

RESEARCH ARTICLE

# Isoproterenol Acts as a Biased Agonist of the Alpha-1A-Adrenoceptor that Selectively Activates the MAPK/ERK Pathway

Alicja J. Copik<sup>1‡b</sup>, Aleksander Baldys<sup>3‡d</sup>, Khanh Nguyen<sup>2‡e</sup>, Sunil Sahdeo<sup>1‡h</sup>, Hoangdung Ho<sup>2‡f</sup>, Alan Kosaka<sup>2</sup>, Paul J. Dietrich<sup>2</sup>, Bill Fitch<sup>2‡g</sup>, John R. Raymond<sup>3</sup>, Anthony P. D. W. Ford<sup>1‡a</sup>, Donald Button<sup>1‡c</sup>, Marcos E. Milla<sup>1\*</sup>

**1** Biochemical Pharmacology, Inflammation Discovery, Roche Palo Alto LLC, 3401 Hillview Drive, Palo Alto, CA 94304, United States of America, **2** Discovery Technologies, Roche Palo Alto LLC, 3401 Hillview Drive, Palo Alto, CA 94304, United States of America, **3** Nephrology Division, Department of Medicine, Medical University of South Carolina, and Medical and Research Services, Ralph H Johnson Veterans Affairs Medical Center, Charleston, South Carolina 29425, United States of America

\* [mmilla22@its.jni.com](mailto:mmilla22@its.jni.com)

‡a. Current address: Afferent Pharmaceuticals, 2755 Campus Dr. Suite 100, San Mateo, CA 94403, United States of America

‡b. Current address: Burnett School of Biomedical Sciences, University of Central Florida College of Medicine, 6900 Lake Nona Blvd., Orlando, FL 32827, United States of America

‡c. Current address: Acorda Therapeutics, Inc. 15 Skyline Drive, Hawthorne, NY 10532, United States of America

‡d. Current address: Simens 1275 York Ave, New York, NY 10065, United States of America

‡e. Current address: Anshin BioSolutions, 5201 Great America Pkwy, Suite 320, Santa Clara, CA 9505, United States of America

‡f. Current address: Genentech Inc., 1 DNA Way, South San Francisco, CA 94080, United States of America

‡g. Current address: Department of Anesthesia, Stanford University School of Medicine, 300 Pasteur Drive, Stanford, California 94305, United States of America

‡h. Current address: Janssen Pharmaceuticals, 3210 Merryfield Row, San Diego, CA 92121, United States of America



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## Abstract

The  $\alpha_{1A}$ -AR is thought to couple predominantly to the  $G\alpha_q$ /PLC pathway and lead to phosphoinositide hydrolysis and calcium mobilization, although certain agonists acting at this receptor have been reported to trigger activation of arachidonic acid formation and MAPK pathways. For several G protein-coupled receptors (GPCRs) agonists can manifest a bias for activation of particular effector signaling output, *i.e.* not all agonists of a given GPCR generate responses through utilization of the same signaling cascade(s). Previous work with  $G\alpha_q$  coupling-defective variants of  $\alpha_{1A}$ -AR, as well as a combination of  $Ca^{2+}$  channel blockers, uncovered cross-talk between  $\alpha_{1A}$ -AR and  $\beta_2$ -AR that leads to potentiation of a  $G\alpha_q$ -independent signaling cascade in response to  $\alpha_{1A}$ -AR activation. We hypothesized that molecules exist that act as biased agonists to selectively activate this pathway. In this report, isoproterenol (Iso), typically viewed as  $\beta$ -AR-selective agonist, was examined with respect to activation of  $\alpha_{1A}$ -AR.  $\alpha_{1A}$ -AR selective antagonists were used to specifically block Iso evoked signaling in different cellular backgrounds and confirm its action at  $\alpha_{1A}$ -AR. Iso induced signaling at  $\alpha_{1A}$ -AR was further interrogated by probing steps along the  $G\alpha_q$ /PLC,  $G\alpha_s$  and MAPK/ERK pathways. In HEK-293/EBNA cells transiently transduced

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with  $\alpha_{1A}$ -AR, and CHO- $\alpha_{1A}$ -AR stable cells, Iso evoked low potency ERK activity as well as  $Ca^{2+}$  mobilization that could be blocked by  $\alpha_{1A}$ -AR selective antagonists. The kinetics of Iso induced  $Ca^{2+}$  transients differed from typical  $G\alpha_q$ -mediated  $Ca^{2+}$  mobilization, lacking both the fast  $IP_3R$  mediated response and the sustained phase of  $Ca^{2+}$  re-entry. Moreover, no inositol phosphate (IP) accumulation could be detected in either cell line after stimulation with Iso, but activation was accompanied by receptor internalization. Data are presented that indicate that Iso represents a novel type of  $\alpha_{1A}$ -AR partial agonist with signaling bias toward MAPK/ERK signaling cascade that is likely independent of coupling to  $G\alpha_q$ .

## Introduction

Adrenoceptors (AR) belong to the large family of G protein-coupled receptors (GPCRs), also known as seven-transmembrane receptors (7-TMRs), which transduce extracellular stimuli into cellular responses. Adrenoceptors respond to the endogenous catecholamines norepinephrine and epinephrine, and mediate critical functions of the central and peripheral nervous systems. They were initially subdivided into two main types,  $\alpha$ - and  $\beta$ -, based on the rank orders of potency of norepinephrine, epinephrine and Iso as well as the physiological outcome of the response (contraction vs. relaxation) [1,2]. With the discovery of new synthetic and more selective ligands, new receptor subtypes have been identified within each of the two groups.  $\beta$ -AR now includes  $\beta_1$ ,  $\beta_2$ , and  $\beta_3$ -subtypes while  $\alpha$ - is subdivided into  $\alpha_1$ - and  $\alpha_2$ - [3–6]. Introduction of molecular cloning confirmed the existence of these genetically and pharmacologically distinct subtypes of  $\beta$ -AR and allowed a final classification of the  $\alpha_1$ - subgroup into  $\alpha_{1A}$ -,  $\alpha_{1B}$ - and  $\alpha_{1D}$ - [7] and  $\alpha_2$ - into  $\alpha_{2A}$ -,  $\alpha_{2B}$ - and  $\alpha_{2C}$ - [8] ARs.

Iso has been one of the most commonly used agonists for differentiation of  $\alpha$ - and  $\beta$ -ARs. At low concentrations (1–100 nM) Iso causes smooth muscle relaxation through its action at  $\beta$ -ARs, a property that prompted its introduction for the treatment of asthma, chronic bronchitis and emphysema. Even though very selective for the  $\beta$ -AR class, several groups reported that Iso, at high doses (4  $\mu$ M and higher) also evoked  $\alpha$ -mediated responses leading to the contraction of smooth muscles of rabbit aorta and posterior vena cava as well as of rat vas deferens [9–14]. High doses of Iso were also shown to increase blood pressure in rabbits [1], and cause arterial hypertension in anesthetized cats and dogs [15,16]. The involvement of  $\alpha$ -AR in mediating the physiological effects of Iso was implicated in these and other studies by the ability of antagonists dibenamine, phenoxybenzamine or phentolamine to block responses [11,14,15].

More recently, observations of  $Ca^{2+}$  mobilization responses in rat parotid acinar cells in response to high concentrations of Iso (1–200  $\mu$ M) led to a long running debate of how  $Ca^{2+}$  is involved in cAMP-mediated amylase release, and whether this response is mediated solely by  $\beta$ -AR [17,18]. Subsequent studies in rat parotid acinar cell preparations revealed prazosin sensitivity for the Iso-mediated  $Ca^{2+}$  mobilization response, indicating Iso activation of  $\alpha$ -AR [19,20] although the subtype involved was not identified. Thus, although compelling historical precedents exist for Iso agonism at  $\alpha_1$ -ARs, no studies focused on the signaling mechanisms or  $\alpha_1$ -AR receptor subtypes involved. The use of Iso in basic and clinical studies would clearly benefit from greater mechanistic understanding of Iso-mediated signaling via  $\alpha$ -ARs.

Iso binds with relatively high affinity to all three  $\beta$ -AR subtypes ( $K_i$ : 0.22  $\mu$ M at  $\beta_1$ -, 0.46  $\mu$ M at  $\beta_2$ - and 1.6  $\mu$ M at  $\beta_3$ -AR in presence of GTP; 0.02  $\mu$ M at  $\beta_2$ -AR in its absence [21,22]), acting as a high intrinsic efficacy (full) agonist. Thus, Iso-bound  $\beta$ -AR couples to  $G\alpha_s$  leading to stimulation of adenylyl cyclase, cAMP production, and phosphorylation of protein kinase A

(PKA). In addition to the activation of this “canonical” pathway, Iso is highly efficacious at inducing  $\beta_2$ -AR mediated signaling via G-protein coupled receptor kinases-(GRK) and  $\beta$ -arrestin. This leads to receptor phosphorylation, recruitment of c-Src and activation of MAPK signaling pathways among others (reviewed in [23]).

It has also been shown that Iso at higher doses (above 100 nM) induces G protein-independent signaling at  $\beta_2$ -ARs [24]. In mouse embryonic fibroblasts (MEFs), stimulation of  $\beta_2$ -AR by Iso results in a biphasic concentration-dependent increase in extracellular signal-regulated protein kinase (ERK) activity. The high potency phase was found to be dependent on  $G\alpha_s$  while the low potency phase was not. Sun *et al.* combined the use of MEF cells from various knock-out mice with biophysical studies testing the interaction of the purified components ( $\beta$ -AR and Src) to show that the low potency phase reflects direct interaction and activation of Src by the Iso-activated  $\beta_2$ -AR. The high concentrations of Iso used in this and several other studies [18,25,26] may also lead to activation of  $\alpha$ -ARs with signaling consequences that are not well-characterized at the molecular level. Thus, there is a great need to explore the mechanism of Iso signaling through  $\alpha$ -ARs.

We investigated the Iso initiated signaling in transiently transduced HEK-293/EBNA cells expressing quasi-physiological levels of  $\alpha_{1A}$ -AR. Since HEK-293 cells endogenously express  $\beta_2$ -AR, this system gave us the ability to monitor Iso activation of several cellular events in the presence and absence of  $\alpha_{1A}$ -AR [27]. In untransduced cells, we observed Iso-induced monophasic cAMP and ERK activation as well as  $Ca^{2+}$  mobilization with efficacy within the range expected for this agonist acting at  $\beta_2$ -AR. In contrast, in cells transiently transduced with  $\alpha_{1A}$ -AR, Iso evoked biphasic concentration-dependent activation of ERK activity as well as  $Ca^{2+}$  mobilization. The high potency phase of the concentration-effect relation was sensitive to a  $\beta_2$ -selective antagonist, while the low potency phase was blocked by application of  $\alpha_{1A}$ -AR-selective antagonists. Iso was found to be an agonist at recombinantly expressed  $\alpha_{1A}$ -AR subtype in a manner which recruits signaling mechanisms distinct from those seen with NE or selective  $\alpha_{1A}$ -AR agonists. Our data suggest that Iso induces a  $\alpha_{1A}$ -AR-mediated signaling mode biased toward MAPK/ERK and likely independent  $G\alpha_q$ . We also show evidence indicating that this signaling mode involves receptor internalization.

## Materials and Methods

### Materials and reagents

Reagents were purchased from the following commercial suppliers: A-61603, xamoterol, procaterol and fenoterol from Tocris (Ellisville, MO); norepinephrine, prazosin, phentolamine, ICI 118551, atenolol, salbutamol, propranolol, probenecid, BSA and glucose from Sigma-Aldrich (St. Louis, MO); isoproterenol and oxymetazoline from MP Biomedicals (Irvine, CA), sodium butyrate from Alfa Aesar (Ward Hill, MA); HEPES, HBSS, 3-isobutyl-1-methylxanthine (IBMX) from Axxora (San Diego, CA) and Fluo3-AM from Invitrogen (Carlsbad, CA). [ $^3H$ ]-Prazosin and [ $^{125}I$ ]-CYP were purchased from Perkin Elmer (Boston, MA). Crude membranes were prepared from transduced or transfected HEK-293/EBNA cells as published [28] with one modification. Cells were resuspended in the lysis buffer without sucrose and broken using a Polytron homogenizer (3  $\times$  30 s pulses).

### Cell culture and transient transductions

Cell-based experiments were performed using suspension-adapted HEK-293/EBNA cells [27], HEK293 or CHO cells. HEK-293/EBNA cells were grown in Free Style 293 serum free medium (Invitrogen) and maintained in an incubator at 37°C, 7% CO<sub>2</sub> atmosphere with constant shaking at 150 rpm. Prior to experiments, cells were transduced with baculovirus strains designed

for transient expression of  $\alpha_{1A}$ -AR or aldehyde oxidase (negative control). This was performed by incubating cells with virus (MOI 100–150) for 3 to 4 h, followed by exchange into fresh serum free growth medium supplemented with 4 mM sodium butyrate (NaBu). Cells were grown for another 14 to 18 h and then examined for agonist-evoked responses in transient  $\text{Ca}^{2+}$  release, IP accumulation or pERK activation assays. The surface receptor expression density was determined by flow cytometry via immunofluorescence labeling of an N-terminal HA epitope tag on the receptor and radioligand binding in partially purified membranes.

HEK293 cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen Gibco, Carlsbad, CA) at 37°C in a humidified atmosphere of 95% air and 5%  $\text{CO}_2$ .

### $\text{Ca}^{2+}$ transient response assay

Cells were resuspended in Hank's balanced salt solution (Invitrogen) supplemented with 2 mM  $\text{CaCl}_2$ , 10 mM HEPES pH 7.4, 2.5 mM probenecid, plus 1g/L each of glucose and bovine serum albumin (BSA), and seeded in poly-D-lysine coated 96-well black plates with transparent bottom (Costar) at a density of 50,000 cells/0.1 mL per well. Cells were then incubated for 1 h at 37°C with an additional 0.1 mL of buffer containing 4  $\mu\text{M}$  fluo3-AM that was diluted from a stock solution containing 1 mM fluo3-AM dye in DMSO with 10% pluronic acid. After dye loading incubation, plates were washed twice with 100  $\mu\text{L}$  of buffer and refilled with 100  $\mu\text{L}$  of assay buffer. In experiments testing the effect of antagonists, 25  $\mu\text{L}$  of buffer from wells of washed cells were replaced with 25  $\mu\text{L}$  of vehicle or 4 $\times$  antagonist solution. Pre-incubation of ligand with dye-loaded cells proceeded for 5–30 minutes immediately prior to measurements of agonist-evoked responses. Agonist-evoked  $\text{Ca}^{2+}$  mobilization responses of cell populations were monitored at room temperature simultaneously in all wells of the assay plate using the plate-imaging fluorometric reader FLIPR (MDS Analytical Technologies, Sunnyvale, CA). Measurements consisted of recording baseline fluorescence signal for 10 seconds followed by addition of the test substance and 1–2 minute readings of  $\text{Ca}^{2+}$  transient responses as reported by changes in  $\text{Ca}^{2+}$  dye fluorescence (excitation 488 nm, emission 510–570 nm). The amplitudes of  $\text{Ca}^{2+}$  transient responses are reported as  $\Delta F/F_0$ , or fold-change in  $\text{Ca}^{2+}$  dye fluorescence relative to the baseline signal  $\Delta F/F_0 = (F - F_0)/F_0 + 1$  (where  $\Delta F$  is maximal fluorescence intensity observed following agonist application,  $F_0$  = baseline fluorescence, or average intensity measured over the 10 s interval prior to agonist application). Image acquisition rates were varied from 1 Hz during the first 100 s of measurements to 0.5 Hz for the remaining time of the recording.

