Substance P-stimulated interleukin-8 expression in human colonic epithelial cells involves Rho family small GTPases

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Interaction of the neuropeptide substance P (SP) and its neurokinin-1 receptor (NK-1R) plays an important role in the pathophysiology of intestinal inflammation. SP is known to stimulate production of interleukin (IL)-6 and IL-8 in the U-373-MG human astrocytoma cell line via activation of p38 MAPK (mitogen-activated protein kinase) and nuclear factor (NF)- κ B, respectively. However, the signalling mechanisms by which SP-NK-1R interaction induces NF- κ B activation and IL-8 expression are still not clear. In this study we demonstrate that SP stimulates IL-8 secretion and IL-8 promoter activity in the NCM460 non-transformed human colonic epithelial cell line transfected with NK-1R cDNA. Our results indicate that inhibition of endogenous Rho family proteins (RhoA, Rac1 and

Cdc42) by their respective dominant negative mutants significantly decreases SP-induced IL-8 secretion and IL-8 promoter activity. We also demonstrate that SP rapidly activates RhoA, Rac1 and Cdc42 and that co-expression of the dominant negative mutants of RhoA, Rac1 and Cdc42 in NK-1R cDNA-transfected NCM460 cells significantly inhibits SP-induced NF- κ B-dependent gene expression. These results demonstrate that Rho family small GTPases RhoA, Rac1 and Cdc42 are novel signal transducers for SP-stimulated IL-8 expression.

Key words: G-protein-coupled receptor, inflammation, neuropeptide, signal transduction.

INTRODUCTION

The neuropeptide substance P (SP), a member of the tachykinin family, originally isolated by Chang and Leeman [1], is distributed in the central and the peripheral nervous system. In the intestine, SP is localized in sensory neurons [2], enteric nerves [3], enteroendocrine cells [4], and macrophages [5]. SP mediates several diverse intestinal responses, including motility [6], mucosal permeability [7,8] and chloride secretion [9]. SP, acting through its neurokinin-1 receptor (NK-1R), also plays an important role in the pathogenesis of intestinal inflammation of diverse aetiologies [5,8,10–12]. The mechanism of SP-NK-1R participation in intestinal inflammatory states involves release of mediators associated with inflammation [13,14]. Along these lines, SP can directly stimulate production of cytokines, including interleukin (IL)-1, IL-6, and IL-8, and tumour necrosis factor (TNF) α [5,15–19].

SP stimulates a number of intracellular signalling molecules such as the mitogen-activated protein kinase (MAPK) members, extracellular signal-regulated protein kinases (ERK1/2) which are required for cell proliferation [20] and p38 MAPK that is involved in IL-6 expression [17] in the U-373-MG human astrocytoma cell line. In addition SP also activates the transcription factor, nuclear factor (NF)- κ B and NF- κ B-dependent expression of the potent neutrophil chemotactic factor IL-8 in U373-MG cells [21]. It is well documented that NF- κ B is essential for the expression of genes involved in inflammation [22]. NF- κ B consists of homodimers and heterodimers of the Rel family proteins, including p50, p52, p65, c-Rel, and Rel-B, of which the

heterodimer of p65 and p50 is the predominant form present in most cell types. The activity of NF- κ B is regulated by association with a family of inhibitory proteins called I κ Bs, in particular I κ B α . NF- κ B is normally sequestered as an inactive form in the cytoplasm by I κ B inhibitory proteins. Upon exposure of cells to stimuli such as TNF or IL-1 β , a kinase cascade is initiated, which results in activation of I κ B kinases. The I κ B kinases then phosphorylate I κ B α and the phosphorylated I κ B α is subsequently degraded by the ubiquitin-dependent proteasome. The degradation of I κ B α leads to the nuclear translocation of NF- κ B and the activation of NF- κ B responsive genes.

