

# Cross-talk from $\beta$ -Adrenergic Receptors Modulates $\alpha_{2A}$ -Adrenergic Receptor Endocytosis in Sympathetic Neurons via Protein Kinase A and Spinophilin\*

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**Background:** Cross-talk between GPCRs is an important but undercharacterized mechanism regulating receptor responsiveness.

**Results:** Co-activation of  $\beta$  and  $\alpha_2$ ARs accelerates  $\alpha_{2A}$ AR endocytosis in a PKA- and spinophilin-dependent fashion.

**Conclusion:**  $\beta$ AR-mediated signaling modulates  $\alpha_{2A}$ AR endocytosis via PKA-dependent disruption of  $\alpha_{2A}$ AR/spinophilin interaction.

**Significance:** Cross-talk from  $\beta$  to  $\alpha_2$ ARs may have important implications in basal adrenergic tone and the pharmacology of commonly used adrenergic therapeutics.

Inter-regulation of adrenergic receptors (ARs) via cross-talk is a long appreciated but mechanistically unclear physiological phenomenon. Evidence from the AR literature and our own extensive studies on regulation of  $\alpha_{2A}$ ARs by the scaffolding protein spinophilin have illuminated a potential novel mechanism for cross-talk from  $\beta$  to  $\alpha_2$ ARs. In the present study, we have characterized a mode of endogenous AR cross-talk in native adrenergic neurons whereby canonical  $\beta$ AR-mediated signaling modulates spinophilin-regulated  $\alpha_{2A}$ AR endocytosis through PKA. Our findings demonstrate that co-activation of  $\beta$  and  $\alpha_{2A}$ ARs, either by application of endogenous agonist or by simultaneous stimulation with distinct selective agonists, results in acceleration of endogenous  $\alpha_{2A}$ AR endocytosis in native neurons. We show that receptor-independent PKA activation by forskolin is sufficient to accelerate  $\alpha_{2A}$ AR endocytosis and that  $\alpha_{2A}$ AR stimulation alone drives accelerated endocytosis in spinophilin-null neurons. Endocytic response acceleration by  $\beta/\alpha_{2A}$ AR co-activation is blocked by PKA inhibition and lost in spinophilin-null neurons, consistent with our previous finding that spinophilin is a substrate for phosphorylation by PKA that disrupts its interaction with  $\alpha_{2A}$ ARs. Importantly, we show that  $\alpha_2$ AR agonist-mediated  $\alpha_{2A}$ AR/spinophilin interaction is blocked by  $\beta$ AR co-activation in a PKA-dependent fashion. We therefore propose a novel mechanism for cross-talk from  $\beta$  to  $\alpha_2$ ARs, whereby canonical  $\beta$ AR-mediated signaling coupled to PKA activation results in phosphorylation of spinophilin, disrupting its interaction with  $\alpha_{2A}$ ARs and accelerating  $\alpha_{2A}$ AR endocytic responses. This mechanism of cross-talk has significant implications for endogenous adrenergic physiology and for therapeutic targeting of  $\beta$  and  $\alpha_{2A}$ ARs.

The physiological phenomenon of cross-talk between  $\beta$ - and  $\alpha_2$ -adrenergic receptor (AR)<sup>2</sup> subtypes has long been indicated by reports in the G protein-coupled receptor (GPCR) literature. Inter-regulation of  $\beta$  and  $\alpha_2$ ARs has been described in *in vitro* cell models (1–5), *in vivo* central (6–12), and peripheral (13) nervous systems and rodent development (14). Despite this accumulation of evidence, a clear picture of the mechanisms underlying AR cross-talk has yet to emerge, particularly as regards the unidirectional influence of  $\beta$ AR activity on  $\alpha_2$ AR function. Such information is vital given that the ARs are an important GPCR family responsible for mediating responses to the endogenous agonists epinephrine (Epi) and norepinephrine. These receptors exhibit wide distribution in the body and have myriad well appreciated functions, with most cell types expressing some combination of AR subtypes. Perhaps unsurprisingly, there is significant overlap in both the physiology and pharmacology of the ARs (15–17). Given that overlap, any new insights into AR inter-relationships and the mechanisms underlying AR cross-talk will contribute to a better understanding of adrenergic physiology and pharmacology.

We have previously carried out extensive studies on the function of the  $\alpha_{2A}$ AR subtype and its regulation by non-G protein-interacting partners. Our work has identified the scaffolding protein spinophilin (18–20) as an  $\alpha_{2A}$ AR interacting partner (21–23), and we have characterized a novel regulatory mechanism whereby spinophilin serves as a functional antagonist at the  $\alpha_{2A}$ AR to the traditional GPCR-interacting partners GPCR kinase and arrestin (24). We have demonstrated the importance of this regulatory mechanism both *in vitro* and *in vivo*, finding that a number of agonist-dependent  $\alpha_{2A}$ AR-mediated

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<sup>2</sup> The abbreviations used are: AR, adrenergic receptor; ALB, albuterol; CFP, cyan fluorescent protein; DOB, dobutamine; Epi, epinephrine; FLIM, fluorescence lifetime imaging; GPCR, G protein-coupled receptor; ISO, isoproterenol; PKI, protein kinase A inhibitor; SAL, salmeterol; SCG, superior cervical ganglion; YFP, yellow fluorescent protein; MEF, mouse embryonic fibroblast; ANOVA, analysis of variance.

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responses are enhanced and/or accelerated in the absence of spinophilin (24–26). Intriguingly, our work has also pinpointed spinophilin as a potential link between  $\alpha_{2A}$  and  $\beta$ ARs. Spinophilin is known to be a substrate for phosphorylation by PKA (27), and we have shown that this modification disrupts spinophilin/ $\alpha_{2A}$ AR interaction and accelerates agonist-driven receptor endocytosis (28). Meanwhile, canonical  $\beta$ AR signal transduction results in activation of PKA downstream of  $G\alpha_s$ -containing heterotrimeric G proteins (29). Therefore, in the present study, we hypothesize that co-activation of  $\beta$ ARs will accelerate agonist-driven  $\alpha_{2A}$ AR endocytosis via PKA-dependent phosphorylation of spinophilin, disrupting its interaction with  $\alpha_{2A}$ ARs.

In the present study, we have elected to utilize endocytosis of endogenous receptors as a functional readout that can be examined cleanly and specifically through the use of our previously reported novel epitope-tagged  $\alpha_{2A}$ AR knock-in mouse model (30). By culturing from the superior cervical ganglia (SCG), we can obtain a 98% pure population of adrenergic sympathetic neurons (31), allowing us to investigate endocytic responses in a native cell type with endogenous expression of  $\alpha_{2A}$ ARs,  $\beta$ ARs, and interacting protein partners. Furthermore, endocytosis is itself an important GPCR response, under tight and complex control, which is intimately involved in determining acute and long term neuronal responsiveness to both endogenous neuro-modulators and exogenous therapeutics (32, 33). Indeed, our past findings have underscored the importance of spinophilin/arrestin-regulated endocytosis for  $\alpha_{2A}$ AR signal transduction (24, 34).

Our results indicate that co-activation of  $\alpha_{2A}$  and  $\beta$ ARs, either by application of endogenous agonist or by simultaneous stimulation with distinct selective agonists, results in an acceleration of  $\alpha_{2A}$ AR endocytosis in native adrenergic neurons. This acceleration occurs in a PKA- and spinophilin-dependent fashion, whereas a similar acceleration of agonist-driven  $\alpha_{2A}$ AR endocytosis is observed either with receptor-independent activation of PKA by forskolin or in spinophilin-null neurons. We further show that  $\beta$ AR co-activation disrupts agonist-dependent  $\alpha_{2A}$ AR/spinophilin interaction in a PKA-dependent fashion. In sum, our data establish a novel mechanism for unidirectional cross-talk from  $\beta$  to  $\alpha_{2A}$ ARs affecting  $\alpha_{2A}$ AR responsiveness in a setting with significant physiological and pharmacological importance.

### EXPERIMENTAL PROCEDURES

**Animals**—Mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited animal resources program facility at the University of Alabama at Birmingham in accordance with the Animal Welfare Act and the 1989 amendments to that act. All studies followed protocols approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee. The generation of HA-tagged  $\alpha_{2A}$ AR knock-in (30), spinophilin-null (35), and arrestin3-null (36) mice has been previously described. These transgenic lines were backcrossed over 10 generations to a pure C57BL/6 genetic background. HA- $\alpha_{2A}$ AR mice were crossed with spinophilin-null mice to generate a HA<sup>+/+</sup>/spinophilin<sup>-/-</sup> line (which will be referred to as Sp<sup>-/-</sup> in the interest of

simplicity), as well as with arrestin3-null mice to generate a HA<sup>+/+</sup>/arrestin3<sup>-/-</sup> line (which will be referred to as Arr3<sup>-/-</sup> in the interest of simplicity).

**Primary Culture of SCG Neurons**—SCG neurons were cultured from mouse pups at postnatal day 4–6 as described previously (30, 37) with slight modifications. Briefly, SCG were dissected and placed into Hanks' balanced salt solution (Invitrogen) containing 25 mM glucose and 20 mM HEPES, pH 7.3, and subjected to enzymatic digestion with 3 mg/ml collagenase and 1 mg/ml trypsin (Sigma). Neurons were dissociated by trituration with a fire-polished siliconized Pasteur pipette and, after a preplating step to reduce non-neuronal cell types in the final culture, plated onto coverslips treated with poly-D-lysine and laminin (Sigma). Growth medium was L-15 base medium (Invitrogen) plus 10% Nu-Serum (Clontech), 30% glucose, 2% GlutaMAX (Invitrogen), 1% insulin/transferrin/selenium supplement (Invitrogen), 25 ng/ml nerve growth factor (Sigma), and 24 mM NaHCO<sub>3</sub>. Medium changes were on days *in vitro* 1, 4, and 6, with the addition of 10  $\mu$ M 5-fluoro-2'-deoxyuridine (Sigma) on days 1 and 4 to control non-neuronal cell growth, and 1  $\mu$ M yohimbine ( $\alpha_{2A}$ AR antagonist; Sigma) on days 4 and 6 to protect surface  $\alpha_{2A}$ ARs. For immunofluorescent staining, neurons were plated at a ganglion to coverslip ratio of 1:1. All experiments were performed on day *in vitro* 8, a time point at which  $\alpha_{2A}$ ARs have robust somatodendritic and axonal surface expression in SCG neurons (37).

