Amino Acids (2015) 47:1455–1464
DOI 10.1007/s00726-015-1965-6

ORIGINAL ARTICLE

Regulation of β2-adrenergic receptor cell surface expression by interaction with cystic fibrosis transmembrane conductance regulator-associated ligand (CAL)

Longyan Yang[1] · Junfang Zheng[1] · Ying Xiong[1] · Ran Meng[1] · Qian Ma[1] · Hua Liu[1] · Hui Shen[1] · Shuai Zheng[1] · Songlin Wang[1,2] · Junqi He[1]

Received: 3 May 2014 / Accepted: 13 March 2015 / Published online: 16 April 2015

Abstract The beta-2 adrenergic receptor (β2AR), a member of GPCR, can activate multiple signaling pathways and is an important treatment target for cardiac failure. However, the molecular mechanism about β2AR signaling regulation is not fully understood. In this study, we found that cystic fibrosis transmembrane conductance regulator-associated ligand (CAL) overexpression reduced β2AR-mediated extracellular signal-regulated kinase-1/2 (ERK1/2) activation. Further study identified CAL as a novel binding partner of β2AR. CAL is associated with β2AR mainly via the third intracellular loop (ICL3) of receptor and the coiled-coil domains of CAL, which is distinct from CAL/β1AR interaction mediated by the carboxyl terminal (CT) of β1AR and PDZ domain of CAL. CAL overexpression retarded β2AR expression in Golgi apparatus and reduced the receptor expression in plasma membrane.

Handling Editor: I. Greger.

L. Yang and J. Zheng contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00726-015-1965-6) contains supplementary material, which is available to authorized users.

✉ Junqi He
jq_he@ccmu.edu.cn

[1] Department of Biochemistry and Molecular Biology, Capital Medical University, No. 10 Xitoutiao, You An Men, Beijing 100069, People's Republic of China

[2] Molecular Laboratory for Gene Therapy and Tooth Regeneration, Capital Medical University School of Stomatology, Beijing 100050, China

Keywords Adrenergic receptor · PDZ homology · Coiled-coil domain · CAL · Intracellular loop

Abbreviations

βARs	Beta-adrenergic receptors
CAL	Cystic fibrosis transmembrane conductance regulator-associated ligand
CT	Carboxyl terminal
ERK	Extracellular signal-regulated kinase
GST	Glutathione S-transferase
ICL	Intracellular loop
PDZ	PSD-95/discs-large/ZO-1 homology

Introduction

Beta-adrenergic receptors (βARs) play important roles in cardiovascular, respiratory, metabolic, reproductive and central nervous systems. Pharmacological stimulation or blockade of βAR signaling is the therapeutic mainstay in cardiogenic shock, hypertension, ischemia, arrhythmias, and heart failure (Dorn and Liggett 2008). β2AR is one of the primary βAR subtypes responsible for cardiac response. Activation of β2AR promotes cardiomyocyte hypertrophy and thus plays a vital role in the development of human heart failure (Heineke and Molkentin 2006; Nunn et al. 2010). Therefore, the study on the regulatory mechanism of β2AR pathway activation would improve the understanding of heart failure pathogenesis.

The regulation of βARs carboxyl terminal (CT)-binding protein on βARs function was widely concerned by researchers. Compared to the multiple regulatory mechanisms of β1AR pathway by its various CT-binding proteins (He et al. 2004; Hu et al. 2000, 2003; Xu et al. 2001), the regulation of β2AR pathway by its CT-binding protein

was rarely reported (Hall et al. 1998; Yang et al. 2010). We previously reported that cystic fibrosis transmembrane conductance regulator-associated ligand (CAL, also known as GOPC or PIST), a PSD-95/discs-large/ZO-1 homology (PDZ) domain-containing protein, interacted with β1AR-CT via its PDZ domain to regulate the surface expression of β1AR, but it did not directly bind with β2AR-CT (He et al. 2004). However, in this study we found that CAL retarded β2AR-mediated ERK1/2 activation. Further study revealed that CAL was associated with β2AR via a novel mechanism distinct from PDZ domain of CAL and β1AR-CT mediated interaction. CAL over expression could also reduce cell surface expression of β2AR.

Materials and methods

Preparation of plasmids and fusion proteins

Flag-β2AR and Hemagglutinin (HA)-CAL plasmids were used as previously reported (Hall et al. 1998; He et al. 2004). β2AR-CT (the last 100 amino acids of β2AR), β2AR-the third intracellular loop (ICL3, the amino acids 221-274 of β2AR), CAL-coiled coil (CC, the amino acids 83-200 of CAL), and CAL-PDZ (the amino acids 288-371 of CAL) were amplified by PCR and subcloned into pGEX-4T-1 vector for expression as GST fusion proteins. CAL-CC was also subcloned into pET-30b vector for expression as His fusion protein. These constructs were verified by DNA sequencing. Fusion proteins were prepared as reported before (Yang et al. 2010).

Cell culture, transfection and cell treatment

COS-7 and HEK293 cells were maintained in complete medium (Dulbecco's modified eagle medium plus 10 % fetal bovine serum and 1 % penicillin/streptomycin) in a 37 °C/5 % CO_2 incubator. Transfections were performed following the protocol reported before (Yao et al. 2012). COS-7 cells were serum starved overnight, then treated with 10 μM β2AR agonist, isoproterenol (ISO, Sigma-Aldrich Chemical Corp., St. Louis, MO, USA) for 5 min at 37 °C, or β2AR selective agonist salmeterol (Tocris Cookson, Ballwin, MO, USA) for 10 min at 37 °C, or pretreated with β2AR selective antagonist ICI118,551 (Tocris Cookson) for 30 min before treatment with salmeterol to detect the effect of CAL expression on β2AR-mediated ERK signal transduction pathway.

GST pull-down assay

GST fusion proteins pull-down assay was performed as previously reported (Sun et al. 2013). The cells were lysed or mouse hearts were homogenized in ice-cold lysis buffer. Purified GST fusion proteins were incubated with lysates from cells or tissues, respectively. The eluted proteins were analyzed by Western blotting.

Co-immunoprecipitation (Co-IP)

Co-IP was performed as described previously (Yang et al. 2010). Briefly, transfected COS-7 cells were harvested and mouse hearts were homogenized in ice-cold lysis buffer. Lysate supernatants were incubated with anti-Flag M2 mouse monoclonal antibody affinity gel (Sigma, MO) for transfected cells, anti-β2AR antibody or IgG coupled with protein G plus/protein A-agarose (Merck Millipore, Hong Kong, China) for mouse heart lysates. After washing, the immunoprecipitated proteins were analyzed by Western blotting with monoclonal anti-HA or anti-CAL antibody.

