The NK1 Receptor Localizes to the Plasma Membrane Microdomains, and Its Activation Is Dependent on Lipid Raft Integrity*

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The spatial targeting of receptors to discrete domains within the plasma membrane allows their preferential coupling to specific effectors, which is essential for rapid and accurate discrimination of signals. Efficiency of signaling is further increased by protein and lipid segregation within the plasma membrane. We have previously demonstrated the importance of raft-mediated signaling in the regulation of smooth and skeletal muscle cell contraction. Since G protein-coupled receptors (GPCRs) are key components in the regulation of smooth muscle contraction-relaxation cycles, it is important to determine whether GPCR signaling is mediated by lipid rafts and raft-associated molecules. Neurokinin 1 receptor (NK1R) is expressed in central and peripheral nervous system as well as in endothelial and smooth muscle cells and involved in mediation of pain, inflammation, excocrine secretion, and smooth muscle contraction. The NK1 receptor was transiently expressed in HEK293 and HepG2 cell lines and its localization in membrane microdomains investigated using biochemical methods and immunofluorescent labeling. We show that the NK1 receptor, similar to the earlier described β2-adrenergic receptor and G proteins, localizes to lipid rafts and caveolae. Protein kinase C (PKC) is one of the downstream effectors of the NK1 activation. Its active form translocates from the cytoplasm to the plasma membrane. Upon stimulation of the NK1 receptor with Substance P, the activated PKC relocated to lipid rafts. Using cholesterol extraction and replenishment assays we show that activation of NK1 receptor is dependent on the microarchitecture of the plasma membrane: NK1R-mediated signaling was abolished after cholesterol depletion of the receptor-expressing cells with methyl-β-cyclodextrin. Our results demonstrate that reorganization of the plasma membrane has an effect on the activation of the raft-associated NK1R and the downstream events such as recruitment of protein kinases.

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The plasma membrane is segregated into domains, which incorporate specific lipid and protein moieties. These lateral assemblies of cholesterol and glycosphingolipids form dynamic structures termed lipid “rafts” or microdomains, and are associated with characteristic sets of proteins, linked by glycosylphosphatidylinositol (GPI) anchors to their extracellular face and/or multiple acylated cytoplasmic proteins to the inside of the cell. Membrane rafts can self-associate to form higher order structures with higher physical stability and constitute hubs of signaling activity (1). Over the last few years, a wide array of signaling and receptor proteins have been found to associate with lipid rafts, which further strengthened this concept (2). Signal transduction across the plasma membrane is a fundamental aspect of cellular communication in which G protein-coupled receptors (GPCRs) play a major role. Molecular regulation by these seven transmembrane receptors results in the transmission of an extracellular signal from the receptors via heterotrimeric (α-, β-, and γ-subunit-containing) G proteins to effector molecules. Each cell expresses a number of different GPCRs, which may share their downstream signaling partners. Since GPCRs remain major targets for pharmacological manipulation, their activation and blockade by specific agonists and antagonists have been extensively investigated. Most of this work has been aimed at studying the receptor-ligand interaction, disregarding the downstream events of GPCR signaling, yet many G protein-coupled receptors have the capacity to interact with multiple G proteins and thus regulate more than one effector pathway (3). Such observations contradict the paradigm that receptors and other integral membrane proteins are randomly distributed in the plasma membrane. Indeed, fluorescence labeling experiments have demonstrated that the distribution of G proteins is not random and that both βγ- and α-subunits display limited mobility (4). Since the receptors themselves interact with various scaffolding molecules, (5), it is highly likely that GPCRs form a part of the pre-assembled and relatively stable signal transduction complexes within the plasma membrane.

G protein-coupled receptors play an important role in the regulation of smooth muscle (SM) contraction and relaxation, as well as other important processes such as growth, remodeling, and inflammation (6). A number of different agonists activate G protein-coupled receptors to stimulate adenyl cyclase (AC), increase cAMP formation, and promote relaxation in vascular smooth muscle (7). β-Adrenergic receptors are the primary therapeutic targets in airways since their activation promotes relaxation of airway smooth muscle and combats acute asthma attacks. The Gα2-coupled receptors, on the other hand, are the principal mediators of SM contraction. They

The abbreviations used are: GPI, glycosylphosphatidylinositol; GPCR, G protein-coupled receptor; PLC, phospholipase C; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; SM, smooth muscle; NK1R, neurokinin 1 receptor; MBCD, methyl-β-cyclodextrin; PMA, 4β-phorbol 12-myristate 13-acetate; HEK, human embryonic kidney cells; Ab, antibody; SP, substance P.
activate PLC, which causes an increase of the intracellular Ca\(^{2+}\) through IP\(_3\)-mediated release from sarcoplasmic calcium stores. The raise in Ca\(^{2+}\) concentration promotes Ca-calmodulin to activate MLCK and start the actin-myosin cross-bridge cycling and contraction.

The evidence is emerging, that many components of the GPCR signal transduction chains in muscle cells are localized to specific membrane domains. Oh and Schnitzer (8) have shown that Go\(_q\) subunits of G proteins concentrate in caveolae, whereas Go\(_s\) and Go\(_i\) target lipid rafts. Consistent with the presence of G proteins in rafts, several members of the GPCR family, as well as certain channel proteins, AC, PLC, and activated PKC have been shown to localize to rafts and caveolae (9, 10, 11, 12). Often raft localization is a dynamic process as it has been shown that the \(\beta\)-adrenergic receptor leaves caveolae upon ligand binding, whereas muscarinic acetylcholine receptor and B2 bradykinin receptor that are uniformly distributed throughout the plasma membrane localize to caveolae only in the presence of ligand (13, 14).

