

Full Paper

Identification of Cysteine-Rich Epidermal Growth Factor–Like Domain 1 α (CRELD1 α) as a Novel α_{1A} -Adrenoceptor–Down-Regulating Protein and Establishment of an α_{1L} -Adrenoceptor–Expressing Cell Line

Atsushi Nishimune^{1,2,†}, Fumiko Suzuki^{1,2,†}, Hatsumi Yoshiki¹, Shigeru Morishima¹, and Ikunobu Muramatsu^{1,2,*}

¹Division of Pharmacology, Department of Biochemistry and Bioinformative Sciences, and ²Organization for Life Science Advancement Programs, School of Medicine, University of Fukui, 23-3 Matsuoka-Shimoaizuki, Eiheiji, Fukui 910-1193, Japan

Received April 5, 2010; Accepted April 21, 2010

Abstract. Two distinct α_1 -adrenoceptor phenotypes (α_{1A} - and α_{1L} -ARs) are known to originate from a single ADRA1A(α_{1a}) gene by an as-yet-unknown mechanism. We hypothesized that an α_{1a} -AR–interacting protein could generate the α_{1L} -AR phenotype and we sought to identify such a protein and to examine its effects on the expression of α_{1A} and α_{1L} phenotypes. Cysteine-rich epidermal growth factor–like domain 1 α (CRELD1 α) was first identified using a yeast two-hybrid approach as an α_{1a} -AR–interacting protein. Transfection of α_{1a} -AR cDNA alone yielded Chinese hamster ovary (CHO) cells expressing α_{1A} -ARs having a predominant high affinity site for prazosin, with a low proportion (<10%) of prazosin-low affinity sites (α_{1L} -AR). Knockdown of endogenous CHO-CRELD1 α [α_{1a} -CKD(α_{1A} -enhanced) cells] enhanced the expression of α_{1A} -AR, whereas over-expression of CRELD1 α reduced α_{1A} -AR expression, yielding α_{1a} -COE(α_{1L} -dominant) cells expressing a high proportion (50%) of the α_{1L} -AR phenotype. The ligand binding and functional agonist and antagonist profiles in α_{1a} -CKD(α_{1A} -enhanced) and α_{1a} -COE(α_{1L} -dominant) cell lines were entirely in accord with the α_{1A} -AR and α_{1L} -AR phenotypes observed in intact tissues. CRELD1 α down-regulates expression of the α_{1A} -AR, thereby enhancing the proportion of expression of the α_{1L} -AR phenotype. The α_{1L} -AR–expressing α_{1a} -COE(α_{1L} -dominant) cell line reflects accurately the phenotype of this AR observed in vivo and will facilitate development of α_{1L} -AR–targeted drugs.

Keywords: cysteine-rich epidermal growth factor–like domain 1 α (CRELD1 α), α_{1L} -adrenoceptor, α_{1A} -adrenoceptor, phenotype pharmacology

Introduction

There are three distinct subtypes of α_1 -adrenoceptors (ARs), (α_{1A} , α_{1B} , and α_{1D}) corresponding to the ADRA1A(α_{1a}), ADRA1B(α_{1b}), and ADRA1D(α_{1d}) genes, respectively (nomenclature according to ref. 1). The pharmacological profiles of these α_1 -AR subtypes, all typified by a high affinity (subnanomolar K_D) for prazosin (a prototypic, selective α_1 -AR antagonist) have been verified for the recombinantly expressed receptors,

which reflect the same phenotype observed for these subtypes (α_{1A} , α_{1B} , and α_{1D}) in intact tissues (2 – 5).

In addition to these ‘classical α_1 -ARs’, functional studies with intact tissues have identified a unique α_1 -AR characterized by a low-affinity for prazosin, as observed in blood vessels, the iris dilator muscle, and in the lower urinary tract. These data point to the existence of an additional α_1 -AR subtype, referred to as the “ α_{1L} -AR”, reflecting its unique low affinity for prazosin (6 – 11). The expression of the α_{1L} -AR phenotype in tissues has since been confirmed independently by a number of functional studies, but attempts to identify a gene or gene splice variant that can account for the expression of this unusual AR phenotype have so far been unsuccessful.

[†]These authors equally contributed to this work.

*Corresponding author. muramatu@u-fukui.ac.jp

Published online in J-STAGE on May 28, 2010 (in advance)

doi: 10.1254/jphs.10093FP

Recent studies with α_1 -AR gene-knockout mice revealed that the ADRA1A(α_{1a}) gene plays an obligatory role in the expression of the α_{1L} -AR phenotype because the α_{1L} -AR phenotype in tissues recognized to express the α_{1L} -AR could no longer be detected in α_{1a} -AR-null (ADRA1A^{-/-}) mice (12, 13). These knockout mouse studies unequivocally demonstrated that the α_{1L} -AR is a receptor phenotype that is derived in the some way from the ADRA1A(α_{1a}) gene. Therefore, at least in tissues examined thus far (cerebral cortex, vas deferens, and prostate) where the α_{1L} -AR can be detected pharmacologically, it is now clear that the ADRA1A(α_{1a})-derived polypeptide is an essential ingredient for generating the α_{1L} -AR (12, 14).

One can speculate that the α_{1L} -AR phenotype might arise from the alternative splicing of the ADRA1A(α_{1a}) gene. However, this possibility has already been rigorously tested to demonstrate that all functional splice variants of the ADRA1A(α_{1a}) gene, when expressed in cells individually yield only the α_{1A} -AR phenotype and not α_{1L} -AR phenotype (15, 16). Therefore, the mechanism(s) for generating the α_{1L} -AR phenotype arising from a single ADRA1A(α_{1a}) gene is still an open question.

In our previous work, we found that the pharmacological profile of the α_{1L} -AR detected intact tissues could not be detected in membrane preparations from the tissues; only the characteristics of the 'classical α_{1A} -AR' were detected upon tissue homogenization (12, 17–20). Nonetheless, it proved possible to detect the α_{1L} -AR in 'tissue fragments' that had not been subject to rigorous homogenization (21). Thus, the process of tissue disruption was able to explain the failure of previous attempts to demonstrate α_{1L} -ARs in the radioligand binding studies using isolated membrane fractions (8, 22).

Based on these observations, we suggested that tissue homogenization disrupted the interaction of the α_{1A} -AR with a labile regulatory protein that could, when expressed along with the α_{1A} -AR, generate the α_{1L} -AR low-prazosin-affinity phenotype, in keeping with the 'mobile' or 'floating' receptor paradigm of receptor function discussed some time ago (23). We therefore hypothesized that tissues exhibiting the pharmacological characteristics of the α_{1L} -AR would contain an auxiliary protein that leads to a phenotypic change of the α_{1A} -AR into the α_{1L} -AR phenotype (14). Since extracellular, transmembrane and intracellular sequences of ARs are all involved in ligand binding and signaling (24), we could not predict the receptor domain at which the interacting protein might interact with the α_{1A} -AR.

To test our hypothesis we therefore turned to a yeast two-hybrid approach using the entire α_{1a} -AR open reading frame (ORF) as bait to screen a cDNA library from a

tissue (human prostate) known to express the α_{1L} -phenotype. Upon identifying an α_{1a} -AR-interacting protein from that library, we then went on to evaluate its ability, upon co-expression with the α_{1a} -AR, to generate a cell line that would exhibit the α_{1L} -AR phenotype. Here, we report that cysteine-rich epidermal growth factor-like domain 1 α (CRELD1 α) is a novel α_{1a} -AR-interacting protein, which when expressed along with the α_{1a} -AR, yields a cell line expressing the α_{1L} -AR phenotype in sufficient abundance for pharmacological characterization. A preliminary account of this work has been presented to The Japanese Pharmacological Society (25).

Materials and Methods

Plasmids

For the yeast two-hybrid experiment, the bait plasmid pGBT7- α_{1a} -AR (Clontech, Mountain View, CA, USA) was used as bait, and pGADT7 (Clontech) was used to express prey constructs. Plasmids used for transient or stable expression in Chinese hamster ovary (CHO) cells are listed below. Note that antibiotics and their concentration for maintaining stably transfected cell lines are indicated in parentheses. p α_{1A} -AR-IRES-Puro^r (10 μ g/ml puromycin) (IRES: intra-ribosomal entry site) is for human ADRA1A (α_{1a} -AR) expression. The full ORF for human CRELD1 α was amplified from the Marathon-ready human brain cDNA library (Clontech) by polymerase chain reaction (PCR) and subcloned to create pCR3.1-CRELD1 α (1.5 mg/ml G-418). pSilencer4.1-CMV-siCRELD1 α (hygromycin B, 100 μ g/ml) (Ambion, Austin, TX, USA) was used for the RNAi experiment. p α_{1a} -AR-IRES-CRELD1 α was used for transient expression of the two proteins simultaneously. p α_{1a} -AR-IRES-(empty) was used as a negative control for the transient simultaneous expression experiment.

