Modulation of regulatory of mechanisms of intestinal ion secretion by TNFa and NPY
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Chapter two

TNFα potentiates the ion secretion induced by muscarinic receptor activation in HT29cl.19A cells

Judith C.J. Oprins, Helen P. Meijer, Jack A. Groot

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Abstract

Chronic gastrointestinal diseases, such as ulcerative colitis and Crohn’s disease are characterized by severe diarrhea. Mucosal biopsies of these patients show enhanced levels of cytokines, secreted by infiltrated inflammatory cells. In this study we investigated the effect of the cytokine tumor necrosis factor α (TNFα) on ion secretion in human intestinal epithelial cells. The conventional microelectrode technique in the cell line HT29cl.19A was used, which allows for simultaneous measurements of transepithelial (Vt) and intracellular potential differences across the apical membrane (Va). Preincubation (2-78 hours) with 10 ng/ml TNFα did not change basal secretory activity. However, the secretory response to the muscarinic receptor agonist carbachol was strongly increased after exposure to TNFα. Application of the PKC inhibitor GF109203X inhibited the response to carbachol as well as the TNFα potentiated response, indicating that PKC mediates the effect of carbachol in this cell line. Propranolol, a substance which inhibits the phospholipase D pathway, strongly reduced the response to muscarinic stimulation and its potentiation by TNFα. The results indicate that activation of PLD is involved in ion secretion induced by muscarinic receptor activation and that TNFα can potentiate this pathway.

Introduction

Chloride secretion across intestinal epithelium plays a key role in regulating water secretion into intestinal lumen. The chloride secretion is under close regulation by hormonal, neural and paracrine mediators. An increased chloride secretion can result in severe diarrhea, due to excessive water transport from blood to lumen. Inflammatory bowel diseases (IBD) like ulcerative colitis and Crohn’s disease are characterized by diarrhea. The underlying pathophysiological mechanisms for the diarrhea remain unknown. IBD-patients show an increase in cytokines in the intestinal wall, which are secreted by infiltrated inflammatory cells [1, 2].

Antibodies against TNFα, one of the elevated cytokines, have been applied in animal models of experimental colitis. These studies suggested a role for antibodies against TNFα in the treatment of inflammatory bowel disease [3]. In a multicenter, double blind, placebo controlled trial, a single infusion of a monoclonal antibody (cA2) against this cytokine appeared to be an effective treatment in patients with Crohn’s disease [4]. This indicates the importance of TNFα in the disease. Several studies have shown that cytokines are able to alter ion transport and barrier properties of intestinal epithelium [5, 6], which could contribute to the diarrhea.

TNFα may mediate activation of the ion secretion in intestinal epithelium. But data on this matter are quite scarce. In human distal colon TNFα is shown to increase ion
secretion via an increased release of prostaglandins by subepithelial cells [7]. In porcine ileum a similar study was performed, which also showed an indirect effect of TNFα on the ion secretion [8].

Several epithelial cells are known to express TNFα receptors and, especially when the receptor density is increased by IFNγ they respond to TNFα directly by modulating the permeability of the tight junctions [9]. However, direct effects of TNFα on ion secretion are not widely studied. This study aimed to get more insight in the possible direct effect of TNFα on human intestinal epithelium. With intracellular electrophysiological techniques, the effect of human recombinant TNFα on the basal as well as the secretagogue-induced ion secretion in human colonic epithelial cells HT29cl.19A was determined.

The data show that TNFα is able to potentiate the secretion induced by the secretagogue carbachol, an activator of the Ca^{2+}/PKC mediated pathway, but not the secretion induced by forskolin, an activator of the cAMP pathway. The results indicate that carbachol-induced secretion involves activation of the phospholipase D (PLD) pathway and that the potentiation by TNFα occurred via that pathway. As far as we know this is the first study in intestinal epithelial cells, which shows a direct effect of TNFα on carbachol-induced ion secretion and of the involvement of PLD in the intestinal secretory response.

Material and Methods

Cell culture

HT29cl.19A cells were cultured as described previously [10]. Briefly, the human intestinal epithelial cell line HT29cl.19A, passage number 12-28, 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 cm² flasks at 37 °C in 5 % CO₂/95 % O₂ and passaged weekly. For electrophysiological experiments, cells were subcultured on Falcon filters (25 mm diameter), from which medium was replaced every other day. Confluency was reached 7 days after seeding, and the cells were used between 13 and 26 days after seeding. TNFα incubations were performed in culture media during the indicated time.

