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Spinophilin Is Indispensable for the α_{2B} Adrenergic Receptor-Elicited Hypertensive Response

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Abstract

The α_2 adrenergic receptor (AR) subtypes are important for blood pressure control. When activated, the α_{2A} subtype elicits a hypotensive response whereas the α_{2B} subtype mediates a hypertensive effect that counteracts the hypotensive response by the α_{2A} subtype. We have previously shown that spinophilin attenuates the α_{2A}AR-dependent hypotensive response; in spinophilin null mice, this response is highly potentiated. In this study, we demonstrate that spinophilin impedes arrestin-dependent phosphorylation and desensitization of the α_{2B}AR subtype by competing against arrestin binding to this receptor subtype. The Del301-303 α_{2B}AR, a human variation that shows impaired phosphorylation and desensitization and is linked to hypertension in certain populations, exhibits preferential interaction with spinophilin over arrestin. Furthermore, Del301-303 α_{2B}AR-induced ERK signaling is quickly desensitized in cells without spinophilin expression, showing a profile similar to that induced by the wild type receptor in these cells. Together, these data suggest a critical role of spinophilin in sustaining α_{2B}AR signaling. Consistent with this notion, our *in vivo* study reveals that the α_{2B}AR-elicited hypertensive response is diminished in spinophilin deficient mice. In arrestin 3 deficient mice, where the receptor has a stronger binding to spinophilin, the same hypertensive response is enhanced. These data suggest that interaction with spinophilin is indispensable for the α_{2B}AR to elicit the hypertensive response. This is opposite of the negative role of spinophilin in regulating α_{2A}AR-mediated hypotensive response, suggesting that spinophilin regulation of these closely related receptor subtypes can result in distinct functional outcomes *in vivo*. Thus, spinophilin may represent a useful therapeutic target for treatment of hypertension.

Introduction

The discovery of a plethora of G protein-coupled receptor (GPCR) interactions with non-G protein partners has led to the view that GPCRs do not function in isolation, but in complex protein networks which impact receptor trafficking, signaling and pharmacology. Among GPCR interacting proteins, G protein-coupled receptor kinases (GRKs) and arrestins are considered "universal regulators" and are the most extensively studied (e.g. recently reviewed in [1–6]). GRK-catalyzed phosphorylation, which leads to subsequent arrestin binding, represents a major mechanism for homologous desensitization of GPCRs. In addition to terminating G protein coupling, β-arrestins (arrestin 2 and 3) mediate receptor trafficking and scaffold cellular signaling cascades. Our previous studies identified spinophilin as an endogenous antagonist of arrestin, impeding multiple arrestin-mediated regulations of the receptor in cultured cells and *in vivo* [7]. Spinophilin is a ubiquitously expressed protein, and competes against arrestin for binding to the α_{2A} adrenergic receptor (AR) third intracellular (3i) loop [8]. Interestingly, multiple α_{2A}AR-elicited central responses are dampened in arrestin 3 deficient mice, but these responses are potentiated in spinophilin deficient mice where arrestin functions are unimpeded [7, 9, 10]. This suggests that arrestin 3 promotes, whereas spinophilin attenuates, α_{2A}AR-dependent processes *in vivo*.

Although closely related to the α_{2A}AR, the α_{2B}AR subtype exhibits distinct trafficking profiles [11–14] and induces separate physiological responses [15–17] from the α_{2A}AR. In response to α_2 adrenergic ligands, the α_{2B}AR mediates the hypertensive effect [18], which counteracts the hypotensive response elicited by the α_{2A} subtype [19]. The α_{2B}AR is also required for development of salt-induced hypertension [20–22]. In humans, a common polymorphism of the α_{2B}AR gene, Del301-303, has been linked to early onset hypertension in a Swedish population [23, 24]. Unlike the α_{2A} subtype, the α_{2B}AR 3i loop contains a highly acidic stretch of amino acids (aa294-309 [25]), which promotes GRK phosphorylation [25, 26]. Consistently, the Del301-303 α_{2B}AR exhibits reduced phosphorylation and desensitization profiles [27]. The α_{2B}AR interacts with both β-arrestins [13, 28] and spinophilin [29]. How spinophilin and β-arrestins regulate the α_{2B}AR-mediated *in vivo* responses and how Del301-303 may affect the receptor's interaction with these proteins remain to be investigated.

In the present study, we demonstrated that spinophilin impeded β arrestin-dependent α_{2B}AR phosphorylation and desensitization by competing against arrestin binding to the receptor. Compared to the wild type (WT) α_{2B}AR, the Del301-303 α_{2B}AR exhibited diminished binding affinity to arrestin 3 but enhanced interaction with spinophilin. Moreover, ERK signaling induced by this polymorphic variant was prolonged. Intriguingly, Del301-303 α_{2B}AR-induced ERK signaling was quickly desensitized in cells without spinophilin expression, showing a profile similar to that induced by the WT receptor in these cells. Together, these data suggest a critical role of spinophilin in sustaining α_{2B}AR signaling. Furthermore, the α_{2B}AR-elicited hypertensive response is diminished in spinophilin deficient mice, but the same response is enhanced in arrestin 3 deficient mice where the α_{2B}AR has a stronger binding to spinophilin. These data strongly suggest that the interaction with spinophilin is indispensable for α_{2B}AR to elicit the hypertensive response.

Methods and Materials

Reagents and drugs

Rat anti-HA rat monoclonal antibody (Roche); mouse HA.11 monoclonal antibody (Covance); rabbit anti-spinophilin antibody (Upstate); phospho- and total-p42/44 antibodies (Cell Signaling Technology); rabbit anti-GFP monoclonal antibody (Santa Cruz Biotechnology); mouse

anti-myc antibody (Clontech); rabbit anti-GRK2 polyclonal antibody (Santa Cruz Biotechnology); anti-mouse IRDye 800CW and anti-rabbit IRDye 680RD (LI-COR) immobilized protein G-agarose (Pierce); arrestin 3 polyclonal antibodies (generously provided by Dr. Benovic, Thomas Jefferson University). All other chemicals were reagent-grade, and were purchased from Sigma-Aldrich or Fisher Chemicals.

Animals

Spinophilin deficient ($Sp^{-/-}$), arrestin 3 deficient ($Arr3^{-/-}$) and their respective corresponding wild type (WT) mice in the same genetic background were obtained and maintained as described previously [7]. Mice were housed in the Association for Assessment and Accreditation of Laboratory Animal Care-accredited Animal Resources Program at the University of Alabama at Birmingham. Experimental procedures are in accordance with Animal Welfare Act and the 1989 amendments to this Act. All protocols were approved by University of Alabama Institutional Animal Care and Use Committee.

Cells

HEK293 and CosM6 cells were originally from American Type Culture Collection (ATCC). Immortalized arrestin 2 and 3 double knockout ($Arr2,3^{-/-}$) and the corresponding WT ($Arr2,3^{+/+}$) mouse embryonic fibroblasts (MEFs) were generated [30] and generously provided by Dr. Lefkowitz's laboratory. $Sp^{-/-}$ and the corresponding $Sp^{+/+}$ MEFs were generated previously as described in [31]. Cells were cultured in 5% CO_2 at 37°C in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 100 units/ml of penicillin and 10 μg/ml of streptomycin (Invitrogen). For mouse embryonic fibroblasts (MEFs), 2mM glutamine was added to the culture medium. Parental cell lines used in this study have no detectable endogenous expression of α_2AR subtypes.