Yeast two-hybrid screening

The yeast two-hybrid screening of the human prostate cDNA library in pACT2 (Clontech) was performed by using entire ORF of human α_{1a} -AR bait as previously described (26). The complete ORF for human CRELD1 α was obtained from the Marathon-ready human brain cDNA library (Clontech) by PCR and subcloned into pCR3.1 (Invitrogen, Carlsberg, CA, USA).

Truncation mapping of the interaction site

A set of deletion mutants of CRELD1 α (in the pGADT7 vector, as shown in Fig. 1a) and full-length α_{1a} -AR (in the pGBKT7 vector) or CRELD1 α (211–420) and a set of deletion mutants of α_{1a} -AR (Fig. 1b) were co-transformed into a yeast strain Y187, and the β -galactosidase activity in each clone was examined as

previously described (26).

CHO cell culture and transfection

CHO cells were used throughout for the heterologous reconstitutions of α_{1A} -ARs and/or α_{1L} -ARs. CHO cells were maintained in DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) FBS (Invitrogen), 2 mM L-glutamine, non-essential amino acids, and a penicillin-streptomycin mixture. All transfections were conducted using FuGene 6 reagent (Roche, Carlsbad, CA, USA) following the manufacturer's instructions.

CHO cells transfected with human α_{1A} -AR by FuGene 6 (Roche) were selected by puromycin (10 μ g/ml) (InvivoGen, San Diego, CA, USA) to isolate a cell line stably expressing the α_{1A} -AR. This cell line (α_{1A} -plain cells) was further transfected with pCR3.1-human CRELD1 α and selected using G-418 (1.5 mg/ml) to obtain a cell line [α_{1A} -COE(α_{1L} -dominant)] that stably overexpresses human CRELD1 α along with the α_{1A} -AR. The α_{1A} -AR cell line (α_{1A} -plain cell) was also transfected with pSilencer 4.1-CMV siRNA CRELD1 α to obtain a cell line [α_{1A} -CKD(α_{1A} -enhanced)] with knocked-down/silenced CRELD1 α expression, along with stably expressed α_{1A} -AR.

RNAi of CHO-CRELD1 α and transfection

To knockdown CRELD1 α endogenously expressed in CHO cells, the nucleotide sequence of CHO-CRELD1 α was first determined and then hairpin siRNAs targeting the intracellular loop between transmembrane (TM) I and TM II of CHO-CRELD1 α was subcloned into the pSilencer vector (Ambion). The α_{1A} -AR-expressing cell line (α_{1A} -plain cell) was transfected with the CHO-CRELD1 α siRNA vector and selected using 1.5 mg/ml hygromycin. One of the α_{1A} -AR-expressing cell lines with silenced CHO-CRELD1 α [α_{1A} -CKD(α_{1A} -enhanced)] was used for further study. The sequences of the siRNA for CHO-CRELD1 α and scrambled siRNA were 5'-GGCGACCTAGTGTTTACC-3' and 5'-GCTGTCA GCTAGTCGCTA-3', respectively.

Immunoblot analysis and antibodies

Equal amounts (12 μ g) of the lysed membrane fraction of the cells were subjected to immunoblot analysis and the blot densities for CRELD1 α were normalized to those of the β -actin observed in each sample as described previously (26). The affinity purified CRELD1 α -specific rabbit antibody and mouse anti- β -actin antibody were from Sigma-Aldrich.

Whole cell binding experiments

Whole cell binding experiments were performed as previously described (20). In brief, CHO cells stably

transfected with α_{1A} -AR with or without CRELD1 α in pIRES (Clontech) vector were harvested with Krebs incubation buffer (135.7 mM NaCl, 5.4 mM KCl, 1.2 mM MgCl₂, 2.0 mM CaCl₂, 1.2 mM NaH₂PO₄, 10.5 mM NaHCO₃, and 11.5 mM D-glucose, pH 7.4). The cells were incubated with [³H]-silodosin for 30 min at 37°C in 1 ml of a Krebs incubation buffer. In binding saturation experiments, [³H]-silodosin at concentrations 50 – 1000 pM were used. Binding competition experiments were performed with 500 pM [³H]-silodosin. Reactions were terminated by rapid filtration using a Brandel cell harvester onto Whatman GF/C filters, and the trapped radioactivity was measured. Non-specific binding was defined as the binding in the presence of 10 μ M phentolamine (20).

Measurement of calcium transients [Ca^{2+}]_i

[Ca^{2+}]_i measurement was performed as described previously (26). Briefly, CHO cells were loaded with 5 μ M Fura-2 AM (Dojindo, Kumamoto) together with 0.02% (w/v) Pluronic F-127 and 1.4 mM probenecid for 45 min, washed, and then resuspended in Ca²⁺ assay buffer with 1.4 mM probenecid and 3% (v/v) FBS. [Ca^{2+}]_i was measured by Fura-2 ratio fluorometry using a CAF-110 fluorescence spectrophotometer (JASCO, Tokyo) (26, 27). Fura-2 fluorescence was measured at 37°C with stirring. During the stimulation with an agonist, the cells were illuminated alternatively (100 Hz) at excitation wavelengths of 340 and 380 nm, respectively, by means of two monochromators in the light path of a 75-W Xe lamp. Fluorescence emission from the cells was measured at 510 nm by a photomultiplier. The fluorescence intensity at each excitation wavelength (F_{340} and F_{380} , respectively) and the ratio of these two fluorescence values ($R_{340/380}$) were recorded with a time constant of 250 ms and stored on a computer. At the end of the experiments, the maximum fluorescence ratio was determined by adding Triton X-100. The minimum fluorescence ratio was determined by adding 10 mM ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA). The intracellular calcium concentration ([Ca^{2+}]_i) was calculated, as described (27), using the formula [Ca^{2+}]_i (nM) = $K_d \times [(R - R_{min}) / (R_{max} - R)] \times (S_{f2} / S_{b2})$, where K_d (224 nM at 37°C) is the dissociation constant of Fura-2 for Ca²⁺; R is the ratio of fluorescence of the sample at 340 to that at 380 nm; R_{min} and R_{max} represent the ratios of fluorescence at the same wavelengths in the presence of zero and saturating Ca²⁺, respectively; and S_{f2}/S_{b2} is the ratio of fluorescence of Fura-2 at 380 nm in zero Ca²⁺ to that in saturating Ca²⁺, respectively.

Functional studies with rat prostate and tail artery

Male Wistar rats (about 300 g) were anesthetized with

sodium pentobarbital (50 mg/kg) and sacrificed. The prostate and tail artery were isolated and cleaned in a modified Krebs-Henseleit solution (112.0 mM NaCl, 5.4 mM KCl, 1.2 mM MgCl₂, 2.0 mM CaCl₂, 1.2 mM NaH₂PO₄, 25.5 mM NaHCO₃, and 11.5 mM D-glucose, pH 7.4) aerated with 95% O₂ and 5% CO₂ at 4°C. The present study was performed according to the Guidelines for Animal Experiments, University of Fukui.

Strips of prostate and ring preparations of tail artery were placed in organ baths containing a modified Krebs-Henseleit solution (37°C). Noradrenaline and other test agonists were applied cumulatively and the isometric tension changes were recorded through a force transducer. Desipramine (0.3 μ M), deoxycorticosterone acetate (5 μ M), and propranolol (1 μ M) were added to inhibit neural and extraneural reuptake of noradrenaline and to block β -ARs, as described elsewhere (17). In the tail artery, rauwolscine (0.1 μ M) and L-NAME (100 μ M) were also added to block possible activation of α_2 -ARs (28) and NO release from endothelial cells, respectively (17).

Transcript level assays via quantitative real-time RT-PCR (qPCR)

Total RNA was extracted from the cerebral cortex, tail artery, or prostate derived from adult male rat by using the RNeasy Tissue Mini Kit (Qiagen, Hilden, Germany) and digested with DNase (amplification grade I, Invitrogen) following the manufacturer's instructions. Two micrograms of DNase treated RNA were reverse transcribed in a 50- μ l reaction, using pd(N)₆ and anchored-d(T)₁₈ primers (Transcriptor First Strand cDNA Synthesis Kit; Roche Diagnostics, Indianapolis, IN, USA). The resultant first-strand cDNA was applied to PCR, which was performed using a HotStarTaq Master Mix Kit (Qiagen). A negative control without reverse transcription was run in parallel to confirm the completion of genomic DNA digestion. Expression levels of the transcript for GAPDH were used for the normalization of *Adra1a* and *Creld1* transcript levels. The following primers were employed: *Gapdh*-forward (5'-ACC ATC TTC CAG GAG CGA G-3'), *Gapdh*-reverse (5'-CTA AGC AGT TGG TGG TGC AG-3'), *Adra1a*-forward (5'-TTC TCA GTG AGG CTG CTC AA-3'), *Adra1a*-reverse (5'-GGT TTC CGA AGG CTT GAA AT-3'), *Creld1*-forward (5'-ACG AGC AGT GTG AAA ACA CG-3'), and *Creld1*-reverse (5'-ATA GCT GCC ACA GCT CCA AT-3').

