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Desensitization of the Neurokinin-1 Receptor (NK1-R) in Neurons: Effects of Substance P on the Distribution of NK1-R, $G_{\alpha q/11}$, G-Protein Receptor Kinase-2/3, and β-Arrestin-1/2

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Observations in reconstituted systems and transfected cells indicate that G-protein receptor kinases (GRKs) and β-arrestins mediate desensitization and endocytosis of G-protein–coupled receptors. Little is known about receptor regulation in neurons. Therefore, we examined the effects of the neurotransmitter substance P (SP) on desensitization of the neurokinin-1 receptor (NK1-R) and on the subcellular distribution of NK1-R, $G_{\alpha q/11}$, GRK-2 and -3, and β-arrestin-1 and -2 in cultured myenteric neurons. NK1-R was coexpressed with immunoreactive $G_{\alpha q/11}$, GRK-2 and -3, and β-arrestin-1 and -2 in a subpopulation of neurons. SP caused 1) rapid NK1-R–mediated increase in $[Ca^{2+}]_i$, which was transient and desensitized to repeated stimulation; 2) internalization of the NK1-R into early endosomes containing SP; and 3) rapid and transient redistribution of β-arrestin-1 and -2 from the cytosol to the plasma membrane, followed by a striking redistribution of β-arrestin-1 and -2 to endosomes containing the NK1-R and SP. In SP-treated neurons $G_{\alpha q/11}$ remained at the plasma membrane, and GRK-2 and -3 remained in centrally located and superficial vesicles. Thus, SP induces desensitization and endocytosis of the NK1-R in neurons that may be mediated by GRK-2 and -3 and β-arrestin-1 and -2. This regulation will determine whether NK1-R–expressing neurons participate in functionally important reflexes.

INTRODUCTION

The biological effects of neurotransmitters that interact with G-protein–coupled receptors (GPCRs)[1] are attenuated by 1) agonist removal from the extracellular fluid by reuptake and degradation, 2) agonist-induced receptor desensitization by uncoupling activated receptors from heterotrimeric G-proteins to terminate the signal, and 3) agonist-stimulated receptor endocytosis, which depletes the plasma membrane of high-affinity receptors (reviewed in Böhm *et al.*, 1997a). These mechanisms are important because they prevent the uncontrolled stimulation of cells that will otherwise result in prolonged activation and possibly disease.

G-protein receptor kinases (GRKs) and β-arrestins participate in both receptor desensitization and endocytosis. In the presence of receptor agonists, GRK-2 and -3 phosphorylate many GPCRs (Benovic *et al.*, 1989, 1991; Kwatra *et al.*, 1993; Pippig *et al.*, 1993). Subsequently, β-arrestin-1 and -2 interact with GRK-phosphorylated receptors to disrupt their association with heterotrimeric G-proteins and terminate signal transduction (Lohse *et al.*, 1990; Attramadal *et al.*, 1992; Pippig *et al.*, 1993). GRK-mediated phosphorylation is also necessary for endocytosis of certain GPCRs

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[1] Abbreviations used: ACh, acetylcholine; β_2-AR, β_2-adrenergic receptor; Cy3, cyanine 3.18; FITC, fluorescein isothiocyanate; GPCR, G-protein–coupled receptor; GRK, G-protein receptor kinase; NK1-R, neurokinin-1 receptor; SP, substance P.

(Tsuga *et al.*, 1994; Ferguson *et al.*, 1995; Menard *et al.*, 1996; Ruiz-Gomez and Mayor 1997). In addition, β-arrestins participate in endocytosis by acting as clathrin adaptor proteins (Ferguson *et al.*, 1996; Goodman *et al.*, 1996). In unstimulated cells, GRK-2 and -3 and β-arrestin-1 and -2 are principally localized in the cytosol and upon agonist stimulation redistribute to the cell surface and vesicles where they interact with GPCRs to mediate desensitization and endocytosis (Ferguson *et al.*, 1996; Goodman *et al.*, 1996; Barak *et al.*, 1997; Ruiz-Gomez and Mayor 1997). However, most studies on the function and trafficking of GRK-2 and -3 and β-arrestin-1 and -2 were done in reconstituted systems or transfected cells that overexpress these proteins and the GPCRs of interest. It is not known whether they are coexpressed in neurons with the receptors they are thought to regulate and whether agonist-induced redistribution of these proteins occurs in neurons that naturally express these proteins at physiological levels.

One GPCR that may be regulated by GRKs and β-arrestins is the substance P (SP) or neurokinin-1 receptor (NK1-R). SP and the NK1-R are widely expressed in the central and peripheral nervous systems where they participate in several important reflexes (reviewed in Otsuka and Yoshioka 1993). Stimulation of pain receptors in the periphery induces the release of SP from afferent nerve endings in the dorsal horn (Duggan *et al.*, 1988), which interacts with the NK1-R on spinal neurons to transmit signals to higher centers (Mantyh *et al.*, 1995). Intestinal distention releases SP from enteric neurons (Donnerer *et al.*, 1984), which binds to the NK1-R on myenteric neurons and thereby contributes to the ascending contractile limb of the peristaltic reflex (Maggi *et al.*, 1994). Upon binding SP, the NK1-R activates phospholipase-Cβ, resulting in formation of inositol trisphosphate, which mobilizes intracellular Ca^{2+}, and diacylglycerol, which activates protein kinase C. Observations from reconstituted systems and using cross-linkers indicate that the NK1-R couples to $G_{\alpha q/11}$ (Kwatra *et al.*, 1993; Macdonald *et al.*, 1996), but it is not known whether the NK1-R couples to this G-protein in neurons.

Cellular responses to SP are rapidly attenuated by NK1-R desensitization and endocytosis (Gaddum 1953; Bowden *et al.*, 1994; Garland *et al.*, 1994, 1996; Grady *et al.*, 1995, 1996b; Mantyh *et al.*, 1995). GRKs and β-arrestins may mediate NK1-R desensitization and endocytosis, because GRK-2 and -3 phosphorylate the NK1-R in a reconstituted system (Kwatra *et al.*, 1993), and disruption of β-arrestins abrogates NK1-R desensitization in *Xenopus* oocytes (Sasakawa *et al.*, 1994a). However, the importance of GRK-2 and -3 and β-arrestin-1 and -2 in desensitization and endocytosis of the neuronal NK1-R has not been established, and it is not known whether SP induces alterations in the subcellular distribution of these proteins in a manner consistent with regulation of the neuronal NK1-R. The mechanisms that desensitize SP signaling in neurons are likely to be important, for they will determine the ability of NK1-R to participate in functionally important reflexes, including peristalsis and pain transmission.

We studied desensitization of the SP signaling in neurons from the myenteric plexus of the guinea pig small intestine. The aims were 1) to establish that SP stimulates Ca^{2+} mobilization in neurons by activating the NK1-R; 2) to determine the timing and concentration dependency of desensitization and resensitization of Ca^{2+} mobilization to repetitive stimulation by SP; 3) to verify that myenteric neurons expressing the NK1-R also express $G_{\alpha q/11}$, GRK-2 and -3, and β-arrestin-1 and -2; and 4) to determine whether SP induces alterations in the subcellular distribution of GRK-2 and -3 and β-arrestin-1 and -2 in a manner that would indicate that they may mediate desensitization and endocytosis of the NK1-R in neurons.

MATERIALS AND METHODS

Dispersion and Culture of Myenteric Neurons

Newborn male guinea pigs (Duncan-Hartley, Simonsen, Gilroy, CA) were killed with sodium pentabarbitone (200 mg/kg, i.p.). Myenteric neurons were dissociated using a modification of previously described procedures (Grady *et al.*, 1996b; Moneta *et al.*, 1997). The longitudinal muscle layer and attached myenteric plexus of the whole small intestine was placed in oxygenated Krebs bicarbonate buffer (in mM: 118 NaCl, 5.9 KCl, 22.7 $NaHCO_3$, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.4 NaH_2PO_4, pH 7.4) containing 0.1% glucose, 100 U/ml penicillin, and 100 μg/ml streptomycin. Tissue was digested in this buffer containing (mg/100 ml) 166 collagenase IA, 133 protease type IX, 16 DNase I (Sigma, St. Louis, MO), and 30 BSA for 60 min at 37°C. The digest was centrifuged (1000 × *g*, 10 min, 4°C), and the resuspended pellet was sequentially filtered through stainless steel screens of 30, 60, and 150 mesh (Small Parts, Miami Lakes, FL). Neurons collected at the 60 and 150 screens were pelleted and resuspended in culture medium (Earle's medium 199 containing 10% NuSerum (Collaborative Research, Bedford, MA), 100 U/ml penicillin, 100 μg/ml streptomycin, 110 μg/ml Na pyruvate, 2 mM glutamine, 5 mg/ml glucose, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, and 12.5 mM HEPES, pH 7.4). The medium was supplemented with 2.5 μg/ml fungizone for the first 3 d and 20 μM cytosine arabinoside for days 2 and 3. Neurons were plated on collagen-coated glass coverslips and cultured for 7–14 d in 95% air/5% CO_2 at 37°C. Neurons were studied at days 7–14 of culture.

Measurement of $[Ca^{2+}]_i$ in Neurons

Myenteric neurons were washed and incubated in physiological salt solution (in mM: 137 NaCl, 4.7 KCl, 0.56 $MgCl_2$, 2 $CaCl_2$, 1.0 Na_2HPO_4, 10 HEPES, 2.0 L-glutamine, and 5.5 D-glucose, pH 7.4) containing 0.1% BSA, 5 μM fura-2 AM, and 0.2% pleuronic for 20 min at 37°C (Garland *et al.*, 1996). They were rinsed in physiological salt solution-BSA, mounted in a microincubator (1-ml volume) on the stage of a Zeiss (Thornwood, NY) Axiovert 100 TV microscope, and perfused with physiological salt solution-BSA at 1 ml/min at 37°C. Agonists and antagonists were directly added to the perfusate. Neurons were observed with a Zeiss Fluar 20× objective (numerical aperture, 0.75), and fluorescence was detected in individual neurons using an intensified charge-coupled device video camera (Stanford Photonics, Stanford, CA) and a video microscopy acquisition program (Axon Instruments, Foster City, CA). Fluorescence was measured at 340 and 380 nm excitation and 510 nm emission.

The ratio of the fluorescence at the two excitation wavelengths, which is proportional to the $[Ca^{2+}]_i$, was determined for the soma of the neurons. All observations were repeated on at least three different neuron cultures.

Generation and Characterization of Fluorescent Peptides

SP and the NK1-R-selective agonist [Sar^9 $MetO_2^{11}$]-SP (Drapeau *et al.*, 1987) were labeled with Cyanine 3.18 (Cy3) and purified exactly as described (Bunnett *et al.*, 1995). We have previously reported the selectivity of Cy3-SP (Bunnett *et al.*, 1995; Grady *et al.*, 1995, 1996b). The selectivity of Cy3-[Sar^9 $MetO_2^{11}$]-SP as a ligand for the NK1-R was evaluated using rat kidney epithelial cells stably expressing that rat NK1-R (KNRK-NK1-R cells). KNRK-NK1-R cells or untransfected KNRK cells were incubated in DMEM containing 1% BSA (DMEM-BSA) and 190 nM Cy3-[Sar^9 $MetO_2^{11}$]-SP for 60 min at 4°C. Cells were fixed in 4% paraformaldehyde in 100 mM PBS (pH 7.4) for 20 min at 4°C, and observed by fluorescence microscopy. Specificity of binding was also examined by preincubating cells with 1 μM unlabeled [Sar^9 $MetO_2^{11}$]-SP or with 10 μM CP96345 (NK1-R-selective antagonist) for 30 min before addition of the labeled peptide. The biological activities of Cy3-[Sar^9 $MetO_2^{11}$]-SP and [Sar^9 $MetO_2^{11}$]-SP were compared by measuring Ca^{2+} mobilization in neurons.

Agonist-induced Trafficking of NK1-R, $G_{\alpha q/11}$, GRK-2 and -3, and β-Arrestin-1 and -2 in Neurons

Neurons were incubated in DMEM-BSA containing 100 nM SP or Cy3-SP or 190 nM Cy3-[Sar^9 $MetO_2^{11}$]-SP for 2 h at 4°C for equilibrium binding, as described (Grady *et al.*, 1995, 1996b). They were washed in DMEM-BSA at 4°C and either fixed immediately or incubated in SP-free medium at 37°C for 30 s to 30 min to permit receptor endocytosis and trafficking to proceed. They were fixed with 4% paraformaldehyde in 100 mM PBS (pH 7.4) for 20 min at 4°C. All observations were repeated on at least three different neuron cultures.