Plasmid and primers

Constructions of plasmids pGFP-Arr3 [8], pCMV4-Myc-Sp [29, 32, 33], and pcDNA3-GRK2 [7] were described previously. The pGFP-Arr3-R170E construct expressing arrestin 3 with R to E mutation at R170 was generated by quickchange PCR mutagenesis using a primer pair, 5'-CTCAGGAGCAAACTGTACCTTCTCGATGATAAGCCGCACGGAGTT and 5'- AACTCC GTGCGGCTTATCATCGAGAAGGTACAGTTTGCTCCTGAG. pcDNA3.1- HA-α_{2B} expressing wild type human α_{2B}AR with N-terminal 3xHA tag was purchased from UMR cDNA Resource Center (clone ID: AR0A2BTN00). pcDNA3.1-HA-Del301-303 expressing N-terminal tagged human α_{2B}AR with deletion of amino acid 301–303 was generated by overlap PCR mutagenesis using two primer pairs, the 5'-CGGGGTACCACCATGTACCCATACG ATGTT and 5'-ACACTCTTCCTCCTCCTCCTCCTCTTCAGCTTCATCCT pair, and the 5'-GAGGATGAAGCTGAAGAGGAGGAGGAGGAGGAGGAAGAGTG and 5'-ATAC CGCTCGAGTCACCAGGCCGTCTGGGTCC pair. All constructs were confirmed by sequencing.

Transfection

Cells were transfected with indicated plasmid using Lipofectamine 2000 (invitrogen) according to manufacturer's instruction. MEFs were transduced with a retroviral construct encoding HA-α_{2B}AR as described previously [31, 34].

Intact cell receptor phosphorylation

Intact cell phosphorylation was described previously [8]. Briefly, cells were incubated for 1 hr with [^{32}p] orthophosphate (0.1mCi/ml) in phosphate-free, serum-free DMEM at 37°C. Following agonist treatment, cells were harvested in lysis buffer (1% Triton x-100, 0.05% SDS, 1mM EDTA, 1mM EGTA, 10mM NaF, 10mM sodium pyrophosphoate, and protease inhibitors). Detergent-soluble extracts were then subjected to immunoprecipitation assay with a rat anti-HA antibody.

Co-immunoprecipitation

Co-immunoprecipitation was performed as described [8, 35]. In brief, cells were harvested in ice-cold lysis buffer (10 mM Tris HCl, pH 8.0, 0.3% Nonidet P-40, 10% glycerol, 5 mM EDTA, 5 mM EGTA supplemented with protease inhibitors). Soluble extracts were then subjected to immunoprecipitation assays using a rat anti HA antibody.

ERK1/2 activation

Kinase activation for ERK1/2 was determined by measuring the level of phosphorylated kinase and normalizing the value to the total protein level of ERK1/2. Phospho- and total-ERK1/2 were detected and analyzed by LI-COR odyssey Fc dual-mode western blot system. Mouse anti-phospho-p44/42 MAPK (T202/Y204) (Cell signaling) and donkey anti-mouse IRDye 800CW (LI-COR) were used to detect activation of ERK1/2 by green fluorescence channel. Rabbit anti-p44/42 MAPK (Cell signaling) and goat anti-rabbit IRDye 680RD (LI-COR) were used to detect total ERK1/2 by red fluorescence channel.

Measurement of cardiovascular responses

Measurement of cardiovascular responses was performed as described previously [9]. Mice were anesthetized with 100mg/kg ketamine and 10 mg/kg xylazine. Catheterized left femoral artery was used to measure arterial pressure while the right jugular vein was used for anesthetic administration. Arterial blood pressure was recorded with a pressure transducer (BIOPAC's AcqKnowledge 3.8.2, BioPac, Goleta, CA) continually in conscious and free moving mice 24 hr after the surgery. Twenty minutes after the baseline measurement, 0.1mg/kg UK14,304 was administered through bolus injection into the right jugular vein.

Data analysis

Data are expressed as mean ± SEM. Unpaired Student's *t*-tests were performed to determine differences between two groups. All plots were generated using GraphPad Prism.

Results

Interactions of the α_{2B}AR with spinophilin and β-arrestins are mutually exclusive

We first confirmed that β-arrestins and spinophilin compete for interaction with the α_{2B}AR in cells. MEFs express endogenous arrestins and spinophilin, and we readily detected interactions of the α_{2B}AR with endogenous arrestin 3 (Fig 1A, left) and spinophilin (Fig 1C, left), which were enhanced by epinephrine stimulation. In spinophilin deficient ($Sp^{-/-}$) MEFs, the epinephrine-promoted interaction between the α_{2B}AR and arrestin 3 was markedly increased, as compared to that in the corresponding $Sp^{+/+}$ MEFs prepared from WT mice with the same genetic background (Fig 1A, right, and Fig 1B). Similarly, the association of the α_{2B}AR with

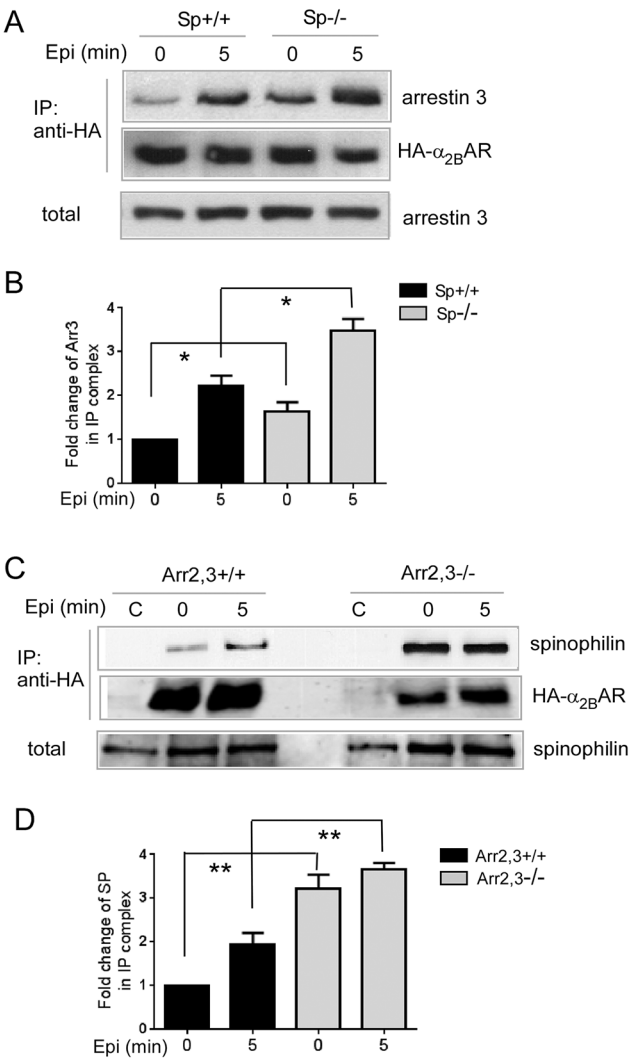

Fig 1. The endogenous arrestin and spinophilin competes for interaction with the α_{2B}AR. (A) Interaction between α_{2B}AR and the endogenous arrestin 3 was enhanced in $Sp^{-/-}$ MEFs. $Sp^{-/-}$ and corresponding WT ($Sp^{+/+}$) MEFs expressing HA-α_{2B}AR were stimulated with 100μM epinephrine (plus 1 μM propranolol to block βARs) for indicated time points. Cell lysates were subjected to IP assays using an HA antibody. (B) Quantitation of the fold change of arrestin 3 in the IP complex isolated from cells with or without stimulation. Data were mean ± SEM. n = 4 for each condition. *, $p<0.05$ by unpaired Student's *t* test, $Sp^{-/-}$ *vs*. $Sp^{+/+}$. (C) Interaction between α_{2B}AR and the endogenous spinophilin was enhanced in $Arr2,3^{-/-}$ MEFs. $Arr2,3^{-/-}$ and corresponding WT ($Arr2,3^{+/+}$) MEFs expressing HA-α_{2B}AR were stimulated with 100μM epinephrine (plus 1μM propranolol to block βARs). Lane C (control) refers to MEFs ($Arr2,3^{+/+}$ or $Arr2,3^{-/-}$) without HA-α_{2B}AR overexpression. (D) Quantitation of the fold change of spinophilin in the IP complex isolated from cells with or without stimulation. Data were mean ± SEM. n = 4 for each condition. **, $p<0.01$, $Arr2,3^{-/-}$ *vs*. $Arr2,3^{+/+}$.

