Endothelin-converting Enzyme 1 and β-Arrestins Exert Spatiotemporal Control of Substance P-induced Inflammatory Signals*

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Although the intracellular trafficking of G protein-coupled receptors controls specific signaling events, it is unclear how the spatiotemporal control of signaling contributes to complex pathophysiological processes such as inflammation. By using bioluminescence resonance energy transfer and superresolution microscopy, we found that substance P (SP) induces the association of the neurokinin 1 receptor (NK1R) with two classes of proteins that regulate SP signaling from plasma and endosomal membranes: the scaffolding proteins β-arrestin (BARRs) 1 and 2 and the transmembrane metalloproteases ECE-1c and ECE-1d. In HEK293 cells and non-transformed human colonocytes, we observed that G protein-coupled receptor kinase 2 and βARR1/2 terminate plasma membrane Ca2+ signaling and initiate receptor trafficking to endosomes that is necessary for sustained activation of ERKs in the nucleus. βARRs deliver the SP-NK1R endosomes, where ECE-1 associates with the complex, degrades SP, and allows the NK1R, freed from βARRs, to recycle. Thus, both ECE-1 and βARRs mediate the resensitization of NK1R Ca2+ signaling at the plasma membrane. Sustained exposure of colonocytes to SP activates NF-κB and stimulates IL-8 secretion. This proinflammatory signaling is unaffected by inhibition of the endosomal ERK pathway but is suppressed by ECE-1 inhibition or βARR2 knockdown. Inhibition of protein phosphatase 2A, which also contributes to sustained NK1R signaling at the plasma membrane, similarly attenuates IL-8 secretion. Thus, the primary function of βARRs and ECE-1 in SP-dependent inflammatory signaling is to promote resensitization, which allows the sustained NK1R signaling from the plasma membrane that drives inflammation.

Alterations in the subcellular distribution of G protein-coupled receptors (GPCRs)4 are critically important for signal transduction. Receptors at the plasma membrane are necessary for detection of extracellular stimuli and for plasma membrane-delimited signaling events, including coupling to heterotrimeric G proteins, activation of second messengers, and transactivation of receptor tyrosine kinases. Activated receptors translocate to endosomes, where they can signal by G protein-dependent and -independent mechanisms (1, 2). The movement of receptors back to the cell surface enables the recovery of plasma membrane signaling, whereas receptor trafficking to lysosomes and degradation terminates their ability to signal. The relevance of receptor trafficking to complex pathophysiological processes, such as inflammation, is unknown.

The interaction of GPCRs with β-arrestins (BARRs) at plasma and endosomal membranes regulates receptor signaling and trafficking (3). At the plasma membrane, G protein-coupled receptor kinases (GRKs) can phosphorylate activated receptors, thereby increasing receptor affinity for βARRs. βARRs disrupt receptor interaction with G proteins, which desensitizes signaling, and couple receptors to clathrin and AP2, which mediates receptor endocytosis. By recruiting downstream signaling proteins such as ERK to endosomes, βARRs also transduce G protein-independent but βARR-dependent signaling of internalized receptors (2). The dissociation of receptors from βARRs is required for receptor recycling and for resensitization of plasma membrane signaling (4).

The abbreviations used are: GPCR, G protein-coupled receptor; β-ARR, β-arrestin; GRK, G protein-coupled receptor kinase; SP, substance P; NK1R, neurokinin 1 receptor; BRET, bioluminescence resonance energy transfer; SMS, Sar9Met(O2)11-SP; IR, immunoreactivity; EGFR, EGF receptor; PP2A, protein phosphatase 2A.
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The availability of agonists at plasma and endosomal membranes also regulates receptor trafficking and signaling. In the case of receptors for neuropeptides, membrane metalloproteases control the interaction of GPCRs with their agonists at the cell surface and in endosomes. The cell surface peptidase nephrilysin degrades the proinflammatory neuropeptide substance P (SP) in the extracellular fluid, which restricts SP interaction with the neurokinin 1 receptor (NK1R) and attenuates SP-induced inflammation in multiple tissues (5, 6). The endosomal peptidase endothelin-converting enzyme 1 (ECE-1) degrades SP in acidified early endosomes, which triggers dissociation of the SP-NK1R-βARR complex. This process induces NK1R recycling and resensitization of G protein-dependent plasma membrane signaling (7–9) but terminates βARR-dependent endosomal ERK signaling (10).

