1 itself (Tran et al., 2016). CaM is involved in many processes downstream of α_{1A} adrenoreceptor activation. However, it is entirely unknown if CaM regulates α_{1A} adrenoreceptor activities at the receptor level. Here, we report novel interaction between CaM and this receptor at the juxtamembranous region of SMD4 (SMD_{4JM}), a segment that encompasses its NLS. We characterized the binding properties and examined the functional role of this interaction using pharmacological and loss-of-function mutagenesis studies.

2. Materials and Methods

2.1. Materials

RNeasy kit was purchased from Qiagen. High Capacity cDNA reverse transcription kit was from Applied Biosystems (Foster City, CA). Restriction enzymes and competent cells were purchased from New England Biolabs (Ipswich, MA). PHUSION PCR reaction mix was from Thermo Fisher (Waltham, MA). Fetal bovine serum and isopropyl β -D-1-thiogalactopyranoside were obtained from Sigma-Aldrich (St. Louis, MO). HisPur cobalt resin was from Thermo Fisher (Waltham, MA). Phenyl Sepharose CL-4B resin was from GE Healthcare Life Sciences (Milwaukee, MI). DMEM culture medium was from Caisson Laboratories (Smithfield, UT). Polyethylenimine and penicillin/streptomycin were from ThermoFisher (Carlsbad, CA). Fura-2/AM was purchased from Teflabs (Austin, TX). Ionomycin and XRhod-5F were obtained from ThermoFisher Scientific (Carlsbad, CA). Rabbit monoclonal anti-HA antibody (C29F4) and anti-phospho-p44/42 MAPK mouse monoclonal antibody (L34F12) were from Cell Signaling Technology (Danvers, MA). Anti-ERK1/2 antibody was from Santa Cruz Biotechnology (SC514302, Santa Cruz, CA). Protease inhibitor cocktail was from EMD Millipore (Burlington, MA). Phenylmethane sulfonyl fluoride was from Sigma-Aldrich (St. Louis, MO). Amido Black B was from Bio-Rad (Hercules, CA). A61603 and norepinephrine were from Cayman Chemical (Ann Arbor, MI).

2.2. Molecular biology

Total mRNA was isolated from human embryonic kidney (HEK) 293 cells using the RNeasy kit. Total cDNA was reverse transcribed from this mRNA using the SuperScript III One-Step RT-PCR kit (Invitrogen, Carlsbad, CA). Full-length human α_{1A} adrenoreceptor was PCR amplified from this cDNA and incorporated in a pcDNA3.1 mammalian expression vector previously used for G protein-coupled estrogen receptor 1 (Tran et al., 2015). The mKate2- α_{1A} adrenoreceptor construct was generated by PCR amplifying mKate2 from the pDONR-P1P4-mKate2 plasmid (a gift from the Alanas and Santalucia laboratories, [Addgene.org](https://addgene.org)) while incorporating the BamHI and KpnI restriction sites flanking mKate2. This fragment was then inserted upstream of α_{1A} adrenoreceptor in the pcDNA3.1 plasmid. The HA epitope was generated using primers spanning the entire HA tag sequence with flanking BamHI and KpnI restriction sites and then inserted upstream of α_{1A} adrenoreceptor as with mKate2 above

to generate the HA- α_{1A} adrenoceptor construct. The combined substitutions K353Q/L356A, located in SMD4_{JM} of α_{1A} adrenoceptor downstream the nuclear localization signal (NLS), were generated by sequential site-directed mutagenesis. To generate FRET-based biosensors with the NLS (a.a. 334 – 349) or SMD4_{JM} (a.a. 333 – 361), these fragments were PCR amplified from the full-length wild-type or K354Q/L356A mutant sequences, introducing a KpnI and AgeI restriction site pair at the N- and C-termini, respectively. The resulting fragments were then introduced between ECFP and EYFP_C fluorophores in the same framework of previously published FRET-based biosensors (Ehlers et al., 2018; Tran, 2014; Tran et al., 2016). All constructs were verified by DNA sequencing (University of Missouri – Columbia).

2.3. Expression and purification of FRET biosensors and CaM

We termed FRET biosensors for α_{1A} adrenoceptor BS α_{1A} -AR_x, where x denotes the amino acid sequence of the insert representing potential CaM-binding sequence under examination. pET bacterial vectors encoding BS α_{1A} -AR_x contained a 6-histidine tag at the C-terminus of the ECFP moiety. The vectors were expressed in BL21(DE3) E. Coli competent cells and purified using Cb²⁺ resin affinity chromatography as described in detail previously (Ehlers et al., 2018; Tran, 2014; Tran et al., 2016). The pET-CaMI plasmid was expressed and purified as described recently (Ehlers et al., 2018; Tran, 2014; Tran et al., 2016).