The upstream signalling pathways involved in NF- κ B activation in response to many stimuli have been extensively studied. Recently it was shown that NF- κ B can also be activated by the Rho family proteins [23]. Rho family proteins belong to the Ras superfamily of small GTPases and include RhoA, -B, -C, -E, -G, Cdc42, Rac-1, Rac-2 and TC10. RhoA, Cdc42 and Rac1, the most studied Rho family proteins, are involved in a variety of cellular responses, including cytoskeletal changes and gene expression, stimulated by many factors [24]. RhoA is known to be important for G-protein-coupled receptor signalling [25,26]. Involvement of the Rho family proteins in inflammatory responses such as IL-8 production has also been suggested [27–29]. Other studies also indicate that the effect of RhoA on inflammatory gene expression may involve NF- κ B activation [23,29–31]. However, whether RhoA, Rac1 and Cdc42 play any role in SP signalling is not known.

In this study we demonstrate that SP stimulates IL-8 secretion and IL-8 promoter activity in the NCM460 non-transformed

Abbreviations used: GPCR, G-protein-coupled receptor; IL, interleukin; LacZ, β -galactosidase; MAPK, mitogen-activated protein kinase; NF, nuclear factor; NK, neurokinin; NK-1R, neurokinin-1 receptor; SP, substance P; SRE, serum response element; TNF, tumour necrosis factor.

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human colonic epithelial cell line transfected with NK-1R cDNA. To examine whether Rho family GTPases are involved in this response, we overexpressed the dominant negative forms of RhoA, Rac1 and Cdc42 (RhoA-19N, Rac1-17N and Cdc42-17N) by using retroviral infection. Our results indicate that blockade of endogenous RhoA, Rac1 and Cdc42 by their respective dominant negative mutants significantly decreases SP-induced IL-8 secretion and IL-8 promoter activity. We also demonstrate that SP strongly activates RhoA, Rac1 and Cdc42 and overexpression of the dominant negative mutants of RhoA, Rac1 and Cdc42 in NK-1R cDNA-transfected NCM460 cells significantly inhibits SP-induced NF- κ B-dependent reporter gene expression. To our knowledge, this is the first report that Rho family GTPases are involved in SP signalling pathways.

MATERIALS AND METHODS

Preparation of retroviruses and infection of NCM460 cells

The human NK-1R cDNA was isolated from plasmid pMAMneo-Blue-NK1 (kindly provided by Dr Nigel Bunnet, University of California, San Francisco, U.S.A.) and then subcloned into the retroviral vector pMMP (kindly provided by Dr Richard C. Mulligan, Children's Hospital, Harvard Medical School, Boston, U.S.A.). The new plasmid (pMMP-NK-1R) was used to prepare NK-1R-expressing retroviruses as described below.

The 293T cells (7×10^4 cells/cm²) were incubated with 10 ml of Dulbecco's modified Eagle's medium/10% (v/v) foetal bovine serum for 24 h (replaced with fresh medium 4 h before transfection). The plasmids pMD-gag-pol, pMD-VSVG (both provided by Dr Richard C. Mulligan) and pMMP-NK-1R were combined in a ratio of 3:1:4 (w/w/w) and used to prepare transfection mixtures using Effectene Transfection Reagent (Qiagen, Valencia, CA, U.S.A.). The media were filtered through 0.45 μ m filters, 48 h after transfection, and the supernatants were used either immediately or stored at -80°C .

To infect NCM460 non-transformed human colonic epithelial cells [32], they were incubated with 2 vol. of filtered virus-containing supernatant and 1 vol. of fresh growth medium for 16–24 h in the presence of 10 μ g/ml polybrene (Sigma, St. Louis, MO, U.S.A.). The infected cells (named NCM460-NK-1R cells) were used in this study.