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spinophilin in response to epinephrine treatment was significantly enhanced in MEFs with no β-arrestin expression ($Arr2,3^{-/-}$), as compared to that in the corresponding $Arr2,3^{+/+}$ MEFs (Fig 1C, right, and Fig 1D). We obtained similar results with other α_2 agonists, including clonidine and UK14,304 (data not shown). Additionally, in the absence of arrestin, the basal interaction between α_{2B}AR and spinophilin was also dramatically enhanced (Fig 1C and 1D). Together, these data demonstrate that interactions of the α_{2B}AR with spinophilin and β-arrestins are mutually exclusive.

Spinophilin attenuates α_{2B}AR phosphorylation through competition against arrestin in cells

We previously demonstrated that stable phosphorylation of the α_{2B}AR requires β-arrestins [7]. Consistently, when arrestin 3 was overexpressed in CosM6 cells, which have a relatively low level of endogenous arrestins compared to other cell lines such as HEK cells and MEFs [7, 36, 37], the levels of α_{2B}AR phosphorylation following epinephrine stimulation were dramatically enhanced compared to the control (Fig 2A and 2B). On the other hand, when spinophilin was overexpressed in HEK293 cells, epinephrine-induced α_{2B}AR phosphorylation was markedly reduced compared to the control (Fig 2C and 2D). This result is consistent with the notion that spinophilin competes against β-arrestins for binding to the α_{2B}AR and impedes the arrestin effect in promoting receptor phosphorylation.

However, spinophilin may regulate α_{2B}AR phosphorylation through mechanisms other than competing against arrestin, given that it contains multiple functional domains in addition to the receptor binding region [38, 39]. To address this, we examined whether the receptor binding region of spinophilin (Sp156-444) alone can sufficiently regulate α_{2B}AR phosphorylation. In HEK293 cells overexpressing Myc-Sp156-444, the levels of α_{2B}AR phosphorylation in response to agonist stimulation were significantly reduced compared to those in control cells expressing the empty vector (Fig 3A and 3B). This effect on α_{2B}AR phosphorylation caused by the receptor binding region of spinophilin is comparable to that caused by the full length spinophilin (comparing Fig 2D and Fig 3B). Furthermore, in CosM6 cells (which express a low level of endogenous β-arrestins), overexpression of Myc-Sp156-444 failed to alter agonist-induced α_{2B}AR phosphorylation (Fig 4C). This suggests that the inhibitory effect of the receptor binding domain of spinophilin on α_{2B}AR phosphorylation requires a relatively high level of arrestin expression to be detected. Taken together, these data strongly support that spinophilin attenuates α_{2B}AR phosphorylation through competition against β-arrestins in cells.

Spinophilin counteracts arrestin-dependent desensitization of α_{2B}AR-induced ERK1/2 activation

We next examined the effect of spinophilin and β-arrestins on α_{2B}AR signaling. In MEFs expressing both spinophilin and arrestins, activation of the α_{2B}AR by clonidine induced transient ERK1/2 activation, which was desensitized after 20 min of stimulation (Fig 4). In MEFs without β-arrestin expression (Arr2,3$^{-/-}$), ERK1/2 signaling was prolonged after a 30-min treatment (Fig 4A and 4B) as compared to that in the corresponding Arr2,3$^{+/+}$ MEFs, suggesting that β-arrestins are required for terminating α_{2B}AR signaling. On the other hand, in cells without spinophilin expression (Sp$^{-/-}$), α_{2B}AR-induced ERK1/2 activation was quickly desensitized at the 10 min time point (Fig 4C and 4D). This desensitization of ERK1/2 signaling is much faster than that in the corresponding Sp$^{+/+}$ MEFs, suggesting an opposing effect of spinophilin on arrestin-dependent desensitization. We obtained similar results with other α_2 agonists, including epinephrine and UK14,304 (data not shown). The activation rate of α_{2B}AR-induced ERK1/2 signaling seemed not altered in Arr2,3$^{-/-}$ or Sp$^{-/-}$ cells. This is different from what we have previously observed for the α_{2A}AR-induced ERK1/2 activation, which was accelerated in Sp$^{-/-}$ cells but slowed in Arr2,3$^{-/-}$ cells [7]. Taken together, our data suggest that reciprocal regulation by spinophilin and β-arrestins can have differential impacts on signaling evoked by the closely related α_2AR subtypes.