**Immunofluorescent Staining**—Internalization of HA- $\alpha_{2A}$ ARs was assessed by a prelabeling method that has been well described previously (28, 30). All staining experiments detected HA-tagged  $\alpha_{2A}$ ARs. As an initial step prior to antibody prelabeling/drug treatments, neurons were washed thoroughly to remove yohimbine. HA- $\alpha_{2A}$ ARs were detected with HA.11 primary antibody (Covance, 1:100 dilution), which was used for a 20-min prelabeling of surface  $\alpha_{2A}$ AR population at room temperature prior to agonist stimulation. Cells were then permeabilized, blocked, and incubated with AlexaFluor 488-conjugated goat anti-mouse secondary antibody (Invitrogen, 1:1,000 dilution) for 1 h at room temperature. Images were obtained using a Zeiss LSM 710 confocal microscope (Carl Zeiss) at 63 $\times$  magnification. For quantitative assessment of receptor internalization, images were analyzed with MetaMorph software (Molecular Devices) to determine total and intracellular fluorescent intensities as described previously (22). A "relative internalization unit" for stimulated cells was then calculated as a ratio of intracellular to total fluorescent intensity normalized to matched unstimulated controls (30). A minimum of 12–14 neurons collected over at least three independent samples were analyzed for each data group, with the exception of clonidine + SAL ( $n = 10$ ).

For the HA- $\alpha_{2A}$ AR double-labeling experiment, surface receptors were prelabeled as above. Nonpermeabilized neurons were then incubated with AlexaFluor 488-conjugated anti-mouse secondary antibody (1:250 dilution) for 1 h at room temperature to saturate prelabeled surface receptors. After permeabilization/blocking, neurons were incubated with AlexaFluor 594-conjugated secondary antibody (Invitrogen, 1:1,000 dilution) for 1 h at room temperature to detect prelabeled cytosolic (endocytosed) receptors.

Immunostaining of LAMP1 was performed together with the prelabeling method to detect both HA- $\alpha_{2A}$ ARs and LAMP1. After HA prelabeling and permeabilization/blocking, neurons were incubated with anti-LAMP1 primary antibody (University of Iowa Hybridoma Bank, 1:400 dilution) overnight at 4 °C. The cells were then subjected to secondary labeling with AlexaFluor 488-conjugated anti-mouse and AlexaFluor 594-conjugated anti-rat (Invitrogen) antibodies (1:1,000 dilution) for 1 h at room temperature.

For adenylyl cyclase activation, neurons were pretreated with forskolin or vehicle ( $\text{Me}_2\text{SO}$ ), and forskolin/vehicle was maintained during stimulation with Epi (Sigma, 100  $\mu\text{M}$  final). Epi stimulation was done either alone (for simultaneous activation of  $\beta$  and  $\alpha_{2A}$ ARs) or in combination with the non-subtype-selective  $\beta$ AR antagonist propranolol (Sigma, 1  $\mu\text{M}$  final) for activation of  $\alpha_{2A}$ ARs only. For  $\beta$ AR co-activation, neurons were pretreated for 10 min with the non-subtype-selective agonist isoproterenol (ISO, 100  $\mu\text{M}$  final) or one of several agonists with varying selectivity for  $\beta_1$  versus  $\beta_2$ ARs: dobutamine (DOB, 1  $\mu\text{M}$  final), albuterol (ALB, 1  $\mu\text{M}$  final), or salmeterol (SAL, 100 nM final). ISO/DOB/ALB/SAL was maintained during stimulation with the  $\alpha_2$ AR agonist clonidine (Sigma, 1  $\mu\text{M}$  final). For PKA inhibition, neurons were subjected to a 10-min pretreatment with myristoylated protein kinase A inhibitor 14–22 amide (PKI, Calbiochem, 8.3  $\mu\text{M}$  final), with PKI then maintained through ISO pretreatment and clonidine stimulation. Previous enzymological evidence indicates that this PKI concentration achieves effectively complete inhibition of PKA (38, 39). Prazosin (Sigma, 1  $\mu\text{M}$  final) was included in all experiments to block potential activation of  $\alpha_1$  and  $\alpha_{2B/C}$ AR subtypes.

**Determination of Arrestin Dependence**—For these experiments, SCG neurons were cultured from our HA<sup>+/+</sup>/arrestin3<sup>-/-</sup> line (referred to as Arr3<sup>-/-</sup> in the interest of simplicity) described above. Arrestin redistribution was examined through the use of a rabbit polyclonal antibody against endogenous arrestin2 (a generous gift of Dr. Jeffrey Benovic, Thomas Jefferson University) and AlexaFluor 594-conjugated anti-rabbit secondary antibody (Invitrogen, 1:1,000 dilution). Arrestin2 knockdown was achieved by lentiviral constructs encoding shRNA against mouse arrestin2, purchased from Open Biosystems and packaged using the ViraPower Lentiviral Packaging System (Invitrogen) according to the manufacturer's instructions. Arrestin3-null SCG neurons were transduced on day *in vitro* 3, and experiments were performed on day *in vitro* 8 as above.

**FLIM-FRET**—FLIM-based FRET experiments were utilized to directly observe  $\alpha_{2A}$ AR/spinophilin interaction in live cells according to a previously described method (34). The C-terminally CFP-tagged  $\alpha_{2A}$ AR construct has been reported (34), and the N-terminally YFP-tagged spinophilin construct was prepared by PCR amplification and cloning of cDNA encoding spinophilin into the pEYFP-C1 vector (Clontech), with the construct verified by sequencing prior to use. HEK293 cells were transiently transfected using Lipofectamine 2000 (Sigma) with the plasmids containing either CFP- $\alpha_{2A}$ AR alone (2  $\mu\text{g}/60\text{-mm}$  plate) or in combination with YFP-spinophilin (1  $\mu\text{g}/60\text{-mm}$  plate). For each treatment group, five or six individual cells from two or three independent samples were imaged and ana-

lyzed. FLIM-FRET efficiency ( $E$ ) was calculated as:  $E = 1 - (t_{\text{FRET}}/t_{\text{CFP}})$ , where  $t_{\text{FRET}}$  and  $t_{\text{CFP}}$  are CFP lifetime values obtained from cells expressing CFP and YFP together and CFP alone, respectively (34). Cells were stimulated with clonidine plus prazosin at 1  $\mu\text{M}$ , with  $\beta$ AR co-activation achieved as described above.

**Mouse Embryonic Fibroblasts (MEFs)**—The isolation of MEFs from spinophilin-null mouse mice and generation of a spinophilin-null MEF line stably expressing HA-tagged  $\alpha_{2A}$ ARs have been previously described (40). Spinophilin-null MEFs were transfected using Lipofectamine 2000 with plasmids encoding GFP-tagged wild-type spinophilin (referred to as WT-Sp) or GFP-tagged mutant spinophilin lacking PKA phosphorylation sites (Sp94A, 177A, referred to as mut-Sp), 3  $\mu\text{g}/60\text{-mm}$  plate. Transfected MEFs were split to coverslips on a 24-well culture plate ~24 h post-transfection, and immunostaining for  $\alpha_{2A}$ AR endocytosis was performed 48 h post-transfection as described above, with the substitution of AlexaFluor 594-conjugated anti-mouse (Invitrogen) secondary labeling. MEF cells were visualized via confocal microscopy using a Nikon A1 scope (Nikon) at 60 $\times$  magnification.

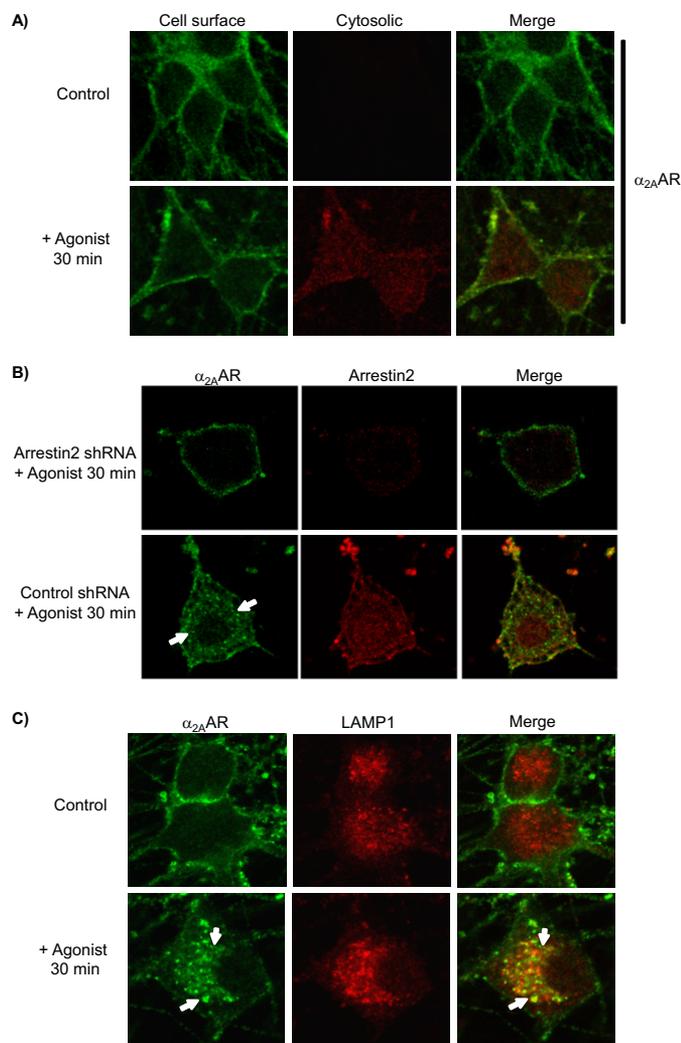
**cAMP Production Assay**—cAMP production in MEFs was measured using the AlphaScreen cAMP assay kit (PerkinElmer Life Sciences) according to the manufacturer's instructions. A total of  $2 \times 10^4$  cells/assay well were used, and cells were exposed to 100  $\mu\text{M}$  ISO for 30 min at room temperature to determine cAMP response mediated by endogenous  $\beta$ ARs. MEFs isolated from WT and spinophilin-null mice of matched genetic background were compared in parallel. AlphaScreen cAMP reporter readings were acquired using a Synergy 2 microplate reader (Biotek). Relative cAMP production was calculated by converting absolute value change in raw AlphaScreen signal to a fold change over control cells.