Data analyses

Binding data in saturation and competition experiments were analyzed using PRISM software (ver. 3; GraphPad Software, San Diego, CA, USA). The number

of α_1 -ARs was presented as the maximal binding capacity per mg of total tissue protein (fmol/mg of total tissue protein). In saturation binding studies, data were fitted by a one-site saturation binding isotherm. In competition studies, the data were first fitted to a one-site model and then a two-site model, and if the residual square sums were significantly lower for a two-site fit of the data than for a one-site fit (P -value <0.05 as determined by the F -test), then a two-site model was accepted (12).

In functional studies, antagonist affinity estimates (pK_B values) were obtained by plotting the data according to Schild analysis. When the straight lines had a slope of unity, the pA_2 value estimated was taken as the pK_B value. When a single concentration of an antagonist was tested, the pK_B value was also determined for a single concentration of the antagonist by the concentration-ratio method (29).

Drugs

The chemicals used were as follows: [³H]-silodosin (1.81 TBq/mmol) and 1-(3-hydroxypropyl)-5-[(2R)-({2-[2-(2-(2,2,2-trifluoroethoxy)phenoxy]ethyl}amino)propyl]indoline-7-carboxamide [silodosin (formerly known as KMD-3213)] (Kissei Pharmaceutical Co., Ltd., Matsumoto); 2-[(5-chloro-3-isopropyl-2-methylphenyl)methyl]-4,5-dihydro-1H-imidazole hydrochloride (MK017) and *N*-[6-chloro-3-(4,5-dihydro-1H-imidazol-2-ylmethoxy)-2-methylphenyl]methanesulfonamide hydrochloride (Ro 115-1240) (Nippon Chemiphar Co., Ltd., Tokyo). The other drugs were obtained from commercially available sources. Subtype selectivity of the used drugs are as follows (9, 17, 18, 30): prazosin: α_{1A} -, α_{1B} -, and α_{1L} -AR antagonist; silodosin: α_{1A} - and α_{1L} -AR antagonist; phentolamine: nonselective antagonist; MK017: α_{1A} - and α_{1L} -AR agonist (I. Muramatsu, unpublished observations); Ro 115-1240: α_{1A} - and α_{1L} -AR partial agonist; *N*-[2-(2-cyclopropylmethoxyphenoxy)ethyl]-5-chloro- α,α -dimethyl-1H-indole-3-ethamine hydrochloride (RS-17053): α_{1A} -AR antagonist; 5-methylurapidil: α_{1A} -AR antagonist.

Results

Identification of CRELD1 α as a novel α_{1A} -AR-interacting protein

As a result of screening a human prostate cDNA library with full-length human α_{1A} -AR as bait, we isolated a cDNA fragment encoding the carboxyl (C)-terminal region (211–420) of the α -isoform of the CRELD1 protein (Accession No. WI11041) (31).

We searched for the domains of CRELD1 α responsible for its interaction with the α_{1A} -AR by the yeast two-hybrid assay. Deletion of the C-terminal 14 amino acids of

CRELD1α completely abolished its interaction with the α_{1A}-AR (Fig. 1a). Although the C-terminal region itself (CRELD1α 407 – 420) was not sufficient to interact with α_{1A}-AR, it appeared that this 14 amino acid sequence is essential for the CRELD1α–α_{1A}-AR interaction. The sequence comprising amino acids 244 – 420 was the minimum site of the interaction. This sequence contains the second FURIN domain, the Ca-EGF-like domain, two TM domains, and the C-terminal tail.

We also searched for the domain(s) of α_{1A}-AR responsible for the interaction with CRELD1α (Fig. 1b). The third cytoplasmic loop and a portion of the TM 1 – 5 domains were necessary for the interaction. The C-terminal intracellular region was not necessary. Although the amino (N)-terminal extracellular region per se did not harbor the interaction, the fact that the inclusion of this region strengthened the yeast two-hybrid signal suggested some additional roles of the region for the interaction.

Effects of CRELD1α on α_{1A}-AR expression

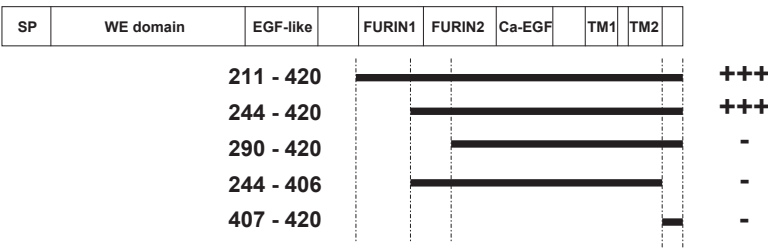
At first, we simply transfected α_{1A}-AR cDNA into CHO cells and prepared its stably expressing cell line (designated here as “α_{1A}-plain cells”). The abundance of specific [³H]-silodosin binding sites in the α_{1A} cells was 1360 ± 130 fmol/mg protein and the affinity was 145 ± 27 pM (n = 6). Most of the binding sites showed a high affinity for prazosin (pK_i = 10.5 ± 0.3), but a small proportion of sites (12 ± 3% of total receptor density) showed a low affinity for prazosin (pK_i = 7.7 ± 0.2), suggesting concomitant expression of α_{1A}- and α_{1L}-ARs in the α_{1A}-plain cells, in spite of a predominance of the α_{1A}-AR

phenotype (Fig. 2b, filled triangles).

Next, the effects of CRELD1α on the expression of the α_{1A}- and α_{1L}-ARs in the α_{1A}-plain cell line were examined. During the course of this study, we realized that the CHO cells express CRELD1α endogenously. Therefore, we used CRELD1α siRNA to knock-down endogenous CRELD1α in the α_{1A}-plain cell line, and we generated a stable cell line [(α_{1A}-CKD(α_{1A}-enhanced) cells] wherein the suppression of endogenous CRELD1α expression was confirmed by immunoblotting (See Fig. 2 legend for results). As shown in Fig. 2a, the abundance of [³H]-silodosin binding sites was markedly increased in the α_{1A}-CKD(α_{1A}-enhanced) cells. Thus, the competition curve for prazosin was essentially monophasic (Fig. 2b, open circles), and the binding sites showed high-affinity for prazosin (pK_i = 9.7 ± 0.1); that is, the α_{1A}-CKD(α_{1A}-enhanced) cells exhibited primarily the α_{1A}-AR phenotype profile, although the resistant component to 30 nM prazosin was still detected (5 ± 2% of total specific binding) (Fig. 2a). This result suggested that knockdown of CRELD1α in the α_{1A}-CKD(α_{1A}-enhanced) cells specifically enhanced the expression of α_{1A}-AR without completely eliminating the expression of α_{1L}-AR. This cell line [α_{1A}-CKD(α_{1A}-enhanced)] exhibited a predominant α_{1A}-AR phenotype compared with the original α_{1A}-plain cell line.

Next, we transfected human CRELD1α into the α_{1A}-plain cell line and established a CRELD1α-overexpressing cell line [α_{1A}-COE(α_{1L}-dominant) cells]. In this cell line, the abundance of [³H]-silodosin binding sites was strikingly depressed to about 20% of the total density of the specific binding sites in the original α_{1A} cell line (Fig.

a.



b.

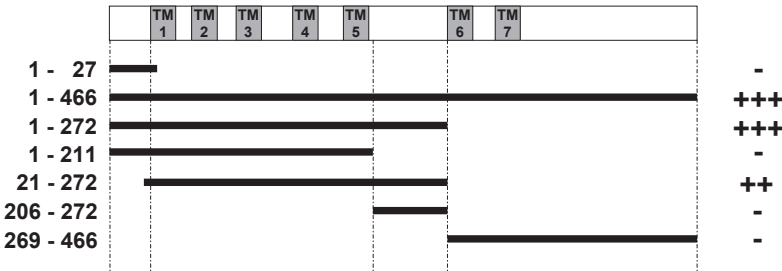


Fig. 1. Interaction between α_{1A}-AR and CRELD1α. a) Truncation mapping of the α_{1A}-AR interaction site on CRELD1α. The results of a yeast two-hybrid binding assay between full-length of α_{1A}-AR and series of truncation mutants of CRELD1α are shown in the right panel. Note that CRELD1α 244 – 420 but not 244 – 406, which is lacking the C-terminal 14 amino acids, interacts with α_{1A}-AR. b) Truncation mapping of the CRELD1α interaction site on α_{1A}-AR. The results of a yeast two-hybrid binding assay between CRELD1α (244 – 420) and series of truncation mutants of α_{1A}-AR are shown.

