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Phosphodiesterase 4 Mediates Interleukin-8-Induced Heterologous Desensitization of the β_2-Adrenergic Receptor

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Abstract

Acute respiratory distress syndrome (ARDS) is a life-threatening illness characterized by decreased alveolar-capillary barrier function, pulmonary edema consisting of proteinaceous fluid, and inhibition of net alveolar fluid transport responsible for resolution of pulmonary edema. There is currently no pharmacotherapy that has proven useful to prevent or treat ARDS, and two trials using beta-agonist therapy to treat ARDS demonstrated no effect. Prior studies indicated that IL-8-induced heterologous desensitization of the beta2-adrenergic receptor (β_2-AR) led to decreased beta-agonist-induced mobilization of cyclic adenosine monophosphate (cAMP). Interestingly, phosphodiesterase (PDE) 4 inhibitors have been used in human airway diseases characterized by

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AUTHOR CONTRIBUTIONS

T Rich, S. Leavesley and B. Wagener designed research; T. Rich, S. Leavesley, A. Brandon, C. Evans, and B. Wagener performed research; T. Rich and S. Leavesley contributed analytic tools; T. Rich, S. Leavesley, A. Brandon, C. Evans and B. Wagener analyzed data; B. Wagener wrote the initial manuscript draft; T. Rich, S. Leavesley, A. Brandon, C. Evans, S. Raju and B. Wagener edited and finalized the manuscript draft for submission.

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low intracellular cAMP levels and increases in specific cAMP hydrolyzing activity. Therefore, we hypothesized that PDE4 would mediate IL-8-induced heterologous internalization of the β_2-AR and that PDE4 inhibition would restore beta-agonist-induced functions. We determined that CINC-1 (a functional IL-8 analog in rats) induces internalization of β_2-AR from the cell surface, and arrestin-2, PDE4, and β_2-AR form a complex during this process. Furthermore, we determined that cAMP associated with the plasma membrane was adversely affected by β_2-AR heterologous desensitization. Additionally, we determined that rolipram, a PDE4 inhibitor, reversed CINC-1-induced derangements of cAMP and also caused β_2-AR to successfully recycle back to the cell surface. Finally, we demonstrated that rolipram could reverse CINC-1-mediated inhibition of beta-agonist-induced alveolar fluid clearance in a murine model of trauma-shock. These results indicate that PDE4 plays a role in CINC-1-induced heterologous internalization of the β_2-AR; PDE4 inhibition reverses these effects and may be a useful adjunct in particular ARDS patients.

Keywords

Acute lung injury; FRET; internalization; recycling; trauma

INTRODUCTION

Acute respiratory distress syndrome (ARDS) is a life-threatening illness that is characterized by decreased alveolar-capillary barrier function, pulmonary edema consisting of proteinaceous fluid and inhibition of net alveolar fluid transport that is responsible for resolution of pulmonary edema (1–3). Furthermore, the medical costs relating to this specific diagnosis are staggering (4). While advances in bundled care and improved ventilator strategies have improved mortality rates in recent years, there is currently no pharmacotherapy proven useful to prevent or treat ARDS (5). Specifically, at least two human trials have attempted to use beta-agonist therapy to treat ARDS and failed (6, 7) despite pre-clinical evidence supporting the use in ARDS patients (8–11). Importantly, one of the trials was stopped for futility (7) and the other did demonstrate effects on ARDS, but also increased mortality in the group treated with intravenous β_2-agonists (6). Furthermore, a meta-analysis evaluating multiple trials using β_2-agonists to treat ARDS has revealed no utility to this therapy in isolation (12). Part of the reason that sole use of β_2-agonsits have failed as an ARDS therapy may lie within the fact that there are multiple possible inciting events, or endotypes, leading to ARDS including, but not limited to, bronchopneumonia and trauma-hemorrhage (13).

Interestingly, recent publications indicate that pro-inflammatory cytokines and chemokines thought to be responsible for dysfunctional mechanisms found during the early phase of ARDS are differentially released by various inciting events (14–16); interleukin-8 (IL-8) and angiopoietin-2 are among the pro-inflammatory cytokines released after trauma-hemorrhage. Previous studies have demonstrated that increased levels of IL-8 inhibit net ion and fluid transport in alveolar epithelial cells (17) and that transforming growth factor-beta-1 (TGF-β1) may act synergistically to further inhibit vectorial transport (18). These studies indicated that IL-8 induced heterologous desensitization of the beta2-adrenergic receptor (β_2-AR)

leading to decreased beta-agonist-induced mobilization of cyclic adenosine monophosphate (cAMP) and subsequent inactivation of Na^+ and Cl^- ion transport.

Phosphodiesterases (PDE) are enzymes that degrade cyclic nucleotides that are important to signal transduction and are emerging as integral contributors to signal specificity, including the control of local, subcellular concentrations of cAMP (19). PDE families show distinct kinetic and regulatory properties with some specifically hydrolyzing cAMP (PDE4, 7, 8), cAMP or cGMP (PDE2, 3, 10, 11) or only cGMP (PDE5, 6, 9) (20). PDE3 and PDE4 account for 60% of PDE activity in the lung epithelium (21), while PDE4 was found to be the major contributor for cAMP-PDE activity in alveolar epithelial cells (22). Additionally, PDE4 plays a critical role in the regulation of cAMP mobilization by the β2-AR signaling pathway (21, 23–27). Thus, PDE4 inhibitors have been used to treat human airway diseases, such as chronic obstructive pulmonary disease (COPD) that are characterized by low intracellular cAMP levels and increased cAMP hydrolyzing activity, in order to increase β_2-AR agonist-mediated bronchodilatation (28–31). Furthermore, while roflumilast has been effective in improving lung function and decreasing morbidity in COPD (32), therapies using β_2-AR agonists and PDE4 inhibitors have been shown to act synergistically to improve overall function (33). Finally, PDE4 inhibitors have been shown to attenuate the severity of acute lung injury caused by *Escherichia coli* endotoxin or cardiopulmonary bypass in rats (34, 35).

In this study, we hypothesized that PDE4 would mediate IL-8-induced heterologous internalization of the β_2-AR in alveolar epithelial cells that leads to decreased net alveolar fluid transport and that PDE4 inhibition would restore beta-agonist-mediated functions. We exposed alveolar epithelial cells to cytokine-induced neutrophil chemoattractant-1 (CINC-1)—a functional IL-8 analog that models IL-8-mediated acute lung injury in the rodent (36–38). First, we examined the more specific mechanisms by which CINC-1 desensitizes the β_2-AR at the cell membrane. Next, we demonstrated that CINC-1-induced internalization of the β_2-AR involves direct binding of both arrestin-2 and PDE4. Furthermore, we examined which specific pools of intracellular cAMP were adversely affected by β_2-AR heterologous desensitization. Additionally, we determined that rolipram (a PDE4 inhibitor) reverses CINC-1-induced derangements in temporal and spatial cAMP mobilization. Next, we revealed that not only does CINC-1 induce internalization of the β_2-AR from the cell surface, but the β_2-AR cannot successfully recycle back to the cell surface in the process of receptor resensitization; however, treatment of cells with rolipram both prevents CINC-1-induced internalization of the β_2-AR and promotes its recycling after the receptor has been internalized. Finally, we demonstrated that rolipram could reverse CINC-1-mediated inhibition of beta-agonist-induced alveolar fluid clearance (AFC) in a murine model of trauma-shock.

MATERIALS AND METHODS

Reagents.

CINC-1, terbutaline and mouse monoclonal ANTI-FLAG BioM2 antibody (Ab) were purchased from Sigma (St. Louis, MO, USA); ADRB2 polyclonal Ab, FITC conjugated (H-20) was purchased from Bioss (Woburn, MA, USA); GAPDH monoclonal Ab (14C10)

was purchased from Cell Signaling; PIK-90 was purchased from Cayman Chemical (Ann Arbor, MI, USA); protein A–Sepharose beads were purchased from Pierce (Rockford, IL, USA); PDE 4D polyclonal Ab (14613) was purchased from Abcam (Cambridge, MA, USA); rolipram was purchased from MP Biomedicals (Waltham, MA, USA); Odyssey Blocking Buffer, IRDye® 680RD and 800CW Donkey anti-Rabbit IgG Abs and IRDye® 680 RD and 800CW Donkey anti-Mouse IgG Abs were purchased from LI-COR (Lincoln, NE, USA); Primocin was purchased from InvivoGen (San Diego, CA, USA); Precision Red Advanced Protein Assay was purchased from Cytoskeleton (Denver, CO, USA); all other reagents were purchased from Fisher Scientific (Waltham, MA, USA).

Rat Type II Alveolar Cell Isolation.

All animal interventions were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Alabama at Birmingham. Rat type II alveolar (ATII) cells were isolated as previously described (39) with modifications. Rats were anesthetized with ketamine/xylazine and lungs were subjected to elastase digestion, mincing and further incubation with DNase. Fetal bovine serum (FBS) was added, and cells were separated via sequential filtration through a 100μm and 40μm mesh. Cells were gently centrifuged, resuspended in SFM in Petri dishes, and incubated at 37°C with 5% CO_2 for 1 hour. Cells in suspension were collected, centrifuged, and plated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 100 units/ml penicillin/streptomycin and 100μg/ml Primocin (InvivoGen, San Diego, CA, USA). Cells were ready for experimentation 72–96 hours later, and purity is >95% by Trypan Blue staining.

Cell Culture.

ATII cells were maintained in DMEM supplemented with 10% FBS, 100 units/ml penicillin/streptomycin and 100μg/ml Primocin. L2 rat lung alveolar epithelial cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA) and were propagated and maintained in F-12K medium supplemented with 10% FBS and 100 units/ml penicillin/streptomycin at 37°C with 5% CO_2 per ATCC instructions.

Western Blotting.

Western blotting of lysates and immunoprecipitations (IP) were performed as previously described (40). Lysates or IPs were resolved with 10% SDS–PAGE (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Burlington, MA, USA) and blocked for 1 hour with Odyssey Blocking Buffer. Blotting was carried out using 1:1000 dilution of M2 mouse anti-FLAG Ab, 1:500 dilution of β_2-AR Ab, 1:500 dilution of PDE4D Ab or 1:1000 dilution of GAPDH Ab, accordingly, in Odyssey Blocking Buffer overnight at 4°C. Blots were washed three times with Tris-buffered saline with 0.1% v/v Tween 20 (TBS-T) and incubated with appropriate secondary Abs in Odyssey Blocking Buffer for 45 minutes at room temperature. Blots were again washed three times with TBS-T. Bands were visualized and densitometry was performed using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) and ratios expressed as mean ratio ± SEM β_2-AR or PDE4D/arrestin-2 immunoprecipitated, respectively, and normalized to zero time-points.

β2-AR Internalization.

β2-AR internalization was measured as previously described (40, 41). L2 cells were grown to confluence, harvested by trypsinization and resuspended in serum-free medium (SFM). Cells were incubated with 10 ng/ml CINC-1 at 37°C, and aliquots were removed and placed in cold SFM at 0, 30, 60, 120, 240 and 360 minutes. In some experiments, cells were pre-treated with desired inhibitor (or appropriate vehicle) before the addition of CINC-1. Cells were washed extensively with cold SFM to remove excess CINC-1. Cells were then resuspended in cold SFM containing 1:200 FITC-conjugated ABRB2 polyclonal antibody and analyzed using a Beckman-Coulter Cytomics FC500 (Beckman Coulter, Brea, CA, USA) at appropriate wavelengths. Live cells were gated using forward- and side-scatter parameters. Live cells were gated, and mean channel fluorescence (MCF) was measured in FL-1 to determine the amount of cell surface receptor. Non-specific background was determined by measuring MCF of L2 cells without antibody and assaying as described. Non-specific binding was subtracted before further analysis. MCF from unstimulated cells represents 100% β_2-AR cell surface expression. Cell surface expression from stimulated cells was calculated by dividing the MCF following treatment by the MCF from unstimulated cells.