Western blotting

Western blotting was performed as described before (Zheng et al. 2010a). Briefly, sample aliquots corresponding to 25 μg of protein were resolved using 10 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membrane. The blots were blocked for at least 30 min and then incubated with primary antibody in the blot buffer for 1 h at room temperature or overnight at 4 °C. The blots were then washed three times with 10 ml of the blot buffer each and incubated for 30 min at room temperature with a horseradish peroxidase (HRP)-conjugated secondary antibody in the blot buffer. Finally, the blots were washed three more times with 10 ml of the blot buffer each and visualized by enzyme-linked chemiluminescence. The primary antibody specific for Flag was purchased from Sigma. Polyclonal anti-HA and anti-His antibodies were purchased from MBL (Medical and biological laboratories, Nagoya, Japan). Anti-β2AR and anti-CAL were bought from Santa Cruz (Dallas, TX, USA) and Prosci Research (Loveland, CO, USA), respectively. The phospho-ERK1/2 assay was performed as previously described (Zheng et al. 2010b). The primary antibodies specific for phospho-ERK1/2 (Thr 202/Tyr 204) and total ERK1/2 were bought from Cell Signaling Technology (Beverly, MA, USA). HRP-conjugated IgG secondary antibodies were purchased from Amersham Biosciences (Piscataway, NJ, USA). Western blot was quantified using the NIH Image 1.62 (Zheng et al. 2010a).

Immunofluorescence microscopy

Immunofluorescence co-localization was performed as described previously (Yang et al. 2010). Briefly, plasmids were transfected into HEK293 cells. After fixation and

permeabilization, cells were stained with mouse anti-Flag antibody and rabbit anti-GM130 antibody (Abcam, Cambridge, UK) for 45 min, followed by Alexa Fluor 488-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR, USA) and Alexa Fluor 647-conjugated anti-rabbit IgG (Molecular Probes) for 30 min. Then, Alexa Fluor 594-conjugated anti-HA (Molecular Probes) was incubated for 1 h and Hoechst 33258 (5 μg/mL) for 5 min. The slides were visualized by a confocal microscope (Leica Microsystems, LAS AF-TCS SP5, Heidelberg, Germany).

Receptor cell surface expression analysis

Receptor cell surface expression analysis was performed as reported before (Cheng et al. 2011). Briefly, HEK293 cells were transiently transfected with constructs of Flag-β2AR plus HA-CAL or Flag-β2AR alone, respectively. Cells were pre-labeled with anti-Flag antibody or normal mouse IgG alone as control in cold medium. After washing, the cells were solubilized in lysis buffer. The lysates were incubated with protein A-coupled agarose beads overnight at 4 °C and collected. Then, the captured proteins were eluted from the beads using loading buffer and separated by SDS-PAGE, immunoblotted with anti-Flag antibody as primary antibody.

On-Cell Western

On-Cell Western was performed as described previously (Chan et al. 2009). Briefly, HEK293 cells were transiently transfected with constructs of Flag-β2AR plus HA-CAL or Flag-β2AR alone, respectively, and split into a 96-well plate. The cells were then incubated with 200 μl HBS/BSA. After fixation and blocking, the cells were incubated with primary antibody overnight at 4 °C. Subsequently, secondary antibody (IRDye™ 800CW DonkeyαRabbit 1:800, Rockland, Gilbertsville, PA, USA) was added and the cells were imaged using the Odyssey (LI-COR Inc., Lincoln, NE, USA).

Statistical analyses

The results of western blotting were semi-quantitatively analyzed by densitometry using Image J software. All data were presented as mean ± SD and statistical significance was analyzed by independent sample *T* test. Differences were considered significant when $P < 0.05$.

Results

CAL reduces β2AR-mediated ERK activation

CAL was reported to inhibit the β1AR expression in cell surface (He et al. 2004), so we further studied if β1AR-mediated ERK signaling could also be regulated by CAL expression. Meanwhile, β2AR was set up as a negative control since β2AR-CT was reported not binding with CAL (He et al. 2004). Unexpectedly, our results showed that β2AR-mediated ERK1/2 activation was significantly reduced by CAL overexpression ($P < 0.05$, Fig. 1a). When β2AR was expressed alone, ISO-stimulated ERK1/2 activation increased to more than 3.3-fold over that of control cells. Co-expression of CAL significantly reduced ISO-stimulated β2AR-mediated ERK1/2 activation up to 57.6 % compared with that of β2AR-alone expressing cells. HA-CAL alone overexpression did not decrease ERK activation of cells compared with that of control cells (Supplemental Fig. S1). These data suggest that CAL is involved in regulating the signaling of β2AR.

To verify these results, we further explored if CAL inhibited ERK activation induced by a selective β2AR agonist, salmeterol. Results showed that salmeterol stimulated ERK activation in a dose-dependent manner (Supplemental Fig. S2), and salmeterol-induced ERK activation was significantly inhibited by ICI118,551, a selective β2AR antagonist (Supplemental Fig. S3), indicating that salmeterol selectively induced β2AR-mediated ERK signaling activation in COS-7 cells, which was consistent with previous reports (Kaya et al. 2012; Qian et al. 2009; Tan et al. 2007). When β2AR was overexpressed in COS-7 cells, salmeterol-induced ERK activation increased to approximately 2-fold over that of control cells ($P < 0.01$, Fig. 1b). However, when co-overexpressed with CAL, salmeterol-induced ERK activation was robustly retarded ($P < 0.05$, Fig. 1b). These data indicate that CAL specifically inhibits β2AR-mediated ERK activation.

CAL is a novel binding partner of β2AR

CAL, an adaptor protein, usually exerts its function via interaction with its binding partners (Cheng et al. 2004). CAL expression retarded β2AR-mediated ERK1/2 activation, indicating that β2AR might interact with CAL, even though β2AR-CT was reported not binding with it (He et al. 2004). Thus, we further detected the interaction of full-length β2AR and CAL by co-immunoprecipitation assay. We found that full-length HA-CAL was evidently co-immunoprecipitated with full-length Flag-β2AR (Fig. 2a). To rule out the possibility that co-immunoprecipitation between CAL and β2AR was mediated by HA-tag, we transfected COS-7 cells with constructs of CAL alone, or β2AR and CAL; subsequently, cells were solubilized and incubated with IgG or anti-β2AR antibody coupled to affinity gel. Co-IP of CAL with β2AR was evident and there was no CAL signal detectable when IgG coupled to affinity gel was used (Fig. 2b).

