Functional Neurokinin 1 Receptors for Substance P Are Expressed by Human Vascular Endothelium

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Summary

Substance P (SP), a neurotachykinin, is important in a number of inflammatory processes in which the endothelial cell also plays a critical role. SP receptors have previously been identified only on arterial endothelium, and the scant in vitro evidence for direct effects of SP on human endothelium is based on studies using nonarterial cells. To better understand SP's role in inflammation, we sought to identify functional SP receptors on human endothelium in situ and in culture. Autoradiographic ligand binding to human umbilical cord sections demonstrates the presence of SP binding sites with characteristics of the neurokinin 1 (NK-1) receptor (displacement by GTP analogues and the NK-1 specific antagonist CP-96,345) on human umbilical arterial, but not venous, endothelium. In culture, human umbilical venous endothelial cells (HUVECs) and human aortic endothelial cells express low levels of available SP binding sites. However, HUVECs, which are serum starved and refed, undergo a dramatic increase in SP binding. SP binding to starved/refed HUVECs induces a transient increase in intracellular calcium. This calcium flux is dose dependent over appropriate SP concentrations and can be blocked by NK-1 specific antagonists. The proinflammatory effects of SP may be mediated in part through the NK-1 receptor on endothelium.

Substance P (SP)[1], an 11 peptide neurotachykinin discovered in 1931 by von Euler and Gaddum (1), was characterized originally by its seemingly paradoxical ability to cause contraction of intestinal smooth muscle, but relaxation of vascular smooth muscle. It has since been shown in vascular ring studies that SP causes vascular relaxation through a mechanism consistent with the production of nitric oxide by the endothelium (2, 3). In addition, experimental data have implicated SP's participation in a number of inflammatory processes including vascular leak (4), migration and activation of white blood cells (5–10), and neovascularization (11), all processes in which endothelial interactions are critical. Despite the aforementioned evidence, it is not clear that SP acts directly on the vascular endothelium. For example, the wheal and flare induced by cutaneous injection of SP is mediated in part by release of histamine from mast cells (4). Published evidence for a direct effect in vitro of SP on isolated endothelium is modest. SP has been reported to act as a growth (11) and chemotactic (12) factor for cultured endothelium. Endothelial cultures may also contain monocytes, which have been shown to respond to SP by releasing a number of cytokines such as IL-1 (13) which may have paracrine effects on endothelial cell function. SP has also been reported to induce endothelial leukocyte adhesion molecule 1 (ELAM-1) in microvascular endothelial cells, and in this case, the induction of ELAM-1 was due to mast cell products contaminating the experimental culture system (14).

If SP acts directly on endothelium, it should be possible to identify specific, functional receptors on the endothelial cell. SP binding sites have been identified autoradiographically on endothelium in situ, generally only in the arterial system and with significant interspecies variability (15–17). The neurokonin 1 (NK-1) receptor has the highest affinity of these binding sites for SP, with an apparent K_d of 10^{-10} M (18). This SP receptor is a member of the G-protein–linked, seven transmembrane region family of receptors (19–21). SP also binds, with much lower affinity, to the NK-2 and NK-3 receptors (the receptors for neurokinins A and B) (22), to a member of the heat-shock protein 70 family (23), and possibly to other unidentified molecules (24). The data presented below will demonstrate that a functional NK-1-like receptor is present on human vascular endothelium, and that despite

[1] *Abbreviations used in this paper:* $[Ca^{2+}]_i$, intracellular calcium; HAEC, human aortic endothelial cell; HUVEC, human umbilical venous endothelial cell; NK-1, neurokinin 1; PPT-SP, (D-pro^2, D-phe^7, D-trp^9)-SP; SP, substance P.

the absence of SP binding to venous endothelium in situ, functionally active binding of SP to cultured human umbilical venous endothelial cells (HUVECs) can be induced by serum starvation.

Materials and Methods

Materials. Substance P, (D-pro^2, D-phe^7, D-trp^9)-SP (PPT-SP), leupeptin, chymostatin, bacitracin, and phosphoramidon were obtained from Sigma Immunochemicals (St. Louis, MO). ^{125}I-SP (labeled by the Bolton-Hunter method and purified by HPLC to a sp act of ~2,000 Ci/mmol; 22) was a gift of J. E. Magio (Harvard Medical School, Boston, MA); CP-96,345 [(2S,3S)-*cis*-2-(diphenylmethyl)-*N*-((2-methoxyphenyl)-methyl)-1-azabicyclo (2.2.2) octan-3-amine] was a gift of M. Snider (Pfizer, Groton, CT); Indo-1-AM was from Molecular Probes, Inc. (Eugene, OR); coverglass chamber slides were from Nunc (Naperville, IL); M199 with Earles salts were from Mediatech (Wash. DC); HBSS, calf and fetal bovine serum, RPMI, L-glutamine, and antibiotics were all obtained from Gibco (Grand Island, NY); IM-9 cells came from the American Type Culture Collection (Rockville, MD); and the Anchored Cell Analysis System (ACAS) 570 was from Meridian Instruments, Inc. (Okemos, MI).