Immunofluorescence and Confocal Microscopy

Neurons were incubated in PBS containing 10% normal goat serum and 0.1 or 0.0025% saponin for 10–15 min and incubated with primary antibodies in the same solution overnight at 4°C. Rabbit polyclonal antibodies to murine $G_{\alpha q/11}$ (residues 13–29, 0.5 μg/ml dilution) and human GRK-2 (residues 675–689, 1 μg/ml dilution) were from Santa Cruz Biotechnology (Santa Cruz, CA). A rabbit polyclonal antibody to rat β-arrestin-1 and -2 (residues 333–410 of β-arrestin-2, 1:500 dilution; Attramadal *et al.*, 1992) and a mouse monoclonal antibody to rat GRK-2 and -3 (C-terminal 221 residues, 1:100 dilution; Oppermann *et al.*, 1996) were from R. Lefkowitz (Duke University, Durham, NC). A rabbit polyclonal antibody to the rat NK1-R (residues 393–407, 1,000 dilution) has been fully characterized (Vigna *et al.*, 1994; Grady *et al.*, 1996a). Neurons were washed, incubated with fluorescently labeled secondary antibodies (1:200) for 2 h at 4°C, washed, and mounted. Affinity-purified goat anti-rabbit and goat anti-mouse immunoglobulin G conjugated to fluorescein isothyocyanate (FITC) or Texas Red were from Cappel Research Products (Durham, NC) or Jackson ImmunoResearch Laboratories (West Grove, PA). Where possible, specificity of antibodies was evaluated by preincubation of the diluted antibodies overnight at 4°C with 10-μg/ml concentrations of the peptides used for immunization.

Neurons were observed with a Zeiss Axiovert 100 TV microscope, an MRC 1000 laser scanning confocal microscope (Bio-Rad, Hercules, CA) equipped with a krypton–argon laser, and a Zeiss plan-Apochromat 100× oil-immersion objective with a numerical aperture of 1.4 (∞0.7) (Grady *et al.*, 1996b). Images were collected under Kalman or Accumulate mode using an aperture of 2–4 mm and a zoom of 1–3. Typically, 10–20 optical sections were taken at 0.50- to 0.72-μm intervals through the cells. Under these conditions the resolution of the confocal microscope in the x–y axis was 170–200 nm and in the z axis was 230–400 nm. Images of 768 × 520 pixels were obtained. Images were processed using Adobe Photoshop 4.0 (Adobe Systems, Mountain View, CA) and printed using a Fujix (Elmsford, NY) Pictrography 3000 printer.

Western Blotting

Antibody selectivity was further verified by Western blotting extracts of myenteric neurons in culture. Neurons were solubilized at 4°C in radioimmunoprecipitation assay buffer (1% Triton X-100, 1% sodium deoxycholate in 150 mM PBS) containing a protease inhibitor mixture (Calbiochem, La Jolla, CA). The lysate was passed through a 25-gauge needle to shear DNA and centrifuged (14,000 × *g*, 10 min, 4°C). The supernatant was boiled in Laemmli sample buffer for 5 min and fractionated on a 12% SDS-polyacrylamide gel under denaturing and reducing conditions (25 or 50 μg protein/lane) (Grady *et al.*, 1996a). Proteins were transferred to nitrocellulose. Filters were incubated in 3% BSA in PBS for 1 h and incubated with primary antibodies ($G_{\alpha q/11}$ and β-arrestin-1 and -2, 1:10,000; GRK-2, 1:2,000; GRK-2 and -3 1:5,000, in 1% BSA in PBS) for 1 h at room temperature. They were washed extensively in PBS containing 0.05% Tween 20 and incubated with goat anti-rabbit or anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Santa Cruz Biotechnology; 1:5,000) for 1 h at room temperature. Blots were washed, and bands were detected on film using the Super-Signal detection kit (Pierce, Rockford, IL), according to the manufacturer's directions.

RESULTS

Specificity of Fluorescent Peptides

We have previously reported that Cy3-SP specifically interacts with the NK1-R in transfected cells and myenteric neurons (Bunnett et al., 1995; Grady *et al.*, 1995, 1996b). To evaluate the selectivity of Cy3-[Sar^9 $MetO_2^{11}$]-SP as a ligand for the NK1-R, we incubated transfected KNRK-NK1-R cells with 190 nM peptide for 60 min at 4°C. Cy3-[Sar^9 $MetO_2^{11}$]-SP was localized to the plasma membrane of KNRK-NK1-R cells at 4°C (Figure 1A). The signal was abolished when cells were preincubated for 30 min with 1 μM unlabeled [Sar^9 $MetO_2^{11}$]-SP (Figure 1B) or with a 1 μM concentration of the NK1-R-selective antagonist CP96345 (Figure 1C) before addition of the fluorescent peptide, and there was no binding to untransfected KNRK cells (Figure 1D). Therefore, Cy3-[Sar^9 $MetO_2^{11}$]-SP interacts specifically with the NK1-R.

Specificity of Antibodies

We verified the specificity of antibodies by Western blotting extracts of cultured neurons. The antibody to $G_{\alpha q/11}$ recognized a protein of ~43 kDa, and the antibody to β-arrestin-1 and -2 recognized a broad band of ~55 kDa (Figure 2). The polyclonal antibody to GRK-2 and the monoclonal antibody to GRK-2 and -3 recognized broad bands of ~80–90 kDa (Figure 2). No other proteins were detected. Thus, these antibodies interact specifically with proteins of the predicted molecular masses in myenteric neuron cultures. We also

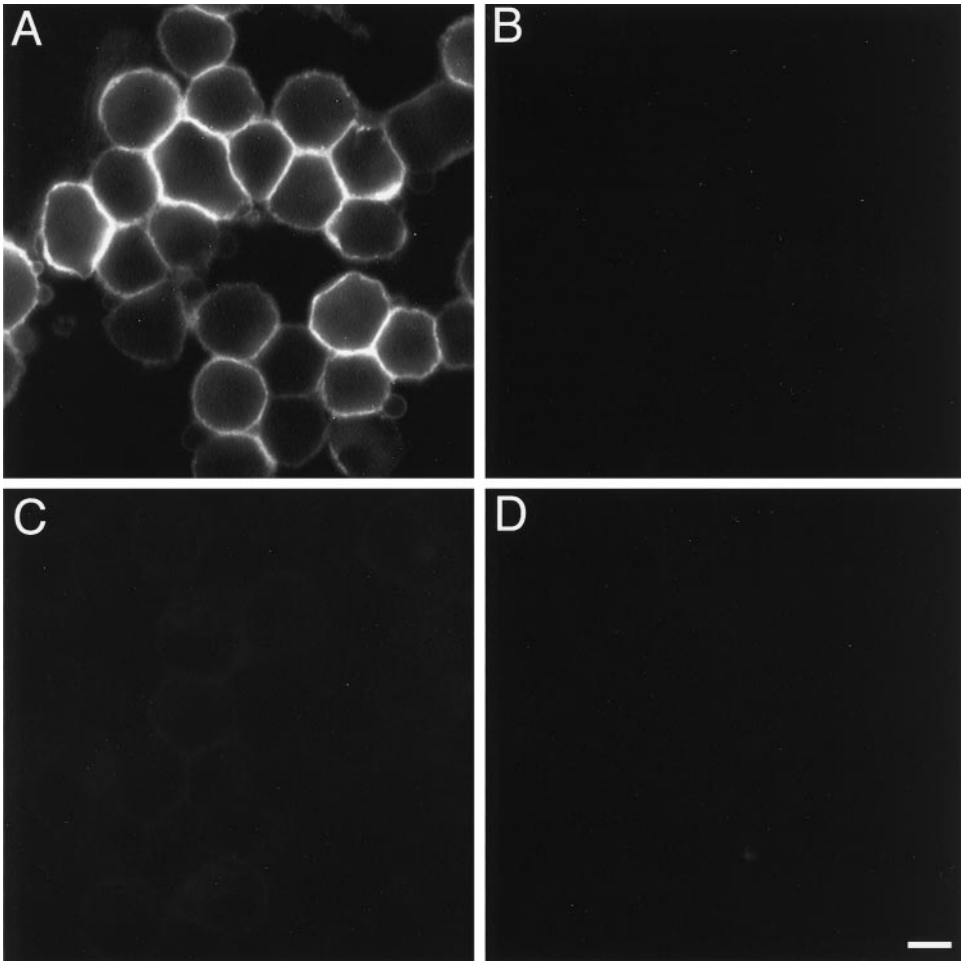

Figure 1. Binding of Cy3-[Sar^9 MetO_2^{11}]-SP to KNRK-NK1-R cells (A–C) or untransfected KNRK cells (D). Cells were incubated with 190 nM Cy3-[Sar^9 MetO_2^{11}]-SP for 1 h at 4°C, fixed, and observed. (A) KNRK-NK1-R cells. (B) KNRK-NK1-R cells preincubated with 1 μM unlabeled [Sar^9 MetO_2^{11}]-SP before addition of Cy3-[Sar^9 MetO_2^{11}]-SP. (C) KNRK-NK1-R cells preincubated with 1 μM CP96345, an NK1-R–selective antagonist, before addition of Cy3-[Sar^9 MetO_2^{11}]-SP. (D) KNRK cells. Bar, 10 μm.

evaluated antibody specificity by immunofluorescence. We have previously established that the NK1-R antibody specifically recognizes the NK1-R in myenteric neurons, because staining is abolished by preincubation of the antibody with the receptor fragments used for immunization (Vigna et al., 1994; Grady *et al.*, 1996a). Staining of neurons with the $G_{\alpha q/11}$ and the GRK-2 antibodies was abolished by preincubation of the diluted antibodies overnight at 4°C with 10-μg/ml concentrations of peptides used for immunization (our unpublished results). The fusion proteins used to generate the antibody to β-arrestin-1 and -2 and GRK-2 and -3 were not available for preabsorption, but these antibodies were affinity purified and have been previously characterized (Attramadal *et al.*, 1992; Oppermann *et al.*, 1996).

SP-induced Mobilization of Intracellular Ca^{2+} in Neurons

We measured Ca^{2+} mobilization in cultured neurons to determine whether they expressed functional neurokinin receptors. Exposure of myenteric neurons to 100 nM SP for 1 min caused a prompt increase in $[Ca^{2+}]_i$ in a small population of cells that declined to basal levels when SP was removed (Figure 3A). When neurons were washed by perfusion and rechallenged with 100 nM SP 5 min after the first exposure, there was an increase in $[Ca^{2+}]_i$ that was only slightly smaller than the first response (Figure 3A). These results indicate that myenteric neurons in culture express functional receptors for SP, and that there is minimal desensitization to a brief exposure to SP.

SP interacts with the NK1-R, NK2-R, and NK3-R, albeit with graded affinity (NK1-R > NK2-R > NK3-R). Because all three neurokinin receptors are expressed in myenteric neurons (Guard and Watson 1987; Yau *et al.*, 1992; Vigna *et al.*, 1994; Sternini *et al.*, 1995; Grady *et al.*, 1996a; Portbury *et al.*, 1996; Mann *et al.*, 1997) and couple to Ca^{2+} mobilization, we used selective antagonists and agonists of the NK1-R to ascertain whether responses to SP were mediated by this receptor. Treatment of neurons with 1 μM

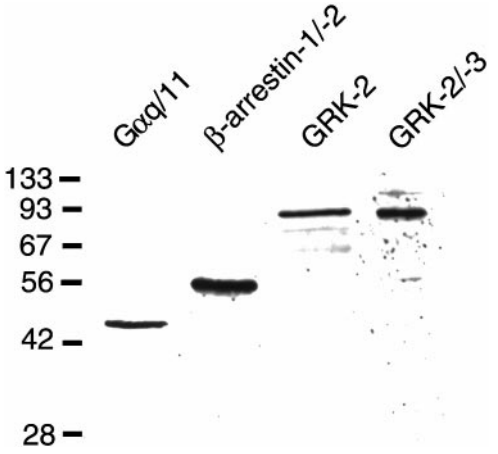

Figure 2. Characterization of antibodies by Western blotting. Extracts of myenteric neurons (50 μg protein/lane for $G_{\alpha q/11}$, β-arrestin-1 and -2, and GRK-2; 25 μg protein/lane for GRK-2 and -3) were separated on 12% SDS-PAGE gels and probed with antibodies to $G_{\alpha q/11}$, β-arrestin-1 and -2, GRK-2, and GRK-2 and -3.

SR140333, a selective antagonist of the NK1-R (Emonds-Alt *et al.*, 1993), abolished the response to a second challenge with 100 nM SP (Figure 3B). This lack of response to the second challenge was not caused by receptor desensitization, because desensitization was minimal under these circumstances (Figure 3A). Neurons that responded to 100 nM SP also responded to 1 μM [Sar^9 $MetO_2^{11}$]-SP, a specific agonist of the NK1-R (Drapeau *et al.*, 1987) (Figure 3C). Therefore, SP stimulates Ca^{2+} mobilization in cultured myenteric neurons by activating the NK1-R.