T84 cells were kindly provided by Dr. H.R. de Jonge, Dept. of Biochemistry, University of Rotterdam. The cells, passage number 21-29, were grown in Dulbecco’s Modified Eagle’s medium and F12 medium in a mixture of 1:1. The medium was supplemented with 10% fetal bovine serum, 8 mg/ml ampicillin and 10 mg/ml streptomycin. The cells were subcultured as described above.
Electrophysiological experiments

The filter was cut from the ring, divided into 4 pieces, and rinsed with mannitol-ringer. One piece was mounted in a small horizontal Ussing chamber, leaving an oblong area of 0.35 cm². The apical and basolateral compartments were continuously perfused with mannitol-Ringer buffer at a temperature of 37 °C and gassed with 5 % CO₂/95 % O₂. The composition of the Ringer’s solution was (in mM): NaCl 117.5, KCl 5.7, NaHCO₃ 25.0, NaH₂PO₄ 1.2, CaCl₂ 2.5, MgSO₄ 1.2, and mannitol 28. In order to increase the driving force for chloride efflux we used a low (0.1 mM) chloride solution containing (in mM): NaCl 0.1, Na-gluconate 117.2, K-gluconate 5.7, CaSO₄ 2.5, MgSO₄ 1.2, NaH₂PO₄ 1.2, NaHCO₃ 25 and Mannitol 28. This solution was applied to the apical side of the monolayer 15 minutes before addition of carbachol. Therefore, the electrophysiological response to carbachol was not affected by junction potentials.

Agar bridges were placed in apical and basolateral compartments and were connected to Ag-AgCl electrodes for monitoring the transepithelial potential difference (Vₜ). An extra Ag-AgCl electrode was placed in the apical bath, serving as a common ground. Apical membrane potential (Vₐ) was measured by impalement with a glass microelectrode, pulled from capillaries (1 mm o.d., Clark Electromedical, Reading, UK) with a Flaming Brown P-87 micropipette puller. The microelectrode was filled with 0.5 M KCl solution. The tip resistance was 100-200 MΩ and the 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-s intervals, in order to calculate the transepithelial resistance (Rₜ) and the fractional resistance [Rₐ/(Rₐ+Rₜ)]. The equivalent short circuit current (Iₛₑ) was calculated from Vₜ and Rₜ.

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

The measurements were corrected for the offset of the electrodes and for the resistances of the fluid and filter without cells. Values are means ± SE. Statistical significance was evaluated using Students t-test.

Histology

Filters containing cells were exposed to 10 ng/ml TNFα during 24 hours, after which they were cut in small pieces. The filters were fixed in 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at room temperature and rinsed three times for 20 minutes each in 0.1 M sodium cacodylate buffer (pH 7.4). The filters were rinsed overnight (4 °C) in 0.1 M sodium cacodylate buffer (pH 7.4) and then processed for routine electron microscopy.
TNFα, bisindolylmaleimide I (GF109203X), 4-ß-phorbol-12,13-dibutyrate (PDBu) and U73122 and U73143 were obtained from Calbiochem. Cycloheximide, propranolol and forskolin were purchased from Sigma, and carbachol is from Brunschwig. TNFα, propranolol and carbachol were dissolved in water. Forskolin and cycloheximide were dissolved in ethanol (maximal concentration in the Ussing chamber 0.1% (v/v)). PDBu and bisindolylmaleimide I were dissolved in DMSO. U73122 and U73143 were dissolved in chloroform. The maximal concentration of the latter two solvents was 0.01% (v/v). Maximal concentrations of carrier solvents were without electrophysiological effect on the cells.

Concentration of bisindolylmaleimide I (1 μM) was chosen because it was without effect on PKA mediated response (see Results). PDBu, U73122, U73143 and cycloheximide concentrations were taken from literature (references in Results). Propranolol concentrations were tested in a range from 100 to 500 μM. At the concentration used in this study the potentiation of the intracellular response to carbachol after exposure to TNFα was fully reversed (see Results).

Results

TNFα potentiates the carbachol-induced secretion

The electrophysiological response to muscarinic receptor activation with carbachol and its relation to chloride secretion in HT29cl.19A cells has been previously reported [11]. A typical electrical response to 100 μM carbachol is presented in figure 1 by the closed dots. The mean values of the electrical parameters and their changes induced by carbachol are shown in the upper part of table 1. For the present description, the response was divided into two phases, which are marked by the two thin lines in figure 1. The data in table 1 were taken at the time points indicated by these lines. The first phase was a fast depolarization of the apical membrane potential $V_a$ together with an increase in fractional resistance $fR_a$, while the transepithelial potential $V_t$ decreased. Because the transepithelial resistance $R_t$ was not affected, the decrease of $V_t$ must be caused by a small but significant decrease of the short circuit current $I_{sc}$. Based on previous experiments [11] this phase is attributed to a sharp increase of the intracellular calcium activity, primarily from the IP$_3$ sensitive intracellular pool, which activates chloride conductances located in the apical, and more prominently, in the basolateral membrane. The second phase was characterized by the repolarization leading to a hyperpolarization of the apical membrane, concomitant with an increase in $V_t$ and a return of the $fR_a$ and $R_t$ to control values. The increase of the $I_{sc}$ during the second phase of the carbachol response is ascribed to opening of basolateral, Ca$^{2+}$-sensitive, K$^+$ channels [11],...
which increase the driving force for Cl\(^{-}\) efflux through apical Cl\(^{-}\) channels. Previous work in HT29cl.19A cells suggests that these channels are different than the Ca\(^{2+}\)-sensitive Cl\(^{-}\) channels and that PKC\(\alpha\) is involved in their activation [11, 12].