The relative importance of plasma membrane and endosomal signaling in determining integrated cellular responses and pathophysiological outcomes is far from clear. βARRs desensitize plasma membrane G protein-dependent signaling, mediate endosomal G protein-independent signaling, and facilitate the intracellular processing of receptors that is necessary for recycling and resensitization of plasma membrane signaling. ECE-1 promotes recycling and resensitization of plasma membrane signaling but terminates endosomal βARR-dependent signaling. Therefore, is plasma membrane or endosomal signaling of primary importance, or is resensitization of cell surface signaling the key event in allowing sustained cellular responses?

To address these questions, we investigated the effects of altered expression and activity of βARRs and ECE-1 on SP-dependent trafficking and inflammatory signaling of the NK1R. This strategy allowed us to study the relative contributions of plasma membrane and endosomal signaling events. Because there is increased mucosal expression of the NK1R in the inflamed intestine (11), we studied human colonocytes overexpressing the NK1R that respond to SP by release of NF-κB-driven proinflammatory cytokines, including IL-8 (12, 13). We defined the importance of βARRs and ECE-1 in regulating plasma membrane, endosomal, and inflammatory signaling. Our results show that the principal role of βARRs and ECE-1 is to resensitize the plasma membrane NK1R signaling that is necessary for sustained SP-induced inflammation.

**EXPERIMENTAL PROCEDURES**

*cDNAs*—cDNAs encoding human NK1R flanked by 5′ BamHI and 3′ Xhol restriction sites were generated using Phusion high-fidelity PCR (New England Biosciences, Ipswich, MA) using the primers CCAGGATCATGGATAACGTC-CTTCTCATTG (sense) and CCGCTCAGGGCCAGCATGT-CTAGAG (antisense). Products were digested with BamHI and Xhol and ligated into pcDNA3-RLuc8 using the BamHI and Xhol sites. The bioluminescence resonance energy transfer (BRET) sensors (human) NK1R-RLuc8, βARR1-YFP, βARR2-YFP, ECE-1a-d-YFP, KRas-Venus, Rab5a-Venus, and Rab11-Venus were generated as described previously (9, 14, 15). The FRET sensors CytoEKAR and NucEKAR (16) were from Addgene (Cambridge, MA) (plasmids 18680 and 18681, respectively). ECE-1a-d-GFP, βARR1-GFP, βARR2-GFP and βARR319–418-GFP have been described previously (9, 17).

**Cell Lines**—For BRET, HEK293 cells were transfected with 1 μg of NK1R-RLuc8 and 4 μg of βARR1-YFP, βARR2-YFP, ECE-1a-d-YFP, KRas-Venus, Rab5a-Venus, or Rab11-Venus (14, 15). After 24 h, cells were plated in poly-d-lysine-coated 96-well plates and cultured overnight. For FRET, HEK293 and NCM-NK1R cells were plated in poly-d-lysine-coated 96-well plates and grown to 50% confluency. HEK293 cells were transfected with 55 ng/well HA-NK1R and 40 ng/well CytoEKAR or NucEKAR. NCM-NK1R cells were transfected with 200 ng/well CytoEKAR or NucEKAR. FRET was assessed 48 h after transfection following serum restriction (0.5% FBS overnight). Stable NCM-NK1R cells were generated and maintained as described previously (12). NCM-NK1R cells were transiently transfected with ECE-1a-d-GFP, βARR1– or 2-GFP, or βARR319–418-GFP as described previously (9, 17).

**BRET**—BRET was measured at 465–505 and 515–555 nm using a LUMistar Omega (BMG LabTech, Offenburg, Germany). HEK cells were equilibrated in Hanks’ balanced salt solution at 37 °C. For kinetic experiments, coelenterazine h was added (5 μM), and BRET was measured for 2 min before and 10 min after challenge with SP or vehicle. For end point experiments, cells were challenged with SP or vehicle, washed, and recovered for various times. Coelenterazine h was added 10 min before BRET detection.

**FRET**—FRET was analyzed using a GE Healthcare INCell 2000 analyzer. For emission ratio analysis, cells were excited sequentially using a FITC filter (490/20) with emission measured using dsRed (605/52) and FITC (525/36) filters and a polychroic optimized for the FITC/dsRed filter pair (Quad4). Images of 14 wells were collected at 1-min intervals. HEK cells were equilibrated in Hanks’ balanced salt solution at 37 °C. Baseline emission ratio images were captured for 4 min. Cells were challenged with SP (1 nM) or vehicle, and images were captured for 20 min. At the end of every experiment, cells were stimulated with phorbol 12,13 dibutyrate (200 nM, 10 min, positive control) to generate a maximal increase in ERK phosphorylation, and images were captured for 4 min. Data were analyzed using ImageJ. Emission ratio image stacks (baseline, stimulated, positive control) were collated and aligned using a StackCreator script written by Cameron Nowell (Monash). Cells were selected, and fluorescence intensity was measured over the combined stack. Background intensity was subtracted, and FRET data were plotted as the change in the dsRed:FITC emission ratio relative to the baseline for each cell (F/F0). Cells with >10% change in F/F0 after stimulation with phorbol 12,13 dibutyrate were selected for analysis.