Tachykinin receptors NK1–3 preferentially couple to Go\(_q\) and are widely expressed in the central and peripheral nervous system, as well as in the target organs including respiratory, genitourinary and gastrointestinal tracts. NK1 receptor, which has Substance P (SP) as its ligand, mediates smooth muscle contraction, water and ion secretion, and inflammatory response (15). NK1 has been extensively studied regarding its membrane topology, ligand binding, signal transduction, and tissue distribution (16, 17). One of the puzzling aspects of the NK1-mediated signaling is its ability to couple to two different signaling pathways: Go\(_q\), which activates PLC\(\beta\), thereby initiating inositol phosphate formation and intracellular Ca\(^{2+}\) release, and Go\(_i\) pathway, leading to activation of AC and formation of cAMP. Using mutational analysis and generation of NK1-G protein fusions, Holst et al. (18) have shown that the heterogeneous pharmacological phenotype of this receptor, which becomes evident at whole cell binding experiments, is a reflection of two complexes of NK1 with either Go\(_q\) or Go\(_i\), and that these complexes do not readily interchange. Based on this evidence, the authors suggested the existence of NK1 receptors in different membrane microdomains.

We investigated the plasma membrane localization of the NK1 receptor, and show that this receptor is localized to lipid rafts. Disruption of rafts following cholesterol extraction with methyl-\(\beta\)-cyclodextrin (MBCD) leads to the dissociation of the NK1R signaling complexes and receptor desensitization. Binding of SP to the NK1R causes receptor activation, and induces protein kinase C (PKC), which in turn inhibits the SP signaling by phosphorylating the receptor (19). We show that activation of the NK1R stimulated translocation of PKC into lipid rafts, where it interacts with the receptor and causes its desensitization. Extracellular signal-regulated kinases 1 and 2 (ERK1/2) are known to be among the downstream effectors of the NK1R signaling (20, 21, 22). Here we study the effects of lipid raft dissociation on the NK1R-mediated activation of ERK1/2, and show that the SP-dependent ERK1/2 phosphorylation does not occur in MBCD-extracted cells expressing the NK1R.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Anti-annexin 1, 2, 4, and 6, anti-Lyn, anti-PKC\(\alpha\) monoclonal antibodies, anti-caveolin polyclonal antibody were from Transduction Laboratories, anti-Go\(_q\), NK1R, ERK1/2, and phospho-ERK1/2 polyclonal antibodies were from Santa Cruz Biotechnology, anti-\(\beta\)-actin, anti-FLAG antibodies, as well as MBCD, MBCD-cholesterol, filipin, and 4\(\beta\)-phorbol 12-myristate 13-acetate (PMA) were from Sigma. Peroxidase-conjugated secondary anti-mouse and anti-rabbit antibodies and the ECL detection kit were from Amersham Biosciences. Restriction endonucleases and T4 DNA ligase were pur chased from New England Biolabs. Fluo-3 was from Molecular Probes, Inc. The radiolabeled NK1R-selective antagonist \([\text{H}]\)SR-140333 was from PerkinElmer Life Sciences, and selective NK1 receptor antagonist L-733,460 was from Tocris.

**Cell Culture and Transfections**—HEK293 and HepG2 cells, as well as human aortic smooth muscle cell primary cultures (HAoSMC) (Promo cell, Heidelberg) and human myometrium smooth muscle cultures were maintained in Dulbecco’s modified Eagle’s medium containing 2 mm glutamine, 100 units of penicillin/ml, 100 \(\mu\)g of streptomycin/ml, 10% fetal calf serum. All cells were grown in 5% CO\(_2\) at 37 °C in a humidified incubator. HEK293 and HepG2 cells were transiently transfected with pCI-NK1R plasmid using electroporation (Bio-Rad), maintained at 37 °C for 48 h, and analyzed by SDS-PAGE and Western blotting with anti-NK1R antibodies. HAoSMC, which endogenously express NK1R were used without transfection.

**Immunohistochemistry**—Immunolabeling of the fixed, permeabilized cells with anti-FLAG monoclonal antibody, and anti-caveolin 1 polyclonal antibodies was performed as described (23). For immunostaining using the native (unfixed) conditions, transfected cells were seeded onto glass coverslips, and 48 h post-transfection washed with ice-cold Na\(^+-\)Tyrode’s solution (140 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), 10 mM glucose, and 10 mM HEPES; pH 7.4) containing 0.05% NaN\(_3\), and incubated for 1 h at 4 °C with the primary anti-FLAG mAb diluted in the same buffer. After washing and incubation with the Alexa-488-conjugated anti-mouse and anti-caveolin polyclonal antibodies, Eugene, OR secondary antibody using the same conditions, the plasma membrane of the non-fixed cells was stained for 5 min with concanavalin A conjugated with tetramethylrhodamine (40 \(\mu\)g/ml, Molecular Probes). Finally the cells were washed and fixed with 4% paraformaldehyde buffered with Na\(^+-\)Tyrode’s solution before mounting. Negative controls were generated by applying a non-binding primary antibody. Samples were examined in a Zeiss LSM META 510 confocal microscope.