Autoradiography. Umbilical cords were collected shortly after delivery in sterile containers and stored at 4°C until use. 1–2-cm segments were frozen, cut into 15-μm sections, and then thaw-mounted onto gelatin-coated slides. The slides were incubated for 10 min in a solution of 0.005% polyethylenimine, and then for 2 h at room temperature in a 50-mM Tris-buffered solution at pH 7.4 containing 100 pM ^{125}I-SP (with or without cold SP or other competitive agents as indicated), 2 mg/ml chymostatin, 4 mg/ml leupeptin, 1 mg/ml bacitracin, 0.05% BSA, and 10 μM MnCl (22). The slides were then washed in Tris buffer and placed in apposition to β-max hyperfilm for 5–10 d to produce images for analysis.

Quantitative Binding Assays. Umbilical venous cells were harvested with collagenase and cultured in M199 with 20% bovine serum as previously described (25). Primary cultures were grown on 100-mm culture plates and passaged onto 24-well 2 cm^2 plates and grown to confluence. Cells used between passages 1–3 were identical in SP binding and contained <1% monocytes. For cell starvation experiments, the culture media was changed to 2% bovine serum while the cells were subconfluent. These cells were maintained for at least 48 h in low-serum media, and then refed with media containing 20% bovine serum 4 h before experimental manipulation. Human aortic endothelial cells (HAECs) were cultured as previously described (26). IM-9 cells (a lymphoblastoid cell line known to express high levels of NK-1 receptor; 27) were grown to a concentration of 10^{-5}–10^{-6} cells/ml in RPMI with 15% fetal bovine serum. Binding assays were carried out at 4°C for 1 h in HBSS with 10 mM Hepes at pH 7.4, 2 mg/ml chymostatin, 4 mg/ml leupeptin, 1 mg/ml bacitracin, 10^{-5} M phosphoramidon, 0.5% human serum albumin, and varying concentrations of ^{125}I-SP. Nonspecific binding was determined in the presence of 10^{-6} M CP-96,345, an NK-1 specific antagonist (28). Free ^{125}I-SP concentrations were determined at the end of the experiment by counting samples of the cell-free supernatant. For bound SP, the cell layer was washed twice with HBSS, solubilized with 1 N NaOH, and counted in the same manner. Binding to IM-9 cells was assayed with cells in suspension (10^6/ml). Free and bound counts were determined by pelleting the IM-9 cells in a microcentrifuge at 10,000 *g* for 5 min and counting an aliquot of the supernatant and washed cell pellet. For some experiments, to assess ligand degradation, the reaction mixture was aspirated at the end of the endothelial binding assay, centrifuged, and the cell-free supernatant reused to assay binding to IM-9 cells. NK-1 specific binding was defined as the difference in total and nonspecific (CP-96,345 nondisplaceable) binding. At the concentrations of ^{125}I-SP used, cold SP was only slightly more effective than CP-96,345 at displacing the radioligand from endothelial and IM-9 cells.

Intracellular Calcium (Ca^{2+})$_i$ Measurements. Passaged endothelial cells were grown to confluence on glass coverslip chamber slides, and in some experiments, starved/refed as described above. The cells were incubated with 4 mM Indo-1 acetoxymethyl ester (dissolved in culture media with 20% serum) for 1 h and then washed and covered with HBSS with 0.5% human albumin and 10 mM Hepes at pH 7.4. After 1 h (to allow metabolism of Indo-1 to its cell-impermeant free acid), the slide was placed on the incubated stage of the laser cytometer (model ACAS 570; Meridian Instruments, Inc.) (29) and maintained at 37°C throughout the experiment. The cell layer was brought into sharp focus under phase-contrast with a 100× inverted oil-immersion lens. The cells were excited by a laser at 360 nm, and fluorescence measured at 480 (free Indo-1) and 405 nm (Indo-1 bound to calcium), while aliquots of various agonists (dissolved in buffer identical to that which covered the cells) were added to the chambers. Cells were scanned by automated movement of the stage to produce a two-dimensional image of a field of cells every 30 s, or a single line through several cells every 0.5 s. Relative calcium values were determined from the ratio of fluorescence at the two wavelengths, 405/480. To determine absolute values for intracellular calcium concentration, a standard curve was constructed using 4 mM Indo-1 free acid in a high potassium buffer [10 mM 3-(*N*-morpholino)propanesulfonic acid, 115 mM KCl, 20 mM NaCl, 1 mM EGTA] containing 40% ethanol (30).