Transfection and Plasmids.

Transient transfection of L2 cells was performed with Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer instructions. Arrestin-2-FLAG plasmid (40) and pmEpac2 and cytEpac3 plasmids (42–44) were previously described.

Co-Immunoprecipitation.

Co-IP was performed as previously described (40). L2 cells transiently transfected with FLAG-tagged arrestin-2 were grown to confluence. Cells were serum starved for 30 minutes and stimulated for the designated times with 10 ng/ml CINC-1 in SFM at 37°C. Medium was removed and cold co-IP lysis buffer [1ml of 1% v/v Triton-X-100, 150mm NaCl, 10mm Tris–HCl pH 7.4 supplemented with protease inhibitor cocktail (Calbiochem, San Diego, CA, USA)] was added immediately. Lysates were collected, incubated on ice for 30 minutes and centrifuged at maximum speed for 30 minutes at 4°C. An aliquot from each tube was set aside for determining pre-IP levels of proteins by Western blot. For IPs, 25μl of protein A–Sepharose was washed 3× with co-IP lysis buffer. Beads were rotated for at least 1 hour at 4°C in 250μl of co-IP lysis buffer with 1:1000 biotinylated M2 anti-FLAG antibody. Beads were washed with co-IP lysis buffer, and lysates were added and rotated overnight at 4°C. The following day, beads were washed again with co-IP lysis buffer, 40μl of 2× sample buffer was added, and immunoprecipitated proteins were released by boiling for 5 minutes.

cAMP Measurement.

L2 cells were grown to confluence and serum-starved for 30 minutes. Cells were then exposed to terbutaline (20μM) for 5 minutes to activate cAMP. For some experiments, cells were pre-treated with CINC-1 (10ng/ml) or CINC-1 and rolipram (10μM) for 30 minutes before the addition of terbutaline. After treatment with terbutaline, cells were lysed

and assayed with a cAMP ELISA (R&D Systems, Minneapolis, MN, USA) according to manufacturer instructions.

FRET Measurement of cAMP.

FRET for measurement of localized cAMP was performed as previously described (26). Briefly, coverslips with confluent L2 cells transiently transfected with pmEpac2 or cytEpac2 were placed in 300μl of oxygenated Locke's medium (154mM NaCl, 5.6mM KCl, 2.2mM CaCl2, 1mM MgCl2, 6mM NaHCO3, 10mM glucose, 2mM HEPES) containing 0.05% BSA in a temperature-controlled (37 °C), modified Sykes-Moore Chamber mounted on a Nikon TE2000 inverted fluorescence microscope (Nikon Instruments, Inc., Melville, NY, USA). Cells were imaged under a 100× epifluorescence objective using a Xenon light source. Images were captured using NIS Elements 5.0 Imaging Software (Nikon Instruments, Inc., Melville, NY, USA). Cyan fluorescent protein (donor) fluorescence was measured using an excitation filter with 430–455 nm bandpass and an emission filter with of 470–490 bandpass. Yellow fluorescent protein (acceptor) fluorescence was measured using an excitation filter with 500–520 nm bandpass and an emission filter with of 535–565 nm bandpass. FRET was measured using an excitation filter with 430–455 nm bandpass (donor excitation) and an emission filter with 535–565 nm bandpass (acceptor emission). Average FRET intensity was estimated from corrected images. Changes in FRET are calculated as a percentage of basal FRET (%ΔF).

β2-AR Recycling.

β2-AR recycling was measured as previously described (40). L2 cells were grown to confluence, harvested by trypsinization and resuspended in SFM. An aliquot was removed to measure total cell surface receptor. The remaining cells were stimulated with 10 ng/ml CINC-1 in SFM at 37°C for 4 hours and were then washed extensively to remove excess unlabelled ligand. Half of the remaining cells were resuspended in pre-warmed SFM at 37°C to allow the FPR to recycle and were then moved to pre-chilled SFM at 1 or 2 hours. The other half was kept on ice to measure post-internalization cell surface receptor levels. All aliquots were then resuspended in SFM containing 1:200 FITC-conjugated ABRB2 polyclonal antibody and assayed by flow cytometry. For analysis, assayed cells were gated for live cells using forward and side scatter parameters. Live cells were gated, and MCF was measured in FL-1 to monitor cell surface expression of the β_2-AR. Non-specific background was determined by measuring MCF of L2 cells without antibody and assaying as described. Non-specific binding was subtracted before further analysis. MCF from unstimulated cells represents 100% β_2-AR cell surface expression. Cell surface expression from stimulated cells was calculated by dividing the MCF following treatment by the MCF from unstimulated cells.

Trauma-Hemorrhage Model.

The TH-resuscitation mouse model was performed as previously described (45). C57BL/6 male mice were anaesthetized by inhalation of 5% isoflurane in air and isoflurane was then reduced to the minimal concentration for maintenance. The abdomen and groins were shaved and washed with 10% povidone-iodine, and a 2 cm midline laparotomy was performed to induce soft tissue trauma. Both femoral arteries were cannulated with catheters

(Braintree Scientific, Braintree, MA, USA). Systemic arterial pressure was continuously monitored through one arterial line while hemorrhage and resuscitation were performed via the other. Mice were bled over 30 minutes to a mean arterial pressure of 30 ± 5 mmHg. This blood pressure was maintained for another 30 minutes. All incision sites are bathed with 2% lidocaine for analgesia and re-bathed as needed. At the end of the 60 minutes hemorrhagic shock period, animals were resuscitated over 30 minutes with fresh packed red blood cells (pRBC) in a 1:1 ration with plasma. After resuscitation, mice were placed into cages with a heating pad for 1 hour and then moved to standard housing.

Alveolar Fluid Clearance.

Alveolar fluid clearance was measured as previously described (17, 46). The mice were anesthetized by an intramuscular injection of ketamine/xylazine. The mice were quickly tracheotomized and bled before fluid instillation. Each mouse was instilled with 0.4ml of 5% BSA/physiological saline solution containing 5μCi ^{125}I. After instillation into the airways, a first aliquot was taken as the reference sample. The tracheotomy cannula was immediately connected to a continuous positive-pressure circuit delivering air with a positive pressure of 8 cm H_2O. The mice were subjected to continuous positive pressure for 30 minutes, after which duplicate aliquots were sampled from the distal airspace. All the samples were weighed, and radioactivity was measured using a γ-counter. The distal airways fluid clearance is expressed as the percentage of alveolar fluid volume cleared during 30 minutes by the following equation: distal airways fluid clearance = $1 - (P_{Instilled}/P_{Final}) \times 100$, where $P_{Instilled}$ is the initial ^{125}I-albumin concentration, and P_{Final} is the final ^{125}I-albumin concentration.

Statistical Analysis.

All data are summarized as mean ± SEM. Statistical analysis was performed using GraphPad Prism, Version 8.3.0 for Windows, GraphPad Software, (La Jolla, CA, USA). The normal distribution was verified using the Kolmogorov-Smirnov test. For normally distributed data, a Student t-test was used to compare two experimental groups. Bonferroni correction, controlling for false positive error rate, was used to adjust for multiple comparisons. A *p*-value of <0.05 was considered statistically significant. All statistical comparison of means was bilateral (two-tailed tests).

RESULTS

CINC-1 induces β_2-AR heterologous desensitization via receptor internalization from the cell surface in a phosphoinositide 3-kinase-dependent manner.

Previous publications have demonstrated heterologous desensitization of β_2-AR-induced cAMP mobilization in both a respiratory syncytial virus (47) and trauma-hemorrhage (17) models. In each study, their respective pulmonary insults 1) increase CINC-1 (rodent IL-8 functional analog) in the lung; 2) demonstrate that CINC-1 inhibition prevents heterologous desensitization; and 3) demonstrate that desensitization occurs in a protein kinase C-zeta-dependent manner. To further elucidate the mechanisms of β_2-AR heterologous desensitization by CINC-1, we examined whole cell β_2-AR expression after CINC-1 exposure (Figure 1). First, we isolated rat primary type II alveolar cells, cultured

them to confluence and exposed them to CINC-1 for 6 hours. Western blotting (Figure 1A) and quantification of densitometry (Figure 1B) demonstrate that whole cell β_2-AR expression does not change after extended exposure to CINC-1. Furthermore, we examined this response in an immortalized type II alveolar cell line that can create a physiologic barrier when cultured at an air-liquid interface and faithfully represents an alveolar epithelial cell phenotype (L2, ATCC; hereafter known as L2 cells) (48, 49). Again, Western blotting (Figure 1C) and densitometry (Figure 1D) indicate no CINC-1-mediated effects on total β_2-AR expression. These data indicate that CINC-1 does not induce heterologous desensitization of the β_2-AR via degradation of the receptor itself.

While our results indicate that β_2-AR degradation is not the mechanism of heterologous desensitization, GPCR signaling can be desensitized by multiple methods including, but not limited to, receptor trafficking and protein binding (50, 51). In a previous report, it was revealed that β_2-AR remained on cell membranes at 30 minutes, but not at 6 hours (17). This report did not answer specific questions about the kinetics and timing of internalization in relation to other receptor events. Therefore, we measured β_2-AR internalization in response to CINC-1 exposure (Figure 2) using flow cytometry (40, 41). We measured this response in both primary and immortalized alveolar epithelial cells. β_2-AR on both primary (Figure 2A) and L2 (Figure 2B) alveolar cells internalizes from the cell surface in response to CINC-1. Internalization of the receptor can be seen in both cell types after 30 minutes and appears maximal after a few hours. About 25% of total cell surface receptor is internalized; this receptor internalization maximum is similar to that seen in cells exposed to β_2-AR agonists (52). Previous studies have indicated that heterologous desensitization of the β_2-AR is phosphoinositol-3-kinase (PI3K)-dependent (17). Thus, we determined whether CINC-1-mediated β_2-AR internalization is PI3K-dependent. L2 cells pre-treated with PIK90 (a PI3K family inhibitor) did not internalize β_2-AR in response to CINC-1 (Figure 2C). Together, these data indicate that one method of β_2-AR heterologous desensitization via CINC-1 is mediated by PI3K.

Heterologous internalization of the β_2-AR by CINC-1 involves β_2-AR binding of arrestin-2 and PDE4.

Archetypal GPCR activation, internalization and desensitization each involve a variety of proteins that bind the GPCR tail to interrupt G protein signaling and initiate trafficking (51, 53). Interestingly, two proteins that are involved with β_2-AR internalization and desensitization of cAMP signaling are arrestins and PDEs. One of the interactions that takes place during homologous desensitization is arrestin-2 binding to the C-terminus of the β_2-AR coupled with PDE4D binding to arrestin-2. This may be one of the mechanisms by which CINC-1 causes heterologous internalization of the β_2-AR (54). To determine whether arrestin-2 and/or PDE4D bind the β_2-AR after exposure to CINC-1, we over-expressed FLAG-tagged arrestin-2 in L2 cells and performed co-IP (40) over a time course of CINC-1 stimulation (Figure 3). First, our data indicate that there is a minimal amount of β_2-AR, arrestin-2 and PDE4D binding at baseline (time zero). This has been described previously for β_2-AR and arrestin-2 (55). Second our data indicate that shortly after exposure to CINC-1, the β_2-AR, arrestin-2 and PDE4D bind together at rates significantly above

baseline interaction. These data indicate a potential role for both arrestin-2 and PDE4D in the heterologous internalization and desensitization of the β_2-AR induced by CINC-1.

CINC-1 desensitizes β_2-AR-mediated cAMP signaling at the plasma membrane that is resensitized by PDE4 inhibition.