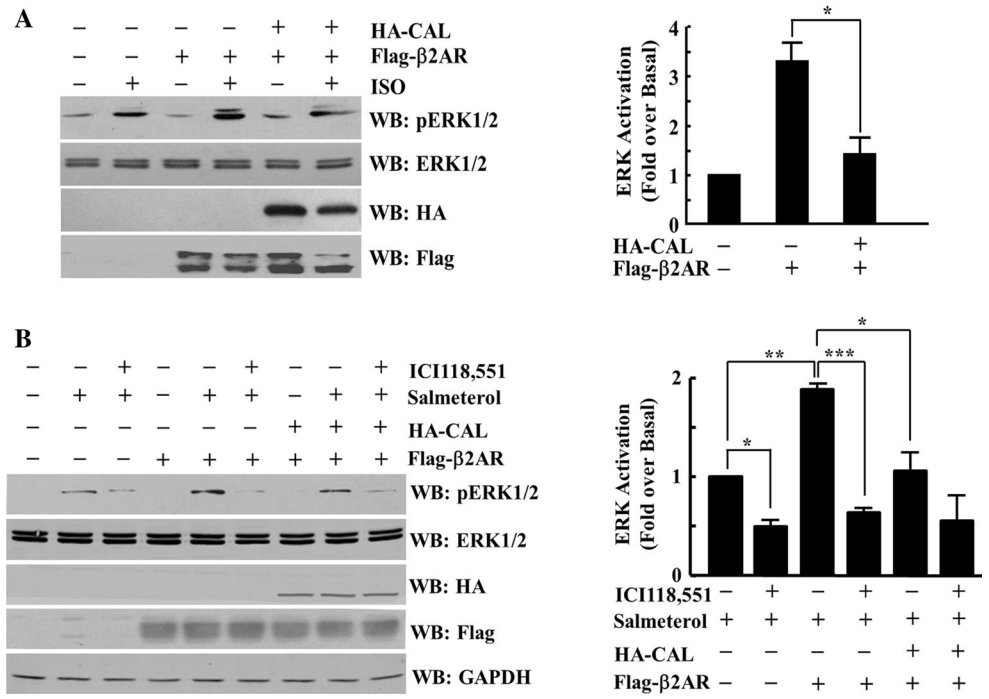

Fig. 1 CAL inhibits β2AR-mediated ERK activation. **a** CAL attenuated β2AR-mediated ERK phosphorylation. COS-7 cells were transfected with constructs of Flag-β2AR alone or in combination with HA-CAL. Cells were treated with serum-free medium overnight after 48 h of transfection and then stimulated with ISO. **b** CAL inhibited salmeterol-stimulated ERK activation mediated by β2AR. Constructs of Flag-β2AR or Flag-β2AR/HA-CAL were transiently transfected into COS-7 cells. After serum deprivation, the cells were stimulated with 1 μM salmeterol for 10 min at 37 °C or pretreated with 10 μM ICI118,551 for 30 min before salmeterol stimulation. ERK1/2 activation was detected with the western blotting. The effect of CAL on β2AR-mediated ERK1/2 activation was quantified. ERK1/2 activation was normalized to total ERK1/2 expression. Then, the results of normalized pERK in agonist/antagonist-stimulated cell were divided by those of agonist/antagonist-unstimulated same cells, and again it was calculated by dividing pERK in receptor over-expressing cells by that of control cells to obtain the final ratio of ERK activation. There was a significant decrease in pERK immunoreactivity (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) in cells co-transfected with Flag-β2AR and HA-CAL than in cells transfected with Flag-β2AR alone. *Data* and *error bars* represent mean ± standard deviation (SD) for three independent experiments

In addition to the transfection experiments, we also studied the interaction of endogenous β2AR and CAL in mouse heart tissue. Mice were killed, and their hearts were solubilized, lysates were incubated with β2AR antibody or IgG linked to protein A/G-agarose beads. Co-precipitated CAL was detected by Western blotting with anti-CAL. Robust co-immunoprecipitation of CAL with β2AR from heart tissue was observed (Fig. 2c). These results indicate that CAL is also a novel binding partner of β2AR.

The coiled-coil (CC) domains of CAL interact with the third intracellular loop of β2AR

The protein sequence analysis shows that CAL contains two CC domains and one PDZ domain (Fig. 3a). Both the CC domains and PDZ domain have been demonstrated to mediate protein–protein interaction, respectively (He et al. 2004; Tash et al. 2012; Watanabe et al. 2013). To elucidate the structural determinant of the interaction between CAL and β2AR, GST pull-down assay was performed using GST-CAL-CC and GST-CAL-PDZ proteins, respectively, to pull down lysates of COS-7 cells transfected with Flag-β2AR. Results showed that the CC domains of CAL, but not its PDZ domain, mediated its interaction with β2AR (Fig. 3b), which is distinct from PDZ domain-mediated CAL/β1AR interaction (He et al. 2004).

Lines of evidence indicated that GPCR associates with its binding proteins not only via its CT, but also via its intracellular loops (ICLs) (Borroto-Escuela et al. 2011; Chen et al. 2011; Dong et al. 2012; Marion et al. 2006; Woods 2010). Interactions with G proteins occur mainly in ICLs of the receptors. ICL1 and ICL2 in β2AR are short and lack of extensive helicity to support transducer anchoring (Parker et al. 2011), so ICL3 is the primary binding region of G-proteins for β2AR (Kling et al. 2013). β2AR was also reported to bind with its other partners via its ICL3 (Shiina et al. 2000), so we proposed that CAL/β2AR interaction might also act through its ICL3. To test this hypothesis, we used GST-β2AR-ICL3 or GST-β2AR-CT fusion proteins, respectively, to pull down HA-CAL protein expressed in