Incubation during 48 hours with TNF\(\alpha\) (10 ng/ml bilaterally) significantly increased \(R_t\), whereas the other basal electrical parameters were not changed. However, it appeared to be much more difficult to obtain stable intracellular recordings. The open dots in figure 1 show the change in electrophysiological parameters induced by carbachol after 48 hours exposure to TNF\(\alpha\). The mean values of 9 experiments are given in the lower part of table 1.

**Table 1:** Effect of 48 hours incubation with 10 ng/ml TNF\(\alpha\) on the changes in electrical parameters induced by 100 \(\mu\)M carbachol

<table>
<thead>
<tr>
<th></th>
<th>(V_t), mV</th>
<th>(R_t), (\Omega).cm(^2)</th>
<th>(V_a), mV</th>
<th>(fR_a)</th>
<th>(I_{sc}), (\mu)A.cm(^{-2})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>baseline</td>
<td>4.5±0.3</td>
<td>181±11</td>
<td>-53±2</td>
<td>0.67±0.02</td>
<td>25±2</td>
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<td>Carbachol phase 1</td>
<td>3.3±0.3</td>
<td>175±10</td>
<td>-32±3</td>
<td>0.77±0.02</td>
<td>20±2</td>
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<tr>
<td>Change in phase 1</td>
<td>-1.1±0.2</td>
<td>-5±2</td>
<td>21±3</td>
<td>0.10±0.03</td>
<td>-6±1</td>
</tr>
<tr>
<td>Carbachol phase 2</td>
<td>5.5±0.5</td>
<td>171±10</td>
<td>-55±3</td>
<td>0.69±0.02</td>
<td>33±3</td>
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<tr>
<td>Change in phase 2</td>
<td>2.2±0.3</td>
<td>-4±2</td>
<td>-23±3</td>
<td>-0.08±0.01</td>
<td>14±2</td>
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<td><strong>TNF(\alpha)</strong></td>
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<td>baseline</td>
<td>5.0±0.6</td>
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<td>-48±2</td>
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<td>Carbachol phase 1</td>
<td>4.3±0.5</td>
<td>211±7#</td>
<td>-31±4</td>
<td>0.79±0.01</td>
<td>21±3</td>
</tr>
<tr>
<td>Change in phase 1</td>
<td>-0.7±0.2</td>
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<td>17±4</td>
<td>0.16±0.02</td>
<td>-3±1#</td>
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<tr>
<td>Carbachol phase 2</td>
<td>15±0.8*</td>
<td>152±10</td>
<td>-31±2*</td>
<td>0.44±0.05*</td>
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<tr>
<td>Change in phase 2</td>
<td>11±0.5*</td>
<td>-59±4*</td>
<td>0.6±2*</td>
<td>-0.35±0.06*</td>
<td>85±10*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Changes in electrophysiological parameters after application of 100 \(\mu\)M carbachol in absence or presence of TNF\(\alpha\). Changes during phase 1 and 2 are measured at time points indicated by 2 thin lines in figure 1. For transepithelial measurements, \(n=9\) for the 2 groups. For intracellular measurements [apical membrane potential \(V_t\) and fractional apical resistance \(fR_a\)], \(n=8\) for control monolayers, and \(n=4\) for TNF\(\alpha\) exposed monolayers. \(V_t\) transepithelial potential difference; \(R_t\) transepithelial resistance; \(I_{sc}\), short circuit current. * \(P<0.05\) compared with control carbachol response; # \(P<0.001\) compared with control carbachol response.
Figure 1. Changes in electrophysiological parameters of HT29cl.19A after addition of 100 μM carbachol in absence (close dots) or presence (open dots) of 48 h of TNFα (10 ng/ml). Two tracings (control and TNFα-treated group), represent experiments in which intracellular recordings could be obtained. Two thin lines divide responses into phases 1 and 2 as described in Results. $V_t$, transepithelial potential; $V_a$, intracellular potential; $fR_a$, fractional apical resistance; $R_t$, transepithelial resistance; $I_{sc}$, short circuit current. For statistics see Table 1.

The sharp depolarization in phase 1 and the increase of $fR_a$ were not affected by the exposure to TNFα, however the first extracellular potential change (basolateral side negative) and the change in $I_{sc}$ were smaller. The second phase showed large differences due to TNFα exposure; the depolarization of $V_a$ was prolonged, concurrent with a strong decrease in the $fR_a$ (0.35 vs. 0.08 in control) and $R_t$ (28 % vs. 2 % in control). The carbachol-induced increase of $I_{sc}$, with respect to basal values, was much larger after treatment with TNFα.
To examine whether the increased current was due to a chloride efflux or a sodium influx, we replaced chloride in the apical bath by gluconate, thereby increasing the chloride gradient across the apical membrane. Under this condition monolayers exposed to TNFα responded to carbachol with larger changes of \( V_t \) (175 ± 29 %) and \( I_{sc} \) (150 ± 19 %) compared to the same monolayers exposed to TNFα in normal Ringer (100 %; n=4). This indicates that the cytokine potentiated the carbachol-induced Cl efflux across the apical membrane.

**Dose response curve of TNFα**

To define the dose dependence of the action of TNFα filters were incubated during 24 hours with different concentrations of TNFα varying between 1 and 100 ng/ml. Figure 2 shows the effects of the different concentrations TNFα on the carbachol-induced \( I_{sc} \). The optimal potentiation takes place at a concentration of 10 ng/ml TNFα. Therefore in the following experiments this concentration was used in the following experiments.