**ECE-1 and βARR Expression**—Expression of endogenous ECE-1 and βARR isoforms was assessed in NCM460 cells by RT-PCR and Western blotting (8, 9, 18, 19).

**Inhibitors**—Cells were treated with SM-19712 (10 μM), UBO-QIC (100 nM), GF109203X (1 μM), AG1478 (1 μM), PD98059 (25 μM), rolletrin (1 μM), foscirecin (300 nM), or vehicle (30–60 min preincubation, inclusion throughout).
siRNA—siRNA sequences with 3’ dTdT overhangs (Dharmacon, Lafayette, CO) were as follows: human bARR1, 5’-AGC-CUUUCUGCGGAGAAU-3’; human bARR2, 5’-GGACCG-CAAAAGGUUUGUG-3’; and control non-targeting, 5’-UUC-UCCGAAUGUCAGCU-3’ (20). Cells were transfected with 20–60 pmol siRNA (X-tremeGene, Roche Applied Science, or Lipofectamine RNAiMAX, Invitrogen). Cells were studied after 48–72 h. BARR expression was determined by Western blotting (18) or quantitative real-time RT-PCR using primer sets for bARR1 (Hs00244527_m1) or bARR2 (Hs00188826_m1) (Applied Biosystems, Carlsbad, CA). Levels of mRNA were normalized to 18 S mRNA using the primer set Hs99999901_s1, and results were expressed as fold change compared with control.

Localization of Alexa-tagged Sar\(^3\)Met(O\(^2\)Met\(^2\)SP, ECE-1, bARRs, and Early Endosomal Antigen 1—To localize the NK\(_r\), we incubated NCM-NK\(_r\)R cells with Sar\(^3\)Met(O\(^2\)Met\(^2\)SP (SMSP), an NK\(_r\), agonist (21), tagged with either Alexa Fluor 568 or 594 (Alexa-SMSP). Cells were incubated with Alexa-SMSP (10 nm, 60 min, 4 °C), washed, incubated for 30 min at 37 °C, and fixed (4% paraformaldehyde and 100 mM PBS (pH 7.4), 20 min, 4 °C). Cells were blocked in PBS containing 1% normal goat serum and 0.1% saponin and incubated with primary antibodies overnight at 4 °C as follows: rabbit antibody to the N terminus of human ECE-1 (Invitrogen, 1:400), mouse secondary antibodies overnight at 4 °C as follows: rabbit antibody to the N terminus of human ECE-1 (Invitrogen, 1:400), mouse antibody to bARR1 (BD Transduction Laboratories, 1:100), and mouse antibody to early endosomal antigen 1 (EAA-1) (BD Transduction Laboratories, 1:200) (9, 18). Cells were washed and incubated with fluorescent secondary antibodies (1:400, 2 h, room temperature). As a control for specificity, rabbit anti-ECE-1 antibody was preincubated with the immunizing peptide (25 μg/ml peptide and 1 μg/ml antibody, 3 h, 4 °C). Cells were observed using a LSM 510 Meta confocal microscope with a Plan Apo ×63 (numerical aperture 1.4) objective. Images were collected at a zoom of 1–3, and three to five optical sections were taken at intervals of 0.39 μm. For superresolution microscopy, cells were observed using a Leica DMi60000 ground state depletion microscope equipped with a HXC PL APO ×100 (numerical aperture 1.47) objective, a SuMo stage, an Andor iXon 3987 camera (Andor, Belfast, UK), and LAS AF software (version 3.2.0.9652, Leica).

\([\text{Ca}^{2+}],\text{Assays—}[\text{Ca}^{2+}],\) was measured in NCM-NK\(_r\)R cells using Fura/2-AM in a FlexStation III microplate reader (Molecular Devices, Sunnyvale, CA) as described previously (9, 18). To assess resensitization, cells were challenged with SP (1 or 10 nm, 10 min) or vehicle (control), washed, and recovered for 0–150 min at 37 °C. \([\text{Ca}^{2+}],\) was measured in response to a second challenge of SP (10 nm) (9, 18).

NF-κB p65 Activation—NCM-NK\(_r\)R cells were incubated with SP (10 nm) or vehicle (control) for 45 min, and phosphorylation of NF-κB p65 was determined by Western blotting (13).