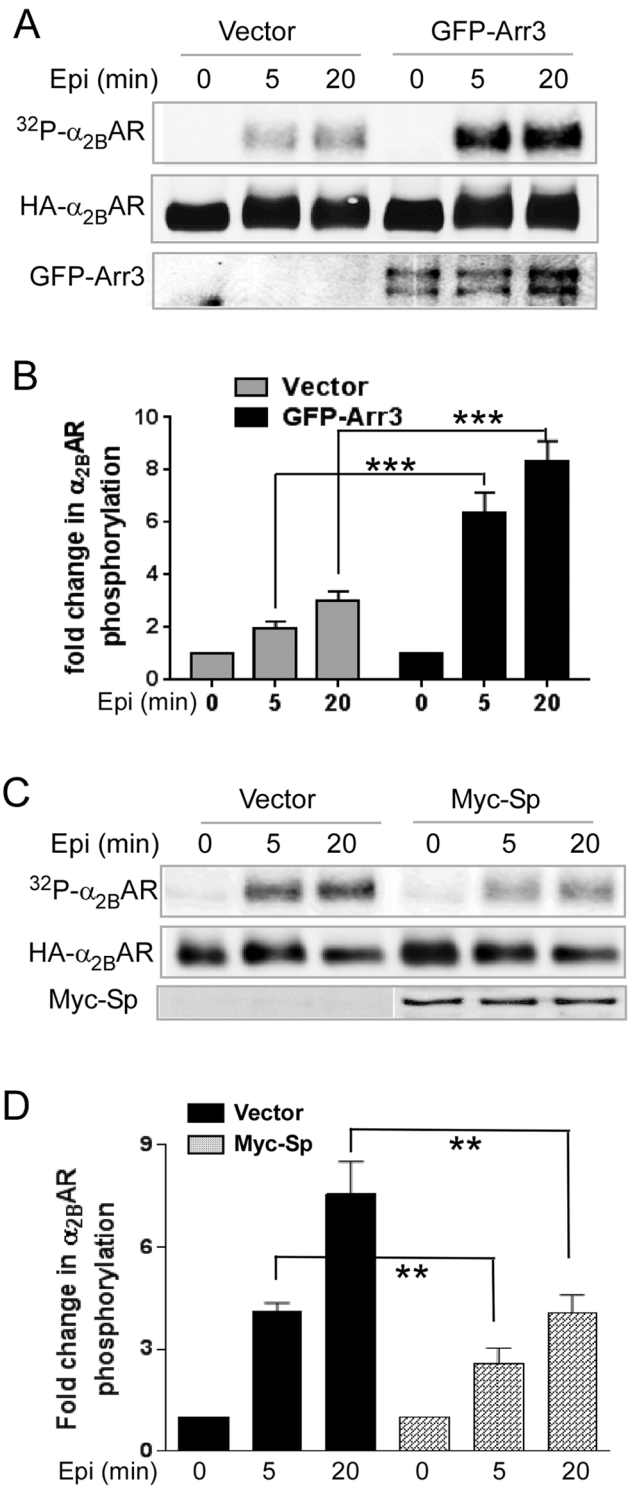

Fig 2. Arrestin 3 and spinophilin reciprocally regulate agonist-induced α_{2B}AR phosphorylation. (A) CosM6 cells co-expressing HA-α_{2B}AR together with GFP-tagged arrestin 3 (GFP-Arr3) or GFP alone (vector) were stimulated with 100μM epinephrine (plus 1μM propranolol to block βARs) for indicated time points. Overexpression of GFP-Arr3 increased the phosphorylation level of α_{2B}AR following epinephrine stimulation. (B) Quantitation of agonist-induced fold change in α_{2B}AR phosphorylation. Data were mean ± SEM. n = 5 for each condition. ***, $p<0.001$ by unpaired Student's *t* test, GFP-Arr3 *vs*. vector control. (C) HEK293 cells co-expressing HA-α_{2B}AR with or without Myc-spinophilin were stimulated. Overexpression of Myc-spinophilin

(Myc-Sp) reduced the phosphorylation level of α_{2B}AR following epinephrine stimulation. (D) Quantitation of agonist-induced fold change in α_{2B}AR phosphorylation. Data were mean ± SEM. n = 3 for each condition. **, $p<0.01$, Myc-Sp *vs*. vector control.

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The Del301-303 α_{2B}AR exhibits preferential interaction with spinophilin over arrestin

The human variation Del301-303 α_{2B}AR displays reduced phosphorylation and desensitization following agonist treatment [27]. Given the importance of β-arrestins for α_{2B}AR phosphorylation and desensitization demonstrated above, we predicted that this receptor would show impaired interaction with β-arrestins. Indeed, while epinephrine markedly enhanced the amount of arrestin 3 associated with the WT α_{2B}AR, such treatment failed to increase the association of arrestin 3 with the Del301-303 α_{2B}AR (Fig 5A and 5B). Impaired arrestin binding to the Del301-303 α_{2B}AR may be a consequence of the decreased phosphorylation level of this receptor. Alternatively, Del301-303 may cause conformational changes that reduce its binding to arrestins independent of receptor phosphorylation. To address this possibility, we examined the ability of Del301-303 α_{2B}AR to interact with the phosphorylation-insensitive arrestin 3 (Arr3R170E). Replacement of Arg170 with a Glu results in constitutive binding of arrestin 3 to agonist-activated GPCRs even in the absence of receptor phosphorylation [40, 41]. Epinephrine treatment significantly enhanced interaction of Arr3R170E with the WT α_{2B}AR (Fig 5C and 5D). However, such treatment had no effect on Arr3R170E interaction with the Del301-303 α_{2B}AR (Fig 5C and 5D). These data suggest that Del301-303 changes the conformation of the receptor leading to a diminished affinity for β-arrestin binding.

Based on the reciprocal effect of spinophilin and arrestin on receptor interaction, we predicted that the Del301-303 α_{2B}AR would have a higher affinity for spinophilin binding. As expected, interaction of spinophilin with the Del301-303 α_{2B}AR was significantly increased when compared to that with the WT α_{2B}AR (Fig 6). Taken together, our data suggest biased interaction of the Del301-303 α_{2B}AR with spinophilin.

Spinophilin is essential for sustaining the prolonged ERK1/2 signaling elicited by the Del301-303 α_{2B}AR

The above data suggest that the diminished affinity of the Del301-303 α_{2B}AR for β-arrestin binding likely underlies impaired desensitization of signaling elicited by this receptor variant. Since the Del301-303 α_{2B}AR showed increased interaction with spinophilin, we further sought to address whether spinophilin binding to this receptor plays a role in sustaining its prolonged signaling. We therefore examined the kinetics of ERK1/2 signaling elicited by the WT or Del301-303 α_{2B}AR in $Sp^{-/-}$ and the corresponding $Sp^{+/+}$ MEFs. Consistent with the reduced desensitization of the Del301-303 α_{2B}AR signaling reported previously [27], ERK1/2 activation elicited by the Del301-303 α_{2B}AR was prolonged when compared to that elicited by the WT α_{2B}AR in $Sp^{+/+}$ MEFs (Fig 7A and 7B). Strikingly, in $Sp^{-/-}$ MEF, we failed to observe any difference in ERK1/2 activation kinetics induced by the Del301-303 versus the WT α_{2B}AR (Fig 7C and 7D). In both cases, ERK1/2 signaling was quickly desensitized (Fig 7C and 7D). These data suggest that spinophilin is required for sustaining the prolonged ERK1/2 signaling elicited by the Del301-303 α_{2B}AR.