**Time dependence of TNFα action**

To define the time dependence of the potentiating effect of TNFα cells were incubated with 10 ng/ml TNFα during different times, varying between half an hour and 78 hours. Figure 3 shows the relation between the carbachol-induced \( I_{sc} \) and the time of exposure to TNFα. The maximal change in \( I_{sc} \) is presented as the percentage of the carbachol response of monolayers not exposed to TNFα (=100 %). Till 2 hours of incubation TNFα did not show a significant potentiation. However, incubation of 2.5 hours with TNFα enhanced the carbachol response significantly (206 ± 7 %), and with increasing incubation times, the potentiating effect of TNFα on the carbachol response became larger (r=0.70). After 78 hours of incubations the
response was increased to over 900%. We decided to use in some of the experiments a shorter exposure time for TNFα since this permitted more stable intracellular measurements.

**Figure 3.** Time dependence of potentiating effect of TNFα on carbachol response. Monolayers were exposed to 10 ng/ml TNFα for between 0 and 78 h, and maximal change in $I_{sc}$ after addition of carbachol was measured. Data are expressed as percentage change in $I_{sc}$ compared with change in $I_{sc}$ after carbachol alone (100%). Longer incubation times with TNFα resulted in larger changes in $I_{sc}$ with a correlation coefficient of $r=0.70$.

**Histology**

Figure 4a and 4b are typical electron micrographs (3100x) of HT29cl.19A monolayers with (4b) or without (4a) exposure to 10 ng/ml TNFα during 48 hours. No differences are seen as compared with time matched control monolayers. A propidium iodide staining combined with a Hoechst 33258 was used to check for apoptosis. Even after 48 h exposure to TNFα no indication was found for TNFα-induced apoptosis (not shown).

**Figure 4.** Electronmicrographs of monolayers with or without exposure to TNFα for 48 h. a: representative control monolayer. b: representative monolayer exposed to 10 ng/ml TNFα for 48 h. No morphological differences are seen between the monolayers. The photographs are orientated with the filter side down. Magnification: X3,100.
TNFα action is dependent on protein synthesis

We examined whether the action of TNFα was dependent on protein synthesis using the protein synthesis inhibitor cycloheximide. We incubated the cells (bilatterally) for 1 hour prior to TNFα exposure with 10 μg/ml cycloheximide [13]. The cells were then exposed to TNFα in the presence of the inhibitor during 4 hours and the maximal change in $I_{sc}$ induced by 100 μM carbachol was measured. Figure 5 shows the effects of cycloheximide on the carbachol response with or without exposure to TNFα. The increase in $I_{sc}$ induced by carbachol in control monolayers was not affected by incubation with cycloheximide ($7.1 \pm 1.2 \mu A.cm^{-2}$ cycloheximide vs. $5.8 \pm 1.5 \mu A.cm^{-2}$ control, n=4, p>0.1). However, in the presence of cycloheximide the potentiating effect of TNFα was completely abolished ($31.8 \pm 11.2 \mu A.cm^{-2}$ TNFα vs. $6.5 \pm 1.1 \mu A.cm^{-2}$ cycloheximide + TNFα, n=4, p<0.05). This, together with the time lag suggests that TNFα potentiates the carbachol response via a mechanism, which requires protein synthesis.

![Figure 5](image)

PKC is involved in the electrical response to carbachol

We examined the involvement of PKC in the response to carbachol with and without exposure to TNFα by using an inhibitor of PKC, bisindolylmaleimide I (GF109203X), which is specific for PKC, but, in high concentrations it can also inhibit PKA [14]. We tested whether GF109203X could inhibit specifically PKC, but not PKA in the HT29cl.19A cells. Figure 6 shows the effects of bilateral application of consecutive 0.1 μM and 1 μM GF109203X on the changes in $V_a$ induced by PKA and PKC activation. Forskolin stimulates PKA-activated Cl⁻ channels, via direct activation of adenylyl cyclase. Application of 1 μM PDBu (4-β-phorbol-12,13-dibutyrate) results in activation of PKC [15]. Addition of forskolin or PDBu, although with different time constants, resulted in a depolarization of $V_a$, due to the increase in apical chloride conductance [16]. Application of GF109203X did not affect the
forskolin-induced depolarization. However, 1 μM GF109203X reduced the depolarization of $V_a$ induced by PDBu. Since the full effect of GF109203X required about 20 minutes, in the following experiments preincubation was performed during 30 minutes. As shown in table 2, 1 μM GF109203X did not show an effect on the basal electrical parameters.

Figure 6. Effect of GF109203X (bisindolylmaleimide I) on changes in $V_a$ after addition of forskolin (10 μM, basolateral) and PDBu (1 μM, apical). GF109203X was added to both sides of the cells in concentrations of respectively 0.1 μM and 1 μM. See results for effects of preincubation of the cells with GF109203X on forskolin- and PDBu-induced changes in $I_{sc}$.