IL-8 Assays—NCM-NK\(_r\)R cells were incubated with SP (10 nm) or vehicle (control) for 4–8 h. IL-8 in medium was measured by ELISA (12).

Statistics—Data are presented as mean ± S.E. of triplicate observations from \(n > 3\) experiments. Differences were assessed using Student’s t test for two comparisons and one- or two-way analysis of variance followed by Bonferroni post test and Student-Newman-Keul test for multiple comparisons. \(p < 0.05\) was considered significant.

RESULTS

SP Promotes NK\(_r\),R Association with bARRs and ECE-1 at the Plasma Membrane and in Endosomes of HEK293 Cells—We analyzed the interactions between NK\(_r\), bARRs, and ECE-1 in defined cellular compartments using BRET. It was not possible to coexpress at adequate levels two BRET constructs in NCM460 colonicocytes. Therefore, we examined the interactions between NK\(_r\), bARRs, or ECE-1 in HEK293 cells in which the NK\(_r\),R could be coexpressed with bARR or ECE-1 isoforms at suitable levels for BRET measurements. In HEK293 cells expressing NK\(_r\),R-RLuc8 and bARR1-YFP or bARR2-YFP, SP stimulated a rapid (1-min), sustained (>10 min), and concentration-dependent increase in BRET between NK\(_r\),R-RLuc8 and bARR1-YFP or bARR2-YFP (EC\(_{50}\) for both isoforms, 2.5 nm) (Fig. 1, A and B).

To quantitatively assess SP-induced NK\(_r\)R trafficking between the plasma membrane and early and recycling endosomes, we assessed BRET between NK\(_r\),R-RLuc8 and Venus-tagged BRET acceptors that are localized to the plasma membrane (KRas) and early (Rab5a) or recycling (Rab11) endosomes. SP (1 nm and 10 nm) decreased NK\(_r\),R-RLuc8 and KRas-Venus BRET (Fig. 1C) and increased NK\(_r\),R-RLuc8 and Rab5a-Venus BRET (Fig. 1D) in a concentration-dependent manner. To assess NK\(_r\)R trafficking after SP removal, cells were challenged with SP (1 nm, 10 min) and washed, and then BRET was measured 15–120 min after SP exposure. Cells were treated with cycloheximide (100 μm) to prevent new receptor synthesis. After SP removal, NK\(_r\),R-RLuc8 and KRas-Venus BRET continued to decrease, and NK\(_r\),R-RLuc8 and Rab5a-Venus BRET continued to increase for 15 min (Fig. 1F). Thereafter, BRET between NK\(_r\),R-RLuc8 and Rab11-Venus or KRas-Venus increased and NK\(_r\),R-RLuc8 and Rab5a-Venus BRET declined. Thus, SP stimulates NK\(_r\)R trafficking from the plasma membrane to early and then recycling endosomes, which deliver the NK\(_r\),R back to the plasma membrane.

To determine whether the NK\(_r\),R associates with ECE-1 isoforms, we expressed NK\(_r\),R-RLuc8 and ECE-1a-d-YFP in HEK293 cells. SP (1 nm) decreased NK\(_r\),R-RLuc8 and ECE-1a-YFP BRET and increased NK\(_r\),R-RLuc8 and ECE-1d-YFP BRET (Fig. 1F). SP did not affect NK\(_r\),R-RLuc8 and ECE-1b-YFP BRET but increased NK\(_r\),R-RLuc8 and ECE-1c-YFP BRET (Fig. 1G). These results are consistent with NK\(_r\)R trafficking from ECE-1a at the plasma membrane to associate with ECE-1d in early endosomes (9, 22). The NK\(_r\),R preferentially associates with ECE-1d over ECE-1b, both of which are present in endosomes.

To determine whether the association of ECE-1 with the NK\(_r\),R in endosomes controls the interactions between the NK\(_r\),R and bARRs, we examined the effects of the ECE-1 inhibitor SM-19712 on NK\(_r\),R and bARR BRET. SM-19712 caused a 2-fold increase in SP (1 nm)-induced BRET between NK\(_r\),R-RLuc8 and bARR1-YFP or bARR2-YFP (Fig. 2A). In contrast, the Go\(_q\), inhibitor UBO-QIC and the PKC inhibitor GF109203X did not affect SP-induced BRET between NK\(_r\),R-RLuc8 and bARR1-YFP or bARR2-YFP (Fig. 2B). These inhibitors also had
no effect on SP-induced endocytosis of the NK1R, determined by measuring the decrease in NK1R-RLuc8 and KRas-Venus BRET in response to SP (1 nM) (Fig. 2C). Considered together, these results are consistent with the hypothesis that SP induces association of the NK1R with ECE-1, notably ECE-1d, in endosomes. Endosomal ECE-1 can degrade SP and, thereby, disrupt NK1R interaction with ARRs. Furthermore, the NK1R/ARR interaction and NK1R trafficking to endosomes are independent of Gq-PKC activation.