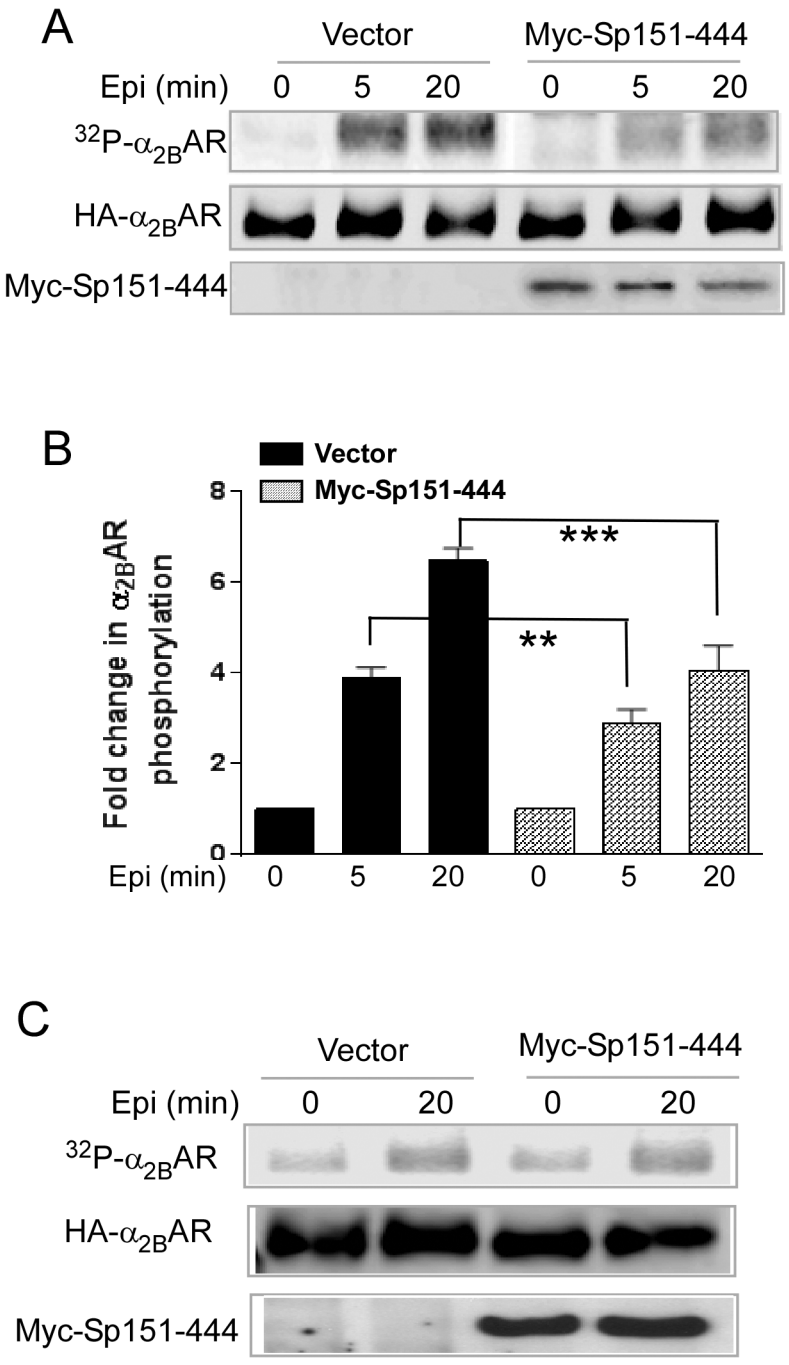

Fig 3. Overexpression of spinophilin aa151-444 sufficiently attenuates α_{2B}AR phosphorylation. (A) HEK293 cells co-expressing HA-α_{2B}AR t.ogether with or without Myc-Sp151-444 were stimulated with 100μM epinephrine (plus 1μM propranolol to block βARs) for indicated time points. (B) Quantitation of agonist-induced fold change in α_{2B}AR phosphorylation. Data were mean ± SEM. n = 4 for each condition. **, $p<0.01$; ***, $p<0.001$ by unpaired Student *t* test, Myc-Sp151-444 *vs*. vector control. (C) Overexpression of Sp151-444 showed no effect on α_{2B}AR phosphorylation in CosM6 cells, which have a low level of endogenous arrestin expression. CosM6 cells co-expressing HA-α_{2B}AR together with or without Sp151-444 were stimulated. Representative blots from multiple independent experiments are shown.

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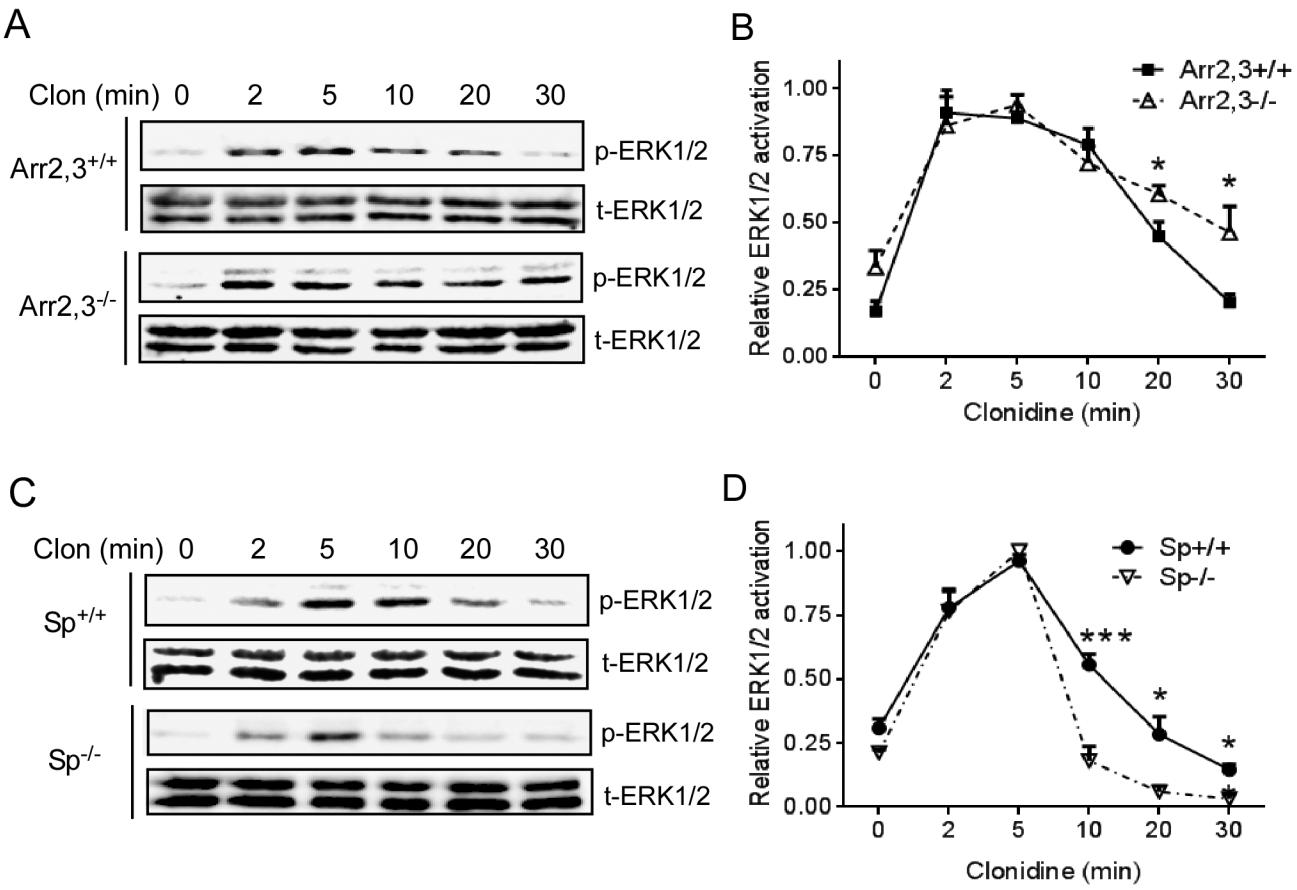

Fig 4. Spinophilin and arrestin reciprocally regulate α_{2B}AR-induced ERK1/2 activation kinetics in MEFs. (A) Arr2,3$^{-/-}$ and corresponding WT (Arr2,3$^{+/+}$) MEFs expressing HA-α_{2B}AR were stimulated with 1μM clonidine for indicated time points. Phospho- and total-ERK1/2 were detected by Western blots. Representative blots from multiple independent experiments are shown. (B) Quantitation of ERK1/2 activation in Arr2,3$^{+/+}$ or Arr2,3$^{-/-}$ MEFs at indicated time points. The relative ERK1/2 activation at each time point was expressed as a ratio to the peak level of ERK1/2 activation in the same experiment, which was arbitrarily defined as 1.0. Data were mean ± SEM. n = 4 for each condition. *, p<0.05, Arr2,3$^{-/-}$ *vs*. Arr2,3$^{+/+}$. (C) Sp$^{-/-}$ and corresponding WT (Sp$^{+/+}$) MEFs expressing HA-α_{2B}AR were stimulated. Representative blots for phospho- and total ERK1/2 from multiple independent experiments are shown. (D) Quantitation of ERK1/2 activation in Sp$^{+/+}$ or Sp$^{-/-}$ MEFs at indicated time points. Data were mean ± SEM. n = 7 for data collected in Sp$^{+/+}$ cells and n = 4 for Sp$^{-/-}$ cells. *, p<0.05; ***, p<0.001, Sp$^{-/-}$ *vs*. Sp$^{+/+}$.