The increase of $I_{sc}$ induced by forskolin was not different with or without 30 minutes of preincubation with the inhibitor (43 ± 5 μA.cm$^{-2}$ and 47 ± 11 μA.cm$^{-2}$ respectively, n=9 resp. 4). However the $I_{sc}$ induced by application of 1 μM PDBu in the presence of the inhibitor was significantly reduced by 64 % from 12 ± 2 μA.cm$^{-2}$ to 5 ± 1 μA.cm$^{-2}$ n=6, p<0.05). Thus 30 minutes exposure to 1 μM GF109203X specifically inhibits PKC in the HT29cl.19A. GF109203X was therefore used to test whether PKC was involved in the response to carbachol and its potentiation by TNFα. The cells were preincubated for 30 minutes with 1 μM GF109203X, after which the effect of carbachol and of exposure to TNFα was measured. After TNFα exposure, one part of the filter was used to measure the potentiated carbachol response. The second part of the filter was preincubated for 30 minutes with 1 μM GF109203X, after which the effect of carbachol was measured.

Table 2. effects of 30 minutes incubation 1 μM GF109203X on basal electrical parameters

<table>
<thead>
<tr>
<th></th>
<th>$V_t$, mV</th>
<th>$R_t$, Ω.cm$^2$</th>
<th>$V_a$, mV</th>
<th>$fR_a$</th>
<th>$I_{sc}$, μA.cm$^{-2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>3.2 ± 0.7</td>
<td>215 ± 20</td>
<td>-58 ± 2</td>
<td>0.56 ± 0.06</td>
<td>15 ± 3</td>
</tr>
<tr>
<td>GF109203X</td>
<td>3.5 ± 0.7</td>
<td>224 ± 28</td>
<td>-59 ± 2</td>
<td>0.64 ± 0.02</td>
<td>17 ± 3</td>
</tr>
</tbody>
</table>

Values are means ± SE.
Figure 7 shows the relative decrease of the carbachol-induced increase in I_{sc} after preincubation with GF109203X with or without exposure to TNFα. In the presence of the inhibitor, the maximal change in I_{sc} after carbachol alone was significantly reduced with 71 ± 14 %. After exposure to TNFα the potentiated carbachol response was inhibited with 64 ± 6 %. We conclude that PKC activation is involved in the normal carbachol-induced secretion as well as in the TNFα-potentiated response in this cell line.

![Figure 7. Effect of 30 min of preincubation with 1 μM GF109203X (bilateral) on the I_{sc} induced by carbachol with or without exposure to TNFα. Data are presented as percentage changes compared with pairwise control experiments without the inhibitor. Menas ± SE from 4 or 5 monolayers. Cells were exposed to TNFα between 4 and 48 hours. * P<0.05 compared with carbachol; # P<0.05 compared with carbachol + TNFα.](image)

**TNFα does not affect the PDBu- and the forskolin-induced secretion**

One mechanism by which TNFα could potentiate the effect of carbachol is a direct upregulation of PKC. To investigate this possibility, we preincubated the cells with TNFα between 6 and 24 hours, and we measured the I_{sc} induced by the PKC activator PDBu. The change in I_{sc} induced by 1 μM PDBu alone was 9.9 ± 1.2 μA.cm^{-2} and after TNFα 9.3 ± 0.7 μA.cm^{-2}; n=5, resp. 4). In HT29cl.19A the secretory response activated by PDBu is mediated by the same Cl⁻ channels as the PKA activated channels namely the CFTR [17]. To test whether TNFα had an effect on the PKA-mediated activation of the apical chloride channels, we applied forskolin. The I_{sc} induced by 10 μM forskolin alone was 43 ± 5μA.cm^{-2} (n=9) After exposure to TNFα for 48 hours the I_{sc} induced by forskolin was 51 ± 3μA.cm^{-2} (n=4) which is not significantly different.

**Phospholipase D activation is involved in the carbachol-mediated secretory response.**

The foregoing results indicate that the potentiation by TNFα must be sought upstream from PKC. One possibility is that the effect is based on a stronger activation of PKC by an
increased production of diacylglycerol (DAG). DAG can be produced by several reactions: first, the hydrolysis of PIP\textsubscript{2} by phosphoinositide phospholipase C (PI-PLC) (this pathway has been demonstrated for muscarinic and histaminic receptor activation in intestinal epithelial cells) [18]; secondly, hydrolysis of phosphatidyl choline (PC) by PC-PLC or, thirdly, hydrolysis of phospholipids to phosphatidic acid (PA) by phospholipase D (PLD) and further to DAG by phosphatidate phosphohydrolase (PAP) (reviewed in [19]). However, as far as we know, the two last mechanisms are not documented in intestinal epithelial cells. To test the possible involvement of the PI-PLC pathway we attempted to block PLC by U73122, a putative PLC-PIP\textsubscript{2} specific antagonist [20]. However, addition of this compound (10 \mu M) did not show inhibitory effects and the negative control U73343 did show the same quantitative effects on $V_a$. Thus this compound could not be used as a tool to investigate the role of PI-PLC. The formation of DAG by PAP, and therefore the involvement of PLD can be blocked by 100 \mu M propranolol [21]. Using thin layer chromatography of $^{32}$P\textsubscript{s} incubated monolayers, it was found that propranolol (100 \mu M) increased the level of PA, indicating an inhibition of the degradation of PA to DAG, (manuscript in preparation). To check whether PLD-dependent DAG formation and therefore increased PKC activation is involved in the (TNF\alpha-potentiated) carbachol response, we examined the effect of propranolol on the carbachol response with or without exposure to TNF\alpha. Table 3 shows that 10 minutes exposure to 100 \mu M propranolol of the apical side of the cells slightly changed the basal electrical parameters. A small hyperpolarization occurred together with an increase in $fR_a$, indicating an increase in basolateral $K^+$ conductance. However, no significant effect was seen on transepithelial parameters. (At 500 \mu M or higher propranolol reduced the transepithelial resistance.) Propranolol at 100 \mu M did not have any effect on forskolin and PDBu-induced secretion (results not shown).

