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ORIGINAL ARTICLES

Neurokinin-1 Receptor (NK-1R) Expression is Induced in Human Colonic Epithelial Cells by Proinflammatory Cytokines and Mediates Proliferation in Response to Substance P

TRIONA GOODE,[1] TERRY O'CONNOR,[1] ANN HOPKINS,[2] DEREK MORIARTY,[2] GEARLD C. O'SULLIVAN,[3] J. KEVIN COLLINS,[1] DIARMUID O'DONOGHUE,[2] ALAN W. BAIRD,[2] JOE O'CONNELL,[1]* AND FERGUS SHANAHAN[1]

[1]*Department of Medicine, National University of Ireland, Cork, Ireland*
[2]*Department of Pharmacology, National University of Ireland, Dublin, Ireland*
[3]*Department of Surgery, National University of Ireland, Cork, Ireland*

We have previously shown that the receptor for substance P (SP), neurokinin-1 receptor (NK-1R), is a marker of human mucosal but not peripheral mononuclear cells. In the present study, we investigate NK-1R expression in the human colonic mucosa in vivo, particularly in the epithelial cells. We investigate the influence of proinflammatory Th1 cytokines and SP on expression and function of NK-1R in colonic epithelial cells in vitro. Using in situ hybridization to detect NK-1R mRNA, and immunohistochemistry to detect NK-1R protein, colonic epithelial cells were found to express NK-1R in vivo. In contrast, colon epithelial cell lines (Caco-2, HT29, SW620, T84) were negative for NK-1R mRNA and protein. However, stimulation with a proinflammatory cytokine cocktail containing IFN-γ, TNF-α, and IL-1β, caused induction of NK-1R expression. Expression of NK-1R in human colonic epithelial cells in vivo may therefore reflect cytokine conditioning by the mucosal microenvironment. SP did not alter ion transport in monolayers of cytokine-treated T84 cells. While SP stimulated epithelial ion transport in colonic mucosae ex vivo, this was not a direct effect of SP on the epithelial cells, and appeared to be neurally mediated. However, SP (10^{-10}–10^{-8} M) elicited a dose-dependent proliferative effect on cytokine-stimulated, but not unstimulated, SW620 cells. Proliferation of the epithelial cells in response to SP was mediated specifically via cytokine-induced NK-1R, since an NK-1R-specific antagonist (Spantide 1) completely blocked SP-mediated proliferation in the cytokine-treated cells. Our results therefore demonstrate that proinflammatory cytokines induce expression of NK-1R in human colonic epithelial cell lines, and that SP induces proliferation of the epithelial cells via cytokine-induced NK-1R. J. Cell. Physiol. 197: 30–41, 2003.

The neuropeptide substance P (SP) is known to have multiple effects on gastrointestinal function: these include regulation of motility, blood flow, epithelial ion transport, and immune function (Pernow, 1983). The major source of SP in the intestine is neuronal. SP-immunoreactivity has been identified in extrinsic sensory neurons (Maggi, 1990), and in intrinsic enteric neurons of the myenteric and submucosal plexuses (Sundler et al., 1977). In the human colon, SP nerve fibres ramify throughout the lamina propria and form complex networks beneath the epithelium (Keast et al., 1985b). Many individual SP fibres terminate in close apposition to the epithelial basement membrane (Brodin et al., 1983). Neurons are not the exclusive source of mucosal SP. Enteroendocrine cells immunoreactive for SP have been described in the epithelium of the small and large intestine of several species, including man (Sokolski and Lechago, 1984; Keast et al., 1985b). Human colonic eosinophils (Metwali et al., 1994), rat ileal macro-

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TG and T. O'C contributed equally and share first authorship.

*Correspondence to: Dr. Joe O'Connell, Department of Medicine, NUIC, Clinical Sciences Building, University Hospital, Cork, Ireland. E-mail: j.oconnell@ucc.ie.

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phages (Castagliuolo et al., 1997), and mouse colonic glia (Bernstein and Vidrich, 1994) have also been shown to produce SP.

The anatomical juxtapositioning of SP nerve fibres with epithelial cells suggests neuropeptide–epithelial intercommunication. Many studies implicate SP as a mediator of ion secretion in the small intestine (Kachur et al., 1982; Keast et al., 1985a; Mathison and Davison, 1989; Parsons et al., 1992). Kachur et al. (1982) reported that SP evoked anion secretion by acting directly on epithelial cells. Other findings conclude that SP also modulates ion secretion indirectly, by acting on enteric neurons (Keast et al., 1985a; Mathison and Davison, 1989). Similar observations were noted in the guinea pig (Kuwahara and Cooke, 1990), and canine colon (Rangachari et al., 1990). We have found that antagonists of the receptor for SP, the neurokinin-1 receptor (NK-1R), inhibit neurally and immunologically triggered secretory responses in human and rat colonic tissue ex vivo (Moriarty et al., 2001a,b). Additional modulatory influences of SP on epithelial function include stimulation of migration and proliferation of airway epithelial cells (Kim et al., 1995), and of corneal epithelium (Reid et al., 1993; Nakamura et al., 1997). Such findings provide evidence for a role for SP in epithelial wound healing in these tissues.

SP mediates it's biologic effects by preferentially binding to NK-1R (Regoli et al., 1987). We have previously documented the cellular sites of expression of NK-1R in human colonic mucosa (Goode et al., 1998). We found that in contrast to peripheral blood mononuclear cells (PBMC), lamina propria mononuclear cells (LPMC), lymphoid follicles, and epithelial cells express NK-1R mRNA and protein. While isolated LPMC expressed NK-1R, PBMC did not express NK-1R, even after in vitro activation; hence NK-1R is a marker of human mucosal but not peripheral mononuclear cells (Goode et al., 1998). While NK-1R mRNA could be detected in isolated human monocytes and macrophages using nested RT-PCR (Ho et al., 1997), subsequent analysis using quantitative-competitive (qc) RT-PCR revealed that the expression level was negligible (Goode et al., 2000a). Others have described SP binding sites in germinal centers of lymph nodules and in epithelium of human (Gates et al., 1988) and canine (Mantyh et al., 1988b) gastrointestinal tract. NK-1R mRNA has also been detected in isolated colonocytes from the guinea pig (Cooke et al., 1997), and in isolated canine colonic crypts (Khan et al., 1995). Mantyh et al. reported elevation of SP binding sites in colonic lymphoid follicles and small blood vessels of patients with ulcerative colitis, Crohn's disease (Mantyh et al., 1988a), and *Clostridium difficile*-induced pseudomembranous colitis (Mantyh et al., 1996). We have found increased NK-1R mRNA levels in colonic biopsies from human inflammatory bowel disease patients (Goode et al., 2000b). Increased levels of NK-1R have also been associated with other inflammatory diseases, such as rheumatoid arthritis (Krause et al., 1995). Formalin-induced hind paw inflammation has been shown to upregulate NK-1R mRNA in dorsal horn neurons (Schafer et al., 1993; Krause et al., 1995). Recently, Pothoulakis et al. (1998) demonstrated upregulation of NK-1R expression in rat intestinal epithelium following intraluminal administration of *Clostridium difficile* toxin A, the causative agent of antibiotic-associated enteritis.

In this study, we investigated whether human colonic epithelial cells express NK-1R in vivo and in vitro. We examined whether a proinflammatory microenvironment influences epithelial NK-1R expression. We also sought to elucidate the physiological function of colonic epithelial NK-1R. Our findings show that while human colonic epithelial cells do not express NK-1R in vitro, they do express NK-1R in vivo; we provide evidence that this may reflect cytokine conditioning. We report that NK-1R expressed by epithelial cells is not functionally coupled to intracellular signaling pathways involved in ion transport. However, we demonstrate that following an inflammatory insult, NK-1R-expressing epithelial cells proliferate in response to SP.