**Isolation of Lipid Rafts and Determination of Their Lipid and Protein Content**—Lipid rafts were prepared by detergent extraction on ice and flotation on sucrose gradients. Pieces of frozen pig or mouse brain tissue (approx 100 mg each) were finely sliced and homogenized on ice in BW buffer (20 mM imidazole, 40 mM KCl, 2 mM MgCl\(_2\), pH 7.0) using a Dounce homogenizer. Cultured cells were scraped off the dish and homogenized by sonication. Following 30 min of incubation of the homogenates on ice in the presence of 0.4% Triton X-100 and 0.2 \(\mu\)M Ca\(^{2+}\), the sucrose content of the homogenate was adjusted to 40% by the addition of 80% sucrose in BW buffer. A 30–50% discontinuous sucrose gradient was layered over the lysate. The gradients were centrifuged for 16 h at 35,000 rpm in SW50.1 rotor at 4 °C. Seven 650-\(\mu\)l fractions were collected from the top of the gradient, and SDS-PAGE followed by Western blotting was performed to examine their protein content. Lipid composition of the fractions was analyzed by thin-layer chromatography (TLC). When present, the lipid rafts were found to be floating at the 5–30% interface, corresponding to fractions 3 and 4 of the gradient.

**Isolation of Microsomal Membranes**—Cultured cells (6\(\times\)10\(^6\)) expressing NK1R were scraped from the dish, washed three times with 5 ml of buffer A (60 mM KCl, 2 mM MgCl\(_2\), 0.2 mM CaCl\(_2\), 20 mM imidazole, pH 7.0) on ice. Cells were homogenized, and homogenates subjected to low speed centrifugation (10,000 \(\times\) g, 30 min). The supernatants were filtered through glass wool, and centrifuged at high speed (50,000 \(\times\) g, 120 min). The pellets were washed three times with 1 ml of buffer B (120 mM KCl, 0.2 mM CaCl\(_2\), 20 mM imidazole, pH 7.0) with intervening centrifugations at 10,000 \(\times\) g for 30 min and resuspended in 100 \(\mu\)l of the same buffer. Protein concentration was measured and adjusted to 4 mg/ml across the preparations. Samples of equal volume were then analyzed by SDS-PAGE and Western blotting. 5'-Nucleotide Activity in Gradient Fractions—5'-Nucleotidase was assayed using 5'-AMP as a substrate, as described previously (23). Lipid rafts were isolated by flotation in sucrose gradient and 40 \(\mu\)l of each fraction taken for the 5'-nucleotidase assay. Protein concentration in fractions was determined using BCA assay (Sigma) as described by the manufacturer, to estimate the amount of protein taken into each 5'-nucleotidase assay. 5'-Nucleotidase activity in each fraction was monitored as P, release, which was measured spectrophotometrically at a wavelength of 820 nm (OD\(_{820}\)). Activity was expressed as an optical density at 820 nm per \(\mu\)g of protein. Data represent the average of three independent experiments.

**Intracellular Calcium Measurements**—NK1R receptor signaling was measured using SP-induced Ca\(^{2+}\) mobilization. For calcium imaging recordings, the cultured cells expressing NK1R were loaded with Fluo-3/AM (Molecular Probes) for 30 min at room temperature. Single wavelength measurements at 520 nm (488 nm excitation) were performed on a laser scanning confocal microscope (LSM 510 META, Zeiss,
Lipid Raft Localization of the NK1 Receptor

RESULTS

Expression of the NK1 Receptor in Heterologous Systems and in Vivo—The NK1R was expressed in pCI vector (Promega) following its PCR amplification from human bladder cDNA with receptor-specific primers as described previously (25). The NK1R was tagged with the FLAG tag at the N terminus using the forward primer 5'-TATAACTCGAGATGGACTACAAGGATTTAGATGATGACAAGGATAACGTCCTCCCGGTGGAC-3' and the reverse primer 5'-TATAACTCGAGATGGACTACAAGGATTTAGATGATGACAAGGATAACGTCCTCCCGGTGGAC-3'. Epitope tagging had been shown to have no effect on the receptor signaling and localization (26). The receptor was transiently expressed in HEK293 and HepG2 cells using electroporation and its presence and plasma membrane localization confirmed by Western blot and immunofluorescence with both anti-tag and anti-receptor antibodies.

