INFLAMMATION AND INFLAMMATORY BOWEL DISEASE

# Tumour necrosis factor α potentiates ion secretion induced by histamine in a human intestinal epithelial cell line and in mouse colon: involvement of the phospholipase D pathway

**J C J Oprins, C van der Burg, H P Meijer, T Munnik, J A Groot**

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See end of article for authors' affiliations

Correspondence to: J C J Oprins, Swammerdam Institute for Life Sciences, University of Amsterdam, PO Box 94084, 1090 GB Amsterdam, the Netherlands; oprins@science.uva.nl

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**Background and aims:** Patients suffering from inflammatory bowel disease show increased levels of the mast cell products histamine and tumour necrosis factor α (TNF-α). Treating these patients with antibodies against TNF-α diminishes the symptoms of diarrhoea. In this study, the effect of TNF-α on ion secretion induced by the mast cell mediator histamine in HT29cl.19A cells and mouse distal colon was investigated and the possible second messengers involved were studied.

**Methods:** Electrophysiology of filter grown HT29cl.19A cells and isolated mouse distal colon was used to monitor the secretory response to histamine with and without prior exposure to TNF-α for 3–24 hours. Phospholipase D (PLD) activity and phosphatidic acid levels were analysed by $^{32}P_i$ labelling of HT29cl.19A cells.

**Results:** In both experimental systems TNF-α was found to potentiate ion secretion induced by histamine. Phospholipid analysis of HT29cl.19A cells revealed that histamine activates the PLD pathway. Furthermore, TNF-α pretreated cells were found to have decreased phosphatidic acid levels, the intermediate product of the PLD pathway, which indicates upregulation of the enzyme phosphatidic acid phosphatase.

**Conclusions:** The mast cell products TNF-α and histamine synergistically stimulate ion secretion in intestinal epithelium via upregulation of the PLD pathway.

Patients suffering from inflammatory bowel diseases (IBD) such as ulcerative colitis or Crohn's disease experience severe diarrhoea. The underlying mechanism of the diarrhoea remains uncertain. Patients show increased release of mast cell products such as histamine and also tumour necrosis factor α (TNF-α).[1 2 3] A number of studies have supported a predominant role for the cytokine TNF-α in the pathogenesis of IBD. In bowel mucosa of IBD patients, mRNA levels of TNF-α are increased[4] as well as concentrations of the protein in blood and stool.[5 6] Moreover, TNF-α secretion from infiltrated inflammatory cells is enhanced in biopsies from these patients.[3] Antibodies against this cytokine appear to be successful in the therapy of patients with Crohn's disease resulting in diminished symptoms of the disease.[7] These data imply that TNF-α plays an important role in the pathogenesis of diarrhoea in these patients.

Chloride secretion across the intestinal epithelium plays an important role in water secretion into the lumen of the intestine. Increased chloride secretion can result in severe diarrhoea due to excessive water secretion into the lumen. In several models it was shown that histamine increased chloride secretion. Histamine is thought to stimulate ion secretion in colonic epithelium via increased prostaglandin release or via the augmenting effects of endogenously released neurotransmitter.[8–11] In the intestinal epithelial cell line T84, histamine increases chloride secretion via increased intracellular calcium levels due to activation of the phospholipase C pathway by the histamine receptor.[12] This pathway resembles that of muscarinic receptor activation and in HT29cl.19A cells intracellular electrophysiological effects of histamine resemble those of carbachol (see below).

Recently, we showed that TNF-α potentiated carbachol induced chloride secretion in HT29cl.19A cells in a protein synthesis dependent manner. We deduced that the potentiation could be a result of increased protein kinase C (PKC) activity as a consequence of increased 1,2-diacylglycerol (DAG) levels due to upregulation of the phospholipase D (PLD) pathway.[13] This proposition was corroborated by analysis of membrane phospholipids.[14] To our knowledge, this was the first time a potentiating effect of TNF-α on epithelial ion secretion had been shown.

Based on the similarities between activation of muscarinic receptors and histamine receptors, our aim was to study if TNF-α could also potentiate the effect of the mast cell mediator histamine. Because it is important to validate the results obtained in cell lines in native tissue, we also studied the effect of TNF-α and histamine on ion secretion in mouse isolated distal colon.

The present data showed that TNF-α potentiates ion secretion induced by histamine in HT29cl.19A cells via de novo protein synthesis and most probably by upregulating the PLD pathway, thereby generating enhanced DAG levels and subsequent PKC activation. Furthermore, we demonstrated that TNF-α also augments the secretory response to histamine in mouse distal colon indicating the generality of the observed effect in the human cell line.

The present results suggest a synergy between the mast cell products histamine and TNF-α in stimulating ion secretion in

**Abbreviations:** TNF-α, tumour necrosis factor α; PKC, protein kinase C; DAG, 1,2-diacylglycerol; PLD, phospholipase D; PA, phosphatidic acid; PAP, PA phosphatase; PBut, phosphatidylbutanol; IBD, inflammatory bowel disease; $V_t$, transepithelial potential; $V_a$, intracellular potential; $I_{sc}$, short circuit current; $R_t$, transepithelial resistance; $fR_a$, fractional resistance; $PIP_2$, phosphatidylinositol 4,5-bisphospate; TTX, tetrodotoxin; IFN-γ, interferon γ; $IP_3$, inositol 1,4,5-trisphosphate.

intestinal epithelium, and this may contribute to symptoms in patients with IBD.

## MATERIAL AND METHODS

### Cell culture

HT29cl.19A cells were cultured as described previously.[15] Briefly, the human intestinal epithelial cell line HT29cl.19A, passages 17–29, was grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 8 mg/l ampicillin, and 10 mg/l streptomycin. Cells were seeded in 25 $cm^2$ flasks at 37°C in 5% $CO_2$–95% $O_2$ and passaged weekly. For the experiments, cells were subcultured on Falcon filters (25 mm Ø), and medium was replaced every other day. Confluency was reached seven days after seeding, and filters with cells were used between 13 and 19 days post seeding. TNF-α incubations were performed in culture media during the indicated times.