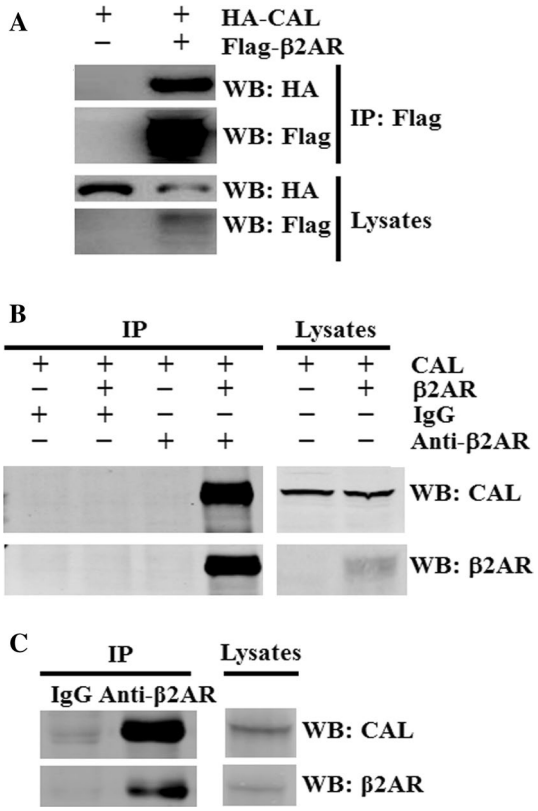

Fig. 2 CAL is a novel binding partner of β2AR. **a** CAL co-immunoprecipitated with β2AR. COS-7 cells transfected with constructs of HA-CAL alone, or Flag-β2AR and HA-CAL, were solubilized and incubated with anti-Flag antibody coupled to affinity gel. Co-IP of HA-CAL with Flag-β2AR was evident and it was not detectable when HA-CAL was expressed alone. **b** CAL co-immunoprecipitated with β2AR. COS-7 cells transfected with constructs of CAL alone, or β2AR and CAL, were solubilized and incubated with IgG or anti-β2AR antibody coupled to affinity gel. Co-IP of CAL with β2AR was evident and there was no detectable CAL when CAL was expressed alone. **c** β2AR interacts with CAL in native tissues. Solubilized lysates from homogenized mouse heart tissues were immunoprecipitated with anti-β2AR and analyzed by Western blotting with anti-CAL antibody

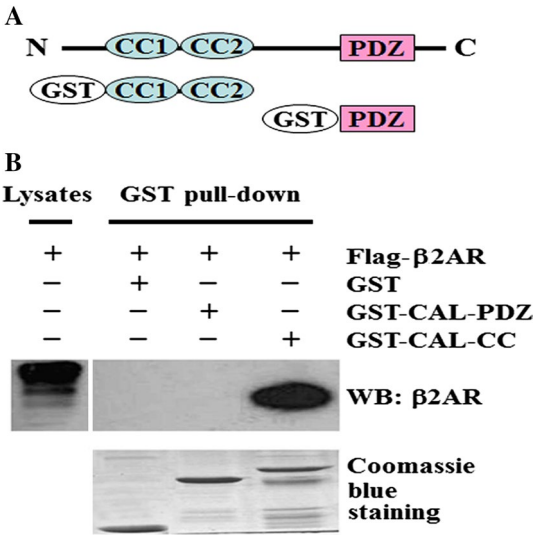

Fig. 3 CAL associates with β2AR via its coiled-coil (CC) domains. **a** Schematic representation of full-length CAL, GST-CAL-PDZ and GST-CAL-CC. **b** CAL interacted with β2AR via its CC domains. Purified Glutathione S-transferase (GST) fusion proteins, GST-CAL-CC or GST-CAL-PDZ were used in pull-down with lysates of COS-7 cells transfected with Flag-β2AR. Precipitates were subjected to Western blotting with the anti-β2AR antibody (*top panel*). The data are representative of three independent experiments

COS-7 cells. As shown in Fig. 4b, a robust HA-CAL was detected from the GST-β2AR-ICL3 pull-down complex, while relatively weaker HA-CAL signal was detected from the GST-β2AR-CT pull-down complex, indicating that β2AR interacts with CAL mainly via its ICL3.

To further rule out the noise of over-expressed exogenous protein from the interaction, tissue lysates from the hearts of mice were pulled down using GST-β2AR-ICL3 and GST-β2AR-CT fusion proteins, respectively. Consistently, endogenous CAL strongly interacted with GST-β2AR-ICL3, but weakly interacted with GST-β2AR-CT (Fig. 4c). Therefore, ICL3 of β2AR mainly mediates CAL/β2AR interaction.

Additionally, to verify the direct interaction between CAL-CC and β2AR-ICL3, GST pull-downs were performed with purified proteins. Results showed that a robust His-CAL-CC signal was detected from the GST-β2AR-ICL3 pull-down complex, while no His-CAL-CC signal was detected from the GST-β2AR-CT pull-down complex (Fig. 4d), demonstrating that the direct interaction between β2AR and CAL is mediated by β2AR-ICL3 and CAL-CC.

CAL entraps β2AR in the Golgi apparatus and retards cell surface expression of β2AR

The interaction of CAL with β2AR was further examined by immunofluorescence and confocal microscopy. HEK293 cells were transfected with Flag-β2AR in the presence or absence of HA-CAL. Images showed that β2AR localized primarily to the plasma membrane (Fig. 5a), whereas CAL was punctually distributed in the cytoplasm (Fig. 5d), when they were transfected individually. Cytoplasmic punctual co-localization of Flag-β2AR and HA-CAL was detected when CAL was co-transfected with β2AR (Fig. 5g–h), which indicated that a significant fraction of total β2AR was colocalized with CAL in the cytoplasm. Due to the Golgi localization of CAL, we speculated that Flag-β2AR and HA-CAL punctually co-localized in Golgi apparatus at cytoplasm.

To further elucidate the subcellular localization of β2AR and CAL, fluorescence confocal microscopy studies were performed again. In the same way, HEK293 cells were transfected with constructs of Flag-β2AR in the presence