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The α_{2B}AR-elicited hypertensive response is enhanced in arrestin 3 null mice but diminished in spinophilin null mice

Given the reciprocal effects of spinophilin and β-arrestins in regulating α_{2B}AR phosphorylation and desensitization, we sought to further determine the role of these proteins in regulating the α_{2B}AR-elicted hypertensive response. We first compared the level of increase in blood pressure (a response known to be elicited by the α_{2B}AR [18]) following administration of an α_2 agonist, UK14,304, in Arr3$^{-/-}$ and the corresponding WT (Arr3$^{+/+}$) mice in the same genetic background. In Arr3$^{-/-}$ mice, UK14,304 induced a significantly higher increase in the mean arterial pressure (MAP) compared to that in Arr3$^{+/+}$ mice (Fig 7A and 7B). Furthermore, this α_{2B}AR-elicited hypertensive response appeared to last for a longer time in Arr3$^{-/-}$ mice compared to WT mice (Fig 7A), and the peak area under the hypertensive curve (AUC) was more than twice of that in WT mice (Fig 7C). These data suggest that the α_{2B}AR-elicited hypertensive response is enhanced and prolonged in the absence of arrestin 3, supporting an *in vivo* role of arrestin 3 in desensitizing this effect.

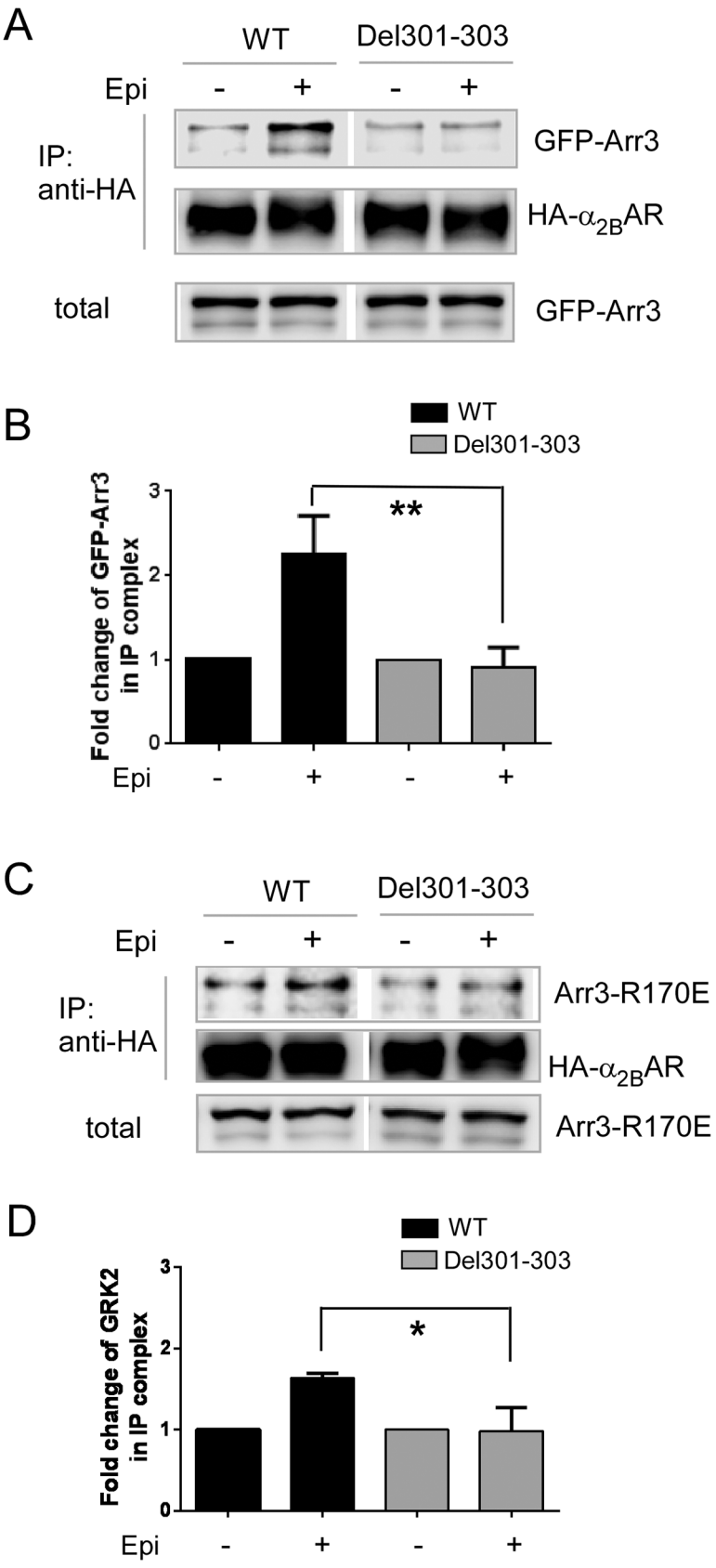
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Fig 5. The Del301-303 α_{2B}AR shows impaired interaction with arrestin 3. (A) Agonist treatment failed to promote interaction of the Del301-303 α_{2B}AR with arrestin 3. Cells co-expressing GFP-tagged arrestin3 (GFP-Arr3) with HA-tagged WT α_{2B}AR or Del301-303 α_{2B}AR were stimulated with 100μM epinephrine (plus 1μM propranolol to block βARs), and the interaction between arrestin and either WT or Del301-303 α_{2B}AR was examined by co-IP assays. (B) Quantitation of the agonist-induced fold change of GFP-Arr3 in the IP complex with the WT or Del301-303 α_{2B}AR. n = 3–4 for each condition. **, $p<0.01$, WT *vs*. Del301-303. (C) Del 301–303 α_{2B}AR was unable to interact with constitutively active mutant arrestin3 R170E following agonist stimulation. Cells co-expressing GFP-Arr3R170E together with WT or Del301-303 α_{2B}AR were stimulated with 100μM epinephrine (plus 1μM propranolol). (D) Quantitation of the agonist-induced fold change of GFP-Arr3R170E in the IP complex with the WT or Del301-303 α_{2B}AR. n = 3–4 for each condition. *, $p<0.05$, WT *vs*. Del301-303.

