Expression and coupling of neurokinin receptor subtypes to inositol phosphate and calcium signaling pathways in human airway smooth muscle cells

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Mizuta K, Gallos G, Zhu D, Mizuta F, Goubaeva F, Xu D, Panettieri RA Jr, Yang J, Emala CW Sr. Expression and coupling of neurokinin receptor subtypes to inositol phosphate and calcium signaling pathways in human airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 294: L523–L534, 2008. First published January 18, 2008; doi:10.1152/ajplung.00328.2007.—Neuropeptide tachykinins (substance P, neurokinin A, and neurokinin B) are present in peripheral terminals of sensory nerve fibers within the respiratory tract and cause airway contractile responses and hyperresponsiveness in humans and most mammalian species. Three subtypes of neurokinin receptors (NK1R, NK2R, and NK3R) classically couple to Gq protein-mediated inositol 1,4,5-trisphosphate (IP3) synthesis and liberation of intracellular Ca2+, which initiates contraction, but their expression and calcium signaling mechanisms are incompletely understood in airway smooth muscle. All three subtypes were identified in native and cultured human airway smooth muscle (HASM) and were subsequently overexpressed in HASM cells using a human immunodeficiency virus-1-based lentivirus transduction system. Specific NKR agonists (NK1R, [Sar9,Met(O2)11]-substance P; NK2R, [β-Ala8]-neurokinin A(4–10); NK3R, senktide) stimulated inositol phosphate synthesis and increased intracellular Ca2+ concentration ([Ca2+]i) in native HASM cells and in HASM cells transfected with each NKR subtype. These effects were blocked by NKR-selective antagonists (NK1R, L-732138; NK2R, GR-159897; NK3R, SB-222200). The initial transient and sustained phases of increased [Ca2+]i were predominantly inhibited by the IP3 receptor antagonist 2-aminoethoxydiphenyl borate (2-APB) or the store-operated Ca2+ channel antagonist SKF-96365, respectively. These results show that all NKR subtypes are members of the seven-transmembrane domain receptor superfamily that couple to Gq proteins.

Classically, the expression of NK1R and NK2R has been described in both the nervous system and peripheral target organs, including human airways, whereas the expression of NK3R was believed to be more limited to the central and peripheral nervous system (31, 34). Furthermore, it has traditionally been thought that only the NK3R mediated direct contraction of airway smooth muscle, especially in human airways (3). However, recently it was reported that in small-diameter human bronchi, tachykinins also cause contraction via NK1R stimulation (4). Moreover, functional evidence for the presence of NK3R exists in both animal and human airways (24, 38, 40, 43). Although most functional studies suggest that the NK3R subtype is not directly involved in airway smooth muscle constriction (2, 13, 14, 53), an NK3R antagonist was mildly potent at inhibiting a neurokinin A-induced contraction in human bronchus (51).

Early studies using RNase protection assays detected mRNA encoding the NK3R and NK2R but not the NK1R in human bronchi (6). However, more recently, mRNA encoding the NK3R was identified by RT-PCR in human bronchi (48), although the specific lung cell type on which the NK3R is expressed is unclear. The NK1R and NK3R proteins were immunohistochemically detected in human bronchial smooth muscle of central airways, but the presence of the NK3R protein was not evaluated (34). Thus the expression and functional coupling of the NK3R on human airway smooth muscle cell is unclear.

In airway smooth muscle, increases in intracellular Ca2+ concentration ([Ca2+]i) are mediated by intracellular release from sarcoplasmic reticulum (SR) via both ryanodine and inositol 1,4,5-trisphosphate (IP3) receptors (7) and by influx of extracellular Ca2+, which can occur via voltage-gated and receptor-gated channels, as well as in response to SR Ca2+ depletion (store-operated Ca2+ entry; SOCE) (11, 35, 49). Although NKR agonist-induced IP3 accumulation is thought to play a predominant role in the initiation of increases in [Ca2+]i and the initiation of contraction in airway smooth muscle (4, 17), detailed intracellular Ca2+ signaling mechanisms induced by activation of NKR subtypes are not fully understood in HASM.
In the present study, we questioned whether all three known subtypes of tachykinin receptors are expressed in airway smooth muscle cells and whether each of these subtypes is capable of coupling to the activation of inositol phosphate synthesis and elevation of intracellular calcium, and we hypothesized that the predominant mechanism of increased intracellular Ca\(^{2+}\) occurred via activation of IP\(_3\) receptors.