Results

Autoradiography. ^{125}I-SP bound to frozen sections of human umbilical cords (Fig. 1). Specific binding, displaceable by excess unlabeled SP, was confined to the luminal surface of the arteries, and was not seen in the umbilical veins. Occasional arteries (arrow in Fig. 1) that showed no specific binding were found on histologic examination to have been denuded of endothelium. This binding of SP to the arterial endothelium was dependent on the concentration of ^{125}I-SP over the range of 10–1,500 pM (data not shown). At 100 pM, ^{125}I-SP binding was inhibited by coincubation with the NK-1 specific antagonist CP-96,345 at a concentration of 10^{-6} M, as well as with the nonhydrolyzable GTP analogues guanosine 5′-*O*-(2-thiodiphosphate) (GPS) and 5′-guanylylimidodiphosphate (GPP) (Fig. 2).

SP Binding to Cultured Endothelium. HUVECs, as well as HAECs, demonstrated very low levels of specific binding of ^{125}I-SP at 150 pM that was displaceable by CP-96,345 (Fig. 3). Since the level of specific binding was so low, other culture conditions were evaluated in an attempt to increase receptor expression. When the HUVECs were serum starved for 48 h, there was a marked increase in NK-1 specific binding (Fig. 3). This binding reached equilibrium by 60 min (Fig. 4 *a*) and was saturable at concentrations (K_d~0.6 nM) (Fig. 4 *b*) compatible with the reported K_d of 0.1 nM in porcine aortic endothelial membranes (18). To assure that the low level of binding to nonstarved cells was not due to degrada-

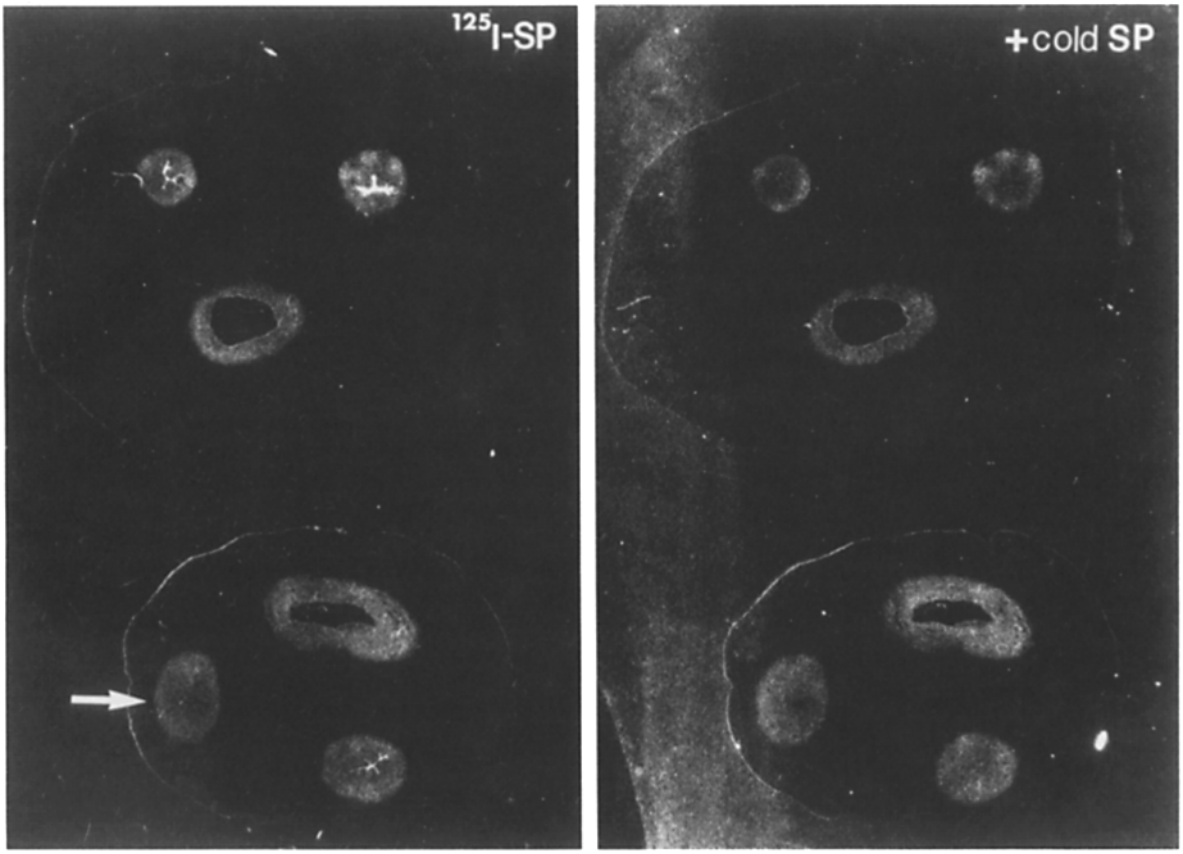

Figure 1. Representative autoradiograms of human umbilical cords (total n = 10). (*Left*) Frozen sections of two cords incubated with 100 pM ^{125}I-SP. (*Right*) Serial sections of the same cords incubated under the same conditions except with the addition of an excess (10^{-6} M) of unlabeled SP. The white silver grains represent areas of ^{125}I-SP binding. Note the intense signal at the luminal surface of the two arteries in each cord which is displaced by cold SP, and the absence of such signal in the single, large veins. An occasional artery (*arrow*) lacked specific binding, and such arteries were found on histologic staining to have been denuded of endothelium.

