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Direct observation of substance P-induced internalization of neurokinin 1 (NK_1) receptors at sites of inflammation

(desensitization/tachyphylaxis/endosomes/receptor-mediated endocytosis/respiratory tract)

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ABSTRACT **Substance P (SP) can cause plasma leakage at sites of inflammation by binding to neurokinin type 1 (NK_1) receptors on the surface of endothelial cells. Internalization after ligand binding could reduce the number of NK_1 receptors on the cell surface and thus participate in the desensitization and resensitization of the inflammatory response to SP. By using an antibody to the receptor, we directly observed SP-induced internalization of NK_1 receptors into endosomes in endothelial cells of postcapillary venules in the rat tracheal mucosa. In the absence of SP, an average of 15 immunoreactive endosomes were present per endothelial cell. After an intravenous injection of SP, the number of immunoreactive endosomes peaked at 107 per cell at 3 min and gradually returned to the baseline by 120 min. In parallel experiments we observed that when cultured cells transfected with the NK_1 receptor were exposed to rhodamine-SP and an antibody to an extracellular Flag epitope of the NK_1 receptor, the SP was internalized with the receptor antibody. Both in the cultured cells and in the endothelial cells of intact animals, the prompt SP-induced internalization was accompanied by rapid, long-lasting desensitization to SP. These studies suggest that internalization of NK_1 receptors by endothelial cells may be one of the mechanisms that limit the amount of plasma leakage at sites of inflammation.**

Substance P (SP), a neuropeptide released from sensory nerves in the airway mucosa, skin, and other tissues, triggers neurogenic inflammation, in which plasma leakage is a prominent feature (1). This leakage normally is transient and undergoes rapid desensitization, but in some pathological conditions it can be greatly intensified (2, 3). Many of the inflammatory actions of SP, including plasma leakage, are mediated by neurokinin 1 (NK_1) receptors (4), which are seven-transmembrane-domain receptors structurally related to thrombin receptors and β_2-adrenergic receptors. These receptors are rapidly desensitized after exposure to agonists and then gradually become resensitized (5–7). The receptors are internalized after ligand binding, which may contribute to desensitization and resensitization of the cellular responses (5, 6, 8–10).

By depleting NK_1 receptors from the cell surface, internalization could diminish the responsiveness of vascular endothelial cells to SP and thereby limit the magnitude or duration of plasma leakage. However, it is not known whether SP induces the internalization of NK_1 receptors on endothelial cells. In the present study, we used an antibody to the NK_1 receptor to address this issue on endothelial cells of the rat tracheal mucosa. In parallel experiments on cells transfected with the rat NK_1 receptor, we determined whether SP was internalized together with its receptor, using fluorescently labeled SP and an antibody to an extracellular Flag epitope. We also investigated the time course of the disappearance and reappearance of radiolabeled SP binding sites on the surface of the transfected cells, as indices of the internalization and recycling of NK_1 receptors. Finally, we correlated the time course of NK_1 receptor internalization with the desensitization and resensitization of the response of endothelial cells and the transfected cells to SP.

MATERIALS AND METHODS

Protocols for Studies of Rats. For studies of NK_1 receptor internalization, pathogen-free male F344 rats, anesthetized with sodium pentobarbital (50 mg/kg, i.p.), were injected with SP (5 μg/kg, i.v.; Peninsula Laboratories) or vehicle (5 mM acetic acid in 0.9% NaCl) followed by the particulate tracer Monastral blue (30 mg/kg, i.v.; Sigma) (11). From 1 to 240 min after the injection of SP or vehicle, the tissues were fixed by vascular perfusion of 4% paraformaldehyde, and the tracheas were processed as whole mounts for NK_1 receptor immunohistochemistry (12, 13).

For the studies of plasma leakage, anesthetized rats were injected with SP (5 μg/kg, i.v.) and 1–10 min later with Evans blue (30 mg/kg, i.v.). One minute after the Evans blue, the rats were perfused with 1% paraformaldehyde (14). Evans blue extracted from the tracheas was measured by spectrophotometry (14). To determine whether this response to SP was diminished by repeated exposure, anesthetized rats were injected with SP (5 μg/kg, i.v.) followed 3 min to 8 hr later by SP (5 μg/kg, i.v.) and Evans blue. Five minutes later, the rats were perfused.