MATERIALS AND METHODS

Specimens

Colonic tissue was obtained from patients undergoing surgical resection for colorectal adenocarcinoma. Only histologically normal tissue from an uninvolved area of the colon was used. All protocols were approved by the University Teaching Hospitals Ethics Committee of each of the participating Hospitals.

Cell culture

Human Caco-2, HT29, SW620, and T84 colonic epithelial cell lines were obtained from American Type Culture Collection (Rockville, MD). T84 cells were cultured in a 1:1 (vol/vol) mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium, supplemented with 10% fetal calf serum, 2 mM glutamine, non-essential amino acids, and antibiotics. Caco-2, HT29, and SW620 cells were cultured in DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

For RT-PCR analysis, cells were cultured in 6-well tissue culture plates (Costar, Cambridge, MA) until they reached confluency. Cells were serum-starved for 24 h, and then incubated with a proinflammatory cytokine cocktail containing IFN-γ, TNF-α, and IL-1β (R + D, Abingdon, UK), each at a concentration of 10 ng/ml, in serum-free media. Monolayers were also stimulated with SP (Bachem California, Torrance, CA) at graded concentrations ranging from 10^{-10} to 10^{-6} M in serum-free media. Monolayers were also treated with lipopolysaccharide (LPS) (Sigma, St. Louis, MO) at graded concentrations ranging from 1 ng/ml to 1 μg/ml in serum-free media.

For immunocytochemical analysis, cells were cultured on glass chamber slides (Nunc, Naperville, IL). Cells were serum-starved for 24 h, incubated with the same proinflammatory cytokine cocktail as used above, and then fixed in 4% paraformaldehyde for 20 min.

Handling of SP

SP was dissolved in degassed sterile water containing 0.5% BSA, and 0.1 M acetic acid. Stock solutions were aliquoted at 10^{-3} M in polypropylene microcentrifuge tubes and frozen at −70°C until use. Working concentrations were made up in polypropylene tubes that had

been treated with 5% BSA in water for a least 1 h before use, to prevent peptide binding to the plastic.

Detection of NK-1R mRNA expression by RT-PCR

Total RNA was isolated by phenol chloroform extraction of guanidium isothiocyanate lysates (Chomczynski and Saachi, 1987). cDNA was synthesized using approximately 100 ng of total RNA, 9 U of AMV reverse transcriptase (Promega Corp., Madison, WI), 40 U of RNAsin (Promega Corp.), 500 μM dNTPs, and either 500 nM SPR-specific antisense primer GGATTTCATTTCCAGCCCCT or 125 nM random hexanucleotide primers (Boehringer Mannheim GmbH, Mannheim, Germany) per 30 μl reaction for 90 min at 42°C.

NK-1R PCR was performed on the specific-primed cDNA using the following sense and antisense primers, respectively: TGACCGCTACCACGAGCAAGTCTC and ATAGTCGCCGGCGCTGATGAAG corresponding to nucleotides 699–722 and 993–972 of human NK-1R cDNA respectively. β-actin control PCR was performed on the random-primed cDNA using the following sense and antisense primers respectively: CCTTCCTGGGCATGGAGTCCTG and GGAGCAATGATCTTGATCTTC corresponding to nucleotides 794–815 and 995–975 of human β-actin cDNA respectively. PCR primers were designed using the DNASTAR Lasergene Primerselect program (DNASTAR Inc., Madison, WI). Primers were selected that showed no significant homology to any other genes in the EMBL DNA sequence database. Primer pairs were chosen to span introns in their genomic sequences, thus ensuring mRNA-specific amplification.

PCR was performed on 1% of the cDNA using a final concentration of 1.5 μM $MgCl_2$, 50 μM dNTPs, 0.1 μM each primer, and 1 U of Taq DNA polymerase (Promega Corp.) per 50 μl reaction. Thermal cycling programs were as follows: NK-1R PCR: denaturation at 96°C for 15 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 min 30 sec, for 40 cycles followed by a final extension at 72°C for 10 min; β-actin PCR: denaturation at 96°C for 15 sec, annealing at 55°C for 30 sec, and extension at 72°C for 3 min for 35 cycles. Negative controls were performed by either omitting reverse transcriptase from cDNA synthesis or by omitting cDNA from the PCR amplifications. As a positive control, RNA from cells known to abundantly express NK-1R mRNA was used: IM-9 B lymphoblastoid cell line. Hot start PCR was employed to increase the specificity of the amplification. PCR products were analyzed by electrophoresis through 2% agarose gels and viewed under UV light after ethidium bromide staining; Hae III-digested ϕX174 DNA size markers were used. Product specificities were confirmed by DNA sequence analysis using an ABI Prism 310 Genetic Analyzer (Perkin-Elmer, Norwalk, CT).

Immunohistochemical/immunocytochemical localization of NK-1R

The NK-1R antibody was raised in rabbits against a synthetic peptide (MDNVLPVDSDLSP) corresponding to the extracellular N-terminal amino acids 1–13 of human NK-1R. The IgG fraction was affinity purified on an AH-Sepharose column to which the peptide had been coupled. Antibody specificty was confirmed by extensive radioimmunoassays and Western blotting.

Immunohistochemistry was performed on paraffin-embedded, surgically resected human colon sections (4 μm thick), mounted on aminopropylethoxysilane-treated slides. After deparaffinization and rehydration, sections were microwave irradiated in 10 mM citrate buffer, pH 6.0, at 350 W for 5 min to retrieve antigens. Sections were immediately cooled in TBS (50 mM Tris-HCl, pH 7.6; 150 mM NaCl; 5 mM KCl) and then washed twice for 5 min in wash buffer (TBS; 0.001% saponin). Endogenous peroxidase activity was quenched with 0.9% hydrogen peroxide in distilled water for 30 min. Sections were then washed in wash buffer containing 1% normal goat serum (used for all subsequent wash steps). Non-specific binding sites were blocked by incubation in wash buffer containing 5% normal goat serum for 1 h. Sections were washed twice and then incubated overnight at 4°C with the rabbit polyclonal anti-human NK-1R-specific IgG at a dilution of 1:50 in 50 mM Tris-HCl pH 7.6; 1% normal goat serum. Antibody binding was localized using a biotinylated goat anti-rabbit IgG followed by avidin–biotin conjugated horseradish peroxidase (Vectastain Elite ABC detection kit, Vector Laboratories, Burlingame, CA). Antibody binding was visualized using 3′,3′ diaminobenzidine (Vector Laboratories) yielding a brown reaction product. Staining with isotype-matched rabbit IgG was performed as a negative control. A specificity control was performed involving preadsorption of NK-1R antibody with the immunizing peptide (100 μM) for 16 h at 4°C prior to staining of sections.

Cultured epithelial cells were processed for immunocytochemistry as detailed above, except that the deparaffinization, rehydration, and antigen retrieval steps were omitted.

Simultaneous localization of NK-1R and the leukocyte common antigen (CD45) by double immunohistochemical staining

Sections were first processed for NK-1R immunohistochemistry. Following incubation with DAB, sections were washed twice in TBS and then incubated for 1 h at room temperature with mouse anti-human CD45-specific IgG (DAKO A/S, Denmark) at a dilution of 1:70 in RPMI; 10% FCS; 0.15 mM sodium azide. Antibody binding was localized using alkaline phosphatase-conjugated rabbit anti-mouse IgG (DAKO), followed by alkaline phosphatase-conjugated anti-alkaline phosphatase (APAAP) complexes (DAKO). Antibody binding was visualized using Fast Blue RR chromagen (Sigma Chemical Co.) yielding a purple reaction product.