**METHODS**

**Materials.** Cells were cultured in SmGM-2 smooth muscle medium (Lonza, Walkersville, MD). Fluoro-4 AM and Phorbcin F-127 were obtained from Molecular Probes (Eugene, OR). \(\text{[Sar}^9,\text{Met(O}2\text{)}^{11}\text{]}\)-substance P (SM-SP), \([\text{CO}_2\text{-}95\%\text{ air.}\) ng/ml amphotericin B; Lonza) at 37°C in an atmosphere of 5% CO\(_2\)–95% air.

**RT-PCR of NKRs in native HASM.** Studies were approved by Columbia University’s Institutional Review Board and deemed not human subjects research under 45 CFR 46. Human trachea came from two sources. Snap-frozen tracheas obtained from lung transplant donors in accordance with procedures approved by the University of Pennsylvania Committee on Studies Involving Human Beings as previously described (47). For the measurement of [Ca\(^{2+}\)], the cells were grown to confluence on 96-well plates in culture medium (SmGM-2 supplemented with 5% FBS, 5 µg/ml insulin, 1 ng/ml human fibroblast growth factor, 500 pg/ml human epidermal growth factor, 30 µg/ml gentamicin, and 15 ng/ml amphotericin B; Lonza) at 37°C in an atmosphere of 5% CO\(_2\)-95% air.

**RT-PCR of ryanodine receptors.** Total RNA was extracted from primary cultures and NKr-transduced HASM cells as described above. Total RNA from human heart and skeletal muscle were components of the human total RNA Master Panel II (Clontech) and were used as positive controls. The Advantage RT-for-PCR kit was used as described, utilizing primers that transversed at least one intron for each ryanodine receptor (Ryr) subtype: [Ryr1: sense primer, 5′-ATG GCC AGC AAG ATG ACA ATG ACG-3′; antisense primer, 5′-GGC GTT CAA TCC TCT GCC ACG TAC AAT CCA TAA AAG-3′; reverse primer, 5′-CCC TCC TCC AGC ACG AAT ACG ACC AT-3′; Ryr2: forward primer, 5′-GGC GTT CAA TCC TCT GCC ACG TAC AAT CCA TAA AAG-3′; reverse primer, 5′-AGC GCC ACG AAT AGC ACA TCT CCA TCT CCA GGA TAC CGT TGG TCA GTG TCA TGC T-3′; antisense primer, 5′-ATG GCC AGC AAG ATG ACA ATG ACG-3′; Ryr3: sense primer, 5′-ATG GCC AGC AAT AGC ACA TCT CCA TCT CCA GGA TAC CGT TGG TCA GTG TCA TGC T-3′; antisense primer, 5′-AGC GCC ACG AAT AGC ACA TCT CCA TCT CCA GGA TAC CGT TGG TCA GTG TCA TGC T-3′]. PCR products were analyzed as described above.

**Production of lentiviral vectors and transduction of cultured HASM cells.** Plasmids (pcDNA 3.1+) containing the coding sequence for the human (hu) NK,R, NK,R, and NK,R were purchased from the UMR cDNA Resource Center (University of Missouri at Rolla). Coding regions were excised (EcoRI and XbaI for NK,R and NK,R) and subcloned into these same restriction sites in the lentiviral vector Δu6-pLL-IREs-EFGP. Because of the presence of an internal EcoRI restriction endonuclease recognition site, huNK,R was excised from pcDNA 3.1+ using HindIII and XbaI and subcloned into pBlueScript. Subsequently, it was excised using HindIII (blunt) and NotI and subcloned into EcoRI (blunt) and NotI in Δu6-pLL-IREs-EFGP. When expressed, this vector yields two independent proteins, enhanced green fluorescent protein (EGFP) and red fluorescent protein (RFP), and both proteins can be detected by fluorescence microscopy. Cells were transduced with the lentivirus vector at a multiplicity of infection of 100 to 2000. Subsequently, it was excised using HindIII (blunt) and NotI in Δu6-pLL-IREs-EFGP. When expressed, this vector yields two independent proteins, enhanced green fluorescent protein (EGFP) and red fluorescent protein (RFP), and both proteins can be detected by fluorescence microscopy. Cells were transduced with the lentivirus vector at a multiplicity of infection of 100 to 2000.
inositol-free and serum-free DMEM (Chemicon, Temecula, CA), plates were washed three times 
[37°C, 500 /H9262l of Hanks’ balanced salt solution (HBSS) with 10 mM LiCl]. Incubation of cells with NKR-
selective agonists (0.01 nM-10 /H9262M; NK1R, SM-SP; NK2R, β-ala-
NKA; NK1R, senktide) in a final volume of 300 /H9262l at 37°C for 30 min
was performed in the absence and presence of NKR antagonists (100 /H9262µM) (NK1R, L-732138; NK1R, GR-159897; NK1R, SB-222200).
Reactions were terminated, and [3H]inositol and total [3H]inositol
phosphates were recovered using chromatography (21).