## RESULTS

**Epinephrine-mediated  $\alpha_{2A}$ AR Endocytosis in Native Neurons Is Arrestin-dependent and Accelerated in Spinophilin-null Neurons**—SCG neurons exhibit extensive expression of both  $\alpha_{2A}$ AR and  $\alpha_{2C}$ AR subtypes (37) in addition to  $\beta$  and  $\alpha_1$  AR subtypes. To focus on the  $\alpha_{2A}$  subtype, we have made use of our HA-tagged  $\alpha_{2A}$ AR knock-in mouse model for clean and specific detection of  $\alpha_{2A}$ ARs, and we have included appropriate AR blockers (prazosin for  $\alpha_1$  and  $\alpha_{2B/C}$  ARs, propranolol for  $\beta$ ARs) to ensure stimulation of  $\alpha_{2A}$ ARs alone. We have previously demonstrated that  $\alpha_{2A}$ AR expression level, distribution, localization, and functional properties are unaltered in the knock-in line (30). Additionally, we have made exclusive use of immunostaining and imaging methods to assay endocytosis, because SCG culture yields (less than 2,000 cells/ganglion) limit our ability to perform other biochemical or ELISA-based techniques.

As shown in Fig. 1A, stimulation with Epi (plus prazosin/propranolol) drives endocytosis of endogenously expressed  $\alpha_{2A}$ ARs in the native SCG neurons. Our primary antibody prelabeling plus double secondary antibody labeling method (see "Experimental Procedures") detects internalized receptors by the appearance of cytosolic staining in stimulated but not control cells.

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**FIGURE 1. Evaluation of arrestin-dependent agonist-stimulated  $\alpha_{2A}$ AR endocytosis in native neurons.** All immunostaining experiments detect endogenously expressed HA- $\alpha_{2A}$ ARs. *A*, neurons were subjected to primary antibody prelabeling of surface  $\alpha_{2A}$ ARs prior to stimulation, followed by saturation of labeled surface receptors with AlexaFluor488-conjugated secondary labeling, and then permeabilization and cytosolic AlexaFluor594-conjugated secondary labeling. Cytosolic labeling was seen only in stimulated cells, indicating endocytosis of pre-labeled surface  $\alpha_{2A}$ ARs. *B*, endocytosis was assayed in arrestin3-null neurons, transduced with either arrestin2 shRNA (to achieve complete ablation of arrestins) or control shRNA. Analysis revealed that arrestin2 shRNA resulted in a  $69 \pm 5.8\%$  reduction in arrestin2 immunoreactivity.  $\alpha_{2A}$ ARs were detected by primary antibody prelabeling followed by permeabilization and AlexaFluor488-conjugated secondary labeling. In control but not arrestin2 shRNA cells, agonist stimulation resulted in significant  $\alpha_{2A}$ AR endocytosis, indicated by the appearance of intracellular punctae containing internalized receptors (arrows), as well as a partial co-localization of  $\alpha_{2A}$ ARs with arrestin2. *C*, endocytosis was additionally observed by monitoring  $\alpha_{2A}$ AR co-localization with the late endolysosomal pathway marker LAMP1; prelabeling of  $\alpha_{2A}$ ARs was done as in *A*, with the addition of co-immunostaining for LAMP1. In agonist-stimulated but not unstimulated control neurons, endocytosis was seen as indicated by the appearance of intracellular punctae which exhibited co-localization with LAMP1 (arrows). Confocal images are representative of at least three independent samples.

The nonvisual arrestins (arrestin2 and 3, also known as  $\beta$ -arrestin1 and 2) are key mediators of classical GPCR endocytosis via clathrin-coated pits (32, 41, 42). To determine whether this classical endocytosis was being driven for endogenous  $\alpha_{2A}$ ARs in SCG neurons, we cultured neurons from arrestin3-null mice; we have found no difference in  $\alpha_{2A}$ AR density between arrestin3-null and WT mice (34). Those cells were then transduced

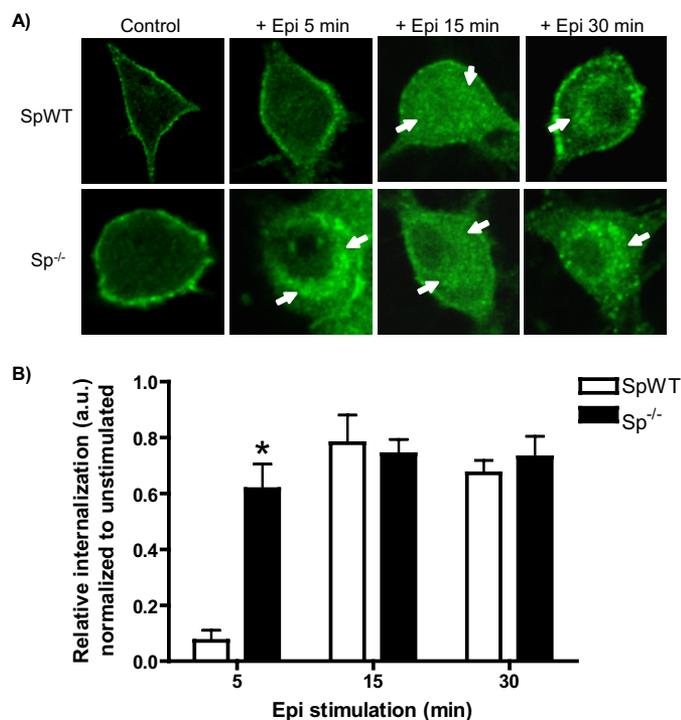
with either arrestin2 shRNA (to achieve ablation of both arrestins) or control shRNA. We then utilized our single prelabeling method (see “Experimental Procedures”), which allows us to monitor the initial surface  $\alpha_{2A}$ AR population. Agonist stimulation drove significant  $\alpha_{2A}$ AR endocytosis, indicated by the appearance of characteristic intracellular punctae containing internalized receptors, in control shRNA neurons but not in arrestin2 shRNA neurons (Fig. 1*B*). These data demonstrate that arrestins are required for agonist-mediated endocytosis of endogenously expressed  $\alpha_{2A}$ ARs.

To provide further confirmation of our ability to observe  $\alpha_{2A}$ AR endocytosis in SCG neurons via our prelabeling method, we performed co-immunostaining for lysosomal-associated membrane protein 1 (LAMP1), a late endolysosomal pathway marker. As shown in Fig. 1*C*, following agonist stimulation, pre-labeled internalized  $\alpha_{2A}$ ARs (intracellular punctae seen clearly in the *lower left panel*) exhibit partial co-localization with LAMP1 (*lower right panel*), strongly suggesting that they are internalized receptors that have entered the endolysosomal pathway. Collectively, these results establish agonist-mediated arrestin-dependent endocytosis of endogenously expressed  $\alpha_{2A}$ ARs in our native SCG cell model.

We have previously established a clear regulatory mechanism for  $\alpha_{2A}$ AR endocytosis involving functional antagonism of arrestin functions by spinophilin (24). However, this regulatory mechanism has not been reported for endogenously expressed  $\alpha_{2A}$ ARs in native SCG neurons. We therefore compared the kinetics of endocytosis induced by Epi (plus prazosin/propranolol) in neurons with and without spinophilin expression (SpWT and Sp<sup>-/-</sup>, respectively). As would be predicted by our regulatory model, we observed a clear acceleration of  $\alpha_{2A}$ AR endocytosis in spinophilin-null neurons, with significantly enhanced endocytosis at the 5-min time point in Sp<sup>-/-</sup> versus SpWT neurons (Fig. 2). Two-way ANOVA revealed significant effects of genotype ( $p = 0.0045$ ) and time ( $p < 0.0001$ ) and a significant genotype  $\times$  time interaction ( $p < 0.0001$ ). It should be noted that our past work indicates no difference in  $\alpha_{2A}$ AR density between spinophilin-null and WT mice (25). These results are consistent with our previous findings in heterologous cells and can likely be attributed to unimpeded agonist-dependent binding of arrestins to the  $\alpha_{2A}$ AR in the absence of opposition from spinophilin.

*Co-activation of  $\beta$  and  $\alpha_{2A}$ ARs Accelerates  $\alpha_{2A}$ AR Endocytosis in a PKA-dependent Fashion*—Given the preponderance of evidence suggesting  $\beta/\alpha_{2A}$ AR cross-talk described at the outset, and endogenous expression of a full range of AR subtypes in the adrenergic SCG neurons, we decided to investigate whether simultaneous activation of both  $\beta$  and  $\alpha_{2A}$ ARs would affect the  $\alpha_{2A}$ AR endocytic response.

We began by attempting to more closely model the physiological setting with Epi stimulation in the absence  $\beta$ AR blockade, although prazosin was maintained in these experiments. Under these conditions, both  $\beta$  and  $\alpha_{2A}$ ARs will be co-activated by Epi. As shown in Fig. 3, Epi stimulation in the absence of propranolol (*i.e.*, non-subtype-selective  $\beta$ AR blockade) drove significant  $\alpha_{2A}$ AR endocytosis at the early time points of 5 and 10 min. When propranolol was added, this endocytosis was effectively blocked at 5 min and significantly attenuated at 10