Protocols for Studies of Transfected KNRK Cells. Kirsten murine sarcoma virus-transformed rat kidney epithelial (KNRK) cells were transfected with the cDNA encoding the rat NK_1 receptor and an N-terminal, extracellular Flag epitope (12). To examine the internalization of the NK_1 receptor, cells were incubated with SP (10 nM) in Hanks' balanced salt solution containing 0.1% bovine serum albumin (HBSS/BSA) at 4°C for 60 min, washed, warmed to 37°C for 0–16 hr, fixed, and processed for immunohistochemistry (12). To determine whether SP was internalized with its receptor, cells were incubated with an antibody to the Flag epitope (10 μg/ml, Flag M2; International Biotechnologies) at 37°C for 60 min, washed, and incubated with rhodamine-SP (100 nM) and fluorescein-labeled goat anti-mouse IgG (1:200; Cappel) at 4°C for 60 min. The cells were washed, warmed at 37°C for 10 min, and then fixed.

SP (0.5 mg) was labeled with rhodamine by incubation with carboxytetramethylrhodamine succinimidyl ester (0.2 mg; Molecular Probes) in 200 μl of 0.2 M sodium bicarbonate (pH

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Abbreviations: NK_1, neurokinin 1; SP, substance P.

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8.0) for 2 hr at room temperature. Rhodamine-SP, separated from unlabeled SP by reverse-phase high-pressure liquid chromatography, was fully functional as assessed by Ca^{2+} mobilization in the transfected cells (EC_{50} of ≈0.3 nM). Binding of rhodamine-SP to the cells was considered specific because it was abolished by pretreatment with the NK_1 receptor antagonist CP-96,345 (1 μM).

The rate of internalization of SP was quantified by incubating cells with [^{125}I]Bolton–Hunter reagent-labeled SP (10 pM, 2000 Ci/mmol; Amersham; 1 Ci = 37 GBq) in HBSS/BSA at 4°C for 60 min, warming the cells to 37°C for 0–60 min, and then separating the internalized label from the cell surface label with an acid wash (12, 15). Nonspecific binding was measured in cells pretreated with 1 μM SP (15).

The time course of the reappearance of NK_1 receptors at the cell surface was determined in cells that were incubated with SP (10 nM) or vehicle (control) in HBSS/BSA at 4°C for 60 min, washed, warmed to 37°C for 10 min for receptor internalization, and then incubated at 37°C for an additional 0–240 min to permit possible recycling. The cells were then incubated with [^{125}I]Bolton–Hunter-SP (10 pM) at 4°C for 60 min to measure total equilibrium binding (12). Some cells were pretreated with cycloheximide (10 μM; Sigma) at 37°C for 60 min to determine whether the reappearance of cell surface binding required protein synthesis.

SP-induced Ca^{2+} mobilization was studied in cells with fura-2 acetoxymethyl ester (12). The desensitization and resensitization of this response were examined in cells that were exposed to SP (10 nM) or vehicle (control) at 37°C for 1 min, washed, and 0–240 min later exposed to SP again for 1 min. Some cells were treated with thapsigargin (4 μM; Sigma) to determine whether the SP-induced desensitization resulted from the depletion of Ca^{2+} stores (16).

Immunohistochemistry. NK_1 receptors were identified with a rabbit polyclonal antibody to the C-terminal 15 amino acids of the rat NK_1 receptor (12). After fixation, tracheas were permeabilized with 0.3% Triton X-100 in phosphate-buffered 0.9% NaCl (PBS/Triton) and pinned flat. The tracheas were incubated with 5% normal goat serum in PBS/Triton for 1 hr and then with the NK_1 receptor antibody (1:10,000) in PBS/Triton containing 1% normal goat serum and 0.01% thimerosol (Sigma) for 48 hr at 4°C (13). The tissues were incubated with biotinylated secondary antibody (1:200; Vector Laboratories) for 24 hr, incubated with peroxidase-avidin–biotin complex (1:100; Vector), and developed with hydrogen peroxide and diaminobenzidine (13). Some specimens were postfixed with osmium tetroxide and uranyl acetate and embedded for electron microscopy (11). After fixation, the transfected KNRK cells were incubated with the NK_1 receptor antibody (1:1000) or with the Flag antibody (10 μg/ml) for 4 hr at 37°C and processed for immunohistochemistry (12).