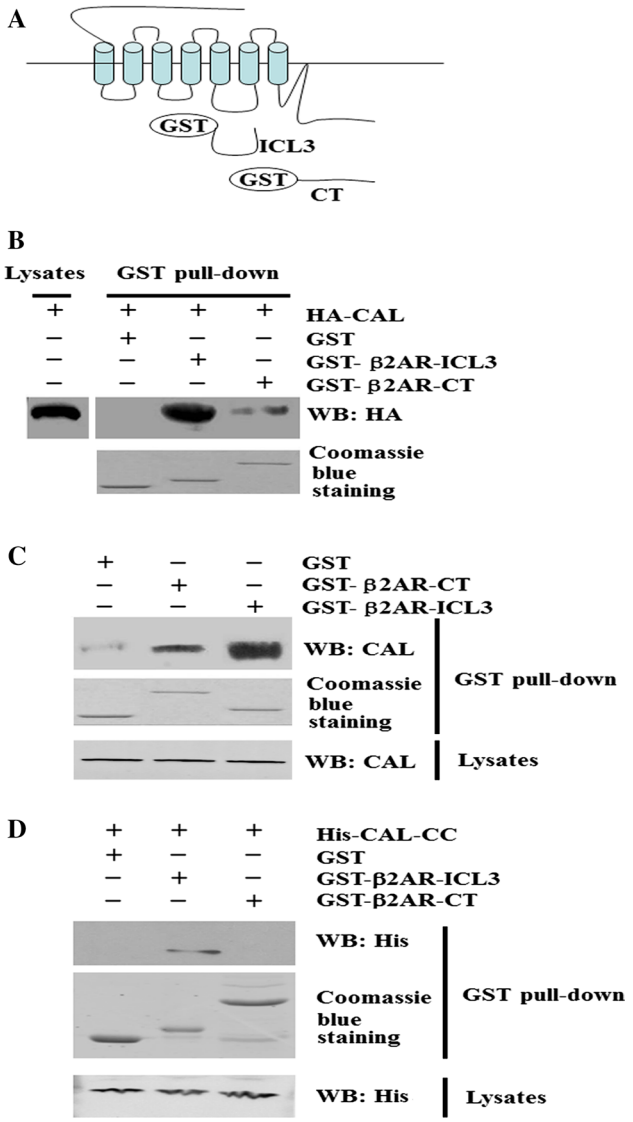

Fig. 4 β2AR associates with CAL via its third intracellular loop (ICL3). **a** Schematic representation of β2AR, GST-β2AR-ICL3 and GST-β2AR-carboxyl terminal (CT). **b** Exogenous CAL interacted with β2AR mainly via the ICL3 of receptor. Purified GST and GST-β2AR-ICL3 or GST-β2AR-CT proteins were used to pull down lysates of COS-7 cells transfected with HA-CAL followed by western blotting with anti-HA antibody. GST-β2AR-ICL3 pulled down significantly more CAL than GST-β2AR-CT. Coomassie Blue staining demonstrated equal loading of the fusion proteins. **c** Endogenous CAL interacted with β2AR-ICL3. Purified GST, GST-β2AR-ICL3 or GST-β2AR-CT proteins were used in pull-down assay with lysates of homogenized mouse heart tissues. Precipitates were visualized with the anti-CAL antibody. Consistent with exogenous protein pull-down experiments, GST-β2AR-ICL3 fusion protein pulled down significantly more CAL proteins than GST-β2AR-CT. **d** Purified β2AR-ICL3 directly interacted with His-CAL-CC fusion protein. Purified GST, GST-β2AR-ICL3 or GST-β2AR-CT proteins were used in pull-down with His-CAL-CC fusion protein. Precipitates were visualized with the anti-His antibody. His-CAL-CC protein can be clearly detected in GST-β2AR-ICL3 fusion protein pull-down complex, but not in GST-β2AR-CT pull-down complex. Coomassie Blue staining demonstrated equal loading of the fusion proteins

or absence of HA-CAL. Images showed that β2AR localized primarily to the plasma membrane did not overlap with the expression of the Golgi-associated protein GM130 (Fig. 5a–c), whereas CAL punctually distributed in the cytoplasm overlapped with GM130 (Fig. 5d–f) when they were transfected individually, indicating that β2AR localized primarily to the plasma membrane and CAL localized in Golgi apparatus. When CAL was co-transfected with β2AR, the majority of β2AR was expressed in cytoplasm and co-localized with CAL, overlapping with GM130 (Fig. 5g–j), which indicated that a significant fraction of β2AR was co-localized with CAL in the Golgi apparatus and CAL overexpression led to the entrapment of β2AR in cytoplasmic Golgi apparatus.

The finding of colocalization study showed that CAL co-localized with β2AR in Golgi apparatus, and indicated that CAL expression entrapped β2AR in the cytoplasm and reduced the expression of β2AR at cell plasma membrane. So we further studied reduced expression of β2AR at cell plasma membrane by CAL overexpression using receptor cell surface expression quantitative analysis. HEK293 cells were transfected with Flag-β2AR in the presence or absence of HA-CAL. Subsequently, live HEK-293 cells were surface-labeled at 4 °C with Flag-specific antibody to analyze surface receptor. Lysates were prepared after anti-Flag antibody labeling, and receptor-antibody complexes were immunoabsorbed onto protein A-agarose beads. The immunoabsorbed proteins were then eluted and probed with anti-Flag-specific antibodies. Immunoblot analysis comparing detergent lysates prepared from Flag-β2AR only-transfected cells versus both Flag-β2AR and HA-CAL-transfected cells identified that Flag-β2AR expression at plasma membrane was significantly reduced after co-transfected with HA-CAL (Fig. 6a).

Meanwhile, we further verified the reduced expression of β2AR at cell plasma membrane by CAL using On-Cell Western. HEK293 cells were transfected with Flag-β2AR in the presence or absence of HA-CAL and split into 96-well plates. Subsequently, primary anti-Flag antibody following fluorescent secondary antibody was added. The results of On-Cell Western also showed that the cell surface expression of β2AR was reduced by approximately 40 % in CAL-overexpressing cells when compared with that of Flag-β2AR-transfected alone (Fig. 6b). Taken together, these results demonstrate that CAL overexpression leads to reduced expression of β2AR at cell plasma membrane.

Discussion

In this study, we found that CAL overexpression regulated β2AR-mediated ERK activation. Further investigation identified a novel interaction of β2AR/CAL and the structural