2.4. Characterization of α_{1A} adrenoceptor biosensor – CaM interactions

Detection of direct interactions between CaM and SMD4_{JM} in α_{1A} adrenoceptor were initially determined by titrating in incremental amounts of purified CaM into a mixture of 500 nM BS α_{1A} -AR_x, 0.1 mg/mL BSA, and 2 mM CaCl₂ in a quartz cuvette (Hellma Analytics, Plainview, NY). Biosensor responses were measured using a QuantaMaster™-40 spectrofluorometer (Photon Technology International Inc., Edison, NJ). Based on FRET principles, we employed three criteria for positive interaction between Ca²⁺-CaM and BS α_{1A} -AR_x: 1) an increase in donor emission, 2) a corresponding decrease in acceptor emission, and 3) crossing of the emission spectra at the isoemissive point (~510 nm) in response to different concentrations of Ca²⁺-CaM (Tran, 2014). Ca²⁺ dependency of the interactions was determined by testing biosensor response in the presence of saturating Ca²⁺ concentration or 0.25 mM Br₂BAPTA and no added Ca²⁺. Biosensor fractional saturation upon addition of Ca²⁺-CaM was determined by the equation

$$BS_{fract} = \frac{R - R_{min}}{R_{max} - R_{min}} \quad (1)$$

where BS_{fract} is BS α_{1A} -AR fractional saturation, R is the ratio between donor and acceptor emission intensities. R_{min} is the ratio in the unbound state, and R_{max} is the ratio in the maximal bound state. BS α_{1A} -AR_x fractional saturation was plotted as a function of free CaM. Dissociation constants were obtained by $BS_{fract} = \frac{R - R_{min}}{R_{max} - R_{min}}$ fitting BS_{fract} as a function of free Ca²⁺-CaM to the equation

$$BS_{fract} = \frac{[BS] - [CaM] - K_d - \sqrt{([BS] + [CaM] + K_d)^2 - 4[BS][CaM]}}{2[BS]} \quad (2)$$

where BS_{fract} , $[BS]$ and $[CaM]$ are $BS\alpha_{1A}$ -AR fractional response upon Ca^{2+} -CaM additions, and the total concentrations of $BS\alpha_{1A}$ -AR_x and CaM in the mixture, respectively.

$$Ca^{2+}(\mu M) = 1.6 \times \frac{F - F_{min}}{F_{max} - F} \quad (3)$$

where 1.6 is the in vitro K_d value (μM) of XRhod-5F for Ca^{2+} ; F_{min} and F_{max} are XRhod-5F's fluorescence intensities measured at $\lambda 600$ nm under nominally Ca^{2+} -free and Ca^{2+} -saturating conditions, respectively. Ca^{2+} sensitivity of $BS\alpha_{1A}$ -AR_x-CaM interactions were determined as the $EC_{50}Ca^{2+}$ values, derived from fits of BS_{fract} as a function of free Ca^{2+} using the equation

$$BS_{fract} = \frac{[Ca^{2+}]_{free}^n}{[Ca^{2+}]_{free}^n + [EC_{50}(Ca^{2+})]^n} \quad (4)$$

where BS_{fract} was from the equation (1); n is the Hill coefficient.

2.5. Cell culture and transfection

Human embryonic kidney (HEK) 293 cells were purchased from AddexBio (T0011001, initial passage 10) and cultured in DMEM medium with 10% fetal bovine serum and 1% penicillin/streptomycin in 90% humidified condition with 5% CO_2 at $37^\circ C$. Transfection was carried out as described earlier (Ehlers et al., 2018). Plasmid DNAs were incubated with polyethylenimine (PEI) at room temperature at a PEI-DNA mass ratio of 1.5:1 for 20 min in serum-free and antibiotic-free DMEM. HEK293 cells grown to 60% confluency were incubated with 1:5 vol/vol DNA-PEI complex in DMEM containing 2% FBS for 6 h. DNA-PEI complex was next removed, FBS was increased to 10% and cells were cultured for another 12 h prior to experiments.

2.6. Immunoblotting

Four h prior to agonist treatment, transfected HEK293 cells were serum-starved in DMEM containing no FBS and cultured under regular conditions. Treatment with vehicle or agonist was carried out at room temperature in Modified Tyrode's buffer (in mM: 150 NaCl, 2.7 KCl, 1.2 KH_2PO_4 , 1.2 $MgSO_4$, 10 HEPES, 1.5 $CaCl_2$, pH 7.4) containing 5 mM dextrose. Following treatment and cell lysis, lysate was centrifuged for 10 min at $21,000 \times g$, the supernatant was collected, followed by triplicate determination of protein content using a Pierce™ BCA assay (Thermo Scientific, Carlsbad, CA). All lanes were loaded with equal amounts of total protein, adjusted for detectability with each antibody. To assess ERK1/2

phosphorylation, following detection of phosphorylated ERK1/2, the same membrane fragments were stripped and reprobed for total ERK1/2 expression levels. After incubation with secondary antibody, membrane fragments were developed with enhanced chemiluminescence in a ChemiDoc™ XRS+ imaging system (Bio-Rad). Densitometric values of phosphorylated ERK1/2 bands were obtained using the Image Lab 5.0 software (Bio-Rad) and corrected for those of total ERK1/2 bands and normalized to control values.