There was no detectable staining of vascular endothelial cells or transfected KNRK cells when the primary antibodies were preabsorbed with the NK_1 receptor-(393–407)-peptide or the Flag peptide (1 or 10 μM, 16 hr at 4°C) or when the primary antibodies were replaced by normal rabbit serum or PBS/Triton (12).

Tracheal whole mounts were examined by differential interference contrast microscopy to survey the vasculature (11) and resolve individual endosomes in endothelial cells. Intracellular sites of NK_1 receptor immunoreactivity in endothelial cells were identified by transmission electron microscopy. The colocalization of rhodamine-SP and NK_1 receptor immunoreactivity in transfected cells was examined by fluorescence microscopy, and the intracellular location of receptors was verified by confocal microscopy.

Quantification of Immunoreactive Endosomes in Endothelial Cells. Morphometric measurements were made on digital color video images of postcapillary venules in tracheal whole mounts (11). Immunoreactive endosomes, magnified ×3000, were counted in 15-μm circular regions of the endothelium of 20 postcapillary venules per trachea. Values are expressed as the number of endosomes per endothelial cell, based on a luminal surface area of 406 μm^2 per cell (11).

RESULTS

Internalization of NK_1 Receptors by Endothelial Cells. In the tracheas of rats that did not receive SP, postcapillary venules had no Monastral blue labeling and had faint NK_1 receptor immunoreactivity, which was on the surface of the endothelial cells and in occasional cytoplasmic granules, measuring 0.1–0.8 μm (Fig. 1 *A* and *C*). After SP, postcapillary venules were labeled with extravasated Monastral blue and had much more immunoreactivity due to an increased number of cytoplasmic granules in endothelial cells (Fig. 1 *B* and *D*). The immunoreactive granules were found by electron microscopy to be endosomes (Fig. 2*A*). Without SP, an average of 15 immunoreactive endosomes per endothelial cell were present in postcapillary venules (Fig. 3*A*). After SP, the number increased to 50 per cell at 1 min, peaked at 107 per cell at 3 min, and returned to the baseline by 120 min (Fig. 3*A*).

Internalization of NK_1 Receptors by KNRK Cells. In cells incubated with SP at 4°C, NK_1 receptor immunoreactivity (Fig. 1*E*) and Flag immunoreactivity were confined to the cell surface. After 3 min at 37°C, numerous immunoreactive granules were present in the cytoplasm, and less immunoreactivity was present on the cell surface (Fig. 1*F*). The immunoreactive granules, which were identified as endosomes by confocal microscopy (Fig. 2*B*), were still present after 1, 2, and 4 hr at 37°C but were replaced at 8 and 16 hr by cell surface immunoreactivity.

To determine whether SP was internalized with the NK_1 receptors, we incubated cells with the Flag antibody and then with rhodamine-SP and a fluorescein-labeled secondary antibody. When the cells were at 4°C, both rhodamine-SP and Flag immunoreactivity were confined to the cell surface. After 10 min at 37°C, rhodamine-SP and Flag immunoreactivity were colocalized in endosomes (Fig. 2 *C* and *D*). The Flag antibody alone did not induce receptor internalization.

In cells kept at 4°C for 60 min, most of the specifically bound [^{125}I]Bolton–Hunter-SP was on the cell surface (surface, 24% of total counts; internalized, 3%). When cells were warmed to 37°C, internalization occurred rapidly and was maximal at 10 min (surface, 3%; internalized, 17%; Fig. 3*B*). The internalization of [^{125}I]Bolton–Hunter-SP coincided with the appearance of immunoreactive endosomes.

Exposure to unlabeled SP caused a long-lasting loss of equilibrium binding of [^{125}I]Bolton–Hunter-SP on the cell surface (Fig. 3*C*). Binding immediately after the 10-min period for SP-induced receptor internalization was <10% of that found in the vehicle-treated cells. The equilibrium binding gradually recovered and after 120 min was 92% of that in vehicle-treated cells. This recovery was unaffected by the presence of cycloheximide and thus was not due to synthesis of new receptors.