### IP and cAMP Accumulation Assays

Virally transduced HEK-293/EBNA cells were washed by centrifugation at 150  $\times$  g for 8 min, before resuspension in assay buffer (20 mM HEPES, 10 mM glucose, 1.8 mM  $\text{CaCl}_2$ , 0.5 mM  $\text{MgSO}_4$ , in HBSS 1X buffer, 50 mM LiCl) at  $10^8$  cells/mL. Cells were placed in 384-well black polystyrene plates (Costar 3912) at 10  $\mu\text{L}$ /well, and incubated with 10  $\mu\text{L}$  of antagonist or vehicle at room temperature. After 10–20 minutes, 10  $\mu\text{L}$  of agonist was added followed by incubation for 5 to 30 minutes. Stimulation was stopped by addition of lysis buffer. Second messenger levels were determined using a homogeneous immunoassay method with time-resolved FRET detection (IP-One HTRF, Cisbio International) according to protocols provided by the supplier. Sample fluorescence was measured with a Nanoscan plate reader (IOM, Berlin). Each data point represents the average of quadruplicate determinations and each experiment was performed at least two times independently.

## Detection of IP<sub>1</sub>, IP<sub>2</sub> and IP<sub>3</sub> inositol phosphates

Virally transduced HEK-293/EBNA cells were resuspended in growth medium and seeded in poly-D-lysine coated 96-well black plates at a density of 100,000 cells/0.1 mL per well. Cells were allowed to attach for 2 hours at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. After that, the medium was replaced with agonist solution in assay buffer (20 mM HEPES, 10 mM glucose, 1.8 mM CaCl<sub>2</sub>, 0.5 mM MgSO<sub>4</sub>, in HBSS 1X buffer, 50 mM LiCl) and cells were incubated at 37°C for 30 min. Stimulation was stopped by exchange of assay buffer with lysis buffer (100 μL 0.1N HCl/MeOH), and lysis proceeded for 30 min at 4°C. Cellular levels of inositol phosphates were determined using a Thermo LC-MS detection system following a published protocol [29]. Chromatography was performed employing a BioBasic column AX (50 × 2.1 mm, Thermo). The mobile phase consisted of solvent A: 10 mM ammonium acetate, pH 6 in 30/70 acetonitrile/water, and solvent B: 1 mM ammonium acetate, pH 11 in 30/70 acetonitrile/water. A gradient solvent system was used starting with 0% solvent B, and analytes were eluted by increasing solvent B to 100% over 3 min. Each data point represents the average of triplicate determinations and each experiment was performed at least three times independently.

## MAPK activation assays

MAP kinase activation was monitored using the bead proximity-based AlphaScreen assay to detect phosphorylated ERK (*SureFire* p-ERK, PerkinElmer, Boston, MA). Virally transduced HEK-293/EBNA cells were seeded in 96 well plates with serum-free growth medium at 50,000 cells/well, followed by incubation for six hours at 37°C in 7% CO<sub>2</sub>. Cells were then stimulated with vehicle or agonist for 5 min at 37°C in 7% CO<sub>2</sub>. Agonist response was terminated following rapid removal of the medium by adding 50 μL per well of *SureFire* lysis buffer, followed by incubation of cells for 10 min at room temperature. Plates were stored at –80 C prior to analysis for p-ERK levels. Processing of cells for phospho-ERK detection was performed using an AlphaScreen *SureFire* p-ERK assay kit (PerkinElmer, Boston, MA) according to specifications from the manufacturer. Briefly, 10 μL of lysate were transferred to 384 well plates (OptiPlate) and combined with 17 μL of *SureFire* buffer containing AlphaScreen beads. Plates were incubated for 2 h at room temperature and the fluorescence signal was recorded using a Fusion plate reader (PerkinElmer), adjusted to standard AlphaScreen settings.

## Radioligand binding studies

Ligand binding was monitored using membranes prepared from NaBu-treated untransduced HEK-293/EBNA cells or cells virally transduced with the α<sub>1A</sub>-AR construct. [<sup>3</sup>H]-prazosin and [<sup>125</sup>I]-CYP were used as radioligands and 100 μM phentolamine or 10 μM propranolol as respective non-radioactive competitors to determine non-specific binding. For competition binding assays, unlabeled ligands (A-61603, NE, and Iso) were used to compete [<sup>3</sup>H]-prazosin or [<sup>125</sup>I]-CYP binding. Reactions were set up as described previously [27]. Affinity (pK<sub>i</sub>) values were calculated from IC<sub>50</sub> values using the Cheng-Prusoff correction [30].

## Immunofluorescence staining and confocal microscopy

HEK293 cells were transiently co-transfected with FLAG-tagged α<sub>1A</sub> – AR and one of the following GFP-tagged proteins: Rab5 (Rab5 Q79L) variant, Rab11 (Rab11 S25N) variant, β-arrestin-1 or β-arrestin-2. Rab5 and Rab11 were kindly provided by Dr. Marino Zerial (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany). Following serum-deprivation for 24h, cells were stimulated with 1 μM A-61603 or 1 mM ISO for 2h.



After treatment, cells were fixed with freshly prepared 3.7% paraformaldehyde in PBS for 15 min at room temperature. Subsequently, cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 5 min, followed by nonspecific binding site blocking with 3% normal serum (Santa Cruz Biotechnology) in PBS for 1 h. Incubation with Alexa Fluor-568 conjugated anti-FLAG antibodies in blocking solution was done as directed by the manufacturer. Confocal microscopy was performed using a Zeiss LSM 510 META laser scanning microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a 60X objective, using the following laser wavelengths: excitation at 488 nm and emission at 505–530 nm; excitation at 543 nm and emission at 560–615 nm.

## Reversible biotinylation of Cell Surface Proteins

HEK293 cells transiently transfected with  $\alpha_{1A}$  – AR were serum deprived for 24h prior to treatments. Cells were washed with ice-cold PBS and incubated with 0.5 mg/mL of cell-impermeable sulfo-NHS-biotin (Pierce) for 30 min at 4°C to label surface proteins, followed by washing with 15 mM glycine to quench excess, unreacted biotin. Cells were further washed with PBS and incubated in serum-free medium at 37°C for 1 h, then treated with vehicle, 1  $\mu$ M A-61603 or 1 mM ISO for 5, 30, or 60 min. After treatment, cells were rinsed briefly with ice-cold PBS, and either collected following stripping of cell surface biotin (intracellular receptors), or collected without biotin stripping (total cell surface and intracellular receptors). Stripping of cell surface biotinylated receptors was performed at 4°C by washing cells three times for 5 min each with ice-cold GSH cleavage buffer (50 mM GSH, 75 mM NaCl, 1mM EDTA, 1% BSA, 0.075 N NaOH). Cellular proteins were extracted with Triton lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, and 5 mM EDTA] supplemented with protease inhibitor cocktail III (EMD Calbiochem, San Diego, CA), 1 mM PMSF, and phosphatase inhibitors (HALT phosphatase inhibitor cocktail, Pierce). Equal amounts of proteins (0.5 mg) were precleared by incubation for 30 min at 4°C with 30  $\mu$ L of protein A/G Agarose beads (Santa Cruz Biotechnology). After brief centrifugation, supernatants were removed and incubated overnight at 4°C with 50  $\mu$ L of streptavidin-agarose beads (Novagen, Madison, WI). Samples were then centrifuged and washed three times with 1 mL of Triton lysis buffer. Proteins were eluted from the beads using Laemmli sample buffer, followed by analysis via SDS-PAGE and Western blotting.

## Data Analysis

Experiments were carried out in independent replicates (n indicated in figure legends). Graphs shown reflect either pooled or representative data (see figure legends). For concentration-response analysis, results from  $Ca^{2+}$  mobilization experiments were plotted as  $\Delta F/F_0$  vs. agonist concentration and fit to a sigmoidal concentration-response equation using the GraphPad Prism software package. In cases where biphasic concentration-response curves were observed data were fit to a two-site concentration-response model:

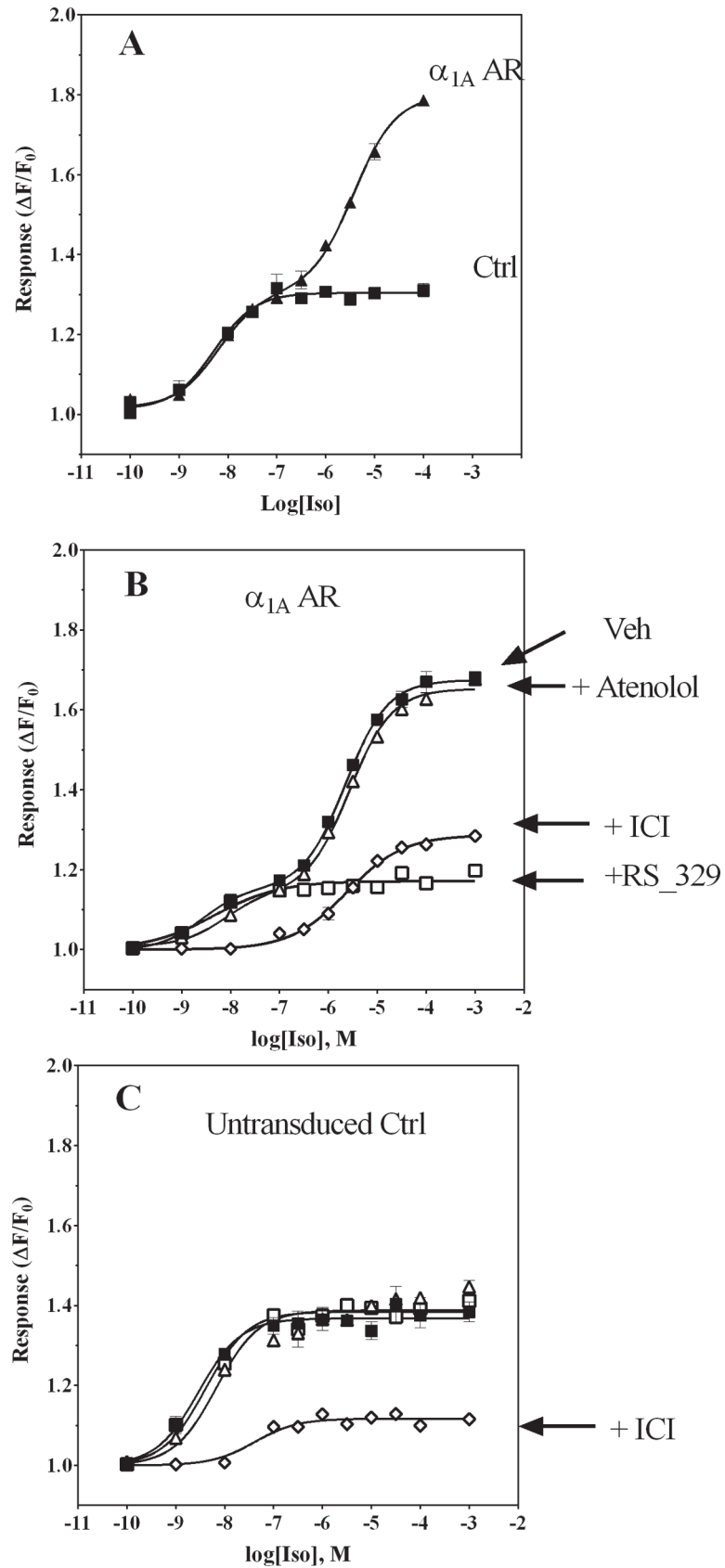
$$F(X) = R_0 + (R_{max} - R_0) [Fr_1 / (1 + 10^{X - \log EC_{50,1}}) + (1 - Fr_1) / (1 + 10^{X - \log EC_{50,2}})] \quad (1),$$

where X is the logarithm of the agonist concentration,  $R_0$  and  $R_{max}$  are respectively the response minimum and maximum;  $Fr_1$  is the response fraction attributable to receptor characterized by half effective concentration of the first response phase ( $EC_{50,1}$ ), with the remainder response attributable to the second half effective concentration ( $EC_{50,2}$ ). For radioligand binding experiments,  $B_{max}$  values were calculated from maximum binding using the equation  $B_{max} = (SB \times (IC_{50} + [L]))/[L]$ , where SB is the specific binding expressed as fmol per mg of membrane protein, and [L] is the ligand concentration.

## Results

### Iso induces biphasic concentration-response behavior in Ca<sup>2+</sup> mobilization in $\alpha_{1A}$ -AR transduced HEK-293/EBNA cells

In baculovirus-transduced HEK-293/EBNA cells homogeneously expressing low levels of  $\alpha_{1A}$ -AR close to the ones observed in primary cells ( $\sim 400$  fmol/mg protein, [27]), the  $\beta$ -AR selective agonist Iso evoked transient responses in Ca<sup>2+</sup> mobilization. A plot of the peak amplitude of the Ca<sup>2+</sup> transient, *i.e.* maximal  $\Delta F/F_0$ , as a function of agonist concentration yielded a biphasic concentration-response curve (Fig. 1A). This suggested the presence of two distinct mechanisms mediating the observed Ca<sup>2+</sup> response. The half-maximal amplitude of the high potency phase was observed at 4.0 nM Iso, whereas the half-maximal amplitude of the lower potency phase occurred at 2.6  $\mu$ M Iso (Table 1). Since HEK-293/EBNA cells are known to express  $\beta_2$ -AR endogenously ( $\sim 100$ – $300$  fmol/mg protein [27,31]), we examined if the parental cells responded to Iso in a similar fashion. Application of Iso to HEK-293/EBNA cells transduced with negative control vector (aldehyde oxidase) resulted in monophasic concentration-dependent Ca<sup>2+</sup> mobilization with  $EC_{50} = 4 \pm 1$  nM (Fig. 1A, Table 1), equivalent to the  $EC_{50}$  determined for the high potency phase of the biphasic dose-response curve in  $\alpha_{1A}$ -AR\_HEK-293/EBNA cells. Furthermore, the maximal response ( $\Delta F/F_0 = 1.3$ ) matched that of the first, high potency phase observed in  $\alpha_{1A}$ -AR-expressing cells, derived from fitting data to a biphasic concentration-response equation ( $\Delta F/F_0 = 1.3$ ). This finding indicates that Iso occupancy of endogenous  $\beta_2$ -AR leads to a small magnitude Ca<sup>2+</sup> transient response in untransduced HEK-293/EBNA cells and potentially in the  $\alpha_{1A}$ -AR-transduced ones. Since the Iso  $EC_{50}$  in non-transduced cells corresponds to the higher potency phase of the biphasic Iso response observed in  $\alpha_{1A}$ -AR\_HEK-293/EBNA cells, we used receptor-selective antagonists to further characterize the observed Ca<sup>2+</sup> response. The lower potency phase of response to Iso was sensitive to the highly selective  $\alpha_{1A}$ -AR antagonist, RS100329 ( $pK_B = 9.6$  for  $\alpha_{1A}$ -AR) (10 nM, Fig. 1B) [32]. The magnitude of the Iso response in presence of RS100329 ( $\Delta F/F_0 = 1.2$ ) matched the magnitude of the high potency response phase observed in vehicle pretreated cells, as determined using the two-site model ( $\Delta F/F_0 = 1.2$ ). Consistent with this, the potency of Iso in the presence of RS100329 ( $EC_{50} = 4.6$  nM) corresponded well to the potency calculated for the high potency phase of biphasic dose-response curves in cells pretreated with vehicle ( $EC_{50} = 4.0$  nM, Table 1). This finding indicates virtually complete blocking of the low potency phase by RS100329 with no effect on the high potency phase. On the other hand, pretreatment of  $\alpha_{1A}$ -AR\_HEK-293/EBNA cells with the  $\beta_2$ -AR selective antagonist ICI 118,551 (10 nM) resulted in monophasic concentration-response to Iso (Fig. 1B). The calculated potency of Iso in the presence of this  $\beta_2$ -AR selective antagonist ( $EC_{50} = 2.4$   $\mu$ M) matched the  $EC_{50}$  of 2.6  $\mu$ M determined for the low potency phase of the biphasic response to Iso in vehicle-treated cells. Interestingly, the maximal response amplitude for Iso in the presence of ICI 118,551 ( $\Delta F/F_0 = 1.3$ , estimated using a monophasic concentration-response model) was lower than the amplitude of the lower potency phase observed in vehicle treated cells ( $\Delta F/F_0 \sim 1.5$ , estimated using a biphasic concentration-response model). This suggests some level of synergy when Iso simultaneously occupies  $\alpha_{1A}$ -AR and  $\beta_2$ -AR. The  $\beta_1$ -AR selective antagonist atenolol (1  $\mu$ M) had no effect on the Iso concentration-response relationship (Fig. 1B). In untransduced HEK-293/EBNA cells, response to Iso was blocked by 10 nM ICI118551, a  $\beta_2$ -selective antagonist, but not by the  $\alpha_{1A}$ -AR-selective antagonist RS100329 (Fig. 1C). Thus, the biphasic Ca<sup>2+</sup> transient concentration-response relationship observed with Iso seems to require co-expression of both the  $\alpha_{1A}$ - and  $\beta_2$ -adrenoceptors.