Overexpression of dominant negative mutants of RhoA, Rac1 and Cdc42

The plasmids containing the dominant negative mutants of RhoA, Rac1 or Cdc42 (RhoA-19N, Rac1-17N or Cdc42-17N) were provided by Dr Margaret M. Chou (University of Pennsylvania, Pennsylvania, U.S.A.). The DNA fragments corresponding to RhoA-19N, Rac1-17N and Cdc42-17N were isolated by PCR and subcloned into the pCMBP retroviral vector [33]. The identity of the DNA fragments was confirmed by DNA sequencing analysis. The retroviral constructs were used to make the viruses expressing RhoA-19N, Rac1-17N or Cdc42-17N as described above and also used in the transient transfection experiments for all promoter assays.

IL-8 measurements

IL-8 protein levels in colonic-epithelial-cell-conditioned media were determined by a double-ligand ELISA using goat anti-(human IL-8) (R&D Systems Inc., Minnesota, MN, U.S.A.) as described previously [34]. Results were expressed as means $\pm$ S.E.M. (ng/ml).

Luciferase assay

Cells were seeded in 12-well plates (0.2×10^6 cells/well) overnight and transiently transfected using Effectene Transfection Reagent (Qiagen) with the IL-8 promoter luciferase construct [35], the serum response element (SRE)-luciferase construct (Clontech, Palo Alto, CA, U.S.A.) or the NF- κ B promoter construct (a gift from Dr Tom Mitchell and Dr Bill Sugden, University of Wisconsin, Madison, WI, U.S.A.) [36] with a control luciferase construct pRL-TK (Promega, Madison, WI, U.S.A.) and/or other DNA constructs as indicated. Transfected cells were serum-starved for 24 h followed by exposure to SP for 4 h. Firefly and *Renilla* luciferase activities in cell extracts were measured using a Dual-Luciferase Reporter Assay System (Promega). The relative luciferase activity was then calculated by normalizing IL-8 promoter-driven firefly luciferase activity to control *Renilla* luciferase activity.

RhoA activation assay

RhoA activity was measured based on the procedure described by Ren et al. [37]. Briefly, cells were washed twice with PBS, and then incubated with lysis buffer A (50 mM Tris, pH 7.2, 500 mM NaCl, 1% (v/v) Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM MgCl₂, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 1 mM PMSF) for 10 min at 4°C . Cell lysates were centrifuged at 1000 *g* for 3 min. To prepare the beads to precipitate cellular RhoA in the GTP-bound form, bacteria containing the plasmid pGST-TRBD (grown to $A = 0.8$) were treated with 1 mM isopropyl β -D-thiogalactoside (IPTG) for 2 h and the bacteria lysate containing GST-TRBD fusion protein was incubated with glutathione-Sepharose-4B beads (Amersham Biosciences, Upsala, Sweden). An equal amount of the GST-TRBD beads was incubated with the fresh supernatants for 45 min on ice, and the beads were then washed with AP buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 1% Triton X-100, 10 mM MgCl₂, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 1 mM PMSF). The beads were then boiled with 2 $\times$ SDS sample buffer [100 mM Tris/HCl, pH 6.8, 200 mM dithiothreitol, 4% (v/v) SDS, 20% (v/v) glycerol, 0.1% Bromophenol Blue] [38] and equal volumes of the samples were subjected to Western-blot analysis using a monoclonal antibody against RhoA (Santa Cruz). To normalize the amount of GTP-bound Rho to total amount of Rho, equal volumes of cell lysates were subjected to Western-blot analysis using the RhoA monoclonal antibody.