We exposed neurons to graded concentrations of SP to determine the concentration dependency of receptor activation. Different neurons were used for each SP concentration that was tested. The threshold concentration for detectable increases in $[Ca^{2+}]_i$ was 0.1 nM SP, and the EC_{50} was ~10 nM (Figure 4A). This concentration is unexpectedly high, because SP stimulates Ca^{2+} mobilization in transfected cell lines expressing the NK1-R with an EC_{50} of 0.66 nM (Vigna *et al.*, 1994). However, because neurons were mounted in a chamber with a 1-ml volume and then were perfused with SP-containing solution at a rate of 1 ml/min, it is likely that the SP concentrations in the chamber were less than those in the perfusate. Similarly, when cultures were exposed to graded concentrations of SP, there was also a graded increase in the number of neurons in which there was a detectable increase in $[Ca^{2+}]_i$ (Figure 4B).

After neurons were exposed to SP, we challenged them with 100 μM acetylcholine (ACh), to determine whether the NK1-R–positive neurons also expressed cholinergic receptors. At the end of experiments, neuronal cultures were challenged with 55 mM KCl, which depolarizes neurons, increasing $[Ca^{2+}]_i$,

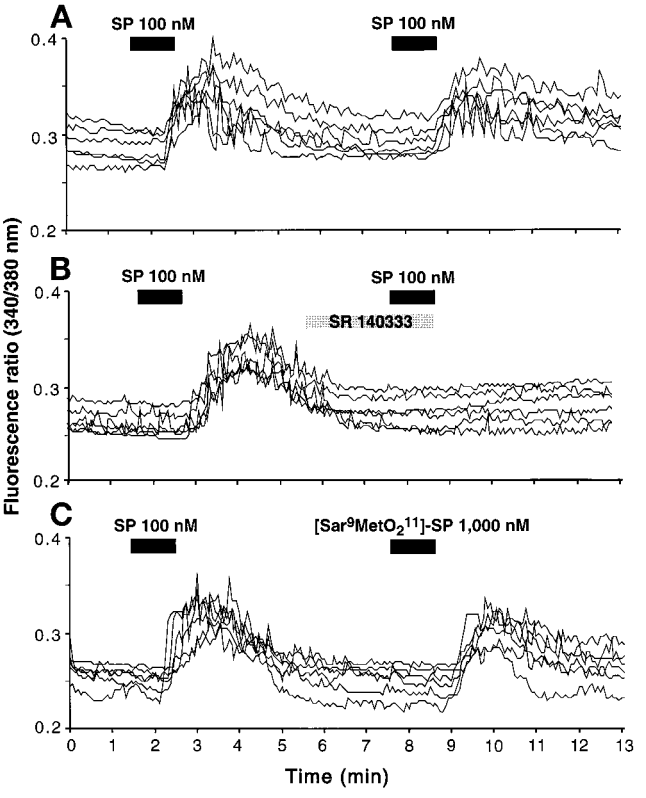

Figure 3. SP-induced Ca^{2+} mobilization in myenteric neurons. (A) Neurons were exposed to SP for 1 min, washed, and then exposed again to SP 5 min later. (B) Neurons were exposed to SP for 1 min, washed, and then exposed again to SP 5 min later in the presence of the NK1-R antagonist SR 140333. (C) Neurons were exposed to SP for 1 min, washed, and then exposed again to the NK1-R–selective agonist [Sar^9 $MetO_2^{11}$]-SP 5 min later. Each trace shows the 340:380 nm fluorescence ratio, which is proportional to $[Ca^{2+}]_i$, for a single neuron, and observations were repeated on at least three different coverslips.

whereas nonneuronal cells do not possess voltage-gated Ca^{2+} channels and would not respond (Simeone *et al.*, 1996). Of the cells that responded to 100 nM SP with increased $[Ca^{2+}]_i$, 87.2 ± 4.6% (mean ± SE, n = 47 neurons) also responded to 100 μM ACh and 55 mM KCl. This result indicates that most neurons that express the NK1-R also express cholinergic receptors. However, only 40.6 ± 4.3% (54 neurons) of the KCl-responsive neurons also responded to 100 nM SP, whereas 63.2 ± 3.2% (n = 84 neurons) of the KCl-responsive neurons responded to 100 μM ACh. This observation indicates that there is a higher proportion of cholinergic neurons than SP-responsive neurons.

Desensitization of SP-induced Mobilization of Intracellular Ca^{2+} in Neurons

We examined desensitization of Ca^{2+} mobilization to repetitive exposure of neurons to SP. When neurons

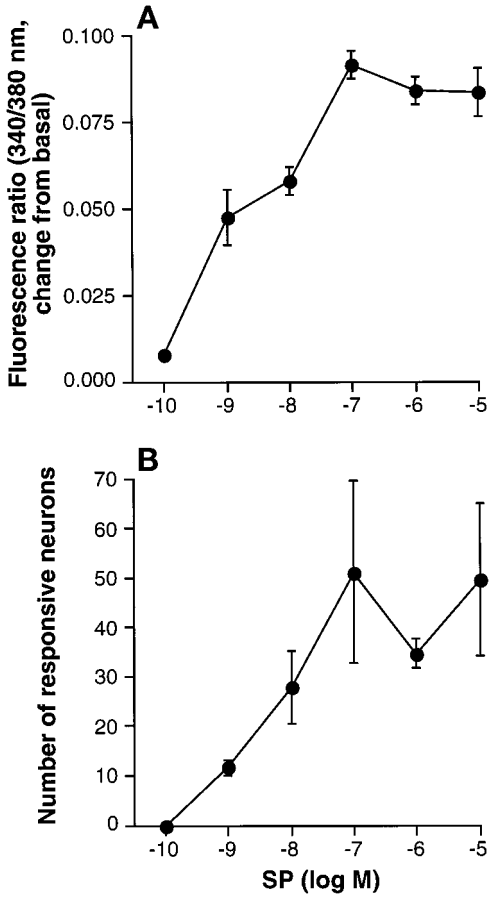

Figure 4. Effects of graded concentrations of SP on Ca^{2+} mobilization in myenteric neurons. Neurons were exposed to a single concentration of SP for 1 min, and the maximal increase in the 340:380 nm fluorescence ratio (A) and the number of responsive neurons per field (B) were measured. Results in A are the mean ± SE of measurements from n = 133 neurons. Results in B are the mean ± SE of measurements from n = 3 coverslips. Different neurons were used for each SP concentration tested.

were exposed to 100 nM SP for only 1 min and then perfused with SP-free medium for 5 min, there was minimal desensitization to a second exposure to 100 nM SP (Figure 3A). However, when neurons were exposed to 100 nM SP for 5 min, washed, and then exposed to 100 nM SP 10 min later, there was a minimal Ca^{2+} response to the second challenge (Figure 5, A and B, II and III), indicating strong desensitization. Therefore, the extent of desensitization depends on the duration of SP exposure. Neurons that had been exposed to SP under conditions that desensitized the NK1-R still responded to 100 μM ACh and 55 mM KCl (Figure 5, A and B, IV and V). This finding indicates that SP-induced desensitization is specific for the NK1-R, and not cholinergic receptors, and that the diminished Ca^{2+} response to a second SP challenge is not due to depletion of stores of intracellular Ca^{2+}.

To examine the concentration dependency of desensitization, we exposed neurons to graded concentrations of SP or carrier (control) for 5 min, washed them, and challenged neurons with 100 nM SP 10 min later. Graded concentrations of SP caused a graded desensitization (Figure 6A). Desensitization was detected after exposure to 0.1 nM SP and was maximal to 100 nM SP, which caused almost complete desensitization at 10 min Desensitization was half-maximal to ~10 nM SP. Therefore, the extent of desensitization of SP-induced Ca^{2+} mobilization depends on both the SP concentration and time of exposure.

Resensitization of SP-induced Mobilization of Intracellular Ca^{2+} in Neurons

To determine the time course for resensitization of SP-induced Ca^{2+} mobilization, we exposed neurons to 100 nM SP or carrier (control) for 5 min, washed them, and challenged neurons with 100 nM SP 10–30 min later. When the interval between SP exposure was 10 min, there was complete desensitization (Figures 5, A and B, III, and 6B). When the interval was 30 min, there was complete resensitization of SP-induced Ca^{2+} mobilization, and recovery was ~50% complete after ~22 min.

Localization of the NK1-R, $G_{\alpha q/11}$, GRK-2 and -3, and β-Arrestin-1 and -2 in Neurons

SP stimulated an increase in $[Ca^{2+}]_i$ that was attenuated in the continued presence of SP and that desensitized to repetitive challenge with agonist. We have previously reported that SP also induces endocytosis of the NK1-R in myenteric neurons (Grady et al., 1996b). Observations in reconstituted systems and using membrane preparations indicate that $G_{\alpha q/11}$ may couple to the NK1-R and mediate signal transduction (Kwatra *et al.*, 1993; Macdonald *et al.*, 1996), but it is not known whether it is coexpressed with the receptor in neurons or whether SP alters its subcellular distribution. GRK-2 and -3 and β-arrestin-1 and -2 mediate desensitization and endocytosis of several GPCRs for hormones and neurotransmitters (Böhm *et al.*, 1997a), and studies of reconstituted systems and transfected cells suggest that these proteins also regulate the NK1-R (Kwatra *et al.*, 1993; Sasakawa *et al.*, 1994a). However, it is not known whether they are coexpressed in neurons with the NK1-R. Furthermore, these cytoplasmic proteins must be targeted to receptors in the plasma membrane upon agonist stimulation. Therefore, we determined the subcellular localization of the NK1-R, $G_{\alpha q/11}$, GRK-2 and -3, and β-arrestin-1 and -2 in myenteric neurons and examined whether $G_{\alpha q/11}$, GRK-2 and -3, and β-arrestin-1 and -2 were expressed in the same neurons as the NK1-R. We also investigated whether NK1-R agonists altered the subcellular localization of $G_{\alpha q/11}$, GRK-2

and -3, and β-arrestin-1 and -2 in a manner consistent with regulating desensitization and endocytosis of the NK1-R.

Localization of NK1-R, $G_{\alpha q/11}$, GRK-2 and -3, and β-Arrestin-1 and -2 in Unstimulated Neurons

We localized the NK1-R, $G_{\alpha q/11}$, GRK-2 and -3, and β-arrestin-1 and -2 in myenteric neurons without exposure to SP by immunofluorescence and confocal microscopy to assess the subcellular distribution of these proteins in unstimulated neurons. The NK1-R was detected in a substantial subpopulation of myenteric neurons (Figure 7A). Immunoreactivity was mainly detected at the plasma membrane of the soma and neurites (Figure 7A, arrowheads), although the NK1-R was also detected in some vesicles in both locations (Figure 7A, arrows). Intracellular NK1-R may be newly synthesized receptor or internalized receptor, because we have previously reported that these cultured neurons secrete SP, which stimulates NK1-R endocytosis (Grady et al., 1996b). $G_{\alpha q/11}$ was detected in most myenteric neurons, where it was mainly confined to the plasma membrane of the soma, and neurites, with minimal intracellular stores (Figure 7B, arrowheads). There was cytoplasmic and punctate staining within the soma and neurites of most myenteric neurons with antibodies to GRK-2 and to GRK-2 and -3 (Figure 7C, arrows). There was also cytoplasmic and punctate staining within the soma and neurites of most myenteric neurons with the antibody to β-arrestin-1 and -2, although the punctate staining was less pronounced than with the GRK antibodies (Figure 7D, arrows). The punctate staining with antibodies to GRK-2 and -3 and β-arrestin-1 and -2 suggests that these proteins are present in vesicles that are distributed throughout the cell, but electron microscopy is required to fully define their subcellular distribution. In sharp contrast to the distribution of the NK1-R and $G_{\alpha q/11}$, which were mostly confined to the plasma membrane of unstimulated neurons, GRK-2 and -3 and β-arrestin-1 and -2 were not prominently detected at the cell surface but were present in intracellular locations, although some vesicles containing GRK-2 and -3 were in close proximity to the plasma membrane. Presumably, GRK-2 and -3 and β-arrestin-1 and -2 translocate to the plasma membrane to interact with surface receptors. Notably, whereas the NK1-R was detected in a subpopulation of myenteric neurons, most myenteric neurons expressed $G_{\alpha q/11}$, GRK-2 and -3, and β-arrestin-1 and -2, which suggests that these proteins interact with many other GPCRs.