tion of the ligand during incubation with HUVECs, the following control experiments were carried out with IM-9 cells (which are known to express high levels of NK-1 receptor; 27). Parallel binding experiments on IM-9 cells were performed by either reusing the supernatant at the end of HUVEC binding experiments or using fresh ^{125}I-SP dilutions under the same conditions. Both gave similar binding curves to IM-9 cells showing a K_d between 0.2 and 1 nM, with 30,000–90,000 sites per cell (n = 3), consistent with previous reports (27).

$[Ca^{2+}]_i$ Measurements. To demonstrate that this receptor is functionally active, changes in $[Ca^{2+}]_i$ in response to SP were examined. HUVECs exposed to SP demonstrated an immediate, transient increase in $[Ca^{2+}]_i$, and once a cell had responded it would not respond to a higher concentration of SP (Fig. 5 *a*). This response was blocked by CP-96,345 (vide infra) or the partial agonist PPT-SP, neither of which affected the response to unrelated agonists such as thrombin (Fig. 5 *b*) or histamine (data not shown). The number of endothelial cells manifesting a rise in $[Ca^{2+}]_i$ was dose dependent over the range of 10^{-11}–10^{-8} M SP (Fig. 6). The top right panel of Fig. 6 shows a summary of the calcium response of 23 cells in a single representative experiment with each vertical line indicating the addition of 1 log higher concentration of SP, from 10^{-10} to 10^{-8} M. No additional cells could be recruited by concentrations $>10^{-8}$ M, and no cells responded in the presence of 10^{-6} M CP-96,345 (Fig. 7). Whereas only 10% of unstarved cells in an experimental field responded to maximal concentrations of SP, about 50% of starved/refed cells did respond (Fig. 7). Refeeding alone is not effective in increasing responses, as the protocol for our calcium determinations involves exposure of the cells to fresh media 2–3 h before the experiment. Conditions of growth such as endothelial growth factors, variation in passage number, time in culture, or degree of confluence did not influence calcium responses (data not shown). Unstarved HUVECs cultured in the presence of IL-1β or IFN-γ, as well as unstarved HAECs (n = 4 for each group) all demonstrated <10% of cells responding to SP.

Discussion

The autoradiograms of human umbilical cords demonstrate specific SP binding to the arterial endothelium, but not to