Rac1- and Cdc42-activation assay

Activity of Rac 1 and Cdc42 was estimated using a similar procedure to that described for RhoA activity measurements. Briefly, cells were washed and then incubated with lysis buffer A for 10 min at 4°C and cell lysates were centrifuged at 1000 *g* for 3 min. To prepare the beads for the pull-down assay of cellular Rac1/Cdc42 in the GTP-bound form, bacteria containing plasmid pGST-PBD (kindly provided by Dr R. A. Cerione, Cornell University, NY, U.S.A.), were incubated until D_{600} reached 0.8. The bacteria were treated with 1 mM IPTG for 2 h and the bacterial lysates containing GST-PBD fusion protein were incubated with glutathione-Sepharose-4B beads. Subsequently, equal amounts of the GST-PBD beads were incubated with the cell lysates for 45 min on ice and the beads were washed with AP buffer. The beads were then boiled with 2 $\times$ SDS sample buffer and equal volumes of the samples were subjected to Western-blot analysis using a monoclonal antibody against Rac1 or Cdc42 (Upstate Biotechnology, Lake Placid, NY, U.S.A.). To normalize the amount of GTP-bound Rac1 or Cdc42 to total

amount of Rac1 or Cdc42, equal volumes of cell lysates were also subjected to Western-blot analysis using the Rac1 or Cdc42 monoclonal antibody.

Statistical analyses

Results were expressed as means $\pm$ S.E.M. Data were analysed using the SIGMA-STATTM professional statistics software program (Jandel Scientific Software, San Rafael, CA, U.S.A.). Analyses of variance with protected *t* test (ANOVA) were used for intergroup comparison.

RESULTS

SP stimulates IL-8 expression in human colonocytes expressing NK-1R

Our laboratory and others have demonstrated that SP–NK-1R interaction plays an important role in the pathophysiology of intestinal inflammation [8,12,14,39,40]. Moreover, SP has been associated with IL-8 secretion in the pathogenesis of experimental pancreatitis [41]. SP can directly stimulate IL-8 expression in human U-373 MG astrocytoma cells, which express endogenous NK-1R in a NF- κ B-dependent manner [21]. Thus, we examined whether SP directly stimulates IL-8 secretion in colonic epithelial cells and determined the mechanism of this response as it relates to intestinal inflammation. For this purpose, we used a non-transformed human colonic epithelial cell line, NCM460 [32]. This cell line was previously used to study the molecular mechanisms by which neurotensin induces IL-8 expression [33]. First, we examined whether SP stimulates IL-8 secretion in the NCM460 cells. This demonstrated that SP did not stimulate IL-8 secretion (SP 10^{-6} M, from 4–24 h) or MAPK phosphorylation (SP 10^{-6} M, from 2–30 min) in NCM460 cells (results not shown). Since expression of the NK-1R is dramatically increased, in various cell types including epithelial cells, during small intestinal and colonic inflammation [10,42–44], we sought to ectopically express NK-1R cDNA in NCM460 cells by retroviral infection (the resulting cells were named NCM460-NK-1R cells). The abundance of NK-1R in the NK-1R-expressing cell line was measured by the radioactive-ligand binding assay with the highly selective NK-1R ligand [³H][Sar⁹,Met(O₂)¹¹]SP as described previously [33]. The number of NK-1R appeared to be approximately 320000 receptors per cell, which is twice as many as that in the human UC11 astrocytoma cell line that expresses endogenous NK-1R [45], suggesting that the levels of NK-1R expressed by our retroviral expression system are close to the physiological levels. To determine whether SP could stimulate IL-8 expression in NCM460-NK-1R cells, the cells were treated with SP for various times at a concentration of 10^{-7} M (Figure 1A) or at various concentrations for 4 h (Figure 1B), and IL-8 was measured in the conditioned media by ELISA. The results show that SP stimulated IL-8 secretion in a time- and dose-dependent manner, with a maximal induction at 16 h after SP treatment (Figures 1A and 1B). IL-8 levels were reduced after 24 h (Figure 1A). The average levels of stimulation of IL-8 secretion by SP in NCM460-NK-1R cells were similar to that induced by SP in the U373MG human astrocytoma cell line [21]. We then examined whether SP-induced IL-8 protein production occurred at the transcriptional level using an IL-8 promoter-driven reporter assay. The results showed that SP strongly stimulated IL-8 promoter activity (Figure 1C).

