Substance P (SP), 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 neurokinin 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 neurokinsins 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
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-pro2, D-phe7, D-trp9)-SP (PFTSP), leupeptin, chymostatin, bacitracin, and phosphoramidon were obtained from Sigma Immunochemicals (St. Louis, MO). 125I-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)-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); coverslip 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% polyethyleneimine, and then for 2 h at room temperature in a 50-mM Tris-buffered solution at pH 7.4 containing 100 pM 125I-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 MnCl2 (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 passed onto 24-well 2-cm2 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 confluence on glass coverslip chamber slides, and grown to confluence. Cells used between passages 1–3 were main-

Results

Autoradiography. 125I-SP bound to frozen sections of human umbilical cords (Fig. 1). Specific binding, displacable 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 125I-SP over the range of 10–1,500 pM (data not shown). At 100 pM, 125I-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) (GSP) and 5′-guanylylimidodiphosphate (GPP) (Fig. 2).

SP Binding to Cultured Endothelium. HUVECs, as well as HAECs, demonstrated very low levels of specific binding of 125I-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 (Kd = 0.6 nM) (Fig. 4 b) compatible with the reported Kd 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 degra-
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\(^{-4}\) 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.

Discussion

The autoradiograms of human umbilical cords demonstrate specific SP binding to the arterial endothelium, but not to
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 non-hydrolyzable 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 $K_d \approx 630$ pM with $B_{max} \approx 16,000$ molecules per cell. Nonspecific binding represented $\approx 50\%$ of total binding. All experiments were conducted at $4^\circ$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 $[Ca^{2+}]$. 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 $[\text{Ca}^{2+}]_i$ 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) $[\text{Ca}^{2+}]_i$ of the field of cells. The image was generated by determining...
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 \([\text{Ca}^{2+}]\). 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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