Measurement of [Ca2+]. Confluent native HASM cells or HASM
cells stably transfected with the NK1R, NK2R, or NK3R in 96-well
plates were incubated in modified HBSS (in mM: 138 NaCl, 5.3 KCl,
2.5 CaCl2, 0.4 MgSO4, 0.49 MgCl2, 0.34 NaH2PO4, 4.2 NaHCO3,
0.44 KH2PO4, 5.5 dextrose, and 20 HEPES, pH 7.4) at 100
/H9262l/well containing 5 /H9262M fluo-4 AM (DMSO vehicle final concentration:
0.5%), 0.05% Pluronic F-127 (DMSO vehicle final concentration:
0.25%), and 2.5 mM probenecid for 30 min at 37°C. Once the cells
were loaded, the cells were washed twice with modified HBSS
containing 2.5 mM probenecid and left for an additional 30 min at
room temperature to allow complete deesterification of the intracel-
lular acetoxy methyl esters. This buffer was exchanged (100
/H9262l/well) just before the measurement of fluorescence was started. The fluores-
cence was then continuously recorded every 5 s at excitation wave-
lengths of 485 nm and emission wavelengths of 528 nm using a
microplate reader (Synergy HT; BioTek Instruments, Winooski, VT).
Triplicate wells were simultaneously measured, and values were
averaged for each data point. After a stable baseline was established
for the first 10 min, the cells were pretreated with inhibitors [10 /H9262µM
verapamil, an L-type voltage-gated Ca2+ channel blocker; 10
/H9262µM SKF-96365, a SOCC blocker; 10 /H9262µM 2-APB, an IP3 receptor antag-
onist; 100 /H9262µM ryanodine, a ryanodine receptor antagonist; or 10 mM
caffeine, a ryanodine receptor agonist that leads to Ca2+ depletion
in the SR] or vehicle (modified HBSS) for 10 min. Baseline was
acquired for 2 min just before addition of NKR-selective agonists. The
cells were then incubated with 1 /H9262µM NK-selective agonist (SM-SP,
β-ala-NKA, or senktide), and the fluorescence was recorded for 10
min. In all studies, the fluorescence after treatments is presented as the
percent change from baseline fluorescence.

Measurement of store-operated Ca2+ entry. These experiments
were designed to examine the effects of 2-APB, caffeine, or ryanodine
on store-operated Ca2+ entry (SOCE) in HASM. SOCE was evaluated
using previously described protocols (5, 46, 49). Extracellular Ca2+
was removed by exposure to 0-Ca2+ modified HBSS containing 1
mM EGTA for 10 min. Cells were exposed to 1 µM verapamil and 10

Fig. 1. Endogenous neurokinin receptor (NKR) expression in human airway smooth muscle (HASM) cells. Representative RT-PCR analysis of total RNA using
primers that specially recognize mRNA for each NKR subtype [NK1R (A), NK2R (B), and NK3R (C)] in freshly dissected human tracheal airway smooth muscle
(ASM) and in primary cultures of HASM cells. Human brain cDNA was used as a positive control.

Fig. 2. NKR subtype-selective agonist effects on inositol phosphate synthesis. Effects of selective NKR agonists [Sar8,Met(O2)11]-substance P (SM-SP; 10
/H9262µM) for NK1R (A), [β-Ala8]-neurokinin A (β-ala-NKA; 10 µM) for NK2R (B), and senktide (10 µM) for NK3R (C) on total inositol phosphate synthesis in both
native cultured HASM and NKR-transduced cultured HASM (HASM-huNK1R, -huNK2R, -huNK3R) cells. Data are means ± SE, presented as percentages of
basal values. *P < 0.05; ***P < 0.001 compared with basal. ###P < 0.001 compared with NKR agonist in native HASM. Numbers in parentheses indicate
the number of experiments.
mM KCl to inhibit L-type Ca$^{2+}$ channels and depolarize cells, respectively. Cells were then exposed to 10 μM cyclopiazonic acid [CPA; an inhibitor of SR Ca$^{2+}$-ATPase (SERCA)] in 0-Ca$^{2+}$ modified HBSS with 1 mM EGTA, resulting in passive SR depletion with continued SR Ca$^{2+}$ leak (likely from both IP$_3$- and ryanodine-sensitive SR stores). As in previous studies (5, 46), a gradual elevation of [Ca$^{2+}$]i was typically noted that eventually reached a plateau or started trending downward (because plasma membrane Ca$^{2+}$ efflux was not inhibited). Pretreatment with SKF-96365 (10 μM; positive control inhibitor), 2-APB (10 μM), ryanodine (100 μM), or caffeine (10 mM) was performed, and then CaCl$_2$ (to achieve a final extracellular Ca$^{2+}$ concentration of 2.5 mM) was rapidly reintroduced (in the continued presence of verapamil, KCl, and CPA) and the peak fluorescence was measured.