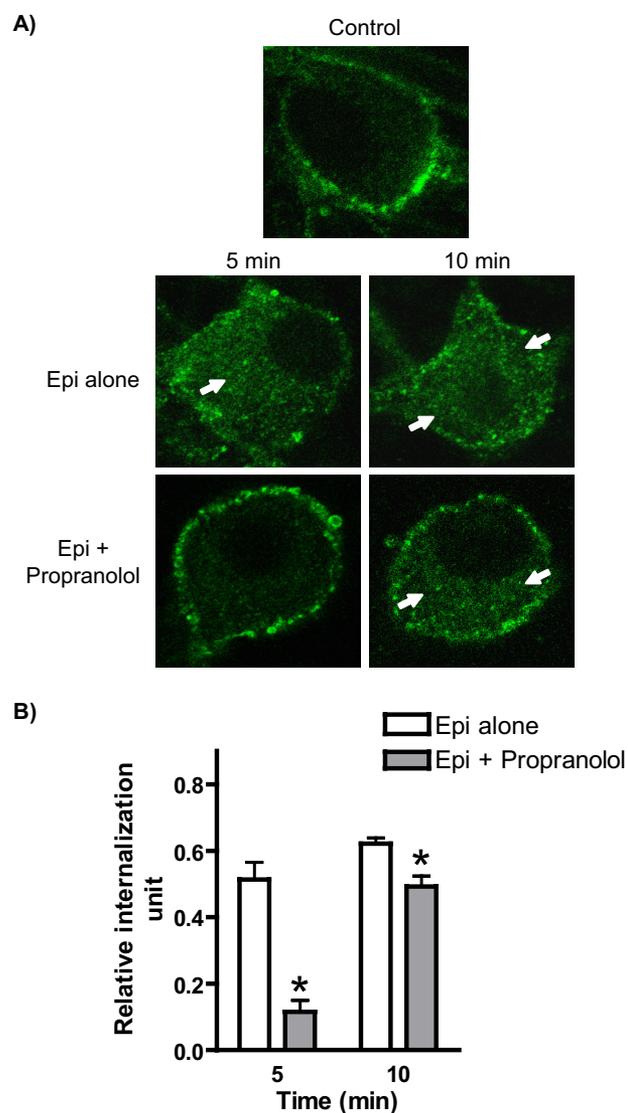


**FIGURE 2. Epinephrine-mediated  $\alpha_{2A}$ AR endocytosis is regulated by spinophilin.** *A*, neurons with (SpWT) and without (Sp<sup>-/-</sup>) spinophilin expression were stimulated by application of Epi (100  $\mu$ M, plus prazosin and propranolol) as indicated. Neurons were subjected to  $\alpha_{2A}$ AR prelabeling method as in Fig. 1 (*B* and *C*). Endocytosis, indicated by the appearance of intracellular punctae containing internalized receptors (arrows), was observed beginning at 5 min in Sp<sup>-/-</sup> neurons and at 15 and 30 min in both SpWT and Sp<sup>-/-</sup> neurons, consistent with endocytic acceleration in the absence of spinophilin. *B*, quantitation of agonist-mediated  $\alpha_{2A}$ AR endocytosis in Epi-stimulated SpWT and Sp<sup>-/-</sup> neurons, with relative internalization determined as described under "Experimental Procedures." Confocal images are representative of at least three independent samples, and quantitation was performed over at least 12–14 neurons. \*,  $p < 0.01$ , SpWT versus Sp<sup>-/-</sup>.

min (Fig. 3), indicating that the endocytic enhancement was dependent on  $\beta$ AR activation and suggesting the existence of modulatory cross-talk from  $\beta$  to  $\alpha_{2A}$ ARs. Two-way ANOVA revealed significant effects of propranolol exposure and time ( $p < 0.0001$ ) and a significant propranolol  $\times$  time interaction ( $p = 0.0002$ ).

To further confirm the ability of  $\beta$ AR co-activation to regulate  $\alpha_{2A}$ AR endocytosis via cross-talk, we next investigated the ability of  $\beta$ AR stimulation by  $\beta$ -specific agonists to affect  $\alpha_{2A}$  agonist-induced  $\alpha_{2A}$ AR endocytosis. Based upon our previous experience with the therapeutic partial  $\alpha_2$  agonist clonidine, we chose to focus on stimulation for 10 and 30 min, time points when clonidine drives little detectable  $\alpha_{2A}$ AR endocytosis and a maximal endocytic response, respectively.

We first utilized the non-subtype-selective  $\beta$ AR agonist ISO as a tool for co-activation. Importantly, we failed to detect any significant endocytosis of endogenous  $\alpha_{2A}$ ARs with ISO treatment alone (Fig. 4*A*). In comparison with clonidine stimulation alone, we found a dramatic enhancement of  $\alpha_{2A}$ AR endocytosis at the 10-min time point with ISO co-treatment (Fig. 4, *B* and *C*). Intriguingly, this endocytic enhancement was prevented by the PKA inhibitor PKI, raising the possibility that canonical cAMP signaling by  $\beta$ ARs is involved. We observed no further enhancement of cloni-

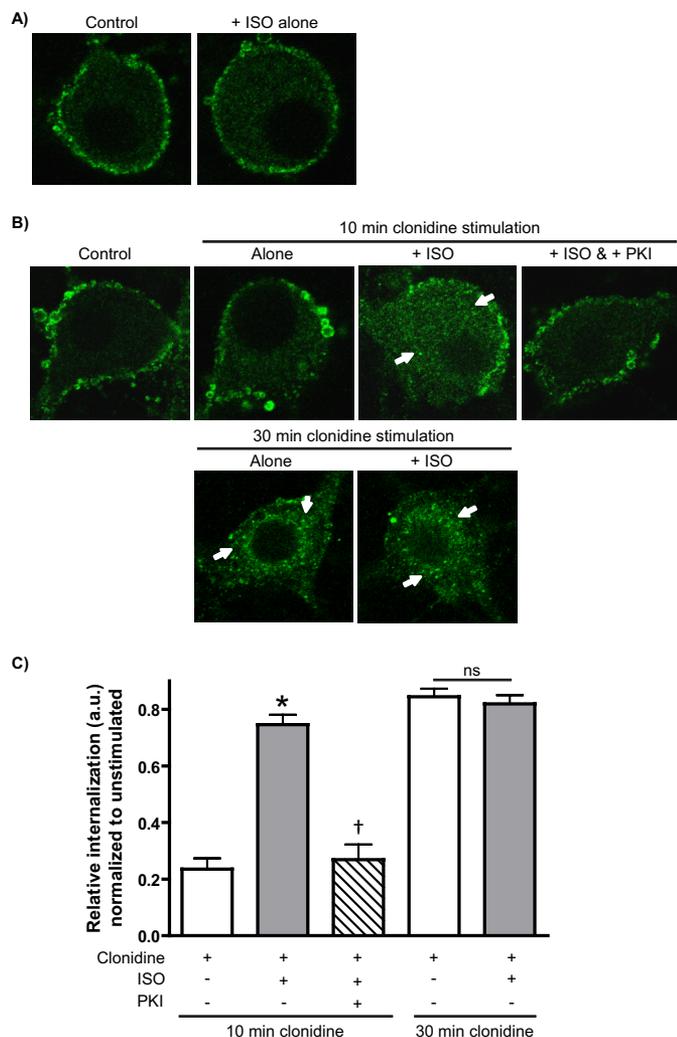


**FIGURE 3. Simultaneous activation of  $\alpha_{2A}$  and  $\beta$ ARs by application of Epi results in accelerated endocytosis of endogenously expressed  $\alpha_{2A}$ ARs in native neurons.** *A*, neurons (SpWT, subjected to  $\alpha_{2A}$ AR prelabeling as in Fig. 1, *B* and *C*) were stimulated by application of Epi (plus prazosin) in the absence or presence of the  $\beta$ AR antagonist propranolol, conditions that allow for activation of both  $\alpha_{2A}$  and  $\beta$ ARs or  $\alpha_{2A}$ ARs alone, respectively. Significant endocytosis, as indicated by the appearance of intracellular punctae containing internalized receptors (arrows), was observed in response to Epi alone at both 5 and 10 min, whereas Epi together with propranolol drove observable endocytosis only at 10 min. *B*, quantitation of agonist-mediated  $\alpha_{2A}$ AR endocytosis in Epi-stimulated neurons the absence or presence of propranolol, with relative internalization determined as described under "Experimental Procedures." Internalization by Epi alone was significantly attenuated by propranolol at both 5 and 10 min. Confocal images are representative of at least three independent samples, and quantitation was performed over at least 12–14 neurons. \*,  $p < 0.01$  versus Epi alone.

dine-induced  $\alpha_{2A}$ AR endocytosis by ISO at the 30-min time point (Fig. 4, *B* and *C*), indicating that  $\beta$ AR co-activation does not increase the efficacy of agonist-mediated  $\alpha_{2A}$ AR endocytosis but rather accelerates the kinetics.

We additionally performed  $\beta$ AR co-activation using a panel of clinically relevant agonists with varying degrees of  $\beta_1$  versus  $\beta_2$ AR subtype selectivity. Our results indicated that DOB, ALB, and SAL were all capable of enhancing clonidine-induced  $\alpha_{2A}$ AR endocytosis (Fig. 5). DOB and ALB were slightly less effective than the non-subtype-selective ISO at enhancing

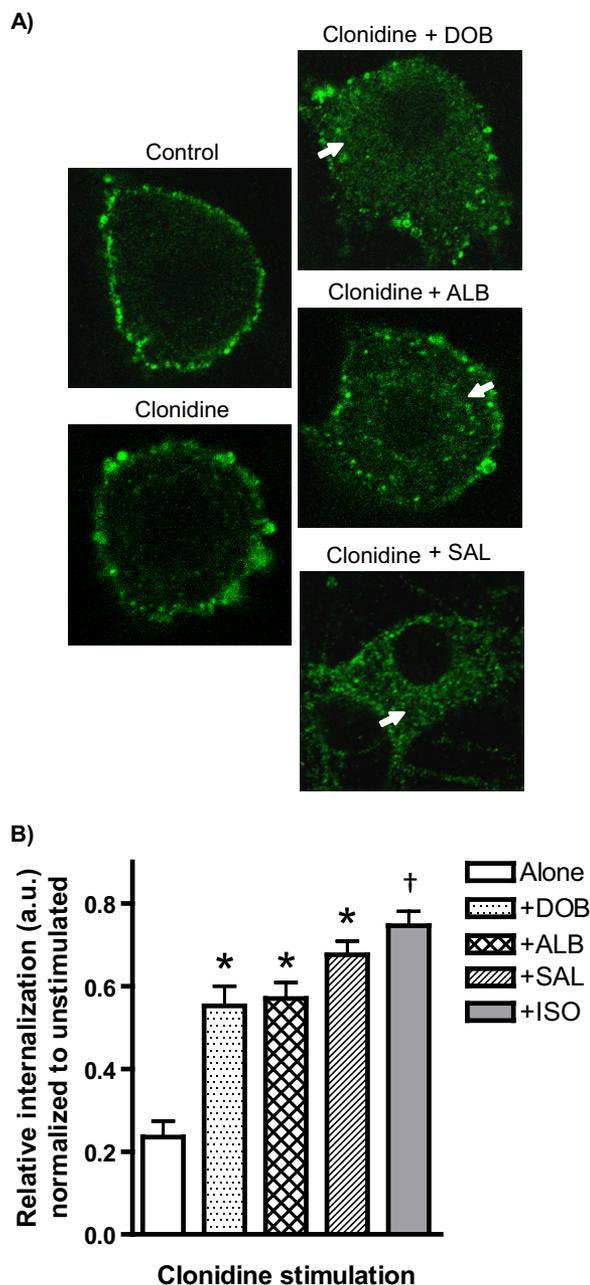
## Modulation of $\alpha_{2A}$ AR Endocytosis by Cross-talk from $\beta$ ARs