### Electrophysiological experiments with HT29cl.19A cells

The filter was cut from the ring, divided into four pieces, and rinsed with mannitol-Ringer. One piece was mounted in a small horizontal Ussing chamber, leaving an oblong area of 0.35 $cm^2$. The apical and basolateral compartments were continuously perfused with mannitol-Ringer buffer at a temperature of 37°C and gassed with 5% $CO_2$–95% $O_2$. The composition of Ringer's solution was (in mM): NaCl 117.5, KCl 5.7, $NaHCO_3$ 25.0, $NaH_2PO_4$ 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2, and mannitol 28. Agar bridges were placed in apical and basolateral compartments and were connected to Ag-AgCl electrodes for monitoring the transepithelial potential difference ($V_t$). An extra Ag-AgCl electrode was placed in the apical bath, serving as a common ground. Apical membrane potential ($V_a$) was measured by impalement with a glass microelectrode, pulled from capillaries (1 mm od; Clark Electromedical, Reading, UK) with a Flaming Brown P-87 micropipette puller. The microelectrode was filled with 0.5 M KCl solution. Tip resistance was 100–200 MΩ and tip potential 2–5 mV. Current electrodes (Ag-AgCl) were placed in the walls of both compartments; these were used to apply bipolar current pulses from a floating current source of 10 and 50 μA at 30 second intervals to calculate transepithelial resistance ($R_t$) and fractional resistance $fR_a$ ($fR_a = R_a/(R_a+R_b)$). The equivalent short circuit current ($I_{sc}$) was calculated from $V_t$ and $R_t$.

The potentials were measured differentially with M-4A electrometer probes (W-P-Instruments, New Haven, USA). Potential differences were continuously recorded on a multipen recorder and on a computer using custom made software.

Measurements were corrected for the offset of the electrodes and for the resistances of the fluid and filter without cells.

### Phospholipid analysis in HT29cl.19A cells

Cells were labelled for 24 hours with $1.85\times10^6$–$3.7\times10^6$ Bq $^{32}P_i$ at the mucosal side and were washed with Ringer's solution to remove unincorporated $^{32}P_i$. Cells were stimulated for 30 minutes with 100 μM histamine in the presence of 0.05% 1-butanol (v/v). The concentration of 1-butanol and the time of incubation were chosen based on results described in a previous study.[14] Reactions were stopped with 0.1 N HCl. Cells are frozen for 30 minutes (−20°C) and scraped off the filters, and lipids were extracted as described previously.[16]

Extracted lipids were separated on heat activated TLC plates using an ethyl acetate solvent system (ethyl acetate/*iso*-octane/formic acid/water (13:2:3:10, by vol)).[17] The TLC plate was exposed to a photographic film overnight and levels of $^{32}P$ labelled phosphatidic acid (PA) and phosphatidylbutanol (PBut) were quantified by phosphoimaging (Storm, Molecular Dynamics).

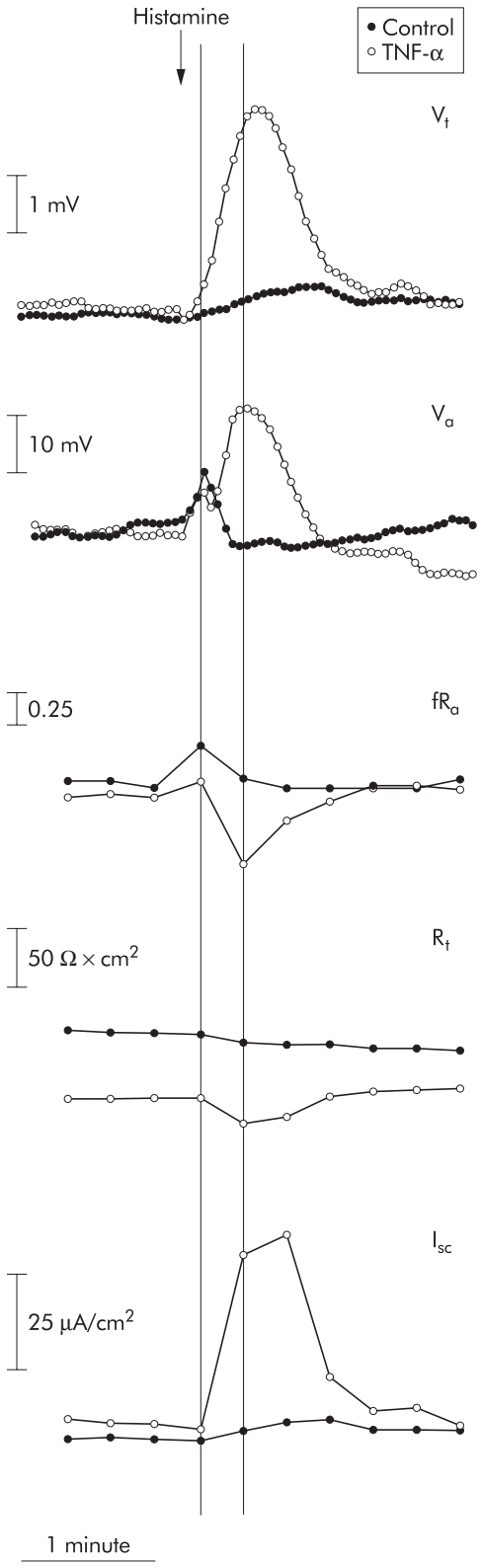


**Figure 1** Changes in the electrophysiological parameters of HT29cl.19A cells after addition of 100 μM histamine in the absence or presence of 10 ng/ml tumour necrosis factor α (TNF-α). Two tracings (control and TNF-α treated groups) represent experiments in which intracellular recordings were obtained. Two thin lines divide the response into phases 1 and 2, as described in the results. $V_t$, transepithelial potential; $V_a$, intracellular potential; $fR_a$, fractional apical resistance ($fR_a = R_a/(R_a+R_b)$); $R_t$, transepithelial resistance; $I_{sc}$, short circuit current. (For statistics see table 1.)