**Figure 1. Biphasic concentration-response relationship for Iso-mediated Ca<sup>2+</sup> responses in α<sub>1A</sub>-AR transduced HEK-293/EBNA cells.** Ca<sup>2+</sup> transient responses (expressed as ΔF/F<sub>0</sub>) were measured as a function of Iso concentration in fluo3-loaded cells by fluorometric plate imaging (FLIPR). HEK-293/EBNA cells were first exposed for 3–4 h to baculovirus encoding α<sub>1A</sub>-AR or an irrelevant negative control protein (aldehyde oxidase), then cultured in fresh medium containing 4 mM NaBu for 18 h prior to use in experiments as described in [Materials and Methods](#). **Panel A:** Control cell (■) Ca<sup>2+</sup> transient responses exhibited a monophasic increase in amplitude, whereas responses in cells transiently expressing α<sub>1A</sub>-AR (▲) revealed a biphasic concentration-response relationship, with large response amplitude at high Iso concentrations. **Panel B:** Blockade of Iso responses by pre-treatment of cells with antagonists selective for β-ARs or α<sub>1A</sub>-ARs reveals roles for both receptors in mediating Ca<sup>2+</sup> transient responses in HEK-293/EBNA cells transiently expressing α<sub>1A</sub>-AR. Although the β<sub>2</sub>-AR-selective antagonist ICI 118551 at 10 nM (◊) abolished responses to low Iso concentrations, responses to high Iso concentrations were attenuated by only ~ 50%. The α<sub>1A</sub>-AR-selective antagonist RS100329 (□) blocked only responses to high Iso concentrations. Neither phase of the concentration-response profile was affected by the β<sub>1</sub>-AR-selective antagonist atenolol (1 μM, Δ). Vehicle-treated cell responses represented by ■. **Panel C:** In non-transduced control cells, only the β<sub>2</sub>-AR-selective antagonist ICI 118551 at 10 nM (◊) was effective at blocking responses to Iso: both α<sub>1A</sub>-AR-selective RS100329 (10 nM, □) and β<sub>1</sub>-AR-selective atenolol (1 μM, Δ) were ineffective. Each data point represents an average of duplicate determinations; results shown are representative of experiments repeated at least 3 times.

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### Iso induces Ca<sup>2+</sup>-mobilization in α<sub>1A</sub>-AR transduced HEK-293/EBNA cells with atypical, slower kinetics

It has been shown previously that the kinetics of intracellular Ca<sup>2+</sup> accumulation mediated by α vs. β<sub>2</sub>-AR are very different, due to the distinct sources of Ca<sup>2+</sup> involved with each pathway downstream from the receptor. The Gα<sub>q</sub>-mediated α<sub>1</sub>-AR transient is very fast in onset relative to the Gα<sub>s</sub>-mediated β<sub>2</sub>-AR transient [31,33–35]. To further dissect Iso-mediated contributions to observed transients, we analyzed the kinetics of Ca<sup>2+</sup> responses to Iso and norepinephrine (NE) in α<sub>1A</sub>-AR\_HEK-293/EBNA transduced and untransduced cells ([Fig. 2](#)). Stimulation of α<sub>1A</sub>-AR\_HEK-293/EBNA cells with 100 μM Iso (a concentration that produces high agonist

**Table 1. Pharmacological parameters determined from IP and cAMP accumulation, Ca<sup>2+</sup> mobilization and p-ERK1/2 assays performed with transiently transduced HEK-293/EBNA cells.**

Cell line	Agonist	K <sub>i</sub> (μM)	EC <sub>50</sub> IP (μM)	EC <sub>50</sub> Ca <sup>2+</sup> (μM)	EC <sub>50</sub> cAMP (μM)	EC <sub>50</sub> p-ERK (μM)	
α <sub>1A</sub> -AR HEK293/EBNA	NE	<sup>1</sup> 3.1	<sup>1</sup> 0.90 ± 0.20	<sup>1</sup> 0.070 ± 0.010	<sup>2</sup> 0.67 ± 0.04	0.09 ± 0.04	
	A-61603	<sup>1</sup> 0.13	<sup>1</sup> 0.040 ± 0.020	<sup>1</sup> 0.003 ± 0.001	<sup>2</sup> 0.019 ± 0.005	0.0008 ± 0.0003	
	Iso	1	<sup>5</sup> 0.44	<sup>3</sup> BQL	<sup>4</sup> 0.004 ± 0.002	0.021 ± 0.020	<sup>4</sup> 0.005 ± 0.004
		2	93	BQL	<sup>4</sup> 2.6 ± 2.0	BQL	<sup>4</sup> 6.0 ± 2.0
	NE	<sup>1a</sup> 34	BQL	<sup>1</sup> 0.50 ± 0.10	4.9 ± 0.04	<sup>6</sup> ND	
HEK293/EBNA	A-61603	<sup>1a</sup> 24	BQL	<sup>1</sup> 0.003 ± 0.001	BQL	BQL	
	Iso	<sup>5</sup> 0.45	BQL	0.004 ± 0.002	0.026 ± 0.020	0.001 ± 0.001	

Data were fitted to a sigmoidal dose-response or two-site concentration-response model using the GraphPad Prism software package to determine EC<sub>50</sub> values, expressed as mean ± standard error.

<sup>1</sup>[27]

<sup>1a</sup> [<sup>125</sup>I]-CYP used as radioligand

<sup>2</sup>Values determined in assays performed with HEK-293/EBNA cells not treated with NaBu and transduced with baculovirus construct yielding high pmol level expression of α<sub>1A</sub>-AR

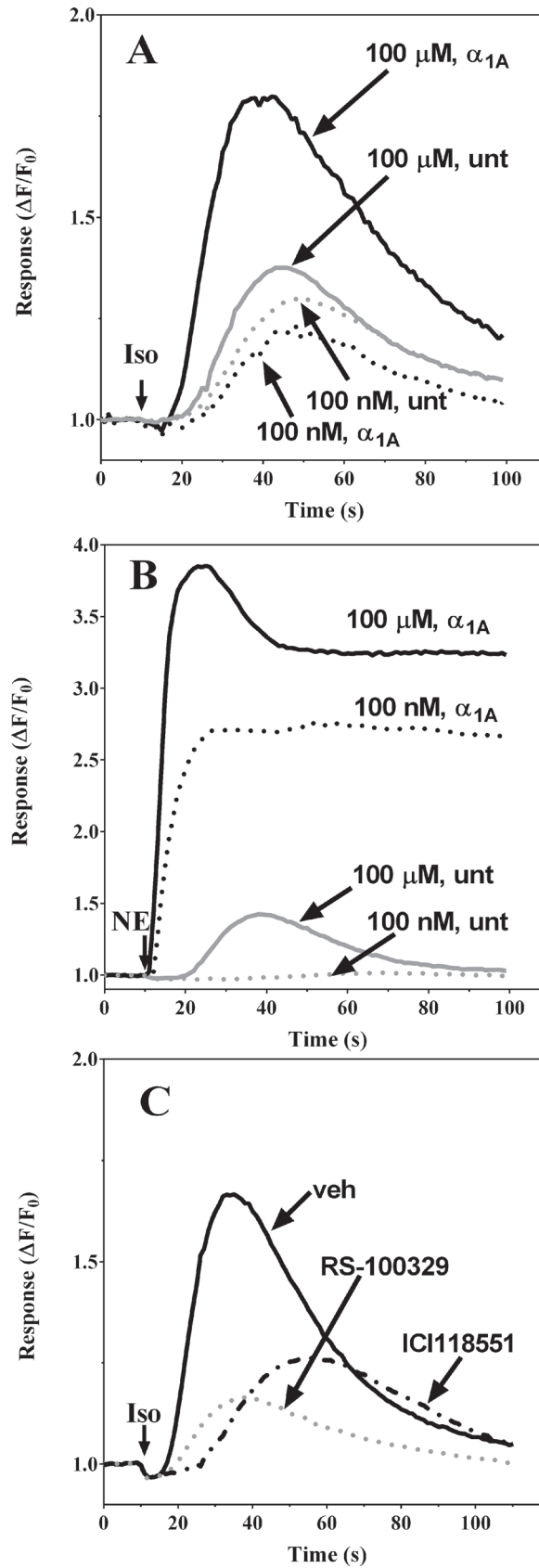
<sup>3</sup>BQL: Below quantifiable levels

<sup>4</sup>EC<sub>50</sub> values for the Ca<sup>2+</sup> mobilization and p-ERK assays were determined from fitting data to a biphasic concentration-effect model (E<sub>q</sub> 1, [Materials and methods](#) section).

<sup>5</sup>K<sub>i</sub> of Iso at β<sub>2</sub>-AR and α<sub>1A</sub>-AR were determined as described in [Materials and Methods](#) by radioligand competition binding methods using [<sup>125</sup>I]CYP and [<sup>3</sup>H]-Prazosin, respectively, as the radioligands.

<sup>6</sup>ND –not determined

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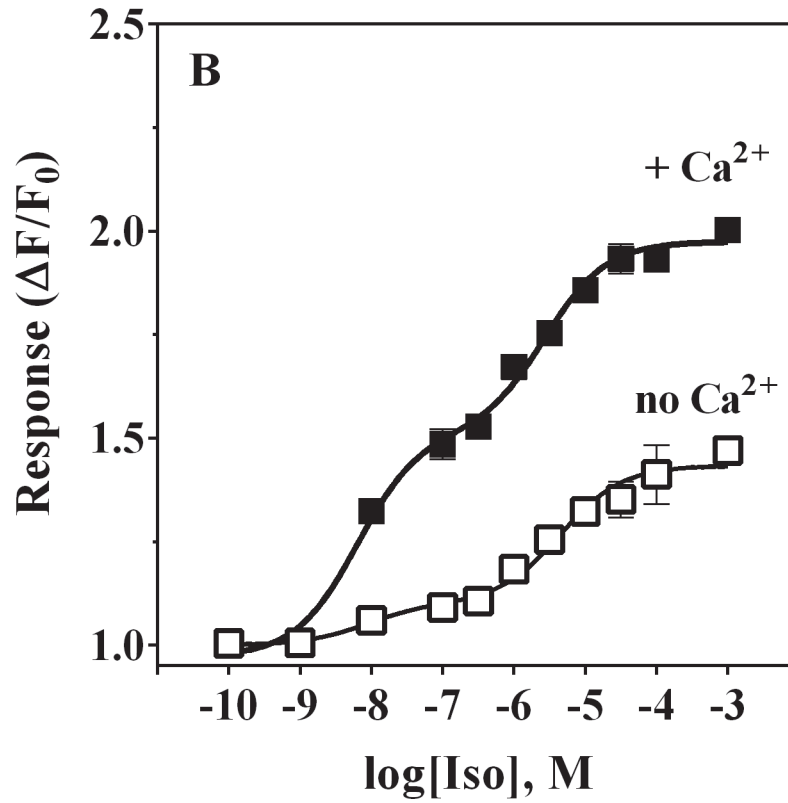
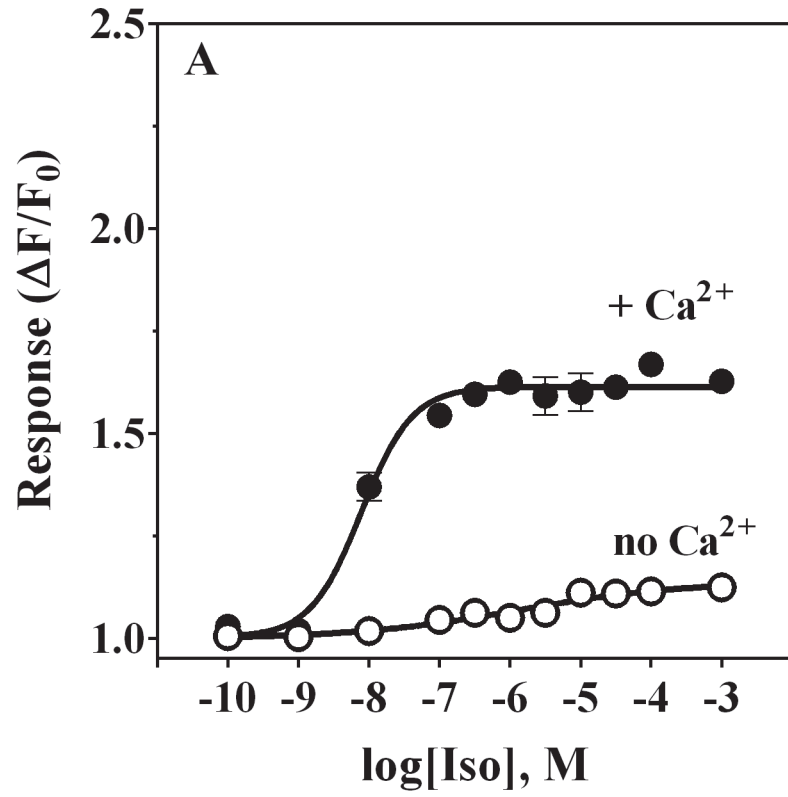
**Figure 2. The Iso-induced  $\text{Ca}^{2+}$  mobilization response in  $\alpha_{1A}$ -AR transduced HEK-293/EBNA cells is slower in onset and shorter in duration than the response to NE.**  $\text{Ca}^{2+}$  transient response kinetics (expressed as  $\Delta F_i/F_0$ ) are shown for fluo3-loaded cells monitored fluorometrically following addition of Iso or NE. HEK-293/EBNA cells were first exposed to recombinant baculoviral strains (3–4 h) encoding either  $\alpha_{1A}$ -AR or an irrelevant negative control protein (aldehyde oxidase), then cultured in fresh culture medium containing 4 mM NaBu for 18 h prior to use in experiments as described in [Materials and Methods](#). **Panel A:** Slow onset of the Iso agonist response which returns to baseline within a 2 min interval is evident in representative traces from  $\alpha_{1A}$ -AR transduced HEK-293/EBNA cells (black lines) and negative control cells (gray lines) during responses elicited by addition of Iso ( $\downarrow$ ) at 100 nM (dashed lines) or 100  $\mu\text{M}$  (solid lines). **Panel B:** Representative  $\text{Ca}^{2+}$  transients in  $\alpha_{1A}$ -AR transduced HEK-293/EBNA cells showing rapid onset and sustained NE response following application of NE ( $\downarrow$ ) at 100 nM (dashed lines) or 100  $\mu\text{M}$  (solid lines) to  $\alpha_{1A}$ -AR transduced (black lines) or negative control-transduced cells (gray lines). **Panel C:**  $\alpha_{1A}$ -AR transduced HEK-293/EBNA cells exhibit distinct kinetics for Iso-mediated responses via occupancy of  $\beta$ -AR vs  $\alpha_{1A}$ -AR transduced, as revealed by monitoring of responses following pre-treatment of cells for 20 min with vehicle (solid black line), 10 nM RS-100329 (dashed gray line) or 10 nM ICI118551 (dashed black line). Representative traces are shown for responses to 100  $\mu\text{M}$  Iso ( $\downarrow$ ).