Previous studies have indicated that heterologous desensitization of the β_2-AR by CINC-1 leads to decreased mobilization of cAMP. PDEs break down cAMP to provide spatiotemporal control of secondary messenger signaling and delicately regulate the integration of multiple cellular signals that may occur in parallel (19, 56, 57). Furthermore, we have described that PDE4D binds to arrestin-2 shortly after cell exposure to CINC-1 (Figure 3). Inhibition of PDEs is known to prolong cAMP activation and is a well-described therapy in chronic obstructive pulmonary disease (COPD) and asthma (28–31). Therefore, we determined whether rolipram (a PDE4 inhibitor) would inhibit CINC-1-mediated desensitization of cAMP mobilization. L2 cells were pre-treated with CINC-1 and/or rolipram, and terbutaline-induced cAMP mobilization was measured (Figure 4A). Our results confirm that pre-treatment of L2 cells with CINC-1 inhibits β_2-AR-mediated cAMP mobilization similar to prior reports (17, 18). Interestingly, pre-treatment of L2 cells with CINC-1 and rolipram reverses the effects of CINC-1 on terbutaline-induced cAMP mobilization.

While inhibition of CINC-1-induced effects on global cAMP mobilization is essential, PDEs regulate temporospatial cAMP signaling with more precision. Therefore, we hypothesized that CINC-1 would inhibit unique, subcellular pools of cAMP and that PDE4 inhibition would be able to reverse these effects specifically. To better determine the subcellular localization of cAMP desensitization, we used FRET-based exchange protein activated by cAMP (Epac) probes that have been previously described (42–44). These probes induce FRET when cAMP is absent, and when bound to cAMP, the FRET efficiency decreases. Furthermore, one of the probes is palmitoylated to keep it at the cell membrane where it measures FRET while the other probe measures FRET within the cytosol. L2 cells were transiently transfected with the aforementioned probes, and cells were pre-treated with CINC-1 (or vehicle) for 30 minutes before cAMP was activated with terbutaline (Figures 4B and 4C). When cells containing the plasma membrane-bound or cytosolic Epac FRET tracer are activated with terbutaline, cAMP mobilization in both areas is quick and maximizes after a few minutes. Pre-treatment with CINC-1 in L2 cells transfected with the cytosolic Epac FRET tracer shows no effect on cAMP mobilization (Figure 4B). However, in L2 cells transfected with the plasma membrane-bound Epac FRET tracer, pre-treatment with CINC-1 effectively ceases activation of cAMP in those regions (Figure 4C). We hypothesized that PDE4 inhibition could restore CINC-1-mediated inhibition of plasma membrane-localized cAMP mobilization. Therefore, we pre-treated L2 cells transfected with the plasma membrane-bound Epac FRET tracer with CINC-1 and/or rolipram and measured plasma membrane-localized cAMP activation (Figure 4D). Interestingly, rolipram did indeed reverse CINC-1-mediated inhibition of terbutaline-induced cAMP mobilization at the plasma membrane. Taken together, these results indicate that CINC-1 inhibits specific, subcellular pools of β_2-AR-induced cAMP and that these effects can be reversed with PDE4 inhibition.

PDE4 inhibition restores β_2-AR receptor expression at the cell surface after heterologous desensitization by CINC-1 and inhibits CINC-1-induced β_2-AR receptor internalization.

Receptor desensitization and resensitization involves internalization from and recycling to the cell surface to control temporospatial signaling via binding of various trafficking proteins (58–60). We have shown that CINC-1 induces heterologous desensitization of the β_2-AR, in part, by receptor internalization and inhibition of plasma membrane-located cAMP mobilization. Next, we questioned whether CINC-1 could inhibit β_2-AR recycling as a mechanism of heterologous desensitization. L2 cells were exposed to CINC-1 for 4 hours to maximize internalization of the β_2-AR. Cells were washed thoroughly to remove excess CINC-1 and returned to 37°C. In cells exposed to vehicle alone, β_2-AR did not return to the cell surface and may have continued to internalize β_2-AR to a small degree (Figure 5A). Because rolipram can reverse alterations in plasma membrane-located cAMP mobilization, we tested whether it would restore β_2-AR recycling. Interestingly, in cells returned to 37°C and exposed to rolipram, β_2-AR did indeed return to the cell surface (Figure 5A).

Because PDE4 inhibition can restore β_2-AR recycling and reverse alterations in CINC-1-induced heterologous desensitization of the β_2-AR, we theorized that PDE4 inhibition would be able to inhibit CINC-1-induced β_2-AR internalization. To this end, L2 cells were pre-treated with rolipram (or vehicle) before inducing β_2-AR internalization with CINC-1 (Figure 5B). L2 cells pre-treated with rolipram had significantly less β_2-AR internalization in response to CINC-1 compared to cells pre-treated with vehicle alone.

PDE4 inhibition restores trauma-mediated inhibition of β_2-AR-induced alveolar fluid clearance.

To this point, we have described some of the trafficking and signaling mechanisms of CINC-1-induced β_2-AR heterologous internalization. Clinical significance of these mechanisms within the lung is based on its ability to increase AFC and resolve pulmonary edema. Trauma releases IL-8 into patient lungs and may be one of reason why patients are more susceptible to ARDS thereafter. Similarly, it has been described in animal models that trauma-shock increases CINC-1 in rodent lungs and that CINC-1 inhibition restores AFC (17, 47). To better understand whether PDE4 inhibition could restore CINC-1-induced β_2-AR heterologous desensitization and improve pulmonary edema, we employed our murine trauma-hemorrhage model (Figure 6). First, we measured AFC in mice that were not exposed to trauma and shock but were exposed to the individual drugs to test their effects on baseline AFC (Figure 6A). Baseline AFC in control mice was ~7–8%. Treatment of mice with terbutaline doubled the AFC rate in control mice. Pre-treatment of mice with rolipram for 30 minutes had no effect on baseline AFC. Our next groups of mice were exposed to trauma and hemorrhagic shock (hereafter referred to as “trauma-shock), and AFC was measured after pre-treatment with the aforementioned therapies (Figure 6B). Mice that underwent trauma-shock again had baseline AFC of ~7–8% that was comparable to controls. When trauma-shock mice were treated with terbutaline 30 minutes before AFC measurement, there was no increase above baseline, in contrast to observations in control mice. However, in trauma-shock mice pre-treated with rolipram, terbutaline was able to significantly increase AFC. These data indicate that PDE4 inhibition can reverse trauma-shock-mediated inhibition of terbutaline-induced AFC.

DISCUSSION

ARDS is a life-threatening illness that is characterized by decreased alveolar-capillary barrier function, pulmonary edema consisting of proteinaceous fluid, and inhibition of net alveolar fluid transport that is responsible for resolution of pulmonary edema (3). While advances in bundled care and improved ventilator strategies have improved mortality rates in recent years, there is currently no pharmacotherapy that has proven useful to prevent or treat ARDS (5). Studies indicate that IL-8 induced heterologous desensitization of the β_2-AR leads to decreased beta-agonist-induced mobilization of cAMP and subsequent inactivation of Na^+ and Cl^- ion transport (17, 47). In our study, we demonstrate that CINC-1-induced heterologous desensitization of the β_2-AR is due to receptor internalization from the cell surface rather than receptor degradation. Our data also indicate that exposure of L2 cells to CINC-1 leads to binding of the β_2-AR by arrestin-2 and PDE4, important components of the β_2-AR's internalization and signaling machinery. Interestingly, we found that CINC-1 inhibited β_2-AR-mediated cAMP mobilization at the plasma membrane but not in the cytosol, and that once the β_2-AR was internalized, it had impaired recycling back to the cell surface. Finally, we demonstrated that PDE4 inhibition could alleviate defects in CINC-1-induced defects in cAMP mobilization, β_2-AR trafficking, and alveolar fluid clearance after trauma/hemorrhage. It is important to note that we also used both primary ATII and L2 alveolar cells to replicate key flow cytometry experiments. This study joins a litany of recent studies that use L2 cells to investigate the physiologic roles of alveolar cells (61–63). This is important because L2 cells are more easily transfected and genetically modified making mechanistic studies on the cellular level more amenable. Taken together, these data reveal a deeper mechanism underlying a key pathophysiologic element of an ARDS endotype caused by release of IL-8 and provides a potential therapy to reverse the dysfunction within this endotype.

Our first goal of this study was to examine the mechanisms of IL-8-induced heterologous desensitization of the β_2-AR. Two prior studies have demonstrated that respiratory syncytial virus (47) or trauma-hemorrhage (17) lead to CINC-1 release that inhibits β_2-AR-mediated mobilization of cAMP. Both of these studies indicated that there was less β_2-AR on isolated plasma membranes after exposure to CINC-1. However, neither study revealed deeper mechanisms of β_2-AR heterologous desensitization. We revealed in both primary ATII alveolar and L2 cells that exposure to CINC-1 had no effect on total β_2-AR cellular levels via Western blot. Interestingly, exposure of both primary alveolar and L2 cells to CINC-1, but not vehicle led to internalization of 20–25% of β_2-AR on the cell surface. Additionally, heterologous internalization began shortly after 30 minutes and was maximal after a few hours. Importantly, this amount of β_2-AR internalization is similar to that seen when cells are exposed to a natural ligand, such as epinephrine (52) suggesting shared mechanisms. In support of such overlap, we found that CINC-1-induced heterologous internalization of the β_2-AR is PI3K-dependent. This is important as the aforementioned previous studies indicate that β_2-AR desensitization of cAMP mobilization is PI3K-dependent.

Next, based on the central role played by cAMP signaling, we demonstrated that when L2 cells are exposed to CINC-1 and β_2-AR internalizes, receptors also form complexes with arrestin-2 and PDE4. It is well known that arrestin-2 binds β_2-AR upon activation

with a cognate ligand (64–66). Furthermore, studies have indicated that PDE4 binds to arrestin-2 as it binds β_2-AR and internalizes to early endosomes leading to phosphorylation and activation of extracellular signal-regulated kinase (ERK) 1/2 (67–69). It is interesting to see that heterologous internalization of the β_2-AR uses similar machinery as the archetypal internalization system. This may indicate a situation in which a ligand that binds one receptor (CINC-1 and IL8R) can hijack and force the internalization and desensitization of another GPCR (in this case, β_2-AR). Furthermore, we show that β_2-AR, arrestin-2, and PDE4 form a complex before exposure to CINC-1. This has been demonstrated in binding studies with β_2-AR and arrestin-2 (55), but not with PDE4 to our knowledge.

Once we detected CINC-1-induced heterologous internalization and binding of β_2-AR by arrestin-2 and PDE4, we hypothesized that this would have specific effects on individual pools of intracellular cAMP. PDE4 is well-known as a regulator of localized cAMP mobilization to control spatiotemporal signaling (19, 56, 57). Furthermore, previous studies indicate that CINC-1 inhibits β_2-agonist-mediated mobilization of global cAMP levels. Our data indicate that plasma membrane-associated cAMP mobilization is inhibited by exposure of L2 cells to CINC-1, but cytosol-associated cAMP appears unaffected, indicating finely regulated and sequestered signaling events. This early inhibition of plasma membrane-associated cAMP is consistent with our other data as: 1) there is very early β_2-AR/arrestin-2/PDE4 complex formation after CINC-1 exposure, and 2) heterologous internalization of the β_2-AR is significantly increased at 30 minutes. Therefore, it is possible that CINC-1 initiates formation of the β_2-AR/arrestin-2/PDE4 complex at the cell surface and degrades these pools of cAMP early. Long-term heterologous desensitization of the β_2-AR may be due to a lack of receptors at the cell surface. Our flow cytometry data indicate that after internalization, the β_2-AR does not recycle back to the cell surface in significant amounts which may make it unavailable to β_2-AR ligands.