Localization of NK1-R, $G_{\alpha q/11}$, GRK-2 and -3, and β-Arrestin-1 and -2 in SP-stimulated Neurons

To determine whether neurons expressing the NK1-R also expressed $G_{\alpha q/11}$, GRK-2 and -3, and β-arrestin-1 and -2, and to examine agonist-induced trafficking of these proteins, we incubated neurons with Cy3-SP and localized the NK1-R, $G_{\alpha q/11}$, GRK-2 and -3, and β-arrestin-1 and -2 by immunofluorescence. Neurons were incubated with 100 nM Cy3-SP for 2 h at 4°C and immediately fixed or were washed, incubated for 30 s to 30 min at 37°C, and then fixed. In control experiments we verified that incubation of neurons at 4°C without exposure to SP followed by warming to 37°C did not cause redistribution of the NK1-R, $G_{\alpha q/11}$, GRK-2 and -3, and β-arrestin-1 and -2 (our unpublished results). This observation indicates that trafficking is due to SP and not to a temperature change.

NK1-R

To verify that neurons that bound Cy3-SP expressed the NK1-R, the NK1-R was localized by immunofluorescence using a secondary antibody conjugated to FITC. After incubation for 2 h at 4°C, Cy3-SP was principally detected at the cell surface of the soma and neurites, and there was minimal internalization (Figure 8A, arrowheads). The NK1-R was also located at the plasma membrane of the soma and neurites (Figure 8B, arrowheads) and in small vesicles of the same neurons that bound Cy3-SP (Figure 8C). Warming to 37°C caused a marked redistribution of Cy3-SP and the NK1-R. After incubation at 37°C for 30 s to 2 min, Cy3-SP and the NK1-R were colocalized in small, superficial vesicles located at or immediately beneath the cell surface of the soma and neurites, with little detectable surface localization (our unpublished results). After 2–10 min, Cy3-SP was detected in vesicles immediately beneath the cell surface and in a perinuclear location (Figure 8D, arrows). Vesicles containing Cy3-SP had the same size, shape, and location as vesicles containing the NK1-R (Figure 8E, arrows), as indicated by superimposition of confocal images, where yellow denotes colocalization (Figure 8F). We have previously shown that these vesicles also contain the transferrin receptor, which identifies them as early endosomes (Grady et al., 1995, 1996b). Colocalization persisted for 30 min, when the Cy3-SP signal in the soma became diffuse, probably because of ligand degradation in lysosomes. Thus, the NK1-R is expressed by myenteric neurons in culture that bind Cy3-SP, and Cy3-SP binding to the soma and neurites is rapidly followed by internalization of the ligand and its receptor into the same early endosomes.

$G_{\alpha q/11}$

We incubated neurons with Cy3-SP to detect functional NK1-R and localized $G_{\alpha q/11}$ by immunofluorescence. $G_{\alpha q/11}$ was detected at the plasma membrane of the soma and neurites of most myenteric neurons. Neurons that bound Cy3-SP, and presumably express the NK1-R, also expressed $G_{\alpha q/11}$. At 4°C, Cy3-SP

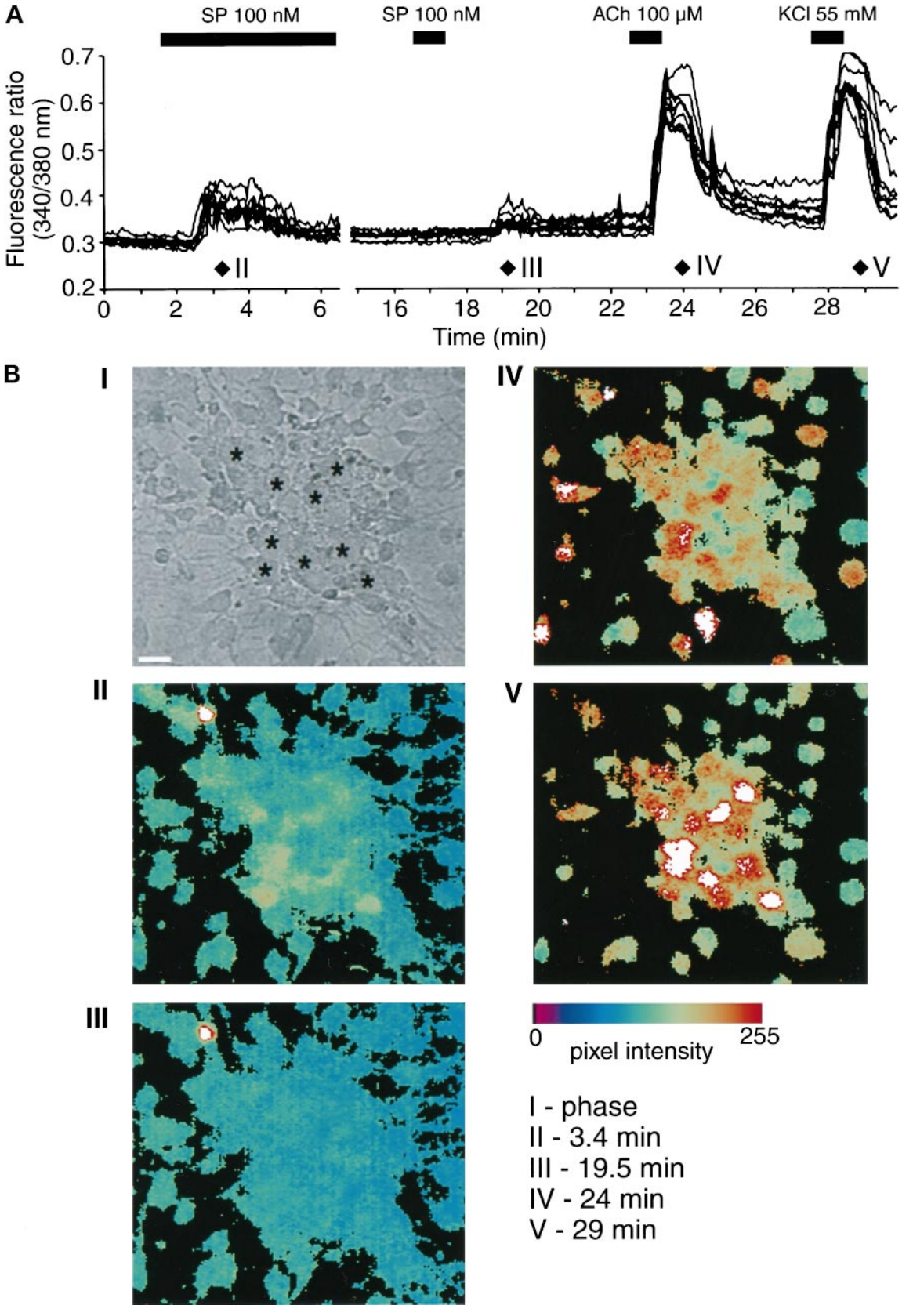
A
SP 100 nM
SP 100 nM
ACh 100 μM
KCl 55 mM
Fluorescence ratio (340/380 nm)
0.7
0.6
0.5
0.4
0.3
0.2
II
III
IV
V
0
2
4
6
16
18
20
22
24
26
28
Time (min)
B
I
II
III
IV
V
0
255
pixel intensity
I - phase
II - 3.4 min
III - 19.5 min
IV - 24 min
V - 29 min

Figure 5.

(Figure 8G) and $G_{\alpha q/11}$ (Figure 8H) were colocalized at the plasma membrane (Figure 8I, arrowheads). Warming to 37°C caused endocytosis of Cy3-SP but did not alter the localization of $G_{\alpha q/11}$. After 1–10 min at 37°C, Cy3-SP was present in endosomes (Figure 8J, arrows), whereas $G_{\alpha q/11}$ remained at the cell surface (Figure 8K, arrowheads), and there was no detectable colocalization (Figure 8L). Thus, $G_{\alpha q/11}$ is expressed by many myenteric neurons, some of which bind Cy3-SP and express the NK1-R. $G_{\alpha q/11}$ is suitably located to couple with the NK1-R at the cell surface, but its widespread distribution in many neurons suggests that $G_{\alpha q/11}$ also couples to other GPCRs. The association between $G_{\alpha q/11}$ and the NK1-R is transient because the NK1-R rapidly internalized after stimulation with SP, whereas $G_{\alpha q/11}$ remained at the plasma membrane. This finding suggests that the NK1-R in endosomes no longer interacts with $G_{\alpha q/11}$.

GRK-2 and -3

We incubated neurons with Cy3-SP to detect functional NK1-R and localized GRK-2 by immunofluorescence. GRK-2 was detected in most myenteric neurons. A subpopulation of these neurons also bound Cy3-SP and, thus, presumably express the NK1-R. When neurons were incubated with Cy3-SP at 4°C, Cy3-SP bound to the cell surface (Figure 9A, white arrowheads). GRK-2 was mainly detected in the cytosol and in a punctate distribution that suggests localization in uniformly distributed vesicles (Figure 9B, yellow arrow). However, GRK-2 was also detected in vesicles at or in close proximity to the plasma membrane (Figure 9B, yellow arrowheads) in the vicinity of binding sites for Cy3-SP (Figure 9C). Warming to 37°C caused endocytosis of Cy3-SP but did not markedly alter the subcellular distribution of GRK-2. After 30 s at 37°C, Cy3-SP was detected in endosomes immediately beneath the plasma membrane (Figure 9D, white arrowheads). GRK-2 was still prominently localized in central vesicles (Figure 9E, yellow arrow) and was also detected in vesicles at or in close proximity to the plasma membrane (Figure 9E, yellow arrowheads) that were distinct from endosomes containing Cy3-SP (Figure 9F). After 2–10 min, Cy3-SP was detected in endosomes in the soma and neurites (Figure 9G, white arrows), and GRK-2 was detected in the cytoplasm and in uniformly distributed vesicles (Figure 9H, yellow arrows), with no detectable staining of the plasma membrane or colocalization with Cy3-SP in endosomes (Figure 9I). In a similar manner we simultaneously localized the NK1-R using a rabbit antibody and GRK-2 and -3 using a mouse antibody that recognizes both kinases after exposure of neurons to unlabeled SP. The results of these experiments (our unpublished results) were similar to those obtained using Cy3-SP and the GRK-2 antibody. Our results indicate that GRK-2 and -3 are expressed by many myenteric

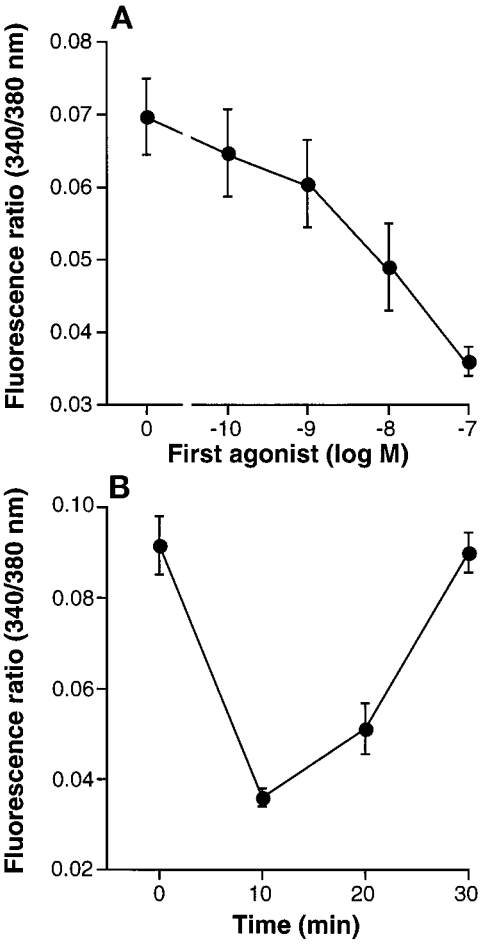

Figure 6. (A) Effects of graded concentrations of SP on desensitization of SP-induced Ca^{2+} mobilization in myenteric neurons. Neurons were exposed to graded concentrations of SP or carrier (control) for 5 min, washed, and then challenged with 100 nM SP 10 min later. The maximal increase in the 340:380 nm fluorescence ratio to the second SP exposure was determined. (B) Time course for resensitization of SP-induced Ca^{2+} mobilization. Neurons were exposed to 100 nM SP or carrier (control) for 5 min, washed, and then challenged with 100 nM SP 10, 20, or 30 min later. The maximal increase in the 340:380 nm fluorescence ratio to the second SP exposure was determined. Results are the mean ± SE of measurements from n = >100 neurons, and observations were repeated on at least three different coverslips at each agonist concentration.

Figure 5 (facing page). Desensitization of SP-induced Ca^{2+} mobilization in myenteric neurons. Neurons were exposed to SP for 5 min, washed, and then exposed again to SP 10 min later. Neurons were washed and then exposed to ACh and then KCl. (A) Each trace shows the 340:380 nm fluorescence ratio, which is proportional to $[Ca^{2+}]_i$, for a single neuron. (B, I) Phase image of neurons. *, Neurons that responded to the first dose of SP. (B, II–V) Pseudocolor images of the 340:380 fluorescence ratio for these neurons at the indicated times. ♦ in A indicates the times at which these images were obtained. Bar, 10 μm.