RhoA activation is required for SP-induced IL-8 expression

To determine whether the small GTPase RhoA is involved in SP-induced IL-8 expression, we first determined whether SP activates

Figure 1 NT stimulates IL-8 secretion and gene transcription in non-transformed human colonocytes expressing NK-1R

(A) NCM460 cells stably transfected with NK-1R (NCM460-NK-1R) were incubated with serum-free M3D media (INCELL Co., San Antonio, TX, U.S.A.) for 24 h and then treated with SP (10^{-7} M) for the indicated time points. Conditioned media were then collected and IL-8 was measured by ELISA. (B) NCM460-NK-1R cells were rendered quiescent, treated for 4 h with SP at the indicated concentrations, and IL-8 was measured in the conditioned media by ELISA. (C) NCM460-NK-1R cells were transiently transfected with human IL-8 promoter-luciferase construct and then serum-starved for 24 h before 4 h stimulation with SP (10^{-7} M). Relative IL-8 promoter activity was measured as described in the Materials and methods section.

RhoA in NCM460-NK-1R cells. RhoA activity was measured using a pull-down assay as described in the Materials and methods section. Cells were treated with SP, and cell extracts were obtained and incubated with the RhoA-binding-domain-GST-fusion protein conjugated to Sepharose beads. The bound protein was eluted and subjected to Western-blot analysis using a monoclonal antibody against RhoA. The results showed that SP strongly stimulated RhoA-GTP loading as early as 1 min after SP treatment (Figure 2A). Next we examined whether RhoA activation is involved in SP-induced IL-8 expression by overexpressing dominant negative RhoA (RhoA-19N) in NCM460-NK-1R cells. To determine the effect of RhoA-19N on SP-induced IL-8 secretion, quiescent cells infected with a β -

Figure 2 SP-induced IL-8 expression requires RhoA activation

(A) Quiescent NCM460-NK-1R cells were treated with SP (10^{-7} M) for the indicated times. Cell extracts were used to determine the levels of GTP-bound RhoA as described in the Materials and methods section. The results represent one of three independent experiments. (B) Quiescent NCM460-NK-1R cells infected with LacZ- or RhoA-19N-expressing viruses were treated with SP (10^{-7} M) for 4 h and the levels of IL-8 protein in the conditioned media were measured by ELISA. (C) NCM460-NK-1R cells were transiently transfected with IL-8 promoter reporter construct together with LacZ- or RhoA-19N-expressing plasmid. The transfected cells were serum-starved and treated with SP (10^{-7} M) for 4 h. Cell extracts were prepared to measure IL-8 promoter activity, expressed as mean $\pm$ S.E.M. (relative luciferase activity, $n = 3$). * indicates $P < 0.001$, versus LacZ-expressing cells with SP stimulation. Results are from a single experiment each with triplicate determinations, representative of three separate experiments.

galactosidase (LacZ)-expressing virus (control) or a RhoA-19N-expressing virus were treated with SP for 4 h and IL-8 was measured in the conditioned media. We found that RhoA-19N significantly decreased SP-induced IL-8 secretion (Figure 2B). We also examined whether RhoA-19 affects SP-induced IL-8 promoter activity. Cells were co-transfected with LacZ- or RhoA-19N-expressing constructs together with the IL-8 promoter reporter construct plus an internal control DNA. The transfected cells were rendered quiescent, treated with SP for 4 h, and luciferase reporter activity was determined in cell extracts. Our