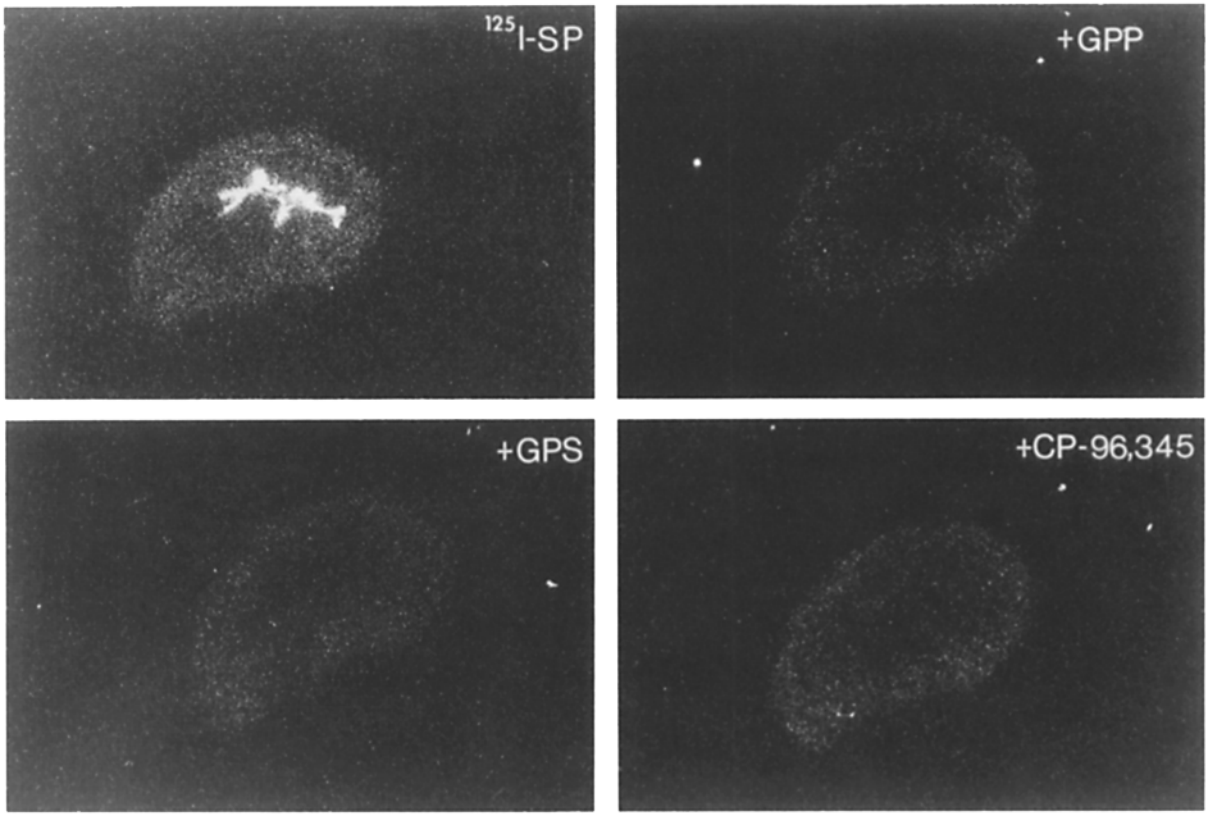

Figure 2. Representative autoradiogram of one umbilical artery incubated with: 100 pM ^{125}I-SP again showing binding to the luminal surface; ^{125}I-SP plus 5′-guanylylimidodiphosphate (GPP 1 mM); ^{125}I-SP plus guanosine 5′-*O*-(2-thiodiphosphate) (GPS 1 mM); ^{125}I-SP plus CP-96,345 (1 mM); each showing displacement of labeled SP.

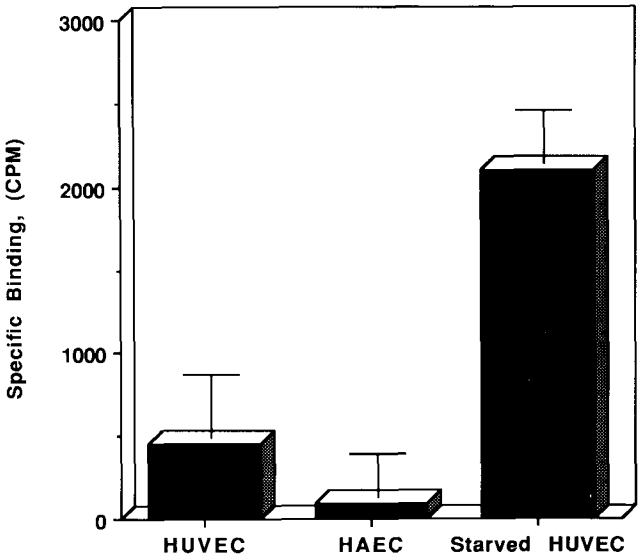

Figure 3. Results of NK-1 specific ^{125}I-SP binding (150 pM for 1 h) to HUVECs (n = 12), HAECs (n = 11), and HUVECs after starving/refeeding as described in Materials and Methods (n = 5). NK-1 specific binding is determined from the difference in binding in the presence or absence of the NK-1 specific inhibitor CP-96,345. Nonspecific binding (in cpm) for each group, respectively, was 1,916 ± 172, 1,781 ± 90, and 2,433 ± 132. All experiments were conducted at 4°C in the presence of protease inhibitors as described in Materials and Methods. Error bars represent the SE of the mean.

the umbilical vein from which cultured cells are most commonly obtained. These binding sites are G-protein–linked as indicated by the displacement by GPS and GPP. These nonhydrolyzable GTP analogues presumably block binding of SP by disrupting the normal association of the G-protein complex with the cytoplasmic tail of the receptor, resulting in a conformational change in the receptor's extracellular domain (18). The NK-1, NK-2, and NK-3 receptors are all G-protein–linked, and all bind SP, but the binding in this case was completely blocked by CP-96,345 at 10^{-6} M. This compound, when tested against SP, has a pA_2 (the negative log of the half-maximal inhibitory concentration) for the NK-1 receptor of 9.5, but only 4.3 and 5.6 for the NK-2 and NK-3 receptors, respectively, so it would not be expected to cause complete displacement of SP from NK-2 or NK-3 receptors at the concentration used (31). These data argue that the binding sites are the NK-1 receptor.

Despite the apparent lack of autoradiographically demonstrable venous binding sites in situ, cultured venous endothelial cells did show specific binding in a pattern consistent with the presence of NK-1 receptors. The level of binding to HUVECs was very low under standard culture conditions. However, binding was much higher in cells that had been starved and refed. In agreement with this observation, the number of HUVECs responding to SP with an increase in $[Ca^{2+}]_i$ also increased dramatically after starving/refeeding.