Table 3: effects of 10 minutes incubation with 100 \mu M propranolol on basal electrical parameters

<table>
<thead>
<tr>
<th></th>
<th>$V_m$, mV</th>
<th>$R_t$, $\Omega \cdot \text{cm}^2$</th>
<th>$V_a$, mV</th>
<th>$fR_a$</th>
<th>$I_{sc}$, \mu A.cm\textsuperscript{-2}</th>
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<tbody>
<tr>
<td>baseline</td>
<td>4.0 ± 0.4</td>
<td>201 ± 9</td>
<td>-55 ± 0</td>
<td>0.70 ± 0.00</td>
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<tr>
<td>Propranolol</td>
<td>4.8 ± 0.4</td>
<td>234 ± 9*</td>
<td>-63 ± 2*</td>
<td>0.76 ± 0.01*</td>
<td>21 ± 2</td>
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</tbody>
</table>

Values are means ± SE. * $P<0.05$ compared with baseline.

Figure 8 shows the effect of propranolol on the change of $V_a$ after addition of carbachol in TNF\alpha treated cells. The prolonged depolarization induced by carbachol in the presence of
TNFα (open dots) was completely abolished after propranolol treatment (closed dots) and is similar to registrations of $V_a$ after carbachol application without TNFα (see figure 1).

Figure 8. Example of intracellular recording of effect of 10 min of preincubation with 100 μM propranolol (apical) on changes in $V_a$ induced by addition of carbachol after exposure to TNFα, as compared with tracing without propranolol. Decreased depolarization and its shorter duration is reflected in a decrease of $I_{sc}$ shown in figure 9. Cells were exposed to TNFα for 4 hours.

Figure 9 shows the relative change in $I_{sc}$ with respect to the carbachol induced change of $I_{sc}$ (100%). After preincubation with propranolol the carbachol-induced increase of $I_{sc}$ was reduced (from 14 ± 2.0 to 7.4 ± 1.7 μA.cm$^{-2}$, n=6, p<0.05). Propranolol completely abolished the potentiating effect of TNFα on the carbachol response (57 ± 7 μA.cm$^{-2}$ carbachol after TNFα versus 4.2 ± 0.3 μA.cm$^{-2}$ in the presence of propranolol, n=6, p<0.001).

Figure 9. Effect of propranolol on $I_{sc}$ induced by carbachol with or without exposure to TNFα. Data are presented as percentage compared with the carbachol-induced response without propranolol (mean ± SE from 6-9 monolayers). Cells were exposed to TNFα between 4 and 48 hours. Means ± SE of absolute values of changes are given in Results. * P<0.05 compared with carbachol; # P<0.001 compared with carbachol + TNFα.

T84 cells

Exposure of T84 cells to 10 ng/ml TNFα for 24 hours did not change basal secretory activity ($I_{sc}$= 6.82 ± 1.50 μA/cm$^{-2}$ control vs. 6.30 ± 1.8 μA/cm$^{-2}$ TNFα exposed monolayers). Also
no change in basal $R_t$ has been found after exposure to TNF$\alpha$ (588 ± 88 $\Omega\cdot$cm$^2$ control vs. 656 ± 99 $\Omega\cdot$cm$^2$ TNF$\alpha$). Addition of 100 $\mu$M carbachol resulted in an increase of $I_{sc}$ of 82 ± 25% (n=3) as compared with basal values. After 24 hours exposure to TNF$\alpha$ the change in $I_{sc}$ was 68 ± 10% (n=3), which is not significantly different than in control monolayers.

The carbachol induced increase of $I_{sc}$ in these cells appeared not to be affected by the PKC inhibitor GF109203X ($I_{sc}$ induced by carbachol in the presence of GF109203X = 121 ± 17% as compared to control values). The membrane potential $V_a$ is in T84 cells −34 ± 0.6 mV (n=3), which is lower than in HT29cl.19A cells, suggesting a higher basal chloride conductance. Addition of carbachol resulted in a hyperpolarization of $V_a$ with −18 ± 2 mV (n=3), which occurred together with the increase in $I_{sc}$.