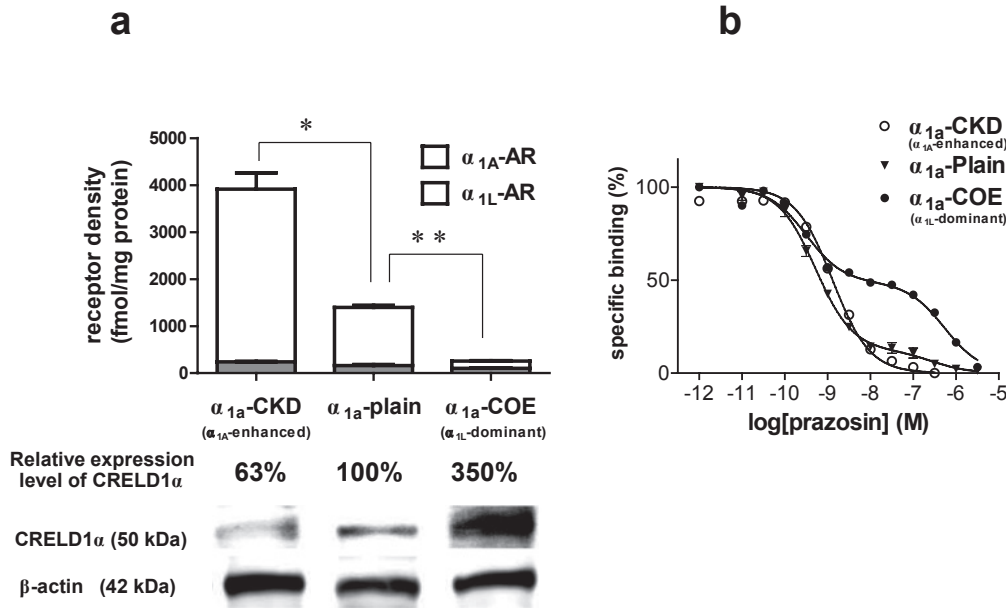


Fig. 2. Effects of CRELD1 α on α_1 -AR expression in α_{1a} -plain cells. The α_{1a} -AR cDNA was stably transfected in CHO cells (α_{1a} -plain cells). Endogenous CRELD1 α in α_{1A} cells was knockdown in the α_{1a} -CKD(α_{1A} -enhanced) cells, so that the relative level of CRELD1 protein is reduced to $63 \pm 5\%$ ($n = 4$) compared to the α_{1A} cells, or human CRELD1 α was over-expressed in α_{1a} -plain cells, α_{1a} -COE(α_{1L} -dominant) cells, in which the relative level of CRELD1 protein is augmented to $353 \pm 54\%$ ($n = 3$) compared to the α_{1a} -plain cells. Total receptor density for each cell line is as follows (units in fmol/mg protein): 3914 ± 349 [α_{1a} -CKD(α_{1A} -enhanced) cells], 1399 ± 42 (α_{1a} -plain cells), 257 ± 6 [α_{1a} -COE(α_{1L} -dominant) cells]. The binding of [3 H]-silodosin was estimated in each cell line. a) Abundance of [3 H]-silodosin binding sites in each cell line. Open and closed parts in each column represent α_{1A} -AR and α_{1L} -AR, respectively, which were separated by the sensitivity to 30 nM prazosin. Ordinate: absolute values of [3 H]-silodosin binding sites. Mean \pm S.E.M. of 5 – 8 experiments. * $P < 0.05$, ** $P < 0.01$. Representative CRELD1 immunoblot images for these three cell lines are shown. b) Competition curves for prazosin at 1000 pM [3 H]-silodosin binding sites in three cell lines. Ordinate: total specific binding of [3 H]-silodosin taken as 100%. Mean \pm S.E.M. of 3 – 5 experiments. Computer-aided analysis estimated the relative content of α_{1L} -AR as follows: 0% in α_{1a} -CKD(α_{1A} -enhanced) cells, 12% in α_{1A} , and 47% in α_{1a} -COE(α_{1L} -dominant) cells. Note that these values are lower than the relative content of α_{1L} -AR shown in panel a, which is the results of mere subtraction of specific [3 H]-silodosin binding competing with 30 nM of prazosin.

2a). On the other hand, the abundance of α_{1L} -AR sites with a low-affinity for prazosin did not change appreciably. Therefore, in the α_{1a} -COE(α_{1L} -dominant) cell line, the relative proportion of α_{1L} -AR sites, relative to the α_{1A} -AR high-affinity prazosin binding sites, was increased to up to 47% (Table 1), resulting in a clear biphasic competition curve for prazosin (Fig. 2b, filled circles). From these results, we considered that the α_{1a} -COE(α_{1L} -dominant) cells expressed a sufficient abundance of the α_{1L} -AR phenotype for further study, even though the overall expression level was comparatively low.

Table 1 summarizes the binding affinities for various antagonists in the α_{1a} -CKD(α_{1A} -enhanced) and the α_{1a} -COE(α_{1L} -dominant) cells. In the α_{1a} -CKD(α_{1A} -enhanced) cells, all tested antagonists competed monotonically in the binding assay. The estimated high affinities for antagonists in this cell line were in accord with the α_{1A} -AR antagonist profile observed in intact tissue (e.g., Schild plot) and membrane binding assays. On the other hand, prazosin, RS-17053, and 5-methylurapidil showed two

distinct (high and low) affinities in α_{1a} -COE(α_{1L} -dominant) cells that express a high proportion of the α_{1L} -AR, suggesting the coexistence of two distinct receptor phenotypes (i.e., α_{1A} - and α_{1L} -ARs).

Ca²⁺ responses to noradrenaline in α_{1a} -CKD(α_{1A} -enhanced) and α_{1a} -COE(α_{1L} -dominant) cell, and antagonist pharmacology

Since α_{1A} - or α_{1L} -ARs were clearly identified as independent entities in the two cell lines by the radioligand binding approach, pharmacological profiles of the expressed receptor phenotypes were also examined in a functional study using a ligand-mediated elevation of intracellular calcium (Ca^{2+} transients) as an index of response. Noradrenaline (10 μM) produced a transient increase in intracellular Ca^{2+} in both the α_{1a} -CKD(α_{1A} -enhanced) and α_{1a} -COE(α_{1L} -dominant) cell lines. This response was completely inhibited by pretreatment with YM-254890 [1 μM , a recognized Gq α inhibitor; (32)] (data not shown). This result suggested that the α_1 -AR–

Table 1. Binding affinities for various antagonists in two cell lines

Antagonist	α_{1A} -CKD(α_{1A} -enhanced) cell	α_{1A} -COE(α_{1L} -dominant) cell	
	pKi	pKi _{high}	pKi _{low}
Prazosin	9.7 \pm 0.1	10.2 \pm 0.1	6.9 \pm 0.2 (47%)
RS-17053	8.7 \pm 0.1	9.5 \pm 0.5	7.0 \pm 0.1 (62%)
5-Methylurapidil	9.3 \pm 0.1	8.9 \pm 0.1	6.2 \pm 0.2 (45%)
Silodosin	9.6 \pm 0.1*	9.4 \pm 0.1*	

%, proportion of low affinity sites. pKi_{high} and pKi_{low}: negative logarithm of the equilibrium constants (pKi) at high and low affinity sites for tested drugs. Competitive binding experiments with whole cell preparations of α_{1A} -CKD(α_{1A} -enhanced) and α_{1A} -COE(α_{1L} -dominant) cells were carried out at 1000 pM [3 H]-silodosin. *The values for silodosin represent the pK_D obtained from saturation experiments with [3 H]-silodosin. Data represent the mean \pm S.E.M. of 4 – 6 experiments.

evoked $[Ca^{2+}]_i$ increase was mediated by Gq α in both cell lines and that this mechanism was not affected by either knockdown or overexpression of CRELD1 α . Figure 3 shows the following: 1) the concentration–response curves for the Ca^{2+} response triggered by noradrenaline in both cell lines and 2) the prazosin antagonism against the noradrenaline response. In α_{1A} -CKD(α_{1A} -enhanced) cells, the concentration–response curves for noradrenaline were shifted to the right in parallel by increasing concentrations of prazosin. Thus, the Schild slope (0.95 ± 0.04) was close to unity, suggesting that the Ca^{2+} responses were mediated through a single type of receptor. The estimated pK_B value for prazosin was high (9.1 ± 0.1), consistent with the α_{1A} -AR profile. In contrast, in α_{1A} -COE(α_{1L} -dominant) cells, the competition by prazosin to block the noradrenaline response was apparently complex: a rightward and downward shift at 1 nM prazosin followed by a slightly rightward shift at 3 and 10 nM prazosin and then a more evident rightward shift at higher concentrations of prazosin. Thus, the estimated Schild slope estimated (0.80 ± 0.11) deviated significantly from unity, showing two-step antagonism. This result suggested that the concentration–response curves for noradrenaline in α_{1A} -COE(α_{1L} -dominant) cells reflect two distinct receptors having different affinities for prazosin (probably α_{1A} - and α_{1L} -ARs). Such two-step antagonism was reported previously in intact tissues (30, 33). Furthermore, an insurmountable inhibition was produced by prazosin in α_{1A} -COE(α_{1L} -dominant) cells, in contrast with the surmountable antagonism observed in the α_{1A} -CKD(α_{1A} -enhanced) cells (Fig. 3: a and b). Details of this insurmountable inhibition by prazosin will be discussed below (see Discussion). We took advantage of the insurmountable inhibition to mask the α_{1A} -AR in α_{1A} -COE(α_{1L} -dominant) cells selectively with the use of 10 nM prazosin so as to evaluate the residual α_{1L} -ARs in the following experiments (32).