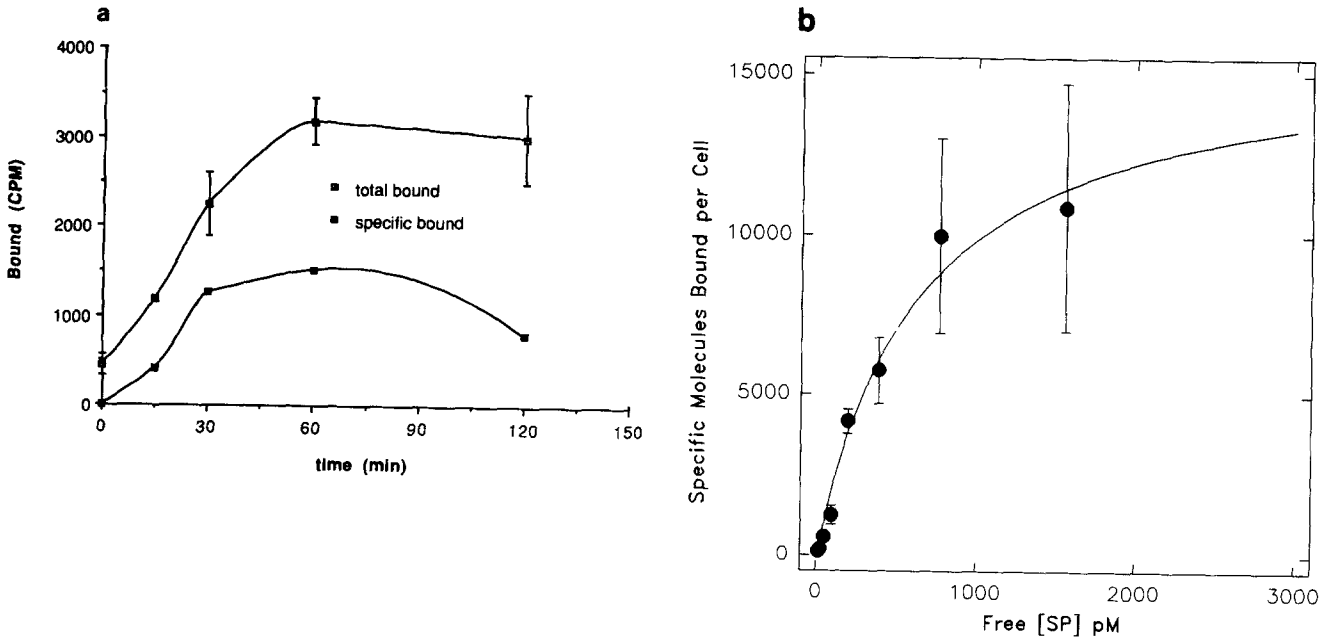

Figure 4. Representative binding curves of ^{125}I-SP incubated with starved/refed HUVECs. (*a*) Time course of both total and specific binding at 100 pM ^{125}I-SP. (*b*) Best fit curve of specific binding (determined by subtracting nonspecific binding in the presence of 10^{-6} M CP-96,345) was generated with Sigmaplot and gave a K_d ~630 pM with B_{max} ~16,000 molecules per cell. Nonspecific binding represented ~50% of total binding. All experiments were conducted at 4°C in the presence of protease inhibitors. Error bars represent the SE of the mean of triplicate determinations.

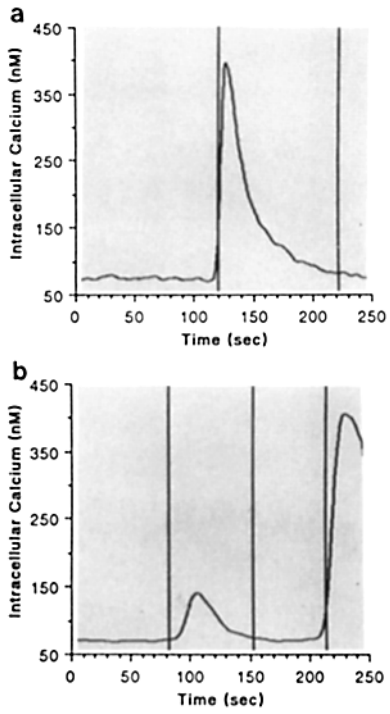

Figure 5. Changes in intracellular calcium in single HUVECs scanned at 0.5-s intervals. (*a*) The immediate response of a single cell exposed to 10^{-7} M SP at the first vertical line, with a failure to respond to the subsequent addition of 10^{-6} M SP at the second line. No response to a second addition was seen even after several minutes, and similar results were obtained starting with submaximal concentrations of SP. (*b*) Another single cell which underwent a transient increase in calcium upon exposure to 10^{-4} M PPT-SP (a partial SP antagonist) at the first line, and then failed to respond to 10^{-6} M SP added at the second line. The cell was still able to respond to the addition of 0.5 U/ml thrombin at the third line.