Different pig and mouse tissues, as well as uterus (HU SMC) and HAO-SMC smooth muscle primary cell cultures were assayed for the expression of the NK1R. We found that total mouse and pig brain lysates, as well as HAO-SMC react with anti-NK1R antibodies in Western blot (see below, Fig. 1). Our cultures of HU SMC did not express the NK1R. Endogenous expression of the NK1R in HAO-SMC was further confirmed by challenging these cells with Substance P and examining the intracellular Ca2+ release by calcium imaging. Certain proportion of HAO-SMC responded to SP application by elevation of [Ca2+]i (data not shown). The fact that not all the cells were responsive to SP, despite a relatively high expression level of the NK1R as judged by Western blotting and immunofluorescence with both NK1 antibodies and the antibodies against raft-associated proteins annexin 2 and Lyn. The amounts of 5'-nucleotidase, a GPI-linked raft-associated protein, were examined by activity assay (C). The lipid rafts and associated proteins, as well as the NK1 receptor, float to the interface between 5 and 30% sucrose forming a peak in fractions 3 and 4.
Lipid Raft Localization of the NK1 Receptor

The NK1R is localized to the low density plasma membrane microdomains—Lipid rafts were prepared following detergent extraction and flotation on sucrose gradients as described previously (27). Since detergent concentration has an important effect on the degree of extraction of raft components, Triton-X100 concentrations ranging from 0.1 to 2% were tested and raft integrity controlled by monitoring the presence of known raft-specific proteins and extraction of the others. After incubation of the lysates on ice in the presence of Ca\(^{2+}\), lipid rafts were isolated by flotation in sucrose gradients as described previously (28). Our earlier experiments (24) demonstrated that lipid rafts prepared using extraction with 0.4% Triton X-100 on ice for 30 min effectively removed the non-raft proteins annexin 1 and RhoA, together with glycerophospholipids. In contrast, the majority of raft-associated proteins (Lyn, G\(\alpha_q\), annexins 2 and 6, caveolin) and lipids (cholesterol, sphingomyelin) were concentrated at the interface between 5 and 30% sucrose following overnight centrifugation of the sucrose gradient. The endogenous NK1 receptor in pig (Fig. 1A) and mouse brain extracts (Fig. 1B) and in the HaosMC cultures (Fig. 1C) localized to the low density fractions 3 and 4 of the gradient. The raft markers annexin 2, Lyn, caveolin, and the GPI-anchored 5' -nucleotidase showed similar localization patterns. The NK1R, heterologously expressed in the HEK293 cells, localized to low density microdomains, in the same fractions as the raft-associated proteins Lyn, annexin 6, and 5' -nucleotidase (Fig. 2A). Therefore, interaction with caveolin was not necessary for the lipid raft targeting of the NK1R. The detergent extraction and sucrose gradient flotation experiments used here did not allow us to precisely determine the co-localization of the NK1R with the other raft-associated proteins. Indeed, the fact that some of the raft markers form peaks at slightly different gradient fractions than NK1R might be a reflection of the heterogeneity of the raft population. This might be especially true in the case of annexins (see distribution of annexin 6 in Fig. 2B), which are not integral membrane proteins and whose interaction with lipid rafts depends on additional factors, such as [Ca\(^{2+}\)].

The NK1R signaling activates PKC\(\alpha\) and induces its translocation to lipid rafts—HEK293 cells, expressing the NK1 receptor, were either left untreated, or exposed to PMA (2 \(\mu\)M) for 15 min to activate PKC; Ca\(^{2+}\)-ionomycin (10 \(\mu\)M) to cause Ca\(^{2+}\) influx; or Substance P (3 nM) to activate the NK1 receptor. Following the treatments, lipid rafts were prepared and the

\(^2\) E. Babiychuk, personal communication.
presence of PKCα in the gradient fractions analyzed by SDS-PAGE and Western blot. Lipid microdomains floated in the low density gradient fractions 3 and 4, as shown using caveolin as a control. In untreated cells, the majority of PKCα localized to the high density fractions 6 and 7, however, activation by PMA or Ca²⁺ increase promoted its association with the lipid rafts in fractions 3 and 4, in accordance with the previously published observations in cardiomyocytes (9). Similarly, Ca²⁺ mobilization by NK1 signaling to 3 nM SP resulted in translocation of PKCα into rafts (Fig. 3A).

Activation of PKC with phorbol esters strongly inhibits signaling by several GPCR, including the NK1R (19, 31, 32). The subsequent translocation of the activated PKCα to lipid rafts might be important for the inhibitory effect of PKCα on SP-induced Ca²⁺ mobilization in transfected HEK293 cells. HEK293 cells expressing the NK1R were loaded with Fluo-3 to study the NK1-mediated intracellular Ca²⁺ release. When the cells were pretreated with 2 μM PMA to activate PKC, their ability to activate the receptor-mediated intracellular Ca²⁺ release was greatly reduced (Fig. 3B). PKC phosphorylates the C-tail of the NK1R, therefore uncoupling it from the G proteins (19). It is conceivable that relocation of the activated PKC into the raft population where NK1R is residing might facilitate this process.

**NK1R Raft Localization Is Disrupted by Cholesterol Extraction with MBCD, and Restored by Cholesterol Repletion**—Extraction of cholesterol from living cells with methyl-β-cyclodextrin leads to the dispersion of lipid rafts and loss of their detergent resistance. The effects of MBCD should manifest themselves in a loss of raft-associated molecules, including the NK1R, from the low density gradient fractions. Replenishment of cholesterol in the depleted membranes can be then achieved by incubating the cells with an MBCD:cholesterol complex. This treatment should reconstitute the lipid microdomains and restore their protein composition. HEK293 cells expressing the NK1 receptor were either left untreated (Fig. 4A), cholesterol extracted with 4% MBCD for 30 min (Fig. 4B), or following MBCD extraction incubated for 1 h with 5 mg/ml cholesterol/MBCD complex to replenish cholesterol (Fig. 4C). Lipid rafts were prepared as described above and their protein content analyzed by Western blot with antibodies against the NK1R and the raft-associated proteins Lyn, caveolin, annexin 2, and Goq. Cholesterol extraction was monitored by TLC, and its amounts estimated using ONE-Dscan 1.0 software (Fig. 4D). MBCD extraction caused significant reduction of the amount of total cholesterol, especially in the gradient fractions 3 and 4 at the 5–30% sucrose interface, and removal of most of the NK1R, and the other raft-associated molecules (annexins 2, Goq, Lyn, and caveolin) from the low density gradient fractions, into the high density fractions 6, 7, and pellet. Repletion of cholesterol for 1 h restored its contents in the plasma membrane (Fig. 4D), and at least partially re-established the lipid rafts as judged by the protein composition of the low density fractions 3 and 4.