To this point, we have delineated cellular mechanisms of heterologous internalization and desensitization. Next, we demonstrated that these processes have significance in *in vivo* by using our trauma-shock model to measure AFC. Without shock, β_2-agonists can recruit movement of ions leading to net fluid movement out of the lungs. Physiologic AFC is not increased by PDE4 inhibition alone implying limited role for cAMP-dependent pathways in healthy conditions. However, after trauma-shock, terbutaline, a β_2-AR agonist, is not able to recruit ion transport and remove fluid out of the lungs in mice. Furthermore, a previous study revealed that patients with acute lung injury have increased IL-8 within their lungs (17). These findings are consistent with CINC-1's ability to trigger heterologous desensitization and internalization—if β_2-AR is not present on the surface of cells, or if its activation is otherwise inhibited, alveolar cells cannot mobilize cAMP and activate ion and fluid movement that stimulates net fluid transport out of the lungs. Again, when this process was reversed by PI3K inhibition with PIK-90, terbutaline was able to increase fluid transport in edematous lungs of mice after trauma-shock.

Most interestingly, PDE4 inhibition alleviated or prevented many of the problems that were caused by CINC-1. For example, heterologous internalization of the β_2-AR, decreased β_2-agonist-induced mobilization of plasma membrane-associated cAMP, inhibition of β_2-AR recycling, and inhibition of alveolar fluid transport were all reversed by treatment with

rolipram. Restoration of cAMP signaling and fluid transport is more easily understood as cAMP mediates both of these processes. How rolipram pre-treatment reverses CINC-1-induced inhibition of heterologous β_2-AR internalization is not entirely clear, but PDE4 has been demonstrated to be integral to epinephrine-induced β_2-AR internalization (67, 70, 71). Furthermore, the mechanism(s) by which restoration of cAMP and/or PDE4 inhibition may improve recycling of the β_2-AR back to the cell surface is poorly understood. However, defective β_2-AR resensitization is critical to asthma pathophysiology (58), and other studies have indicated that impaired GPCR recycling is related to arrestin-2 binding to other trafficking proteins (40, 41). Finally, roflumilast, a clinically-used cousin of rolipram, is used in patients with COPD along with β_2-agonists (32, 33). When used in conjunction, there is improved clinical efficacy and outcomes for patients. Given that β_2-agonist therapy in ARDS patients has not been clinically effective (6, 7), it would be interesting to determine whether co-therapy with roflumilast would improve alveolar fluid transport and provide relief from pulmonary edema.

There are five primary limitations in this study. First, during our internalization studies, we did not use any beta-blocking drugs in conjunction with CINC-1. This could have delineated whether CINC-1 actually binds the β_2-AR to initiate internalization and desensitization. However, previous studies have demonstrated that CINC-1 does induce heterologous desensitization of the β_2-AR via PI3K-, Protein kinase C-, and G protein receptor kinase 2-dependent mechanisms which are downstream of IL-8/CINC-1 (17, 47). Additionally, β_2-AR antagonists, such as carvedilol, can induce internalization of the β_2-AR (72, 73). Second, to determine whether β_2-AR, arrestin-2, and PDE4 would form a complex after exposure to CINC-1, we over-expressed arrestin-2-FLAG in L2 cells. This is not an uncommon procedure, and it is one of the benefits of using immortalized L2 alveolar cells for such studies. While it does enrich the amount of arrestin-2 that is immunoprecipitated, it could change the stoichiometry of the reaction making complex formation more favorable. We feel that the benefits of this study outweigh the drawbacks of such a procedure as complex formation was significantly increased after exposure of these cells to CINC-1. Third, while previous publications have clearly indicated that CINC-1 decreases AFC and we show that PDE4 inhibition is important to reversing the effects of CINC-1 *in vitro* and *in vivo*, neither study clearly indicates that β_2-AR expression is decreased on *in vivo* epithelial cells. Future studies will investigate this important link in the mechanisms of IL-8/CINC-1/trauma-shock-mediated lung injury. Fourth, we used CINC-1 as a model of IL-8-induced ARDS in rat cells. IL-8 is known to be important in ARDS pathophysiology in humans and there is no direct analog of IL-8 in rodents. However, CINC-1 is a commonly used model for IL-8-induced ARDS in rodents as it carries out many of the same functions, including use of similar signaling pathways and neutrophil recruitment (74). Finally, we used rolipram to inhibit PDE4 in all of our *in vitro* and *in vivo* studies instead of clinically-used roflumilast or apremilast. However, rolipram does inhibit many PDE4 family members rendering the findings of our study are clinically applicable.

In summary, we provide new evidence on the mechanism(s) of heterologous internalization and desensitization of a GPCR. Our evidence demonstrates that IL-8 can cause heterologous internalization and desensitization of specific cAMP intracellular pools mediated by β_2-AR activation. Furthermore, our data indicate that PDE4 inhibition via rolipram can inhibit

IL-8-induced heterologous internalization of the β_2-AR and can resensitize the β_2-AR as well. PDE4 inhibition is a cornerstone of treatment of human airway diseases such as COPD and asthma (28–32); additionally, they are often used in concert with β_2-agonists (33) and prior clinical studies using β_2-agonists to treat ARDS have shown no effect (6, 7) and a Phase 2 clinical trial is studying the effects of PDE4 inhibition in ARDS patients (clinicaltrials.gov NCT04429555). One mechanism by which this therapy may be effective is if PDE4 inhibitors can resensitize the β_2-AR and improve its activation of fluid resorption systems within the lung. There may be specific ARDS endotypes that have IL-8 release as a component of its pathophysiology that make PDE4 inhibition a suitable therapy and implements precision medicine. Future studies will continue to explore these avenues and the potential for PDE4 inhibition in other lung diseases such as bacterial pneumonia that can lead to ARDS.

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Abbreviations Used

AFC	alveolar fluid clearance
ALI	acute lung injury
ARDS	acute respiratory distress syndrome
β_2-AR	beta2-adrenergic receptor
cAMP	cyclic adenosine monophosphate
CINC-1	cytokine-induced neutrophil chemoattractant-1
DMEM	Dulbecco’s modified Eagle’s medium
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FRET	Förster resonance energy transfer
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
IL-8	interleukin-8
IP	immunoprecipitation
PAGE	polyacrylamide gel electrophoresis
PDE	phosphodiesterase
PI3K	phosphoinositide 3-kinase

SDS	sodium dodecyl sulfate
SFM	serum-free medium
TGF-β1	transforming growth factor beta-1

REFERENCES

1. Yadav H, Thompson BT, and Gajic O (2017) Fifty Years of Research in ARDS. Is Acute Respiratory Distress Syndrome a Preventable Disease? Am J Respir Crit Care Med 195, 725–736 [PubMed: 28040987]
2. Derwall M, Martin L, and Rossaint R (2018) The acute respiratory distress syndrome: pathophysiology, current clinical practice, and emerging therapies. Expert Rev Respir Med 12, 1021–1029 [PubMed: 30431366]
3. Huppert LA, Matthay MA, and Ware LB (2019) Pathogenesis of Acute Respiratory Distress Syndrome. Semin Respir Crit Care Med 40, 31–39 [PubMed: 31060086]
4. Bice T, and Carson SS (2019) Acute Respiratory Distress Syndrome: Cost (Early and Long-Term). Semin Respir Crit Care Med 40, 137–144 [PubMed: 31060095]
5. Peck TJ, and Hibbert KA (2019) Recent advances in the understanding and management of ARDS. F1000Res 8
6. Gao Smith F, Perkins GD, Gates S, Young D, McAuley DF, Tunnicliffe W, Khan Z, Lamb SE, and investigators, B.-s. (2012) Effect of intravenous beta-2 agonist treatment on clinical outcomes in acute respiratory distress syndrome (BALTI-2): a multicentre, randomised controlled trial. Lancet 379, 229–235 [PubMed: 22166903]
7. National Heart, L., Blood Institute Acute Respiratory Distress Syndrome Clinical Trials, N., Matthay MA, Brower RG, Carson S, Douglas IS, Eisner M, Hite D, Holets S, Kallet RH, Liu KD, MacIntyre N, Moss M, Schoenfeld D, Steingrub J, and Thompson BT (2011) Randomized, placebo-controlled clinical trial of an aerosolized beta(2)-agonist for treatment of acute lung injury. Am J Respir Crit Care Med 184, 561–568 [PubMed: 21562125]
8. Su X, Robriquet L, Folkesson HG, and Matthay MA (2006) Protective effect of endogenous beta-adrenergic tone on lung fluid balance in acute bacterial pneumonia in mice. Am J Physiol Lung Cell Mol Physiol 290, L769–L776 [PubMed: 16284214]
9. McAuley DF, Frank JA, Fang X, and Matthay MA (2004) Clinically relevant concentrations of beta2-adrenergic agonists stimulate maximal cyclic adenosine monophosphate-dependent airspace fluid clearance and decrease pulmonary edema in experimental acid-induced lung injury. Crit Care Med 32, 1470–1476 [PubMed: 15241090]
10. Pittet JF, Wiener-Kronish JP, McElroy MC, Folkesson HG, and Matthay MA (1994) Stimulation of lung epithelial liquid clearance by endogenous release of catecholamines in septic shock in anesthetized rats. J Clin Invest 94, 663–671 [PubMed: 8040320]
11. Perkins GD, McAuley DF, Thickett DR, and Gao F (2006) The beta-agonist lung injury trial (BALTI): a randomized placebo-controlled clinical trial. Am J Respir Crit Care Med 173, 281–287 [PubMed: 16254268]
12. Singh B, Tiwari AK, Singh K, Singh SK, Ahmed A, Erwin PJ, and Franco PM (2014) beta2 agonist for the treatment of acute lung injury: a systematic review and meta-analysis. Respir Care 59, 288–296 [PubMed: 23777655]
13. Reilly JP, Calfee CS, and Christie JD (2019) Acute Respiratory Distress Syndrome Phenotypes. Semin Respir Crit Care Med 40, 19–30 [PubMed: 31060085]
14. Bos LD, Schouten LR, van Vught LA, Wiewel MA, Ong DSY, Cremer O, Artigas A, Martin-Loeches I, Hoogendijk AJ, van der Poll T, Horn J, Juffermans N, Calfee CS, Schultz MJ, and consortium, M. (2017) Identification and validation of distinct biological phenotypes in patients with acute respiratory distress syndrome by cluster analysis. Thorax 72, 876–883 [PubMed: 28450529]

15. Calfee CS, Delucchi K, Parsons PE, Thompson BT, Ware LB, Matthay MA, and Network NA (2014) Subphenotypes in acute respiratory distress syndrome: latent class analysis of data from two randomised controlled trials. Lancet Respir Med 2, 611–620 [PubMed: 24853585]

16. Calfee CS, Janz DR, Bernard GR, May AK, Kangelaris KN, Matthay MA, and Ware LB (2015) Distinct molecular phenotypes of direct vs indirect ARDS in single-center and multicenter studies. Chest 147, 1539–1548 [PubMed: 26033126]

17. Roux J, McNicholas CM, Carles M, Goolaerts A, Houseman BT, Dickinson DA, Iles KE, Ware LB, Matthay MA, and Pittet JF (2013) IL-8 inhibits cAMP-stimulated alveolar epithelial fluid transport via a GRK2/PI3K-dependent mechanism. FASEB J 27, 1095–1106 [PubMed: 23221335]

18. Wagener BM, Roux J, Carles M, and Pittet JF (2015) Synergistic Inhibition of beta2-adrenergic Receptor-mediated Alveolar Epithelial Fluid Transport by Interleukin-8 and Transforming Growth Factor-beta. Anesthesiology 122, 1084–1092 [PubMed: 25591042]

19. Conti M, and Beavo J (2007) Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. Annu Rev Biochem 76, 481–511 [PubMed: 17376027]

20. Houslay MD (2010) Underpinning compartmentalised cAMP signalling through targeted cAMP breakdown. Trends Biochem Sci 35, 91–100 [PubMed: 19864144]