Localization of NK-1R mRNA expression by in situ hybridization

In situ hybridization was performed on paraffin-embedded surgically resected human colon sections (4 μm thick), mounted on aminopropylethoxysilane-treated slides. Following de-paraffinization and rehydration, prehybridization treatments involved washing 2×5 min each in (i) PBS, (ii) PBS; 0.1 M glycine, (iii) PBS; 0.3% triton X-100, and (iv) PBS again. Sections were digested for 30 min at 37°C with proteinase K (10 μg/ml in 100 mM Tris-HCl, 50 mM EDTA, pH 8.0),

fixed for 5 min at 4°C in 4% paraformaldehyde; PBS, and then acetylated for 2×5 min in fresh 0.25% acetic anhydride; 0.1 M triethanolamine (pH 8.0). Sections were incubated at 37°C for 10 min in a prehybridization buffer consisting of 50% formamide in $4 \times$ SSC. A digoxigenin-labelled antisense RNA hybridization probe (324 bp) corresponding to codons 230–338 of the human NK-1R cDNA sequence was synthesized from a recombinant partial NK-1R plasmid clone (O'Connell et al., 1998) by in vitro transcription with digoxigenin-11-UTP (Boehringer Mannheim) and T7 RNA polymerase. The nucleotide sequence of the NK-1R probe showed no significant homology to any other sequences in the EMBL DNA sequence database. Hybridization was performed at 42°C overnight in hybridization buffer (50% formamide; 10% dextran sulphate; 1× Denhardt's reagent; $4 \times$ SSC; 10 mM DTT; 500 μg/ml yeast tRNA, and 100 μg/ml heat-denatured herring sperm DNA) containing 1 ng/ml digoxigenin-labelled riboprobe. After hybridization, sections were washed with increasing stringency to $0.1 \times$ SSC at 37°C. Hybridized probe was detected immunologically using alkaline-phosphatase-conjugated sheep anti-digoxigenin antibody (Boehringer Mannheim) and visualized with NBT-BCIP (purple/black precipitating product). Control slides involved competitive inhibition of hybridization with a 10-fold excess of unlabelled antisense riboprobe. This resulted in a marked reduction in signal intensity, thus confirming specificity of the hybridization.

Tissue preparation for electrophysiology experiments

Human tissue was obtained from patients undergoing colonic resection for adenocarcinoma as described previously (Stack et al., 1995). Segments of tissue were removed from an area marginal to the lesion and were shown to be histologically normal. Smooth muscle was removed by blunt dissection leaving a mucosal sheet consisting of an epithelial layer with attendant lamina propria. The composition of Krebs–Hensleit solution was: 113 mM NaCl; 4.7 mM KCl; 1.2 mM KH_2PO_4; 1.2 mM $MgSO_4 \cdot 7H_2O$; 1.9 mM $CaCl_2 \cdot 2H_2O$; 25 mM $NaHCO_3$, and 12.1 mM D-glucose.

Short-circuit current measurement

Stripped human mucosae were mounted in Ussing chambers with a window area of 0.63 cm^2. Preparations were bathed on either side with 10 ml of standard Krebs–Hensleit solution recirculated in reservoirs maintained at 37°C. The solution was gassed with 95% O_2; 5% CO_2 to maintain pH at 7.4. When mounted, each preparation was connected to a voltage clamp (DVC-1000; World Precision Instruments, Stevenage, Herts, UK) with current and voltage electrodes placed on either side. Preparations were clamped to zero potential difference and were allowed to equilibrate for 30–60 min. Short circuit current (SCC; μA/cm^2) was continuously monitored using a MacLab® digital-to-analog data acquisition system (AD Instruments Ltd., Hastings, UK). In all cases, paired (test and control) preparations of tissue were from the same individual. Since desensitization to SP was a feature, we generated cumulative concentration response curves to three selected concentrations of SP over a wide concentration range. SP challenges were made at 15 min intervals. Tetrodotoxin (1 μM) reduced basal SSC from $46.0 + 8.4$ to $31.6 + 8.6$ μA/cm^2 ($n = 5$; $P < 0.05$).

Electrophysiology of T84 monolayers

For ion transport studies, 10^6 T84 cells were plated on Transwell Clear® porous cell culture inserts (polyester membrane, tissue culture treated, 12 mm diameter, 0.4 μm pore size from Costar). Nutrient medium was provided to both apical and basolateral bathing domains of the monolayers. After 10–14 days of culture, electrophysiological characteristics of confluent monolayers were examined using an EVOM® apparatus (World Precision Instruments) to establish basal parameters. Monolayers which achieved stable resistances of $>800\ \Omega cm^2$ were treated for 12 h with IFN-γ, TNF-α, and IL-1β, each at a concentration of 10 ng/ml. Following incubation with cytokines, monolayers were bathed in Hank's balanced salt solution (HBSS) with 20 mM HEPES (three changes) to permit equilibration of electrophysiological parameters before challenge with drugs. These experiments were performed in a 37°C incubator. Effects of SP and the secretagogues forskolin and carbachol were measured at 1 min intervals. SCC was calculated from the Ohmic relationship. Ion transport responses are expressed as change in (Δ) SCC.

Cell proliferation assay

Serum-free medium [DMEM] was used throughout the proliferation assay. SW620 cells were plated in 6-well flat-bottomed microtitre plates at a density of 1×10^5 cells/well. After 24 h, cells were washed three times, and then incubated for 12 h with or without a proinflammatory cytokine cocktail containing IFN-γ, TNF-α, and IL-1β, each at a concentration of 10 ng/ml. After cytokine pretreatment, cultures were washed three times to remove the cytokines. The cells were then stimulated with SP (10^{-10}–10^{-8} M), plus or minus an NK-1R antagonist (Spantide 1:[D-Arg^1, D-$Trp^{7,9}$, Leu^{11}]-SP; Sigma) at 10^{-8} M for 24 h. Controls were cytokine-pretreated cells incubated without SP. [^{3}H] thymidine (0.5 μCi/well) was included during stimulation. Incorporation of [^{3}H] thymidine into DNA was quantified in a liquid scintillation counter (Beckman, Fullerton, CA). Each experiment was performed in duplicate and repeated three times.

Results are expressed as means + the standard error of the percentage of change in [^{3}H] thymidine incorporation and were calculated as follows:

$$\%\ \text{change} = \frac{\text{c.p.m. of cells with SP} - \text{c.p.m. of cells without SP}}{\text{c.p.m. of cells without SP}} \times 100$$

Data analysis

Matched preparations (test and control) were used throughout. Changes in ion transport are given as peak (ΔSCC) values. Results are expressed as mean ± stanstandard error of mean for n experiments. Statistical comparisons were carried out using two-tailed paired Student's *t*-test, Wilcoxon's signed rank test or by

repeated measures analysis of variance (ANOVA) where appropriate. Differences with $P < 0.05$ were considered to be statistically significant.

RESULTS

In vivo expression of NK-1R by colonic epithelium

We investigated the expression of NK-1R in epithelium of normal human colonic resections. Using immunohistochemistry, NK-1R protein was localized in both surface and crypt epithelial cells (Figs. 1 and 2C). NK-1R was also detected in LPMC. Dual staining for NK-1R and the leukocyte common antigen (CD45) revealed that epithelial NK-1R staining (brown) could not be attributed to intraepithelial lymphocytes (brown/purple) (Fig. 1A). While NK-1R-expressing cells could be detected with some frequency among LPMC, NK-1R-positive cells were more rarely seen among the less frequent intra-epithelial lymphocytes (Fig. 1A and B). Hence, NK-1R detected in the epithelial cells could not be confused with NK-1R detected in occasional intra-epithelial lymphocytes. The specificity of antibody binding was confirmed as staining was competitively inhibited by pre-incubation of the primary antibody with the NK-1R-immunizing peptide (Fig. 2D).