**Western blot analysis of the heavy chain of smooth muscle-specific myosin.** Confluent HASM cells either nontransduced or stably transduced with the NK1R, NK2R, or NK3R were scraped from T75 flasks conditioned with 1 mM EGTA, resulting in passive SR depletion with continued presence of verapamil, KCl, and CPA. Freshly dissected human tracheal airway smooth muscle was homogenized on ice using a Tekmar homogenizer set at top speed for 30 s in the same lysis buffer. After centrifugation (15,000 g, 15 min, 4°C) of the whole cell lysate, the supernatant was saved and protein concentrations were determined. Aliquots of the supernatants were solubilized by heating at 95°C for 5 min in sample buffer (final concentrations: 50 mM Tris-HCl, pH 6.8, 2.5% SDS, 6% glycerol, 2.5% 2-mercaptoethanol, and bromphenol blue) and stored at -20°C. The supernatants of solubilized whole cell or tissue lysates were electrophoresed through a discontinuous SDS-PAGE gel (3% stacking over 5% separating) and transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were blocked for 2 h at room temperature with 5% milk in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBS-T) and then probed overnight while shaking at 4°C with a 1:1,000 dilution of mouse monoclonal antibody directed against the heavy chain of smooth muscle-specific myosin (MAB3570; Chemicon). After being washed three times, PVDF membranes were incubated for 2 h at room temperature with horseradish peroxidase-conjugated secondary anti-mouse antibodies (1:5,000 dilution in 1% milk in TBS-T; Amersham Biosciences NA931V). The signal from the immunoreactive bands was detected using enhanced chemiluminescence (ECL Plus; Amersham Biosciences) according to the manufacturer’s recommendations, developed on film (Kodak Biomax light film; Kodak, Rochester, NY), and detected using Quantity One software (Bio-Rad).

**Filamentous-to-globular actin ratio measurements.** Basal and agonist-induced ratios of filamentous (F) and globular (G) actin were determined as previously described (55), except that a blue fluorescence-tagged phalloidin was used instead of a green FITC-tagged phalloidin because of the coexpression of green fluorescent protein (GFP) in these NKR subtype transduced cells. Cells at 70% confluence on eight-well microscope slides were exposed to vehicle (water) or NKR subtype-specific agonists (10 μM for 5 min) before being fixed by the addition of an equal volume of 7.4% paraformaldehyde in PBS to achieve a final concentration of 3.7% paraformaldehyde. After permeabilization with Triton X-100 (0.5% in PBS, 5 min) and blocking (1% BSA in 0.1% Triton-X100 in PBS, 10 min), cells were stained with Alexa Fluor 530-phalloidin (3 U/ml; Invitrogen) and Alexa Fluor 594-DNase I (10 μg/ml; Invitrogen) in 300 μl of blocking solution in the dark for 20 min. After being washed (twice in 0.1% Triton X-100 in PBS and twice in PBS), a coverslip was mounted and visualized with an inverted fluorescent microscope (Olympus IX-70). Digitized images were quantified as described previously (20).

**Statistical analysis.** Statistical analysis was performed using repeated measures of ANOVA, followed by Bonferroni posttest comparison using Prism 4.0 software (GraphPad, San Diego, CA). Data are means ± SE. *P < 0.05 was considered significant.

**RESULTS**

**Expression of native and overexpressed NKRAs in HASM cells.** Messenger RNA encoding the NK1R, NK2R, and NK3R was detected in total RNA extracted from freshly dissected airway smooth muscle from human bronchi and in primary cell cultures of HASM cells by RT-PCR (Fig. 1). Bands of expected size (162, 310, and 360 bp, respectively) were detected in at least three independent RNA samples from each source and in total RNA from whole human brain used as a positive control.

We first established functional coupling of each tachykinin receptor subtype in native cultured airway smooth muscle cells by using receptor subtype-specific agonist stimulation of inositol phosphate synthesis. In native HASM cells, all three NKR subtypes are expressed.
NKR-selective agonists (10 μM SM-SP, 10 μM β-ala-NKA, and 10 μM senktide) significantly increased inositol phosphate synthesis (SM-SP: P < 0.05, n = 7; β-ala-NKA: P < 0.05, n = 7; senktide: P < 0.05, n = 10) (Fig. 2). We next demonstrated in native cultured airway smooth muscle cells that subtype-selective agonists selective for the NK1R, NK2R, and NK3R increased [Ca2+]i (Fig. 3).