21. Barnes AP, Livera G, Huang P, Sun C, O'Neal WK, Conti M, Stutts MJ, and Milgram SL (2005) Phosphodiesterase 4D forms a cAMP diffusion barrier at the apical membrane of the airway epithelium. J Biol Chem 280, 7997–8003 [PubMed: 15611099]

22. Kolosionek E, Savai R, Ghofrani HA, Weissmann N, Guenther A, Grimminger F, Seeger W, Banat GA, Schermuly RT, and Pullamsetti SS (2009) Expression and activity of phosphodiesterase isoforms during epithelial mesenchymal transition: the role of phosphodiesterase 4. Mol Biol Cell 20, 4751–4765 [PubMed: 19759179]

23. Xin W, Tran TM, Richter W, Clark RB, and Rich TC (2008) Roles of GRK and PDE4 activities in the regulation of beta2 adrenergic signaling. J Gen Physiol 131, 349–364 [PubMed: 18347080]

24. Li X, Huston E, Lynch MJ, Houslay MD, and Baillie GS (2006) Phosphodiesterase-4 influences the PKA phosphorylation status and membrane translocation of G-protein receptor kinase 2 (GRK2) in HEK-293beta2 cells and cardiac myocytes. Biochem J 394, 427–435 [PubMed: 16356165]

25. De Arcangelis V, Liu R, Soto D, and Xiang Y (2009) Differential association of phosphodiesterase 4D isoforms with beta2-adrenoceptor in cardiac myocytes. J Biol Chem 284, 33824–33832 [PubMed: 19801680]

26. Blackman BE, Horner K, Heidmann J, Wang D, Richter W, Rich TC, and Conti M (2011) PDE4D and PDE4B function in distinct subcellular compartments in mouse embryonic fibroblasts. J Biol Chem 286, 12590–12601 [PubMed: 21288894]

27. Richter W, Day P, Agrawal R, Bruss MD, Granier S, Wang YL, Rasmussen SG, Horner K, Wang P, Lei T, Patterson AJ, Kobilka B, and Conti M (2008) Signaling from beta1- and beta2-adrenergic receptors is defined by differential interactions with PDE4. EMBO J 27, 384–393 [PubMed: 18188154]

28. Trian T, Burgess JK, Niimi K, Moir LM, Ge Q, Berger P, Liggett SB, Black JL, and Oliver BG (2011) beta2-Agonist induced cAMP is decreased in asthmatic airway smooth muscle due to increased PDE4D. PLoS One 6, e20000 [PubMed: 21611147]

29. Niimi K, Ge Q, Moir LM, Ammit AJ, Trian T, Burgess JK, Black JL, and Oliver BG (2012) beta2-Agonists upregulate PDE4 mRNA but not protein or activity in human airway smooth muscle cells from asthmatic and nonasthmatic volunteers. Am J Physiol Lung Cell Mol Physiol 302, L334–342 [PubMed: 22101762]

30. Shan WJ, Huang L, Zhou Q, Jiang HL, Luo ZH, Lai KF, and Li XS (2012) Dual beta2-adrenoceptor agonists-PDE4 inhibitors for the treatment of asthma and COPD. Bioorg Med Chem Lett 22, 1523–1526 [PubMed: 22297114]

31. Nino G, Hu A, Grunstein JS, and Grunstein MM (2009) Mechanism regulating proasthmatic effects of prolonged homologous beta2-adrenergic receptor desensitization in airway smooth muscle. Am J Physiol Lung Cell Mol Physiol 297, L746–757 [PubMed: 19666775]

32. Rabe KF (2011) Update on roflumilast, a phosphodiesterase 4 inhibitor for the treatment of chronic obstructive pulmonary disease. Br J Pharmacol 163, 53–67 [PubMed: 21232047]
33. Giembycz MA, and Newton R (2011) Harnessing the clinical efficacy of phosphodiesterase 4 inhibitors in inflammatory lung diseases: dual-selective phosphodiesterase inhibitors and novel combination therapies. Handb Exp Pharmacol, 415–446 [PubMed: 21695651]
34. Miotla JM, Teixeira MM, and Hellewell PG (1998) Suppression of acute lung injury in mice by an inhibitor of phosphodiesterase type 4. Am J Respir Cell Mol Biol 18, 411–420 [PubMed: 9490659]
35. Hamamoto M, Suga M, Nakatani T, Takahashi Y, Sato Y, Inamori S, Yagihara T, and Kitamura S (2004) Phosphodiesterase type 4 inhibitor prevents acute lung injury induced by cardiopulmonary bypass in a rat model. Eur J Cardiothorac Surg 25, 833–838 [PubMed: 15082290]
36. de Magalhaes SF, Manzo LP, de Faria FM, de Oliveira-Fusaro MC, Nishijima CM, Vieira WF, Bonet IJM, Dos Santos GG, Tambeli CH, and Parada CA (2020) Inflammatory pain in peripheral tissue depends on the activation of the TNF-alpha type 1 receptor in the primary afferent neuron. Eur J Neurosci
37. Kelly FL, Weinberg KE, Nagler AE, Nixon AB, Star MD, Todd JL, Brass DM, and Palmer SM (2019) EGFR-Dependent IL8 Production by Airway Epithelial Cells After Exposure to the Food Flavoring Chemical 2,3-Butanedione. Toxicol Sci 169, 534–542 [PubMed: 30851105]
38. Jiao Y, Yuan Y, Lin Y, Zhou Z, Zheng Y, Wu W, Tang G, Chen Y, Xiao J, Li C, Chen Z, and Cao P (2019) Propionibacterium acnes induces discogenic low back pain via stimulating nucleus pulposus cells to secrete pro-algesic factor of IL-8/CINC-1 through TLR2-NF-kappaB p65 pathway. J Mol Med (Berl) 97, 25–35 [PubMed: 30397790]
39. Pittet JF, Koh H, Fang X, Iles K, Christiaans S, Anjun N, Wagener BM, Park DW, Zmijewski JW, Matthay MA, and Roux J (2013) HMGB1 accelerates alveolar epithelial repair via an IL-1beta- and alphavbeta6 integrin-dependent activation of TGF-beta1. PLoS One 8, e63907 [PubMed: 23696858]
40. Wagener BM, Marjon NA, Revankar CM, and Prossnitz ER (2009) Adaptor protein-2 interaction with arrestin regulates GPCR recycling and apoptosis. Traffic 10, 1286–1300 [PubMed: 19602204]
41. Wagener BM, Marjon NA, and Prossnitz ER (2016) Regulation of N-Formyl Peptide Receptor Signaling and Trafficking by Arrestin-Src Kinase Interaction. PLoS One 11, e0147442 [PubMed: 26788723]
42. Agarwal SR, Yang PC, Rice M, Singer CA, Nikolaev VO, Lohse MJ, Clancy CE, and Harvey RD (2014) Role of membrane microdomains in compartmentation of cAMP signaling. PLoS One 9, e95835 [PubMed: 24752595]
43. Wachten S, Masada N, Ayling LJ, Ciruela A, Nikolaev VO, Lohse MJ, and Cooper DM (2010) Distinct pools of cAMP centre on different isoforms of adenylyl cyclase in pituitary-derived GH3B6 cells. J Cell Sci 123, 95–106 [PubMed: 20016070]
44. Nikolaev VO, Gambaryan S, Engelhardt S, Walter U, and Lohse MJ (2005) Real-time monitoring of the PDE2 activity of live cells: hormone-stimulated cAMP hydrolysis is faster than hormone-stimulated cAMP synthesis. J Biol Chem 280, 1716–1719 [PubMed: 15557342]
45. Wagener BM, Hu PJ, Oh JY, Evans CA, Richter JR, Honavar J, Brandon AP, Creighton J, Stephens SW, Morgan C, Dull RO, Marques MB, Kerby JD, Pittet JF, and Patel RP (2018) Role of heme in lung bacterial infection after trauma hemorrhage and stored red blood cell transfusion: A preclinical experimental study. PLoS Med 15, e1002522 [PubMed: 29522519]
46. Carles M, Lafargue M, Goolaerts A, Roux J, Song Y, Howard M, Weston D, Swindle JT, Hedgpeth J, Burel-Vandenbos F, and Pittet JF (2010) Critical role of the small GTPase RhoA in the development of pulmonary edema induced by Pseudomonas aeruginosa in mice. Anesthesiology 113, 1134–1143 [PubMed: 20938335]
47. Davis IC, Xu A, Gao Z, Hickman-Davis JM, Factor P, Sullender WM, and Matalon S (2007) Respiratory syncytial virus induces insensitivity to beta-adrenergic agonists in mouse lung epithelium in vivo. Am J Physiol Lung Cell Mol Physiol 293, L281–289 [PubMed: 17435077]

48. Greenlee MM, Mitzelfelt JD, Yu L, Yue Q, Duke BJ, Harrell CS, Neigh GN, and Eaton DC (2013) Estradiol activates epithelial sodium channels in rat alveolar cells through the G protein-coupled estrogen receptor. Am J Physiol Lung Cell Mol Physiol 305, L878–889 [PubMed: 24097558]
49. Helms MN, Self J, Bao HF, Job LC, Jain L, and Eaton DC (2006) Dopamine activates amiloride-sensitive sodium channels in alveolar type I cells in lung slice preparations. Am J Physiol Lung Cell Mol Physiol 291, L610–618 [PubMed: 16679376]
50. Rajagopal S, and Shenoy SK (2018) GPCR desensitization: Acute and prolonged phases. Cell Signal 41, 9–16 [PubMed: 28137506]
51. Pavlos NJ, and Friedman PA (2017) GPCR Signaling and Trafficking: The Long and Short of It. Trends Endocrinol Metab 28, 213–226 [PubMed: 27889227]
52. Menard L, Ferguson SS, Zhang J, Lin FT, Lefkowitz RJ, Caron MG, and Barak LS (1997) Synergistic regulation of beta2-adrenergic receptor sequestration: intracellular complement of beta-adrenergic receptor kinase and beta-arrestin determine kinetics of internalization. Mol Pharmacol 51, 800–808 [PubMed: 9145918]
53. Gurevich VV, and Gurevich EV (2019) GPCR Signaling Regulation: The Role of GRKs and Arrestins. Front Pharmacol 10, 125 [PubMed: 30837883]
54. Shi Q, Li M, Mika D, Fu Q, Kim S, Phan J, Shen A, Vandecasteele G, and Xiang YK (2017) Heterologous desensitization of cardiac beta-adrenergic signal via hormone-induced betaAR/arrestin/PDE4 complexes. Cardiovasc Res 113, 656–670 [PubMed: 28339772]
55. Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, Caron MG, and Lefkowitz RJ (1999) Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. Science 283, 655–661 [PubMed: 9924018]
56. Willis MJ, and Baillie GS (2014) Arrestin-dependent localization of phosphodiesterases. Handb Exp Pharmacol 219, 293–307 [PubMed: 24292836]
57. Baillie GS, and Houslay MD (2005) Arrestin times for compartmentalised cAMP signalling and phosphodiesterase-4 enzymes. Curr Opin Cell Biol 17, 129–134 [PubMed: 15780588]
58. Gupta MK, Asosingh K, Aronica M, Comhair S, Cao G, Erzurum S, Panettieri RA Jr., and Naga Prasad SV (2015) Defective Resensitization in Human Airway Smooth Muscle Cells Evokes beta-Adrenergic Receptor Dysfunction in Severe Asthma. PLoS One 10, e0125803 [PubMed: 26023787]
59. Gupta MK, Mohan ML, and Naga Prasad SV (2018) G Protein-Coupled Receptor Resensitization Paradigms. Int Rev Cell Mol Biol 339, 63–91 [PubMed: 29776605]
60. Mohan ML, Vasudevan NT, Gupta MK, Martelli EE, and Naga Prasad SV (2012) G-protein coupled receptor resensitization-appreciating the balancing act of receptor function. Curr Mol Pharmacol
61. Wagener BM, Anjum N, Evans C, Brandon A, Honavar J, Creighton J, Traber MG, Stuart RL, Stevens T, and Pittet JF (2020) alpha-Tocopherol Attenuates the Severity of Pseudomonas aeruginosa-induced Pneumonia. Am J Respir Cell Mol Biol 63, 234–243 [PubMed: 32243761]
62. Che P, Wagener BM, Zhao X, Brandon AP, Evans CA, Cai GQ, Zhao R, Xu ZX, Han X, Pittet JF, and Ding Q (2020) Neuronal Wiskott-Aldrich syndrome protein regulates Pseudomonas aeruginosa-induced lung vascular permeability through the modulation of actin cytoskeletal dynamics. FASEB J 34, 3305–3317 [PubMed: 31916311]
63. Wagener BM, Hu M, Zheng A, Zhao X, Che P, Brandon A, Anjum N, Snapper S, Creighton J, Guan JL, Han Q, Cai GQ, Han X, Pittet JF, and Ding Q (2016) Neuronal Wiskott-Aldrich syndrome protein regulates TGF-beta1-mediated lung vascular permeability. FASEB J 30, 2557–2569 [PubMed: 27025963]
64. Lefkowitz RJ (2013) Arrestins come of age: a personal historical perspective. Prog Mol Biol Transl Sci 118, 3–18 [PubMed: 23764048]
65. Bang I, and Choi HJ (2015) Structural features of beta2 adrenergic receptor: crystal structures and beyond. Mol Cells 38, 105–111 [PubMed: 25537861]
66. Weis WI, and Kobilka BK (2018) The Molecular Basis of G Protein-Coupled Receptor Activation. Annu Rev Biochem 87, 897–919 [PubMed: 29925258]