2.7. Measurement of intracellular Ca^{2+} in cells expressing mKate2- α_{1A} receptor fusions

Intracellular Ca^{2+} concentration was measured in HEK293 cells expressing mKate2 only or mKate2 in fusion with wild-type or mutant full-length α_{1A} adrenoceptor using a method described previously (Ehlers et al., 2018; Tran et al., 2016; Tran et al., 2015). Transfected HEK293 cells were loaded with 4 μM fura-2/AM for 30 min in DMEM medium containing 2% FBS at 37°C. Following removal of fura-2/AM, the cells were equilibrated in modified Tyrode's buffer (composition in mM: 150 NaCl, 2.7 KCl, 1.2 KH_2PO_4 , 1.2 MgSO_4 , 10 HEPES, 1 CaCl_2 , 5 glucose, pH 7.4) for 15 min at room temperature. Fluorescence of mKate2 was first identified and selected in RFP channel. Cells expressing mKate2 fluorescence in the same range were selected, followed by switching of imaging filter cube to the fura-2 channel while cells were kept at the same microscopic field. Excitation of fura-2 was alternated between $\lambda 340$ nm and $\lambda 380$ nm for 100 ms per 1-s cycle from an ultra-high speed wavelength switcher (Lambda DG-4, Sutters Instruments) at 1-ms switching interval. Emission of fura-2 at $\lambda 510$ nm was collected via an EMCCD camera (DU-885, Andor Technology).

2.8. Statistical Analysis

Data are expressed as means $\pm$ S.E.M. Statistical analysis was done using Student's *t* test or one-way ANOVA followed by a Tukey *post-hoc* test where appropriate. Statistical significance was determined as $P < 0.05$.

3. Results

3.1. Identification of CaM binding site on α_{1A} adrenoceptor at the juxtamembranous region of submembrane domain 4 (SMD4_{JM})

To test CaM interaction with SMD4_{JM} in α_{1A} adrenoceptor, we used a FRET biosensor-based approach that we previously reported to identify and characterize new CaM-binding domains in 7TM receptors (Ehlers et al., 2018; Tran, 2014; Tran et al., 2016). Fig. 1A shows the topographic amino acid sequence of SMD4, in which the known nuclear localization signal is shown in blue. The diagram in Fig. 1B re-encapsulates our method to identify CaM-binding domains on 7-pass transmembrane receptor. A sequence of interest from a submembrane domain is inserted between an enhanced CFP (ECFP)-enhanced citrine YFP (EYFP_c) FRET donor-acceptor pair. We named such biosensors BS α_{1A} -AR_x, where x denotes the amino acid numbering of the sequence to be tested for CaM binding. In the absence of CaM, proximity between the donor and acceptor allows for robust FRET when the ECFP moiety is excited at 430 nm, generating a spectrum with two separate peaks (475 nm for ECFP, and 535 nm for EYFP_c, Fig. 1B, left and right panels). In the presence of CaM, when there is specific interaction with the insert sequence, FRET will be disrupted,

causing (1) a large increase in ECFP (475 nm) emission, (2) a large decrease in EYFP_C (535 nm) emission, and (3) crossing of the spectra at the isoemissive point (510 nm) (Fig. 1B, middle and right panels). In the absence of specific interaction, up to 700 μ M CaM does not cause any change in FRET signal (Tran, 2014). The approach has helped identify CaM-binding domains that do not conform to any known binding motifs (Ehlers et al., 2018; Tran, 2014). Our aim here was to identify the sequence that demonstrated the highest affinity for CaM in the SMD4_{JM} region of α_{1A} adrenoceptor. Fig. 1C shows a Coomassie gel of three BS α_{1A} -AR_X's. Initial screening indicated that segment 333-361 (SMD4_{JM}) directly interacts with CaM in the presence of saturating Ca²⁺; further lengthening of the insert did not increase binding affinity (not shown), while shortening of the insert reduced affinity. Fig. 1D shows the fractional saturation of BS α_{1A} -AR₃₃₃₋₃₆₁ upon incremental addition of Ca²⁺-saturated CaM. Clear spectral changes were observed that fulfilled the three criteria described in Fig. 1B for direct interaction with CaM. Since this domain encompasses the receptor's known NLS (a.a. 334–349), we tested if the NLS alone binds CaM. Using the same method, BS α_{1A} -AR₃₃₄₋₃₄₉ binds CaM directly, but with a 3-fold lower affinity (Fig. 1E). Fits of biosensor fractional saturation (Eq. 1, Experimental Procedures) as a function of CaM to Eq. 2 (Experimental Procedures) yielded K_d values of 1.06 ± 0.04 and 3.05 ± 0.14 μ M for BS α_{1A} -AR₃₃₃₋₃₆₁ and BS α_{1A} -AR₃₃₄₋₃₄₉, respectively (Fig. 1F).