To enhance the expression of each receptor subtype to facilitate signaling studies, we transduced primary cell lines of HASM cells with lentivirus encoding each of the human NKR subtypes along with coexpressed GFP. Transfected HASM cells exhibited bright green fluorescence 3–5 days after transduction (Fig. 4). Approximately 70–80% of the HASM cells routinely expressed GFP at 3–5 days.

Confirmation of smooth muscle cell phenotype in transduced cells. Two independent studies were performed to confirm that both native and NKR-transduced HASM cells retained a smooth muscle phenotype. We confirmed the expression of myosin by immunoblotting and confirmed activation of the actin cytoskeleton (19, 20, 54) by NKR agonists. An antibody specific for the heavy chain of smooth muscle myosin detected a 204-kDa protein in solubilized lysates of freshly dissected HASM and in native cultured HASM cells as well as cultured HASM cells transduced with the NK,R subtype (Fig. 5A).

Similarly, activation of the actin cytoskeleton, a process required for smooth muscle contraction, was induced by the respective specific agonists for each NKR subtype (Fig. 5B). In NK1R-transduced HASM cells, 10 μM SM-SP for 5 min increased the basal F/G actin ratio from 2.6 ± 0.13 to 3.6 ± 0.25 (n = 11, P = 0.003).

In NK2R-transduced HASM cells, 10 μM β-ala-NKA for 5 min increased the basal F/G actin ratio from 2.3 ± 0.11 to 3.5 ± 0.26 (n = 12, P = 0.0004). In NK3R-transduced HASM cells, 10 μM senktide for 5 min increased the basal F/G actin ratio from 2.3 ± 0.08 to 3.0 ± 0.24 (n = 8, P = 0.016).

Effect of NKR-selective agonists on inositol phosphate synthesis. In these HASM cell lines individually transfected with each of the huNKR subtypes, NKR-selective agonists significantly increased inositol phosphate synthesis (SM-SP: P < 0.001, n = 6; β-ala-NKA: P < 0.001, n = 7; and senktide: P < 0.001, n = 6) in HASM-huNK1R, -huNK2R, and -huNK3R cells, respectively, which was significantly greater than that in native cells (SM-SP: P < 0.001; β-ala-NKA: P < 0.001; and senktide: P < 0.001) (Fig. 2). These increases were virtually abolished by pretreatment with 100 μM NKR-selective antagonists (L-732138, GR-159897, and SB-222200) (P < 0.001, n = 6) in HASM-huNK1R, -huNK2R, and -huNK3R cells, respectively) (Fig. 6).

In all three HASM-huNKR cell lines, NKR-selective agonists (SM-SP, β-ala-NKA, and senktide, 0.01 nM–10 μM) increased both inositol phosphate synthesis and peak [Ca2+]i, mobilization in a concentration-dependent manner. At higher concentrations (10 μM), all relatively selective NKR agonists activated all three NKR subtypes but had markedly lower affinities compared with that to their preferred receptor subtype (Fig. 7). Thus each of the subtype-specific agonists displayed expected selectivity for their respective human NKR subtype.