**Integrity of Lipid Raft Affects the NK1R Response to Substance P**—In view of rafts as pre-assembled signaling platforms, we sought to determine whether the efficiency and magnitude of the NK1R response to SP, measured by receptor-mediated intracellular calcium release, is reduced following the dispersion of rafts by MBCD extraction. HEK293 cells expressing the NK1R were loaded with Fluo-3 and the SP-induced Ca²⁺ mobilization measured by Ca²⁺ imaging. For concentration-response analysis cells were exposed to single applications of graded amounts of SP. (Fig. 5). When the cells were treated with 4% MBCD for 30 min at room temperature to extract cholesterol, washed, and subjected to SP at the concentrations close to EC₅₀ (0.3 nM, (19)), receptor-mediated signaling was almost completely abolished. However, the same cells did respond to the SP challenge, albeit at a much higher concentration (30 nM), and with a reduced magnitude of response (Fig. 5, MBCD). It can be concluded that though the MBCD treatment did not affect the ability of the receptor to couple to its downstream effectors, it caused the receptor to desensitize, possibly by dispersing its coupling to Goq, which is also located in lipid rafts. Addition of MBCD did not change receptor conformation or its ability to interact with its ligand, since short applications of MBCD followed by addition of SP without washing away the detergent did not result in significant alterations of the NK1R-mediated Ca²⁺ mobilization (not shown).

In order to prove that inhibition of the NK1R-mediated signaling was caused by dispersion of lipid raft micro-architecture, and not by removal of cholesterol per se, we repeated these
experiments using filipin. Filipin selectively binds and sequesters cholesterol within the plasma membrane, forming complexes that induce structural disorder (33). The NK1R-expressing cells were loaded with Fluo-3, and then incubated with 3 μg/ml filipin for 1 h prior to SP challenge. Disruption of rafts by filipin, similar to cholesterol extraction by MBCD, reduced the magnitude of NK1R signaling. Effects of filipin were more pronounced at low concentrations of SP, however, they were weaker than after MBCD treatment (Fig. 5, filipin), at 3 and 30 nM SP, filipin-extracted cells showed 50–70% of the control
responses. These data might reflect filipin’s comparatively low efficiency of lipid raft dispersion, which has also been observed by the others (33).

Our biochemical findings presented above indicate that cholesterol repletion during 1 h after extraction partially restored the plasma membrane microdomains in their lipid and protein composition. When MBCD-extracted cells were incubated with MBCD/cholesterol for 1 h, and then exposed to increasing concentrations of SP, their ability to mobilize intracellular Ca\textsuperscript{2+} was at least partially restored. In MBCD-extracted and cholesterol-replenished cells, 0.3 nM SP evoked responses of ~17% of the maximum, compared with ~40% in the non-extracted control and no response in the cholesterol-depleted cells. The saturating concentrations of the agonist (30 nM) produced in the replenished cells the responses of ~60% of the maximum (Fig. 5, MBCD + Chol.). We found that prolonged exposures (up to 3 h) to soluble cholesterol further increased the response, returning it to almost pre-extraction levels (not shown). It can be speculated that though the amount of cholesterol in the plasma membrane is relatively easy to restore, the actual raft assembly i.e. the formation of lipid-protein complexes involved in raft-mediated signaling takes longer to re-establish.

**Cholesterol Extraction Does Not Deplete NKIR at the Cell Surface**—Extraction of the NKIR-expressing cells with MBCD prior to isolation of lipid rafts caused the receptor to relocate from the gradient interface to the high density gradient fractions. To determine whether cholesterol extraction and dispersion of lipid rafts had an effect on the plasma membrane localization of the NKIR, microsomal membranes were isolated from the MBCD-extracted and control HEK293 cells expressing NK1. Membranes were analyzed by Western blotting with anti-NKIR antibodies, as well as the antibodies against caveolin and annexin 2 (Fig. 6A). Extraction of cells with MBCD did not cause the removal of the NKIR from the membrane fraction, as evident by the presence of the receptor in the microsomes. The microsomal membrane preparations contained approximately equal amounts of NKIR in both extracted and control samples, indicating that cholesterol depletion with MBCD has no significant effect on the membrane localization of the receptor.