### Tissue preparation

Female Balb/C mice were used. They had access to food and water ad libitum. The animals were killed by cervical dislocation. The intestine was cut proximal from the caecum and

**Table 1** Electrophysiological parameters of HT29cl.19A cells before and after application of histamine

| | $V_t$ (mV) | $R_t$ ($\Omega \times cm^2$) | $V_a$ (mV) | $fR_a$ | $I_{sc}$ ($\mu A/cm^2$) |
|---|---|---|---|---|---|
| Control | | | | | |
| Baseline | 1.7 (0.7) | 195 (10) | –62 (6) | 0.67 (0.02) | 9.5 (5) |
| Histamine phase 1 | 1.6 (0.7) | 195 (10) | –58 (3) | 0.73 (0.05) | 8.9 (5) |
| Histamine phase 2 | 2.5 (0.8) | 190 (11) | –66 (7) | 0.65 (0.02) | 14 (6) |
| ΔHistamine | 0.8 (0.1) | –5 (2) | –4 (0.4) | –0.02 (0.01) | 5 (1) |
| TNF-α | | | | | |
| Baseline | 2.2 (0.7) | 173 (12) | –57 (2) | 0.70 (0.06) | 13 (4) |
| Histamine phase 1 | 2.1 (0.7) | 173 (12) | –54 (3) | 0.73 (0.05) | 12 (4) |
| Histamine phase 2 | 8.4 (1)** | 154 (11)** | –38 (2)** | 0.47 (0.07)** | 58 (11)** |
| ΔHistamine | 6.2 (0.8)** | –20 (5)** | 19 (3)** | –0.24 (0.05)** | 45 (10)** |

TNF-α, tumour necrosis factor α; $V_t$, transepithelial potential; $V_a$, intracellular potential; $I_{sc}$, short circuit current; $R_t$, transepithelial resistance; $fR_a$, fractional resistance.
Effects of addition of 100 μM histamine to the serosal side of the cells. ΔHistamine represents the difference between phase 2 and baseline.
n=5 for control experiments; n=7 for TNF-α exposed monolayers except for $V_a$ and $fR_a$ (n=5).
Mean incubation time for TNFα (10 ng/ml) was 6–24 hours.
Statistical significance was evaluated using the Students *t* test: **p<0.01 compared with control histamine.

proximal from the rectum. The colon was rinsed with inosine-Ringer to remove any faeces and placed over a thin plastic rod. The distal colon was taken 2 cm distal from the caecum and cut along the mesenteric border across a length of 1 cm. The excised segments were mounted on a sledge[18] and placed in a modified Ussing chamber kept at a temperature of 37°C. The exposed area was 0.2 $cm^2$ and the volume of the serosal and mucosal bath was 2 ml each. The composition of the Ringer's solution was (in mM): NaCl 117.5, KCl 5.7, $NaHCO_3$ 25.0, $NaH_2PO_4$ 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2, inosine 5, β-OH-butyrate 0.5, glutamine 2.5+50 mg/l azlocillin (modified from Grotjohann and colleagues[19]). Ringer's solution in the two compartments was refreshed every 30 minutes to avoid bacterial overflow. The solution was circulated by a gas lift using 5% $CO_2$/95% $O_2$.

### Electrophysiological measurements in distal mouse colon

Agar bridges were placed in apical and basolateral compartments and were connected to Ag-AgCl electrodes for monitoring $V_t$. Platina electrodes were placed in serosal and mucosal chambers and current injections of +10 μA and −10 μA were given to measure $R_t$ of the epithelia. From $V_t$ and $R_t$ the short circuit current $I_{sc}$ was calculated.

Potential difference was continuously recorded on a notebook using custom made software. Measurements were corrected for the offset of the electrodes and for the resistance of the fluid.

### Materials

TNF-α and (San Diego, California, USA) GF109203X (bisindolylmaleimide I) were from Calbiochem. Propranolol, histamine, cycloheximide, azlocillin, tetrodotoxin (TTX), β-OH-butyrate, indomethacin, and amiloride were purchased from Sigma (St Louis, Missouri, USA), and 1-butanol from Brocades (Delft, the Netherlands). Silica 60 TLC plates and reagents for lipid extraction were from Merck (Darmstadt, Germany). [$^{32}$P] Orthophosphate ($^{32}P_i$; carrier free) was from Amersham International (Uppsala, Sweden). All drugs were dissolved in water except for indomethacin and cycloheximide which were dissolved in ethanol with a maximal concentration of 0.1% ethanol in the Ussing chamber. Concentrations of GF109203X, TNF-α, cycloheximide, and 1-butanol were chosen based on earlier studies with this cell line.[13] Concentrations were taken from the literature for histamine, propranolol, indomethacin, and amiloride.[20–22]

### Statistics

Data are presented as means (SEM). Statistical significance was evaluated using a Student's *t* test or a non-parametric test. In the corresponding text, p values are given and in the legends the test is indicated.