Effect of NKR-selective receptor agonists on [Ca2+]i response. In HASM cells, exposure to G protein-coupled receptor (GPCR) agonist such as bradykinin, histamine, and acetylcholine typically resulted in a characteristic “biphasic” [Ca2+]i response, with an initial, significantly higher peak (transient phase) followed by a sustained elevation significantly above the baseline (sustained phase) (39). Likewise, NKR-selective agonists (1 μM SM-SP, 1 μM β-ala-NKA, and 1 μM senktide)
produced the biphasic $[Ca^{2+}]_i$, response in HASM-huNKR cells (Fig. 3, top). To characterize the mechanisms of NKR-mediated $[Ca^{2+}]_i$, mobilization in both the transient and sustained phases, HASM-huNKR cells were pretreated for 5 min with 1) verapamil (10 \mu M), 2) SKF-96365 (10 \mu M), 3) 2-APB (10 \mu M), 4) ryanodine (100 \mu M), or 5) caffeine (10 mM) and then stimulated with NKR agonists. In the transient phase, 2-APB predominantly inhibited the $[Ca^{2+}]_i$, mobilization (57.39 ± 9.65%, $P < 0.001$, $n = 11$; 44.07 ± 5.84%, $P < 0.001$, $n = 8$; and 69.49 ± 8.27%, $P < 0.001$, $n = 8$ in HASM-huNK1R, -huNK2R, and -huNK3R cells, respectively), whereas verapamil (9.65 ± 6.67%, $P < 0.05$, $n = 8$; 11.62 ± 7.28%, $P < 0.05$, $n = 10$; and 13.35 ± 4.74%, $P < 0.01$, $n = 9$ in HASM-huNK1R, -huNK2R, and -huNK3R cells, respectively), SKF-96365 (76.63 ± 4.50%, $P < 0.001$, $n = 9$; 84.19 ± 14.42%, $P < 0.001$, $n = 11$; and 60.67 ± 9.11%, $P < 0.01$, $n = 8$ in HASM-huNK1R, -huNK2R, and -huNK3R cells, respectively), and caffeine (76.13 ± 8.71%, $P < 0.001$, $n = 10$; 83.70 ± 14.74%, $P < 0.01$, $n = 9$; and 60.34 ± 9.85%, $P < 0.01$, $n = 11$ in HASM-huNK1R, -huNK2R, and -huNK3R cells, respectively) inhibited the NKR-selective agonist-induced $[Ca^{2+}]_i$, mobilization, whereas verapamil inhibited less (17.57 ± 6.58%, $P < 0.05$, $n = 8$; 20.10 ± 7.91%, $P < 0.05$, $n = 10$; and 20.98 ± 6.26%, $P < 0.01$, $n = 9$ in HASM-huNK1R, -huNK2R, and -huNK3R cells, respectively) (Fig. 9). Ryanodine also partially inhibited the NKR-selective agonist-induced $[Ca^{2+}]_i$, mobilization in the sustained phase (13.34 ± 1.73%, $P < 0.001$, $n = 11$; 11.78 ± 3.06%, $P < 0.01$, $n = 11$; and 15.44 ± 5.25%, $P < 0.05$, $n = 15$ in HASM-huNK1R, -huNK2R, and -huNK3R cells, respectively) (Fig. 9).

Effect of SKF-96365, 2-APB, ryanodine, and caffeine on SOCE in HASM. The sustained phase is thought to be primarily mediated by the extracellular $Ca^{2+}$ influx through SOCC (35). SKF-96365, 2-APB, ryanodine, and caffeine also inhibited the sustained phase in the present study. To examine whether SOCC contributes to the SKF-96365-, 2-APB-, ryanodine-, or caffeine-induced inhibition of the sustained phase, we examined the effect of SKF-96365 (10 \mu M), 2-APB (10 \mu M), ryanodine (100 \mu M), and caffeine (10 mM) on SOCE in native HASM cells. Exposure to SKF-96365, ryanodine, and caffeine resulted in partial inhibition of SOCE (27.49 ± 2.09%, $P < 0.001$, $n = 7$; 10.07 ± 2.60%, $P < 0.01$, $n = 9$; and 18.51 ± 2.85%, $P < 0.01$, $n = 6$, respectively), whereas exposure to 2-APB did not exert any effect on SOCE (−0.60 ± 4.72%, NS, $n = 5$) (Fig. 10).

Confirmation of expression of ryanodine receptor subtypes in cultured transduced HASM. Messenger RNA encoding all three known subtypes of ryanodine receptors was identified in both native and NKR-transduced HASM cells (Fig. 11). PCR products of expected sizes (142, 127, and 314 bp) were also detected in total RNA isolated from respective positive control tissues (human and mouse skeletal muscle for the grefr1 and grefr3 and human and mouse heart for the grefr2) (Fig. 11).

DISCUSSION

We have demonstrated that all three known subtypes of NKRs are expressed at the mRNA level in native HASM and that all three receptor subtypes couple to the synthesis of inositol phosphates and increase $[Ca^{2+}]_i$. Utilizing independent lentivirus-mediated overexpression of each receptor subtype in primary cultures of HASM cells, we have demonstrated agonist and antagonist specificity of each receptor subtype to

Fig. 5. Confirmation of smooth muscle cell phenotype of NKR-transduced HASM cells. A: representative immunoblot identifying expression of the 204-kDa smooth muscle-specific heavy chain of myosin in freshly dissected human tracheal ASM and in primary (HASM) and transduced (HASM-huNKR) cultures of HASM cells. B: fluorescent staining ratios of filamentous and globular actin (F/G actin) in NKR-transduced HASM cells (HASM-huNK1R, HASM-huNK2R, and HASM-huNK3R) under untreated (control) or subtype-specific agonist (10 \mu M)-treated conditions [NK1 agonist, SM-SP (n = 11); NK2 agonist, β-ala-NKA (n = 12); NK3 agonist, senktide (n = 8)]. In each cell type, subtype-specific activation of the NKR increased F actin, which is a component of smooth muscle cell contraction.
in the three different NKR subtypes with lower affinities than on the preferred receptor (50).