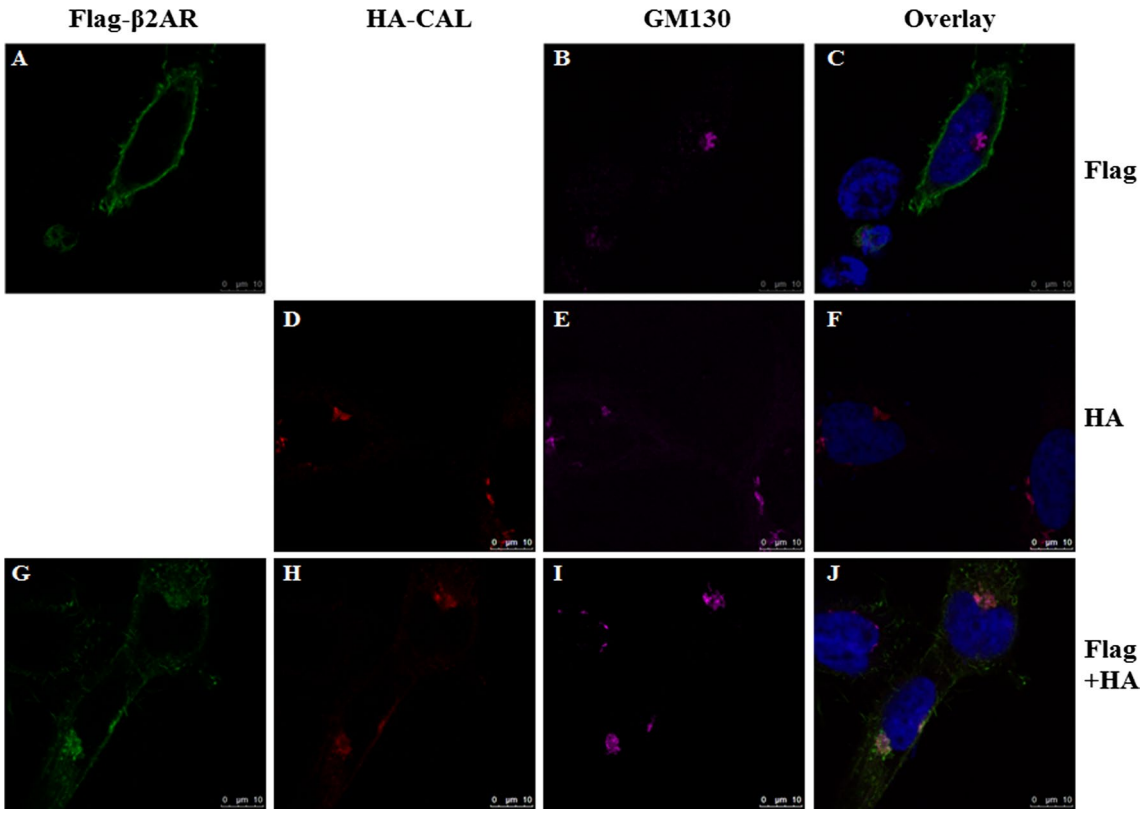

Fig. 5 Entrapment of β2AR in Golgi apparatus of the cytoplasm by interaction with CAL. CAL co-localized with β2AR in Golgi apparatus of HEK293 cells. Constructs of Flag-β2AR and HA-CAL were transfected into HEK293 cells alone or in combination. Flag-β2AR was mainly localized to the plasma membrane and did not overlap with Golgi-associated protein GM130 (**a–c**), while CAL was punctually dispersed in the cytoplasm and overlapped with GM130 when they were expressed alone (**d–f**). While CAL and β2AR were co-expressed, co-localization of CAL and β2AR at cytoplasm and overlapping with GM130 was detected (**g–j**)

determinants of their interaction. The results showed that β2AR interacted with CAL protein mainly via its ICL3, but not CT. CAL interacts with β2AR via its CC domain, but not its PDZ domain. These are substantially distinct from CAL/β1AR binding. CAL overexpression can regulate cell surface expression of β2AR. Thus, this study found a novel protein–protein interaction and demonstrated a new mechanism by which the functional activity of β2AR was regulated.

Similar to some other GPCRs, the carboxyl terminal (CT) of β2AR contains a PDZ-binding motif which has been reported to bind to PDZ domain-containing proteins to regulate receptor activity and expression (He et al. 2004; Zhang et al. 2008). In this study, we found that the CT of β2AR did not evidently mediate its interaction with CAL. CAL, containing two CC domains and one PDZ domain, has been reported to interact with multiple proteins via its different domains. CAL binds to β1AR (He et al. 2004), the cystic fibrosis transmembrane conductance regulator (CFTR) (Amacher et al. 2013), and Frizzled protein 5/8 (Yao et al. 2001) via its PDZ domain, whereas CAL interacts with Syntaxin 6 via its CC domain (Charest et al. 2001). In this study, we found that it was the CC domains of CAL that mediated its interaction with β2AR.

β2AR-mediated ERK1/2 activation can be regulated by the receptor binding with Gα protein or β-arrestin (Daaka et al. 1997; Kobayashi et al. 2005; Shenoy et al. 2006) or heterodimerization of β1AR and β2AR (Lavoie et al. 2002). This study found that β2AR-mediated ERK1/2 activation was regulated by a novel mechanism. That is, CAL overexpression entrapped β2AR in the cytoplasm, which was indicated by the receptor cell surface expression analysis (Fig. 6a) and On-Cell Western (Fig. 6b). Thus, CAL overexpression resulted in attenuated β2AR functional activity and β2AR-mediated ERK1/2 activation.

CAL is known to be predominantly located in the Golgi apparatus and interact with syntaxin 6 (Charest et al. 2001). Syntaxin 6 interacts with the Rab5 effector EEA1 (early endosomal autoantigen) and tethers post-Golgi vesicles to early endosome as well as the regulation of early endosome fusion (Simonsen et al. 1999). Thus, we speculated that the retention and accumulation of β2AR in the cytoplasm resulted from CAL modulation of β2AR anterograde trafficking from the ER-Golgi to the cell surface.

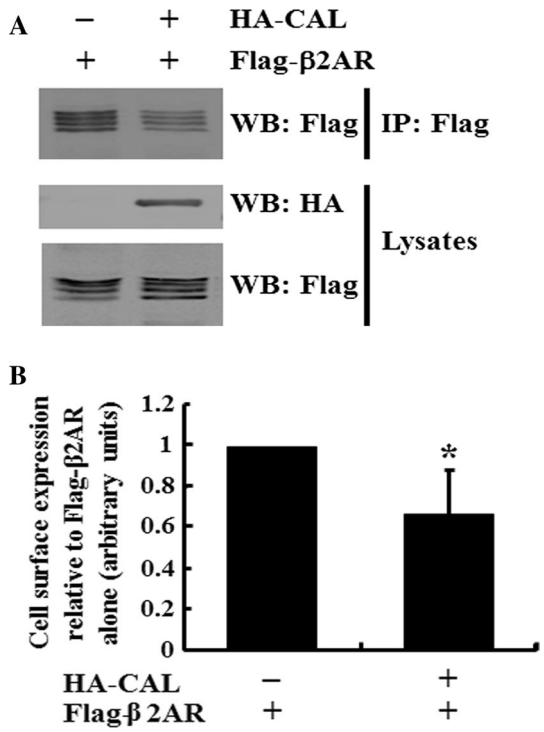

Fig. 6 CAL retards cell surface expression of β2AR. **a** CAL reduced β2AR expression in cell surface. HEK293 cells, transfected with constructs of Flag-β2AR alone, or Flag-β2AR and HA-CAL, were pre-labeled with anti-Flag antibody. After the cells were lysed, anti-Flag antibody-labeled Flag-β2AR was immunoprecipitated by protein A beads and then detected by immunoblotting. **b** CAL expression entrapped β2AR in the cytoplasm. COS-7 cells, transfected with constructs of Flag-β2AR alone, or Flag-β2AR and HA-CAL, were cultured in 96-well plates, subsequently fixed, and incubated with anti-Flag antibody and fluorescent secondary antibody, then detected using the LI-COR Odyssey. Integrated intensities were analyzed and normalized by cell number. Results showed that CAL expression led to the reduced Flag-β2AR expression at plasma membrane

Similar to this study, CAL has also been reported to play a role in the vesicle transport of CFTR (Cheng et al. 2002, 2004) from the Golgi apparatus to the plasma membrane. CAL decreased the cell surface expression of CFTR by reducing the rate of anterograde CFTR trafficking to the plasma membrane and favored retention of the CAL-CFTR complex in the Golgi (Cheng et al. 2002). The Golgi apparatus is required for the processing of complex sugar structures on many proteins and for the sorting of these proteins to their correct subcellular destinations (Farquhar and Palade 1998). Thus, CAL may play a role in the post-translational modification of β2AR, such as glycosylation (Mialet-Perez et al. 2004), thereby regulating the formation of mature β2AR competent for subsequent transport to the cell surface. However, this hypothesis needs to be further explored.