67. Perry SJ, Baillie GS, Kohout TA, McPhee I, Magiera MM, Ang KL, Miller WE, McLean AJ, Conti M, Houslay MD, and Lefkowitz RJ (2002) Targeting of cyclic AMP degradation to beta 2-adrenergic receptors by beta-arrestins. Science 298, 834–836 [PubMed: 12399592]

68. Bolger GB, McCahill A, Huston E, Cheung YF, McSorley T, Baillie GS, and Houslay MD (2003) The unique amino-terminal region of the PDE4D5 cAMP phosphodiesterase isoform confers preferential interaction with beta-arrestins. J Biol Chem 278, 49230–49238 [PubMed: 14500724]

69. Houslay MD, and Baillie GS (2003) The role of ERK2 docking and phosphorylation of PDE4 cAMP phosphodiesterase isoforms in mediating cross-talk between the cAMP and ERK signalling pathways. Biochem Soc Trans 31, 1186–1190 [PubMed: 14641023]

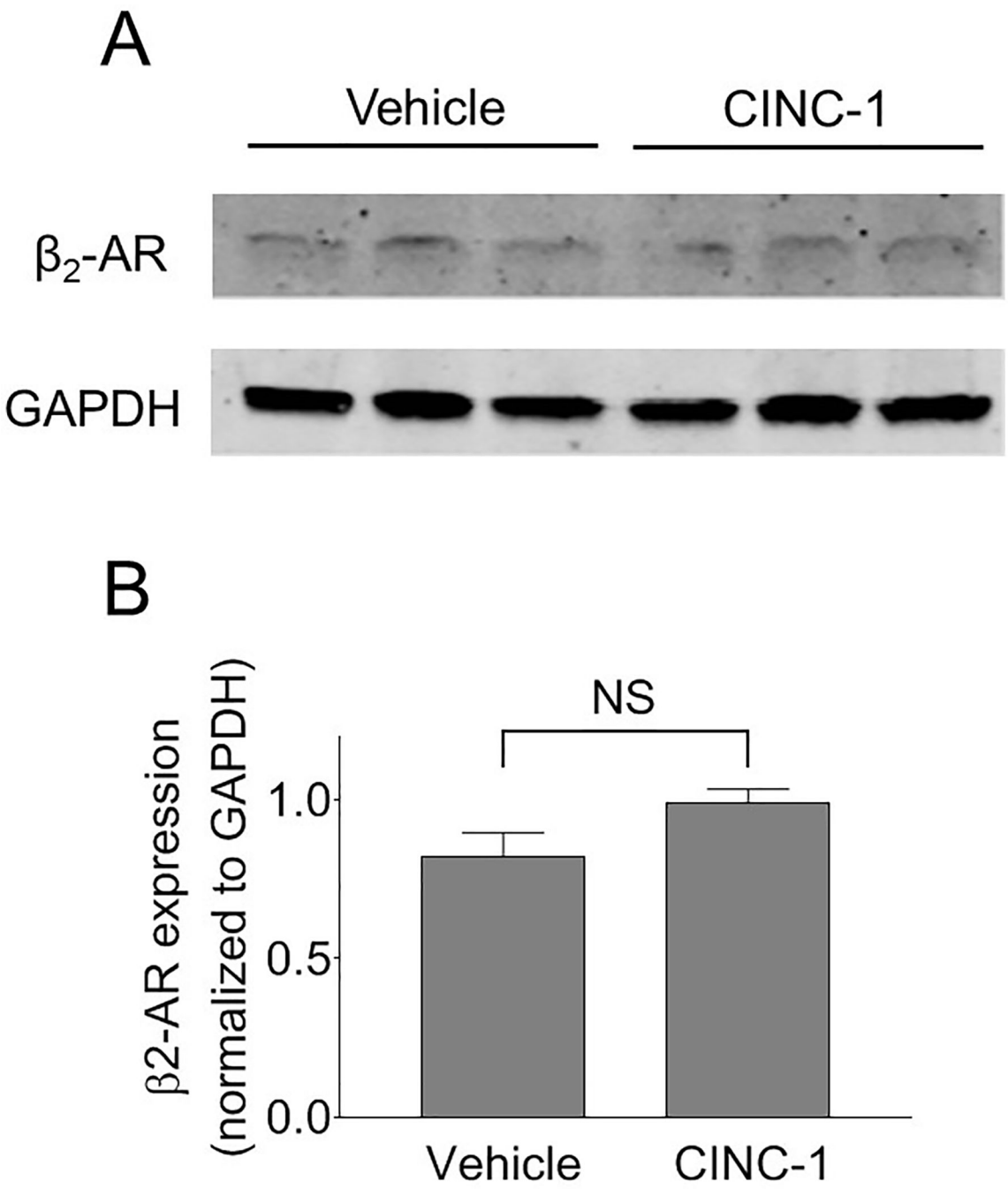
70. Lynch MJ, Baillie GS, Mohamed A, Li X, Maisonneuve C, Klussmann E, van Heeke G, and Houslay MD (2005) RNA silencing identifies PDE4D5 as the functionally relevant cAMP phosphodiesterase interacting with beta arrestin to control the protein kinase A/AKAP79-mediated switching of the beta2-adrenergic receptor to activation of ERK in HEK293B2 cells. J Biol Chem 280, 33178–33189 [PubMed: 16030021]

71. Bolger GB, Baillie GS, Li X, Lynch MJ, Herzyk P, Mohamed A, Mitchell LH, McCahill A, Hundsrucker C, Klussmann E, Adams DR, and Houslay MD (2006) Scanning peptide array analyses identify overlapping binding sites for the signalling scaffold proteins, beta-arrestin and RACK1, in cAMP-specific phosphodiesterase PDE4D5. Biochem J 398, 23–36 [PubMed: 16689683]

72. Wisler JW, DeWire SM, Whalen EJ, Violin JD, Drake MT, Ahn S, Shenoy SK, and Lefkowitz RJ (2007) A unique mechanism of beta-blocker action: carvedilol stimulates beta-arrestin signaling. Proc Natl Acad Sci U S A 104, 16657–16662 [PubMed: 17925438]

73. Carr R 3rd, Schilling J, Song J, Carter RL, Du Y, Yoo SM, Traynham CJ, Koch WJ, Cheung JY, Tilley DG, and Benovic JL (2016) beta-arrestin-biased signaling through the beta2-adrenergic receptor promotes cardiomyocyte contraction. Proc Natl Acad Sci U S A 113, E4107–4116 [PubMed: 27354517]

74. Handa O, Naito Y, and Yoshikawa T (2006) Rat Cytokine-Induced Neutrophil Chemoattractant-1 (CINC-1) in Inflammation. Journal of Clinical Biochemisty and Nutrition 38, 51–58



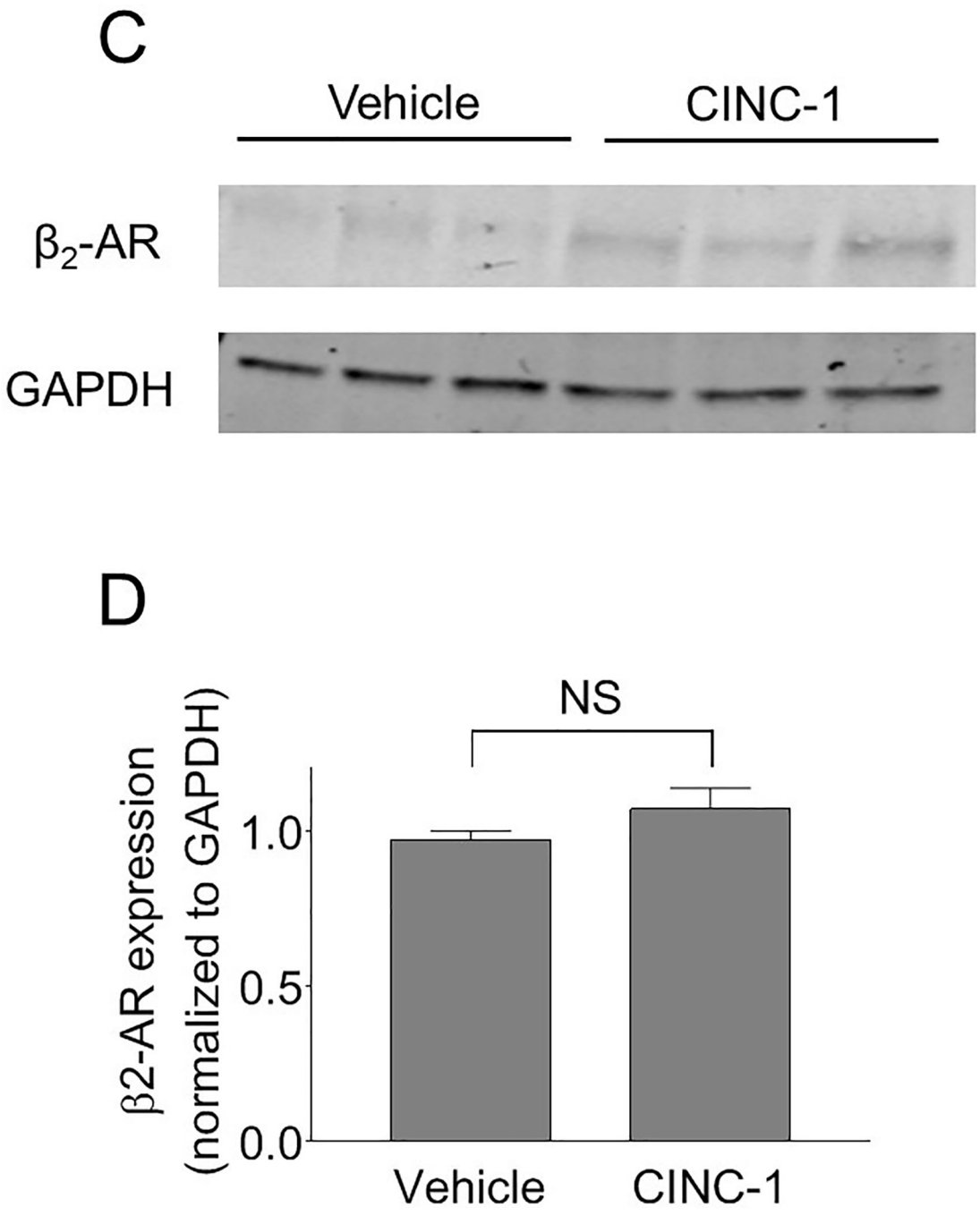


Figure 1. Heterologous desensitization of the β_2-AR by CINC-1 is not dependent upon receptor degradation.