NKR agonists. The expression and functional significance of all three NKR subtypes in native HASM cells has been under debate. Most functional studies in both human and animal models have suggested that the NK1R subtype is the primary receptor subtype that mediates direct smooth muscle constriction (3, 51, 53). Some studies also have suggested a role for the NK2R in HASM constriction (4). However, the role of the NK3R in airways has traditionally been attributed to modulation of peripheral nerve function (9).

To characterize the coupling of NKR to pivotal intracellular signaling pathways in HASM cells, we individually overexpressed the three subtypes of wild-type huNKR in primary cultures of HASM cells (HASM-NK1R, -NK2R, and -NK3R) using an HIV-1-based lentivirus transduction system (29). A three-plasmid expression system was used to generate the plasmids encoding each subtype of the human NRs. This system has three advantages over other systems: it can be used on relatively slowly dividing cells such as airway smooth muscle (41), does not require a cell surface receptor for entry into the target cell but mediates viral entry through lipid binding and plasma membrane fusion (45), and allows for a higher transduction efficiency than most other transfection methods in smooth muscle cells.

We demonstrated that NKR subtype-selective agonists significantly increased inositol phosphate synthesis in both native HASM and HASM-NKR-transfected cells and that these increases were completely blocked by NKR subtype-selective antagonists. Receptor agonists designed to be selective for one receptor subtype typically demonstrate preferred selectivity for receptor subtypes across species because of subtle species differences in receptor protein structure (10, 15, 27). In the current study, we have demonstrated that [Sar²,Met(O₂)³] substance P, [β-Ala⁸]-neurokinin A (4–10), and senktide display the predicted selectivities for the human NK₁, NK₂, and NK₃ receptors, respectively. These results agree with the previous study showing that all tachyklinins can act as full agonists on the three different NKR subtypes but with lower affinities than on the preferred receptor (50).
[Ca\textsuperscript{2+}] increase in human bronchial smooth muscle cell was not impaired by ryanodine (22). Together, these results suggest that ryanodine receptors expressed in HASM cells do not have a significant role in the initial transient phase of NKR-mediated increases in [Ca\textsuperscript{2+}]i.

It has been reported that Ca\textsuperscript{2+} influx via L-type voltage-gated Ca\textsuperscript{2+} channels also takes part in airway smooth muscle contraction (23, 26, 36). In the present study, NKR-selective agonist-induced [Ca\textsuperscript{2+}] increases were partially blocked by verapamil. Likewise, Lin et al. (28) reported that substance P-evoked airway smooth muscle contraction and transient Ca\textsuperscript{2+} mobilization were partially inhibited by verapamil, indicating that the NKR-mediated [Ca\textsuperscript{2+}] increases are associated with activation of voltage-gated Ca\textsuperscript{2+} channels. They further demonstrated that smooth muscle contraction and [Ca\textsuperscript{2+}] increases activated by substance P were also partially blocked under Ca\textsuperscript{2+}-free external conditions (28). Together, these findings indicate that Ca\textsuperscript{2+} influx from the extracellular space is a component of the transient initial phase of NKR-mediated transient [Ca\textsuperscript{2+}] increases. In the present study, SKF-96365 had a small effect on the NKR-mediated transient [Ca\textsuperscript{2+}] increase but a much greater effect on the sustained phase. This finding raises the possibility that SOCE also takes part in the transient [Ca\textsuperscript{2+}] increase. However, a limitation of the interpretation of the results of the present study is that SKF-96365 also has a minor inhibitory effect on voltage-gated Ca\textsuperscript{2+} channels (56).
The sustained Ca\textsuperscript{2+} response is important for maintaining the contractile response to agonist (18) and requires the continued presence of agonist (11, 39). In the sustained phase, SOCE has been considered as the predominant source of Ca\textsuperscript{2+} influx (11, 35). SOCC are Ca\textsuperscript{2+}-permeable channels in the plasma membrane that are open following depletion of intracellular Ca\textsuperscript{2+} stores underlying the so-called “store-operated” or “capacitative” Ca\textsuperscript{2+} entry (35). In the present study, the NKR-selective agonist-induced sustained [Ca\textsuperscript{2+}] increase was significantly blocked by SKF-96365. These findings agree with the previous study (11, 35). However, verapamil also partially blocked this sustained [Ca\textsuperscript{2+}] increase. In contrast, it has been known that agonist-induced sustained [Ca\textsuperscript{2+}] increases in airway smooth muscle are relatively insensitive to inhibitors of voltage-gated Ca\textsuperscript{2+} channel despite their established presence (23, 26, 36). Our findings indicate that voltage-gated Ca\textsuperscript{2+} channels partially contribute to the maintenance of the NKR-mediated sustained [Ca\textsuperscript{2+}] response. We used SKF-96365 as an inhibitor of SOCE and validated this effect in the absence of receptor agonists (Fig. 10). However, SKF-96365 also has
been shown to inhibit receptor-operated Ca\(^{2+}\) entry in airway smooth muscle (37), which could confound the interpretation of the studies of the sustained phase of Ca\(^{2+}\) entry in the presence of NKR agonists (Fig. 9).