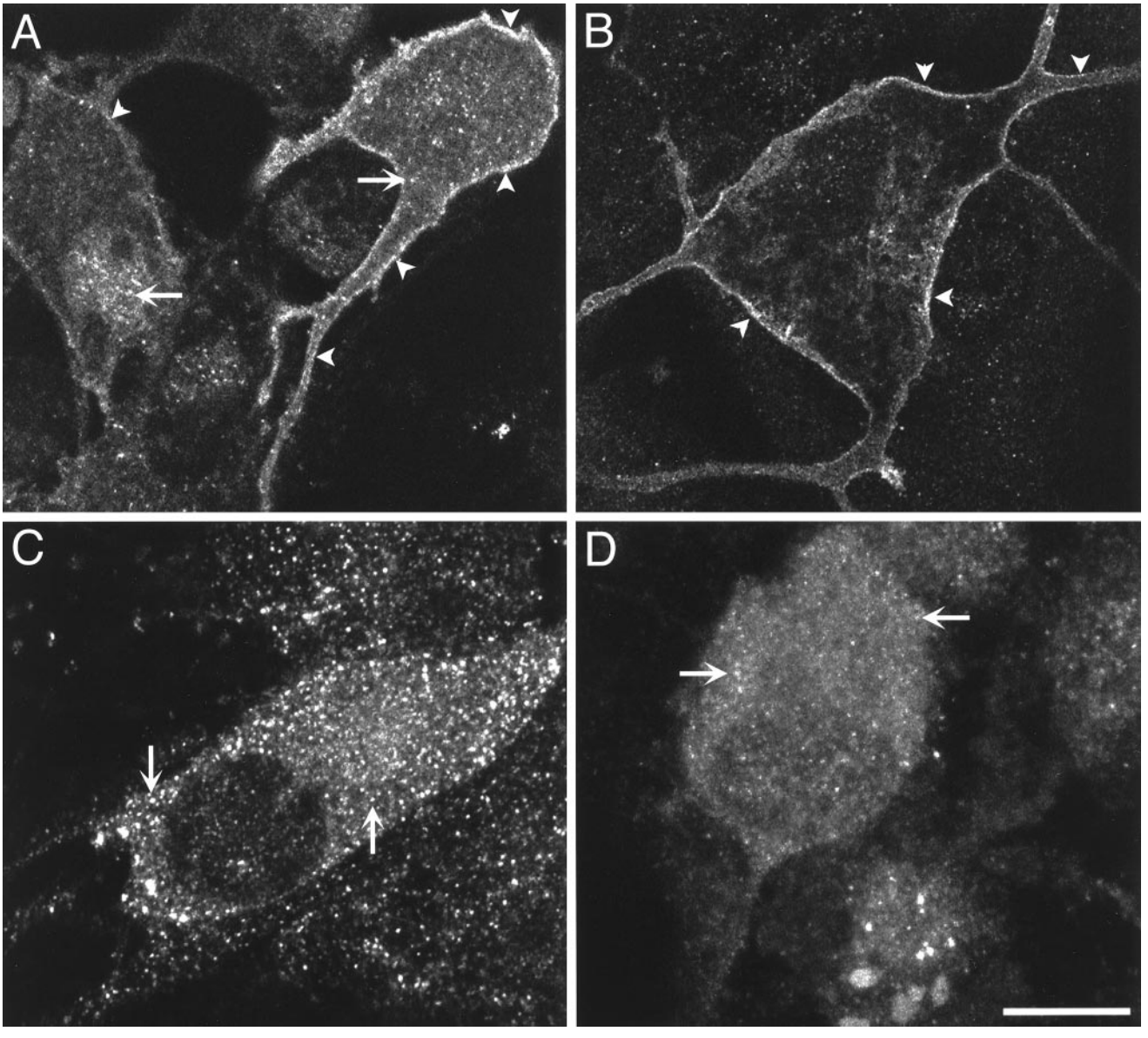

Figure 7. Confocal images showing the localization of immunoreactive NK1-R, $G_{\alpha q/11}$, GRK-2, and β-arrestin-1 and -2 in myenteric neurons not exposed to SP. (A) Localization of the NK1-R at the plasma membrane of the soma and neurites (arrowheads) and in some vesicles (arrows). (B) Localization of $G_{aq/11}$ at the plasma membrane of the soma and neurites (arrowheads). (C) Punctate and cytosolic localization of GRK-2 (arrows). (D) Punctate and cytosolic localization of β-arrestin-1 and -2 (arrows). Bar, 7.5 μm (A and B), 10 μm (C and D).

neurons, some of which bind Cy3-SP and express the NK1-R. Therefore, GRK-2 and -3 are appropriately located to regulate the NK1-R, but their more widespread distribution suggests that these kinases also interact with other GPCRs. Although exposure to SP did not markedly alter the subcellular distribution of GRK-2 and -3, these kinases were detected in vesicles at or close to the cell surface where they may phosphorylate the agonist-occupied NK1-R at the plasma membrane.

Cy3-SP and β-Arrestin-1 and -2

We incubated neurons with Cy3-SP to localize functional NK1-R and localized β-arrestins using an antibody that interacts with β-arrestin-1 and -2. β-arrestin-1 and -2 were expressed by a large number of myenteric neurons. A subpopulation of these neurons also bound Cy3-SP. At 4°C Cy3-SP was detected at the cell surface (Figure 9J, white arrowheads). Although β-arrestin-1 and -2 were detected in the cytosol and in

a punctate distribution (Figure 9K, yellow arrow), as in unstimulated neurons, there was also surface labeling of some neurons (Figure 9K, yellow arrowheads). Thus, β-arrestin-1 and -2 were detected at the plasma membrane in the vicinity of binding sites for Cy3-SP (Figure 9L). Warming to 37°C caused a marked redistribution of Cy3-SP and β-arrestin-1 and -2. After 1–2 min at 37°C, small superficial vesicles containing Cy3-SP in the soma and neurites also contained β-arrestin-1 and -2 (our unpublished results), and there was no detectable Cy3-SP or β-arrestin-1 and -2 at the plasma membrane. After 2–10 min, Cy3-SP (Figure 7M) and β-arrestin-1 and -2 (Figure 7N) were completely colocalized in superficial and perinuclear endosomes (Figure 7O, white arrows). This striking colocalization was apparent until at least 30 min, when the signal for Cy3-SP became more diffuse (our unpublished results). Thus, β-arrestin-1 and -2 are expressed in myenteric neurons that bind Cy3-SP and therefore express the NK1-R, although they are also found in many other neurons. Therefore, β-arrestin-1 and -2 are appropriately located to regulate the NK1-R, but the widespread distribution suggests that they also regulate other GPCRs. SP stimulates the transient localization of β-arrestin-1 and -2 to the plasma membrane and then induces a marked redistribution of β-arrestin-1 and -2 in the soma and neurites to early endosomes containing Cy3-SP and thus the NK1-R. β-Arrestin-1 and -2 may interact with the agonist-occupied NK1-R at the plasma membrane and in early endosomes.

Specific Localization of Functional NK1-R in Myenteric Neurons

SP interacts with NK1-R, NK2-R, and NK3-R, which are all expressed by myenteric neurons (Guard and Watson 1987; Yau et al., 1992; Vigna *et al.*, 1994; Sternini *et al.*, 1995; Grady *et al.*, 1996a; Portbury *et al.*, 1996; Mann *et al.*, 1997). To verify that the SP-induced trafficking was due to specific activation of the NK1-R, we labeled [Sar^9 $MetO_2^{11}$]-SP, a specific agonist of the NK1-R (Drapeau *et al.*, 1987), with Cy3, and used it to selectively activate and localize the NK1-R. To verify that Cy3-[Sar^9 $MetO_2^{11}$]-SP was biologically active, we measured its effects on $[Ca^{2+}]_i$ in myenteric neurons. Cy3-[Sar^9 $MetO_2^{11}$]-SP (1000 nM) stimulated a prompt increase in $[Ca^{2+}]_i$ in myenteric neurons that had previously responded to 100 nM SP (Figure 10A), comparable to that observed with unlabeled peptide (Figure 3C). When cultures were observed by fluorescence microscopy within 5 min of stimulation, Cy3-[Sar^9 $MetO_2^{11}$]-SP was detected at the cell surface and in endosomes in the soma and neurites of responsive neurons (Figure 10B, arrows). Therefore, Cy3-[Sar^9 $MetO_2^{11}$]-SP is biologically active and is internalized by neurons similarly to Cy3-SP.

To confirm that specific activation of the NK1-R caused the striking redistribution of the NK1-R and β-arrestins-1 and -2 that we observed using Cy3-SP, we incubated neurons with 190 nM Cy3-[Sar^9 $MetO_2^{11}$]-SP for 2 h at 4°C, washed them, and incubated neurons at 37°C. The NK1-R and β-arrestin-1 and -2 were localized by immunofluorescence. After 5 min at 37°C, Cy3-[Sar^9 $MetO_2^{11}$]-SP was detected in endosomes in the soma and neurites (Figure 11, A and D). These endosomes also contained the NK1-R (Figure 11B) and β-arrestin-1 and -2 (Figure 11E), as indicated by superimposition of confocal images (Figure 11, C and F, arrows). Thus, specific activation of the NK1-R induces redistribution of the NK1-R and β-arrestin-1 and -2 into the same endosomes. Neurons that bound Cy3-[Sar^9 $MetO_2^{11}$]-SP also contained immunoreactive GRK-2 and -3 and $G_{\alpha q/11}$ (our unpublished results).

DISCUSSION

Neurons expressing the NK1-R in the myenteric plexus of the guinea pig small intestine also express $G_{\alpha q/11}$, GRK-2 and -3, and β-arrestin-1 and -2, which may interact with the NK1-R and regulate neurotransmission by SP. In the absence of exogenous SP, the NK1-R and $G_{\alpha q/11}$ are mainly present at the plasma membrane, whereas GRK-2 and -3 and β-arrestin-1 and -2 have a punctate and cytoplasmic distribution. In the presence of SP, $G_{\alpha q/11}$ may couple to the NK1-R, activate phospholipase-Cβ, and increase $[Ca^{2+}]_i$. SP causes 1) a rapid and transient increase in $[Ca^{2+}]_i$, which rapidly desensitizes and slowly resensitizes to repeated challenge; 2) internalization of the NK1-R into early endosomes containing SP, which depletes the plasma membrane of high-affinity receptors; and 3) rapid and transient redistribution of β-arrestin-1 and -2 from the cytosol to the plasma membrane, followed by a striking and prolonged redistribution of β-arrestin-1 and -2 to endosomes containing the NK1-R and SP. The marked redistribution of β-arrestin-1 and -2 in the presence of SP suggests that these proteins regulate desensitization and endocytosis of the neuronal NK1-R. SP did not markedly alter the subcellular distribution of $G_{\alpha q/11}$ or GRK-2 and -3. However, GRK-2 and -3 were detected in vesicles at or close to the cell surface and may phosphorylate SP-occupied NK1-R at the plasma membrane. To our knowledge our results provide the first evidence that $G_{\alpha q/11}$, GRK-2 and -3, and β-arrestin-1 and -2 are appropriately localized to regulate the NK1-R in neurons.