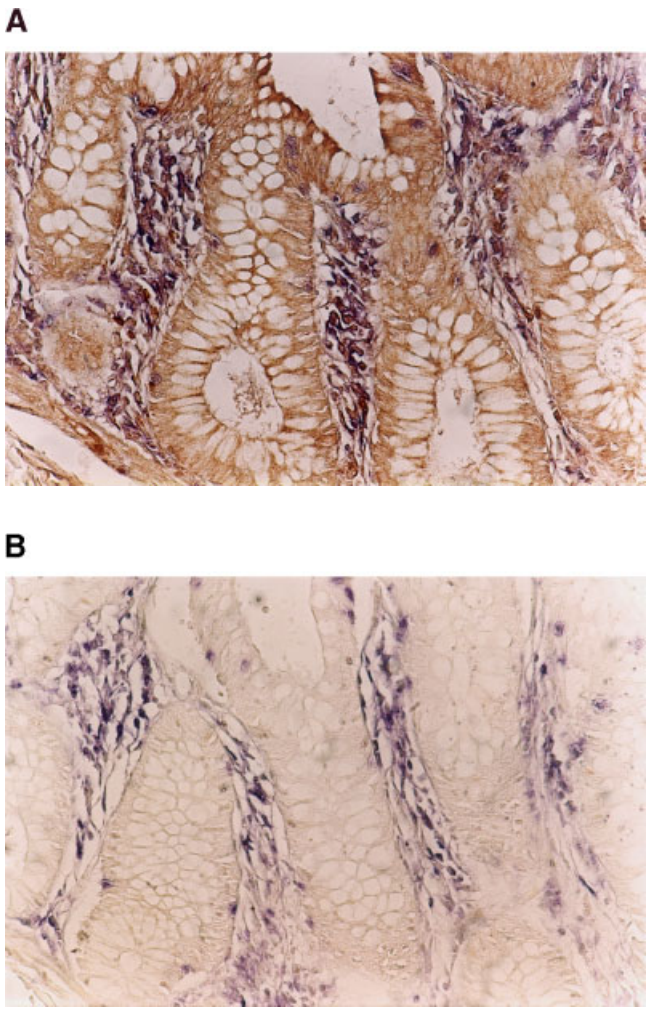

Fig. 1. Immunohistochemical colocalization of NK-1R and the leukocyte common antigen (CD45) in human colonic mucosa. **A**: Dual NK-1R/CD45 immuno-histochemical staining showing NK-1R-positive (brown) surface and crypt epithelial cells, and double positive NK-1R/CD45 (brown/purple) LPMC and intraepithelial lymphocytes. **B**: CD45-immunophosphatase staining (purple) only, showing CD45-positive immunocytes. Magnification, 200×.

In situ hybridization was employed to examine the in vivo expression of NK-1R mRNA by colonic epithelium. Both surface and crypt epithelial cells were shown to express NK-1R mRNA (Fig. 2A). NK-1R mRNA was also evident in LPMC. Hybridization specificity was confirmed, as a 10-fold excess of unlabeled riboprobe resulted in a dramatic reduction in hybridization signal intensity (Fig. 2B). The pattern of NK-1R mRNA expression detected by in situ hybridization closely matched that of NK-1R protein expression detected in consecutive sections by immunohistochemistry (Fig. 2A and 2C). Colocalization of NK-1R mRNA and protein confirmed that colonic epithelial cells express NK-1R in vivo.

Proinflammatory cytokines induce expression of NK-1R in colonic epithelial cells

We examined the human colonic epithelial cell lines Caco-2, HT29, SW620, and T84, for NK-1R mRNA expression by RT-PCR. The RT-PCR assay was controlled by equalization of input RNA for each cell isolation. Comparable amplification efficiencies were achieved in all RNA samples, as evidenced by the uniformity of control β-actin RT-PCR product yields (Fig. 3). All cell lines tested negative for NK-1R mRNA ($n = 5$). However, following incubation with a proinflammatory cytokine cocktail containing IFN-γ, TNF-α, and IL-1β (10 ng/ml each), NK-1R mRNA was detected in Caco-2, SW620, and T84 cells, but not in HT29 cells ($n = 5$) (Fig. 3). Caco-2 and HT-29 are moderately well-differentiated colonic epithelial cell lines derived from primary colonic tumors, and have been widely used as models of normal colonic epithelium. T84 is a colonic epithelial cell line derived from a colon tumor metastasis, and in culture forms monolayers exhibiting tight junctions between adjacent cells. T84 has been widely used as a model for colonic epithelia. SW620 is a colonic epithelial cell line derived from a colonic tumor metastasis. Cytokine induction of NK-1R expression in three of four colonic epithelial cell lines suggests that this response is characteristic of colonic epithelial cells. Although NK-1R expression was not detected in the HT29 cell line, either with or without cytokine treatment, our results demonstrate that normal human colonic epithelial cells express NK-1R in vivo (Figs. 1–2). NK-1R induction was observed after 12 h incubation with the cytokine cocktail for Caco-2 and T84 cells, and after 4 h incubation for SW620 cells. This might suggest increased responsiveness to cytokines in the SW620 cell line, which exhibits a more rapid growth rate than Caco-2 or T84. Stimulation with the same cytokines individually did not induce NK-1R expression in any of the cell lines (not shown). Incubation of the epithelial cell lines with graded concentrations of SP (10^{-10}–10^{-6} M) for 6, 12, or 24 h, did not cause induction of NK-1R mRNA (not shown); hence, SP itself does not appear to influence the expression of it's own receptor in these cells.