For all experiments, alveolar epithelial cells were exposed to vehicle or CINC-1 (10ng/ml) for 6 hours. Cells were then lysed, resolved via SDS-PAGE and blotted with anti-β_2-AR or anti-GAPDH antibodies. Blots and quantification are shown for primary ATII (***A*** and ***B***, respectively) and L2 (***C*** and ***D***, respectively) cells. Experiments for each cell type were run in three independent experiments (n=3), and lysates were combined to make one series of blots.

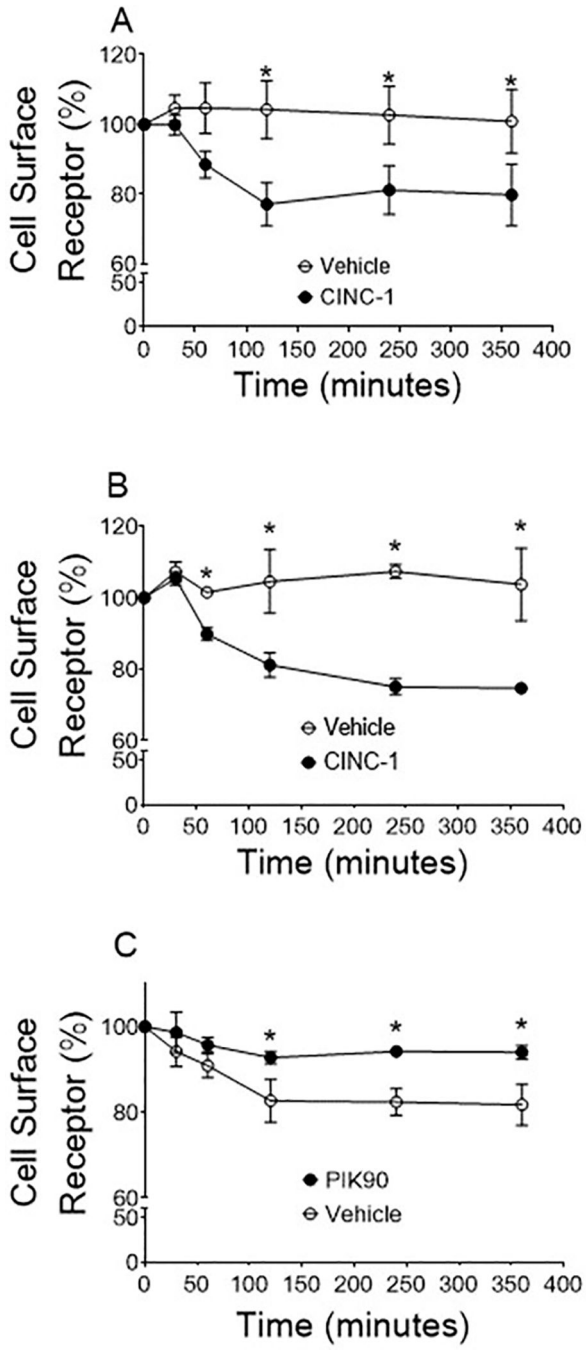


Figure 2. Heterologous desensitization of the β_2-AR by CINC-1 is dependent upon receptor internalization.

For all experiments, alveolar epithelial cells were exposed to vehicle or CINC-1 (10ng/ml) for the times indicated, and β_2-AR internalization was assayed as described in the Methods. β_2-AR internalization was assayed in primary ATII cells (***A***) and L2 cells (***B***), respectively. (***C***) L2 cells were pre-treated with PIK-90 (100nM) or vehicle before exposure to CINC-1. Data are expressed as mean cell surface receptor ± SEM. n=3–4 for all time points assayed; * indicates $p < 0.05$ compared to vehicle at the indicated time point.

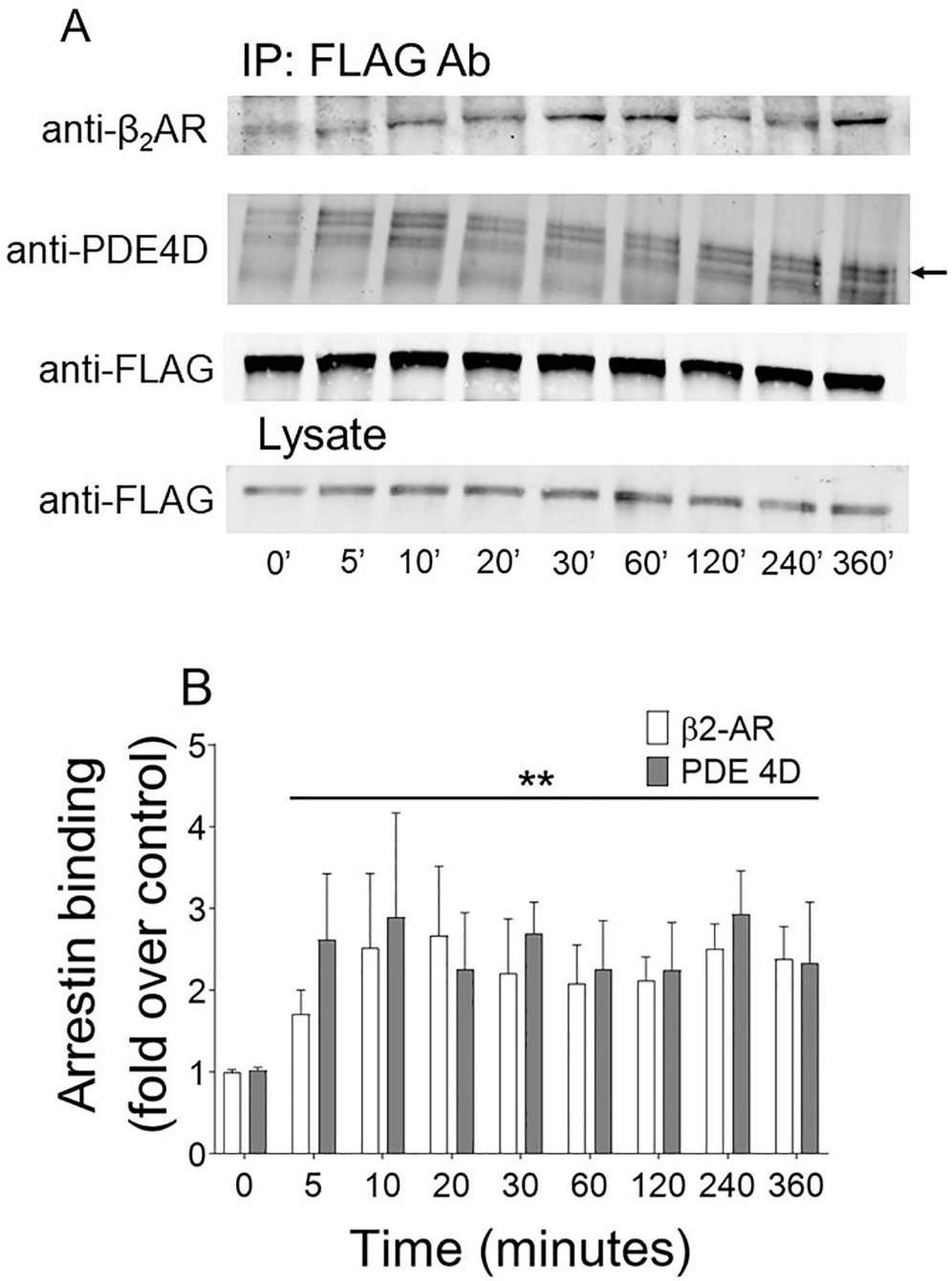


Figure 3. Internalization of the β_2-AR by CINC-1 involves binding of arrestin-2 and PDE4.

For all experiments, L2 cells were transiently transfected with arrestin-2-FLAG. Transfected L2 cells were exposed to CINC-1 (10ng/ml) for the times indicated and lysed in pre-chilled lysate buffer. An aliquot of lysate was preserved, and the remainder of the lysate was immunoprecipitated with anti-FLAG antibody as described in the Methods. Immunoprecipitated and aliquoted lysates were then resolved with SDS-PAGE and immunoprecipitated lysate was blotted with anti-β_2-AR, anti-PDE4D and anti-FLAG antibodies while aliquoted lysate was blotted with anti-FLAG antibodies. (***A***) A

representative blot and (***B***) quantification of densitometry are shown. The arrow designates the bands representing PDE4D. Data are expressed as mean arrestin-2 binding ± SEM, n=3; * indicates $p < 0.05$ compared to zero time point for both β_2-AR and PDE4D binding to arrestin-2.

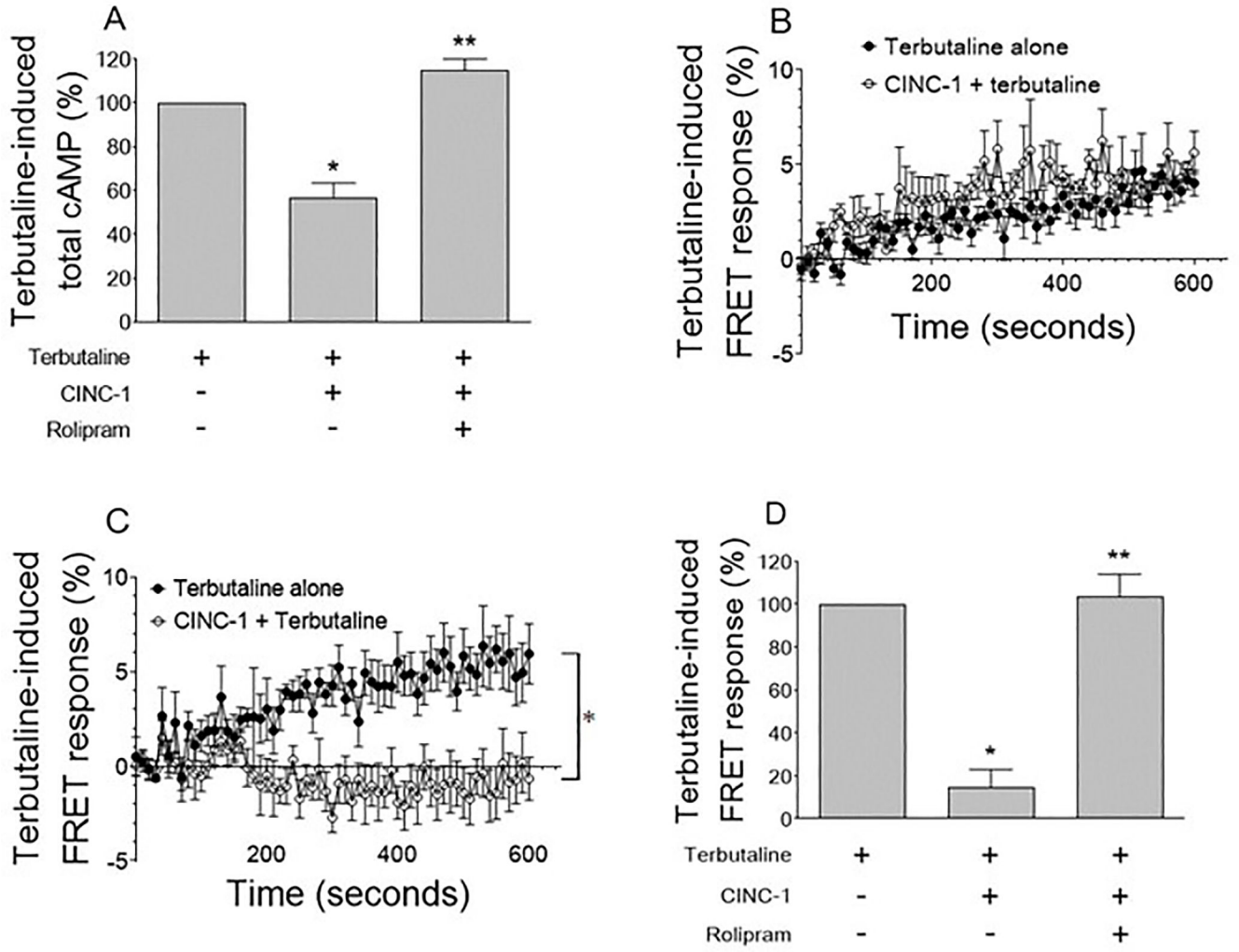


Figure 4. CINC-1 desensitizes β_2-AR-mediated cAMP signaling at the plasma membrane that is resensitized by PDE4 inhibition.