3.2. Ca²⁺ dependency and Ca²⁺ sensitivity of CaM interaction at SMD4_{JM}

The data in Fig. 1 indicated Ca²⁺-dependent interaction between SMD4_{JM} and CaM. CaM interaction with targets can be Ca²⁺-independent and/or Ca²⁺-dependent (Black et al., 2007; Black et al., 2004). To determine the Ca²⁺ dependency of the interactions between CaM the NLS and the full SMD4_{JM}, BS α_{1A} -AR₃₃₄₋₃₄₉ and BS α_{1A} -AR₃₃₃₋₃₆₁ responses were tested to respective saturating concentrations of CaM obtained in Fig. 1 in the presence of saturating concentration of Ca²⁺ (Fig. 2A and C) or absence of Ca²⁺ and presence of 0.25 mM BAPTA (apoCaM) (Fig. 2B and D). Ca²⁺-saturated CaM produced the spectral changes described and observed in Fig. 1. However, apoCaM did not, indicating Ca²⁺-dependency of the interactions.

An important biochemical parameter for Ca²⁺-dependent CaM interaction with a target protein is the Ca²⁺ sensitivity of the association. As the interaction depends at the front end on the formation of Ca²⁺-CaM complexes and thus the amplitude and dynamics of the Ca²⁺ signals produced in cells, this information will allow for assessment of the Ca²⁺ level required to trigger an interaction between CaM and the target and for prediction if the interaction can take place in the cell in response to a stimulus. To determine the Ca²⁺ sensitivity of the interactions between CaM and BS α_{1A} -AR₃₃₃₋₃₆₁ and BS α_{1A} -AR₃₃₄₋₃₄₉, incremental Ca²⁺ was titrated in a mixture of each and respective saturating concentration of CaM determined from CaM titrations (Fig. 1) and a suitable Ca²⁺ indicator. Selection of the Ca²⁺ indicator was based on the proximity between known K_d values for Ca²⁺ of available Ca²⁺ indicators and initial estimates of the EC₅₀(Ca²⁺) values of biosensor-CaM interactions from Ca²⁺ titrations. XRhod-5F was chosen for these biosensors as its affinity for Ca²⁺ falls in the range of the estimated EC₅₀(Ca²⁺) values for interactions between CaM and BS α_{1A} -AR₃₃₃₋₃₆₁ and BS α_{1A} -AR₃₃₄₋₃₄₉. Fig. 3A shows concurrent biosensor and XRhod-5F signals as Ca²⁺ was incrementally added to the biosensor-CaM mixture. In this

set up, excitation/emission spectra of ECFP-EYFP_c are far separated from those of XRhod-5F; in addition, BS α_{1A} -AR and XRhod-5F were alternately excited, so that fluorescence bleed-through was avoided. To verify this, Ca²⁺ was titrated a mixture of BS α_{1A} -AR and XRhod-5F in the absence of CaM and presence of 0.25 mM BAPTA. Only XRhod-5F fluorescence signal was incrementally increased as a function of added Ca²⁺ (lower panel, Fig. 3B); the response of BS α_{1A} -AR_x was only increased upon addition of CaM in the presence of Ca²⁺ (upper panel, Fig. 3B). To determine the Ca²⁺ sensitivity of BS α_{1A} -AR₃₃₄₋₃₄₉ and BS α_{1A} -AR₃₃₃₋₃₆₁, Ca²⁺ was incrementally titrated in a mixture of biosensor, XRhod-5F, 0.25 mM BAPTA, and saturating CaM concentrations determined from Fig. 1. After titrations, fractional saturations of BS α_{1A} -AR_x were then calculated based on Eq. 1, while free Ca²⁺ values were calculated based on Eq. 3 (Experimental Procedures). Fig. 3C shows aggregate plots of biosensor fractional responses as a function of free Ca²⁺. Fits of these plots to Eq. 4 yielded EC50(Ca²⁺) values for BS α_{1A} -AR₃₃₃₋₃₆₁ and BS α_{1A} -AR₃₃₄₋₃₄₉ interactions with CaM to be 0.7 ± 0.03 and 1.34 ± 0.02 μ M.

3.3. CaM binding at SMD4_{JM} is important for α_{1A} receptor-mediated ERK1/2 phosphorylation

To examine the role of CaM binding to SMD4_{JM} in α_{1A} adrenoceptor's function, we generated two substitutions in the SMD4_{JM} CaM-binding sequence outside of the NLS, K353Q/L356A, aiming to reduce CaM binding affinity. The K353Q/L356A SMD4_{JM} was then introduced into a BS α_{1A} -AR_x biosensor format, followed by determinations of CaM binding affinity and Ca²⁺ sensitivity as described in Figs. 1 and 3. BS α_{1A} -AR_{333-361mut} binds Ca²⁺-CaM with an affinity of 3.8 ± 0.57 μ M, a 3.5-fold reduction in affinity from the wild-type value (Fig. 4A, Table-2). The EC50(Ca²⁺) value for CaM binding to BS α_{1A} -AR_{333-361mut} was 1.26 ± 0.05 μ M, a 1.8-fold reduction in Ca²⁺ sensitivity vs the wild-type biosensor (Fig. 4B, Table-2).