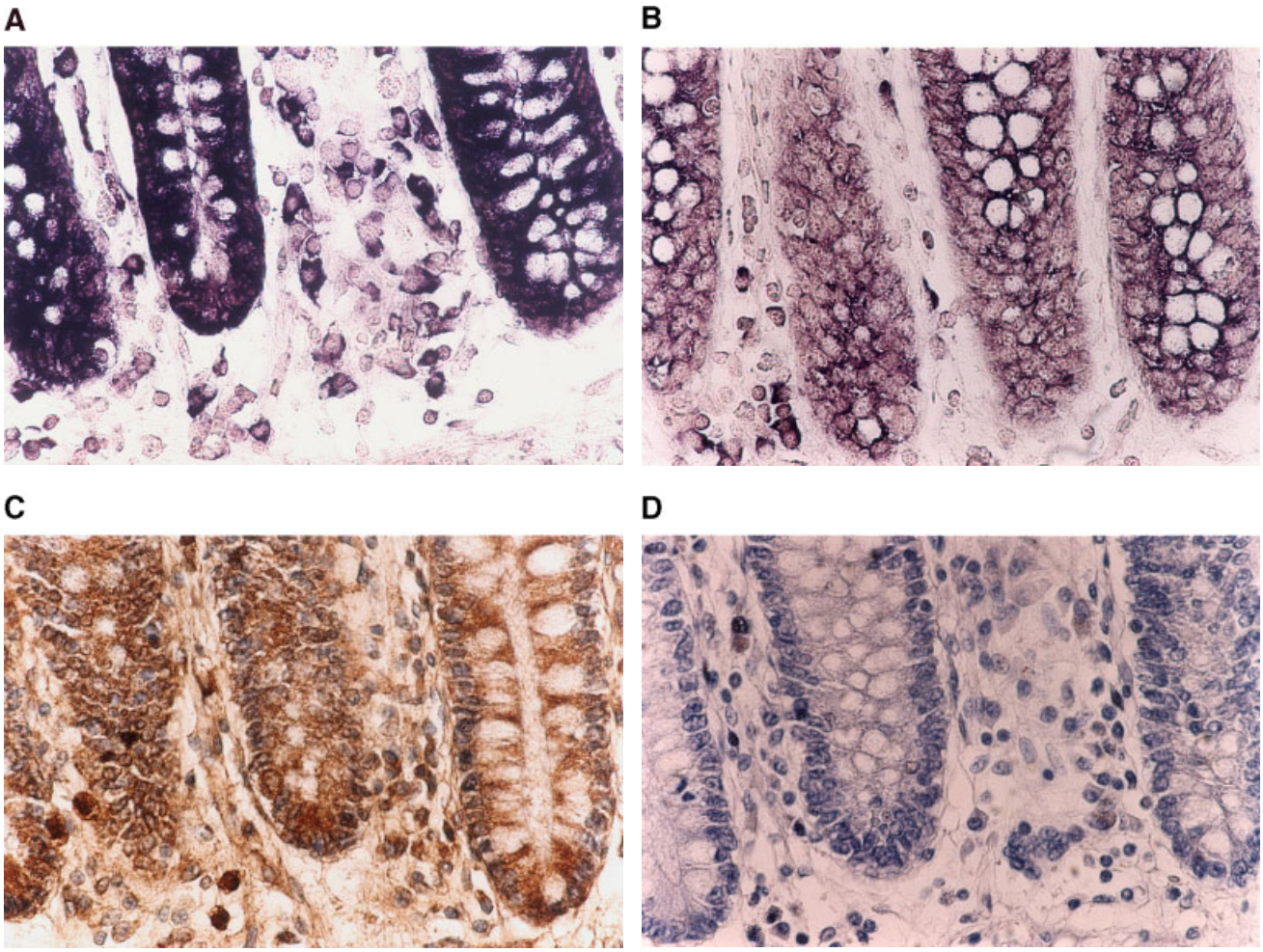

Fig. 2. Expression of NK-1R in normal human colonic mucosa. **A–B** Localization of NK-1R mRNA in normal colonic mucosa by in situ hybridization. A: NK-1R mRNA expression (purple) is detected in crypt epithelial cells and in LPMC. B: Control hybridization with a 10-fold excess of unlabeled riboprobe. Signal intensity is dramatically reduced, confirming specificity of hybridization. **C–D**: Localization of NK-1R protein in normal colonic mucosa (consecutive section) by immunohistochemistry. C: NK-1R immunoperoxidase staining (brown) is present in crypt epithelial cells and in LPMC. D: Control staining. Preincubation of the primary antibody with the immunizing peptide inhibited staining. This confirmed the specificity of antibody binding. Sections were counterstained with haematoxylin (blue). Magnification, 400×.

Upregulation of NK-1R in response to the proinflammatory cytokines was also detected at the protein level. The immunocytochemistry results in Figure 4 show that SW620 cells do not express NK-1R protein. However, following stimulation with the proinflammatory cytokine cocktail containing IFN-γ, TNF-α, and IL-1β, NK-1R protein was detected in SW620 cells. Similar results were obtained for the T84 and Caco-2 cell lines ($n = 3$) (not shown). The specificity of antibody staining was confirmed, as staining of the cytokine-treated cell lines was competitively inhibited by pre-incubation of the primary antibody with the NK-1R-immunizing peptide.

SP stimulates ion transport in voltage clamped human colonic mucosae in vitro

SP, added to the basolateral bathing solution of voltage-clamped colonic mucosae, stimulated a rapid-onset inward current which peaked within 5 min of challenge ($n = 5$). This response was accounted for by electrogenic chloride secretion since it was bumetanide sensitive and virtually abolished in chloride-free solutions. The relationship between SP concentration and response was over a wide range (Fig. 5). Furthermore, SP-induced changes in SCC were significantly attenuated in the presence of tetrodotoxin (1 μM) ($n = 5$), which was without effect upon ion transport responses to the directly acting secretagogue forskolin (Fig. 5; inset). These data suggest that the effect of SP on epithelial chloride ion transport was primarily indirect, and neuronally mediated.

SP failed to stimulate electrogenic ion transport across monolayers of T84 cells

Basal electrical parameters across monolayers were -0.4 ± 0.4 mV and $1{,}448 \pm 139$ Ωcm^2 ($n = 16$). Cytokine treatment for 8 h did not alter resting parameters. After 12 h of exposure to the cytokine cocktail, transepithelial resistance was significantly reduced (905 ± 163 Ωcm^2;

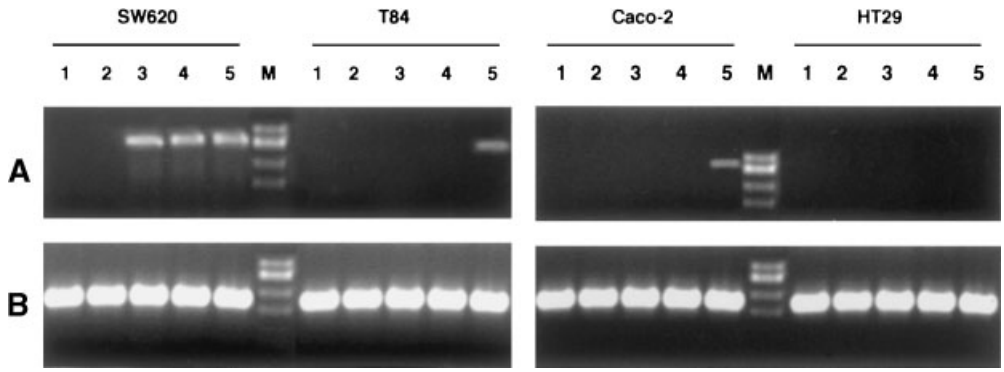

Fig. 3. Proinflammatory cytokines induce NK-1R mRNA expression in three different colonic epithelial cell lines. NK-1R expression was analyzed by RT-PCR in untreated cells (lane 1), and in cells stimulated with a proinflammatory cytokine cocktail containing IFN-γ, TNF-α, and IL-1β (10 ng/ml each) for 30 min, 4, 8, and 12 h (lanes 2–5 respectively). mRNA specific amplification products for NK-1R (295 bp) and β-actin (202 bp) are shown in **A** and **B**, respectively. Equalized input RNA was used for each test, and control β-actin RT-PCR confirmed equal amplification efficiency in all RNA samples. The proinflammatory cytokines induced NK-1R expression in SW620, T84, and Caco-2, but not in HT29, colonic epithelial cells. HaeIII-digested øX174 DNA size markers (M) were used. Data are representative of five experiments.