**Discussion**

In the present study we showed that exposure to TNF$\alpha$ (10 ng/ml) potentiates the chloride secretion induced by muscarinic receptor activation in human intestinal epithelial cells HT29cl.19A. The potentiating effect of TNF$\alpha$ was time dependent; a significant increase of the carbachol-induced short circuit current $I_{sc}$ was observed after exposure to TNF$\alpha$ for at least 2.5 hours. The rather long lag time and the fact that blocking protein synthesis by cycloheximide prevented the potentiating action of TNF$\alpha$, suggest that de novo synthesis of a link or activator in the signaling cascade occurs.

There are many potential sites where TNF$\alpha$ could have augmented the effect of carbachol.

1) The basal Cl$^-$ conductance in the apical membrane may be increased, so that the carbachol dependent increase of K$^+$ conductance in the basolateral membrane had a larger effect. However, the observation that the basal electrophysiological parameters after prolonged exposure to TNF$\alpha$ did not show increased secretory activity, argues against this possibility.

2) TNF$\alpha$ may have increased the number of Cl$^-$ channels in the apical membrane so that activation induced a larger conductance. But activation of the PKA route by forskolin or the PKC route by PDBu did not show potentiation by TNF$\alpha$. Therefore, this possibility is not likely to occur.

3) TNF$\alpha$ may have increased the number of muscarinic receptors. Although we have not tested this possibility directly, the observation that activation by histamine after exposure to TNF$\alpha$ also showed a potentiated secretory response (unpublished observations) indicates that potentiation is in the pathway between receptors and the Cl$^-$ channel.

4) Because in HT29cl.19A cells the carbachol effect is mediated via PKC [11] (and presently illustrated by the effect of GF109203X), it may be that TNF$\alpha$ has upregulated PKC so that a larger pool of PKC molecules is available. This possibility should be studied directly, however, because the effect of PDBu was not different in TNF$\alpha$ exposed cells, this
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possibility is less likely. Additionally, the observation that GF109203X inhibited the PDBu effect and the potentiated carbachol effect with the same percentage suggests that the quantitative relation between the blocker and PKC has not been changed by TNFα. Therefore we hypothesize that the effect of TNFα is between the receptor and PKC. The absence of an effect of exposure to TNFα on the carbachol response in T84 cells may be in line with this assumption. In these cells the mechanism leading to increased Cl\(^-\) secretion is different from the mechanism in HT29cl.19A cells. In T84 cells, the carbachol induced increase of the short circuit current is primarily due to activation of basolateral K\(^+\) channels which lead to an increased driving force for Cl\(^-\) efflux through conducting apical Cl\(^-\) channels [22]. This view is corroborated by the present intracellular potential measurements. The resting apical membrane potential is much lower than in the HT29cl.19A cells suggesting a much larger Cl\(^-\) conductance. The simultaneous hyperpolarization of the apical membrane potential and the increase of transepithelial potential and short circuit current upon carbachol addition indicate activation of basolateral K\(^+\) channels as the underlying mechanism for transepithelial current in this cell line. The refractoriness of the carbachol response to the PKC inhibitor GF109203X confirms the finding that PKC is not involved in the carbachol response in these cells [23]. Therefore, the absence of an effect of TNFα on the electrophysiological effect of carbachol in these cells is an argument in favor of the hypothesis.

5) The activation of the muscarinic receptor after exposure to TNFα may lead to increased activation of phospholipase C, leading to larger amounts of the intracellular messengers IP\(_3\) and the PKC-activator DAG. This possibility should also be studied directly. However, because IP\(_3\) leads to increase of Ca\(^{2+}\) and Ca\(^{2+}\) in turn leads to the activation of the first phase of the carbachol response [11], which was not different after TNFα exposure, this possibility may be less likely. Therefore, another pathway to increase DAG may be involved. The PI-PLC inhibitor U73122 was without effect on the first phase of the carbachol effect and therefore it was not possible to use this inhibitor to show the involvement of other phospholipase(s) in the generation of DAG.

6) Carbachol, after TNFα exposure, may lead to increased production of DAG by activation of the PLD pathway. Although, not described for epithelia TNFα can increase the PLD route in a number of cells [24, 25]. Direct measurements of DAG levels and the analyses of the involvement of the PLD pathway have to be performed. However, in parallel studies it was confirmed that in the presence of butanol, carbachol increased the synthesis of phosphatidylbutanol, a specific product of phospholipase D activation. This indicates that muscarinic receptor activation can increase the PLD activity (manuscript in preparation). Participation of the PLD pathway in the carbachol-response was also indicated by the electrophysiological effect of propranolol which is considered an inhibitor of conversion of the PLD-product phosphatidic acid to DAG by phosphatidic acid phosphatase, PAP [19]. The inhibitive effect of propranolol on PAP in these cells was confirmed by the observation that propranolol increased the \(^{32}\)PA level in \(^{32}\)P\(_t\) exposed cells, as analyzed with thin layer chromatography (manuscript in preparation). Other studies have shown an effect of TNFα on PC-PLC [26, 27,
This route forms DAG directly without PA as an intermediate and does not require PAP. We can not exclude a role for PC-PLC in the (potentiated) carbachol response. However, the large suppression of the carbachol response by propranolol suggests that DAG formation by the PLD route plays an important role in the TNFα-potentiated carbachol response.