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occupancy at both  $\alpha_{1A}$ -AR and  $\beta_2$ -ARs elicited a slow onset  $\text{Ca}^{2+}$  transient response (5–10 second delay from addition of agonist to the rise in  $\text{Ca}^{2+}$ ) that return towards baseline (i.e. decline from peak amplitude >75%) within 100 sec and lacked the sustained phase ([Fig. 2A](#), solid black line). A smaller magnitude response with similar slow onset and no sustained elevated phase was observed in response to 100 nM Iso, a concentration that would achieve high fractional occupancy of  $\beta_2$ -AR, yet insignificant at  $\alpha_{1A}$ -AR ([Fig. 2A](#), dashed black line). Responses to application of 100 nM or 100  $\mu\text{M}$  Iso in untransduced negative control cells are shown by the gray dashed and solid traces, respectively. By contrast, treatment of  $\alpha_{1A}$ -AR\_ HEK-293/EBNA transduced cells with 100 nM NE resulted in an almost immediate  $\text{Ca}^{2+}$  transient onset, with peak response amplitude for the population average attained within 10 seconds post-agonist addition ([Fig. 2B](#), dashed black line). This response was characterized by a sustained plateau phase of elevated cytosolic  $\text{Ca}^{2+}$ , not found in untransduced control cells ([Fig. 2B](#), black lines vs. solid gray trace). Because NE possesses  $\sim 10$ -fold higher affinity at  $\alpha_{1A}$ -AR relative to  $\beta_2$ -AR ( $K_i = 3.1 \mu\text{M}$  at  $\alpha_{1A}$ -AR vs.  $34 \mu\text{M}$  at  $\beta_2$ -AR, [Table 1](#)), the observed response to 100 nM NE is likely driven mostly through occupancy at  $\alpha_{1A}$ -AR ([Fig. 2B](#), dashed black line). Thus, the rapid and sustained  $\text{Ca}^{2+}$  elevation in response to 100 nM NE reveals an ability of this agonist to elicit  $\text{Ca}^{2+}$  transient responses through a mechanism distinct from the slow onset and transient mobilization response induced by Iso. Responses to 100  $\mu\text{M}$  Iso in cells that were preincubated with the  $\alpha_{1A}$ -AR selective antagonist RS100329 or the  $\beta_2$ -AR-selective antagonist ICI 118551 revealed the functional contributions of both receptors ([Fig. 2C](#); gray dashed and black dashed lines, respectively): both antagonists significantly diminished responses and delayed their onset when compared to the vehicle control ([Fig. 2C](#); solid black line). Interestingly, the biggest delay was observed in cells pretreated with the  $\beta_2$ -AR-selective antagonist ICI 118551, a condition that isolates the  $\alpha_{1A}$ -AR component of Iso-mediated  $\text{Ca}^{2+}$  transients ([Fig. 2C](#); black, dashed line).

### Effect of extracellular $\text{Ca}^{2+}$ removal on the $\beta_2$ - and $\alpha_{1A}$ -AR-dependent $\text{Ca}^{2+}$ mobilization response to Iso stimulation

We next addressed whether observed rises in cytosolic  $\text{Ca}^{2+}$  were due to influx of extracellular  $\text{Ca}^{2+}$  by measuring  $\text{Ca}^{2+}$  mobilization in HEK-293/EBNA cells upon exposure to Iso in the presence or absence of external  $\text{Ca}^{2+}$ . Omitting  $\text{Ca}^{2+}$  from the extracellular buffer eliminated Iso-evoked  $\text{Ca}^{2+}$  responses in untransduced HEK-293/EBNA cells ([Fig. 3A](#)). In  $\alpha_{1A}$ -AR\_ HEK-293/EBNA cells, Iso-mediated responses were greatly reduced in amplitude in the absence of extracellular  $\text{Ca}^{2+}$  ([Fig. 3B](#)). However, the low potency phase of the concentration-response



**Figure 3. Iso-induced  $\text{Ca}^{2+}$  mobilization in  $\alpha_{1A}$ -AR transduced HEK-293/EBNA cells is partially dependent on the presence of extracellular  $\text{Ca}^{2+}$ .**  $\text{Ca}^{2+}$  transient responses (expressed as  $\Delta\text{F}/\text{F}_0$ ) were measured as a function of Iso concentration in untransduced control cells (**Panel A**) or  $\alpha_{1A}$ -AR transduced HEK-293/EBNA cells (**Panel B**), either in the presence (filled symbols) or absence (open symbols) of 2 mM  $\text{Ca}^{2+}$  in the assay buffer. Responses in untransduced cells were virtually abolished in the absence of extracellular  $\text{Ca}^{2+}$  (**Panel A**). In  $\alpha_{1A}$ -AR transduced cells, responses to low concentrations of Iso in the absence of extracellular  $\text{Ca}^{2+}$  were also essentially abolished whereas the low potency phase of response was diminished by approximately 50% (**Panel B**). Each data point is an average of duplicate determinations; this experiment was repeated twice.

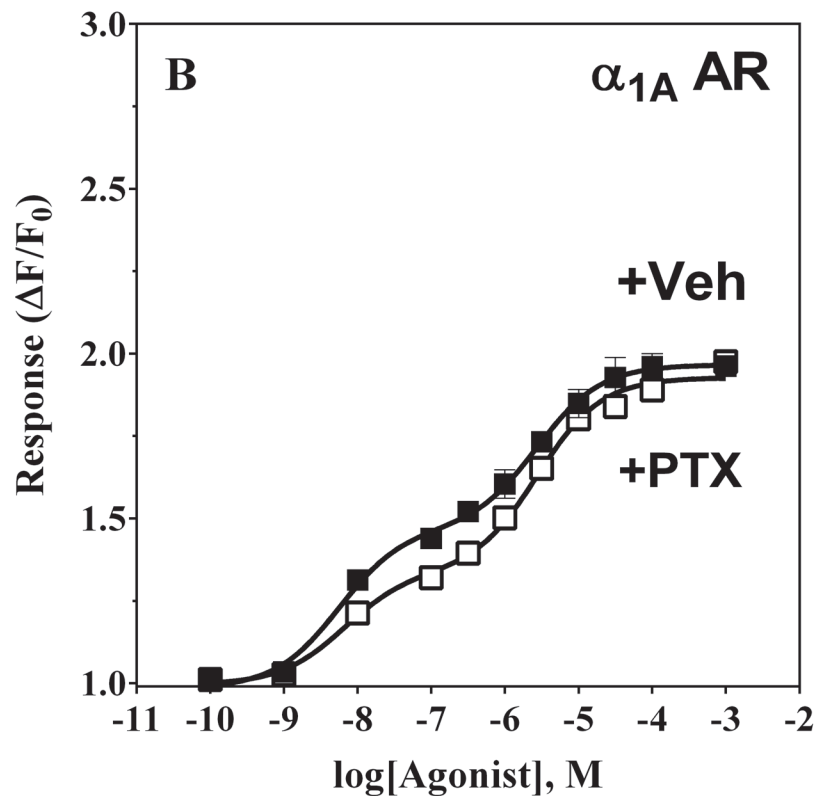
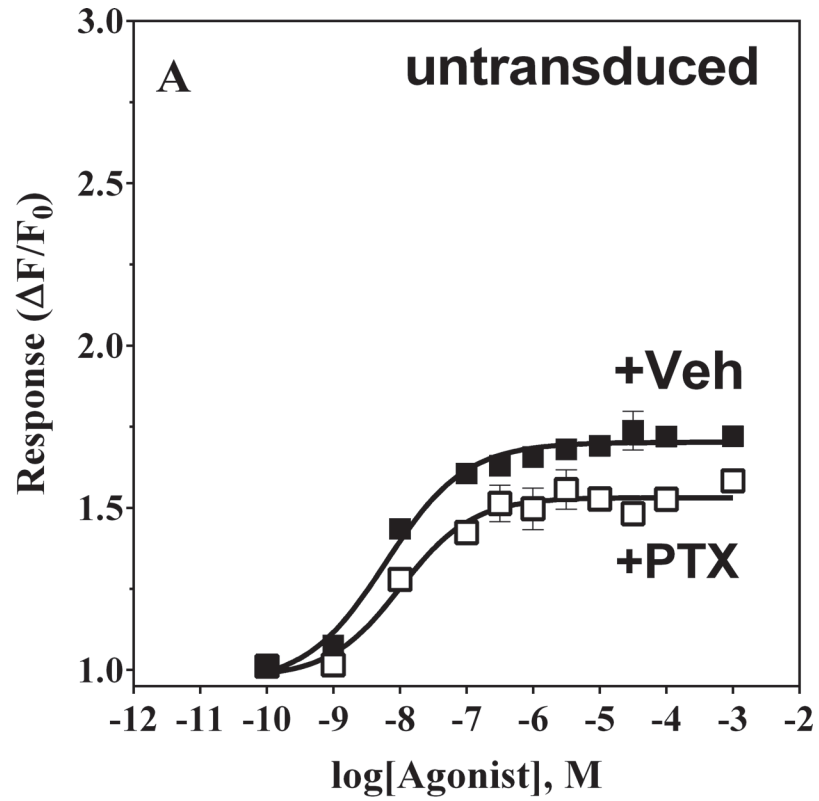
doi:10.1371/journal.pone.0115701.g003

relationship was only partially affected, unlike responses to Iso < 100 nM, which were almost fully inhibited. Fitting of those data to a biphasic concentration-response model yielded  $\text{EC}_{50}$  values of 8.6 nM and 4.5  $\mu\text{M}$  for the high and low potency phases, respectively, close to the  $\text{EC}_{50}$  values of 6.3 nM and 2.5  $\mu\text{M}$  measured in the same cells responding to Iso in the presence of extracellular  $\text{Ca}^{2+}$ . Moreover, the amplitude of the Iso-induced maximal responses under nominally  $\text{Ca}^{2+}$ -free conditions ( $\Delta\text{F}/\text{F}_0 = 1.1$  and 1.3 for the high and low potency phases, respectively) was lower than that determined in the same cells in the presence of extracellular  $\text{Ca}^{2+}$  ( $\Delta\text{F}/\text{F}_0 = 1.5$  and 1.4). Thus, under near-zero  $\text{Ca}^{2+}$  concentration both the Iso response of untransduced cells and the high potency phase of the response in  $\alpha_{1A}$ -AR transduced cells were largely absent. This finding suggests that the observed  $\beta_2$ -AR dependent  $\text{Ca}^{2+}$  mobilization requires an influx of  $\text{Ca}^{2+}$  from the extracellular compartment, likely via activation of cAMP nucleotide gated-channels. As described in the previous section, the time course of intracellular  $\text{Ca}^{2+}$  mobilization in response to 100  $\mu\text{M}$  Iso (a concentration that produces high occupancy of  $\alpha_{1A}$ -ARs) was very similar to that observed for  $\beta_2$ -AR response and dramatically different from typical  $\text{G}\alpha_q$ -initiated signaling canonical for  $\alpha_{1A}$ -AR. Remarkably,  $\text{Ca}^{2+}$  mobilization in response to Iso occupancy of both  $\beta_2$ -AR and  $\alpha_{1A}$ -AR was found to be less sensitive to removal of extracellular  $\text{Ca}^{2+}$  (**Fig. 3B**), indicating intracellular stores as the source of  $\text{Ca}^{2+}$ .

### Iso induced $\text{Ca}^{2+}$ mobilization in $\alpha_{1A}$ -AR transduced HEK-293/EBNA cells does not reflect coupling to $\text{G}\alpha_i$

Receptor coupling to  $\text{G}\alpha_i$ , the most abundant G protein  $\alpha$  subunit, has been shown to lead to activation of PLC activity via the  $\text{G}\beta/\gamma$  subunits and thus, can lead to IP accumulation and release of intracellular  $\text{Ca}^{2+}$  (Reviewed in [36]). Since this pathway is different from  $\text{G}\alpha_q$ -mediated signaling, the kinetics of  $\text{Ca}^{2+}$  mobilization may also differ. We next searched for a role for  $\text{G}\alpha_i$  activation in response to Iso-treatment in untransduced and  $\alpha_{1A}$ -AR transduced cells, pretreated for 18 hours with pertussis toxin or vehicle. Pretreatment of untransduced cells with pertussis toxin (100 ng/mL) resulted in a slight increase in the maximum amplitude of the  $\text{Ca}^{2+}$  transient response at saturating concentrations of Iso ( $\Delta\text{F}/\text{F}_0 = 1.5$  vs. 1.7), with a negligible increase in  $\text{EC}_{50}$  from 6 to 10 nM (**Fig. 4A**). A similar increase was observed in the  $\beta_2$ -AR-dependent phase of the Iso concentration-response curve (from  $\Delta\text{F}/\text{F}_0 = 1.3$  to 1.5) in  $\alpha_{1A}$ -AR expressing cells, also with no change in observed potency ( $\text{EC}_{50} = 6.6$  vs. 5.6 nM) (**Fig. 4B**). Since in these cells induction of cAMP leads to  $\text{Ca}^{2+}$  mobilization [27], increase in  $\text{Ca}^{2+}$  mobilization following PTX pretreatment may be a result of an increase in intracellular cAMP. This observation would be consistent with previous findings indicating that  $\beta_2$ -AR can couple to both  $\text{G}\alpha_s$  and  $\text{G}\alpha_i$  in HEK-293 [37]. On the other hand, in  $\alpha_{1A}$ -AR transduced HEK-293/EBNA cells, pertussis toxin treatment did not significantly affect  $\text{Ca}^{2+}$  mobilization responses to Iso ( $\text{EC}_{50} = 2.8$  vs. 2.7  $\mu\text{M}$  with PTX and  $\Delta\text{F}/\text{F}_0 = 1.6$  vs. 1.5 with PTX), suggesting that coupling to  $\text{G}\alpha_i$  is not necessary for this Iso-mediated function of  $\alpha_{1A}$ -AR (**Fig. 4B**).