Figure 3 SP-induced IL-8 expression requires Rac1 activation

(A) Quiescent NCM460-NK-1R cells were treated with SP (10^{-7} M) for the indicated times. Cell extracts were used to determine the levels of GTP-bound Rac1 as described in the Materials and methods section. The results represent one of three independent experiments. (B) Quiescent NCM460-NK-1R cells infected with LacZ- or Rac1-17N-expressing viruses were treated with SP (10^{-7} M) for 4 h and IL-8 protein was measured by ELISA in the conditioned media. (C) NCM460-NK-1R cells were transiently transfected with IL-8 promoter reporter construct together with LacZ- or Rac1-17N-expressing plasmid. The transfected cells were serum-starved and treated with SP (10^{-7} M) for 4 h. Cell extracts were prepared to measure IL-8 promoter activity, which was expressed as mean $\pm$ S.E.M. (relative luciferase activity, $n = 3$). * indicates $P < 0.01$, Rac1-17N- versus LacZ-expressing cells with SP stimulation. Results are from a single experiment each with triplicate determinations, representative of three separate experiments.

results showed that overexpression of RhoA-19N substantially inhibited SP-induced IL-8 promoter activity (Figure 2C). Together, our results indicate that this small GTPase plays an important role in IL-8 gene transcription in response to SP.

Activation of Rac1 and Cdc42 is required for SP-induced IL-8 expression

Although overexpression of either constitutive Rac1 or Cdc42 stimulates NF- κ B DNA-binding activity [23], evidence that these two proteins are involved in expression of proinflammatory

Figure 4 SP-induced IL-8 expression requires Cdc42 activation

(A) Quiescent NCM460-NK-1R cells were treated with SP (10^{-7} M) for the indicated times. Cell extracts were used to determine the levels of GTP-bound Cdc42 as described in the Materials and methods section. The results represent one of three independent experiments. (B) Quiescent NCM460-NK-1R cells infected with LacZ- or Cdc42-17N-expressing viruses were treated with SP (10^{-7} M) for 4 h and the levels of IL-8 protein in the conditioned media were measured by ELISA. (C) NCM460-NK-1R cells were transiently transfected with an IL-8 promoter reporter construct together with LacZ- or Cdc42-17N-expressing plasmid. The transfected cells were serum-starved and treated with SP (10^{-7} M) for 4 h. Cell extracts were prepared to measure IL-8 promoter activity, expressed as mean $\pm$ S.E.M. (relative luciferase activity, $n = 3$). * indicates $P < 0.01$, versus LacZ-expressing cells with SP stimulation. Results are from a single experiment each with triplicate determinations, representative of three separate experiments.

genes is lacking. To examine whether Rac1 and Cdc42 GTPases play a role in SP-induced IL-8 expression, we first determined whether SP activates them. NCM460-NK-1R cells were treated with SP for various times and cell extracts were used to measure the activity of Rac1 and Cdc42 using a pull-down assay as described in the Materials and methods section. As shown in Figure 3(A) and Figure 4(A), respectively, SP strongly stimulated the formation of both Rac1-GTP and Cdc42-GTP. We also examined whether Rac1 and Cdc42 activation was also involved

Figure 5 SP stimulates Rho-dependent IL-8 promoter activity in both high and low levels of NK-1R expression

NCM460 cells were transiently transfected with either 0.1 or 0.025 μ g of NK-1R expression plasmid/well (12-well plates) plus equal amount of the IL-8 promoter-luciferase construct, the internal control pRL-TK and pCMBP-lacZ, pCMBP-RhoA-19N, pCMBP-Rac1-17N or pCMBP-Cdc42-17N as indicated. The total amount of DNA for each well was made equal by supplementing with pCMBP-LacZ. After 16 h, cells were serum starved for 24 h and then treated with SP (10^{-7} M) for 4 h. Relative IL-8 promoter activity was measured. * denotes $P < 0.01$, and ** denotes $P < 0.001$, versus LacZ-expressing cells with SP stimulation.