## RESULTS

### Electrophysiology in HT29.cl19A cells

#### TNF-α potentiates ion secretion induced by histamine in HT29cl.19A cells

A typical electrical response to 100 μM histamine in HT29cl.19A cells is shown in fig 1. The response is divided into two phases: the first from the time of histamine application to the first vertical thin line (fig 1) and the second as the period thereafter. In table 1, the electrophysiological parameters for the control responses are summarised. They were taken at the two thin vertical lines indicated in fig 1. Application of 100 μM histamine to the serosal side of the cells resulted in fast depolarisation of the apical membrane potential ($V_a$) together with an increase in $fR_a$ during phase 1 without a significant change in $I_{sc}$ or $V_t$. In phase 2, depolarisation of $V_a$ was followed by repolarisation leading to hyperpolarisation, concomitantly with an increase in $V_t$, decrease in $R_t$, and increase in $I_{sc}$. $fR_a$ returned to basal values. To our knowledge, this response has not been described previously in detail. Muscarinic receptor activation by carbachol triggers the same intracellular response (although usually larger) and thus based on earlier studies with carbachol[23] we explain the changes in the parameters as follows. The first phase—depolarisation without a change in $V_t$—is attributed to opening of the chloride channels located in both membranes of the cells which are activated by increased intracellular calcium levels, primarily from the inositol 1,4,5-trisphosphate ($IP_3$) sensitive intracellular pool. The following hyperpolarisation of $V_a$ and increase in $V_t$ and $I_{sc}$ is ascribed to increased calcium dependent potassium conductance located in the basolateral membrane. This increases the driving force for chloride efflux through the apical membrane through chloride channels, which are believed to be activated by PKC-α.[24]

Incubation for 24 hours with 10 ng/ml TNF-α (bilaterally) did not change basal electrophysiological parameters. The parameters, representing seven experiments, are presented in table 1. In fig 1 a typical electrophysiological response to histamine after exposure to TNF-α is shown. After exposure of cells to TNF-α, the first phase depolarisation was not affected. However, instead of repolarisation in the second phase, a prolonged depolarisation of $V_a$ occurred in TNF-α exposed cells. The decrease in $fR_a$ was significantly larger. The change in $V_t$ was increased and the change in $R_t$ was also significantly larger. The histamine induced increase in $I_{sc}$ was ninefold larger after exposure to TNF-α. The effects of exposure to

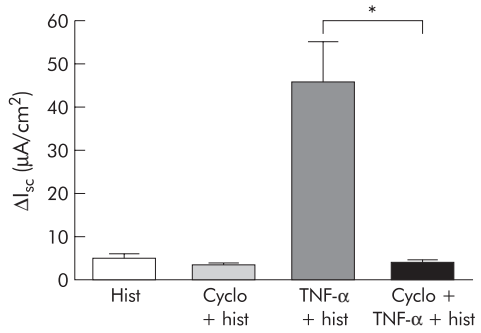


**Figure 2** Effect of cycloheximide on maximal change in short circuit current ($I_{sc}$) induced by histamine (hist) with or without exposure to tumour necrosis factor α (TNF-α). Cells were incubated with 10 μg/ml cycloheximide (cyclo) bilaterally for one hour before TNF-α exposure (4–5 hours). Data are presented as means (SEM) from 3–7 monolayers. Statistical significance was evaluated using the non-parametric Mann-Whitney test: *p<0.05.

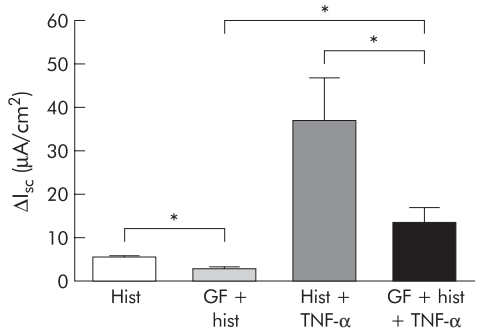


**Figure 3** Effect of GF109203X on the maximal change in short circuit current ($I_{sc}$) induced by histamine with or without exposure to tumour necrosis factor α (TNF-α). Cells were incubated with 1 μM GF109203X (GF) 30 minutes prior to addition of histamine (hist). Data are presented as means (SEM) from 5–6 monolayers. Cells were exposed to TNF-α for 5–24 hours. Statistical significance was evaluated using the Student's *t* test: *p<0.05.

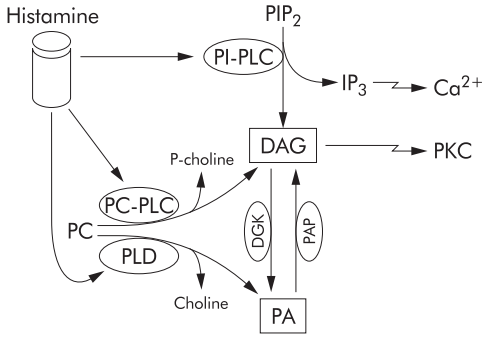


**Figure 4** Schematic representation of the signalling mechanisms involved in histaminic receptor activation. PC, phosphatidylcholine; $PIP_2$, phosphatidylinositol 4,5-bisphosphate; PI-PLC, phosphatidylinositol phospholipase C; PLD, phospholipase D; DAG, 1,2-diacylglycerol; PA, phosphatidic acid; $IP_3$, inositol 1,4,5-trisphosphate; DGK, DAG kinase; PAP, phosphatidic acid phosphatase.

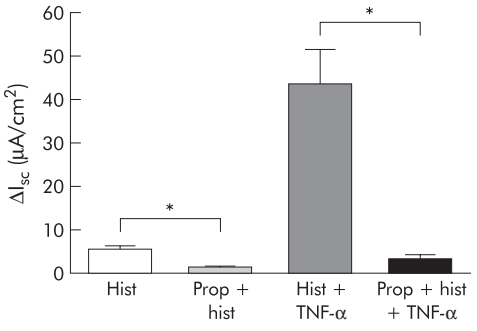


**Figure 5** Effect of propranolol on the maximal change in short circuit current ($I_{sc}$) induced by histamine with or without exposure to tumour necrosis factor α (TNF-α). Cells were incubated with 100 μM propranolol (prop) 10 minutes prior to addition of histamine (hist). Data are presented as means (SEM) from 3–4 monolayers. Cells were exposed to TNF-α for 5–24 hours. Statistical significance of the differences in change in $I_{sc}$ was evaluated using the non-parametric Mann-Whitney test: *p<0.05.