Table 2 shows the functional affinities for various an-

tagonists in α_{1A} -CKD(α_{1A} -enhanced) and α_{1A} -COE(α_{1L} -dominant) cells. In α_{1A} -CKD(α_{1A} -enhanced) cells, concentration-dependent Ca^{2+} responses were competitively inhibited by prazosin (1 – 300 nM), RS-17053 (1 μ M), 5-methylurapidil (100 nM), and silodosin (1 nM). The estimated high affinities for the antagonists were close to those expected for the native α_{1A} -AR. In contrast, in α_{1A} -COE(α_{1L} -dominant) cells, where measurements were done in the presence of 10 nM prazosin to mask the α_{1A} -AR, not only prazosin but also RS-17053 and 5-methylurapidil antagonized the Ca^{2+} responses with lower affinities than those expected for the native α_{1A} -AR. However, even in the presence of 10 nM prazosin, silodosin still inhibited the Ca^{2+} response with a high affinity (pK_B = 9.5 ± 0.2). This profile for antagonists in the α_{1A} -COE(α_{1L} -dominant) cells was entirely consistent with the profile reported for the α_{1L} -AR in native tissues.

Agonist pharmacology in α_{1A} -CKD(α_{1A} -enhanced) and α_{1A} -COE(α_{1L} -dominant) cell lines and in rat tail artery and prostate tissue preparations

Next, agonist pharmacology was examined for the α_{1A} -AR expressed in α_{1A} -CKD(α_{1A} -enhanced) cells and for the α_{1L} -AR in α_{1A} -COE(α_{1L} -dominant) cells (pretreated with 10 nM prazosin to mask the α_{1A} -AR). Figure 4 shows the concentration-dependent Ca^{2+} response curves for three agonists. Noradrenaline showed a higher potency for acting on the α_{1A} -AR compared with the expressed α_{1L} -AR [pEC₅₀: 8.3 ± 0.1 and 6.3 ± 0.1 in α_{1A} -CKD(α_{1A} -enhanced) and α_{1A} -COE(α_{1L} -dominant) cell lines, n = 7 and 8, respectively]. This difference in potency for noradrenaline might be in part due to differences in the expression level of the receptor between the two cell lines (Fig. 2a) and/or their intrinsic profile of two subtypes. Ro 115-1240, originally developed as a partial α_{1A} - and α_{1L} -AR agonist (34), showed agonist activity principally acting on the α_{1A} -AR. In contrast, MK017, a new α_{1A} - and α_{1L} -AR agonist (I. Muramatsu,

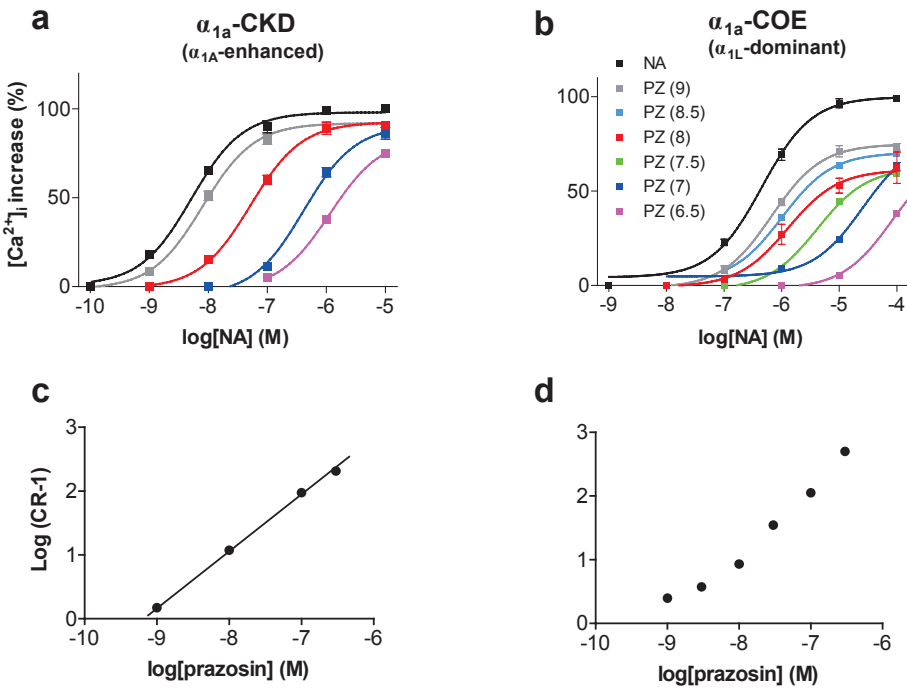


Fig. 3. Concentration–response curves for noradrenaline and effects of prazosin. a and b): Peak amplitudes of Ca^{2+} responses induced by each concentration of noradrenaline before and after prazosin were plotted in α_{1a} -CKD(α_{1A} -enhanced) cells and α_{1a} -COE(α_{1L} -dominant) cells. The maximum Ca^{2+} increases induced by noradrenaline in α_{1a} -CKD(α_{1A} -enhanced) cells and α_{1a} -COE(α_{1L} -dominant) cells (800 ± 60 and 845 ± 23 nM, respectively) were taken as 100%. The values of prazosin described within the figure represent the negative logarithm concentrations. c and d) Schild plot for prazosin. Mean values of 3–5 experiments at each concentration of prazosin are plotted.

Table 2. Functional affinities for various antagonists in two cell lines

Antagonist	α_{1a} -CKD(α_{1A} -enhanced) cell	α_{1a} -COE(α_{1L} -dominant) cell
	pK_B	pK_B
Prazosin	9.1 ± 0.1	7.8 ± 0.2
RS-17053	8.5 ± 0.5	6.9 ± 0.1
5-Methylurapidil	8.9 ± 0.4	7.8 ± 0.1
Silodosin	10.1 ± 0.1	9.5 ± 0.2

Ca^{2+} responses were induced by noradrenaline and the antagonist affinities were evaluated. In α_{1a} -COE(α_{1L} -dominant) cells, 10 nM prazosin was present throughout the experiments to mask α_{1A} -AR. pK_B values for prazosin were calculated by Schild analysis. The pK_B values for 1 μM RS-17053, 100 nM 5-methylurapidil, and 1 nM silodosin were estimated by the concentration-ratio method (29). The data are shown as the mean \pm S.E.M. of 4–6 experiments.

unpublished observations), showed a higher potency than noradrenaline for both α_1 -AR subtypes.

For comparison with the cell-based assays, the agonist pharmacology was also examined in two representative tissues where the contractile responses to noradrenaline have been reported to be mediated mainly through either the α_{1A} -AR (rat tail artery) (28, 35, 36) or the α_{1L} -AR (rat prostate) (37) As shown in Fig. 4, c and d, the relative potencies and efficacies of the three tested agonists were in agreement with the data observed for the recombinantly expressed α_{1A} -AR (Fig. 4a) and α_{1L} -AR (Fig. 4b), respectively.

qPCR assay for CRELD1 α mRNA expression in rat tissues

Finally, we measured expression levels of α_{1a} -AR and CRELD1 α transcripts in several tissues including the rat tail artery and prostate (Fig. 5). CRELD1 α transcripts were detected in all tissues examined. The level of CRELD1 α expression in the prostate (Fig. 5a), where the α_{1L} -AR phenotype is predominantly expressed (Fig. 4d), was much higher than in the rat tail artery (Fig. 5a), where α_{1a} -AR is predominantly expressed (Fig. 4c). Furthermore, there is a clear difference in the ratio of CRELD1 α mRNA relative to ADRA1A(α_{1a}) mRNA in the prostate and brain tissue, compared with the tail artery (Fig. 5b). Since CRELD1 α mRNA is expressed, even in the rat tail artery, the presence of CRELD1 α expression