To assess the role of CaM binding at SMD4_{JM} in α_{1A} adrenoceptor-mediated signaling, wild-type and K353Q/L356A full-length α_{1A} adrenoceptor sequences were fused at the N-termini to a hemagglutinin (HA) epitope tag. Plasmids encoding HA-tagged α_{1A} receptor with wild-type or the K353Q/L355A mutant sequence were then transfected in HEK293 cells. Twenty-four h after transfection, the cells were serum starved for 4 h in DMEM culture media containing no serum, followed by treatment with vehicle or 100 nM A61603, a specific agonist for α_{1A} receptor ($K_d = 9$ nM) (Knepper et al., 1995). Immunoblots for the HA epitope in lysate of the transfected cells showed no immunoactivity in lysates from mock-transfected cells and equal levels of expression of the heterologously expressed receptors, with bands detected at ~42, 51 and 60-kDa levels, with some small differences in the migration patterns of the lower bands for the K353Q/L355A mutant (Fig. 5A, upper blot). The presence of these bands is consistent with previous observations of epitope-tagged α_{1A} receptor overexpressed in HEK293 cells (Vicentic et al., 2002). Post-development Amido Black staining of SDS-PAGE membranes indicate equal loading across samples (Fig. 5A, lower vignette). The same samples were simultaneously immunoblotted for phosphorylated ERK1/2, followed by mild stripping and reprobing of the same membranes for total level of ERK1/2 (Fig 5B, upper and middle blots). Treatment of mock-transfected cells with 100 nM A61603 triggered a 10-fold increase in ERK1/2 phosphorylation. In cells

overexpressing wild-type HA-tagged α_{1A} receptor, a 20-fold increase in ERK1/2 phosphorylation was observed in response to A61603. However, the K353Q/L355A mutations significantly reduced the increase in ERK1/2 phosphorylation stimulated by A61603 to 4-fold (Fig. 5B, histogram).

3.4. CaM binding at SMD4_{JM} is important for α_{1A} adrenoceptor-mediated Ca^{2+} signaling

To further test the role of CaM binding at SMD4_{JM}, we assessed the effect of the K353Q/L356A substitutions on α_{1A} adrenoceptor-mediated Ca^{2+} signals. To this end, the wild-type and mutant receptors were fused at their N termini with the red fluorescent protein mKate2, and the fusions were each expressed in HEK293 cells. Transfected cells were then loaded with fura-2/AM for Ca^{2+} imaging. Transfected cells were first identified in the RFP channels (Fig. 6A & D), followed by switching of the imaging cube to monitor fura-2 signal (Fig. 6B & E). This approach allows for imaging and comparison of signals in both non-transfected and transfected cells in the same microscopic field (Fig. 6C & F). We have previously shown that expression of the mKate2 moiety alone does not interfere with the total Ca^{2+} signals induced by receptor agonist or by inhibitor of the sarcoplasmic/endoplasmic Ca^{2+} -ATPase (Ehlers et al., 2018; Terry et al., 2017). The endogenous α_{1A} receptor agonist norepinephrine (NE) stimulated a large Ca^{2+} signal in cells that did not express the heterologous receptor (black, Fig. 6G). In the same microscopic field, cells expressing the wild-type α_{1A} adrenoceptor in fusion with mKate2 responded to NE with a much higher Ca^{2+} signal (Fig. 6G, red). Cells expressing the mutant K353Q/L356A α_{1A} adrenoceptor in fusion with mKate2, however, responded to NE with a lower Ca^{2+} signal (Fig. 6H, blue) compared to cells that did not express mKate2-K353Q/L356A α_{1A} receptor in the same microscopic field (Fig. 6H, black). In these experiments, care was taken to select cells overexpressing the wild-type and mutant receptor with equal mKate2 fluorescence intensity to allow for valid comparison (Fig. 6I). To compare Ca^{2+} signals produced by cells expressing wild-type vs mutant α_{1A} adrenoceptor, the average fura-2 signals from the cells expressing either construct were divided point-by-point over the entire time course by those from non-expressing cells in the same respective microscopic fields. This exercise yielded an upward deflection time course for cells overexpressing the wild-type α_{1A} receptor, and a downward deflection for cells overexpressing the mutant receptor (Fig. 6J). To quantitate the differences across the time course of the signals, integrated areas under curve were calculated for both the average positive and negative deflections. A comparison of these values showed clear significant differences (Fig. 6K).

4. Discussion

The α_{1A} adrenoceptor plays important roles in the control of vascular tone and cardiac contractility. This study is the first to document a role for Ca^{2+} -dependent interaction between CaM and this receptor in regulating downstream signaling events. The interaction between purified CaM and BS α_{1A} -AR₃₃₃₋₃₆₁ and BS α_{1A} -AR₃₃₃₋₃₄₉ are specific, since we have demonstrated that in the absence of specific interaction with the insert sequence, up to 700 μM CaM does not cause any spectral changes in a biosensor response (Tran, 2014). Our data demonstrated that CaM interacts with the nuclear localization signal in α_{1A} adrenoceptor. However, based on the affinity difference between BS α_{1A} -AR₃₃₄₋₃₄₉ and