**ARRs and ECE-1 Regulate NK1R Trafficking in NCM460 Colonocytes**—To ascertain whether ECE-1 and ARRs regulate NK1R trafficking and signaling in cells that participate in SP-mediated inflammation, we studied non-transformed human colonocytes (NCM460 cells) stably expressing the NK1R (NCM-NK1R cells). The NK1R is up-regulated in colonocytes in the inflamed intestine, and NCM460 cells are widely used to examined neuropeptide-stimulated inflammatory signaling (11). SP stimulated a small and variable increase in [Ca2+]i in non-transfected NCM460 cells (data not shown), and, thus, the NK1R was overexpressed to facilitate studies of regulation and signaling. NCM-NK1R cells expressed endogenous ARR1 and 2 mRNA (Fig. 3A), although ARR1 immunoreactivity (IR) was
more abundant (Fig. 3B). NCM-NK,R cells also expressed mRNA for ECE-1a-d isoforms (Fig. 3C) and ECE-1-IR (Fig. 3D). Thus, NCM460 colonocytes endogenously express isoforms of βARRs and ECE-1 that play a major role in regulating trafficking and signaling of the NK1R.

We incubated NCM-NK,R cells with Alexa-tagged SMSP, an NK,R agonist (21), to localize the NK,R. To determine the role of βARRs in the endocytosis of Alexa-SMSP in NCM-NK,R cells, we transfected cells with βARR1-GFP, βARR2-GFP, or dominant negative βARR1319–418-GFP (23). Incubation of cells with Alexa-SMSP (10 nM) at 4 °C induced translocation of both βARR1-GFP and βARR2-GFP to the plasma membrane (Fig. 4, A and B). After subsequent incubation at 37 °C for 30 min, βARR1-GFP and βARR2-GFP had trafficked with Alexa-SMSP to endosomes (Fig. 4, A and B). In contrast, Alexa-SMSP was not internalized in cells expressing βARR1319–418-GFP (Fig. 4C). Internalized Alexa-SMSP also colocalized with endogenous βARR1-IR in endosomes (Fig. 4D). The localization and SP-induced redistribution of endogenous βARR1-IR was completely consistent with the localization and SP-induced redistribution of βARR1-GFP, and the βARR1 antibody detected βARR1 in Western blot analyses (not shown). These results suggest the specific detection of endogenous βARR. Together, these results suggest that βARRs mediate SP-induced endocytosis of the NK,R and traffic with SP/NK,R to endosomes in NCM-NK,R cells.

To examine the subcellular localization of ECE-1, we transfected NCM-NK,R cells with ECE-1a-d-GFP, ECE-1b-GFP, and ECE-1d-GFP were colocalized predominantly with EEA-1 (Fig. 5, A and B). ECE-1a-GFP and ECE-1c-GFP were mainly at the plasma membrane and, to a lesser extent, colocalized with EEA-1 (Fig. 5, A and B). Endogenous ECE-1-IR, detected with an antibody to ECE-1b/d (9), also colocalized with EEA-1 (Fig. 5A). ECE-1 staining was prevented by preadsorption of the antibody with ECE-1 (Fig. 5C).

After incubation for 60 min at 4 °C, Alexa-SMSP was present at the plasma membrane (Fig. 5D). After washing and recovery for 30 min at 37 °C, Alexa-SMSP was detected in endosomes containing ECE-1a-d-GFP (Fig. 5, D and E). Colocalization of Alexa-SMSP and ECE-1b-d-GFP in endosomes was particularly evident (Fig. 5D). Internalized Alexa-SMSP also colocalized with endogenous ECE-1b/d-IR in EEA-1-positive endosomes (Fig. 5F). Examination by superresolution microscopy confirmed the colocalization of Alexa-SMSP and ECE-1b/d-IR in the same endosomes (Fig. 5G).
ARRs and ECE-1 Control the Spatiotemporal Dynamics of Intracellular NK1R Signaling in NCM460 Colonocytes and HEK293 Cells—To precisely define the spatiotemporal regulation of ERK signaling and assess its regulation by ARRs and ECE-1, we expressed, in NCM460 cells, the NK1R and the FRET biosensors CytoEKAR and NucEKAR, which permit the detection of ERK activity in the cytoplasm or nucleus, respectively (16). We also treated cells with ARR siRNA or inhibitors of ECE-1 (SM-19712), Gq (UBO-QIC), PKC (GF109203X), or epidermal growth factor receptor (EGFR, AG1478) to evaluate the importance of these signaling pathways for SP-stimulated activation of ERK in the cytosol and nucleus.