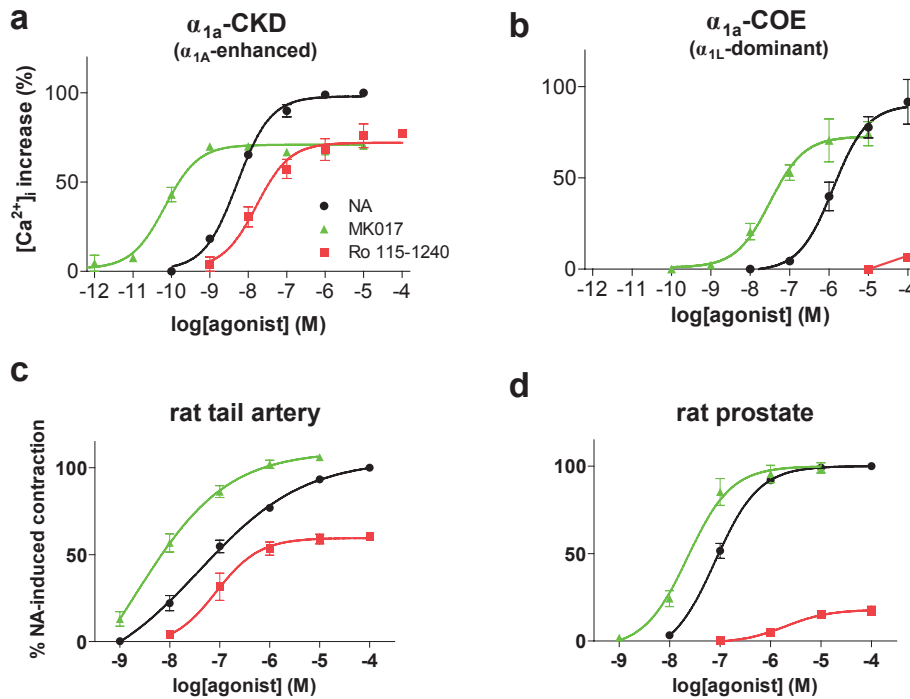


Fig. 4. Functional responses to three agonists in two cell lines and in rat tail artery and prostate. a and b): Ca^{2+} responses induced by noradrenaline, MK017, and Ro 115-1240 in α_{1A} -CKD(α_{1A} -enhanced) and α_{1A} -COE(α_{1L} -dominant) cells are plotted. In α_{1A} -COE(α_{1L} -dominant) cells, the responses were obtained after the treatment with 10 nM prazosin to mask α_{1A} -AR (see text for further explanation). The maximum response to noradrenaline in each cell line was taken as 100%. c and d) Concentration–contraction curves for noradrenaline, MK017, and Ro 115-1240 in rat tail artery and prostate. The maximum contraction induced by noradrenaline in each tissue strip was taken as 100%. Each reported value is the mean \pm S.E.M. of 3 – 5 experiments.

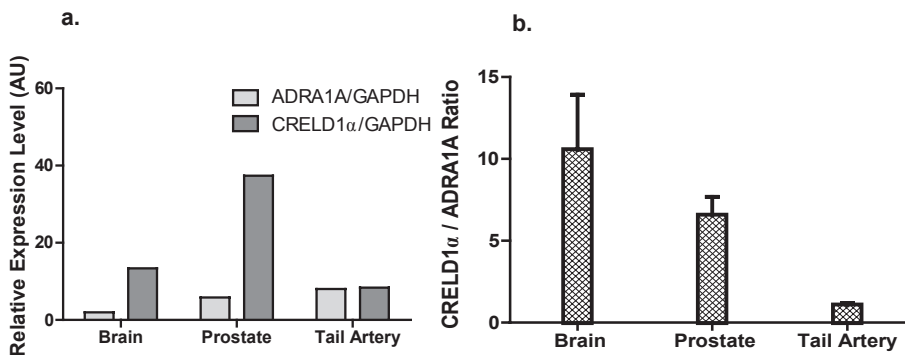


Fig. 5. qPCR analysis of transcript level of ADRA1A(α_{1A}) and CRELD1 α in the native tissues. a) Relative mRNA expression level of ADRA1A (white bars) and CRELD1 α (gray bars) in the brain (cerebral cortex), prostate, and tail artery, normalized to the GAPDH level. b) Relative CRELD1 α expression level is shown as a ratio to the ADRA1A level. Mean \pm S.E.M. of 4 experiments. Note that the relative CRELD1 α /ADRA1A ratio is low in the tissue in which α_{1A} -AR is dominantly expressed (tail artery). Each reported value is the mean \pm S.E.M. of 4 experiments.

alone along with the ADRA1A(α_{1A}) does not yield the α_{1L} -AR phenotype. We suggest that it is the ratio of expression of CRELD1 α relative to the ADRA1A(α_{1A}) expression (CRELD1 α /ADRA1A ratio) that may serve as a predictive index for the expression of the α_{1L} -AR phenotype in a given tissue.

Discussion

We identified CRELD1 α as an α_{1A} -AR-interacting protein modulating the expression and phenotype of the α_{1A} -AR. When CRELD1 α protein production was repressed in an α_{1A} -AR expressing cells [α_{1A} -CKD(α_{1A} -enhanced) cells], the α_{1A} -AR abundance was upregulated; and when overexpressed [α_{1A} -COE(α_{1L} -dominant) cells],

CRELD1 α led to a down-regulation of α_{1A} -AR abundance, and a substantial increase in the proportion of the α_{1L} -AR phenotype was observed. The pharmacological characteristics of the α_{1L} -AR phenotype detected in the α_{1A} -COE(α_{1L} -dominant) cells mirrored faithfully the phenotype of this receptor detected in intact tissues.

CRELD1 α is a double-pass transmembrane protein with large N-terminal and small C-terminal extracellular regions. Its region-terminal domain consists of several domains including EGF-like, Ca^{2+} -EGF-like, and two FURIN domains (30). CRELD1 α is ubiquitously expressed in early development and persists in adulthood in most tissues, but the physiological functions of CRELD1 α especially its relationship to G-protein-coupled receptors (GPCRs) have yet to be studied in any depth.

Since two distinct phenotypes (α_{1A} - and α_{1L} -ARs) arise from a single ADRA1A(α_{1A}) gene, we at first hypothesized that an unknown α_{1A} -AR-interacting protein might be responsible for the α_{1L} -AR phenotype. Thus, when the two-hybrid interaction between CRELD1 α and α_{1A} -AR was demonstrated, we expected that the co-expression of CRELD1 α and α_{1A} -AR would generate the α_{1L} -AR phenotype. However, we found that mechanisms whereby CRELD1 α regulates α_{1L} -AR function are rather complicated.

Indeed, we found that CRELD1 α overexpression along with the α_{1A} -AR could generate a cell line [α_{1A} -COE(α_{1L} -dominant)] in which about 50% of the expressed ARs showed α_{1L} -AR phenotype. However, the expression of CRELD1 α led to a marked down-regulation of the overall abundance of the α_{1A} -AR, whereas the repression of CRELD1 α mRNA led to a significant up-regulation of α_{1A} -AR abundance, compared to the cells expressing the α_{1A} -AR alone. The complex mechanism whereby CRELD1 α leads to an increase in the proportion of the α_{1L} -AR present in cells that nonetheless express a lower overall abundance of the α_{1A} -AR compared with the CRELD1 α knocked-down cells [α_{1A} -CKD(α_{1A} -enhanced)] remains to be determined. However, the qPCR data suggest that the relative expression level of both α_{1A} -AR and CRELD1 α may regulate the predominance of phenotype expression. It remains to be determined whether the mechanism involves a direct or indirect interaction between the α_{1L} -AR and CRELD1 α (or both). The very low proportion of the α_{1L} -AR in cells not transfected with an excess of CRELD1 α might account for the failure to detect this receptor subtype in a number of previous α_{1A} -AR expression studies (2, 16, 38).

It should be also noted that [3 H]-prazosin, having a low-affinity for the α_{1L} -AR phenotype, was used in most of the previous studies. To circumvent this problem, we took advantage of using [3 H]-silodosin as a receptor probe having high-affinity for both α_{1A} -AR and α_{1L} -AR phenotypes (8). The identification of a minor proportion (less than 10%) of radioligand binding subsites using a mathematical analysis of the binding data may be unreliable (39). Notwithstanding, Ford et al. reported a detectable amount of the α_{1L} -AR subtype expressed in their α_{1L} -AR-expressing CHO cell line (40, 41). Thus, from our data, together with previously reported evidence, it appears that in a CHO cell background, the α_{1L} -AR phenotype can be found upon α_{1A} -AR transfection, although the α_{1L} -AR population is quite low when using the wild-type CHO cells.

One of our main goals was to establish a cell line stably expressing the α_{1L} -AR phenotype for further pharmacological studies. As outlined above, although the mechanism is complex, the overexpression of CRELD1 α [α_{1A} -

COE(α_{1L} -dominant) cells] along with the α_{1A} -AR does result in expressing the α_{1L} -AR phenotype. Relative to α_{1A} -AR, the expressed α_{1L} -AR showed significantly lower affinities for prazosin and other α_{1A} -AR selective antagonists, RS-17053 and 5-methylurapidil, but both expressed AR phenotypes showed a high affinity for silodosin (α_{1A} - and α_{1L} -AR antagonist). This antagonist profile agrees with the α_{1L} -AR found in native tissues (8, 9, 12, 42, 43).

The agonist profiles of the reconstituted α_1 -AR phenotypes were also confirmed using a Ca^{2+} transient response. In α_{1A} -CKD(α_{1A} -enhanced) cells (predominantly expressing the α_{1A} -AR phenotype), the agonist-evoked Ca^{2+} response was antagonized not only by prazosin but also by RS-17053 or 5-methylurapidil with appropriately high affinities. On the other hand, in α_{1A} -COE(α_{1L} -dominant) cells, prazosin antagonized the concentration–response curves for noradrenaline in a two-step manner, showing both high and low affinities for prazosin (Fig. 3). Furthermore, prazosin produced insurmountable inhibition in α_{1A} -COE(α_{1L} -dominant) cells. This result might be due to the following: 1) different affinities of prazosin for the α_{1A} - and α_{1L} -ARs, 2) slow dissociation of prazosin from the receptors, 3) the transient nature of the response (here, intracellular release of Ca^{2+}), and 4) a low receptor reserve in α_{1A} -COE(α_{1L} -dominant) cells (44, 45). Amongst these possibilities, it is likely that 4) and 1) for the α_{1A} -AR would lead to insurmountable inhibition. Indeed, such an insurmountable inhibition was not produced by prazosin (1 – 300 nM) in α_{1A} -CKD(α_{1A} -enhanced) cells with a high reserve of α_{1A} -ARs (Fig. 3a).