**Figure 4. Pertussis toxin pretreatment does not impair Ca<sup>2+</sup> responses to Iso.** HEK-293/EBNA cells were exposed for 3 h to a baculoviral strain carrying  $\alpha_{1A}$ -AR or to viral medium. Cells were treated with 100 ng/mL of pertussis toxin (PTX) or vehicle for 18 h prior to experiments. **Panel A:** untransduced HEK-293/EBNA cells exposed to PTX (filled symbols) or vehicle (open symbols) were treated with Iso (■, □) during fluorometric imaging of the Ca<sup>2+</sup>-tracking dye. **Panel B:** untreated (open symbols) or PTX-pretreated (filled symbols)  $\alpha_{1A}$ -AR HEK-293/EBNA cells were stimulated with Iso (■, □) during fluorometric imaging of the Ca<sup>2+</sup>-tracking dye. Each experiment was performed in duplicate two independent times.

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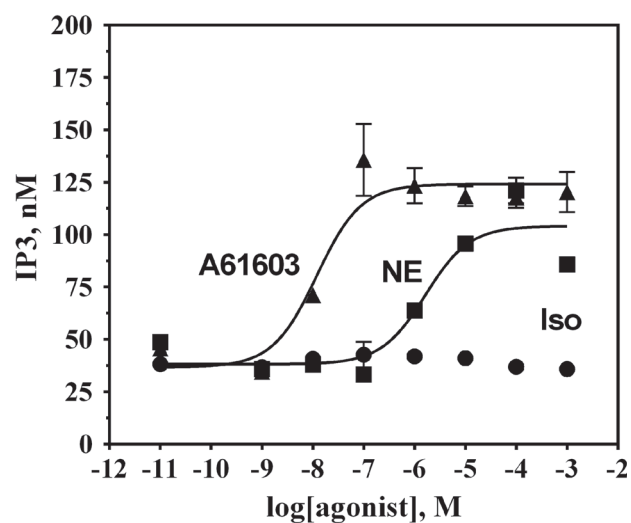
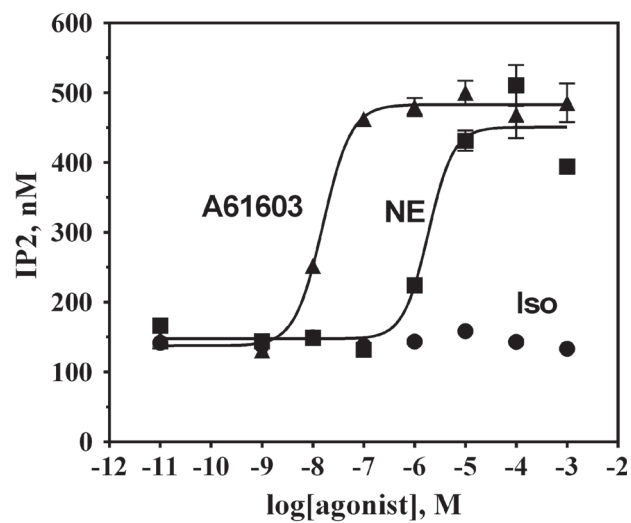
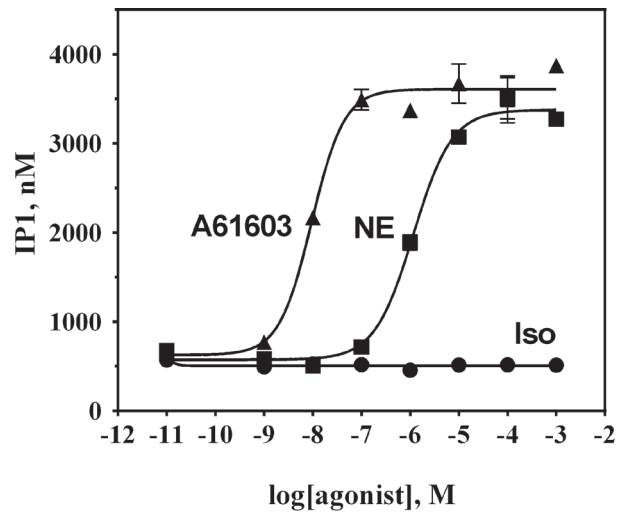
### Iso does not stimulate formation of inositol phosphates (IP<sub>1</sub>, IP<sub>2</sub> or IP<sub>3</sub>) nor IP accumulation in HEK-293/EBNA- $\alpha_{1A}$ AR cells

Given that the  $\alpha_{1A}$ -AR-dependent phase of the Iso concentration-response relationship was found to be only partially dependent on the extracellular Ca<sup>2+</sup> and insensitive to pertussis toxin treatment, we asked whether Iso-occupancy at  $\alpha_{1A}$ -AR results in G $\alpha_q$  activation. In  $\alpha_{1A}$ -AR transduced HEK-293/EBNA cells, the selective  $\alpha_{1A}$ -AR agonist A-61603 elicited concentration-dependent IP accumulation (S1 Fig.). A-61603-stimulated IP accumulation was best described by a single phase sigmoidal equation yielding an EC<sub>50</sub> of 40 nM (Table 1). Furthermore, preincubation of those cells for 5 minutes with 10 nM of the  $\alpha_{1A}$ -AR selective antagonist RS100329 significantly attenuated IP accumulation and produced a large, rightward shift in A-61603 potency. In contrast, when replicate cells were treated with Iso (up to 10 mM) no significant accumulation of IP was detected. Furthermore, IP formation in untransduced cells upon treatment with either A-61603 or Iso was not detectable (data not shown). We employed an LC-MS method to increase our detection sensitivity and to measure individual inositol phosphates rather than total IP<sub>x</sub> accumulation (Fig. 5). In  $\alpha_{1A}$ -AR transduced HEK-293/EBNA both NE and the selective  $\alpha_{1A}$ -AR agonist A-61603, produced concentration-dependent increases in cellular levels of inositol phosphates IP<sub>1</sub>, IP<sub>2</sub> and IP<sub>3</sub> that were best described by a monophasic sigmoidal equation (Fig. 5). On the other hand, when replicate cells were treated with Iso (up to 10 mM), no significant changes in intracellular inositol phosphates were detected. Thus, it appears that Iso occupancy at  $\alpha_{1A}$ -AR biases receptor signaling toward a G $\alpha_q$ -independent pathway.

### Iso induces biphasic concentration-response behavior for p-ERK formation in $\alpha_{1A}$ -AR transduced HEK-293/EBNA cells

Significant efforts have been placed in understanding the role of the mitogen-activated protein kinase (MAPK) cascade in adrenoceptor signaling (*e.g.* in cardiomyocyte physiology, see [38]). We decided to test whether the Iso-mediated signaling that we observed involved this signaling pathway. For this, we examined whether Iso induces ERK1/2 activation. Treatment of  $\alpha_{1A}$ -AR transduced HEK-293/EBNA cells with Iso induced a dose-dependent increase of p-ERK formation (Fig. 6, filled circles). The resulting concentration-response relationship was best fit by a biphasic model. The calculated EC<sub>50</sub> values were 5 nM and 6  $\mu$ M for the high and low potency phases, respectively. In untransduced cells a monophasic relationship was observed (EC<sub>50</sub> = 1 nM, S2 Fig.). Interestingly, in  $\alpha_{1A}$ -AR transduced HEK-293/EBNA cells, A-61603 or NE yielded concentration-dependent activation of ERK that was best fit by a monophasic sigmoidal model. The measured EC<sub>50</sub> values were 0.8 and 90 nM for A-61603 and NE, respectively (Fig. 6, filled triangles or squares, and Table 1). In those cells, A-61603 appeared to be almost 4 times more potent at inducing p-ERK formation than in effecting Ca<sup>2+</sup> mobilization (EC<sub>50</sub> = 3 nM, Table 1), while NE had about the same potency for both response modes.

Iso stimulation of  $\alpha_{1A}$ -AR-HEK-293/EBNA cells resulted in a biphasic concentration-response relationship for both p-ERK formation and Ca<sup>2+</sup> mobilization transients. The high



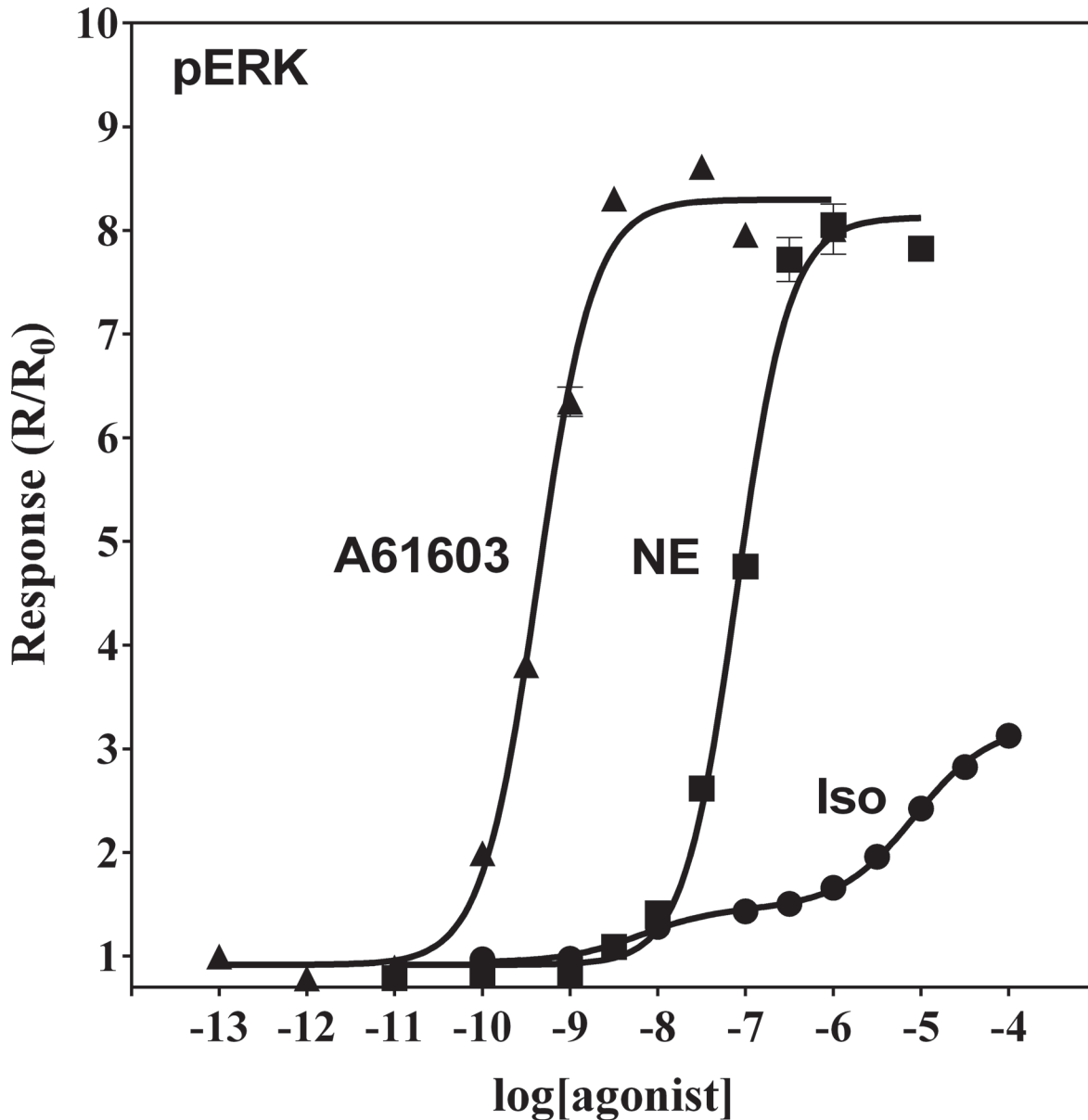
**Figure 5. Inositol phosphates production in  $\alpha_{1A}$ -AR transduced HEK-293/EBNA cells occurs in response to A-61603 and NE, but not in response to Iso.** HEK-293/EBNA cells were exposed to baculovirus encoding  $\alpha_{1A}$ -AR for 3–4 h, then cultured in fresh medium containing 4 mM NaBu for 18 h prior to use in experiments as described in [Materials and Methods](#). IP<sub>1</sub> (top), IP<sub>2</sub> (middle) and IP<sub>3</sub> (bottom) formation was measured in  $\alpha_{1A}$ -AR transduced HEK-293/EBNA cells stimulated with increasing concentrations of A-61603 (▲), NE (■) or Iso (●). IP<sub>1</sub>, IP<sub>2</sub>, and IP<sub>3</sub> levels were determined via LC-MS. Plots are representative of three independent experiments with each data point being the average of triplicates.

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potency phase of response to Iso (via occupancy of  $\beta_2$ -AR) and the low potency phase (via occupancy of both  $\beta_2$ -AR and  $\alpha_{1A}$ -AR) were quite similar for the two different readouts ([Table 1](#)).

### Stimulation of $\alpha_{1A}$ -AR transduced HEK-293/EBNA with A-61603 and Iso leads to an increase in the level of intracellular $\alpha_{1A}$ -AR without recruitment of arrestins

We next investigated whether or not  $\alpha_{1A}$ -AR internalizes upon stimulation with A-61603 or ISO. In several prior studies  $\alpha_{1A}$ -AR has been found to undergo constitutive, ligand independent trafficking and to internalize rather modestly ( $\sim 20\%$ ) upon agonist stimulation [[39–41](#)]. Furthermore this receptor cycling involves clathrin-coated vesicles and entry to early endosomes [[39](#)]. To eliminate some of the experimental challenges and uncertainty related to detection of the modest and transient internalization of  $\alpha_{1A}$ -AR, we employed constitutively active and dominant-negative forms of Rab5 and Rab11, respectively, to the analysis of  $\alpha_{1A}$ -AR endocytosis using confocal microscopy. Constitutively active Rab5 Q79L enhances endocytosis and early endosome fusion, causing the formation of enlarged early endosomes, and its overexpression has been shown to inhibit transferrin recycling. Rab11 S25N is defective in GTP binding and impairs recycling by inhibiting exit from sorting endosomes (early endosomes) to the recycling endosomes and/or plasma membrane. In HEK-293/EBNA cells transfected with  $\alpha_{1A}$ -AR only and stimulated with A-61603 or ISO,  $\alpha_{1A}$ -AR was mostly localized at the plasma membrane, as was the case for the vehicle control ([Fig. 7A](#)). Overexpression of Rab5Q79L caused some intracellular  $\alpha_{1A}$ -AR accumulation in vehicle control cells, indicative of constitutive trafficking, as well as significant  $\alpha_{1A}$ -AR accumulation upon A-61603 or ISO treatment. Overexpression of Rab11 S25N led to some accumulation in A-61603 or ISO-treated cells, not as pronounced as the one observed upon Rab5Q79L overexpression. In vehicle-treated cells,  $\alpha_{1A}$ -AR appeared predominantly at the plasma membrane. To confirm the observation that stimulation with Iso led to internalization of  $\alpha_{1A}$ -AR, we took advantage of a cleavable biotin labeling reagent to discern levels of surface versus internalized  $\alpha_{1A}$ -AR by means of immunoprecipitation ([Fig. 7B](#) and [S3 Fig.](#)). Intracellular  $\alpha_{1A}$ -AR could be detected at low levels in control cells (lane 3, “C+GSH”) indicative of constitutive trafficking. Treatment with A-61603 caused an increase in intracellular  $\alpha_{1A}$ -AR levels after 5, 30, and 60 min of treatment (lanes 4–6, “A-61603+GSH”) relative to control cells (lane 3, “C+GSH”). Similarly, treatment with ISO caused an increase in the intracellular  $\alpha_{1A}$ -AR (lanes 7–8, “ISO+GSH 5 or 30”), as compared to control cells (lane 3, “C+GSH”). We next examined whether Iso-induced internalization of  $\alpha_{1A}$ -AR in HEK-293 cells involved the recruitment of  $\beta$ -arrestins. The ability of the N-terminally FLAG-tagged  $\alpha_{1A}$ -ARs to trigger the translocation of GFP-tagged  $\beta$ -arrestins in those cells was investigated by confocal microscopy. As shown in [Fig. 8](#), in vehicle treated cells, the  $\alpha_{1A}$ -AR was localized mainly at the plasma membrane while both  $\beta$ -arrestins 1 (left panels) and 2 (right panels) were homogeneously distributed throughout the cytoplasmic compartment with no visible co-localization with the surface receptor. In cells expressing the  $\alpha_{1A}$ -AR, stimulation with A-61603 for 30 min did not induce significant translocation of either  $\beta$ -arrestin-1 nor  $\beta$ -arrestin-2, to the plasma membrane. Similarly exposure of the same cells to Iso did not



**Figure 6. MAPK activation in  $\alpha_{1A}$ -AR transduced HEK-293/EBNA cells treated with A-61603, NE or Iso.**  $\alpha_{1A}$ -AR transduced HEK-293/EBNA cells were pre-treated with NaBu for 18 h to induce receptor expression. Cells were stimulated with A-61603 ( $\blacktriangle$ ), NE ( $\blacksquare$ ) or Iso ( $\bullet$ ) for 5 min. Agonist treatment was terminated by addition of of SureFire lysis solution. Samples were analyzed for levels of phospho-ERK using an AlphaScreen SureFire p-ERK assay kit. Plots are representative of four independent experiments, with each data point being the average of triplicates.