(***A***) Confluent L2 cells were exposed to terbutaline (20μM) for 5 minutes, lysed with pre-chilled lysate buffer, and cAMP was measured. For some experiments, cells were pre-treated with CINC-1 (10ng/ml) and/or rolipram (10μM) for 30 minutes before addition of terbutaline. Data are expressed as a mean percentage of terbutaline-induced cAMP levels ± SEM, n=3; * indicates $p < 0.05$ compared to terbutaline only and ** indicates $p < 0.05$ compared to CINC-1 and terbutaline. For (***A-C***), L2 cells were transiently transfected with Epac-FRET plasmids that detect cytosolic (***B***) or plasma membrane (***C, D***) located cAMP and FRET efficiency was measured as described in the Methods. After baseline FRET efficiency was measured, cells were exposed to terbutaline to determine the cAMP response. For some experiments, cells were pre-treated with CINC-1 (10ng/ml) and/or rolipram (10μM) for 30 minutes before addition of terbutaline. In (***B, C***), FRET efficiency is measured every 10 minutes and plotted as mean FRET response ± SEM, n=5–7; * indicates $p < 0.05$ compared to baseline. In (***D***), data is plotted as mean terbutaline-induced FRET response ± SEM, n=5–7; * indicates $p < 0.05$ compared to terbutaline alone and ** indicates $p < 0.05$ compared to CINC-1 and terbutaline.

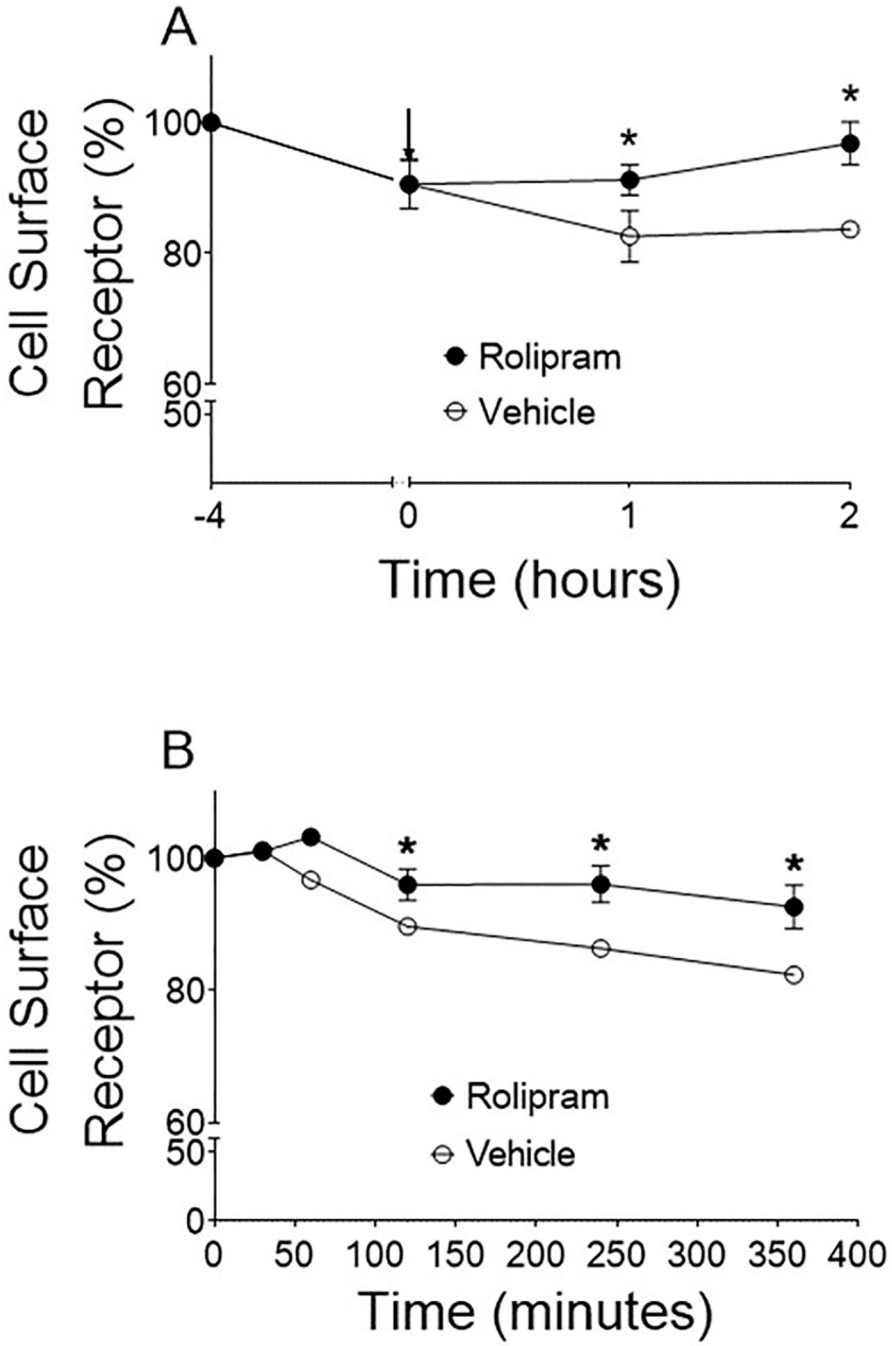


Figure 5. PDE4 inhibition restores β_2-AR receptor expression at the cell surface after heterologous desensitization by CINC-1 and inhibits CINC-1-induced β_2-AR receptor internalization.

(***A***) L2 cells were exposed to CINC-1 (10ng/ml) for four hours and extensively washed in pre-chilled serum-free media. Then, cells were treated with vehicle or rolipram (10μM) and returned to 37°C. Cells were assayed at the times indicated for β_2-AR recycling as described in the Methods. (***B***) L2 cells were pre-treated with rolipram (10μM) or vehicle for 30 minutes. Then, all cells were exposed to CINC-1 (10ng/ml) for the times indicated and β_2-AR internalization was assayed as described in the Methods. Data are expressed as mean

cell surface receptor ± SEM. n=3 for all time points assayed; * indicates $p < 0.05$ compared to vehicle at the indicated time point.

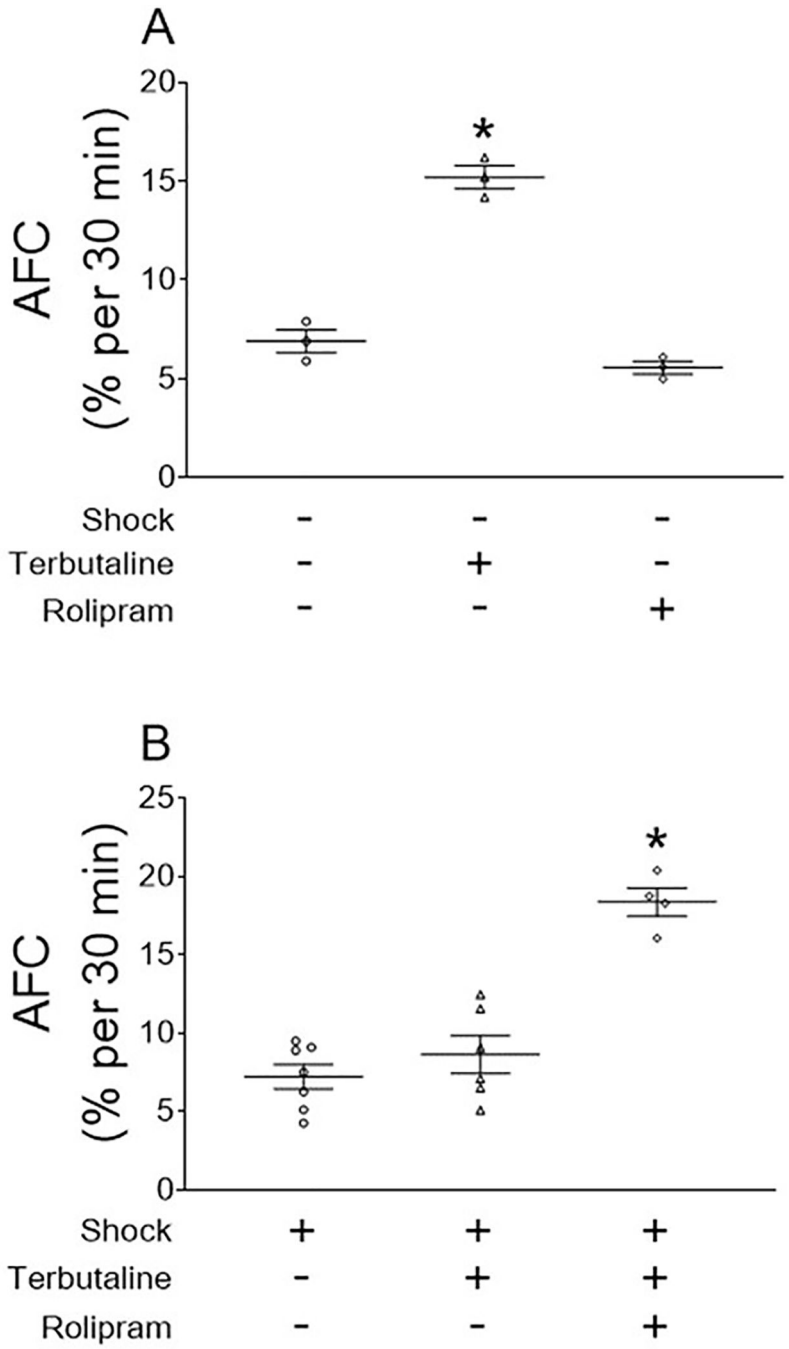


Figure 6. PDE4 inhibition restores trauma-mediated inhibition of β_2-AR-induced alveolar fluid clearance.

For all experiments, AFC was measured as described in the Methods. (***A***) Baseline AFC measurements in mice without exposure to trauma shock. Mice were exposed to no treatment, terbutaline (10^{-5}M), or rolipram (3mg/kg) alone 30 minutes before AFC was measured. Data are expressed as mean AFC ± SEM. n=3 for all conditions assayed; * indicates $p < 0.05$ compared to no treatment and rolipram. (***B***) Mice were exposed to trauma-shock as described in the Methods. Six hours later, AFC was measured. Mice were

exposed to no treatment, terbutaline (10^{-5}M), and/or rolipram (3mg/kg) 30 minutes before AFC was measured. Data are expressed as mean AFC ± SEM. n=3–7 for all conditions assayed; * indicates $p < 0.05$ compared to no treatment and terbutaline alone.