BS α_{1A} -AR₃₃₃₋₃₆₁, full interaction with CaM is predicted to involve the longer SMD4_{JM} segment (a.a. 333 - 361). It is noteworthy that the K_d values for these interactions were obtained with target sequences in biosensor format, and are likely different from those for the interactions between CaM and the holoreceptor, whose purification, as with other 7-pass transmembrane receptors, continues to represent a major challenge. Similar cautions should also be applied to values obtained using isolated peptides corresponding to the NLS or SMD4_{JM}. However, these values, easily and cost-effectively obtained using FRET reporters, provide a reliable parameter for comparison and determination of the precise interaction domains for CaM, as has been demonstrated in the cases of G protein-coupled estrogen receptor 1 and angiotensin II receptor type 1 (Ehlers et al., 2018; Tran, 2014). The Ca²⁺ sensitivity of CaM-SMD4_{JM} interaction was determined by simultaneous measurement of the responses of BS α_{1A} -AR₃₃₃₋₃₆₁ or BS α_{1A} -AR₃₃₄₋₃₄₉ and XRhod-5F in the presence of CaM as Ca²⁺ was titrated to the mixture. Given no bleed-through between the biosensor and XRhod-5F signals (Fig. 3B) and the concurrency of the measurements, the EC50(Ca²⁺) values obtained are highly accurate. Functionally, coupled with knowledge of intracellular Ca²⁺ concentrations, these parameters allow for prediction of the conditions in which CaM-SMD4_{JM} interaction can occur in cells. Thus, both EC50(Ca²⁺) values for CaM-NLS and CaM-SMD4_{JM} interactions are well within free nucleoplasmic and cytoplasmic Ca²⁺ levels (Ljubojevic et al., 2014), supporting a possibility that CaM binding at SMD4_{JM} can regulate α_{1A} receptor activities at both the plasmalemmal and nuclear membranes. The 0.7 μ M EC50(Ca²⁺) value for SMD4_{JM} and its Ca²⁺ titration curve (Fig. 4) suggest that CaM-SMD4_{JM} interaction may begin to occur at resting Ca²⁺ concentrations of 0.15 – 0.2 μ M.

Activation of α_{1A} adrenoceptor has been shown to transactivate the epidermal growth factor receptor, which stimulates pathways downstream of ERK1/2 (Lei et al., 2013; Prenzel et al., 1999). The increase in ERK1/2 phosphorylation induced by 100 nM A61603 can safely be considered as a functional parameter for α_{1A} adrenoceptor, since A61603 binds over 100-fold more specifically to the α_{1A} (K_d = 9 nM) than to the α_{1B} (K_d = 1103 nM) and the α_{1D} isoform (K_d = 1360 nM) (Knepper et al., 1995). At 100 nM, A61603 stimulates a ~10-fold increase in ERK1/2 phosphorylation from basal level in mock-transfected HEK293 cells. This most likely reflects activity of endogenous α_{1A} adrenoceptor, since our α_{1A} receptor cDNA was in fact reverse-transcribed from mRNA isolated from the same HEK293 cells. Compared to their respective control value, overexpression of HA-tagged wildtype α_{1A} receptor is associated with 20-fold increase while the K353Q/L355A mutant receptor is linked to only 4-fold increase in ERK1/2 phosphorylation signal upon treatment with A61603. These observations indicate that the heterologously expressed receptors overrides the endogenous receptor in mediating signaling and provide assurance for the use of behaviors in overexpressing cells to reflect molecular manipulations in the heterologous receptor. The K353Q/L356A substitutions were strategically generated outside of the NLS to avoid potential interference with possible nuclear localization of the receptor. We noted some small differences in the migration patterns of a few lower bands representing the HA-tagged wild-type and mutant receptors in Western blot analysis (Fig. 5A). These differences might be associated with the reduction in CaM binding at SMD4_{JM} and resultant changes in α_{1A} adrenoceptor-mediated signaling. However, we currently do not have a clear explanation for this observation and would require further examination.

Agonist-induced intracellular Ca^{2+} signals are a classical behavior of α_{1A} adrenoceptor activation leading to numerous downstream effects. Our data now show that the function of this receptor can be regulated at the receptor level by Ca^{2+} -dependent interaction with CaM. We utilized an imaging approach that allowed for measurement of Ca^{2+} signals in individual cells overexpressing the wild-type or mutant receptor and comparing with non-expressing cells in the same microscopic fields. The point-by-point ratios between fura-2 signals in cells expressing the mKate2-receptor fusions over those from cells that did not express the fusion in the same microscopic fields guarantees that the comparison was specific for α_{1A} adrenoceptor. Consistent with the ERK1/2 phosphorylation data, NE triggers robust Ca^{2+} signals in mock-transfected HEK293 cells, again indicating endogenous receptor activity, and overexpressing wild-type α_{1A} receptor is associated with significantly higher NE-induced Ca^{2+} signals than is overexpressing the mutant receptor that has a CaM-binding domain with reduced affinity for CaM. The changes in intracellular Ca^{2+} signals are likely to affect many downstream Ca^{2+} -dependent activities in the cell. Since formation of Ca^{2+} -CaM complexes to interact with α_{1A} adrenoceptor requires Ca^{2+} in the first place, a relevant question is how much of this change in intracellular Ca^{2+} affects Ca^{2+} -dependent α_{1A} receptor functions at the receptor level. It is worth noting that the Ca^{2+} signals measured here were total intracellular signals and not sub-PM or sub-nuclear membrane Ca^{2+} , where the receptor may reside. Solving this question quantitatively requires further technical development. However, it has been observed that sub-PM free Ca^{2+} levels can reach 45 μM while cytoplasmic Ca^{2+} rises to only 5 μM upon stimulation with vasopressin in aortic smooth muscle cells (Marsault et al., 1997). Given $\text{EC}_{50}(\text{Ca}^{2+})$ values of 0.7 and 1.26 μM for CaM binding to the wild-type and mutant α_{1A} receptor domains, respectively, it is unlikely that the observed reductions in the mutant α_{1A} receptor functions were due to reductions in Ca^{2+} in the vicinity of the receptor leading to reduced Ca^{2+} -dependent interaction with CaM. Rather, they are likely due to reductions in CaM binding *per se* to SMD4_{JM}, as suggested by the 3-fold reduction in K_{CaM} . In a native tissue, CaM- α_{1A} receptor interaction at this site then would strongly depend on the abundance of free CaM, whose expression has been shown to be limiting across cardiovascular tissues (Luby-Phelps et al., 1995; Song et al., 2008; Tran, 2005; Tran et al., 2003; Wu and Bers, 2007).