NK_1 Receptor Signaling in KNRK Cells. SP caused a rapid rise in intracellular Ca^{2+}, which peaked at 9 sec and returned to baseline with a half-life of 51 sec (Fig. 3*B*). The peak occurred 10 min before [^{125}I]Bolton–Hunter-SP internalization was maximal (Fig. 3*B*) and before immunoreactive endosomes were abundant. When cells were exposed to two SP doses 3 min apart, the second exposure produced only 1% as much Ca^{2+} mobilization as in the controls (Fig. 4*A*). Resensitization of the Ca^{2+} response to SP paralleled the return of total binding and at 120 min was 98% of the control. Exposure to SP had little effect on thapsigargin-induced Ca^{2+} mobilization, indicating that desensitization did not result from depletion of intracellular Ca^{2+} stores.

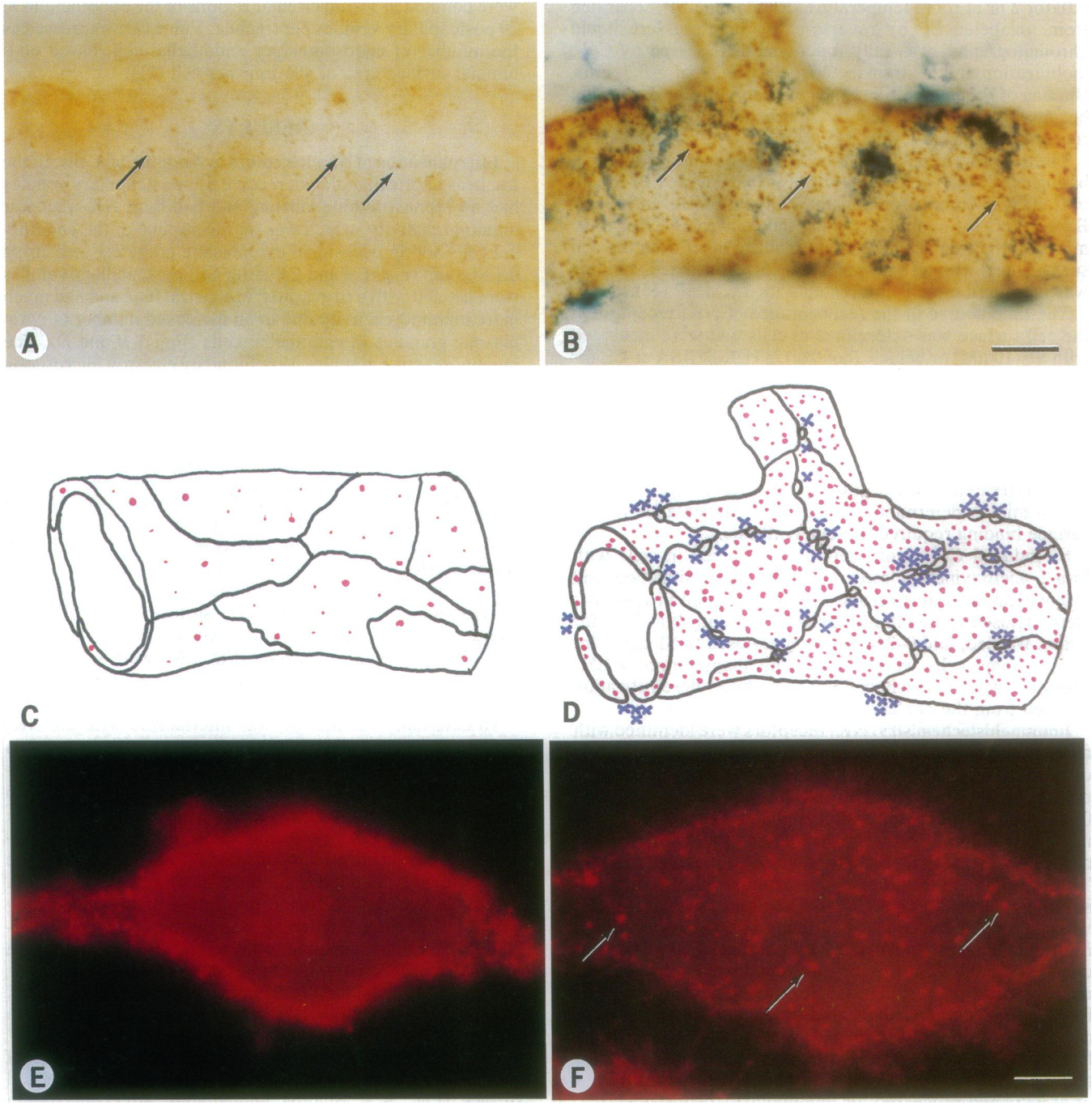

FIG. 1. (*A* and *B*) NK_1 receptor immunoreactivity in endothelial cells of postcapillary venules. Few immunoreactive endosomes (arrows) are observed and no Monastral blue leakage is present in the unstimulated venule (*A*), but 3 min after SP, immunoreactive endosomes (arrows) are abundant and extravasated Monastral blue is present (*B*). (*C* and *D*) Corresponding drawings of endosomes (red dots) and Monastral blue leakage (blue) superimposed on drawings of endothelial cell borders (black lines) and intercellular gaps (openings along cell borders) from silver nitrate-stained postcapillary venules (11). (*E* and *F*) NK_1 receptor immunoreactivity in transfected KNRK cells. After exposure to SP at 4°C for 60 min, immunoreactivity is confined to the cell surface (*E*), but after 3 min at 37°C, most of the immunoreactivity is in endosomes (*F*, arrows). [Bars = 10 μm (*A–D*) or 5 μm (*E* and *F*).]

Plasma Extravasation, Receptor Internalization, and Desensitization of Endothelial Cells. SP-induced leakage of Evans blue peaked within 2 min and then declined rapidly, with a half-life of about 1 min. This decline coincided with the appearance of NK_1 receptor-immunoreactive endosomes in endothelial cells (Fig. 3*A*). When two sequential injections of SP were given 3 min apart, the second dose produced 56% less Evans blue leakage than did a single dose (Fig. 4*B*). Complete resensitization did not occur for 4–8 hr, by which time the number of immunoreactive endosomes had diminished to the baseline.

DISCUSSION

SP triggered the internalization of NK_1 receptors in endothelial cells at sites of plasma leakage and in transfected KNRK cells. In the transfected cells, rhodamine-SP was internalized with the NK_1 receptors, and receptor internalization was accompanied by a rapid decrease in cell surface binding of [^{125}I]Bolton–Hunter-SP, which coincided with desensitization of the response to SP.

By comparing the internalization of NK_1 receptors in endothelial cells *in vivo* with that in cultured cells, we were