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We then compared the level of increase in blood pressure induced by UK14,304 in $Sp^{-/-}$ and the corresponding WT ($Sp^{+/+}$) mice in the same genetic background. UK14,304-induced change in MAP in $Sp^{+/+}$ mice were somewhat higher than that in $Arr3^{+/+}$ mice (7.4 ± 2.5 vs 5.4 ± 2.9, $p = 0.28$), likely due to the difference in genetic background between these lines. In $Sp^{-/-}$ mice, the α_{2B}AR-elicited hypertensive response was greatly diminished (Fig 8A and 8B) and the AUC in these mice was dramatically reduced as compared to those in $Sp^{+/+}$ mice (Fig 8C). These data strongly suggest that the *in vivo* α_{2B}AR responsiveness requires the presence of

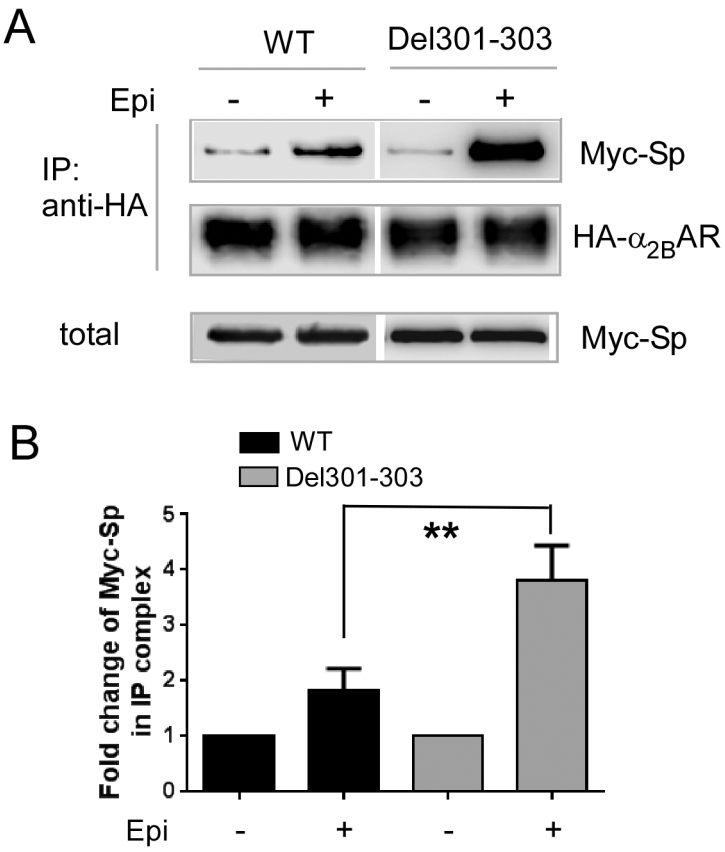

Fig 6. Del301-303 α_{2B}AR has a stronger interaction with spinophilin than WT α_{2B}AR. (A) Cells co-expressing Myc-spinophilin together with HA-tagged WT or Del301-303 α_{2B}AR were stimulated with 100μM epinephrine (plus 1μM propranolol to block βARs). (B) Quantitation of the agonist-induced fold change of Myc-spinophilin in the IP complex with the WT α_{2B} or Del301-303 α_{2B}AR. n = 3–5 for each condition. **, $p<0.01$, WT *vs*. Del301-303 α_{2B}.

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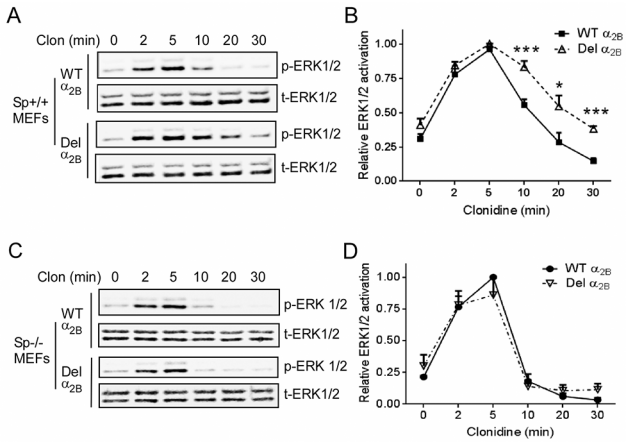

Fig 7. Spinophilin is required for maintaining the sustained ERK1/2 activation induced by the Del301-303 α_{2B}AR in MEFs. (A) $Sp^{+/+}$ MEFs expressing the WT or Del301-303 α_{2B}AR were stimulated with 1μM clonidine for indicated time points. Representative blots show phospho- and total-ERK1/2. (B) Quantitation of ERK1/2 activation in $Sp^{+/+}$ MEFs. The relative ERK1/2 activation at each time point was expressed as a ratio to the peak level of ERK1/2 activation in the same experiment, which was arbitrarily defined as 1.0. n = 7 for the WT α_{2B} group and n = 6 for the Del301-303 α_{2B} group. *, $p<0.05$; ***, $p<0.001$, WT α_{2B} *vs*. Del301-303 α_{2B}. (C) $Sp^{-/-}$ MEFs expressing the WT or Del301-303 α_{2B}AR were stimulated with 1μM clonidine for indicated time points. Representative blots show phospho- and total-ERK1/2. (D) Quantitation of ERK1/2 activation in $Sp^{-/-}$ MEFs. n = 4 for the WT α_{2B} group and n = 3 for the Del301-303 α_{2B} group.

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spinophilin; in mice without spinophilin expression, the α_{2B}AR cannot elicit an effective hypertensive response to α_2 ligands.

Discussion

Using the α_{2A}AR subtype as a model, we previously identified competition between spinophilin and β-arrestins for interaction with the 3i loop of the receptor [8]. In this study, we confirmed the mutually exclusive binding of these proteins to the 3i loop of the α_{2B}AR subtype

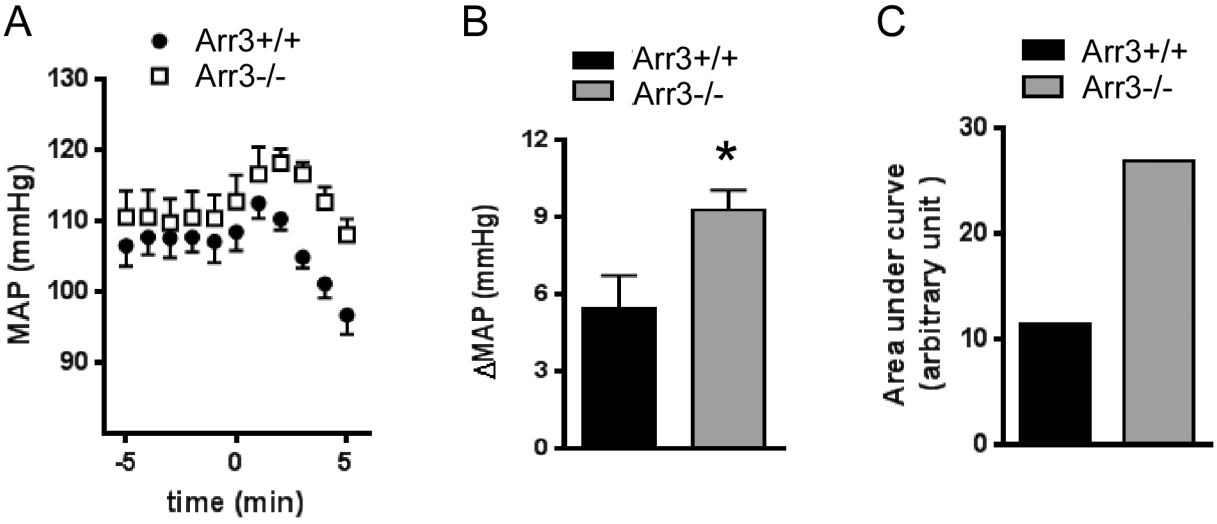

Fig 8. The α_{2B}AR dependent hypertensive response is enhanced in arrestin 3 deficient mice. (A) Mean arterial pressure (MAP) measured in $Arr3^{-/-}$and corresponding $Arr3^{+/+}$ mice in the same genetic background after injection of UK14,304 (0.1mg/kg i.v.). (B) Quantitation of agonist-induced changes in MAP (ΔMAP) over the basal level. (C) Quantitation of area under curve of the hypertensive response curve. n = 5 for each group. *, $p<0.05$, $Arr3^{+/+}$ *vs*. $Arr3^{-/-}$.