The in-cell experiments here were conducted in HEK293 cells, which express robust functional α_{1A} adrenoceptor, as indicated by our cloning of the receptor from their mRNA and their strong responses to α_{1A} receptor agonists both in ERK1/2 phosphorylation and Ca^{2+} signaling. However, care should still be taken in extrapolating the results to other cell systems due to potential differences in receptor expression, Ca^{2+} signaling machinery and CaM abundance.

In summary, we have identified a novel CaM-binding site for CaM at SMD4_{JM} in the α_{1A} adrenoceptor whose interaction with CaM can occur at physiological Ca^{2+} levels and is important for diverse downstream effects produced by α_{1A} receptor activation.

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Fig. 1. CaM interactions with α_{1A} adrenoceptor at the SMD4_{JM} region. (A) Topography of human α_{1A} receptor representing 7th transmembrane domain and SMD4. Known NLS is in blue. Red residues cover the identified CaM-binding region at SMD4_{JM}. (B) FRET-based approach to identify CaM-binding sequence. See text for brief explanation. EYFP_C, citrine enhanced YFP; ECFP, enhanced CFP; CBD, CaM-binding domain. Right panel, biosensor responses in the presence (blue spectrum) or absence (dotted spectrum) of CaM binding; arrows, spectral changes due to FRET alteration upon specific binding. (C) Coomassie gel of

the specified BSA_{1A}-AR_x. (D-E) Spectral changes of BSA_{1A}-AR₃₃₃₋₃₆₁ (D) and BSA_{1A}-AR₃₃₄₋₃₄₉ (E) upon titration of Ca²⁺-liganded CaM starting reaction mixture contained 0.5 μM BSA_{1A}-AR_x, 2 mM Ca²⁺, 0.1 mg/ml BSA in 100 mM KCl, 25 mM Tris, pH 7.4. (F) Fractional saturation of BSA_{1A}-AR₃₃₃₋₃₆₁ and BSA_{1A}-AR₃₃₄₋₃₄₉ as a function of Ca²⁺-CaM. Values are from 6 determinations.

Fig. 2.

Determination of Ca²⁺ dependency of CaM-α_{1A} adrenoceptor interactions. Spectral responses of BSα_{1A}-AR_x in the presence (black spectra) or absence (colored spectra) of added CaM. Specified CaM concentrations were added to a mixture of 0.5 μM BSα_{1A}-AR₃₃₄₋₃₄₉ (A, B) or BSα_{1A}-AR₃₃₃₋₃₆₁ (C, D) in 100 mM KCl, 25 mM Tris, pH 7.4 containing either saturating Ca²⁺ (2 mM) (A, C) or no Ca²⁺ in the presence of 0.25 mM Br2BAPTA (B, D). Data are representative of triplicate determinations for each biosensor.

Fig. 3.

Determination of Ca $^{2+}$ sensitivity for CaM interactions with SMD4 $_{JM}$ or NLS in α_{1A} adrenoceptor. (A) Simultaneous determination of the responses BS α_{1A} -AR $_x$ (upper panel) and XRhod-5F Ca $^{2+}$ indicator (lower panel) to incremental addition of Ca $^{2+}$. Initial reaction mixture contained 0.25 mM Br2BAPTA, 0.5 μ M BS α_{1A} -AR $_x$, 2 μ M XRhod-5F, 0.1 mg/ml BSA, and 100 μ M CaM. (B) Control for bleed-through. Responses of BS α_{1A} -AR $_x$ (upper panel) and XRhod-5F (lower panel) were simultaneously measured as incremental amounts of Ca $^{2+}$ were added to a mixture of 0.25 mM Br2BAPTA, 0.5 μ M BS α_{1A} -AR $_x$, 2 μ M

XRhod-5F, 0.1 mg/ml BSA, and no CaM. Upon saturation of XRhod-5F signal, 100 μ M CaM was added to the mixture. (C) Aggregate plots of fractional saturation for BS α_{1A} -AR₃₃₄₋₃₄₉ and BS α_{1A} -AR₃₃₃₋₃₆₁ as a function of free Ca²⁺. Data are composite of three independent determinations for each biosensor.