**FIGURE 4.  $\beta$ AR co-activation by ISO accelerates  $\alpha_{2A}$ AR agonist-mediated  $\alpha_{2A}$ AR endocytosis in native neurons in a PKA-dependent fashion.** Neurons (SpWT) were subjected to  $\alpha_{2A}$ AR prelabeling method as in Fig. 1 (B and C) with endocytosis indicated by the appearance of intracellular punctae containing internalized receptors (arrows). A, ISO alone was not found to drive any significant  $\alpha_{2A}$ AR endocytosis. B and C, neurons were subjected to the indicated treatments; pretreatment with the non-subtype-selective  $\beta$ AR agonist ISO (100  $\mu$ M) and/or the PKA inhibitor PKI (8.3  $\mu$ M) was done prior to stimulation with the therapeutic partial  $\alpha_{2A}$ AR agonist clonidine (1  $\mu$ M). 10 min of clonidine stimulation alone was not sufficient to drive any significant  $\alpha_{2A}$ AR endocytosis, whereas pretreatment with ISO drove a significant response that was blocked when PKA was inhibited with PKI. No further enhancement of clonidine-mediated  $\alpha_{2A}$ AR endocytosis by ISO was observed at the 30-min time point, consistent with a kinetic acceleration of  $\alpha_{2A}$ AR endocytosis by  $\beta$ AR co-activation. Confocal images (A and B) are representative of at least three independent samples, and quantitation (C) was performed over at least 12–14 neurons. \*,  $p < 0.001$  versus clonidine plus ISO; †,  $p < 0.001$  versus clonidine plus ISO.

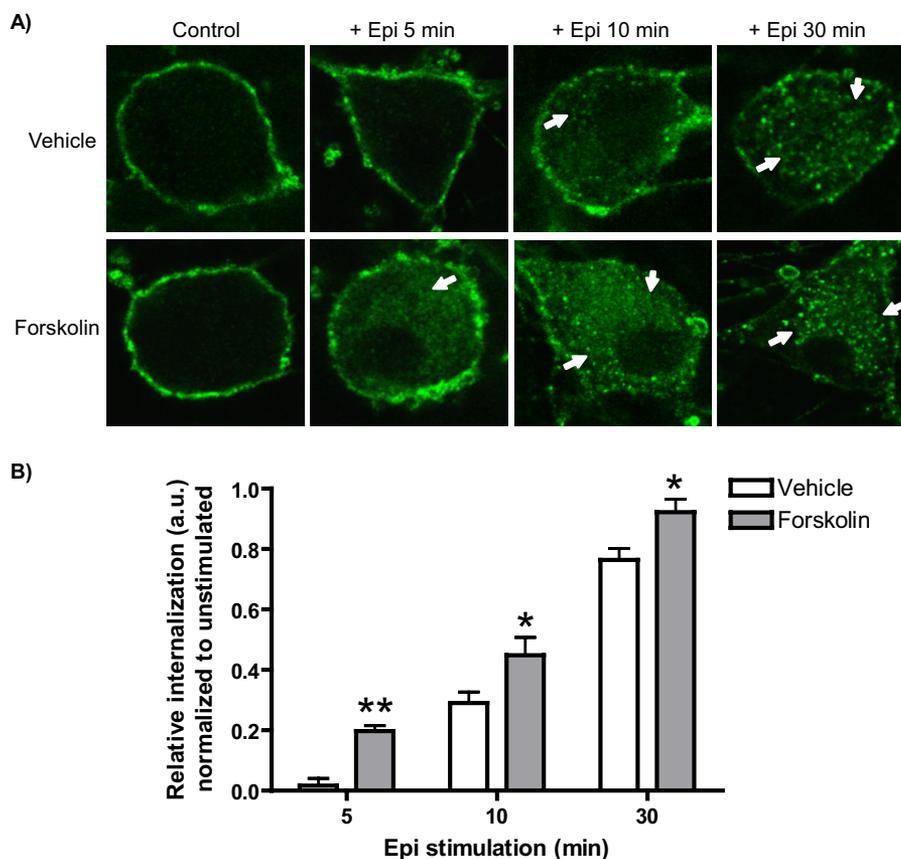
endocytosis, whereas SAL was similarly effective. Although there is some debate regarding the relative selectivity of DOB and ALB, SAL is widely accepted as a potent and highly  $\beta_2$ AR-selective agonist. These results further support that activation of both  $\beta$ AR subtypes is capable of driving the acceleration of  $\alpha_{2A}$ AR endocytosis and that the effects observed in Fig. 4 are not unique to ISO only among the  $\beta$ AR agonists.

Collectively, the above results demonstrate that co-activation of endogenous  $\beta$  and  $\alpha_{2A}$ ARs results in an acceleration of agonist-mediated  $\alpha_{2A}$ AR endocytosis in native neurons. These



**FIGURE 5. Co-activation with  $\beta$ AR subtype-selective agonists accelerates  $\alpha_{2A}$ AR agonist-mediated  $\alpha_{2A}$ AR endocytosis in native neurons.** A, neurons (SpWT) were subjected to treatment with a panel of clinically relevant agonists with varying degrees of  $\beta_1$  versus  $\beta_2$ AR subtype selectivity (1  $\mu$ M DOB, 1  $\mu$ M ALB, or 100 nM SAL) and 10 min of stimulation with clonidine (1  $\mu$ M).  $\alpha_{2A}$ AR endocytosis (detected by prelabeling method), as indicated by the appearance of intracellular punctae containing internalized receptors (arrows), was observed with co-activation by DOB, ALB, and SAL, but not with clonidine alone. B, quantitation of agonist-mediated  $\alpha_{2A}$ AR endocytosis, with relative internalization determined as described under “Experimental Procedures.” Internalization by clonidine alone was significantly enhanced with co-activation by DOB, ALB, and SAL. The data for clonidine plus ISO from Fig. 4 are included for comparison. Confocal images are representative of at least three independent samples, and quantitation was performed over at least 12–14 neurons (except for clonidine plus SAL,  $n = 10$ ). \*,  $p < 0.01$  versus clonidine alone. †,  $p < 0.01$  versus clonidine plus DOB or clonidine plus ALB.

data establish a phenomenon of cross-talk from  $\beta$  to  $\alpha_{2A}$ ARs modulating  $\alpha_{2A}$ AR endocytic responses and suggest that such cross-talk may rely on canonical  $\beta$ AR/ $G_{\alpha_s}$ /cAMP signal transduction linking to PKA activation.



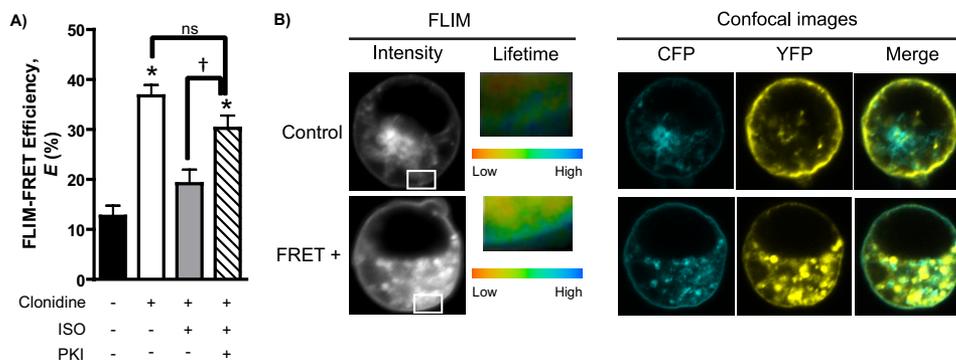
**FIGURE 6. Activation of PKA by forskolin treatment is sufficient to accelerate agonist-mediated  $\alpha_{2A}$ AR endocytosis in native neurons.** *A*, neurons (SpWT) were pretreated with either vehicle or forskolin and then subjected to  $\alpha_{2A}$ AR prelabeling as in Fig. 1B&C followed by stimulation with Epi (100  $\mu$ M, plus prazosin and propranolol) as indicated. Endocytosis, as indicated by the appearance of intracellular puncta containing internalized receptors (arrows), was observed in beginning at 5 min in forskolin-treated but not vehicle-treated neurons and continued to be significant, although less dramatic endocytic enhancement was observed at 15 and 30 min. *B*, quantitation of agonist-mediated  $\alpha_{2A}$ AR endocytosis in Epi-stimulated forskolin- and vehicle-treated neurons, with relative internalization determined as described under "Experimental Procedures." Confocal images are representative of at least three independent samples, and quantitation was performed over at least 12–14 neurons. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  versus vehicle-treated.

**Receptor-independent PKA Activation Is Sufficient to Accelerate Agonist-mediated  $\alpha_{2A}$ AR Endocytosis**—The present data indicate that PKA activation is critically involved in accelerating agonist-mediated  $\alpha_{2A}$ AR endocytosis in SCG neurons (Fig. 4). Indeed, we have previously demonstrated that receptor-independent activation of PKA by forskolin accelerates  $\alpha_{2A}$ AR endocytosis in heterologous cells (28). Such a finding in our native neurons would support our contention that  $\beta$ AR/ $G\alpha_s$ /cAMP signal transduction linking to PKA activation underlies the  $\alpha_{2A}$ AR endocytic acceleration by  $\beta$ AR co-activation.

We therefore ascertained whether forskolin treatment would affect endocytosis of endogenous  $\alpha_{2A}$ ARs in SCG neurons. Consistent with our previous findings, we observed an acceleration of endocytosis as stimulated by Epi (plus prazosin/propranolol) with significantly higher levels of internalization for forskolin-treated cells compared with vehicle controls (Fig. 6). The effect of forskolin was most dramatic at the 5- and 10-min time points, with a diminishing effect at 30 min of Epi stimulation. Two-way ANOVA revealed significant effects of forskolin pretreatment and time ( $p < 0.0001$ ), but no significant forskolin  $\times$  time interaction. These results demonstrate that PKA activation alone in the absence of  $\beta$ AR co-activation is sufficient to accelerate agonist-mediated endocytosis of endogenous  $\alpha_{2A}$ ARs.