After masking the α_{1A} -AR component in α_{1A} -COE(α_{1L} -dominant) cells by treatment with a low concentration of prazosin (10 nM) (32), we could clearly detect the functional α_{1L} -AR response, which showed low affinities for prazosin and the α_{1A} -AR selective antagonists RS-17053 and 5-methylurapidil. These low affinity estimates can not be attributed to an underestimation due to the use of prazosin to mask the α_{1A} -AR because a high affinity for silodosin at α_{1L} -AR was still observed even in the presence of 10 nM prazosin.

Agonist pharmacology also revealed differences between the CHO-expressed α_{1A} - and α_{1L} -ARs. Ro 115-1240 was originally developed as a partial α_{1A} - and α_{1L} -AR agonist (34). In our cell lines, however, Ro 115-1240 was in fact a partial agonist at α_{1A} -AR but was essentially inactive at the α_{1L} -AR. On the other hand, MK017, a new α_{1A} - and α_{1L} -AR agonist, showed a high potency for both AR phenotypes. These agonist profiles of receptors expressed in cell lines were entirely in accord with the phenotypes observed in intact tissue preparations both for the α_{1A} -AR [rat tail artery (19, 28)] and for the α_{1L} -AR [rat prostate (37)]. However, it should be noted that there

are significant differences in the efficacies to MK017 and Ro 115-1240 between native and reconstituted receptors (Fig. 4). These differences probably relate to mechanism(s) that affect the receptor efficacies.

In addition to the classical α_1 -ARs (α_{1A} , α_{1B} , and α_{1D}), it is recognized that α_{1L} -ARs are involved in many physiological responses. Of note, recent studies have concluded that α_{1L} -AR is the functional α_1 -AR found in the lower urinary tract (14). Furthermore, α_{1L} -ARs in the lower urinary tract represent a new target for α_1 -AR antagonists for the treatment of urinary obstruction in men with benign prostatic hypertrophy (9, 46) and for women with urinary incontinence (34, 47). Thus, the α_{1L} -AR-expressing cell line should be of particular utility for the development of α_{1L} -AR-targeted drugs and for the pharmacological study of α_{1L} -ARs.

In the context of our work, one can point to the predictions of the 'mobile' receptor model outlined some time ago (23). It was predicted that interactions of a receptor with different 'effectors' in the membrane would affect ligand affinities. The interaction of CRELD1 α with the α_{1A} -AR is in keeping with that prediction. It is now recognized that GPCRs exist as multi-protein complexes in the plasma membrane. The GPCR-interacting proteins play roles in regulating GPCR signaling, pharmacological properties (potency and efficacy), internalization, and trafficking of receptor-associated scaffolds that mediate signaling (48–51). Most of the GPCR-interacting proteins described to date are intracellularly located, targeting the intracellular loops or C-terminal domain of GPCRs. Among these, the so-called RAMPs were first identified as single-span membrane proteins interacting with the calcitonin-receptor-like receptor. RAMP interactions can modulate the glycosylation of the accompanying GPCRs to regulate their transport and ligand specificity (52, 53). The possibility that the extracellular domains of CRELD1 α might inhibit ligand binding to the α_{1A} -AR should also be noted. Although the mechanism underlying the impact of CRELD1 α on the α_{1A} -AR is as yet unknown, its effects may also be applicable to other GPCRs. In addition, it is noteworthy that we do not yet fully understand the full mechanism that actively converts α_{1A} -ARs into α_{1L} -ARs. These issues merit further study in the future.

In summary, we have identified CRELD1 α as an α_{1A} -AR-interacting protein that modulates the expression and phenotype of this receptor, enabling the identification of the α_{1L} -AR phenotype in a cell expression system. The α_{1A} -COE(α_{1L} -dominant) cell line that we have established may provide a paradigm for evaluating the interactions of CRELD1 α with other GPCRs and should provide for the evaluation of therapeutic agents that selectively affect the α_{1L} -AR.

Acknowledgments

We express great appreciation to Professor Morley D. Hollenberg (University of Calgary, Canada) for his helpful discussions and comments concerning the writing of our manuscript. This study was supported in part by the Grant-in-Aid for Scientific Research from Japan Society of the Promotion of Science (JSPS) (to A. Nishimune, F. Suzuki, S. Morishima, and I. Muramatsu); by the Adaptable and Seamless Technology transfer Program through target-driven R&D from Japan Science and Technology Agency (JST) (To I. Muramatsu); and by the grant from Smoking Research Foundation of Japan (To I. Muramatsu).

References

- 1 Alexander SP, Mathie A, Peters JA. Guide to receptors and channels (GRAC), 3rd edition. *Br J Pharmacol.* 2008;153 Suppl 2: S1–S209.
- 2 Lomasney JW, Cotecchia S, Lefkowitz RJ, Caron MG. Molecular biology of alpha-adrenergic receptors: implications for receptor classification and for structure-function relationships. *Biochim Biophys Acta.* 1991;1095:127–139.
- 3 Hieble JP, Bylund DB, Clarke DE, Eikenburg DC, Langer SZ, Lefkowitz RJ, et al. International Union of Pharmacology. X. Recommendation for nomenclature of alpha 1-adrenoceptors: consensus update. *Pharmacol Rev.* 1995;47:267–270.
- 4 Zhong H, Minneman KP. Alpha1-adrenoceptor subtypes. *Eur J Pharmacol.* 1999;375:261–276.
- 5 Michelotti GA, Price DT, Schwinn DA. Alpha 1-adrenergic receptor regulation: basic science and clinical implications. *Pharmacol Ther.* 2000;88:281–309.
- 6 Flavahan NA, Vanhoutte PM. Alpha-1 and alpha-2 adrenoceptor: response coupling in canine saphenous and femoral veins. *J Pharmacol Exp Ther.* 1986;238:131–138.
- 7 Muramatsu I, Ohmura T, Kigoshi S, Hashimoto S, Oshita M. Pharmacological subclassification of alpha 1-adrenoceptors in vascular smooth muscle. *Br J Pharmacol.* 1990;99:197–201.
- 8 Muramatsu I, Suzuki F, Nishimune A, Anisuzzaman AS, Yoshiki H, Su TH, et al. Expression of distinct alpha1-adrenoceptor phenotypes in the iris of pigmented and albino rabbits. *Br J Pharmacol.* 2009;158:354–360.
- 9 Ford AP, Arredondo NF, Blue DR Jr, Bonhaus DW, Jasper J, Kava MS, et al. RS-17053 (N-[2-(2-cyclopropylmethoxyphenoxymethyl)-5-chloro-alpha, alpha-dimethyl-1H-indole-3-ethanamine hydrochloride], a selective alpha 1A-adrenoceptor antagonist, displays low affinity for functional alpha 1-adrenoceptors in human prostate: implications for adrenoceptor classification. *Mol Pharmacol.* 1996;49:209–215.
- 10 Stam WB, Van der Graaf PH, Saxena PR. Analysis of alpha 1L-adrenoceptor pharmacology in rat small mesenteric artery. *Br J Pharmacol.* 1999;127:661–670.
- 11 Argyle SA, McGrath JC. An alpha(1A)/alpha(1L)-adrenoceptor mediates contraction of canine subcutaneous resistance arteries. *J Pharmacol Exp Ther.* 2000;295:627–633.
- 12 Muramatsu I, Morishima S, Suzuki F, Yoshiki H, Anisuzzaman AS, Tanaka T, et al. Identification of alpha 1L-adrenoceptor in mice and its abolition by alpha 1A-adrenoceptor gene knockout. *Br J Pharmacol.* 2008;155:1224–1234.
- 13 Gray K, Short J, Ventura S. The alpha1A-adrenoceptor gene is required for the alpha1L-adrenoceptor-mediated response in iso-