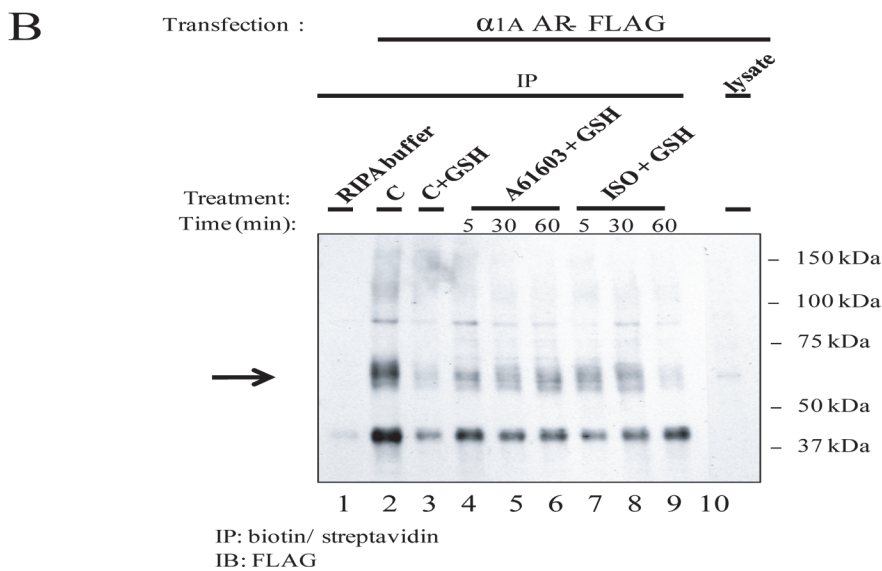
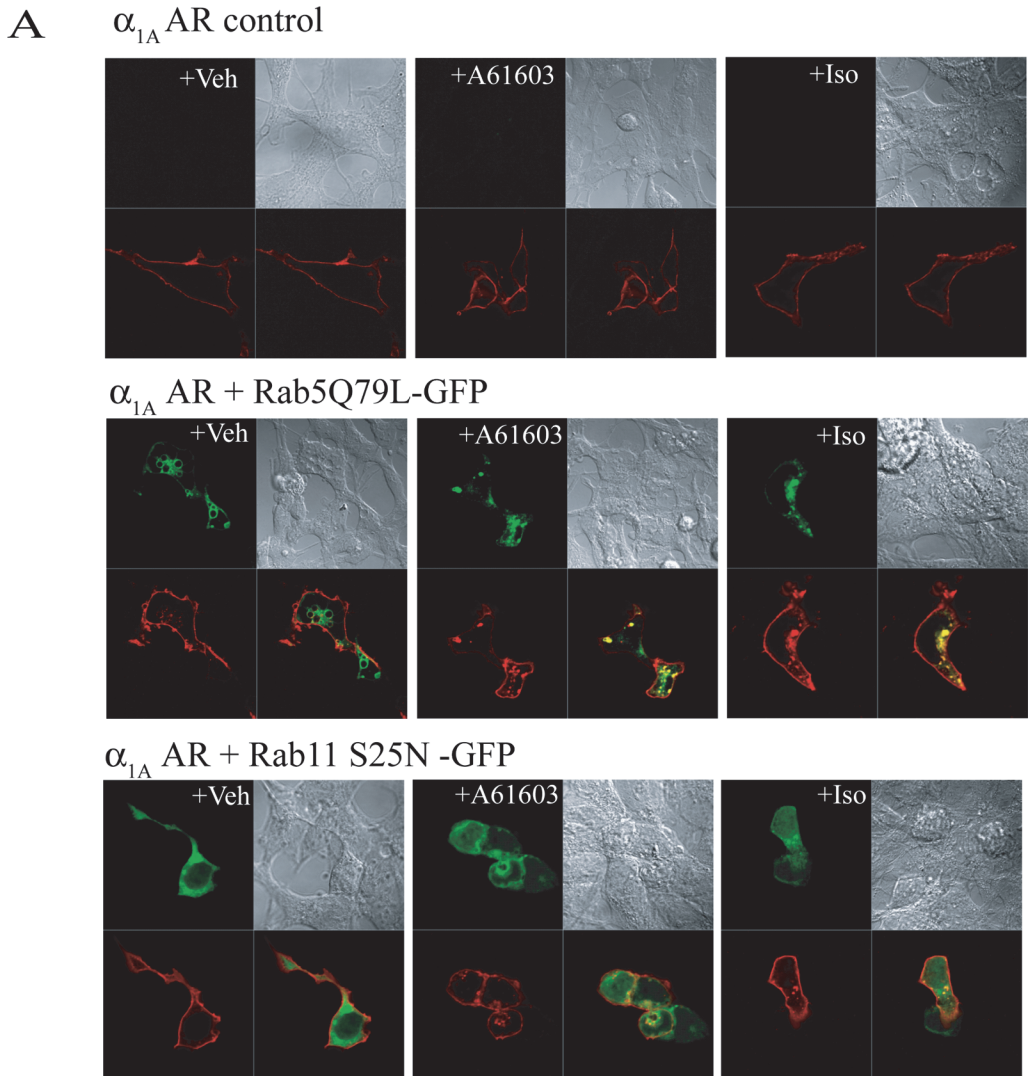
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cause visible  $\beta$ -arrestin rearrangement. Internalization of the  $\alpha_{1A}$ -AR was not detected with either agonist by this method.

Isoproterenol-induced  $Ca^{2+}$  mobilization in  $\alpha_{1A}$ -AR\_CHO cells is not accompanied by detectable increases in intracellular inositol phosphates, yet correlates with activation of the MAPK kinase pathway

Since in HEK-293 cells Iso can stimulate both endogenous  $\beta_2$ -AR as well as transduced  $\alpha_{1A}$ -AR, we next tested the activity of this  $\beta$ -agonist at  $\alpha_{1A}$ -AR in CHO cells stably expressing





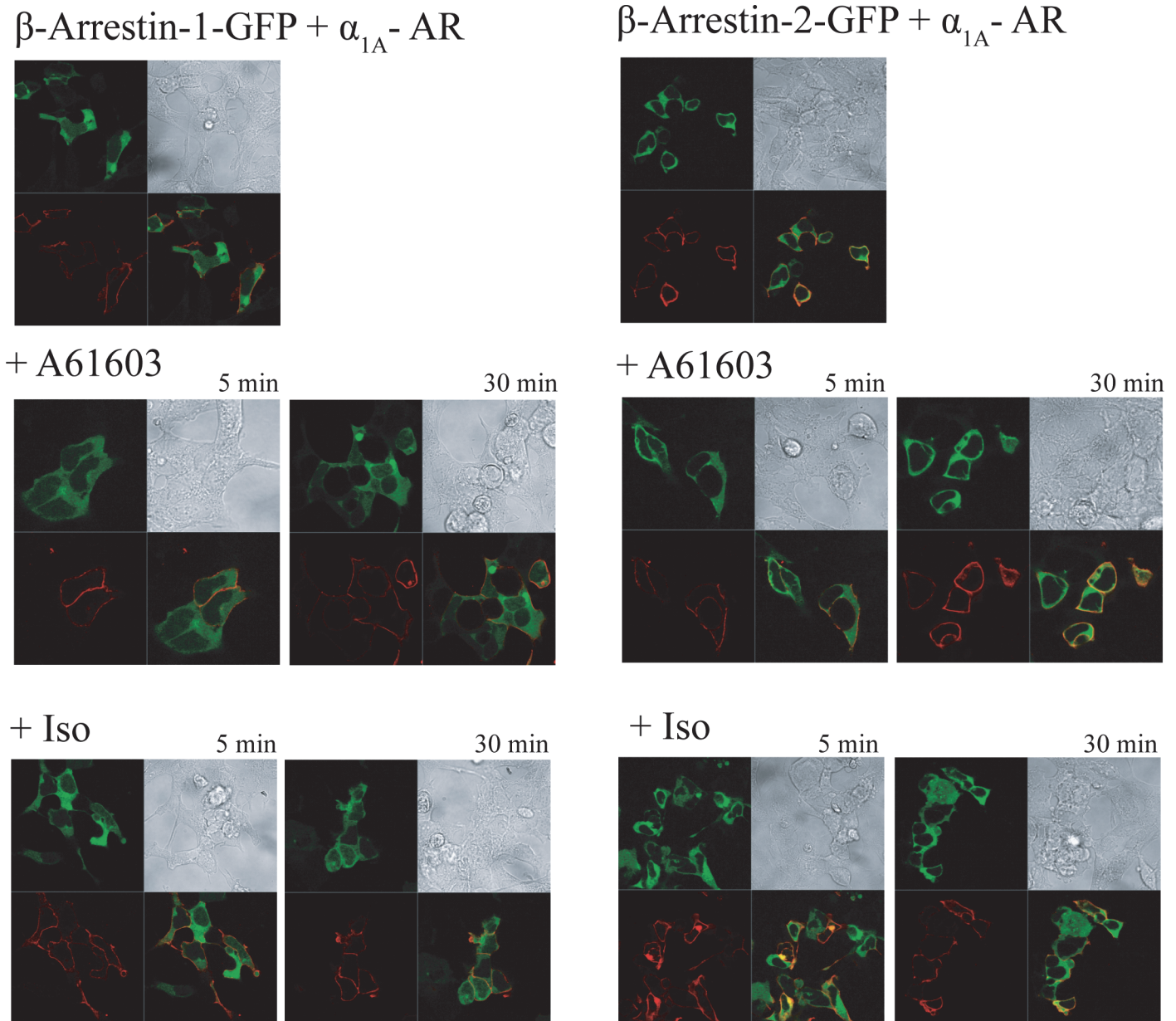
**Figure 7. Stimulation of  $\alpha_{1A}$ -AR transduced HEK-293/EBNA with A-61603 and Iso leads to an increase in intracellular  $\alpha_{1A}$ -AR.** **A.** HEK293 cells were transiently transfected with  $\alpha_{1A}$ -AR only (top panels), or co-transfected with  $\alpha_{1A}$ -AR and Rab5 variant Q79L (middle panels) or Rab11 variant S25N (bottom panels). Following serum-deprivation, cells were stimulated with vehicle, 1  $\mu$ M A-61603 or 1 mM ISO for 2h. Cells were then fixed and analyzed by confocal microscopy. **B.** HEK293 cells were transiently transfected with  $\alpha_{1A}$ -AR. After serum deprivation for 24h, cells were pre-treated with a membrane impermeable, disulfide-cleavable biotin reagent to label plasma membrane  $\alpha_{1A}$ -AR. Cells were then left untreated, or stimulated 1  $\mu$ M A-61603 or 1 mM ISO for 5, 30, or 60 min. After treatment, one dish of control cells was harvested without any further manipulations (C: total  $\alpha_{1A}$ -AR). The remaining seven dishes were divided into one control (C+GSH), three treated with A-61603 (A-61603+GSH) and three treated with ISO (ISO+GSH). They were stripped of surface biotin label using a reducing agent, in order to reveal internalized, labeled  $\alpha_{1A}$ -AR. Samples were then analyzed by immunoprecipitation (IP) with streptavidin followed by immunoblotting (IB) with an anti-FLAG antibody.

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moderate levels of the receptor ( $\sim 1$  pmol/mg of membrane proteins) [42]. The  $\beta$ -AR selective agonist Iso evoked a  $\text{Ca}^{2+}$  transient response in  $\alpha_{1A}$ -AR\_CHO cells (Fig. 9A) that was not observed in CCR5\_CHO cells used as a negative control (S4 Fig.). The peak amplitude of  $\text{Ca}^{2+}$  transient response,  $\Delta F/F_0$ , as a function of agonist concentration yielded a monophasic concentration-response curve with the half-maximal amplitude occurring at 20  $\mu$ M Iso (Fig. 9A). That response to Iso was blocked by pretreatment with 100 nM RO100329, but was insensitive to propranolol at 100 nM. We next examined whether Iso-mediated  $\text{Ca}^{2+}$  mobilization involved formation of inositol phosphates. Stimulation of  $\alpha_{1A}$ -AR\_CHO cells with NE led to concentration-dependent IP accumulation with an  $\text{EC}_{50}$  of 0.6  $\mu$ M (Fig. 9B). On the other hand, no IP accumulation could be detected in the same cells stimulated with Iso at concentrations up to 1 mM. Similarly, in  $\alpha_{1A}$ -AR\_CHO cells both NE, and the selective  $\alpha_{1A}$ -AR agonist A-61603, produced concentration-dependent increases in cellular concentration of inositol phosphates; IP1, IP2 and IP3 (Fig. 9D) that were best described by a single-site sigmoidal equation. In contrast, when replicate cells were treated with Iso (up to 1 mM) no significant changes in intracellular levels of inositol phosphates could be detected. Thus, it appears that Iso occupancy at  $\alpha_{1A}$ -AR biases receptor signaling to a  $\text{G}\alpha_q$ -independent pathway. Yet, similarly to the observations made with  $\alpha_{1A}$ -AR\_HEK-293/EBNA cells, stimulation of  $\alpha_{1A}$ -AR\_CHO cells with Iso resulted in a concentration dependent increase in phospho-ERK formation (Fig. 9C). The amount of p-ERK formed at the maximal concentration of Iso represented 35% of the level observed in these cells when stimulated with saturating concentrations of NE or A-61603. Iso was less potent than NE and A61603 at inducing ERK activation in  $\alpha_{1A}$ -AR\_CHO cells (Iso:  $\text{EC}_{50} = 17 \pm 5$   $\mu$ M; NE:  $\text{EC}_{50} = 0.13 \pm 0.02$   $\mu$ M; A61603:  $\text{EC}_{50} = 0.8 \pm 0.2$  nM). These results indicate that Iso is ineffective at inducing  $\alpha_{1A}$ -AR-mediated  $\text{G}\alpha_q$  coupling and PLC activation, yet shows partial agonist activity at mediating  $\alpha_{1A}$ -AR induced activation of the MAPK signaling cascade.