Because, in addition to the plasma membrane, microsomes might contain some of the membranes derived from the endoplasmic reticulum and Golgi, we sought to determine the amounts of the receptor at the cell surface only. The extracellular N-terminus of the NKIR was tagged with FLAG epitope, and immunofluorescence labeling with anti-FLAG mAb under native, non-permeabilized conditions was used to detect the NKIR localized on the cell surface of the control and MBCD-extracted cells. First, to monitor the presence and localization of the tag, NKIR-expressing HEK293 cells were double-labeled with anti-FLAG mAb and anti-caveolin polyclonal Ab and examined by confocal microscopy (Fig. 6B). The FLAG-tagged NKIR co-localized with caveolin at the plasma membrane of the transfected cells. Then anti-FLAG labeling was performed in the unfixed non-permeabilized cells, which were either untreated (control) or extracted with 2% MBCD. To avoid endocytosis of the antibody-labeled receptor, all incubations were performed at 4 °C in the presence of NaN\textsubscript{3}. Concanavalin A-tetramethylrhodamine conjugate was used as the plasma membrane marker, as well as an additional permeabilization control based on its ability to bind to the glycoproteins either on the plasma membrane alone in non-permeabilized cells, or both on the plasma membrane and endoplasmic reticulum in permeabilized cells. The control and MBCD-extracted cells were labeled with anti-FLAG antibody only at the plasma membrane, and showed no significant difference in the intensity of labeling, or the amount of the labeled cells (Fig. 6B), indicating that cholesterol depletion and dispersion of lipid rafts did not affect the plasma membrane localization of the NKIR.

**Cholesterol Extraction Does Not Significantly Affect the NKIR Ligand Binding**—Further we sought to investigate whether cholesterol extraction with MBCD might influence the conformation of the receptor in a way which would preclude its binding to the ligand and therefore abrogate the receptor-mediated signaling. The whole cell radioligand binding experiments were performed in order to obtain a quantitative estimate of the cell-surface NKIR interacting with its selective antagonist [\textsuperscript{3}H]SR-140333. The binding was receptor-specific: there was no detectable specific binding of the radioligand to the non-transfected HEK293 cells, but only to the cells expressing the NKIR (not shown). When the NKIR-expressing cells were treated with 2% MBCD, there was a significant reduction in the amount of total cholesterol after 30 min of extraction (Fig. 7A). In order to minimize the differences in transfection efficiency, different batches of electroporated cells were mixed before plating, making sure that there was no difference in the expression level of control and MBCD-extracted cells in a particular experiment. The cholesterol-depleted cells were then used in the radioligand binding assays and results compared with the binding observed in the non-extracted control. Three independent experiments were performed, with each data point in triplicate. Shown are the data from a representative experiment (Fig. 7B). In control the $B_{\text{max}}$ was 1248 ± 70 fmol/mg protein, and $K_D$ was 0.36 ± 0.08 nM, compared with the $B_{\text{max}}$ 1080 ± 54 fmol/mg protein and $K_D$ 0.78 ± 0.12 nM observed in the MBCD-extracted cells. It can be concluded that the cholesterol-depleted cells retained the ability to bind [\textsuperscript{3}H]SR-140333 at ~86% of the untreated control, and there was no significant difference in the ligand affinity.

**NKIR-mediated Activation of ERK Is Sensitive to Cholesterol Extraction**—The NK1 receptor signaling induces phosphorylation and activation of the extracellular signal-regulated kinase (ERK). The NK1-dependent activation of ERK was assessed using cholesterol extraction/repletion assays by monitoring the accumulation of the phosphorylated form of the kinases in the cells exposed to SP. Cells were incubated with 30 nM SP for up to 30 min, and activation was determined by Western blotting.
Fig. 6. Cholesterol extraction with MBCD does not deplete the NK1R at the cell surface. A, microsomal membranes were prepared as described under "Experimental Procedures" from the NK1R-expressing HEK293 cells either left untreated (lane 1) or extracted with 4% MBCD for 30 min at 37 °C (lane 2). Samples were analyzed by SDS-PAGE and Western blotting. The amounts of NK1R were assayed using ONE-Dscan 1.0 software, and a mean of two experiments is shown in the graph. B, NK1R-expressing cells were labeled with anti-FLAG mouse monoclonal antibody (red), and anti-caveolin polyclonal antibody (green) before viewing at the confocal microscope. Graphs show the intensity profile of the red and green signals along an arbitrarily selected axis (arrow), demonstrating the co-localization of the signals on the plasma membrane. C, NK1R-expressing cells were either left untreated (control) or extracted with 2% MBCD for 30 min at room temperature (MBCD-extracted) before labeling with anti-FLAG mAb under native conditions (without fixing or permeabilization) as described under "Experimental Procedures." Rhodamine-conjugated concanavalin A was used to stain the plasma membrane. FLAG-tagged NK1R was detected at the cell surface of both control and cholesterol-extracted cells, judged by its co-localization with concanavalin A-rhodamine.
with antibodies against the phosphorylated form of ERK1/2 (pERK1/2) (Fig. 8A). Under basal conditions, there was a low level of phosphorylation of ERK1/2 in HEK293 cells. In the cells expressing the NK1R, application of SP induced an increase of phosphorylation of ERK1/2. When cells were extracted with MBCD for 30 min prior to administration of the NK1R agonist, there was no noticeable elevation of the pERK1/2 amounts above the basal level. When cholesterol, which had been removed by MBCD extraction, was replenished by addition of 5 mg/ml MBCD/cholesterol for 2 h, and the cells exposed to the same concentration of SP, activation of ERK1/2 was restored to the levels observed in the untreated cells (Fig. 8B). In order to prove that the cholesterol extraction disrupts the NK1R-specific activation of ERK, but does not prevent ERK1/2 phosphorylation by other, potentially raft-independent signaling cascades, we assessed the sensitivity of the phorbol ester (PMA)-mediated ERK activation in MBCD-extracted cells. When cholesterol-extracted HEK293 cells were exposed to 5 μM PMA for 30 min, phosphorylation of ERK1/2 was detected at levels similar to the ones in the PMA activated, non-extracted cells (Fig. 8C).