In control experiments (no inhibitors), SP (1 nM) induced a rapid increase in cytosolic ERK phosphorylation within 5 min that declined to basal levels after 20 min (Fig. 6, A—C and G). In contrast, SP stimulated a gradual increase in nuclear ERK phosphorylation that was sustained for 20 min (Fig. 6, D—F and H).

To determine how the NK1R activates ERK in two spatially distinct pools with distinct kinetics, we assessed the effect of siRNA knockdown and of pharmacological inhibitors on SP-induced cytosolic and nuclear ERK phosphorylation. The use of
siRNA allowed selective knockdown of βARR1 and βARR2 mRNA (Fig. 7, A and B), and βARR1 and βARR2 siRNA were combined for effective knockdown of both isoforms (Fig. 7C). βARR1 and βARR2 siRNA had no effect on SP-stimulated phosphorylation of cytosolic ERK (Fig. 6, A and G) but abolished SP-stimulated phosphorylation of nuclear ERK (Fig. 6, D and H). The ECE-1 inhibitor SM-19712 did not affect SP-stimulated cytosolic ERK phosphorylation (Fig. 6, B and G) but increased nuclear ERK phosphorylation ~2-fold (Fig. 6, E and H). Inhibitors of Goq (UBO-QIC), PKC (GF109203X), or EGFR (AG1478) abolished SP-induced cytosolic ERK phosphorylation (Fig. 6, C and G). Inhibition of Goq and PKC, but not EGFR, also prevented SP-induced nuclear ERK phosphorylation (Fig. 6, F and H).

To determine whether these effects were cell-type specific, we similarly examined SP-induced ERK signaling in HEK293 cells expressing NK1R and FRET biosensors. In HEK293 cells, and in NCM460 colonocytes, βARR1 and βARR2 siRNA blocked SP-stimulated phosphorylation of nuclear, but not cytosolic, ERK (Fig. 8, A, D, G, and H). SM-19712 magnified SP-stimulated phosphorylation of nuclear, but not cytosolic, ERK (Fig. 8, B, E, G, and H). UBO-QIC and GF-109203X both blocked SP stimulation of nuclear and cytosolic ERK, and AG1478 selectively prevented SP-induced stimulation of cytosolic ERK (Fig. 8, C, F, G, and H).

Considered together, the results of these experiments to examine mechanisms of ERK signaling support our previous studies showing that βARRs can recruit the NK1R and upstream activators of ERK, including Src and MEKK, to endosomes (10, 24). They are also consistent with the ability of ECE-1 to interact with the NK1R, degrade SP in endosomes, and disrupt the NK1R association with βARR that is necessary for activation of nuclear ERK (9, 10). Our results suggest that the transient cytosolic ERK phosphorylation mediated by the NK1R depends on activation of a Goq-PKC pathway leading to EGFR transactivation. The delayed and sustained phosphor-
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GRK2 and βARRs to NK1-R resensitization, NCM-NK1-R cells were preincubated with vehicle or 1 nM SP because GRKs and βARRs preferentially mediate desensitization to submaximal agonist concentrations. The GRK2 inhibitor CMPD103A (26) increased the magnitude and duration of the initial response to SP (80 ± 11% increase in maximal response compared with vehicle, p < 0.05, Fig. 9C) and inhibited resensitization of SP-induced Ca2+ signaling (20 ± 1.8% of control resensitization, 120-min recovery; p < 0.001; Fig. 9, C and F). BARR1 and βARR2 siRNA caused a small but insignificant increase in the magnitude of the initial SP response (30 ± 24% increase in maximal response compared with vehicle, p = 0.33, Fig. 9D) and inhibited resensitization of SP-induced Ca2+ signaling (43 ± 1.8% of control resensitization; 120-min recovery; p < 0.001; Fig. 9, D and F). The ECE-1 inhibitor SM-19712 did not affect the magnitude or desensitization of the first response to SP (10 nM) but did inhibit resensitization of SP (10 nM)-induced Ca2+ signaling (16 ± 6% of control resensitization; 90-min recovery; p < 0.001; Fig. 9, E and F).

Our results show that GRK2, βARRs, and ECE-1 all promote resensitization of plasma membrane NK1-R signaling in colonocytes. By phosphorylating the NK1-R, GRK2 promotes the association with βARRs, which deliver SP and the NK1-R to endosomes containing ECE-1. ECE-1 can then degrade SP and disrupt the NK1-R/βARR signalingosome, which permits NK1-R recycling.