**$\beta$ AR Co-activation Results in PKA-dependent Disruption of  $\alpha_{2A}$ AR/Spinophilin Interaction**—Having established the clear importance of PKA activity in accelerating endogenous  $\alpha_{2A}$ AR endocytosis in SCG neurons, we sought to further investigate the mechanistic link underlying cross-talk from  $\beta$  to  $\alpha_{2A}$ ARs. Spinophilin is a substrate for PKA phosphorylation at serine residues 94 and 177 (27), and our previous work has demonstrated that this PKA phosphorylation of spinophilin disrupts its ability to interact with and regulate  $\alpha_{2A}$ ARs (28). Furthermore, we have shown that the interaction of  $\alpha_{2A}$ ARs with spinophilin occurs in an agonist-dependent fashion both *in vitro* and *in vivo* (21, 23–25). We therefore postulated that our findings of endocytic acceleration (Figs. 3 and 4) could be explained by  $\beta$ AR co-activation leading to canonical  $G\alpha_s$ /cAMP signal transduction, PKA activation, phosphorylation of spinophilin, and disrupted  $\alpha_{2A}$ AR/spinophilin interaction. To provide direct evidence for this mechanism, we utilized a FLIM-FRET technique to observe the  $\alpha_{2A}$ AR/spinophilin interaction under various conditions in live cells. As shown in Fig. 7, we observed a significant clonidine-dependent interaction between  $\alpha_{2A}$ ARs and spinophilin, indicated by the large increase in FRET efficiency (Fig. 7A). Importantly, this clonidine-dependent interaction was abolished when  $\beta$ ARs were co-activated by ISO treatment, and this effect was in turn reversed with inhibition of

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**FIGURE 7. Agonist-dependent  $\alpha_{2A}$ AR/spinophilin interaction is blocked by  $\beta$ AR co-activation in a PKA-dependent fashion.** *A*, FLIM-FRET efficiency ( $E$ ) values calculated based on CFP lifetime values as described under “Experimental Procedures.” The values were obtained over five or six cells from two or three independent samples. Live HEK293 cells transiently transfected with CFP- $\alpha_{2A}$ AR and YFP-spinophilin constructs were subjected to ISO (100  $\mu$ M) and/or PKI (8.3  $\mu$ M) pretreatment prior to stimulation with clonidine (1  $\mu$ M) as in Fig. 2(A and B). *B*, representative images from nonstimulated control and stimulated FRET positive (FRET+) cells. FLIM images show CFP fluorescent intensity (gray scale) and lifetime values (represented in pseudocolors). *Inset areas* are cell surface regions selected for CFP lifetime analysis. A single measurement point corresponding to a median CFP lifetime value (green in the pseudocolor range) was the data point extracted from each cell. Confocal images show the cellular localization of CFP- $\alpha_{2A}$ AR and YFP-spinophilin. \*,  $p < 0.0001$  versus clonidine alone; †,  $p < 0.0001$  versus clonidine + ISO.

PKA activity by PKI, which rescued the clonidine-dependent interaction (Fig. 7A). Taken together, these data provide support for our postulated mechanism of  $\beta$  to  $\alpha_{2A}$ AR cross-talk and provide a plausible explanation for the observed endocytic acceleration in native neurons.

**Expression of WT and Mutant Spinophilin in Spinophilin-null MEFs Supports Involvement of  $\beta$ AR-mediated Phosphorylation**—Given the impracticality of biochemically assaying for spinophilin phosphorylation in SCG neurons, we elected to provide additional support for our proposed mechanism using MEFs with endogenous  $\beta$ AR expression as a substitute model system. Using transient transfection, we expressed GFP-tagged WT spinophilin (WT-Sp) or GFP-tagged mutant spinophilin with PKA phosphorylation sites at Ser-94 and Ser-177 mutated to Ala (Sp94A, 177A, referred to as mut-Sp) into spinophilin-null MEFs. Based on our extensive experience studying  $\alpha_{2A}$ AR endocytosis, we know that response kinetics are accelerated in MEFs versus SCG neurons, and so we used 5 min of stimulation as the early time point instead of 10 min. As shown in Fig. 8, when compared with nonexpressing control (spinophilin-null) cells, expression of WT-Sp appears to rescue the phenotype of these cells. Endocytosis can be detected in spinophilin-null but not WT-Sp cells after 5 min of clonidine stimulation (Fig. 8B, compare *left* and *middle panels*). Co-stimulation with ISO caused detectable endocytosis in WT-Sp cells but no additional effect in spinophilin-null cells (compare left and middle images; Fig. 8C). By contrast, cells expressing mut-Sp exhibited no  $\alpha_{2A}$ AR endocytosis following 5-min clonidine stimulation alone or in combination with ISO (Fig. 8, B and C, *right panels*). All cells exhibited  $\alpha_{2A}$ AR endocytosis following 30 min of clonidine stimulation (Fig. 8D). These data underline the importance of phosphorylation of spinophilin at the Ser-94/Ser-177 sites to the  $\alpha_{2A}$ AR endocytic acceleration observed with  $\beta$ AR co-activation. We additionally utilized an assay for cAMP production to compare the canonical  $G_{\alpha_s}$ /cAMP response for endogenous  $\beta$ ARs in our spinophilin-null MEFs versus WT MEFs (matched genetic background). As shown in Fig. 8E, MEFs from the spinophilin-null line do not exhibit any deficit in ISO-stimulated cAMP response; in fact,

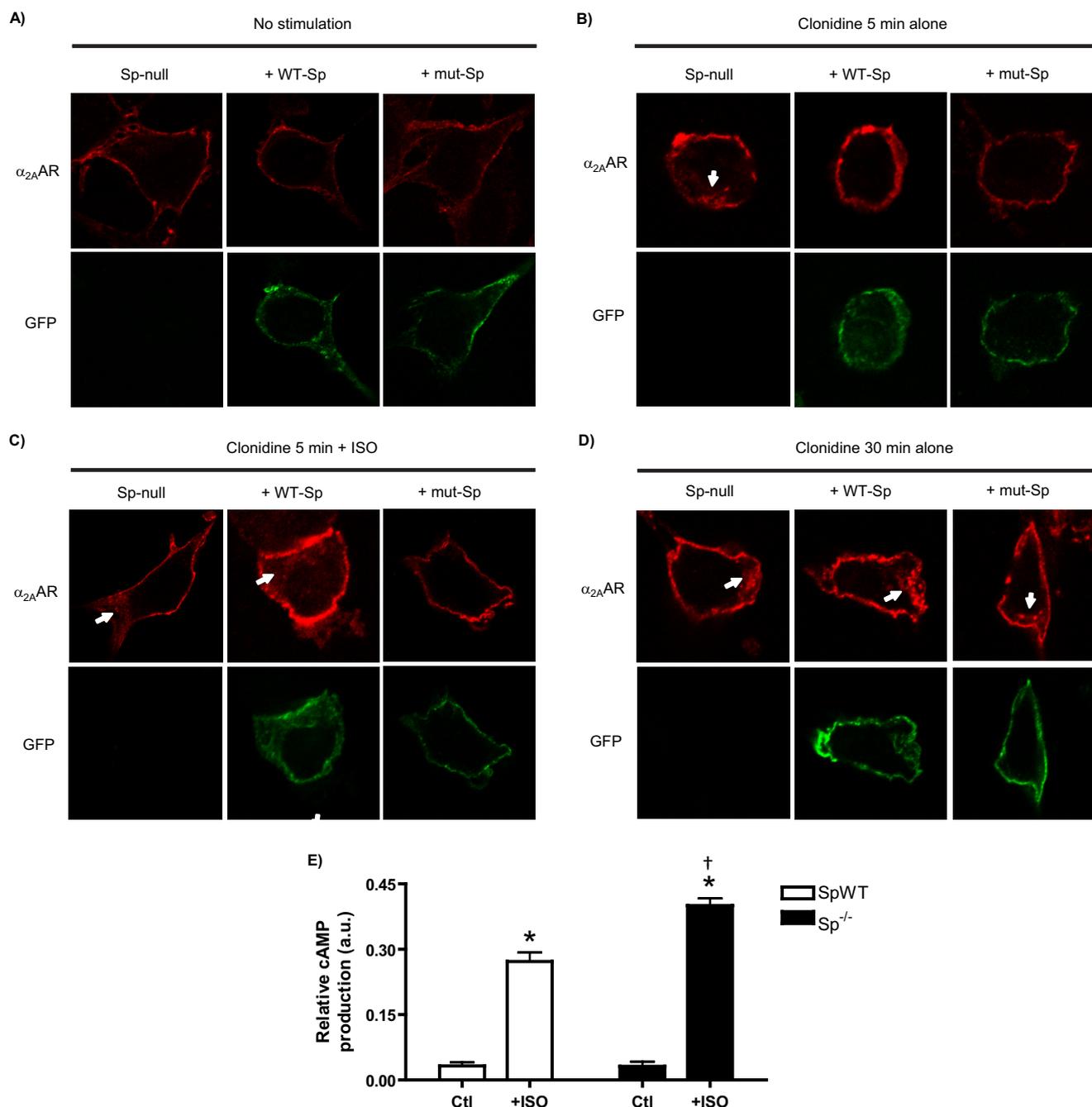
the response to ISO is slightly enhanced in the spinophilin-null cells. This result indicates that loss of spinophilin does not affect  $\beta$ AR responsiveness at the ISO concentration used throughout our study.

**Acceleration of  $\alpha_{2A}$ AR Endocytosis by  $\beta$ AR Co-activation Is Lost in Spinophilin-null Neurons**—As a final step, we sought to provide validation of our proposed mechanism relying on disruption of the  $\alpha_{2A}$ AR/spinophilin interaction in native neurons. We first characterized the kinetics of clonidine-mediated  $\alpha_{2A}$ AR endocytosis in spinophilin-null neurons, finding that endocytosis of endogenous  $\alpha_{2A}$ ARs by clonidine is accelerated in Sp<sup>-/-</sup> versus SpWT neurons (Fig. 9, A and B). Two-way ANOVA revealed significant effects of genotype and time ( $p < 0.0001$ ) and a significant genotype  $\times$  time interaction ( $p < 0.0001$ ).