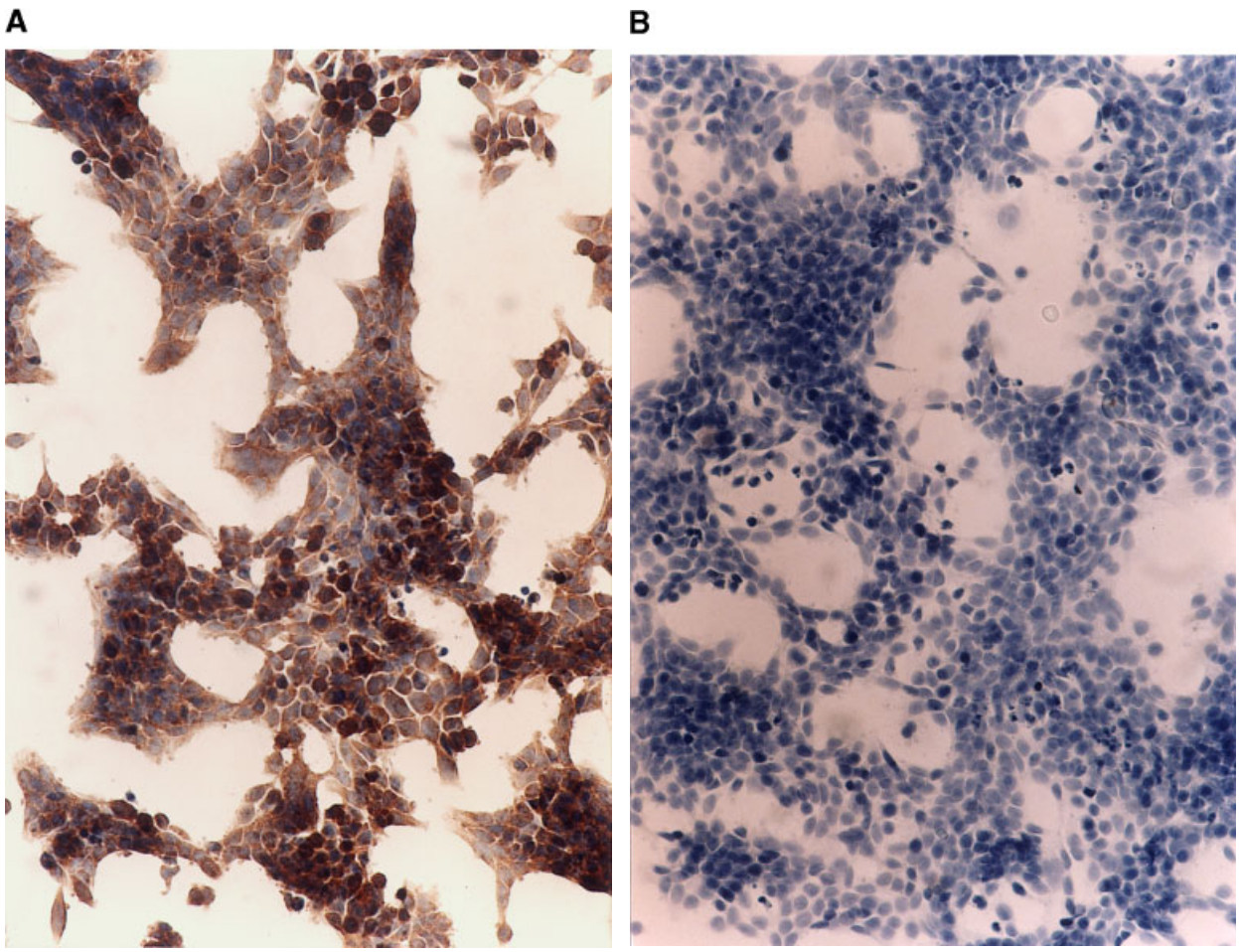

Fig. 4. Proinflammatory cytokines induce NK-1R protein expression in colonic epithelial cell lines. **A**: NK-1R protein expression detected by immunoperoxidase staining (brown) in SW620 colonic epithelial cells following stimulation with the proinflammatory cytokines IFN-γ, TNF-α, and IL-1β (10 ng/ml each) or 12 h. **B**: NK-1R staining is absent from untreated SW620 cells. Cells were counterstained with haematoxylin (blue). Data are representative of three experiments. Magnification, 200×.

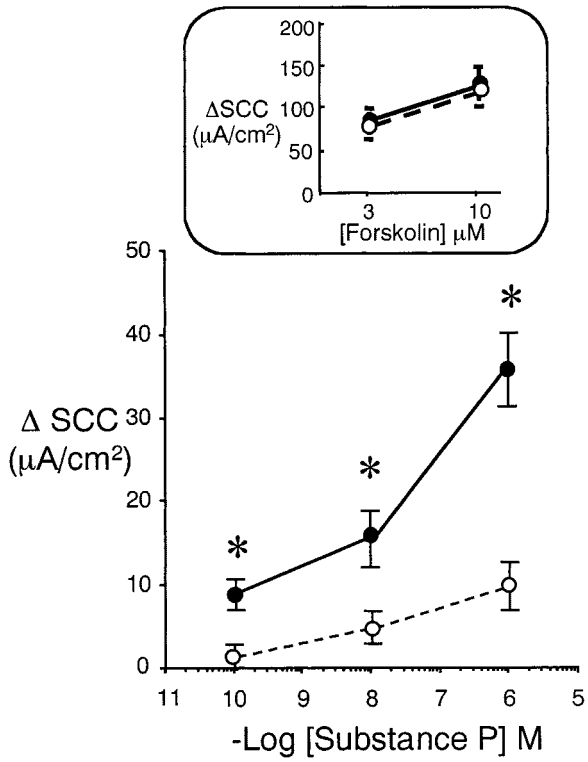

Fig. 5. Substance P (SP) stimulates neuronally mediated ion secretion in human colonic mucosae. In Using chamber electrophysiology experiments using resected human colonic mucosae, SP induced a concentration-dependent inward short circuit current (SCC) (solid circles; solid line). In the presence of tetrodotoxin (1 μM), responses were significantly reduced (open circles; broken line). The same concentration of tetrodotoxin was without effect upon SCC response to the directly acting secretagogue forskolin (10 μM; inset). Data are representative of five experiments.

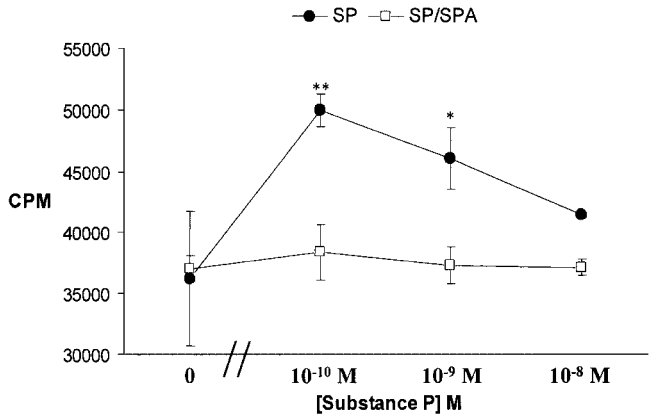

Fig. 6. SP induces proliferation of colon epithelial cells via cytokine-induced NK-1R. [^{3}H] thymidine incorporation into DNA was measured in SW620 colonic epithelial cells stimulated with SP for 24 h. Cells were pre-treated with a proinflammatory cytokine cocktail containing IFN-γ, TNF-α, and IL-1β (10 ng/ml each) for 12 h, to induce expression of NK-1R. SP caused a dose-dependent increase in proliferation of the epithelial cells, maximal at a dose of 10^{-10} M SP (solid circles). Co-treatment with the NK-1R antagonist, Spantide 1, ([D-Arg^1, D-Trp^{7,9}, Leu^{11}]-SP) at a concentration of 10^{-8} M, effectively blocked proliferation at every tested concentration of SP (open squares). This indicated that SP-induced proliferation was receptor mediated. SP did not stimulate proliferation of SW620 cells that did not receive cytokine pretreatment (not shown), which were NK-1R-negative. Data are expressed as a percentage change in proliferation relative to control cultures without SP. Values represent means (± standard error). $^*P < 0.05$; $^{**}P < 0.01$.

n = 8; $P < 0.05$), although transepithelial potential difference (−0.8 ± 0.57; n = 8) was unchanged. In experiments in which cytokines or vehicle-treated controls were used, SP (0.1 nM–5 μM) added to the basolateral bathing solution did not alter transepithelial SCC (not shown). The capacity of individual monolayers to transport chloride ions was examined by challenge with the directly acting secretagogues forskolin and carbachol. Monolayers which were pre-treated with the cytokine cocktail for 12 h, and then challenged with forskolin (3 and 10 μM), gave SCC responses of 15.9 ± 1.5 and 20.7 ± 2.0 μA/cm^2, respectively (n = 8). SCC responses to forskolin (3 and 10 μM) in control monolayers which did not receive the cytokine pre-treatment were not significantly different (22.0 ± 5.9 and 28.7 ± 6.5 μA/cm^2, respectively; n = 7). Similarly, responses to carbachol (10 μM) in cytokine treated monolayers (166 ± 31 μA/cm^2; n = 8) were not different from responses obtained in matched controls (175 ± 35 μA/cm^2; n = 7).