TNF-α on the response to histamine are similar to its potentiating effect on the response to carbachol where we argued that this must be due to increased apical chloride conductance.[13]

## Cycloheximide prevents the potentiating effect of TNF-α

To examine whether the action of TNF-α was dependent on protein synthesis, we incubated cells with the protein synthesis inhibitor cycloheximide one hour prior to TNF-α application and measured $I_{sc}$ induced by histamine. Addition of 10 μg/ml cycloheximide did not affect basal parameters (not shown). Figure 2 shows that $I_{sc}$ induced by histamine was not significantly affected after application of cycloheximide over one hour (5.0 (1) μA/cm² for controls *v* 3.5 (0.4) μA/cm² for cycloheximide; n=3). However, in the presence of cycloheximide the potentiating effect of TNF-α on histamine-induced $I_{sc}$ was completely inhibited (45 (9.6) μA/cm² for TNF-α *v* 3.7 (0.7) μA/cm² for cycloheximide+TNF-α; p<0.001; n=4), as shown in fig 2. These results show that the effect of TNF-α is dependent on de novo protein synthesis.

## Involvement of PKC in the histamine response

To investigate the role of PKC in the secretory response to histamine and in the potentiating effect of TNF-α, cells were preincubated with 1 μM GF109203X on both sides of the cells over 30 minutes. We demonstrated previously that at this concentration GF109203X can be used as an inhibitor of PKC.[13] In fig 3 it is shown that incubating cells with GF109203X prior to addition of histamine decreased histamine induced $I_{sc}$ from 5.6 (0.3) to 3.1 (0.3) μA/cm² (n=5; p<0.005), suggesting a role for PKC in histaminic receptor activation. Pre-exposure with GF109203X decreased the TNF-α potentiated histamine response from 37.3 (9.7) to 13.7 (3.6) μA/cm² (n=6; p<0.05) but the potentiating effect of TNF-α was still present. These results indicate that PKC activation is involved in the secretory response to histamine as well as in the TNF-α potentiated response.

## Involvement of PLD in the histamine response

In our previous study[13] we argued that exposure of cells to TNF-α would make them more susceptible to PKC activation by muscarinic receptor related intracellular messengers—for example, via increased DAG production. DAG can be generated via different pathways. The phosphatidylinositol-PLC pathway has been identified as necessary for histamine receptor activation in intestinal epithelial cells and hydrolyses the membrane lipid phosphatidylinositol 4,5-bisphospate ($PIP_2$) into DAG and $IP_3$.[25] $IP_3$ is related to increased intracellular calcium which determines the first phase of the electrophysiological response. Because this phase was unchanged, we excluded activation of this pathway by TNF-α. Furthermore, DAG can be generated by hydrolysis of structural phospholipids, such as phosphatidylcholine by PLD via the intermediate product PA. PA is subsequently hydrolysed by phosphatidic acid phosphatase (PAP) to DAG. A schematic presentation of the second messenger pathways involved in histaminic receptor activation is presented in fig 4.

To investigate the role of the PLD/PAP pathway in the histamine response, we used propranolol to inhibit PAP.[21] In a previous study we have shown that in these cells levels of PA

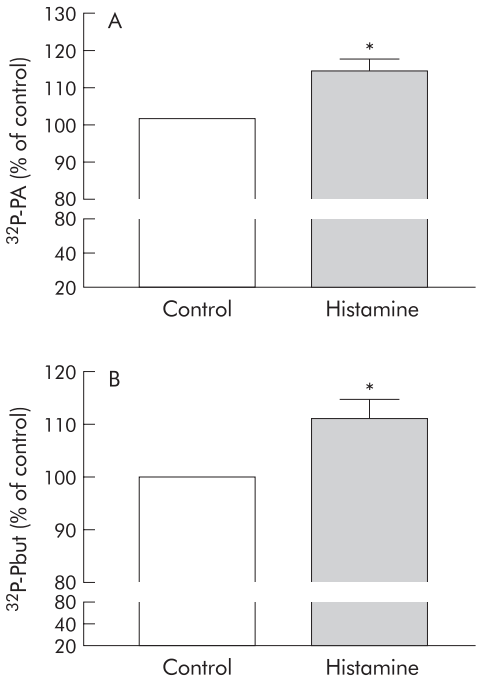


**Figure 6** Effect of histamine on levels of $^{32}$P-phosphatidic acid (PA) (A) and $^{32}$P-phosphatidylbutanol (PBut) (B) in HT29cl.19A cells. Cells were stimulated for 30 minutes with 100 μM histamine in the presence of 0.05% 1-butanol. Data are shown as percentage of control monolayers (100%). The basal $^{32}$P-PA level was 2.32 (0.28)% of total labelled phospholipids and basal $^{32}$P-PBut was 0.38 (0.06)% of total labelled phospholipids. About 20% of the total label was incorporated into phosphatidylcholine. The other labelled phospholipids cannot be distinguished on this system. Data are presented as means (SEM) of five monolayers. Statistical significance was evaluated using the Student's *t* test: *p<0.05.