**DISCUSSION**

This study provides evidence of the lipid raft-specific localization of the neurokinin receptor NK1R in brain tissue and transfected cell lines, and demonstrates the significance of the raft microarchitecture for the NK1R-mediated signaling of the Gαq pathway. Membrane rafts can be grouped into at least two categories: large, stationary ones, which are associated with the caveolar regions and smaller, presumably more flexible ones (34). Nevertheless, the controversy around the raft composition, and size (35, 36, 37), few disagree that local heterogeneities exist within the plasma membrane and that specific protein-protein and protein-lipid interactions can induce partitioning of proteins into separate microdomains. The finding that many raft proteins mediate signal transduction, particularly in T and B cells, (38, 39), led to the hypothesis of rafts as signal transduction platforms (40). Stable caveolar rafts, which have been shown to harbor receptors for certain growth factors (41), might be involved in signaling activities of long duration. But the highly dynamic, Ca2+-regulated association of non-caveolar rafts suggests that they are involved in rapid signaling events. Expression of caveolin in cells normally devoid of this protein resulted in a 50% increase in the amounts of raft cholesterol (35), and there is a distinction between signaling mechanisms in these two types of microdomains (42).

Since the work of Oh and Schnitzer (8) on the raft localization of G proteins, evidence has emerged of the targeting of some G protein-coupled receptors to the same domains. It is interesting to note that, like the NK1R described here, many of these GPCRs couple to Gαq and activate phospholipase C (43, 44, 14). In many cases, a direct interaction of the receptor with caveolin has been detected by co-immunoprecipitation. Here we show that the NK1R does not depend on the presence of caveolin for interaction with lipid rafts: it is localized to the low density gradient fractions, together with Gαq in HepG2 cells, naturally devoid of caveolin. The NK1 receptor is abundant in central and peripheral nervous system, where caveolin is generally expressed at a low level, and caveolae are absent (45). Therefore its ability to associate with rafts independently of caveolin might reflect its natural properties and not be an artifact of a heterologous expression system used in these studies. Upon addition of substance P to the NK1R-expressing cells prior to lipid raft extraction, there was a slight shift of the NK1R distribution into the high density gradient fractions, which might reveal loss of raft binding upon receptor internalization. However, the cells were harvested shortly after addition of the ligand, and no time course experiments performed, therefore this issue awaits further clarification. Extraction with 0.4% Triton X-100 was chosen as optimal for the experimental conditions, and extraction of the non-raft membrane proteins RhoA and annexin 1 as well as lipid composition served as control.

Having demonstrated the raft-specific localization of the NK1R and its effector Gαq, we sought to determine whether downstream NK1R signaling events involved these plasma membrane compartments. Activation of PLC by GPCRs and subsequent intracellular Ca2+ release leads to activation of the Ca2+-sensitive second messenger kinase PKCa. PKCa, which in its inactive state resides in the cytoplasm, translocates to the plasma membrane and has been shown to associate with caveolae (9). Here we show that stimulation of the NK1R expressing cells with SP causes PKCa to relocate from the soluble (40% sucrose) fraction of the gradient to the low density microdomain fractions. PKCa activation by PMA or Ca2+ influx had the same effect. A physiological reflection of the co-localization
FIG. 8. The NK1 receptor signals to ERK in a raft-dependent manner. A, phosphorylation and activation of the ERK induced by the NK1 receptor signaling was assessed by Western blotting with antibodies specifically recognizing the phosphorylated form of ERK 1/2. Cells expressing the NK1 receptor were either left untreated (lane 1) or exposed to the receptor agonist SP (30 nM) for 30 min (lane 2). Cholesterol extraction/repletion assays were employed to examine the involvement of lipid rafts in NK1R-mediated ERK signaling. Cells were extracted with 2% MBCD for 1 h, washed, and exposed to 30 nM SP for 30 min (lane 3) or extracted with MBCD, replenished with 5 mg/ml MBCD-cholesterol for 1 h and then exposed to the same concentration of SP (lane 4). Following treatment, the cells were lysed in SDS-PAGE sample buffer and the amount of phosphorylated ERK 1/2 (p-ERK) was analyzed by Western blotting. Loading was kept equal in each lane as monitored by the amounts of non-phosphorylated ERK2, NK1R, and staining for the total protein with Amido Black. B, amounts of phosphorylated ERK were compared in samples using ONE-Dscan software. Amounts of total protein, expression of NK1, and amounts of non-phosphorylated form of ERK were identical across the samples. Phosphorylated ERK was elevated following the challenge with SP. This effect was abolished if the cells were MBCD extracted before application of SP, and restored after cholesterol repletion. C, HEK293 cells were left untreated (lanes 1 and 2) or extracted with 2% MBCD for 1 h (lanes 3 and 4). Activation of ERK1/2 was examined by Western blotting with phospho-ERK1/2 after exposing the cells to 5 μM PMA (lanes 2 and 4) and compared with the control (no PMA, lanes 1 and 3). Equal loading in each lane was monitored by the amounts of non-phosphorylated ERK2, actin and total protein. PMA-induced phosphorylation of ERK1/2 was not influenced by extraction of cholesterol with MBCD.
of PKC and the NK1R in the same plasma membrane domain is the attenuation of receptor signaling to SP, which was observed after pre-incubation of cells with PMA and is caused by receptor phosphorylation (19).