We then repeated our clonidine plus ISO experiment in the Sp<sup>-/-</sup> neurons. Using the same conditions as in Fig. 4, we found no additional enhancement of clonidine-mediated  $\alpha_{2A}$ AR endocytosis by  $\beta$ AR co-activation in Sp<sup>-/-</sup> neurons (Fig. 9, A and C, SpWT included for comparison). This result indicates that spinophilin is required for  $\beta$ AR co-activation to modulate endogenous  $\alpha_{2A}$ AR endocytosis and supports our proposed mechanism for  $\beta$  to  $\alpha_{2A}$ AR cross-talk relying on  $\beta$ AR signaling to PKA and disruption of  $\alpha_{2A}$ AR/spinophilin interaction.

## DISCUSSION

The present study provides a novel example of adrenergic receptor cross-talk wherein co-activation of endogenously expressed  $\beta$  and  $\alpha_{2A}$ ARs results in accelerated  $\alpha_{2A}$ AR endocytic responses to agonist stimulation in native adrenergic neurons. Our findings support an acceleration of agonist-stimulated  $\alpha_{2A}$ AR endocytosis under  $\beta$ AR co-activating conditions, as well as in other scenarios in which  $\alpha_{2A}$ AR/spinophilin interaction is disrupted or abolished, as seen with forskolin-driven cAMP signaling or in spinophilin-null cells, respectively. Based upon the data presented here and our past findings, we have constructed a working model for PKA- and spinophilin-dependent cross-talk from  $\beta$  to  $\alpha_{2A}$ ARs affecting  $\alpha_{2A}$ AR responsiveness, which is reliant upon  $\beta$ AR- $G_{\alpha_s}$  signal transduction (Fig.



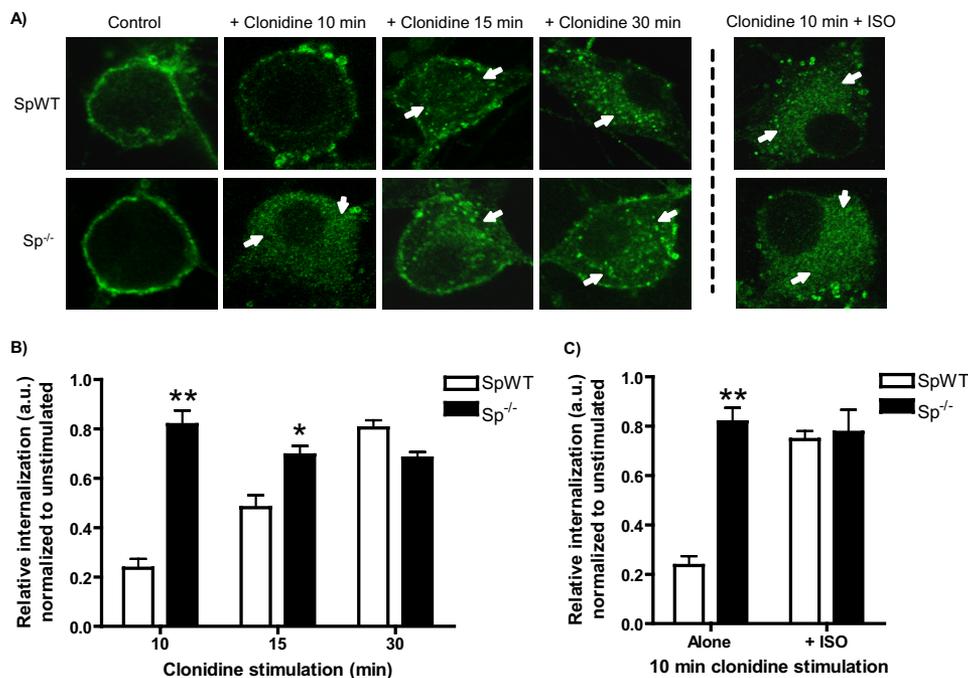
**FIGURE 8. Effects of expressing wild-type (WT-Sp) or PKA phosphorylation mutant (mut-Sp) spinophilin in spinophilin-null MEF cells stably expressing HA- $\alpha_{2A}$ ARs.** A–D, confocal images representative of numerous cells observed over  $n = 3$  independent samples. Spinophilin-null MEFs were transiently transfected with GFP-tagged spinophilin (WT-Sp or mut-Sp) as described under “Experimental Procedures.” Nonexpressing cells were also recorded as controls. MEFs were treated as indicated: no stimulation control (A), 5 min of clonidine stimulation alone (B), 5 min of clonidine + ISO (C), and 30 min of clonidine stimulation alone (D). Cells were then subjected to the antibody prelabeling method for assaying  $\alpha_{2A}$ AR endocytosis as in previous figures, with the substitution of AlexaFluor594-conjugated secondary labeling.  $\alpha_{2A}$ AR endocytosis is indicated by the appearance of intracellular punctae containing internalized receptors (arrows). E, cAMP assay in spinophilin WT (SpWT) and spinophilin-null (Sp<sup>-/-</sup>) MEFs, matched for genetic background. cAMP accumulation was measured in response to 100  $\mu$ M ISO to compare endogenous  $\beta$ AR responsiveness in SpWT versus Sp<sup>-/-</sup> cells.  $n = 3$  replicates were obtained for each group. \*,  $p < 0.001$ , +ISO versus control; †,  $p < 0.01$ , Sp<sup>-/-</sup> versus SpWT. Ctl, control.

10). Importantly, our data support the existence of this cross-talk in a native adrenergic neuronal cell type with endogenous AR expression, and we have further shown that this cross-talk is associated with stimulation by a panel of physiological and clinical  $\beta$  and  $\alpha_{2A}$ AR ligands.

**Simultaneous Activation of  $\beta$  and  $\alpha_{2A}$ ARs Accelerates  $\alpha_{2A}$ AR Endocytosis**—We have demonstrated that an acceleration of agonist-driven  $\alpha_{2A}$ AR endocytosis occurs under various condi-

tions of  $\beta$  and  $\alpha_{2A}$ AR co-activation in native adrenergic neurons. First, we showed that stimulation of neurons with the endogenous nonselective full AR agonist Epi has differential effects in the presence or absence of the  $\beta$ AR antagonist propranolol. In the presence of propranolol, a condition that allows for only  $\alpha_{2A}$ AR activation, Epi drives little or no  $\alpha_{2A}$ AR endocytosis at early time points, particularly at 5 min (Figs. 2 and 3). However, in the absence of propranolol, a condition that allows

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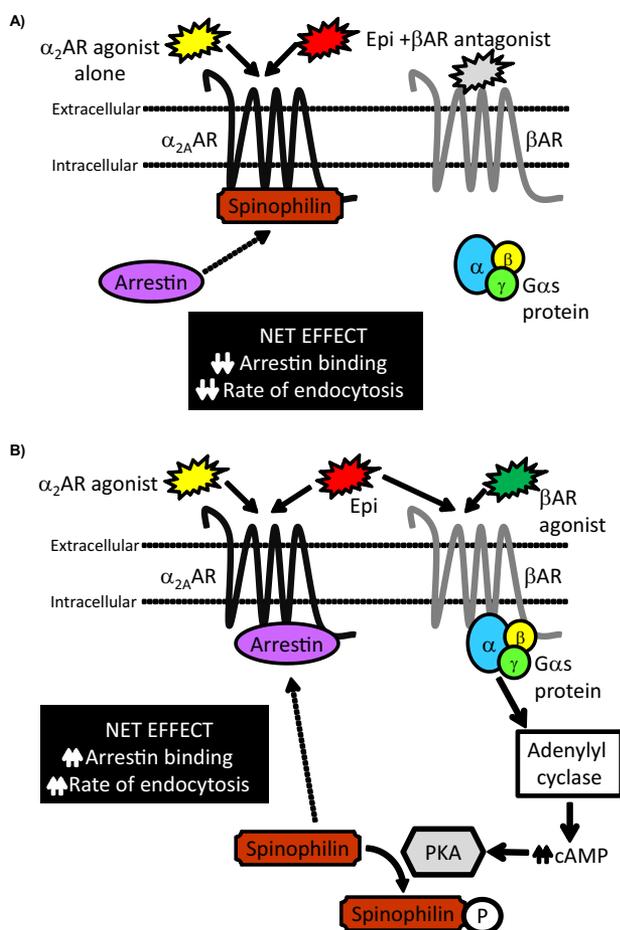
**FIGURE 9. Acceleration of endogenously expressed  $\alpha_{2A}$ AR endocytosis by  $\beta$ AR co-activation requires spinophilin.** *A*, neurons with (SpWT) and without (Sp<sup>-/-</sup>) spinophilin expression were stimulated with clonidine (1  $\mu$ M) as indicated. Neurons were subjected to  $\alpha_{2A}$ AR prelabeling method as in Fig. 1 (*B* and *C*), with endocytosis indicated by the appearance of intracellular punctae containing internalized receptors (arrows). ISO pretreatment to co-activate  $\beta$ ARs was done as in Fig. 4. *B*, quantitation showing an acceleration of clonidine-mediated  $\alpha_{2A}$ AR endocytosis basally in Sp<sup>-/-</sup> neurons, with significantly enhanced internalization at both the 10- and 15-min time points. *C*, quantitation showing that no enhancement of clonidine-mediated  $\alpha_{2A}$ AR endocytosis by  $\beta$ AR co-activation with ISO is observed in Sp<sup>-/-</sup> neurons. Confocal images are representative of at least three independent samples, and quantitation was performed over at least 12–14 neurons. \*,  $p < 0.01$ ; \*\*,  $p < 0.0001$  versus SpWT.

for Epi to simultaneously activate  $\beta$  and  $\alpha_{2A}$ ARs, endocytosis is significantly enhanced at these early time points (Fig. 3). Furthermore, the use of agonists that distinctly target  $\beta$  or  $\alpha_{2A}$ ARs reveals similar findings of cross-talk. Co-stimulation of SCG neurons with the non-subtype-selective  $\beta$ AR agonist ISO (Fig. 4, *B* and *C*) or with agonists of varying subtype selectivity for either  $\beta_1$  or  $\beta_2$ ARs (Fig. 5) together with the  $\alpha_2$ AR agonist clonidine results in an enhancement of early time point  $\alpha_{2A}$ AR endocytosis. Collectively, these results establish the phenomenon of accelerated endogenous  $\alpha_{2A}$ AR endocytic responses to agonist under conditions of  $\beta$ AR co-activation. Although work by the Hall laboratory (1) has established the occurrence of heterodimerization between  $\beta_1$  and  $\alpha_{2A}$ ARs, we believe that dimerization does not provide an adequate mechanistic explanation for our data. In fact, their study as well as our own (Fig. 4*A*) found that stimulation with  $\beta$ -agonist only is not sufficient to drive endocytosis of  $\alpha_{2A}$ ARs, and so our present findings of cross-talk are unlikely to be explained by simple physical co-internalization of  $\beta$  and  $\alpha_{2A}$ ARs.