SP Interacts with the NK1-R in Neurons

SP stimulated a prompt increase in $[Ca^{2+}]_i$ in myenteric neurons. Although we did not determine the

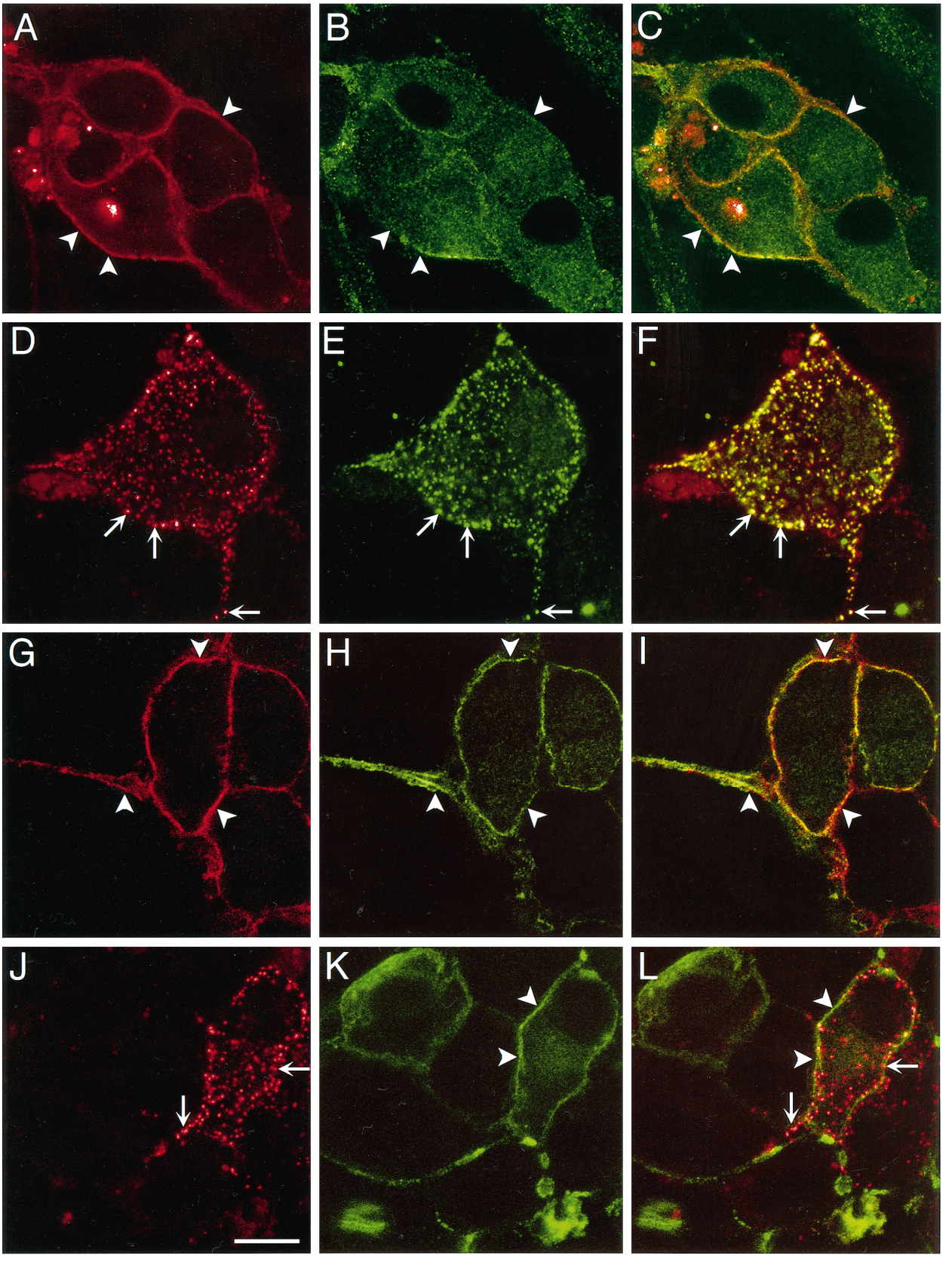

Figure 8.

source of the increase $[Ca^{2+}]_i$, it is likely that Ca^{2+} is mobilized from intracellular pools and also enters from the extracellular fluid, because both intracellular and extracellular Ca^{2+} contribute to SP-induced increases in $[Ca^{2+}]_i$ in transfected cells expressing the NK1-R (Garland et al., 1996). Three observations indicate the SP increases $[Ca^{2+}]_i$ in myenteric neurons by activating the NK1-R. First, SP-stimulated Ca^{2+} responses were abolished by the NK1-R–selective antagonist SR140333 (Emonds-Alt *et al.*, 1993). Second, neurons that responded to SP also responded to [Sar^9 $MetO_2^{11}$]-SP, an NK1-R–selective antagonist (Drapeau *et al.*, 1987). Third, fluorescent SP and [Sar^9 $MetO_2^{11}$]-SP bound to neurons that expressed immunoreactive NK1-R. Our results showing expression of functional NK1-R in myenteric neurons are supported by the results of immunochemical and autoradiography experiments, which have localized the NK1-R to a subpopulation of neurons in the myenteric plexus of the small intestine in guinea pigs and rats (Burcher *et al.*, 1984, 1986; Vigna *et al.*, 1994; Portbury *et al.*, 1995; Sternini *et al.*, 1995; Grady *et al.*, 1996a). On the basis of their morphology and projections these may be interneurons, which participate in cell–cell communication and local integration, or sensory neurons.

SP also interacts with the NK2-R and NK3-R, which are also expressed by myenteric neurons, albeit it with lower affinity than the NK1-R. In guinea pigs, NK2-Rs are preferentially targeted to varicosities at the terminals of descending interneurons, although they are abundantly expressed in the muscularis externa (Portbury *et al.*, 1996). NK3-Rs are localized to a subpopulation of rat myenteric neurons that also express NK1-Rs (Grady *et al.*, 1996a; Mann *et al.*, 1997). These may be sensory neurons, based on their morphology and projections. The NK3-R is also expressed in myenteric neurons from guinea pigs, because the NK3-R–selective agonist senktide stimulates ACh release from myenteric neurons (Yau *et al.*, 1992) and induces ileal contraction by a neural, cholinergic mechanism (Guard and Watson 1987). Despite the expression of the NK2-R and NK3-R by myenteric neurons, both of which mobilize Ca^{2+}, our observations indicate the SP-induced Ca^{2+} mobilization is caused by the NK1-R. Thus, it is possible that we did not use adequate concentrations of SP to excite other receptors or that their expression is lost in culture.

The SP-stimulated NK1-R activates phospholipase Cβ, resulting in generation of inositol trisphosphate and Ca^{2+} mobilization and formation of diacylglycerol and activation of protein kinase C (Otsuka and Yoshioka 1993). The NK1-R couples to pertussis toxin–insensitive G proteins, and experiments with photoactivatable SP analogues and chemical cross-linkers indicate that the NK1-R in rat submaxillary gland membranes couples to $G_{\alpha q/11}$ (Macdonald *et al.*, 1996). Furthermore, addition of purified $G_{\alpha q/11}$ to the NK1-R reconstituted in phospholipid vesicles increases its affinity for SP (Kwatra *et al.*, 1993). G_α subunits directly activate phospholipase-Cβ, which suggests that the NK1-R stimulates phospholipase Cβ through $G_{\alpha q/11}$. Thus, our observation that the NK1-R colocalizes with $G_{\alpha q/11}$ at the plasma membrane of the soma and neurites of myenteric neurons suggests that the neuronal NK1-R also couples to $G_{\alpha q/11}$.

SP-induced Ca^{2+} Responses in Neurons Rapidly Desensitize and Gradually Resensitize

SP-induced Ca^{2+} mobilization in myenteric neurons rapidly desensitized to repeated challenge with SP and slowly resensitized. The extent of desensitization was affected by the duration of exposure and the concentration of SP, suggesting that it depends on the proportion of surface receptors that were activated. This desensitization was not due to depletion of pools of intracellular Ca^{2+}, because exposure to ACh produced robust responses. Therefore, desensitization is likely to be due to a receptor-specific event. Indeed, it is well established that responses to SP that are mediated by the NK1-R strongly desensitize in the intact animal, in tissues, and in cell lines (Gaddum 1953; Bowden et al., 1994; Garland *et al.*, 1996). We have previously shown that exposure of myenteric neurons to SP also causes a rapid loss of high-affinity binding sites for SP at the cell surface of myenteric neurons, which supports our findings (Grady *et al.*, 1996b). This SP-induced loss of high-affinity binding sites from the plasma membrane correlates with endocytosis of the NK1-R and with desensitization of Ca^{2+} mobilization that was observed in the present investigation. Although receptor endocytosis could contribute to desensitization by depleting the plasma membrane of high-affinity receptors that are available to bind hy-

Figure 8 (facing page). Confocal images of myenteric neurons showing SP-induced trafficking of the NK1-R and $G_{\alpha q/11}$. Neurons were incubated with 100 nM Cy3-SP for 2 h at 4°C and either fixed immediately or washed, incubated for 5 min at 37°C, and then fixed. The NK1-R and $G_{\alpha q/11}$ were localized by immunofluorescence using FITC-labeled secondary antibodies. The images at any one horizontal level are of the same neurons, and the images in the right panels are superimpositions of the images in the left and center panels. (A–C) Localization of Cy3-SP (A) and NK1-R (B) to the plasma membrane (arrowheads) of neurons at 4°C. (D–F) Localization of Cy3-SP (D) and NK1-R (E) to the same early endosomes (arrows) of a neuron after 5 min at 37°C. Note that Cy3-SP colocalizes with the NK1-R at the plasma membrane and in early endosomes in the soma and neurites (C and F). (G–I) Localization of Cy3-SP (G) and $G_{\alpha q/11}$ (H) to the plasma membrane (arrowheads) of neurons at 4°C. (J–L) Localization of Cy3-SP (J) to early endosomes (arrows) and $G_{\alpha q/11}$ (K) to the plasma membrane (arrowheads) of neurons after 5 min at 37°C. Note that $G_{\alpha q/11}$ remains at the cell surface of the soma and neurites, whereas Cy3-SP internalizes (I and L). Each image is a sum of one to three single optical sections collected at 0.50–0.72 μm intervals. Bar, 15 μm (A–C), 10 μm (D–L).

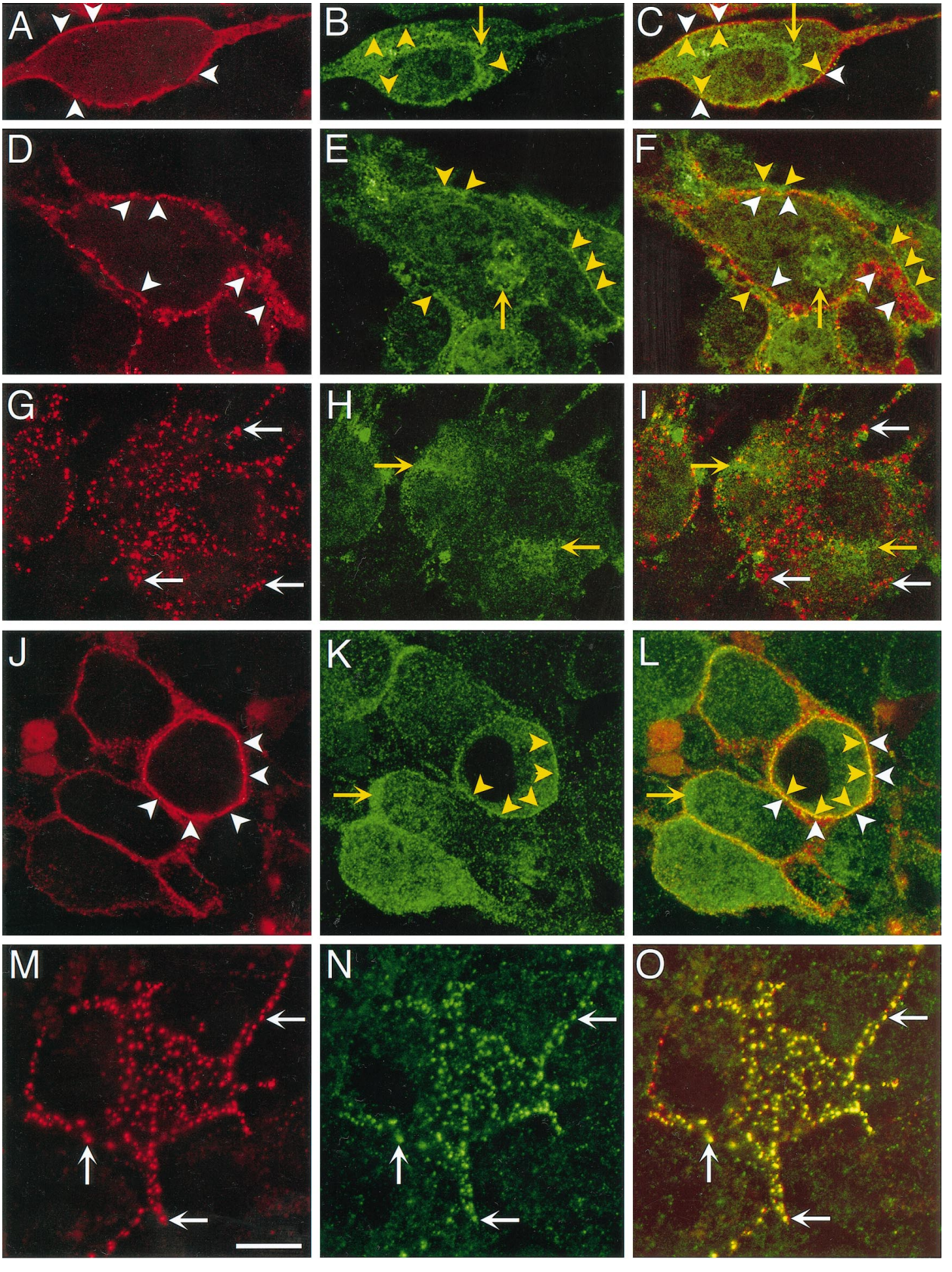

Figure 9.

drophilic ligands in the extracellular fluid, this is not the principal mechanism, because the NK1-R still desensitizes after endocytosis is inhibited (Garland *et al.*, 1996). In a similar manner, the β_2-adrenergic receptor (β_2-AR) desensitizes if endocytosis is blocked by receptor mutation or by using inhibitors (Yu *et al.*, 1993; Barak *et al.*, 1994). Thus, the main mechanism of desensitization of many GPCRs is uncoupling from G-proteins, which involves receptor phosphorylation and association with β-arrestins.