βARR2 and ECE-1 Control SP-induced Inflammatory Signaling in NCM460 Colonocytes—Sustained incubation with SP stimulates NF-κB-dependent generation of IL-8 from colonocytes (12, 13). In NCM-NK1-R cells, SP (10 nM, 45 min) stimulated phosphorylation of NF-κB p65 (Fig. 10A). βARR2 siRNA, but not βARR1 siRNA, reduced NF-κB p65 phosphorylation (Fig. 10A). SM-19712 also suppressed NF-κB p65 phosphorylation (Fig. 10B). SP (10 nM, 8 h) stimulated IL-8 release from NCM-NK1-R cells, and βARR2 siRNA and SM-19712 inhibited SP-stimulated IL-8 secretion (Fig. 10, C and D). βARR1 siRNA did not affect SP-induced IL-8 expression. Thus, βARR2 and ECE-1 promote SP-driven inflammatory signaling in colonocytes, as assessed by NF-κB activation and IL-8 release.

In NCM-NK1-R cells, SP-induced IL-8 expression involves the activation of PKCδ and is blocked by rottlerin, which inhibits multiple kinases (12). Because SP causes ERK activation in NCM-NK1-R cells by a βARR-dependent mechanism that is terminated by endosomal ECE-1, ERK could also regulate IL-8 release. However, PD98059, an inhibitor of the MEK1-ERK pathway, did not affect SP-induced phosphorylation of NF-κB p65 (Fig. 10B) or IL-8 release (Fig. 10E), whereas rottlerin blocked SP-induced phosphorylation of NF-κB p65 (Fig. 10B), suggesting that ERK is not involved in SP-induced IL-8 expression in colonocytes.

Our results show that sustained SP inflammatory signaling requires the presence of the NK1-R at the plasma membrane, which depends on the capacity of ECE-1 and βARRs to promote recycling and resensitization of the NK1-R. However, a substantial proportion of activated, phosphorylated NK1-R remains at the plasma membrane, where protein phosphatase 2A (PP2A)
dephosphorylates and resensitizes non-internalized NK₁R (18). Fostriecin, an inhibitor of PP2A that blocks resensitization of non-internalized NK₁R, strongly inhibited SP-stimulated IL-8 secretion (Fig. 10D). These findings reinforce the conclusion that sustained SP inflammatory signaling requires resensitization of NK₁R at the plasma membrane rather than sustained endosomal NK₁R signaling via ERK.

**DISCUSSION**

Our results provide insights into the relative importance of plasma membrane and endosomal signaling of the NK₁R for SP-induced inflammation. We report that SP stimulates interactions between the NK₁R and two classes of proteins that play a major role in regulating NK₁R signaling at plasma and endosomal membranes: the scaffolding proteins βARR1 and βARR2 and the transmembrane endopeptidases ECE-1a through ECE-1d. βARRs uncouple the NK₁R from G proteins at the plasma membrane, which desensitizes plasma membrane Ca²⁺ signaling, and mediate NK₁R endocytosis, which is required for sustained activation of nuclear ERK. ECE-1d associates with SP/NK₁R in endosomes and degrades SP, which terminates activation of nuclear ERK. By delivering SP/NK₁R to endosomes, βARRs facilitate ECE-1 degradation of SP. Our results suggest that both βARRs and ECE-1 are necessary for resensitization of plasma membrane Ca²⁺ signaling. The sustained stimulation of colonocytes with SP induces NF-κB activation and IL-8 secretion. This inflammatory signaling does not require ERK activation and is independent of endosomal NK₁R ERK signaling. However, ECE-1 inhibition and βARR2 knockdown suppress SP-induced NF-κB and IL-8 signaling, indicating that the principal function of ECE-1 and βARR2 in colonocytes, rather than to participate in endosomal signaling, is to deliver resensitized NK₁R to the plasma membrane, which is required for sustained SP inflammatory signaling. Consistent with this proposal is the finding that PP2A, which resensitizes non-internalizing NK₁R at the plasma membrane, is also nec-
necessary for SP-induced IL-8 secretion. Considered together, our findings suggest that sustained inflammatory signaling of SP during inflammation requires efficient resensitization of the NK$_1$R at the plasma membrane by ARR2-, ECE-1-, and PP2A-dependent mechanisms. Further studies using complimentary genetic and pharmacological approaches are required to define the importance of ARR2, ECE-1, and PP2A for chronic inflammation in vivo.