β2AR is mainly expressed in the heart and respiratory system. CAL is widely expressed in different tissues including the heart. β2AR-mediated ERK1/2 activation plays a pivotal role in the development of myocyte hypertrophic growth and heart failure (Heineke and Molkentin 2006; Nunn et al. 2010). To further verify the modulation of CAL on β2AR-mediated signaling in clinical samples, we searched the expression value of CAL and β2AR activation from Gene Expression Omnibus (GEO) heart failure database [GEO accession (GSE3586)] in patients with dilated cardiomyopathy (DCM, $n = 13$, DCM is a common cause of heart failure). The expression levels of adrenergic pathway gene set, a priori defined set of adrenergic pathway gene (Supplemental Table 1), were used as an indicator for the activation of adrenergic pathway (Martyniuk et al. 2013). We used Gene set enrichment analysis (GSEA; http://www.broad.mit.edu/gsea) to test the correlation of expression levels of CAL and gene sets involved in adrenergic pathway in DCM clinical samples. GSEA revealed that CAL was significantly negatively associated with genes involved in the adrenergic pathway, reminding CAL expression contributed negatively to adrenergic pathway activation. In heart failure (HF) mice model, CAL expression level was significantly downregulated compared with control mice. Interestingly, ERK activation level increased in contrast with reduced CAL and the change of β2AR expression level was not detected (Supplemental Fig. S3). This indicated that the low expression of CAL was potentially related to ERK activation in heart failure. All these data further provide clinical evidence for CAL to inhibit β2AR-mediated signaling, suggesting that the regulation on CAL expression might be a potential target for treating heart failure.

In summary, our results demonstrate that CAL interacts with β2AR mainly via the third intracellular loop (ICL3) of β2AR and the coiled-coil domains of CAL, and this interaction inhibits the cell surface expression of β2AR. These results add CAL as a novel member to β2AR-interacting proteins. The potential effect of this interaction on signaling and functions of β2AR may help to develop potential therapeutic measures for treating β2AR-associated diseases such as heart failure.

Acknowledgments This work was supported by the National Natural Science Foundation of the People's Republic of China (No. 81272887 and 81372739), Beijing Municipal Natural Science Foundation (No. 7131003), the Foundation of Beijing Educational Committee (No. KM201110025002), the Importation and Development of High-Caliber Talents Project of Beijing Municipal Institutions (CIT&TCD201304187). The manuscript revision by Dr. Qiong Qin was highly appreciated.

Conflict of interest We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work; there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

References

Amacher JF, Cushing PR, Bahl CD, Beck T, Madden DR (2013) Stereochemical determinants of C-terminal specificity in PDZ peptide-binding domains: a novel contribution of the carboxylate-binding loop. J Biol Chem 288(7):5114–5126

Borroto-Escuela DO, Tarakanov AO, Guidolin D, Ciruela F, Agnati LF, Fuxe K (2011) Moonlighting characteristics of G protein-coupled receptors: focus on receptor heteromers and relevance for neurodegeneration. IUBMB Life 63(7):463–472

Chan LF, Chung TT, Massoud AF, Metherell LA, Clark AJ (2009) Functional consequence of a novel Y129C mutation in a patient with two contradictory melanocortin-2-receptor mutations. Eur J Endocrinol 160(4):705–710

Charest A, Lane K, McMahon K, Housman DE (2001) Association of a novel PDZ domain-containing peripheral Golgi protein with the Q-SNARE (Q-soluble *N*-ethylmaleimide-sensitive fusion protein (NSF) attachment protein receptor) protein syntaxin 6. J Biol Chem 276(31):29456–29465

Chen AS, Kim YM, Gayen S, Huang Q, Raida M, Kang C (2011) NMR structural study of the intracellular loop 3 of the serotonin 5-HT(1A) receptor and its interaction with calmodulin. Biochim Biophys Acta 1808(9):2224–2232

Cheng J, Moyer BD, Milewski M, Loffing J, Ikeda M, Mickle JE, Cutting GR, Li M, Stanton BA, Guggino WB (2002) A Golgi-associated PDZ domain protein modulates cystic fibrosis transmembrane regulator plasma membrane expression. J Biol Chem 277(5):3520–3529

Cheng J, Wang H, Guggino WB (2004) Modulation of mature cystic fibrosis transmembrane regulator protein by the PDZ domain protein CAL. J Biol Chem 279(3):1892–1898

Cheng SB, Quinn JA, Graeber CT, Filardo EJ (2011) Down-modulation of the G-protein-coupled estrogen receptor, GPER, from the cell surface occurs via a trans-Golgi-proteasome pathway. J Biol Chem 286(25):22441–22455

Daaka Y, Luttrell LM, Lefkowitz RJ (1997) Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A. Nature 390(6655):88–91

Dong C, Nichols CD, Guo J, Huang W, Lambert NA, Wu G (2012) A triple arg motif mediates alpha(2B)-adrenergic receptor interaction with Sec24C/D and export. Traffic 13(6):857–868

Dorn GW 2nd, Liggett SB (2008) Pharmacogenomics of beta-adrenergic receptors and their accessory signaling proteins in heart failure. Clin Transl Sci 1(3):255–262

Farquhar MG, Palade GE (1998) The Golgi apparatus: 100 years of progress and controversy. Trends Cell Biol 8(1):2–10

Hall RA, Premont RT, Chow CW, Blitzer JT, Pitcher JA, Claing A, Stoffel RH, Barak LS, Shenolikar S, Weinman EJ et al (1998) The beta(2)-adrenergic receptor interacts with the Na^+/H^+-exchanger regulatory factor to control Na^+/H^+exchange. Nature 392(6676):626–630

He J, Bellini M, Xu J, Castleberry AM, Hall RA (2004) Interaction with cystic fibrosis transmembrane conductance regulator-associated ligand (CAL) inhibits beta1-adrenergic receptor surface expression. J Biol Chem 279(48):50190–50196

Heineke J, Molkentin JD (2006) Regulation of cardiac hypertrophy by intracellular signalling pathways. Nat Rev Mol Cell Biol 7(8):589–600

Hu LA, Tang Y, Miller WE, Cong M, Lau AG, Lefkowitz RJ, Hall RA (2000) Beta 1-adrenergic receptor association with PSD-95. Inhibition of receptor internalization and facilitation of beta 1-adrenergic receptor interaction with N-methyl-D-aspartate receptors. J Biol Chem 275(49):38659–38666