SP-induced Ca^{2+} mobilization in myenteric neurons recovered when the interval between repetitive exposures to SP was increased, indicating that the NK1-R gradually resensitizes. We have previously shown that the NK1-R recycles in transfected cells and myenteric neurons, and that with time after exposure to SP there is a gradual return of high-affinity binding sites at the plasma membrane (Grady *et al.*, 1995, 1996b). This resensitization of the NK1-R is suppressed by inhibitors of receptor endocytosis, by an inhibitor of vacuolar H^+ ATPase, which causes retention of the receptor in endosomes and prevents recycling, and by phosphatase inhibitors (Grady *et al.*, 1995, 1996b; Garland *et al.*, 1996). Together, these findings suggest that resensitization of the NK1-R entails receptor internalization, receptor processing, which may include dissociation of ligand and β-arrestins and receptor dephosphorylation in acidified endosomes, and receptor recycling. In a similar manner, receptor endocytosis and recycling are necessary for resensitization of the β_2-AR (Yu *et al.*, 1993; Barak *et al.*, 1994), and endosomal acidification is also necessary for dephosphorylation of the β_2-AR (Krueger *et al.*, 1997).

Figure 9 (facing page). Confocal images of myenteric neurons showing SP-induced trafficking of GRK-2 and β-arrestin-1 and -2. Neurons were incubated with 100 nM Cy3-SP for 2 h at 4°C and either fixed immediately or washed, incubated for 30 s or 5 min at 37°C, and then fixed. GRK-2 and β-arrestin-1 and -2 were localized by immunofluorescence using FITC-labeled secondary antibodies. The images at any one horizontal level are of the same neurons, and the images in the right panels are superimpositions of the images in the left and center panels. (A–C) Localization of Cy3-SP (A) and GRK-2 (B) at 4°C. Note that Cy3-SP is confined to the plasma membrane (white arrowheads) and that GRK-2 is also found in central vesicles (yellow arrow) and in superficial vesicles at or close to the plasma membrane (yellow arrowheads) in the vicinity of Cy3-SP binding sites (C). (D–F) Localization of Cy3-SP (D) and GRK-2 (E) after 30 s at 37°C. Note that Cy3-SP is found in very superficial endosomes (white arrowheads) and that GRK-2 is found in central vesicles (yellow arrow) and in superficial vesicles at or close to the plasma membrane (yellow arrowheads) with no colocalization with Cy3-SP (F). (G–I) Localization of Cy3-SP (G) and GRK-2 (H) after 5 min at 37°C. Note that Cy3-SP is found in endosomes in the soma and neurites (white arrows) that are distinct from the punctate and cytosolic distribution of GRK-2 (yellow arrows) (I). (J–L) Localization of Cy3-SP (J) and β-arrestin-1 and -2 (K) at 4°C. Note that Cy3-SP is confined to the plasma membrane (white arrowheads), and that β-arrestin-1 and -2 are also found in the cytosol (yellow arrow) and also at the plasma membrane of some neurons (yellow arrowheads) in the vicinity of Cy3-SP binding sites (L). (M–O) Localization of Cy3-SP (M) and β-arrestin-1 and -2 (N) to the same early endosomes (arrows) of a neuron after 5 min at 37°C. Note the striking colocalization of Cy3-SP and β-arrestin-1 and -2 in the same early endosomes of the soma and neurites (O). Each image is a sum of one to three single optical sections collected at 0.50-μm intervals. Bar, 10 μm (A–I and M–O), 15 μm (J–L).

Neurons Expressing the NK1-R Also Express GRK-2 and -3 and β-Arrestin-1 and -2

We observed that exposure of myenteric neurons to SP caused a marked desensitization of the NK1-R and resulted in its redistribution from the plasma membrane into early endosomes of the soma and neurites. Although GRK-2 and -3 and β-arrestin-1 and -2 have been implicated in both desensitization and endocytosis of many GPCRs, including the NK1-R (Kwatra et al., 1993; Sasakawa *et al.*, 1994a), most of the available information derives from experiments in transfected cells that overexpress these proteins or the receptors of interest and from reconstituted systems (Böhm *et al.*, 1997a). Therefore, it is important to define whether they colocalize in tissues with the receptors they are thought to regulate. We detected GRK-2 and -3 and β-arrestin-1 and -2 in a large number of myenteric neurons in culture. We observed the NK1-R in a subpopulation of neurons by immunofluorescence and by specific binding of Cy3-SP or Cy3-[Sar^9 $MetO_2^{11}$]-SP. All of the neurons that expressed the NK1-R also contained GRK-2 and -3 and β-arrestin-1 and -2, which indicates that they may regulate desensitization and endocytosis of the neuronal NK1-R. However, GRK-2 and -3 and β-arrestin-1 and -2 were also detected in many neurons that did not express detectable NK1-R. This more widespread distribution is expected, because GRK-2 and -3 and β-arrestin-1 and -2 regulate many GPCRs (Böhm *et al.*, 1997a). Support for the role of GRK-2 and -3 in desensitization of multiple receptors is provided by their widespread distribution in many tissues, including the brain, where they are localized in the cytosol and at or near the plasma membrane, with enrichment in postsynaptic densities and in axon terminals (Benovic *et al.*, 1989, 1991; Arriza *et al.*, 1992). β-Arrestin-1 and -2 are also widely distributed in other tissues, including the brain (Lohse *et al.*, 1990; Attramadal *et al.*, 1992). β-Arrestin-2 is present in multivesicular bodies of neurons in the brain, where it may interact with endocytosed receptors.

The Effects of SP on the Subcellular Localization of $G_{\alpha q/11}$, GRK-2 and -3, and β-Arrestin-1 and -2 in Neurons Expressing the NK1-R

SP caused marked alterations in the subcellular distribution of the NK1-R and β-arrestin-1 and -2 in myenteric neurons. These effects were also observed after stimulation of neurons with the NK1-R–selective ago-

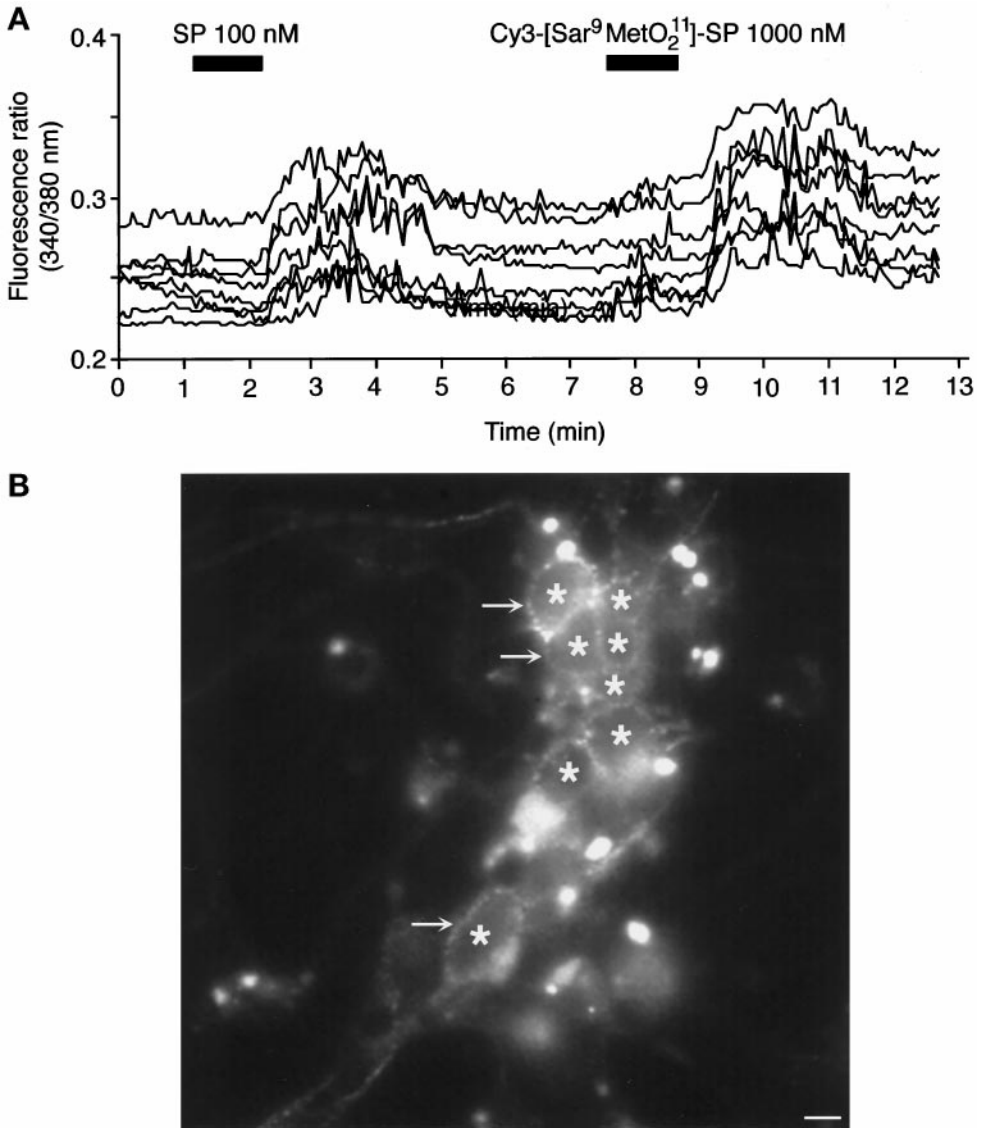

Figure 10. Ca^{2+} mobilization and binding of Cy3-[Sar^9 $MetO_2^{11}$]-SP to myenteric neurons. Neurons were exposed to SP for 1 min, washed, and then exposed to Cy3-[Sar^9 $MetO_2^{11}$]-SP 5 min later. (A) Each trace shows the 340:380 nm fluorescence ratio, which is proportional to $[Ca^{2+}]_i$, for a single neuron. (B) Binding of Cy3-[Sar^9 $MetO_2^{11}$]-SP to the same neurons that were studied in A examined ~5 min after addition of Cy3-[Sar^9 $MetO_2^{11}$]-SP to the cultures. *, Neurons that responded to SP and Cy3-[Sar^9 $MetO_2^{11}$]-SP in A. Observations were repeated on at least three different coverslips. Bar, 10 μm.

nist [Sar^9 $MetO_2^{11}$]-SP, which indicates that they are receptor-specific and not due to activation of the NK2-R or NK3-R by SP.

In unstimulated neurons, immunoreactive NK1-R was mostly confined to the plasma membrane of the soma and neurites. At 4°C, fluorescent SP bound to the NK1-R at the cell surface of the soma and neurites. Warming to 37°C caused internalization of the ligand and its receptor into vesicles that we have previously shown contain the transferrin receptor and are thus early endosomes (Grady *et al.*, 1995, 1996b). In support of our results, SP and the NK1-R colocalize in endosomes of transfected cells until they are sorted in an acidified perinuclear compartment into degradative and recycling pathways, respectively (Grady *et al.*, 1995). In the soma and neurites of unstimulated neurons, immunoreactive GRK-2 and -3 and β-arrestin-1 and -2 were predominantly detected in the cytosol and in a punctate staining pattern that suggests they are localized in vesicles, and there was no detectable localization of these proteins at the plasma membrane. Thus, agonist stimulation must target these proteins to receptors at the cell surface if they are to mediate desensitization and endocytosis of surface receptors.