Interestingly, 2-APB inhibited the NKR-mediated sustained [Ca\(^{2+}\)]\(_i\) increases. Interpretation of these results is confounded by two issues: 1) the dose-dependent specificity of 2-APB, and 2) 2-APB blockade of agonist-mediated SR Ca\(^{2+}\) depletion never allows SOCE to become activated. At low concentrations (1–10 \(\mu\)M), 2-APB inhibits IP\(_3\) receptor-mediated Ca\(^{2+}\) release, whereas it also inhibits SOCE at higher concentrations (25–100 \(\mu\)M). In the present study, 10 \(\mu\)M 2-APB was used to examine the contribution of IP\(_3\) receptors on NKR-mediated [Ca\(^{2+}\)]\(_i\) increases. In the subsequent focused studies on SOCE, this concentration of 2-APB did not inhibit SOCE, indicating that we were selectively studying inhibition of IP\(_3\) receptors using 10 \(\mu\)M 2-APB. The second confounding issue is that when cells are pretreated with 2-APB, preventing NKR-mediated SR Ca\(^{2+}\) release, SOCE never becomes activated. Thus pretreatment with an IP\(_3\) receptor antagonist never allows SOCE to be detected. Since inhibition of SOCE with SKF-96365 had a large effect on the sustained phase of Ca\(^{2+}\) increases, this is likely the primary mechanism of sustained elevations in intracellular Ca\(^{2+}\) following NKR activation.

Caffeine or ryanodine inhibited the NKR-mediated sustained [Ca\(^{2+}\)]\(_i\) increase in all three types of HASM NKR-transfected cells. This effect could be explained by at least two mechanisms: 1) caffeine depletion or ryanodine blockade of SR stores may reduce the amount of SR Ca\(^{2+}\) available for release, and

![Fig. 10. Store-operated calcium entry (SOCE). Effect of SKF-96365 (10 \(\mu\)M), 2-APB (10 \(\mu\)M), ryanodine (100 \(\mu\)M), or caffeine (10 mM) on SOCE in native HASM cells. Data are means ± SE, presented as a percentage of control (ΔF/Δt). Data represent means ± SEM. **P < 0.01 compared with control. Numbers in parentheses indicate the number of experiments.](image)

![Fig. 11. Ryanodine receptor isoform expression. Representative RT-PCR analysis of total RNA using primers that specially recognize mRNA for each ryanodine receptor isoform (Ryr1, Ryr2, Ryr3) in primary cultures of native (HASM) and NK receptor-transduced (HASM-huNK\(_1\)R, HASM-huNK\(_2\)R, HASM-huNK\(_3\)R) HASM cells. Human and mouse skeletal muscle RNA were used as a positive control for Ryr1 and Ryr3 isoforms, and human and mouse heart were used as a positive control for Ryr2.](image)
2) we have independently shown that caffeine or ryanodine has a small effect in attenuating SOCE. However, these effects of caffeine or ryanodine on SOCE may be indirect. They may prevent a portion of SR Ca\(^{2+}\) depletion such that less SOCE is necessary to replenish intracellular Ca\(^{2+}\) levels, and thus less Ca\(^{2+}\) enters the cell upon replacement of Ca\(^{2+}\) in the external medium.

In summary, we have demonstrated that native HASM cells express all three known subtypes of neurokinin receptors, which couple to activation of inositol phosphate and increases in [Ca\(^{2+}\)]\(_i\). Utilizing lentivirus-mediated overexpression of each receptor subtype in primary cultures of HASM cells, we have demonstrated agonist specificity at each receptor subtype and complex regulation of the [Ca\(^{2+}\)]\(_i\) phases following NKR activation. Activation of the SR IP\(_3\) receptor primarily mediates the initial transient phase of elevation of [Ca\(^{2+}\)]\(_i\), whereas the sustained phase is primarily mediated by SOCE. The SR ryanodine receptor plays a minor role in the sustained phase of NKR-mediated increases in [Ca\(^{2+}\)]\(_i\), whereas the L-type voltage-gated Ca\(^{2+}\) channels have minor contributions to both the initial and sustained phases of increases in [Ca\(^{2+}\)]\(_i\). These findings suggest that endogenous tachykinins in airways may have effects on airway smooth muscle that contribute to the asthmatic phenotype.

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