## Discussion

A search on “isoproterenol” in the PubMed database retrieves more than thirty thousand citations. This potent,  $\beta$ -AR-selective agonist has been extensively studied with respect to many aspects of its action at  $\beta$ -ARs. It has been applied frequently as a tool to verify the presence of  $\beta$ -ARs in cell-based systems or to elucidate the mechanism of  $\beta$ -AR signaling in non-recombinant, native cells such as cardiomyocytes, adipocytes and many others, as well as in organ tissue preparations. In several of these studies the concentrations used were in the micromolar range, which may have triggered off-target effects. Dating back to the 1950s, several reports have indicated that Iso at higher concentrations can evoke  $\alpha$ -AR-mediated responses. For instance, Furchgott [9] observed that Iso at concentrations 1–500  $\mu$ M caused contraction of rabbit thoracic aorta, a tissue in which all three  $\alpha_1$ -AR subtypes are expressed (reviewed in [43]). These researchers also noted that the rate of shortening on addition of Iso was much slower relative to epinephrine or norepinephrine, indicating a distinct mechanism of action for

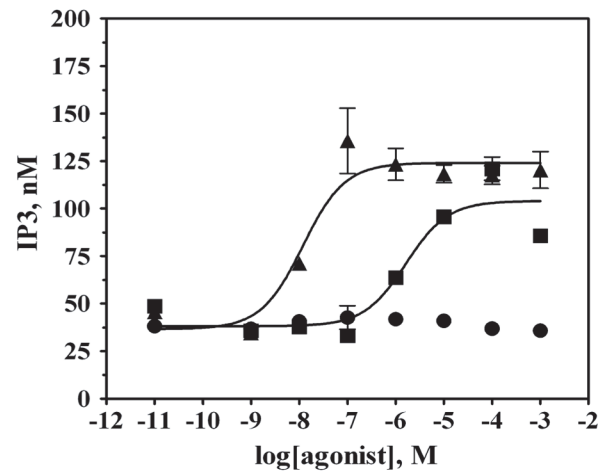
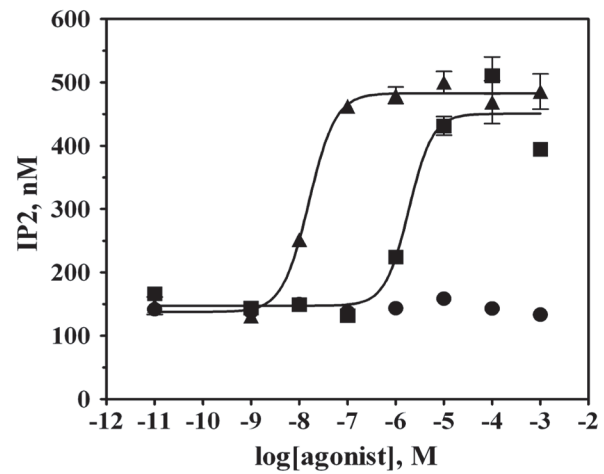
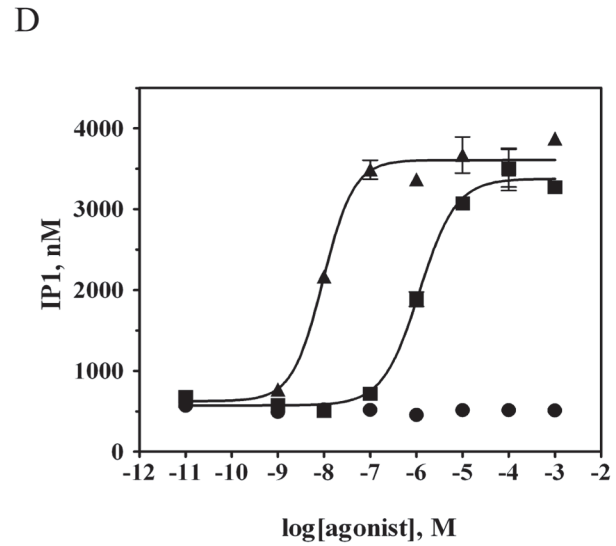
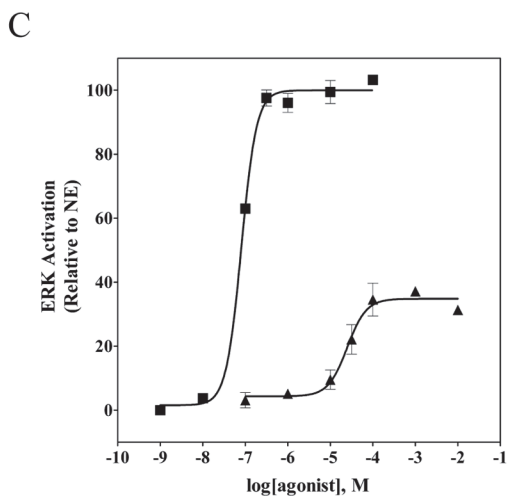
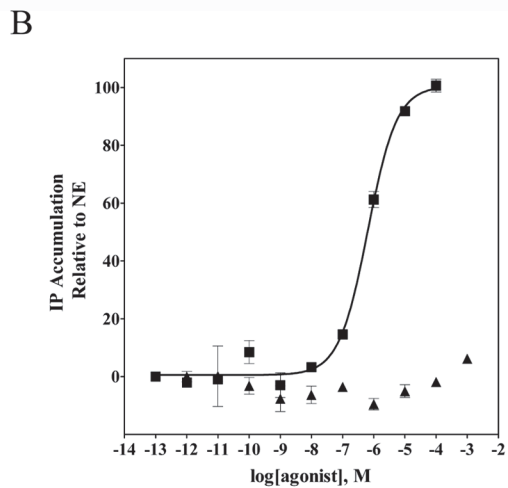
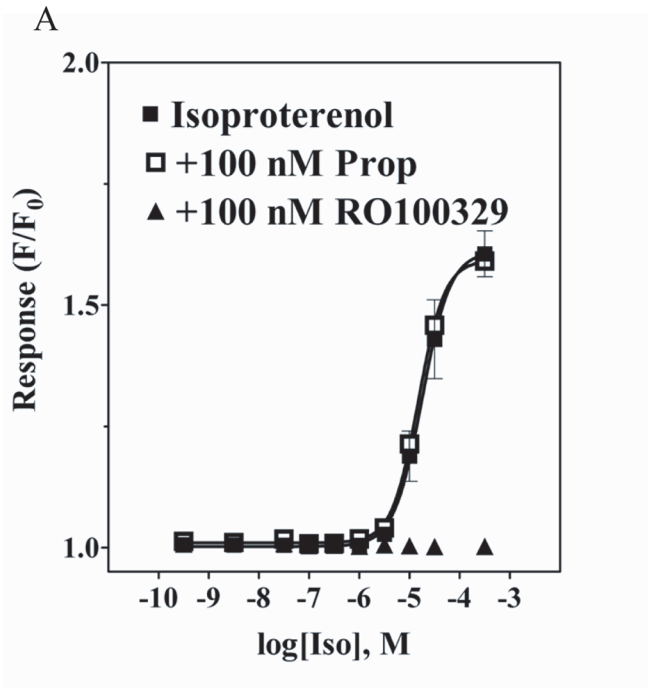


**Figure 8. Treatment of  $\alpha_{1A}$ -AR transduced HEK-293/EBNA with A-61603 and Iso does not trigger intracellular redistribution of arrestins.** HEK293 cells were co-transfected with FLAG-tagged  $\alpha_{1A}$  – AR and GFP- tagged  $\beta$ -arrestin-1 (left panels) or  $\beta$ -arrestin-2 (right panels). Following serum-deprivation for 24h, cells were left untreated (top panels), or stimulated with 1  $\mu$ M A-61603 (middle panels) or 1mM Iso (bottom panels) for the indicated amount of time. Cells were then fixed, permeabilized, stained with Alexa Fluor-568 conjugated anti-FLAG antibodies, and analyzed employing confocal microscopy.

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Iso. The maximal contractile response to Iso was about 75% of that obtained for norepinephrine indicating that Iso acted as a partial agonist.

Our studies of HEK-293/EBNA cells with transient low expression levels of  $\alpha_{1A}$ -ARs cells, to mimic tissues that possess both  $\alpha_{1A}$  and  $\beta$ -ARs, provide for the first time a mechanism to account for Furchgott's original observations. In those cells, a biphasic dose-response relationship was found for Iso-evoked calcium transients. The high potency phase of the response was blocked by non-selective as well as  $\beta_2$ -selective antagonists, while the low potency phase was sensitive only to  $\alpha_{1A}$ -AR-selective antagonists. Remarkably,  $Ca^{2+}$  transient responses to Iso





**Figure 9. Concentration-response behavior to Iso in Chinese hamster ovary cells stably expressing recombinant  $\alpha_{1A}$ -AR.** **A.**  $\text{Ca}^{2+}$  transients (expressed as  $\Delta F/F_0$ ) were measured as a function of Iso concentration in fluo3-loaded cells by fluorometric plate imaging (FLIPR). Responses to Iso were monitored following pre-treatment with either vehicle (■), 100 nM propranolol (□) or 100 nM RO100329 (▲). Inositol phosphate accumulation (**B**) and levels of phospho-ERK (**C**) were measured as a function of increasing concentrations of A-61603 (■) or Iso (▲) and are represented relative to NE. **D.**  $\text{IP}_1$  (top),  $\text{IP}_2$  (middle) and  $\text{IP}_3$  (bottom) formation was measured in CHO cells stably expressing  $\alpha_{1A}$ -AR, stimulated with increasing concentrations of A-61603 (▲), NE (■) or Iso (●).  $\text{IP}_1$ ,  $\text{IP}_2$ , and  $\text{IP}_3$  levels were determined via LC-MS. Plots are representative of three independent experiments with each data point being the average of triplicates.

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mediated by  $\alpha_{1A}$ -ARs exhibited a time course distinctly slower than those seen with prototypical  $\alpha_{1A}$ -AR agonists (NE, A-61603) that are highly efficacious for  $\text{IP}_x$  formation responses and considered typical for  $\text{G}\alpha_q$ -initiated signaling. Responses to Iso showed delayed onset by 5 to 10 seconds and were much slower to rise in amplitude. Moreover, Iso was a partial agonist at  $\alpha_{1A}$ -ARs as recorded by this readout. Finally, the observed  $\text{Ca}^{2+}$  transients exhibited almost complete desensitization, unlike responses to NE. These data taken together indicate a mechanism of action distinct from classical  $\text{G}\alpha_q$  coupling for Iso at the  $\alpha_{1A}$ -AR, and suggest functional selectivity (effector signaling bias) for the activity of this agonist.

Agonist occupancy of any given GPCR may trigger signaling events through activation of more than one physiological response cascade. For instance,  $\alpha_{1A}$ -AR is widely known to couple to phospholipase C. However, this receptor also reportedly mediates activation of several other effectors such as phospholipase D, phospholipase  $\text{A}_2$ , adenylyl cyclase and several members of the MAPK family (reviewed in [44]). Certain synthetic agonists have been found to be capable of biasing GPCR signaling toward a particular proximal effector. This ability of a ligand to direct an activated receptor state to a particular effector output has been termed “functional selectivity” or “effector bias” (for reviews see [45–48]). Within the adrenoceptor family, there is both pharmacological and biophysical evidence for agonist dependent effector bias at  $\beta$ -ARs. Signaling bias has been shown for several ligands at both  $\beta_1$ - and  $\beta_2$ -AR toward adenylyl cyclase and MAPK [49,50].

We observed that Iso occupancy of  $\alpha_{1A}$ -AR caused  $\text{Ca}^{2+}$  transient responses in HEK-293/EBNA cells without observable PLC activation: no formation of individual inositol phosphates or IP accumulation was detected in these cells under the same conditions. Unexpectedly, Iso occupancy of  $\alpha_{1A}$ -AR also mediated ERK1/2 activation, suggesting that  $\text{G}\alpha_q$  coupling is not required for ERK activation by Iso. Adding to the surprise, A-61603 appeared to be significantly more potent at activating ERK as compared to activation of  $\text{G}\alpha_q$ , whereas NE displayed equivalent potencies for both responses. Taken together, the signaling patterns observed with these agonists appear to reveal a spectrum of partially overlapping effector coupling mechanisms. Iso, and the imidazoline A-61603, appear to have a bias toward MAPK activation. Moreover, for A-61603 there seems to be greater receptor reserve or response amplification for the pERK formation response.

The  $\alpha_{1A}$ -AR has also been found to regulate mitogenic responses, but it is not clear if this activity is downstream of  $\text{G}\alpha_q$  engagement [38,51]. Minneman and co-workers have in fact reported NE activation of  $\alpha_{1A}$ -AR-mediated MAPK phosphorylation in rat PC12 cells [51,52], independently of both  $\text{G}\alpha_q$ -induced  $\text{Ca}^{2+}$  mobilization and PKC activity [53]. The same group later showed that deletion of the first three amino acids from the third intracellular loop not only uncouples the receptor from  $\text{G}\alpha_q$ , but also abolishes activation of the MAPK pathway [54]. It was suggested that these two pathways operate independently from one another, yet may depend on similar structural elements of agonist-occupied receptor. Several lines of evidence seem to indicate that in mouse cardiomyocytes, the  $\alpha_{1A}$ -AR-mediated MAPK activation may not be associated with activation of  $\text{G}\alpha_q$  pathway [38,55].  $\alpha_{1A}$ -AR/ $\alpha_{1B}$ -AR double-knock-out mice also developed heart failure after transverse aortic constriction, and reconstitution of



$\alpha_{1A}$ -AR signaling in cardiomyocytes from those animals rescued them from NE-induced apoptosis [56]. The expected IP generation was not detectable in those cardiomyocytes with reconstituted  $\alpha_{1A}$ -AR function or in wild-type mouse cardiomyocytes, pointing at a non-Gq signaling mode for this receptor in myocardial function. More importantly, Huang et al. (2007) reported that  $\alpha_{1A}$ -AR stimulation of an ERK-mediated pathway is critical for cardiomyocyte survival. Our data support those findings as well as provide further evidence indicating that Iso occupancy of  $\alpha_{1A}$ -AR induces an active receptor “state” that leads to MAPK pathway activation without activating the canonical  $G\alpha_q$  pathway.

Our previous studies of  $G\alpha_q$  coupling defective variants of  $\alpha_{1A}$ -AR combined with examination of the effects of Ca(II) channel blockers uncovered cross-talk between  $\alpha_{1A}$ -AR and  $\beta_2$ -AR that leads to potentiation of a  $G\alpha_q$ -independent signaling cascade in response to  $\alpha_{1A}$ -AR activation [27]. Moreover, this signaling event was accompanied by Ca(II) mobilization with unusual kinetics mirroring the kinetics observed for Iso induced Ca(II) mobilization in  $\alpha_{1A}$ -AR\_HEK293/EBNA cells. Interestingly, in the presence of a  $\beta$ -AR-selective antagonist, the amplitude of the  $\alpha_{1A}$ -AR-mediated  $Ca^{2+}$  transient response to Iso (*i.e.* the lower potency phase of the Iso concentration-effect relationship) was not only lower than expected, but also occurred with a significant delay in onset. This may indicate a synergy occurs between these receptors in generating the lower potency phase of response. Depletion of extracellular Ca(II) did not affect Iso induced activation of  $\alpha_{1A}$ -AR indicating that intracellular stores were the source of Ca(II) released. Finally, this signaling cascade did not involve coupling to  $G\alpha_q$ ,  $G\alpha_s$  or  $G\alpha_i$  but led to receptor internalization and activation of ERK1/2. An alternate mechanism for the control of  $Ca^{2+}$  release from the ER involves ryanodine receptors (RyRs). They have been shown to exist in several non-excitabile cell lines, although their functional expression in HEK-293 cells is controversial. RT-PCR analysis of HEK-293/EBNA cells found that significant levels of RyR2 mRNA are present in these cells.