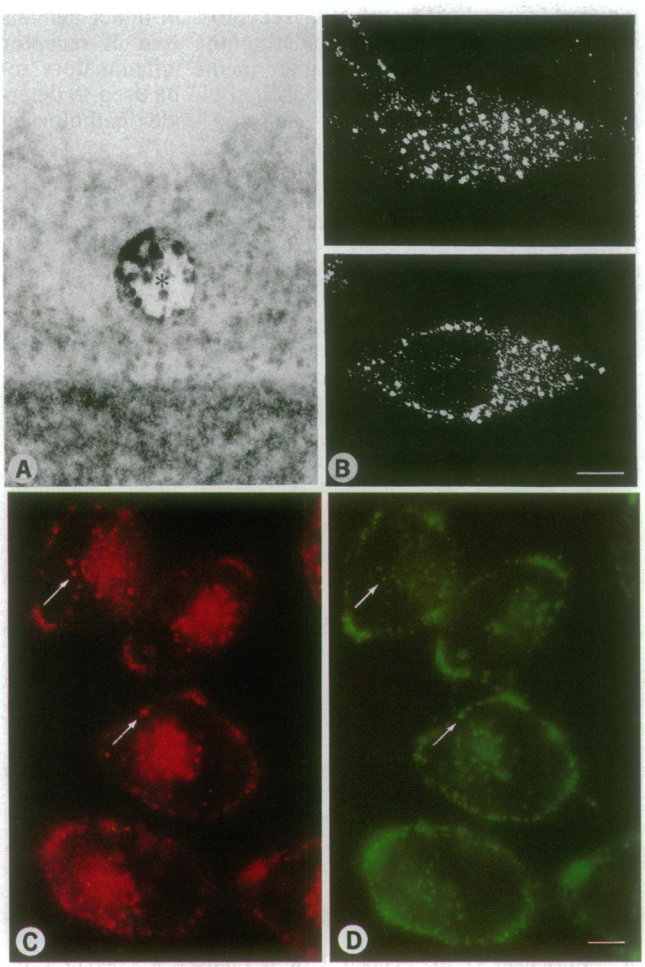

FIG. 2. (*A*) Electron micrograph showing endosome with NK_1 receptor immunoreactivity (asterisk) in tracheal endothelial cell 5 min after SP. (*B*) Confocal microscopic sections showing cytoplasmic localization of NK_1 receptor immunoreactivity at the periphery (*Upper*) and center (*Lower*) of a transfected KNRK cell exposed to SP and then warmed to 37°C for 10 min. (*C* and *D*) Colocalization of rhodamine-SP (*C*) and fluorescein-labeled IgG bound to the Flag antibody (*D*) in endosomes (arrows) in KNRK cells incubated for 10 min at 37°C. [Bars = 0.05 μm (*A*) or 5 μm (*B–D*).]

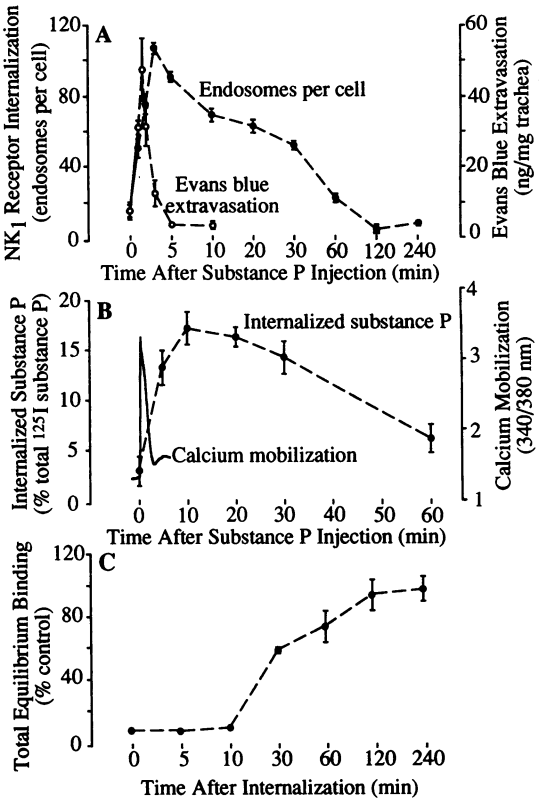

FIG. 3. (*A*) Number of NK_1 receptor immunoreactive endosomes in endothelial cells compared with Evans blue leakage at various times after SP injection (4 rats per group). (*B*) Amount of internalized [^{125}I]Bolton–Hunter-SP in transfected KNRK cells (triplicate observations in six experiments) compared with the transient Ca^{2+} response to SP measured in the same cells with fura-2. (*C*) Reappearance of specific cell surface binding of [^{125}I]Bolton–Hunter-SP on transfected cells, measured after exposure to unlabeled SP or vehicle (control) at 4°C followed by warming at 37°C for 10 min for receptor internalization and 0–240 min for possible receptor recycling (triplicate observations in three experiments). Data are shown as means ± SEM).

able to take advantage of the attributes of both systems. The approach also revealed some differences. The endothelial cells, but not the transfected cells, had conspicuously less immunoreactivity under baseline conditions than after exposure to SP. NK_1 receptors on the surface of the endothelial cells may have been at or below the threshold of detection, whereas the dense aggregates of receptors in endosomes were readily detected. Alternatively, the antibody may have preferentially recognized NK_1 receptors in endosomes of endothelial cells, which could differ in phosphorylation from those in the plasma membrane.