Modulation of  $\alpha_{2A}$ AR endocytosis would be expected to have consequences for cellular  $\alpha_{2A}$ AR responsiveness. First of all, the accelerated endocytosis means a corresponding acceleration of receptor desensitization, with receptors being removed from the cell surface at a faster rate. Furthermore, we have previously shown that removal of spinophilin results in accelerated kinetics of  $\alpha_{2A}$ AR-mediated MAPK signal transduction along with accelerated endocytosis (24). Collectively, these effects will translate into a dramatically altered functional profile for the  $\alpha_{2A}$ AR under conditions of  $\beta$ AR cross-talk.

*$\beta$ AR-stimulated PKA-dependent Phosphorylation of Spinophilin Provides a Mechanistic Basis for Cross-talk with  $\alpha_{2A}$ ARs*—Our previous work has established a regulatory mechanism for  $\alpha_{2A}$ ARs involving interplay between the non-G protein receptor interacting partners spinophilin and arrestin (24, 25). We have further shown that this regulatory mechanism can be disrupted by PKA-dependent phosphorylation of spinophilin, which in turn prevents  $\alpha_{2A}$ AR/spinophilin interaction (28). The present results expand the application of this regulatory mechanism to endogenously expressed  $\alpha_{2A}$ ARs in a native adrenergic neuronal cell type, whereas our previous work was done in heterologous cell systems.

First, we have demonstrated that ligand-dependent  $\alpha_{2A}$ AR endocytosis in SCG neurons is arrestin-dependent (Fig. 1*B*). Additionally, as predicted by our established mechanistic model, the kinetics of this endocytosis are altered in the absence of spinophilin, with an acceleration of the time course observed with application of both the endogenous agonist Epi (Fig. 3) and the therapeutic partial agonist clonidine (Fig. 9, *A* and *B*). Next, we have shown that under conditions of PKA activation, either in a receptor-independent fashion by forskolin stimulation of adenylyl cyclase (Fig. 6) or by stimulation of  $G_{\alpha_s}$ -coupled  $\beta$ ARs (Figs. 4 and 5), the kinetics of agonist-driven  $\alpha_{2A}$ AR endocytosis are accelerated. In the case of  $\beta$ AR agonist-mediated acceleration of endocytosis, we have shown that the effects are blocked by inhibition of PKA (Fig. 4, *B* and *C*) and are not observed in spinophilin-null neurons (Fig. 9, *A* and *C*). In fact, in each case presented here, both PKA activation and genetic deletion of spinophilin have similar effects on  $\alpha_{2A}$ AR



**FIGURE 10. Working model for spinophilin- and PKA-dependent  $\beta$  to  $\alpha_{2A}$ AR cross-talk modulating endogenous  $\alpha_{2A}$ AR endocytosis.** *A*, when  $\alpha_{2A}$ ARs are activated alone, the spinophilin regulatory mechanism will be fully engaged, and  $\alpha_{2A}$ AR responsiveness will be determined by interplay between spinophilin and arrestin binding to the receptor. Relative to co-activating conditions, there would be a net effect of decreased arrestin binding and decreased rate of agonist-stimulated  $\alpha_{2A}$ AR endocytosis. *B*, under  $\beta$  and  $\alpha_{2A}$ AR co-activating conditions, canonical  $\beta$ AR/G $\alpha_s$  signal transduction coupled to PKA activation will result in PKA-dependent phosphorylation of spinophilin. This phosphorylation attenuates the ability of spinophilin to interact with  $\alpha_{2A}$ ARs, which would lead to a predominance of arrestin binding and a net effect of increased rate of agonist-stimulated  $\alpha_{2A}$ AR endocytosis.

responses, with PKA activation by  $\beta$ ARs having no additional effect in the spinophilin-null system. To further support our mechanistic contention, we have provided direct evidence that co-activation of  $\beta$ ARs results in a disruption of ligand-dependent  $\alpha_{2A}$ AR/spinophilin interaction and that this disruption depends critically on PKA activity (Fig. 7). Finally, our experiments utilizing expression of WT and phospho-mutant spinophilin in spinophilin-null MEFs (Fig. 8) highlight the importance of the phosphorylation state of spinophilin to its  $\alpha_{2A}$ AR regulatory function under  $\beta/\alpha_{2A}$ AR co-activating conditions.

Taken together, our present data are strongly supportive of a clear mechanistic link underlying cross-talk from  $\beta$  to  $\alpha_{2A}$ ARs. As described above, we propose a model whereby activation of  $\beta$ ARs results in a disruption of ligand-dependent  $\alpha_{2A}$ AR/spinophilin interaction (Fig. 10). This disruption occurs as a consequence of canonical  $\beta$ AR/G $\alpha_s$  signal transduction linking to activation of PKA, which in turn catalyzes a phosphorylation of spinophilin, disrupting its interaction with  $\alpha_{2A}$ ARs. The

removal of spinophilin from the regulatory scheme would lead to a predominance of  $\alpha_{2A}$ AR/arrestin interaction and, subsequently, to the observed acceleration of agonist-dependent  $\alpha_{2A}$ AR endocytic responses and, potentially, resulting effects on signal transduction. Interestingly, previous reports have suggested the existence of PKA-dependent neuronal current modulation by combinatorial  $\beta/\alpha$  AR stimulation in the CA1 region of hippocampus (9, 10), a brain region with particularly high spinophilin expression and function (18, 35).

**Potential Importance of  $\beta/\alpha_{2A}$ AR Cross-talk to Adrenergic Physiology and Pharmacology**—We believe that our present findings have a number of significant implications for clinically relevant adrenergic physiology and pharmacology. All of the present experiments have utilized clinically relevant therapeutic  $\beta$  and  $\alpha_{2A}$ AR ligands, the effects of which have been examined in a native cell model with endogenous expression of receptors and the players in the proposed mechanism of cross-talk. These drugs, including beta blockers, sympathomimetics, and other adrenergic agonists, have a wide array of clinical applications and are among the most frequently used GPCR-directed therapeutics (17, 43, 44).

Indeed, certain *in vivo* physiological responses to Epi administration are consistent with an enhancement of  $\alpha_{2A}$ AR endocytosis resulting from  $\beta/\alpha_{2A}$ AR cross-talk. As an example, ISO is known to drive a slight hypotensive response, which is not easily explained in terms of  $\beta$ AR activation (17). In light of our data, this response may be explained as a potentiation of  $\alpha_{2A}$ AR signaling drive resulting from basal adrenergic tone. Additionally, the clinical usage of  $\beta_2$ AR-selective agonists to delay preterm labor raises the possibility of developmental relevance for  $\beta/\alpha_{2A}$ AR cross-talk, especially given evidence that administration of the  $\beta_2$ AR-selective agonist terbutaline to pregnant rats affects  $\alpha_{2A}$ AR expression levels in various tissues at different developmental stages (14). This finding could now potentially be explained as a modulation of  $\alpha_{2A}$ AR expression patterns through cross-talk with  $\beta_2$ ARs affecting  $\alpha_{2A}$ AR trafficking and localization. In a long term sense, chronically reduced  $\alpha_{2A}$ AR/spinophilin interaction and corresponding enhancement of arrestin interaction would be expected to potentiate receptor down-regulation, a process that we have previously characterized as occurring in an arrestin-mediated fashion *in vivo* (34).

Another area in which our cross-talk may be relevant is ocular pharmacology. Here, the SCGs are directly involved in the neural circuitry, and  $\alpha_2$ AR agonists have therapeutic benefit in the management of intraocular pressure in glaucoma (45). The clinical application of  $\alpha_2$ AR agonists in this setting has been somewhat limited by side effects, especially sedation, resulting from activation of central  $\alpha_{2A}$ ARs. Given our present findings, it may be advisable to consider combination treatment with low doses of  $\beta$  and  $\alpha_2$ AR agonists, thereby more effectively engaging local  $\alpha_{2A}$ ARs while limiting spillover into more systemic effects. In a broader sense, our results suggest that modulation of  $\alpha_{2A}$ AR responsiveness should be considered in the mechanisms of both beta blockers, which may additionally attenuate basal  $\alpha_{2A}$ AR tone, and  $\beta$ AR agonists, which may additionally potentiate basal  $\alpha_{2A}$ AR tone.

Follow-up studies will be necessary to validate the existence of  $\beta/\alpha_{2A}$ AR cross-talk in *in vivo* physiological settings. Addi-

tionally, the relative importance of our proposed mechanism of cross-talk will vary depending on the relative expression of  $\beta$  and  $\alpha_{2A}$ AR subtypes. Also, more recently appreciated noncanonical  $\beta$ AR signaling mediated by non-G $\alpha_s$ -containing G proteins or arrestins (29) would not be expected to engage the PKA-dependent cross-talk mechanism. Furthermore, the expression of spinophilin is developmentally regulated and varies widely across different tissues (18, 19). At the cellular level, spinophilin localizes exclusively to somatodendritic neuronal compartments (18, 46, 47), precluding the involvement of our regulatory mechanism in axonal terminals. Finally, it should be noted that spinophilin is a multifunctional scaffolding protein with an ever-growing list of physical and regulatory interactions appropriately termed the “spinophilin interactome” (48), raising the possibility that our findings may involve a more complex mechanism than we have proposed here.

Nevertheless, our study has provided new and valuable insight into the physiological and pharmacological inter-relationship among the ARs. Furthermore, we have extended the application of our previous findings on regulation of  $\alpha_{2A}$ ARs by PKA and spinophilin into native adrenergic neurons with endogenous expression of all of the players involved. Integrating our past and present data, we have constructed a novel mechanism for cross-talk between  $\beta$  and  $\alpha_{2A}$ AR subtypes, a mechanism that we believe has great physiological and therapeutic importance.

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