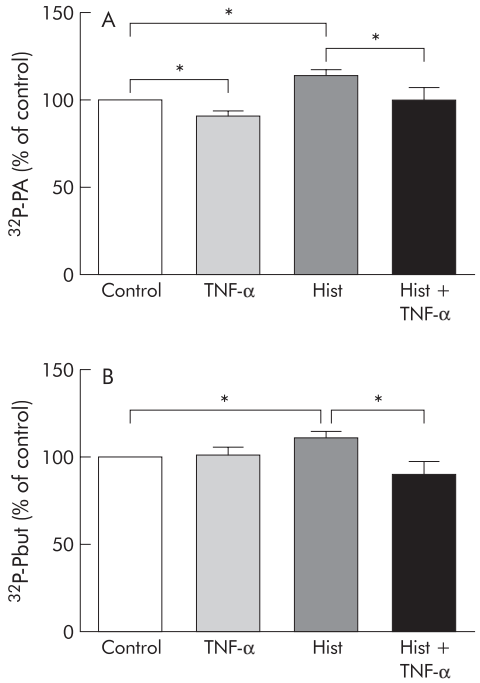


**Figure 7** Effect of tumour necrosis factor α (TNF-α) on basal and histamine induced levels of $^{32}$P-phosphatidic acid (PA) (A) and $^{32}$P-phosphatidylbutanol (PBut) (B). Cells were exposed to 10 ng/ml TNF-α over the 24 hour labelling period. Cells were stimulated with 100 μM histamine (hist). Application of histamine resulted in significantly increased levels of $^{32}$P-PA and $^{32}$P-PBut, as indicated in fig 5. Data are shown as percentage change from control experiments. Data are presented as means (SEM) of five monolayers. Statistical significance was evaluated using the Student's *t* test: *p<0.05.

increase twofold after treatment with propranolol, which confirms the inhibitory action of the substance in these cells.[14] Cells were incubated with 100 μM propranolol on the apical side of the cells for 10 minutes prior to histamine application. The change in $I_{sc}$ induced by histamine is shown in fig 5. In the presence of propranolol the increase in $I_{sc}$ by histamine was reduced from 5.7 (0.6) to 1.6 (0.6) μA/cm$^2$ (n=3; p<0.05). After exposure to TNF-α, $I_{sc}$ induced by histamine was reduced by propranolol from 43.7 (7.7) to 3.3 (1.3) μA/cm$^2$ (n=4; p<0.05). Thus the potentiating effect of TNF-α was completely abolished. These results suggest that the PAP/PLD pathway is involved in the histamine response in these cells and that TNF-α possibly exerts its potentiating effect on histamine induced secretion via upregulation of the PAP/PLD pathway.

## Phospholipid analysis

### PBut and PA formation by histamine

Previous results suggested that the PAP/PLD pathway is involved in histaminic receptor activation and that TNF-α exerts its potentiating effect via this pathway. To investigate this we measured PLD activity. The enzyme PLD has the unique ability to transfer the phosphatidyl group of its substrate to a primary alcohol such as 1-butanol instead of water, forming phosphatidylbutanol (Pbut).[26] Unlike PA, this is a stable product and therefore the relative amount of PBut formed is a measure of PLD activation. After labelling the cells with $^{32}P_i$, cells were stimulated with histamine for 30 minutes in the presence of 0.05 % 1-butanol. Incubation time and concentration were chosen based on results described in a previous study.[14] Incubation with 100 μM histamine resulted in an increase in PA and PBut levels to 114 (3)% and 111 (3)%, respectively (n=5; p<0.05) compared with cells not exposed to histamine (100%) (fig 6A and 6B, respectively). Figure 7A shows that levels of $^{32}$P-PA decreased after 24 hours of exposure to 10 ng/ml TNF-α to 91 (3)% compared with cells without TNF-α (100%; p<0.005; n=5). Also, the increase in $^{32}$P-PA level by histamine was decreased to 88 (6)% (n=5; p<0.01) with respect to histamine alone. Exposure to TNF-α did not affect the level of $^{32}$P-PBut (101 (4)% compared with control 100%; n=5) (fig 7B). However, the presence of TNF-α significantly decreased $^{32}$P-PBut levels to 81 (7)% compared with cells exposed to histamine alone (100%; n=5; p<0.005).

These results suggest that histaminic receptor activation results in activation of the PAP/PLD pathway and that TNF-α upregulates expression or activity of PAP, resulting in decreased PA levels. Reduction in the stable $^{32}$P-PBut levels seems anomalous. A possible explanation will be given in the discussion.

## Electrophysiology of distal colon of the mouse

### TNF-α potentiates the histamine induced secretory response in mouse distal colon

It is important to know whether the previous results can be extrapolated to native tissues. Therefore, the effect of TNF-α on ion secretion induced by histamine in mouse distal colon was studied. We chose the mouse colon as the cell line we used represents colonic epithelia and because mouse recombinant TNF-α was available.

One piece of distal colon was removed from each mouse and mounted in an Ussing chamber, as described in the materials and methods section. To diminish $I_{sc}$, which may be induced by subepithelial and neuronal factors in distal colon, we used the following agents. Amiloride (100 μM) was added immediately after mounting to block mucosal $Na^+$ influx. On the serosal side, TTX 0.3 μM was added to block neuronal activity and indomethacin 1 μM to block ion secretion induced by release of prostaglandins. Figure 8A and B show two typical