Extraction of cholesterol with methyl-β-cyclodextrin leads to dispersion of rafts and is commonly used to study raft dependence of proteins. After incubating the NK1R-expressing cells with MBCD for 1 h, the rafts were destroyed and the NK1R as well as other raft-associated proteins could be extracted with Triton X-100 and appeared in the high density gradient fractions and in the pellet. MBCD treatment almost completely abolished the NK1R signaling as monitored by Ca²⁺ mobilization in response to EC₅₀ concentration of agonist, demonstrating that the raft association of the receptor was necessary for its coupling with Gαᵣ. It is noteworthy that, similar to the NK1R, MBCD treatment removed Gαᵣ from the low density fractions. Cholesterol extraction, and not the adverse effects of MBCD, was responsible for receptor desensitization, since a 10-fold higher concentration of the agonist evoked the signaling whereas the acute application of the detergent had no effect. Filipin, which binds and sequesters cholesterol within the plasma membrane and thus destabilizes lipid rafts, had similar effects as MBCD in reducing the NK1R signaling. Repletion of cholesterol for 1 h after MBCD extraction partially re-established the lipid and protein composition of the rafts, and restored the signaling. The effects of filipin, as well as the observation that removal of cholesterol by MBCD does not deplete the amount of the NK1R at the plasma membrane or influence the ligand binding, suggest that aggregation of the NK1R with its effectors within lipid rafts is important for the efficiency of the NK1R signaling.

Agonist binding to GPCRs and receptor activation are followed by termination of signaling, in order to prevent uncontrolled stimulation, which might ultimately lead to disease. At the receptor level, mechanisms of termination of signal transduction include homologous desensitization by repeated applications of the same agonist, heterologous desensitization to different agonists and removal of receptors from the plasma membrane by endocytosis. Desensitization occurs by uncoupling of receptors from the G proteins following receptor phosphorylation by G protein receptor kinases and PKC/PKA. Desensitization of the NK1 receptor did not require endocytosis; however, resensitization required endocytosis, recycling, and phosphatase activity, suggesting that the NK1 receptor desensitizes by phosphorylation and resensitizes by dephosphorylation in endosomes and recycling. β-Arrestins play a special role in the uncoupling of the phosphorylated receptors from the G proteins and directing them to the clathrin-coated pits for endocytosis (46). The NK1R forms a stable complex with β-arrestins 1 and 2, and the complex internalizes as a unit, which additionally serves to recruit new signaling partners, such as Src, Raf, Erk, Ask1, and Jnk3 (20). This scaffolding complex activates ERK1/2, which in turn is responsible for the anti-apoptotic and proliferative effect of SP, shown in the NK1R transfected cell lines (20), and in neurons (22). Our study demonstrates that phosphorylation of ERK1/2 by activated NK1R is sensitive to cholesterol extraction: the NK1R-expressing cells lost their ability to activate ERK1/2 if their membranes had been extracted with MBCD prior to the challenge with SP. Repletion of cholesterol completely reversed this effect and restored ERK1/2 phosphorylation. Previously, Navratil et al. (42) showed that MBCD-treated cells retained their ability to activate ERK1/2 in response to phorbol ester PMA; therefore, the observed abrogation of the kinase phosphorylation was attributed to the detrimental effect of cholesterol extraction on the NK1R signaling. It is remarkable that both Src and dynamin are essential for ERK1/2 recruitment by the activated NK1R (21). Src non-receptor tyrosine kinase has been shown to constitutively localize to lipid rafts (47, 48), whereas dynamin has been reported to be associated both with clathrin and caveolin and therefore participate in clathrin-dependent and caveolae-dependent endocytosis (49, 50). It is therefore conceivable that the spatial co-localization of the NK1R and its partners Gαᵣ and Src, which is facilitated by their residence in lipid rafts, is essential for coupling of the NK1R signaling and ERK1/2 activation.

The results presented here suggest that low density lipid microdomains are essential for coupling of the NK1 receptor to Gαᵣ, and activation of the signaling pathway, which leads to intracellular calcium release, recruitment of PKC and, ultimately, phosphorylation of ERK1/2. In this work we did not establish the difference between caveolae and non-caveolar rafts, showing only that the presence of caveolin per se was not a requirement for the NK1R association with rafts. However, taking into account the ability of the NK1R to couple to the Gαᵣ as well as the Gαᵣ pathway (51), it is possible that in cells where caveolae are present, they might serve to spatially segregate the two signaling pathways because of their ability to preferentially harbor Gαᵣ and exclude Gαᵣ and Gαᵣ (8). This hypothesis awaits further investigation, however, the data presented here serve as a first indication of the importance of membrane microarchitecture for NK1 receptor-mediated signaling.

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