**Contribution of ECE-1 to SP Inflammatory Signaling**—We show that ECE-1 resensitizes the NK$_1$R at the plasma membrane by βARR2-, ECE-1-, and PP2A-dependent mechanisms. Further studies using complimentary genetic and pharmacological approaches are required to define the importance of βARR2, ECE-1, and PP2A for chronic inflammation in vivo.

**FIGURE 9. Desensitization and resensitization of SP-induced Ca$^{2+}$ signaling in NCM-NK$_1$R colonocytes.** NCM-NK$_1$R cells were preincubated with SP (1, 10 nM, 10 min), washed, recovered for 0–150 min, and challenged with SP (10 nM). A, C, D, and E, changes in [Ca$^{2+}$]. Shown are the effects of the GRK2 inhibitor CMPD103A, βARR1 and βARR2 siRNA, the ECE-1 inhibitor SM-19712, or control (vehicle or control siRNA). B and F, resensitization expressed as percent vehicle (no SP) control. A, three combined traces from one representative experiment. B–F, n = 3 experiments, triplicate observations. **, p < 0.001; ***, p < 0.0001 compared with vehicle or control siRNA.
nuclear ERK in colonocytes and HEK cells, as in primary neurons (8, 10). Although ERK mediates NF-κB activation and IL-8 release in response to some proinflammatory stimulants in colonocytes (28), MEK inhibition did not affect SP-stimulated IL-8 secretion, which, instead, depends on PKCβ and NF-κB in NCM cells (12, 13). The anti-inflammatory effects of an ECE-1 inhibitor are consistent with the inhibitory actions of ECE-1 on ARRs in the assembly of MAPK signalosomes because of their role in endosomal trafficking of the NK1R (22), ECE-1 inhibition could prevent sustained signaling of several neuropeptide mediators of inflammation.

Contribution of βARR to SP Inflammatory Signaling—We report that SP stimulates translocation of βARRs from the cytosol to the plasma membrane, followed by trafficking of SP, NK1R, and βARR1/2 to early endosomes containing ECE-1 through ECE1d. Inhibition of GRK2 and disruption of βARRs enhanced SP-mediated Ca2+ signals and blocked NK1R endocytosis, consistent with the known role of GRKs and βARRs in attenuating plasma membrane signaling and mediating NK1R endocytosis (17, 24). βARR knockdown also attenuated SP-induced activation of nuclear ERK and inhibited resensitization of Ca2+ signaling. These findings support the involvement of βARRs in the assembly of MAPK signalosomes because of their role in endosomal trafficking of the NK1R (24). The results are consistent with receptor endocytosis, intracellular processing, and recycling as mechanisms of resensitization (19).

By selective siRNA knockdown, we probed the role of βARR isoforms in SP-induced inflammatory signaling. Although knockdown of βARR1 and βARR2 was required to inhibit resensitization and internalization-dependent nuclear ERK signaling, βARR2 siRNA alone attenuated SP-stimulated activation of NF-κB and IL-8 release, suggesting that βARR2 promotes SP inflammatory signaling in colonocytes. βARR2 also mediates lipopolysaccharide-stimulated IL-8 secretion (30). One explanation for the proinflammatory action of βARR2 could be the βARR2-dependent recruitment of regulators of NF-κB activation to the NK1R. Similarly, βARR2 recruits CARMA3 to the lysophosphatidic acid receptor to stimulate NF-κB activation (31). Another explanation for the positive role of βARR2 in SP inflammatory signaling may be related to the resensitization of plasma membrane signaling. By mediating endocytosis, βARRs can promote ligand dissociation and receptor dephosphorylation in endosomes, which are required for recycling and resensitization. This possibility is supported by our observation that endosomal ECE-1 induces NK1R resensitization and inflammatory signaling. Our finding that βARRs mediate SP-induced inflammation is supported by reports that βARR1 deletion protects mice from colitis (32), which involves SP/NK1R interactions (11).

A limitation of our study is that it examines the regulation of the NK1R overexpressed in NCM460 colonocytes or HEK293 cells. Although we obtained similar findings with both cell lines, it will be important to confirm that similar mechanisms regulate the endogenous NK1R expressed in functionally relevant cell types and to use complimentary genetic and pharmacological approaches to define the mechanisms of sustained inflammatory signaling by SP and the NK1R.

In summary, our results suggest that the primary function of βARRs and ECE-1 in SP-dependent inflammatory signaling is to maintain plasma membrane signaling through control of NK1R internalization, recycling, and resensitization. These processes may sustain NK1R-driven inflammatory signaling, an important component of the pathophysiology of widespread inflammatory disorders.

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