Autoradiographic data imply differential regulation of NK-1 receptor expression on arterial versus venous endothelium in vivo (15–17), but this difference was not apparent either in SP binding or cell activation once these cells were in culture. Cocks et al. (3) also demonstrated a discordant response between in situ and cultured endothelium. These authors showed vascular relaxation induced by SP stimulation of arterial endothelium in situ. However, when the endothelial cells were cultured onto beads and stimulated with SP, the effluent did not induce relaxation of denuded vascular rings, even though cultured cells did respond to other activators of nitric oxide release (3). In addition, studies that demonstrated that SP stimulated endothelial growth of cultured HUVECs (probably via the NK-1 receptor) (11), were carried out in serum-free media, thus subjecting the cells to conditions similar to our starvation experiments. Whether the results we obtained are due to an actual change in receptor number with starving/refeeding or to some change in the availability or affinity of the receptor, cannot be determined from our data at present.

The data presented indicate that the binding of SP to the cultured endothelium results in a cellular response as manifested by the increase in $[Ca^{2+}]_i$. The rapid response suggests that this is a direct effect rather than a secondary process due to activation of other cells such as monocytes. This response is mediated by the NK-1 receptor as evidenced by a dose dependence over the appropriate concentration range and blocking of the response by CP-96,345 (which did not affect calcium changes induced by other agonists). The technique of individual cell fluorescence measurements allowed us to see a wide cell-to-cell heterogeneity in responsiveness to SP.