Rhodamine-SP and NK_1 receptors were internalized into the same endosomes of the transfected cells. However, the SP is degraded (15), whereas the NK_1 receptors may recycle to the cell surface. Recycling is suggested by the gradual increase in [^{125}I]Bolton–Hunter-SP binding after receptor internalization that occurred even when protein synthesis was blocked by cycloheximide. Alternatively, the increased binding may have resulted from the restoration of SP binding of previously synthesized receptors that were associated with arrestin-like molecules (17).

The results from cultured cells and endothelial cells suggest that the internalization of NK_1 receptors is sufficiently rapid to contribute to the desensitization of cells to SP. Although maximal internalization followed the Ca^{2+} response of KNRK cells and the increase in endothelial permeability, it coincided with the desensitization. The magnitude of the desensitization was less in endothelial cells than in KNRK cells, probably because the endothelial cells were exposed to a lower SP concentration due to rapid enzymatic breakdown of the peptide and the fall in blood pressure produced by SP (18, 19).

The Ca^{2+} response of the desensitized KNRK cells returned to normal within 120 min of the initial exposure to SP. This resensitization coincided with the increase in specific binding of [^{125}I]Bolton–Hunter-SP at the cell surface. Although some internalized NK_1 receptors were still detectable by immunohistochemistry at this time, the number of surface receptors was sufficient to restore ligand binding and Ca^{2+} mobilization to normal. By comparison, resensitization of SP-induced plasma leakage was not complete until 8 hr after the initial exposure, but the number of immunoreactive endosomes in endothelial cells returned to the baseline by 120 min. This discrepancy suggests that additional mechanisms contribute to the prolonged desensitization of endothelial cells to SP.

Other mechanisms, such as NK_1 receptor phosphorylation, may also be responsible for the desensitization of cells to SP. Agonist-induced desensitization of β_2-adrenergic receptors results from receptor phosphorylation by β-adrener-

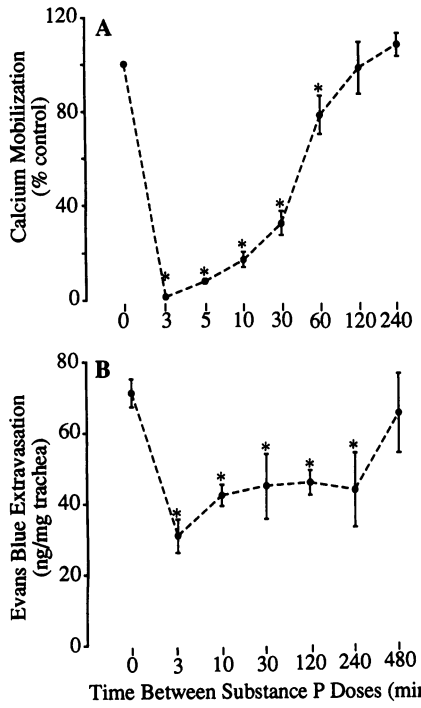

FIG. 4. (*A*) Ca^{2+} response measured with fura-2 in transfected KNRK cells pretreated with SP or vehicle (control) followed in 3–240 min by a second SP exposure. Responses are expressed as percentage of control response (time = 0) (three to six observations per group). (*B*) SP-induced Evans blue leakage in rat trachea. Leakage produced by one dose of SP is shown at time = 0 (control). Other values show leakage for rats pretreated with SP and at 1–480 min thereafter injected with Evans blue and second dose of SP (four to five rats per group). Data are shown as means ± SEM. *, Significantly different from control by analysis of variance and Dunnett's test ($P < 0.05$).

gic receptor kinase rather than from internalization (6). However, the internalization of β_2-adrenergic receptors may be necessary for resensitization (9). NK_1 receptors possess numerous potential phosphorylation sites and are phosphorylated by β-adrenergic receptor kinase (20).

Nonetheless, the rapid removal of NK_1 receptors from the cell surface by internalization would reduce the responsiveness of endothelial cells to SP and could limit the amount of SP-induced plasma leakage. This demonstration of SP-induced internalization of NK_1 receptors in intact animals shows the feasibility of elucidating the role of receptor internalization as a limiting factor in the inflammatory response. Furthermore, this approach could be used to determine whether internalization is altered under pathological conditions where inflammation is intensified.

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