SP stimulates the proliferation of proinflammatory cytokine-pretreated colon epithelial cells

The effect of SP on the proliferation of colon epithelial cells was examined by quantifying the incorporation of [^{3}H] thymidine into DNA in SW620 cells incubated for 24 h with SP. Results are shown in Figure 6 and are representative of three experiments, each performed in duplicate. SP did not stimulate the proliferation of untreated SW620 cells, probably due to lack of NK-1R expression. However, SP significantly increased the incorporation of [^{3}H] thymidine in SW620 cells which had been pretreated with a proinflammatory cytokine cocktail containing IFN-γ, TNF-α, and IL-1β (10 ng/ml each). We have already shown that this treatment induces NK-1R expression in the colon epithelial cells (Figs. 3 and 4). The proliferative response to SP was dose-dependent. A significant increase (28%) in proliferation was observed at 10^{-8} M SP, with the maximal proliferative response (48%) occurring at 10^{-10} M SP (Fig. 6). SP-induced proliferation was mediated specifically via NK-1R; inclusion of the NK-1R-specific antagonist, Spantide 1, at 10^{-8} M, completely inhibited SP-mediated proliferation of the cytokine-pretreated epithelial cells (Fig. 6). In addition to stimulating proliferation of SW620 cells, we found that SP also induced proliferation of T84 epithelial cells, again only after induction of NK-1R by pretreatment with the inflammatory cytokine mix (not shown).

NK-1R expression is not induced by LPS in human colonic epithelial cell lines

The bacterial-derived inflammatory agent LPS has been shown to upregulate NK-1R expression in certain cell types, including rat macrophages (Bost et al., 1992). Since colonic epithelial cells are in proximity with the lumenal bacterial flora, we investigated whether LPS had any influence on NK-1R expression in colonic epithelial cells. We treated the four colonic

epithelial cell lines SW620, T84, CaCo-2, and HT29 with LPS for 8 h at concentrations ranging from 1 ng/ml to 1 μg/ml. Following LPS treatment, NK-1R mRNA was undetectable by RT-PCR in any of the four cell lines (Fig. 7). Hence, our results indicate that LPS stimulation fails to induce NK-1R expression in colonic epithelial cells.

DISCUSSION

In this study, we demonstrate that human colonic epithelial cells express NK-1R in vivo. We show that colonic epithelial cell lines do not express NK-1R in vitro, but can be induced to do so by proinflammatory Th1 cytokines. This suggests that expression of NK-1R in the epithelial cells in vivo is due to cytokine conditioning in the mucosal microenvironment. We demonstrate that SP stimulates proliferation of cytokine-treated epithelial cell lines; proliferation was mediated specifically via cytokine-induced NK-1R, since it was inhibitable by the NK-1R antagonist, Spantide 1. Although SP stimulated ion transport in specimens of intact human colonic mucosae ex vivo, this did not appear to be a direct effect of SP on the epithelial cells; rather, the secretory effect of SP appeared to be neurally mediated, as it was inhibitable by tetrodotoxin. Furthermore, SP did not alter ion transport in monolayers of T84 colonic epithelial cells expressing NK-1R. Hence, while NK-1R expressed by colonic epithelial cells does not appear to be functionally coupled to signal transduction pathways involved in ion transport, NK-1R does function to mediate epithelial proliferation in response to SP.

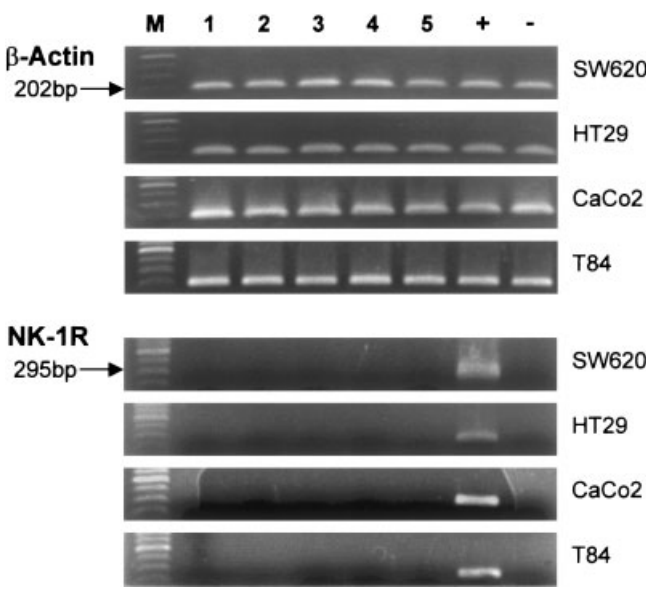

Fig. 7. Lipopolysaccharide (LPS) does not upregulate NK-1R expression in colonic epithelial cell lines. NK-1R expression was analyzed by RT-PCR in SW620, HT29, Caco-2, and T84 colonic epithelial cells following treatment with LPS (1 ng/ml–1 μg/ml) for 8 h. Control β-actin RT-PCR was performed for all RNA samples. HaeIII-digested øX174 DNA size markers (lane M) were used. Lane 1 shows RT-PCR products from control, untreated cells. Lanes 2–5 show RT-PCR products for cells treated with LPS at 1 μg/ml, 100, 10, and 1 ng/ml respectively. As a positive control for NK-1R RT-PCR (Lane +), the B lymphoblastoid cell line, IM-9, was used, which is known to abundantly express NK-1R. As a negative control for NK-1R RT-PCR, peripheral blood mononuclear cells (PBMC's) were used, which do not express NK-1R. LPS failed to induce NK-1R expression in any of the colonic epithelial cell lines tested.

Using immunohistochemistry and in situ hybridization, NK-1R expression was localized in surface and crypt epithelial cells in human colonic resections. This finding agrees with previous reports of NK-1R mRNA expression in isolated colonocytes from the guinea pig (Cooke et al., 1997), in isolated canine colonic crypts (Khan et al., 1995), and in rat intestinal epithelium (Pothoulakis et al., 1998). Others have described SP binding sites in epithelium of the human (Mantyh et al., 1988a) and canine (Mantyh et al., 1988b) gastrointestinal tract. Human airway epithelium has also been shown to express NK-1R mRNA (Bai et al., 1995). The anatomical apposition of SP nerve fibers with colonic epithelium (Brodin et al., 1983) suggests neuropeptide–epithelial communication. Our observation of NK-1R expression by human colonic epithelium provides further evidence for such neuro-epithelial cross-talk.

Results obtained from RT-PCR analysis demonstrated that in the absence of cytokines, human colonic epithelial cell lines did not express NK-1R mRNA. Many studies have reported upregulation of NK-1R during inflammation (Mantyh et al., 1988a; Schafer et al., 1993; Krause et al., 1995; Mantyh et al., 1996). IL-1α, IL-1β, and stem cell factor have been shown to induce expression of NK-1R mRNA in bone marrow fibroblasts and stroma (Rameshwar and Gascon, 1995; Rameshwar et al., 1997). Bost et al. (1992) described upregulation of NK-1R mRNA in response to LPS in rat peritoneal macrophages. Recently, Pothoulakis et al. (1998) demonstrated upregulation of NK-1R in rat intestinal epithelium following intraluminal administration of *Clostridium difficile* toxin A, the causative agent of antibiotic-associated enteritis. It has also been reported that bacterial infection of the gut by organisms such as *C. difficile* stimulates local expression of pro-inflammatory cytokines (Castagliuolo et al., 1998). NK-1R mRNA is also elevated in colonic biopsies from IBD patients (Goode et al., 2000b). We therefore postulated that NK-1R expression by colonic epithelial cells may be influenced by inflammatory mediators.