in SP-induced IL-8 secretion and IL-8 promoter activity by overexpressing dominant negative mutants of Rac and Cdc42 (Rac1-17N and Cdc42-17N). Quiescent NCM460-NK-1R cells, infected with LacZ- (control), Rac1-17N-, or Cdc42-17N-expressing viruses, were treated with SP for 4 h and IL-8 levels measured in the conditioned media. Our results showed that Rac1-17N (Figure 3B) and Cdc42-17N (Figure 4B) significantly decreased SP-induced IL-8 secretion. We further examined whether Rac1-17N or Cdc42-17N affects SP-induced IL-8 promoter activity. Cells were co-transfected with LacZ, Rac1-17N or Cdc42-17N-expressing constructs together with an IL-8 promoter reporter construct. Transfected cells were rendered quiescent, treated with SP for 4 h, and luciferase-reporter activity was measured in the cell extracts. The results showed that overexpression of Rac1-17N (Figure 3C) and Cdc42-17N (Figure 4C) significantly inhibited SP-induced IL-8 promoter activity.

Rho GTPases mediate SP-induced IL-8 promoter activity in both high and low levels of NK-1R expression

It was suspected that high levels of a G-protein-coupled receptor might stimulate a different signal transduction pathway from that induced by low levels of the receptor. Consequently we also conducted the experiment with two different doses of NK-1R expression construct to determine whether SP-induced IL-8 promoter activity was mediated through the Rho pathway in high and low levels of NK-1R. To do this, the parental NCM460 cells were transiently transfected with two different doses (0.1 and 0.025 μ g/well in 12-well plates) plus dominant negative Rho mutants and IL-8-luciferase constructs as indicated. The results indicated that, compared with the control, the higher and the lower doses stimulated IL-8 promoter activity by 26- and 7-fold

Figure 6 SP stimulates SRE-driven gene expression in a Rho GTPase-dependent manner

NCM460-NK-1R cells were transiently transfected with a SRE-luciferase reporter construct together with LacZ-, RhoA-19N-, Rac1-17N- or Cdc42-17N-expressing plasmids. The transfected cells were serum-starved and treated with SP (10^{-7} M) for 4 h. Cell extracts were prepared to measure SRE promoter activity, expressed as mean $\pm$ S.E.M. (relative luciferase activity, $n = 3$).

respectively, which is consistent with reduced levels of NK-1R expression (Figure 5). More importantly our data also show that dominant negative RhoA, Rac1 and Cdc42 all significantly decreased SP-induced IL-8 promoter activity in both higher and lower levels of NK-1R expression (Figure 6). These results suggest that the role of Rho GTPases in SP-stimulated IL-8 gene expression is not due to the artificial effect of overexpressed NK-1R, at least in non-transformed human colonic epithelial cells.

SP stimulates Rho-dependent SRE-driven gene expression

Stimulation of SRE-mediated gene expression is a commonly used parameter of Rho-dependent signalling, and Rho GTPases exert this effect primarily through the transcription factor SRF (serum response factor) [46]. Here we determined whether SP stimulates SRE-driven luciferase activity and examined whether this activity involved Rho GTPases. NCM460-NK-1R cells were transiently transfected with the SRE-luciferase construct together with LacZ-, RhoA-19N-, Rac1-17N- or Cdc42-17N-expressing plasmids. The transfected cells were then treated with SP for 4 h and luciferase-reporter activity was measured. The data show that SP increased luciferase activity by approximately 15-fold and that co-transfection with RhoA-19N, Rac1-17N, or Cdc42-17N significantly reduced SP-induced luciferase activity (Figure 6). These results indicate that SP stimulates this typical Rho-dependent pathway.