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Fig. 4.

Effects of the K353Q/L356A substitutions on the affinity and Ca^{2+} sensitivity for CaM interaction with SMD4_{JM}. (A) Plots of fractional saturations of BS α_{1A} -AR₃₃₃₋₃₆₁ containing wild-type or mutant sequences as a function of free Ca^{2+} -liganded CaM. Data are average of 3 independent determinations. (B) Aggregate plots of fractional saturation of BS α_{1A} -AR_x containing wild-type or mutant sequences in the presence of saturating CaM concentrations (derived from (A)) as a function of free Ca^{2+} . Data are composite of 6 independent determinations for each biosensor.

Fig. 5.

Effects of the K353Q/L356A substitutions on α_{1A} adrenoceptor-mediated ERK1/2 phosphorylation. HEK293 cells transfected with the specified plasmid for 24 h were serum-starved for 4 h prior to treatment with vehicle or 100 nM A61603. (A) Upper panel, Amido black staining of immunoblot demonstrating equal loading; Lower panel, expression of HA-tagged wild-type and mutant α_{1A} adrenoceptor. (B) Upper panel, phosphorylated ERK1/2; lower panel, membrane from ERK1/2 phosphorylation was stripped and reprobed for total ERK1/2. (C) Average relative phosphorylated ERK1/2; densitometric values of the ERK1/2

phosphorylation bands were corrected for those of corresponding total ERK1/2 levels. *, $P < 0.05$ from respective values for untreated samples; ϕ , $P < 0.05$ from A61603-treated cells expressing wild-type α_{1A} receptor; ψ , $P < 0.05$ from mock-transfected cells treated with A61603. $n = 6$ independent experiments.

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Fig. 6. Effects of the K353Q/L356A substitutions on α_{1A} adrenoceptor-mediated Ca^{2+} signals. A – C, Fluorescence of mKate2 (A, D), fura-2 (B, E) and merged (C, F) in HEK293 cell populations transfected with plasmids encoding the mKate2-wildtype α_{1A} receptor and mKate2-K353Q/L356A mutant α_{1A} receptor, respectively; G and H, fura-2 ratio signals in cells expressing mKate2- α_{1A} receptor (red) and cells that did not (black) in the same microscopic fields; I, mKate2 fluorescence intensities in cells expressing the specified mKate2- α_{1A} receptor plasmid; J, relative ratios of fura-2 signals in cells expressing mKate2-

wildtype α_{1A} receptor (red) and mKate2-K353Q/L356A α_{1A} receptor (blue) over those from non-expressing cells in the same microscopic fields; K, integrated area under curve values for data from J. n = 50 cells from 6 independent experiments for each α_{1A} receptor construct. *, $P < 0.05$ vs control.

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Table-1:

Primers

Fragments	Forward primer	Reverse primer
RTPCR for α_{1A} receptor	5'-GAATCCAGTGTCTCTGC-3'	5'-TGGACACTGTAAATCCTG-3'
BamHI- α_{1A} receptor -XbaI	5'-CTCGGGATCCATGGTGTTTCTCTCGGGAA-3'	5'-CTCGTCTAGACTAGACTTTCCTCCCCGTTTC-3'
KpnI-SMD4 _{IM} -AgeI	5'-CTCGGGTACCTTCAAAAAGGCCTTTTCAG-3'	5'-CTCGACCCGGTGCCACCGTGCAAGGGTGTA-3'
AfeI-....SMD4 _{IM} mut....-PflMI	5'AAGAGGCCAAAAAGGCCGCCAGAAATGTCTTTGACAGATCCAGTGTCTCTGCAGACAGCAG-3'	5'-CTGCTGTCTGCAGAGACACTGGATCTGCAAGACATTCTGGCGGCCTTTTTGGCCTCTT-3''
KpnI-SMD4 _{IM} mut-AgeI	5'-CTCGGTACCTTCAAAAAGGCCTTTTCAG-3'	5'-CGATACCGGTGTCAGGGTGTAGCCGCGGCATGCTGGGAAAGACTG-3'
KpnI-NLS-AgeI	5'-CTCGGTACCTTCAAAAAGGCCTTTTCAG-3'	5'-CTCGACCGGTCTGCTTTCTGCAAG-3'
KpnI - HA - BamHI	5'-CTCGGTACCATGTACCCATACGATGTTCCAGAT-3'	5'-CTCGGGATCCAGCGTAATCTGGAACATCGTATGG-3'

Table-2:Apparent K_d and $EC50(Ca^{2+})$ values of $BS\alpha_{1A}-AR_x$

Biosensor	K_d values (μM)	$EC50(Ca^{2+})$ (μM)
$BS\alpha_{1A}-AR_{333-361}$	1.02 ± 0.03	0.70 ± 0.03
$BS\alpha_{1A}-AR_{333-361}$ mut	3.8 ± 0.57	1.26 ± 0.05
$BS\alpha_{1A}-AR_{334-349}$	3.07 ± 0.14	1.34 ± 0.02