Stimulation with a cocktail of the proinflammatory Th1 cytokines IFN-γ, TNF-α, and IL-1β, induced NK-1R expression in three of four colonic epithelial cell lines. Stimulation with the same cytokines individually did not induce NK-1R expression. While little is known about the transcription factors involved in regulating expression of NK-1R, it is well known that the cytokines IFN-γ and TNF-α act synergistically in inducing expression of certain genes, including MHC 1 and IRF 1 (Agresti et al., 1998). Both cytokines synergise in activating the transcription factors NF-κB and IRF-1. IL-1 also synergises with these cytokines in activating NF-κB. The concentrations of cytokines used to induce NK-1R expression (10 ng/ml) were within the physiological range, and below the concentrations that lead to apoptosis of epithelial cells (100 ng/ml). Further work is necessary to elucidate which cytokine-induced transcription factors lead to expression of NK-1R in colonic epithelial cells.

The normal gut mucosa is considered to be in a state of controlled inflammation as opposed to pathological

inflammation (Shanahan, 1994). The gut epithelium is probably exposed to a concert of cytokines, rather than individual cytokines in isolation. LPMC, intraepithelial lymphocytes, mast cells, and subepithelial mesenchymal cells produce a rich repertoire of cytokines (Sartor, 1994; Fiocchi, 1997). Indeed, the epithelium itself plays a pivotal role in mucosal cytokine production and receptivity (Eckmann et al., 1993; Reinecker and Podolsky, 1995). Our observation of NK-1R expression by normal colonic epithelium in vivo probably reflects physiological cytokine conditioning by the mucosal microenvironment. Lack of NK-1R expression in colon epithelial cell lines cultured in vitro may be due be loss of environmental cytokine conditioning.

The bacterial-derived inflammatory agent, LPS has been shown to upregulate NK-1R expression in certain cell types, including macrophages (Bost et al., 1992). LPS produced by bacteria in the gut microbial flora is another inflammatory mediator which could potentially interact with colonic epithelial cells. However, our results indicate that LPS fails to induce NK-1R expression in colonic epithelial cells.

SP and other neurokinin receptor agonists have been extensively characterized as potent secretagogues of mammalian intestinal epithelia (Keast et al., 1985a; Mathison and Davison, 1989; Kuwahara and Cooke, 1990; Rangachari et al., 1990; Parsons et al., 1992; Cox et al., 1993; Cooke et al., 1997). Neurokinin receptors have accordingly been implicated in neural and non-neural ion secretion (Keast et al., 1985a; Mathison and Davison, 1989; Rangachari et al., 1990; Parsons et al., 1992; Cox et al., 1993), and NK-1R antagonists can inhibit secretory responses (Moriarty et al., 2001a,b). Our electrophysiological results clearly demonstrate that SP stimulates chloride ion secretion in human colonic mucosae. Application of tetrodotoxin significantly attenuated the secretory response, thereby indicating that the effect of SP on epithelial ion transport is primarily indirect and neuronally mediated (tetrodotoxin specifically blocks neural ion transport). These observations suggest that the role of SP in ion transport in the human colon is at least as complex as in other mammals (Keast et al., 1985a; Mathison and Davison, 1989; Rangachari et al., 1990; Parsons et al., 1992; Cox et al., 1993). Our findings provide evidence that epithelial NK-1R is not involved in chloride ion secretion.

SP did not stimulate electrogenic ion transport in T84 monolayers, even when treated under conditions which evoke NK-1R expression. This finding corroborates our observations in intact tissue, and confirms that NK-1R expressed by epithelial cells is not functionally coupled to signal transduction pathways involved in ion transport. Furthermore, these data suggest that the prosecretory action of SP in vivo is entirely due to activation of other (non-epithelial) cell types within the lamina propria. Treatment of T84 monolayers with the cytokine cocktail reduced transepithelial resistance after 12 h but did not significantly alter secretory capacity either in response to forskolin or carbachol. These were selected as stimulants of chloride secretion which employ cyclic AMP- and calcium-dependent signal transduction pathways. These findings are in accord with our previous report of cytokine influences on barrier function of T84 monolayers (Taylor et al., 1998). Individual cytokines and lymphocyte-derived supernatant reduced transepithelial resistance but were not cytotoxic (Taylor et al., 1998). Thus, failure of cytokine-treated monolayers to exhibit a secretory response to SP is unlikely to be accounted for by hypo-responsiveness of the epithelium such as that reported as a feature in a mouse model of colitis (MacNaughton et al., 1998).

SP exerted a direct proliferative effect on SW620 cells which had been pretreated with the proinflammatory cytokines IFN-γ, TNF-α, and IL-1β. In contrast, SP had no effect on SW620 cells which did not receive prior cytokine stimulation. Cytokine pre-treatment was already shown to induce expression of NK-1R mRNA. This would suggest that the observed proliferation in response to SP was NK-1R-mediated. To confirm this, we co-treated the cytokine-stimulated SW620 cells with the NK-1R antagonist, Spantide 1, along with SP. The NK-1R antagonist completely blocked SP-induced proliferation in the cytokine-treated SW620 cells. We have also found that SP induced proliferation in T84 colon epithelial cells after cytokine-induction of NK-1R expression (not shown). The proliferative response to SP was dose dependent and maximal at a concentration of 10^{-10} M SP. Others have reported similar dose-response curves whereby the proliferative effect of SP was optimal at a concentration of 10^{-10} M (Ziche et al., 1990b; Rameshwar et al., 1997). Indeed, the selective NK-1R agonist [β-Ala^4, Sar^9, Met$(O_2)^{11}$]-SP(4–11) has been shown to maximally stimulate the proliferation of guinea pig tracheal epithelial cells (Kim et al., 1995), human skin fibroblasts (Ziche et al., 1990a), and bovine capillary endothelial cells (Ziche et al., 1990b) at a concentration of 10^{-10} M.

SP has previously been shown to stimulate the proliferation of guinea pig airway epithelial cells (Kim et al., 1995), rabbit corneal epithelial cells, and mouse lens epithelium (Reid et al., 1993). SP acts as a mitogen for fibroblasts (Nilsson et al., 1985; Ziche et al., 1990a; Rameshwar et al., 1997), smooth muscle cells (Nilsson et al., 1985), endothelial cells (Ziche et al., 1990b), and synoviocytes (Lotz et al., 1987), and is therefore thought to play a significant role in tissue repair. The effect of SP on cell proliferation has been attributed to it's weak homology with acidic fibroblast growth factor (Gimenez-Gallego et al., 1985). The present data suggest a possible role for SP in colonic tissue repair. Indeed it has been reported that experimentally induced colitis in rats is exacerbated by pretreatment with capsaicin (Evangelista and Meli, 1989). It is conceivable that conditions such as an inflammatory response that cause epithelial damage, may stimulate or injure nerves, causing nociceptive release of SP into the site of injury. SP in turn may stimulate the proliferation of NK-1R-expressing epithelial cells, and thus initiate a healing response.

In conclusion, our findings demonstrate expression of NK-1R by human colonic epithelium in vivo, and show that proinflammatory cytokines induce NK-1R expression in colonic epithelial cell lines. This suggests that in vivo epithelial expression of NK-1R reflects cytokine conditioning by the mucosal microenvironment. We also demonstrate proliferation of colonic epithelial cell lines in response to SP, via cytokine-induced NK-1R. Our

results therefore support a role for neuro-epithelial crosstalk via SP in an inflamed milieu, and lend further support to a possible role for SP in the healing phase of mucosal inflammation.

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