Hu LA, Chen W, Martin NP, Whalen EJ, Premont RT, Lefkowitz RJ (2003) GIPC interacts with the beta1-adrenergic receptor and regulates beta1-adrenergic receptor-mediated ERK activation. J Biol Chem 278(28):26295–26301

Kaya AI, Onaran HO, Ozcan G, Ambrosio C, Costa T, Balli S, Ugur O (2012) Cell contact-dependent functional selectivity of beta2-adrenergic receptor ligands in stimulating cAMP accumulation and extracellular signal-regulated kinase phosphorylation. J Biol Chem 287(9):6362–6374

Kling RC, Lanig H, Clark T, Gmeiner P (2013) Active-state models of ternary GPCR complexes: determinants of selective receptor-G-protein coupling. PLoS One 8(6):e67244

Kobayashi H, Narita Y, Nishida M, Kurose H (2005) Beta-arrestin2 enhances beta2-adrenergic receptor-mediated nuclear translocation of ERK. Cell Signal 17(10):1248–1253

Lavoie C, Mercier JF, Salahpour A, Umapathy D, Breit A, Villeneuve LR, Zhu WZ, Xiao RP, Lakatta EG, Bouvier M et al (2002) Beta 1/beta 2-adrenergic receptor heterodimerization regulates beta 2-adrenergic receptor internalization and ERK signaling efficacy. J Biol Chem 277(38):35402–35410

Marion S, Oakley RH, Kim KM, Caron MG, Barak LS (2006) A beta-arrestin binding determinant common to the second intracellular loops of rhodopsin family G protein-coupled receptors. J Biol Chem 281(5):2932–2938

Martyniuk CJ, Prucha MS, Doperalski NJ, Antczak P, Kroll KJ, Falciani F, Barber DS, Denslow ND (2013) Gene expression networks underlying ovarian development in wild largemouth bass (*Micropterus salmoides*). PLoS One 8(3):e59093

Mialet-Perez J, Green SA, Miller WE, Liggett SB (2004) A primate-dominant third glycosylation site of the beta2-adrenergic receptor routes receptors to degradation during agonist regulation. J Biol Chem 279(37):38603–38607

Nunn C, Zou MX, Sobiesiak AJ, Roy AA, Kirshenbaum LA, Chidiac P (2010) RGS2 inhibits beta-adrenergic receptor-induced cardiomyocyte hypertrophy. Cell Signal 22(8):1231–1239

Parker MS, Park EA, Sallee FR, Parker SL (2011) Two intracellular helices of G-protein coupling receptors could generally support oligomerization and coupling with transducers. Amino Acids 40(2):261–268

Qian L, Hu X, Zhang D, Snyder A, Wu HM, Li Y, Wilson B, Lu RB, Hong JS, Flood PM (2009) Beta2 Adrenergic receptor activation induces microglial NADPH oxidase activation and dopaminergic neurotoxicity through an ERK-dependent/protein kinase A-independent pathway. Glia 57(15):1600–1609

Shenoy SK, Drake MT, Nelson CD, Houtz DA, Xiao K, Madabushi S, Reiter E, Premont RT, Lichtarge O, Lefkowitz RJ (2006) Beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor. J Biol Chem 281(2):1261–1273

Shiina T, Kawasaki A, Nagao T, Kurose H (2000) Interaction with beta-arrestin determines the difference in internalization behavor between beta1- and beta2-adrenergic receptors. J Biol Chem 275(37):29082–29090

Simonsen A, Gaullier JM, D'Arrigo A, Stenmark H (1999) The Rab5 effector EEA1 interacts directly with syntaxin-6. J Biol Chem 274(41):28857–28860

Sun C, Zheng J, Cheng S, Feng D, He J (2013) EBP50 phosphorylation by Cdc2/Cyclin B kinase affects actin cytoskeleton reorganization and regulates functions of human breast cancer cell line MDA-MB-231. Mol Cells 36(1):47–54

Tan KS, Nackley AG, Satterfield K, Maixner W, Diatchenko L, Flood PM (2007) Beta2 adrenergic receptor activation stimulates pro-inflammatory cytokine production in macrophages via PKA- and NF-kappaB-independent mechanisms. Cell Signal 19(2):251–260

Tash BR, Bewley MC, Russo M, Keil JM, Griffin KA, Sundstrom JM, Antonetti DA, Tian F, Flanagan JM (2012) The occludin and ZO-1 complex, defined by small angle X-ray scattering and

NMR, has implications for modulating tight junction permeability. Proc Natl Acad Sci USA 109(27):10855–10860

Watanabe S, De Zan T, Ishizaki T, Narumiya S (2013) Citron kinase mediates transition from constriction to abscission through its coiled-coil domain. J Cell Sci 126(Pt 8):1773–1784

Woods AS (2010) The dopamine D(4) receptor, the ultimate disordered protein. J Recept Signal Transduct Res 30(5):331–336

Xu J, Paquet M, Lau AG, Wood JD, Ross CA, Hall RA (2001) Beta 1-adrenergic receptor association with the synaptic scaffolding protein membrane-associated guanylate kinase inverted-2 (MAGI-2). Differential regulation of receptor internalization by MAGI-2 and PSD-95. J Biol Chem 276(44):41310–41317

Yang X, Zheng J, Xiong Y, Shen H, Sun L, Huang Y, Sun C, Li Y, He J (2010) Beta-2 adrenergic receptor mediated ERK activation is regulated by interaction with MAGI-3. FEBS Lett 584(11):2207–2212

Yao R, Maeda T, Takada S, Noda T (2001) Identification of a PDZ domain containing Golgi protein, GOPC, as an interaction partner of frizzled. Biochem Biophys Res Commun 286(4):771–778

Yao W, Feng D, Bian W, Yang L, Li Y, Yang Z, Xiong Y, Zheng J, Zhai R, He J (2012) EBP50 inhibits EGF-induced breast cancer cell proliferation by blocking EGFR phosphorylation. Amino Acids 43(5):2027–2035

Zhang J, Cheng S, Xiong Y, Ma Y, Luo D, Jeromin A, Zhang H, He J (2008) A novel association of mGluR1a with the PDZ scaffold protein CAL modulates receptor activity. FEBS Lett 582(30):4117–4124

Zheng J, Shen H, Xiong Y, Yang X, He J (2010a) The beta1-adrenergic receptor mediates extracellular signal-regulated kinase activation via Galphas. Amino Acids 38(1):75–84

Zheng JF, Sun LC, Liu H, Huang Y, Li Y, He J (2010b) EBP50 exerts tumor suppressor activity by promoting cell apoptosis and retarding extracellular signal-regulated kinase activity. Amino Acids 38(4):1261–1268