Activation of RhoA, Rac1 and Cdc42 mediates SP-induced NF- κ B-dependent gene expression

Previous results indicated that SP-stimulated IL-8 expression in U373MG cells involved a NF- κ B-dependent pathway [21]. Thus, we examined next whether the effect of the dominant negative mutants of Rho-19N, Rac1-17N or Cdc42-17N on SP-induced IL-8 expression in NCM460-NK-1R is mediated through NF- κ B. To do this, cells were transfected with LacZ-, Rho-19N-,

Figure 7 SP-induced NF- κ B-dependent gene expression requires activation of RhoA, Rac1 and Cdc42

NCM460-NK-1R cells were transiently transfected with a NF- κ B promoter reporter construct together with LacZ-, RhoA-19N-, Rac1-17N- or Cdc42-17N-expressing plasmids. The transfected cells were serum-starved and treated with SP (10^{-7} M) for 4 h. Cell extracts were prepared to measure NF- κ B promoter activity, which was expressed as mean $\pm$ S.E.M. (relative luciferase activity, $n = 3$). * indicates $P < 0.01$, versus LacZ-expressing cells with SP stimulation. Results are from a single experiment each with triplicate determinations, representative of three separate experiments.

Rac1-17N- or Cdc42-17N-expressing constructs together with a NF- κ B promoter reporter construct and an internal control plasmid. The transfected cells were serum-starved, treated with SP for 4 h, and NF- κ B promoter activity was measured in cell extracts. We found that overexpression of Rho-19N, Rac1-17N or Cdc42-17N significantly inhibited SP-induced NF- κ B-dependent reporter gene expression (Figure 7).

DISCUSSION

Previous reports indicate that expression of SP and NK-1R is highly induced in several forms of enterotoxin-mediated intestinal inflammation [5,10,39,42,43]. Evidence also suggests a major requirement for NK-1R-mediated release of pro-inflammatory cytokines in the pathogenesis of inflammatory diarrhoea [14]. In this study we utilised the NCM460 non-transformed human colonic epithelial cells to examine the signalling pathway involved in SP-mediated transcriptional activation of the potent chemo-attractant IL-8. Our results demonstrated that exposure of NCM460 cells, infected with NK-1R-expressing viruses to SP, resulted in a time- and dose-dependant increase of IL-8, consistent with previous observations in U373MG cells [21]. Interestingly we report here, for the first time, that SP activates the Rho family GTPases, RhoA, Rac1 and Cdc42, and that activation of these proteins is required for SP-induced IL-8 secretion and gene transcription. In addition, activation of RhoA, Rac1 and Cdc42 mediates SP-induced NF- κ B dependent gene expression. Thus, activation of RhoA, Rac1 and Cdc42 is an important step in SP-induced signalling of NF- κ B activation and IL-8 gene transcription.

An important novel finding of this study was that SP-NK-1R interaction activated the Rho family proteins RhoA, Rac1 and Cdc42 and that this activation was required for SP-mediated

IL-8 production, and NF- κ B-dependent gene expression. This effect does not appear to be due to spurious signalling results from NK-1R overexpression, as significantly reduced expression of NK-1R also stimulates Rho-dependent IL-8 promoter activity. It is known that Rho family proteins mediate many cellular responses stimulated by a variety of extracellular factors, including growth factors, cytokines, and G-protein-coupled receptor (GPCR) ligands [24–26]. For example, RhoA activation was shown to be involved in IL-8 expression stimulated by lipopolysaccharide [27] and IL-1 β expression by bradykinin, a GPCR ligand [29]. Our data indicated that, in addition to RhoA, Rac1 and Cdc42 are also important for pro-inflammatory responses mediated by GPCRs such as the NK-1R. The molecular mechanism(s) whereby Rho family GTPases activate NF- κ B-dependent gene expression is not clear at present. RhoA activation leads to bradykinin receptor-stimulated NF- κ B DNA binding activity in human lung epithelial cells [29]; however, Rac1 regulates IL-1-induced NF- κ B-dependent reporter gene expression without causing I κ B α degradation and nuclear translocation of NF- κ B [47]. Instead, Rac1 activates both p38 and p42/p44, leading to enhanced transactivation by p65 [47]. Further work is needed to identify the intermediate molecules linking activation of Rho GTPases and the NF- κ B pathway in response to SP, and to determine the mechanism of SP-induced activation of Rho GTPases.

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