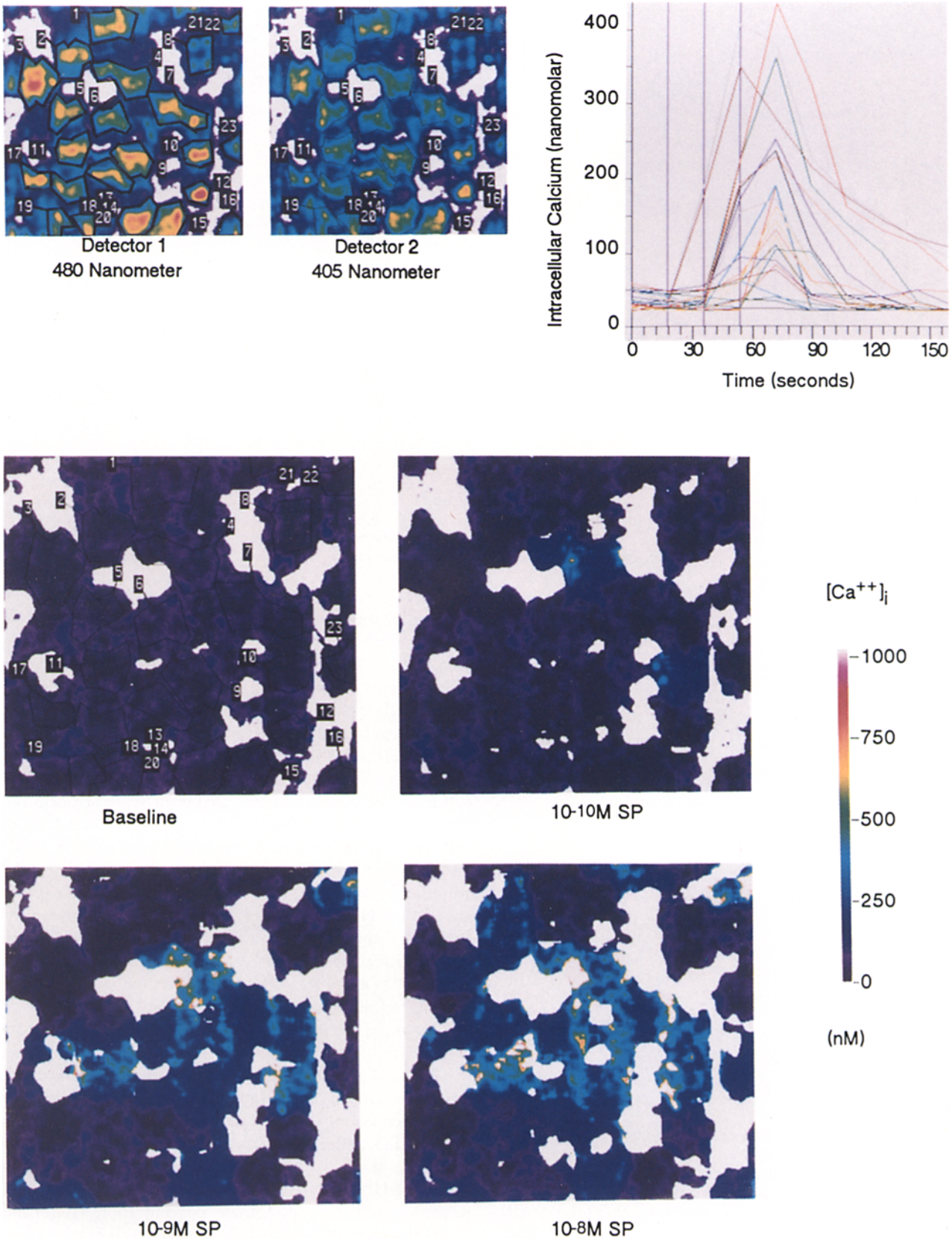

Figure 6. Changes in $[Ca^{2+}]_i$ in multiple individual HUVECs scanned at 30-s intervals. The two small panels (*top left*) show the false-color fluorescent images of the same field of cells in their resting state scanned simultaneously at 480 (free Indo-1) and 405 nm (Indo-1 bound to calcium). The central portion of each cell has been outlined in black and numbered. (*Baseline*) $[Ca^{2+}]_i$ of the field of cells. The image was generated by determining

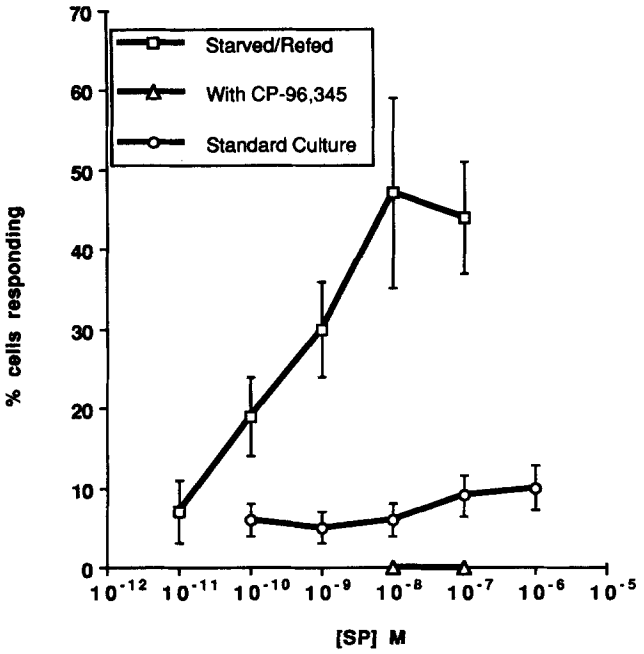

Figure 7. Summary of the calcium response of HUVECs. Data points represent the mean percentage of visualized cells which demonstrated a detectable increase in $[Ca^{2+}]_i$ (always >50 nM) at each concentration of added SP, and error bars are the SE of the mean. For starved/refed cells, n = 15 experiments with an average of 21 cells per experiment; with CP-96,345 (using starved/refed cells) n = 6, average 18 cells per experiment; with standard culture conditions n = 38, average of 11 cells per experiment.

This heterogeneity is seen with other agonists such as thrombin or histamine (Greeno, E., unpublished observation). One can speculate that this heterogeneity results from a wide distribution of NK-1 receptor numbers amongst individual cells that require activation of a fixed number, as opposed to a fixed percentage, of receptors on each cell.

Our data also indicate that the NK-1 receptor undergoes homologous desensitization, since any cell that responds to SP is unable to respond again to SP, but still responds to other agonists such as thrombin or histamine with an increase in $[Ca^{2+}]_i$. Such specific desensitization to SP has been observed previously in vascular relaxation studies (32). This process may occur in a manner analogous to that in other seven-transmembrane domain receptors that undergo homologous desensitization due to phosphorylation of the cytoplasmic tail, since similar phosphorylation sites are present on the NK-1 receptor (20).

In summary, we have demonstrated NK-1 receptors on human vascular endothelium in situ and in culture. These receptors are functionally active in cultured endothelium, and appear to be increased in number and activity in cells that have been starved and refed. This culture system should allow further elucidation of SP's role in endothelial function.

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the ratio of the detector 1 and detector 2 images above it. Changes in color from purple through yellow to red represent increasing $[Ca^{2+}]_i$ as indicated by the scale (*right*), and white represents areas where fluorescence was below the limits of detection of one or both detectors. Each cell is again outlined in black. The three subsequent panels show $[Ca^{2+}]_i$ of the same field of cells (omitting outlining for clarity) as progressively greater concentrations of SP are added at 30-s intervals. The images are 144 × 144 μm. (*Top right*) Summary of the responses of these 23 cells, with each vertical line indicating the addition of a 1 log higher concentration of SP, from 10^{-10} to 10^{-8} M.

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