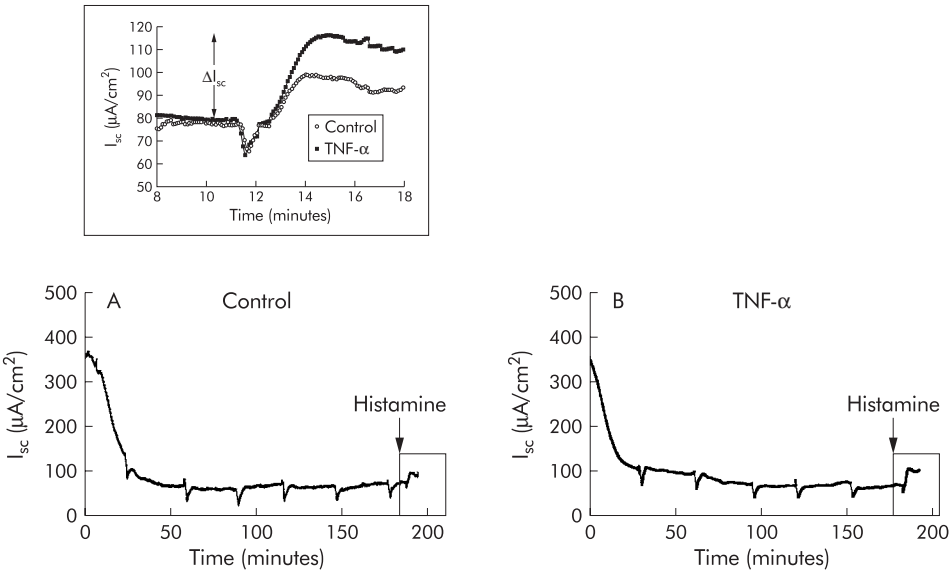


**Figure 8** Changes in short circuit current ($I_{sc}$) across mouse distal colon over a three hour period of measurements for two typical experiments of control (A) and tumour necrosis factor α (TNF-α) exposed tissues (B). At t=0 tissues were mounted and transepithelial potential and transepithelial resistance were continuously recorded. Every 30 minutes Ringer's solution was replaced which shows as a drop in $I_{sc}$ during the recording. Histamine was added at the time point indicated by the arrow. The frames are enlarged in the inset. Two typical recordings of $I_{sc}$ from mouse distal colon of control and TNF-α exposed tissues are presented in the inset. The change in current with respect to baseline levels is indicated by $\Delta I_{sc}$. The recordings represent four experiments for each group. The corresponding electrophysiological data are presented in table 2. Statistical significance was evaluated using the Student's *t* test.

recordings, each representing four experiments for control and TNF-α exposed epithelia (50 ng/ml; three hours), respectively. Every 30 minutes Ringer's solution containing antibiotics was replaced to remove possible accumulated bacteria in the chambers. After changing Ringer's solution, $I_{sc}$ recovers, which can be seen in figure 8A and 8B. In the first hour, $I_{sc}$ decreases rapidly after which it remains at a steady level. No differences in transepithelial potential or resistance were observed between control epithelia and epithelia exposed for three hours to 50 ng/ml TNF-α. In table 2 changes in electrophysiological parameters on histamine application are presented. In the inset in fig 8, enlargement of the frames indicated are presented. Addition of histamine resulted in an increase in $V_t$, preceded by a small decrease in $V_t$. $R_t$ slightly decreased after addition of histamine (−4 (1) Ω/cm²) and $I_{sc}$ increased to 21 (5) μA/cm² with respect to baseline values. After exposure to TNF-α, the change in $V_t$ was increased compared with control epithelia (n=4; $p<0.01$) and the change in $R_t$ was larger than that in control experiments (n=4; $p<0.01$). $I_{sc}$ induced by histamine was 2.6-fold larger than in control epithelia (n=4; $p<0.01$).

**Table 2** Electrophysiological parameters of mouse distal colon before and after application of 100 μM histamine

| | $V_t$ (mV) | $R_t$ (Ω×cm²) | $I_{sc}$ (μA/cm²) |
|---|---|---|---|
| Control | | | |
| Baseline | 2.4 (0.6) | 35 (5) | 67 (8) |
| Histamine | 2.8 (0.7) | 31 (4) | 88 (13) |
| ΔHistamine | 0.4 (0.1) | −4 (1) | 21 (5) |
| TNF-α | | | |
| Baseline | 3.6 (0.6) | 51 (11) | 77 (12) |
| Histamine | 4.8 (1.0) | 38 (7) | 130 (16)** |
| ΔHistamine | 1.1 (0.2)** | −13 (5)** | 54 (7)** |

TNF-α, tumour necrosis factor α; $V_t$, transepithelial potential; $I_{sc}$, short circuit current; $R_t$, transepithelial resistance.
Effects of addition of 100 μM histamine to the serosal side of the tissues with or without exposure to 50 ng/ml TNF-α over three hours (n=4 for the two groups).
Statistical significance was evaluated using the Student's *t* test:
**p<0.01 compared with control histamine.

These results confirm that TNF-α also potentiates histamine induced ion secretion in mouse native tissue.

## DISCUSSION

In the present study we have shown that exposure to 10 ng/ml TNF-α potentiated ion secretion induced by histamine in the colonic epithelial cell line HT29cl.19A. The effect of TNF-α was completely blocked by cycloheximide, which indicates that de novo synthesis of a protein is involved in the action of TNF-α. TNF-α did not affect basal electrophysiological parameters. This, together with our previous finding that TNF-α potentiates ion secretion induced by carbachol,[13] suggests that TNF-α exerts its effect downstream of the histamine receptor. The intracellular recordings show that after exposure to TNF-α activation of chloride conductance, located in the apical membrane, is enhanced. It is unlikely that this chloride conductance is activated by increased calcium levels as the first phase depolarisation is not affected by TNF-α. Moreover, earlier results showed that the response to the calcium ionophore ionomycin was not affected by TNF-α exposure.[13] Apical chloride conductance can also be increased by PKC activation.[27] The present results indicate that PKC activation is involved in the histamine response in these cells and in TNF-α potentiated ion secretion. Because even in the presence of GF109203X (which only partially inhibits PKC[28]) TNF-α exerts its potentiating effect and because we found previously that TNF-α does not affect 4β-phorbol-12,13-dibutyrate induced ion secretion,[13] we hypothesise that TNF-α stimulates the synthesis of a protein involved in the second messenger pathway between histaminic receptor activation and PKC activation.