7) According to work with T84 cells activation of PI-PLC can generate an inhibitory inositol derivative, inositol 3,4,5,6-tetrakisphosphate, that may mediate carbachol-induced inhibition of Cl⁻ secretion [29]. The presence of this mechanism in HT29cl.19A cells is unknown and the possibility that TNFα exposure alleviates a similar inhibition by reducing the synthesis of such a compound remains to be studied. However, TNFα was without effect in the T84 cells and the inhibitory effect of pre-exposure to carbachol on the histamine response in HT29cl.19A cells was not affected by TNFα (unpublished results).

8) In human lymphoma cells TNFα appeared to increase Na⁺ channels [30]. If this was the case in HT29cl.19A cells and the channels were activated by carbachol one would not expect to observe an increased response when the driving force for Cl⁻ was increased. Therefore, the most plausible hypothesis for the effect of TNFα in the potentiation of the effect of muscarinic receptor activation in HT29cl.19A cells is an upregulation of the PLD route leading to increased DAG formation and increased PKC dependent Cl⁻ conductance.

In another clone of the HT29 cells (HT29/B6) TNFα appeared to have no direct effect on the secretory status [7]. However, like in the cl.19A cells the effect of carbachol was potentiated (pers. commun. J.D. Schulzke, F.U. Berlin).

The effects of TNFα on ion secretion in human or animal intestine have not been studied extensively. A difficulty in these studies is that in the presence of so many other cells one can not be sure of the target for the applied TNFα. For example, in human distal unstripped colon [7] and in porcine ileum [8] TNFα increased the short circuit current. This effect could be blocked by indomethacin, indicating the release of prostaglandins. As far as we know no experiments have been reported showing more or less acute effects of TNFα on responsivity to secretagogues in isolated intestine. Pathophysiology gives no clear evidence for effects of TNFα. TNFα is increased in inflammatory intestinal tissue [1, 2] and isolated but not-affected tissue from inflamed intestine appeared hyporesponsive to secretagogues [31]. An explanation for this finding has not been given. It may be that in some studies disruption of the tissue or altered morphology plays a role. Alternatively, the hyporesponsivity may be related to downregulation of one or more steps in the pathway of the secretagogues because the tissue is still in a secretory state or has been in this state for a prolonged period. A recent abstract claims that TNFα is not responsible for the secretory dysfunction caused by inflammation [32].

From cocultures of T84 cells and activated immune cells, there is ample evidence that products from immune cells can modify the epithelial response to secretagogues [5, 33, 34]. The nature of these products or the mechanism of action is not known. As shown in the
present study T84 cells lack the PKC dependent carbachol route and it is feasible that this cell line can not show the potentiating effect of TNFα.

An interesting question therefore is whether in carbachol secretory responses of small or large intestinal enterocytes PKC is involved. Data concerning this question are scarce; but in rabbit ileum PKC appears to be involved in the response to carbachol [35]. Transepithelial permeability appears to be modified by TNFα directly. This effect occurred at high concentrations (100 ng/ml) in Caco2 BBE cells [36] and in HT29/B6 cells [37] and at lower concentrations (10 ng/ml) in HT29cl.19A when TNFα exposure was performed in combination with IFNγ [9]. The cooperative effect of the cytokines may be due to the expression of TNFα receptors triggered by IFNγ [38]. In our laboratory co-exposure of the cells to TNFα and IFNγ made it totally impossible to obtain intracellular recordings and also high concentrations or longer exposure to TNFα alone decreased the success rate of impalements strongly. We have no explanation for this. It appeared not be due to morphological changes as electronmicrographs of monolayers exposed to TNFα for even 48 hours were not different than controls. From the increase of the transepithelial resistance induced by exposure to TNFα, we can conclude that there is no increased permeability for ions. The large, transient, decrease of $R_t$ during the potentiated carbachol response is concomitant with a decrease of $fR_a$ and therefore indicates a transcellular change in conductances. We propose that carbachol after exposure to TNFα may induce an increased activation of PKC and numerous other studies in various cell types have implicated a role for DAG stimulated PKC in the effect of TNFα (reviewed in [39]). Massive stimulation of PKC by phorbol esters with PDBu caused a slowly increasing paracellular permeability for macromolecules in HT29cl.19A cells [40]. It remains to be studied whether TNFα in combination with a secretagogue like carbachol can induce a similar increase of permeability and if so, which isotype of PKC could be involved.

This is the first study, which shows a direct potentiation of receptor-activated ion secretion in intestinal epithelial cells by TNFα. It remains to be studied whether the results in HT29cl.19A cells can be translated to the living animal. If so, TNFα could contribute to the diarrhea in patients with inflammatory bowel disease, especially when the cells are primed by other cytokines like IFNγ to express receptors for TNFα. The potentiation of secretion induced by muscarinic receptor activation (and histamine H1 receptor activation, unpublished observations) suggests that TNFα upregulates a common intermediate in the transduction pathway, and underscores its possible role in mast cell responses. It is conceivable that this intermediate step is also involved in other PKC-dependent secretory mechanisms like induced by for example bacterial toxins [41]. Furthermore our results ask for a place for PLD in the secretory mechanism. These aspects deserve further investigations, which are under way in our laboratory.
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