- lated preparations of the mouse prostate. *Br J Pharmacol.* 2008; 155:103–109.
- 14 Nishimune A, Suzuki F, Yoshiki H, Morishima S, Muramatsu I. alpha-Adrenoceptor pharmacome: alpha-Adrenoceptor and alpha-adrenoceptor in the lower urinary tract. *Int J Urol.* 2010; 31–37.
- 15 Ramsay D, Carr IC, Pediani J, Lopez-Gimenez JF, Thurlow R, Fidock M, et al. High-affinity interactions between human alpha1A-adrenoceptor C-terminal splice variants produce homo- and heterodimers but do not generate the alpha1L-adrenoceptor. *Mol Pharmacol.* 2004;66:228–239.
- 16 Suzuki F, Taniguchi T, Takauji R, Murata S, Muramatsu I. Splice isoforms of alpha(1a)-adrenoceptor in rabbit. *Br J Pharmacol.* 2000;129:1569–1576.
- 17 Hiraizumi-Hiraoka Y, Tanaka T, Yamamoto H, Suzuki F, Muramatsu I. Identification of alpha-1L adrenoceptor in rabbit ear artery. *J Pharmacol Exp Ther.* 2004;310:995–1002.
- 18 Morishima S, Tanaka T, Yamamoto H, Suzuki F, Akino H, Yokoyama O, et al. Identification of alpha-1L and alpha-1A adrenoceptors in human prostate by tissue segment binding. *J Urol.* 2007;177:377–381.
- 19 Morishima S, Suzuki F, Yoshiki H, Md Anisuzzaman AS, Sathi ZS, Tanaka T, et al. Identification of the alpha1L-adrenoceptor in rat cerebral cortex and possible relationship between alpha1L- and alpha1A-adrenoceptors. *Br J Pharmacol.* 2008;153:1485–1494.
- 20 Su TH, Morishima S, Suzuki F, Yoshiki H, Anisuzzaman AS, Tanaka T, et al. Native profiles of alpha(1A)-adrenoceptor phenotypes in rabbit prostate. *Br J Pharmacol.* 2008;155:906–912.
- 21 Muramatsu I, Tanaka T, Suzuki F, Li Z, Hiraizumi-Hiraoka Y, Anisuzzaman AS, et al. Quantifying receptor properties: the tissue segment binding method - a powerful tool for the pharmacome analysis of native receptors. *J Pharmacol Sci.* 2005;98: 331–339.
- 22 Nelson C. The alpha1L-adrenoceptor is an alternative phenotype of the alpha1A-adrenoceptor. *Br J Pharmacol.* 2008;155:1–3.
- 23 Jacobs S, Cuatrecasas P. The mobile receptor hypothesis and “cooperativity” of hormone binding. Application to insulin. *Biochim Biophys Acta.* 1976;433:482–495.
- 24 Piascik MT, Perez DM. Alpha1-adrenergic receptors: new insights and directions. *J Pharmacol Exp Ther.* 2001;298:403–410.
- 25 Suzuki F, Nishimune A, Yoshiki H, Morishima S, Muramatsu I. Alpha 1L-adrenoceptor phenotype revealed through the coexpression of alpha 1A-adrenoceptor and its novel interacting protein, ARIP. *J Pharmacol Sci.* 2010;112 Suppl 1:125P.
- 26 Suzuki F, Morishima S, Tanaka T, Muramatsu I. Snapin, a new regulator of receptor signaling, augments alpha1A-adrenoceptor-operated calcium influx through TRPC6. *J Biol Chem.* 2007; 282:29563–29573.
- 27 Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem.* 1985;260:3440–3450.
- 28 Lachnit WG, Tran AM, Clarke DE, Ford AP. Pharmacological characterization of an alpha 1A-adrenoceptor mediating contractile responses to noradrenaline in isolated caudal artery of rat. *Br J Pharmacol.* 1997;120:819–826.
- 29 Furchgott RF. The classification on adrenoceptors (adrenergic receptors): an evaluation from the standpoint of receptor theory. In: Blaschko H, Muscholl E, editors. *Handbuch der experimentellen Pharmakologie.* New York: Springer; 1972. p. 283–335.
- 30 Muramatsu I, Murata S, Isaka M, Piao HL, Zhu J, Suzuki F, et al. Alpha1-adrenoceptor subtypes and two receptor systems in vascular tissues. *Life Sci.* 1998;62:1461–1465.
- 31 Rupp PA, Fouad GT, Egelston CA, Reifsteck CA, Olson SB, Knosp WM, et al. Identification, genomic organization and mRNA expression of CRELD1, the founding member of a unique family of matricellular proteins. *Gene.* 2002;293:47–57.
- 32 Takasaki J, Saito T, Taniguchi M, Kawasaki T, Moritani Y, Hayashi K, et al. A novel Galphq/11-selective inhibitor. *J Biol Chem.* 2004;279:47438–47445.
- 33 Kenakin TP. Tissue response as a functional discriminator of receptor heterogeneity: effects of mixed receptor populations on Schild regressions. *Mol Pharmacol.* 1992;41:699–707.
- 34 Blue DR, Daniels DV, Gever JR, Jett MF, O’Yang C, Tang HM, et al. Pharmacological characteristics of Ro 115-1240, a selective alpha1A/1L-adrenoceptor partial agonist: a potential therapy for stress urinary incontinence. *BJU Int.* 2004;93:162–170.
- 35 Taki N, Tanaka T, Zhang L, Suzuki F, Israilova M, Taniguchi T, et al. Alpha-1D adrenoceptors are involved in reserpine-induced supersensitivity of rat tail artery. *Br J Pharmacol.* 2004;142: 647–656.
- 36 Rokosh DG, Simpson PC. Knockout of the alpha 1A/C-adrenergic receptor subtype: the alpha 1A/C is expressed in resistance arteries and is required to maintain arterial blood pressure. *Proc Natl Acad Sci U S A.* 2002;99:9474–9479.
- 37 Hiraoka Y, Ohmura T, Oshita M, Watanabe Y, Morikawa K, Nagata O, et al. Binding and functional characterization of alpha1-adrenoceptor subtypes in the rat prostate. *Eur J Pharmacol.* 1999;366:119–126.
- 38 Taniguchi T, Inagaki R, Murata S, Akiba I, Muramatsu I. Microphysiometric analysis of human alpha1a-adrenoceptor expressed in Chinese hamster ovary cells. *Br J Pharmacol.* 1999;127: 962–968.
- 39 Munson PJ, Rodbard D. Ligand: a versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem.* 1980;107:220–239.
- 40 Ford AP, Daniels DV, Chang DJ, Gever JR, Jasper JR, Lesnick JD, et al. Pharmacological pleiotropism of the human recombinant alpha1A-adrenoceptor: implications for alpha1-adrenoceptor classification. *Br J Pharmacol.* 1997;121:1127–1135.
- 41 Daniels DV, Gever JR, Jasper JR, Kava MS, Lesnick JD, Meloy TD, et al. Human cloned alpha1A-adrenoceptor isoforms display alpha1L-adrenoceptor pharmacology in functional studies. *Eur J Pharmacol.* 1999;370:337–343.
- 42 Testa R, Guarneri L, Angelico P, Poggesi E, Taddei C, Sironi G, et al. Pharmacological characterization of the uroselective alpha-1 antagonist Rec 15/2739 (SB 216469): role of the alpha-1L adrenoceptor in tissue selectivity, part II. *J Pharmacol Exp Ther.* 1997;281:1284–1293.
- 43 Van der Graaf PH, Deplanne V, Duquenne C, Angel I. Analysis of alpha1-adrenoceptors in rabbit lower urinary tract and mesenteric artery. *Eur J Pharmacol.* 1997;327:25–32.
- 44 Pediani JD, MacKenzie JF, Heeley RP, Daly CJ, McGrath JC. Single-cell recombinant pharmacology: bovine alpha(1a)-adrenoceptors in rat-1 fibroblasts release intracellular Ca(2+), display subtype-characteristic agonism and antagonism, and exhibit an antagonist-reversible inverse concentration-response phase. *J Pharmacol Exp Ther.* 2000;293:887–895.
- 45 Methven L, Simpson PC, McGrath JC. Alpha1A/B-knockout mice explain the native alpha1D-adrenoceptor’s role in vasocon-

- striction and show that its location is independent of the other α_1 -subtypes. *Br J Pharmacol*. 2009;158:1663–1675.
- 46 Leonardi A, Hieble JP, Guarneri L, Naselsky DP, Poggesi E, Sironi G, et al. Pharmacological characterization of the uroselective α_1 -antagonist Rec 15/2739 (SB 216469): role of the α_1 -adrenoceptor in tissue selectivity, part I. *J Pharmacol Exp Ther*. 1997;281:1272–1283.
 - 47 Taki N, Taniguchi T, Okada K, Moriyama N, Muramatsu I. Evidence for predominant mediation of α_1 -adrenoceptor in the tonus of entire urethra of women. *J Urol*. 1999;162:1829–1832.
 - 48 Brady AE, Limbird LE. G protein-coupled receptor interacting proteins: emerging roles in localization and signal transduction. *Cell Signal*. 2002;14:297–309.
 - 49 Bockaert J, Fagni L, Dumuis A, Marin P. GPCR interacting proteins (GIP). *Pharmacol Ther*. 2004;103:203–221.
 - 50 Lefkowitz RJ, Shenoy SK. Transduction of receptor signals by β -arrestins. *Science*. 2005;308:512–517.
 - 51 Wang H, Westin L, Nong Y, Birnbaum S, Bendor J, Brismar H, et al. Norbin is an endogenous regulator of metabotropic glutamate receptor 5 signaling. *Science*. 2009;326:1554–1557.
 - 52 McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, et al. RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature*. 1998;393:333–339.
 - 53 Poyner DR, Sexton PM, Marshall I, Smith DM, Quirion R, Born W, et al. International Union of Pharmacology. XXXII. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin, and calcitonin receptors. *Pharmacol Rev*. 2002;54:233–246.