In unstimulated neurons, GRK-2 and -3 were prominently localized in the cytosol and in a punctate, possibly vesicular distribution throughout the cell, and this prominent distribution was unaffected by incubation with SP. However, GRK-2 and -3 were detected in vesicles located at or close to the plasma membranes where they may interact with the NK1-R. It is possible that SP induces translocation of GRK-2

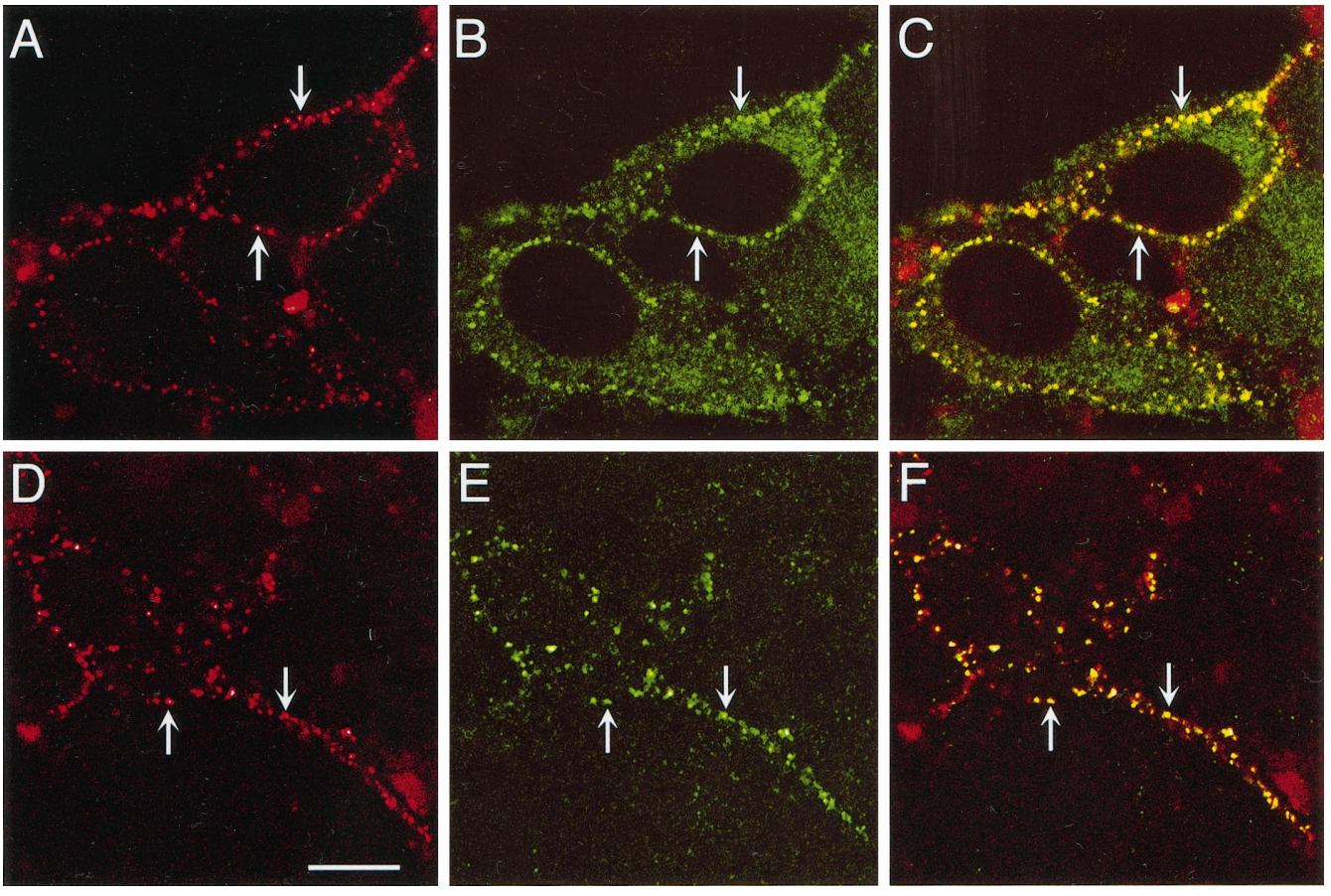

Figure 11. Confocal images of myenteric neurons showing SP-induced trafficking of the NK1-R and β-arrestin-1 and -2. Neurons were incubated with 190 nM Cy3-[Sar^9 MetO_2^{11}]-SP for 2 h at 4°C, washed, incubated for 5 min at 37°C, and then fixed. The NK1-R and β-arrestin-1 and -2 were localized by immunofluorescence using FITC-labeled secondary antibodies. The images at any one horizontal level are of the same neurons, and the images in the right panels are superimpositions of the images in the left and center panels. (A–C) Localization of Cy3-[Sar^9 MetO_2^{11}]-SP (A) and NK1-R (B) to the same early endosomes (arrows) of neurons (C). (D–F) Localization of Cy3-[Sar^9 MetO_2^{11}]-SP (D) and β-arrestin-1 and -2 (E) to the same early endosomes (arrows) of a neuron (F). Each image is a single optical section. Bar, 10 μm.

and -3 to the plasma membrane where they phosphorylate the NK1-R in the presence of $G_{\alpha q/11}$, which was also detected at the cell surface. However, our results do not provide unequivocal support for SP-mediated trafficking of GRK-2 and -3 to the plasma membrane of neurons. Membrane targeting of GRK-2 and -3 entails their interaction with βγ subunits of heterotrimeric G-proteins, a precise mechanism for targeting because free βγ subunits are only found in the plasma membrane at sites of receptor activation (Pitcher *et al.*, 1992). One reason that we did not observe prominent redistribution of GRK-2 and -3 to the plasma membrane may be that the process is very rapid and transient and was complete 30 s after stimulation, the earliest time that we were reliably able to study. Indeed, SP-induced Ca^{2+} mobilization was rapidly attenuated in neurons. Alternatively, immunofluorescence may not be sufficiently sensitive to detect translocation of only a small fraction of total cellular GRK-2 and -3. Support for the suggestion that GRK-2 and -3 phosphorylate the NK1-R in neurons derives from the observations that GRK-2 and -3 phosphorylate the NK1-R in a reconstituted system in the presence of SP and $G_{\alpha q/11}$ (Kwatra *et al.*, 1993), and that truncation of the C-tail of the NK1-R to remove potential phosphorylation sites diminishes homologous desensitization (Sasakawa *et al.*, 1994b). In contrast to the lack of colocalization of GRK-2 and -3 and the NK1-R in endosomes in neurons, GRK-2 colocalizes with the β_2-AR in endosomes after stimulation by agonists (Ruiz-Gomez and Mayor 1997). Thus, the duration of the interaction between receptors and kinases may depend on the receptor and the cell type. GRK-2 and -3 may also participate in receptor endocytosis, because phosphorylation of the β_2-AR and muscarinic m2 receptor by GRK-2 is important for agonist-induced endocytosis (Tsuga *et al.*, 1994; Ferguson *et al.*, 1995; Menard *et al.*, 1996; Ruiz-Gomez and

Mayor 1997). It is not known whether GRK-2 and -3 also contribute to SP-induced endocytosis of the NK1-R, although truncation of the NK1-R to remove potential phosphorylation sites attenuates SP-induced endocytosis (Böhm *et al.*, 1997b).

In unstimulated neurons, immunoreactive β-arrestin-1 and -2 were mainly confined to the cytosol. When neurons were incubated with SP at 4°C, β-arrestin-1 and -2 were detected in the cytosol and at the plasma membrane where they colocalized with the NK1-R. This finding suggests that activation of the NK1-R by SP binding causes translocation of β-arrestin-1 and -2 to the plasma membrane. Warming to 37°C resulted in a striking redistribution of β-arrestin-1 and -2 from the plasma membrane and cytosol to superficial vesicles and early endosomes containing the NK1-R, which suggests that β-arrestin-1 and -2 interact with the internalized NK1-R. In support of our results, many agonists of GPCRs induce rapid translocation of β-arrestin-2 coupled to green fluorescent protein to the cell surface (Barak *et al.*, 1997), and agonists of the β_2-AR also induce redistribution of β-arrestin and β_2-AR into endosomes (Goodman *et al.*, 1996). Colocalization of β-arrestin-1 and -2 with the NK1-R at the cell surface and in endosomes suggests that they interact with GRK-phosphorylated receptors to interdict interaction between the NK1-R and G-proteins and to mediate desensitization. This suggestion is supported by the finding that inositol pentakisphosphate, which disrupts the interactions of arrestins with receptors, attenuates desensitization of the NK1-R (Sasakawa *et al.*, 1994a). β-Arrestins are also important for endocytosis of the β_2-AR. β-Arrestin binds to clathrin with high affinity, and β_2-AR colocalizes with β-arrestin and clathrin in the first-formed endosomes (Ferguson *et al.*, 1996; Goodman *et al.*, 1996). Thus, β-arrestins may serve as adaptor molecules that recruit cellular proteins that facilitate endocytosis of several GPCRs or directly mediate endocytosis themselves. We do not know whether β-arrestin-1 and -2 similarly participate in agonist-induced endocytosis of the NK1-R in neurons. However, we have previously reported that SP-induced endocytosis of the NK1-R in myenteric neurons is clathrin-mediated and that the NK1-R also colocalizes with clathrin in the first-formed vesicles (Grady *et al.*, 1996b). This observation, together with the present finding that the NK1-R colocalizes with β-arrestin-1 and -2 in endosomes, suggest that β-arrestin-1 and -2 also colocalize with clathrin in neurons. We observed that β-arrestin-1 and -2 remained colocalized in the same vesicles as Cy3-SP and thus the NK1-R for up to 30 min after internalization. The significance of this prolonged colocalization remains to be determined. At later times the Cy3-SP signal in the soma became weak, probably because of degradation, so that we were unable to determine the full duration of the colocalization of β-arrestin-1 and -2 and the NK1-R. However, this is an important consideration, for it may determine the ability of recycled receptors to interact with heterotrimeric G-proteins and SP.

In unstimulated neurons, the NK1-R and $G_{\alpha q/11}$ were colocalized at the plasma membrane of the soma and neurites. SP caused rapid endocytosis of the NK1-R, whereas $G_{\alpha q/11}$ remained at the plasma membrane. These observations suggest that the NK1-R couples to $G_{\alpha q/11}$ at the plasma membrane and then rapidly dissociates. The lack of colocalization of the NK1-R and $G_{\alpha q/11}$ in early endosomes suggests that the endocytosed receptor is uncoupled from this signaling pathway. The β_2-AR and $G_{s\alpha}$ similarly colocalize at the plasma membrane of unstimulated cells (Wedegaertner *et al.*, 1996). In contrast to our observations, agonist stimulation causes redistribution of $G_{s\alpha}$ to the cytosol and endocytosis of the β_2-AR.

SP had similar effects on the subcellular distribution of the NK1-R, $G_{\alpha q/11}$, GRK-2 and -3, and β-arrestin-1 and -2 in the soma and neurites in myenteric neurons, which suggests that the NK1-R desensitizes and internalizes similarly in both locations. However, we have previously shown that whereas the NK1-R recycles in the soma, there is no detectable recycling in neurites in the same period (Grady *et al.*, 1996b). One possibility is that endosomes in the neurites are not sufficiently acidic to cause dissociation of the NK1-R and SP, which is required for receptor recycling and ligand degradation (Grady *et al.*, 1995). Endosomes with a pH of $<$6.0 are uncommon in neurites of cultured chick sympathetic neurons (Overly *et al.*, 1995). Another possibility is that retrograde transport to the soma is required for dissociation of the receptor–ligand complex. Indeed, in rat hippocampal neurons, multivesicular bodies mediate transport of endocytosed markers from axons and dendrites to the soma, which contains late endosomes and lysosomes (Parton *et al.*, 1992). Finally, it is possible that retrograde transport of the NK1-R and SP conveys a signal to the cell body, as appears to be the case with neurotensin in the CNS (Burgevin *et al.*, 1992). Together, these results raise the possibility that NK1-R is differentially regulated in the soma and in neurites.

Our protocol for examining agonist-induced trafficking of proteins in neurons involved incubation with fluorescent SP at 4°C followed by washing and warming to 37°C for defined periods. The advantage of this protocol is that incubation at 4°C permits equilibrium binding of SP to the NK1-R and thereby synchronizes neuronal trafficking. At 4°C we observed minimal endocytosis of the NK1-R, but we did detect redistribution of β-arrestin-1 and -2 to the plasma membrane, suggesting that there may be different temperature dependency or energy requirements of these processes. Washing and warming to 37°C resulted in rapid endocytosis of the NK1-R and removal

of β-arrestin-1 and -2 from the plasma membrane and into endosomes. A disadvantage of this protocol is that a temperature change alone may alter the subcellular distribution of proteins. However, the distribution of the NK1-R, $G_{\alpha q/11}$, GRK-2 and -3, and β-arrestin-1 and -2 were unaffected by incubation at 4°C without SP followed by warming to 37°C. This finding indicates that trafficking is due to receptor activation by SP. We also observed endocytosis of Cy3-[Sar^9 $MetO_2^{11}$]-SP at 37°C (Figure 10). In addition, we and others have shown that SP causes endocytosis of the NK1-R in the intact animal under physiological conditions (Bowden *et al.*, 1994; Mantyh *et al.*, 1995).

In summary, we have shown that SP induces rapid desensitization and endocytosis of the NK1-R in myenteric neurons. Neurons expressing the NK1-R also express $G_{\alpha q/11}$, GRK-2 and -3, and β-arrestin-1 and -2, which are therefore appropriately located to interact with the NK1-R. SP alters the subcellular distribution of β-arrestin-1 and -2 in a manner that is consistent with mediating desensitization and endocytosis of the neuronal NK1-R. Regulation of the NK1-R in neurons is of considerable importance, for it will determine the ability of the receptor to participate in functionally important reflexes such as peristalsis and transmission from nociceptors.

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