The diminished electrophysiological response to histamine after propranolol exposure implicates PLD/PAP activation in histaminic receptor activation in these cells. The marked suppression (over 70%) suggests that PLD activation plays a major role in ion secretion induced by histamine. Furthermore, blocking PAP with propranolol completely prevented the potentiating effect of TNF-α, implying that TNF-α acts via this pathway. Measurement of levels of PA and the stable PLD product PBut established that application of histamine leads to activation of PLD in these cells. Activation of the PAP/PLD pathway by histamine has been described previously. In

oligodendroglioma cells and pulmonary endothelium, it was shown that histamine application resulted in PLD activation.[29 30] Interestingly, levels of PA are decreased in the presence of TNF-α. This suggests that TNF-α exerts its effect on the PAP/PLD pathway by upregulation of expression or activity of the protein PAP, thereby increasing turnover from PA to DAG. The increased formation of DAG may activate PKC. Exposure to TNF-α also results in decreased PBut levels induced by histamine. PLD activity can be enhanced by PA[31] and by $PIP_2$, which is formed by the PA dependent PIP kinase.[32] Thus when PA levels are decreased due to TNF-α-induced PAP activity, histamine may stimulate PLD to a lesser extent after TNF-α exposure, resulting in less elevated PBut levels.

Interestingly, in mouse distal colon the histamine response was also potentiated by TNF-α, which indicates the generality of the observed effect in the cell line. Apparently, TNF-α and histamine synergistically stimulate ion secretion in the distal colon also. To our knowledge this is the first study in which a direct effect of TNF-α on ion transport in intestinal tissue has been demonstrated. In porcine ileum and human distal colon, TNF-α was shown to increase basal $I_{sc}$ via increased synthesis of subepithelial prostaglandins.[33 34] We exclude this possibility in the present study as we performed our experiments in the presence of indomethacin, an inhibitor of prostaglandin synthesis.

The effect of histamine on chloride secretion in the colon has been extensively studied in different species. In mouse caecum, rat colon, guinea pig colon, porcine distal colon, and human colon histamine induces a transient increase in $I_{sc}$.[8–11 20] In the guinea pig, histamine acts via augmenting the effects of endogenously released neurotransmitters.[10] Hardcastle and Hardcastle were the first to show that histamine in rat colon induces chloride secretion partly via release of prostaglandins.[9] Also, in mouse caecum histamine causes the majority of its effect through stimulating arachidonic acid metabolism.[20] The increase in histamine related $I_{sc}$ in our study was not due to indirect neuronal stimulation or prostaglandin release as we measured the histamine response after blocking both pathways by TTX and indomethacin, respectively. The response of HT29cl.19A cells and of T84 cells[12] shows that the cell lines express histamine receptors and from our intracellular observations we conclude that histamine triggers a similar intracellular messenger system as carbachol. It remains to be established whether histamine induced ion secretion in mouse distal colon is dependent on the same intracellular messengers. We showed previously that TNF-α also potentiated ion secretion induced by carbachol in HT29cl.19A cells. This would imply that the effects of other mediators, such as bradykinin which uses the same intracellular system, can be potentiated by TNF-α via the PLD pathway.

The role of histamine and TNF-α in patients suffering from IBD has been widely studied but the precise contribution of the inflammatory mediators remains uncertain. Human intestinal mast cells are a major source of histamine and TNF-α.[35] Biopsies from IBD patients show increased levels of histamine and TNF-α.[1 3] The present study revealed that these inflammatory modulators augment synergistically ion secretion in the studied models but the precise contribution to the pathogenesis of the disease remains unknown. Crowe *et al* showed that in inflamed tissue from IBD patients, the secretory response to mast cell activation by anti-IgE was diminished.[36] They postulated that prior activation of mast cell with release of histamine may account for the reduced secretory response to anti-IgE observed in IBD colonic tissue. Others have also shown hyporesponsiveness to other secretagogues in the intestine of animal models for IBD.[37 38] It has been suggested previously that epithelial damage due to cytokines might be the underlying reason for the hyporesponsiveness but under conditions used in the present experiments we found no evidence of damage.[13] According to our data, we hypothesise that hyporesponsiveness may be due to down-regulation of one or more steps in the secretory pathway because of prolonged activity of PKC. Prolonged activation of PKC is known to inhibit basolateral $K^+$ conductance, resulting in an inability to reveal a secretory response to secretagogues. This suggests that the synergistic effect of TNF-α and histamine on ion secretion is more likely involved in an early initiating phase of the disease than longer term. Then, the synergistic effect of histamine and TNF-α on activation of PKC may account for the reduced ion transport in models of IBD.

It has been described previously that TNF-α can also act synergistically with another immunomodulator interferon γ (IFN-γ) in altering epithelial physiology in intestinal epithelial cells (T84 cells).[39 40] This is probably attributed to induction of TNF-α receptors by IFN-γ.[41] Thus it may be that in the presence of both cytokines the effect of secretagogues is even more potentiated. Recently, it has been shown that TNF-α is a stimulus for mast cells to release histamine[42] and because the release process of TNF-α is regulated differentially from degranulation,[43] it may be that TNF-α activates histamine release in an autocrine way.

In summary, TNF-α potentiates ion secretion induced by histamine in HT29cl.19A cells via a protein synthesis dependent mechanism. We hypothesise that TNF-α upregulates expression or the activity of PAP, thereby increasing DAG formation and subsequent PKC activation. This augments ion secretion induced by histamine. Interestingly, in mouse distal colon, TNF-α also potentiated histamine induced ion secretion. It remains to be seen whether the PKC and PAP/PLD pathway are involved in the secretory response to histamine in mouse distal colon and whether this mechanism is involved in the pathogenesis of IBD related phenomena.

**Authors' affiliations**
**J C J Oprins, C van der Burg, H P Meijer, T Munnik, J A Groot,** Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, the Netherlands

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