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(Fig 1), and validated that β-arrestins promoted, whereas spinophilin attenuated, agonist-induced α_{2B}AR phosphorylation (Fig 2). In addition, we demonstrated that the receptor-binding domain of spinophilin sufficiently attenuated α_{2B}AR phosphorylation (Fig 3), which further supports the notion that spinophilin regulates receptor phosphorylation through competition against arrestin binding to the receptor.

Consistent with a role of β-arrestins in desensitizing receptor signaling, we found that α_{2B}AR-mediated ERK1/2 activation was prolonged in cells without β-arrestins expression (Fig 4A and 4B). In cells without spinophilin expression, this signaling process was quickly desensitized (Fig 4C and 4D), presumably due to enhanced arrestin binding to the receptor in these cells. In our previous studies, activation of ERK1/2 signaling by the α_{2A} subtype was accelerated in spinophilin deficient cells but slowed in arrestin deficient cells [7]. However, we did not observe these changes for α_{2B}AR-induced ERK1/2 activation, suggesting that the signaling profiles induced by closely related α_2AR subtypes are differentially regulated by spinophilin and β-arrestins.

The α_{2B}AR mediates the hypertensive response to α_2 ligands [18]. The human Del301-303 α_{2B}AR variant, which exhibits reduced phosphorylation and desensitization profiles *in vitro* [27] (also Fig 7A and 7B) and *in vivo* [42], has been associated with early onset hypertension in a Swedish population [23, 24]. We found that this variant showed impaired interaction with arrestin 3 (Fig 5A). In particular, the Del301-303 α_{2B}AR failed to interact with the constitutive form of arrestin 3 that is insensitive to receptor phosphorylation (Fig 5B), suggesting that the conformational change caused by Del301-303 alters the intrinsic affinity of the receptor to arrestins. By contrast, the Del301-303 α_{2B}AR showed a much stronger interaction with spinophilin (Fig 6). Strikingly, in cells without spinophilin expression, ERK1/2 activation induced by the Del301-303 α_{2B}AR was quickly desensitized with a profile similar to that induced by the WT receptor (Fig 7C and 7D). These data suggest that spinophilin interaction is essential for maintaining the prolonged signaling profile induced by this receptor variant, and further indicate that spinophilin may represent an attractive target in manipulating functions of this polymorphic variant.

We previously found that multiple *in vivo* responses elicited by the α_{2A}AR subtype are potentiated in spinophilin deficient mice, but dampened in arrestin 3 deficient mice where spinophilin binding to the receptor is enhanced [7, 9, 10]. Particularly, we have found that spinophilin attenuates the α_{2A}AR-dependent hypotensive response; in spinophilin null mice, this response is highly potentiated [9]. This is the opposite of what we have observed for responses elicited by the α_{2B}AR subtype in the current study. In spinophilin null mice, the α_{2B}AR-elicited hypertensive response was nearly abolished (Fig 9), whereas in arrestin 3 deficient mice, this response was enhanced and prolonged (Fig 8). Our current data suggest that interaction with spinophilin is indispensable for α_{2B}AR to elicit the hypertensive response. Collectively, our previous and current studies suggest that spinophilin regulation of the closely related α_2AR subtypes can result in distinct functional outcomes *in vivo*. Diminished regulation by spinophilin enhances the hypotensive effect elicited by the α_{2A} subtype [9] while reducing the counteracting hypertensive effect by the α_{2B} subtype (Fig 9). Hence, reducing spinophilin binding to the α_2AR subtypes may represent a useful therapeutic strategy for treatment of hypertension. This strategy may be particularly beneficial to the hypertensive population with the spinophilin-biased variation of α_{2B}AR, Del301-303, given the essential role of spinophilin in sustaining signaling by this receptor variant (Fig 7C and 7D).

We tested multiple α_2 ligands in this study. Although all these ligands promoted binding of both arrestin and spinophilin and gave similar results in our experimental readouts, it should be noted that these ligands likely exhibit different biases for the arrestin pathway, as described for the α_{2C}AR subtype previously [43]. Further investigation is needed to quantitatively

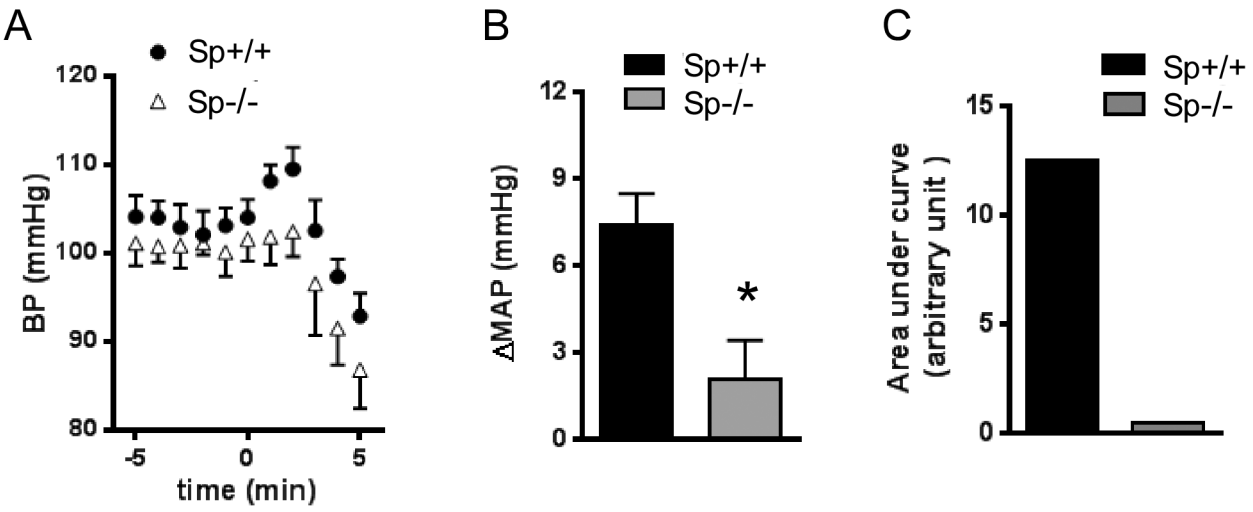

Fig 9. The α_{2B}AR dependent hypertensive response is diminished in spinophilin deficient mice. (A) Mean arterial pressure (MAP) measured in $Sp^{+/+}$ and $Sp^{-/-}$ mice in the same genetic background after UK14,304 injection (0.1mg/kg i.v.). (B) Quantitation of agonist-induced changes in MAP(ΔMAP) over the basal level. (C) Quantitation of area under curve of the hypertensive response curve. n = 5 for each group. *, $p < 0.05$, $Sp^{+/+}$ *vs*. $Sp^{-/-}$.

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compare the functional selectivity of these ligands to the α_{2B}AR, including arrestin and spinophilin recruitment. Our studies suggest that ligands that lead to stronger biased interaction of the α_2AR subtypes with β-arrestins over spinophilin would be more beneficial for hypertension treatment than traditional ligands that can enhance binding of both proteins to the receptor. Identifying such ligands may represent a new direction of therapeutic development for treatment of hypertension.

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Author Contributions

Conceived and designed the experiments: JMW KJ QW. Performed the experiments: PC YC RL NP MG. Analyzed the data: PC JMW KJ QW. Wrote the paper: PC MG KJ QW.

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