Recent reports provide an independent line of evidence for  $\alpha_{1A}$ -AR-mediated activation of endocytic pathway that leads to phosphorylation of ERK1/2, independent of  $G\alpha_q$ /PLC/PKC signaling [57,58]. In one study, dynamin mutants were employed to induce trafficking defects, along with various molecules disrupting actin and tubulin organization. Liu and coworkers show that  $\alpha_{1A}$ -AR induced activation of ERK1/2, but not p38 MAPK, was dependent on cytoskeleton and actin organization. On the other hand,  $\alpha_{1A}$ -AR-induced activation of PKC and C-Raf was not affected by endocytosis disruption. Neither PKC nor PLC inhibition blocked  $\alpha_{1A}$ -AR induced activation of ERK1/2 [57]. More recently, the spatial-temporal characteristics of receptor internalization and ERK1/2 activation in response to stimulation of  $\alpha_{1A}$ -AR in HEK293 were investigated in combination with various inhibitors of PKC, receptor internalization as well as  $\beta$ -arrestin 2 silencing and differential patterns were discovered for Gq/PKC dependent as compared to the  $G\alpha_q$ -independent signaling cascade. The rapid phosphorylation of ERK1/2 was dependent on activation of PKC downstream of  $G\alpha_q$  and resulted in pERK1/2 translocation into the nucleus while sustained activation of ERK1/2 that was limited to cytoplasmic compartment, was independent of PKC but dependent on receptor internalization into acidified endosomes and was mediated by  $\beta$ -arrestin 2 [58]. Together, those findings and our own data provide strong evidence that  $\alpha_{1A}$ -AR can induce activation of ERK1/2 in a manner that is independent of the canonical  $G\alpha_q$ /PLC/PKC signaling cascade but involves receptor internalization. We also found that Iso is a biased agonist that can preferentially stimulate  $\alpha_{1A}$ -AR mediated activation of MAPK signaling pathway through receptor internalization and without induction of  $G\alpha_q$  coupling. Although, our study did not conclusively detect Iso induced  $\alpha_{1A}$ -AR association with  $\beta$ -arrestins after agonist application, involvement of  $\beta$ -arrestin 2 in mediating this response cannot be ruled out. Several groups have demonstrated that agonist-mediated GPCR association with  $\beta$ -arrestin leads to G protein-independent activation of

the MAPK pathway [23]. In fact, it has been shown recently that NE occupied  $\alpha_{1A}$ -AR associates although weakly with both  $\beta$ -arrestin-1 and 2, but coupling to the MAPK pathway was not investigated in this study [59]. Further studies including  $\beta$ -arrestin knock-downs will be needed to fully determine whether or not this signaling mechanism is actually dependent on  $\beta$ -arrestin 2.

On the other hand, it has been recently shown that a genetic variant of the  $\alpha_{1A}$ -AR transactivates EGFR via a  $\beta$ -arrestin1-dependent mechanism similarly to the  $\beta_1$ -AR mediated EGFR transactivation and leads to ERK1/2 activation downstream of EGFR [60–62]. Transactivation of EGFR by  $\beta$ -AR confers cardioprotection [61,62] suggesting that transactivation could be involved in the antiapoptotic and cardioprotective activity of the  $\alpha_{1A}$ -subtype. We have examined this potential mechanism in mediating the Iso induced  $\alpha_{1A}$ -AR stimulation of ERK1/2 using EGFR inhibitor AG1478 and found that activation of ERK was not affected by the inhibition of EGFR (data not shown). Further studies may be needed to fully explore this mechanism.

Our observations differ from those of Sun *et al.*, who reported that Iso evoked biphasic dose-effect relations in MAPK activation [24]. The observed biphasic behavior was attributed to distinct processes by which MAPK activity is modulated, either through regulation of adenylyl cyclase by  $\beta_2$ -AR/ $G_{\alpha_s}$ , or by formation of a macromolecular complex containing  $\beta_2$ -AR and c-Src which directly activates c-Src. The conclusion that  $\beta_2$ -AR was the sole mediator of both phases of the Iso response was based on their complete blockade by 1  $\mu$ M ICI118551, as well as lack of detectable MAPK activation in MEF cells derived from  $\beta_1$ -AR<sup>-/-</sup> and  $\beta_2$ -AR<sup>-/-</sup> mice. Although these findings offer strong evidence for  $\beta_2$ -AR involvement in mediating that response, they do not necessarily rule out a synergy between  $\beta_2$ -AR and  $\alpha_1$ -ARs, if present in those cells.

Interestingly, we observed that in the presence of a  $\beta$ -AR-selective antagonist, the kinetics of Iso-evoked  $\alpha_{1A}$ -AR-mediated Ca(II) transient response was much slower and with the greatest delay (Fig. 2C), and the the amplitude of the  $\alpha_{1A}$ -AR-mediated Ca(II) transient (*i.e.* the lower potency phase of the Iso concentration-effect relationship- Fig. 1B) was lower than expected, indicating synergy between these receptors in generating the low potency response phase. This is consistent with previously published results with wild-type  $\alpha_{1A}$  AR and  $G_{\alpha_q}$  signaling deficient variants expressed in HEK EBNA cells with endogenous expression of  $\beta_2$  AR that showed that  $\beta_2$  AR signaling actually potentiates  $\alpha_{1A}$  AR-mediated Ca(II) response and the potentiation was most striking in cells expressing  $G_{\alpha_q}$  defective mutants [27]. Furthermore, this potentiation of Ca(II) transient was most evident for the signal not blocked by Xestospongin C/2-APB application, and consistent with the non-Gq/ERK signaling cascade.

Nonetheless, Iso can also induce intracellular Ca<sup>2+</sup> mobilization (as well as ERK activation) in CHO cells lacking  $\beta_2$ -AR expression, upon stable expression of  $\alpha_{1A}$ -ARs (Fig. 9) and in  $\alpha_{1A}$ -AR\_ HEK-293/EBNA cells in presence of  $\beta$ -AR antagonists indicating that this signaling is not dependent on  $\beta_2$ -AR. The potency of Iso at mediating the Ca(II) response in  $\alpha_{1A}$ -AR\_ CHO cells ( $EC_{50} = 18 \pm 8 \mu$ M) was somewhat lower than the potency of the second phase observed in HEK-293/EBNA cells ( $EC_{50} = 2.6 \mu$ M). This response was blocked by an  $\alpha_{1A}$ -AR-selective antagonist, but was not sensitive to the  $\beta$ -AR selective antagonist propranolol. On the other hand, in untransduced HEK-293/EBNA cells expressing  $\beta_2$ -ARs which lack  $\alpha_{1A}$ -AR expression, we observed only a high potency, monophasic response to Iso for both intracellular Ca<sup>2+</sup> mobilization and p-ERK formation.

Although, this study utilizes a model system where  $\alpha_{1A}$ -AR was transiently expressed at low, physiological levels together with endogenous  $\beta_2$ -AR in HEK293(EBNA), evidence already exist suggesting that similar mechanism may be utilized in physiologically relevant settings such as *e.g.* cardiomyocytes where both receptors are co-expressed. Sabri *et al* observed that in rat cardiomyocytes ERK activation in response to NE was mostly mediated by  $\alpha_1$ -AR

receptor and only a minor component of ERK was activated by  $\beta$ -AR[63]. In the same cardiomyocytes Isoproterenol induced p38-MAPK, cAMP and enhanced contractile function at concentrations that retained  $\beta$ 2-AR selectivity while ERK1/2 activation required 100 fold higher dose (of 10  $\mu$ M). On the other hand Zinterol induced ERK activation at  $\beta$ 2-AR selective concentrations indicating that receptor reserve was not the problem. Role of other adrenoceptors in mediating ERK1/2 activation in response to ISO was not addressed. In a more recent publication cardioprotective and cardiotoxic effects of Isoproterenol on feline cardiomyocytes *in vitro* and *in vivo* have been reported. The cardiotoxic effect was found to be mediated by PKA and sarcoplasmic Ca(II) overload downstream of  $\beta$ -AR since the effect could be blocked by PKA inhibitory peptide and  $\beta$ -AR antagonist. On the other hand the cardioprotective effect was attributed to ERK activation mediated by EPAC downstream of  $\beta$ -AR [64]. Although strong evidence implicating EPAC in the Iso mediated cardioprotective signaling was presented, the response was induced by high non-selective Iso concentrations (10  $\mu$ M) and could not be fully blocked by  $\beta$ -AR antagonist metoprolol (at 20 mg/kg body weight) implicating potentially involvement of other non- $\beta$ -AR receptor. Interestingly, earlier studies performed with  $\alpha_{1A/B}$ -AR KO mice reported that  $\alpha_{1A}$ -AR induced ERK signaling pathway is cardioprotective and required for cardiomyocyte's survival[38]. The hearts of AB KO mice had worse fibrosis and increased cardiac cell death as compared to those from wild-type littermates when pressure loaded, while NE or Iso treatment of cultured cardiomyocytes from the KO mice resulted in more necrosis and apoptosis. The enhanced susceptibility to cell death could be rescued by re-introduction of  $\alpha_{1A}$ -AR but not  $\alpha_{1B}$ -AR and required activation of ERK1/2. Similarly, in neonatal rat myocytes,  $\alpha_1$ -AR stimulation inhibited apoptosis caused by cAMP, and was abolished by a MEK inhibitor suggesting a role for ERK1/2[65]. Recent evidence points also at potential protective effects of  $\alpha_{1A}$ -AR signaling in the CNS (reviewed in [66]). Chronic  $\alpha_{1A}$ -AR stimulation was shown to increase neurogenesis, enhance learning and memory while also protecting the brain from anoxia and traumatic injury, seizures, and age-dependent neurodegeneration.

In conclusion, we have found that isoproterenol is a low potency agonist at  $\alpha_{1A}$ -ARs, and appears to manifest effector bias when bound at  $\alpha_{1A}$ -AR, inducing activation of a  $G\alpha_q$ -independent signaling cascade. This may explain decades-old observations of unusual properties reported for this ligand. As discussed earlier, in cardiomyocytes, induction of the MAPK pathway via  $\alpha_1$ -ARs has been shown to protect those cells from apoptosis [38]. In contrast, increased  $G\alpha_q$  signaling induced cardiac hypertrophy and loss of  $\beta$ -AR inotropic responsiveness [67], ultimately leading to heart failure [68]. Thus, our discovery of the apparently biased coupling at  $\alpha_{1A}$ -AR of the agonist ISO, adds pharmacological and chemical support for the potential to identify a novel class of therapeutics for treatment of heart failure [56,69]. Although, isoproterenol itself will not be a drug candidate in this respect, it can serve as benchmark for the search of molecules with such pharmacological profile. Meanwhile, caution should be taken while using isoproterenol in non-recombinant, undefined systems, with proper controls and/or selective antagonists included in those experiments to minimize confounds attributable to the involvement of  $\alpha$ -ARs in monitored outcomes.

## Supporting Information

**S1 Fig. Inositol phosphate accumulation in  $\alpha_{1A}$ -AR transduced HEK-293/EBNA cells occurs in response to A-61603 but not in response to Iso.** Inositol phosphate accumulation responses are shown for A-61603 (squares) or Iso (circles) in  $\alpha_{1A}$ -AR transduced HEK-293/EBNA cells after pre-treatment with vehicle (filled symbols) or antagonist (empty symbols). HEK-293/EBNA cells were exposed to baculovirus encoding  $\alpha_{1A}$ -AR for 3–4 hours and then

cultured in fresh media containing 4 mM NaBu for 18 hours prior to use in experiments as described in [Materials and Methods](#). Cells were preincubated for 20 min with vehicle or 10 nM of  $\alpha_{1A}$ -AR antagonist RS-100329 and then exposed to agonist for 10 minutes. IP concentration was determined by homogenous time-resolved immunofluorescence detection method. The plot is representative of two independent experiments with each data point being an average of quadruplicate.

(TIF)

**S2 Fig. MAPK activation in  $\alpha_{1A}$ -AR transduced and mock transduced negative control HEK-293/EBNA cells treated with Iso.** HEK-293/EBNA cells that were transduced with baculovirus bearing aldehyde oxidase (as negative control vector) (☒) or  $\alpha_{1A}$ -AR (●), were pre-treated with NaBu for 18 h to induce receptor expression. Cells were stimulated for 5 min with increasing concentration of Iso. Agonist treatment was terminated by addition of SureFire lysis solution. Samples were analyzed for levels of phospho-ERK using an AlphaScreen SureFire p-ERK assay kit. Plots are representative of two independent experiments, with each data point being the average of triplicates.

(EPS)

**S3 Fig. Stimulation of  $\alpha_{1A}$ -AR transduced HEK-293/EBNA with A-61603 and Iso leads to an increase in intracellular  $\alpha_{1A}$ -AR.** HEK293 cells were transiently transfected with  $\alpha_{1A}$ -AR. After serum deprivation for 24h, cells were pre-treated with a membrane impermeable, disulfide-cleavable biotin reagent to label plasma membrane  $\alpha_{1A}$ -AR. Cells were then left untreated, or stimulated 1  $\mu$ M A-61603(A) or 1mM ISO(I) for 5, 30, or 60 min. After treatment, one dish of control cells was harvested without any further manipulations (C: total  $\alpha_{1A}$ -AR). The remaining seven dishes were divided into one control (C+GSH), three treated with A-61603 (A-61603+GSH) and three treated with ISO (ISO+GSH). They were stripped of surface biotin label using a reducing agent, in order to reveal internalized, labeled  $\alpha_{1A}$ -AR. Samples were then analyzed by immunoprecipitation (IP) with streptavidin followed by immunoblotting (IB) with an anti-FLAG antibody. Bands were quantified by densitometry, normalized to control. Plots are representative of three independent experiments.

(EPS)

**S4 Fig. Concentration-response relation to Iso stimulation in  $\alpha_{1A}$ -AR-free Chinese hamster ovary cells stably expressing recombinant CCR5.**  $Ca^{2+}$  transients (expressed as  $\Delta F/F_0$ ) were measured as a function of Iso concentration (☒) in fluo3-loaded cells by fluorometric plate imaging (FLIPR). Cells were stimulated with CCR5 agonist MIP1 $\alpha$  (∇, positive control) at 1  $\mu$ M concentration to confirm their responsiveness. Plots are representative of two independent experiments with each data point being the average of triplicates.

(EPS)

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## Author Contributions

Conceived and designed the experiments: AJC AB APDWF DB MEM. Performed the experiments: AJC AB KN SS HH. Analyzed the data: AJC AB APDWF DB MEM. Contributed reagents/materials/analysis tools: AJC AB KN SS HH AK PJD BF. Wrote the paper: AJC AB KN SS HH AK PJD BF JRR APDWF DB MEM.

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