Chapter eight

Summarizing discussion

Judith Oprins
Overview

In Part I we report on our study of the effect of the cytokine tumor necrosis factor α (TNFα) on chloride secretion in different models of intestinal epithelium. HT29cl.19A cells were used for intracellular and extracellular electrophysiological measurements of the effects of exposure to TNFα and for the effects of subsequent exposure to the secretagogues carbachol and histamine. We also studied changes in phospholipid composition induced by TNFα and the secretagogues by means of thin layer chromatography. Using the same techniques, we investigated whether TNFα affected chloride secretion via a similar mechanism in another human intestinal epithelial cell line, T84. To extrapolate the results obtained in the cell lines, we studied whether native tissue (mouse distal colon) was responsive to exposure to TNFα. Part II deals with the effect of neuropeptide Y (NPY) on the secretion in HT29cl.19A cells. We studied the mechanisms by means of which NPY could inhibit the chloride secretion induced by several second messenger systems in this cell line.

Part I

Involvement of phospholipase D in chloride secretion

Regulation of chloride secretion in intestinal epithelium has been extensively studied during the last few decades. Detailed knowledge about the second messenger mechanisms and their roles in regulating ion channels and transporters, which contribute to chloride secretion, is being unraveled [1]. The classic messengers cAMP, calcium and to a lesser extent, protein kinase C (PKC), are known potent regulators of chloride secretion. The mechanisms by which they activate or inactivate exchangers, transporters and ion channels, which contribute to the ion transport mechanisms, have been clarified. In this thesis, we provide new evidence of another pathway between receptor and intracellular messengers involved in chloride secretion in intestinal epithelium. It consists of phospholipase D (PLD) and phosphatidic acid phosphatase (PAP) the activation of which generates diacylglycerol (DAG), which in turn can activate protein kinase C (PKC).

It is a well known fact that the activated muscarinic receptor couples to a Gq protein, which can activate PI-PLC [2]. PI-PLC hydrolyzes phosphoinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-trisphosphate (IP3) and DAG. IP3 releases calcium from intracellular stores, whereas DAG activates classic and novel PKC isotypes. Exton et al. described for the first time that this type of G-protein coupled receptor activates another phospholipase: PLD [3]. Activation of PLD results in the formation of phosphatidic acid (PA) and P-choline, following hydrolysis of the structural phospholipid phosphatidylcholine (PC). PA is converted to DAG by PAP and again DAG can activate several PKC isotypes. In addition,
DAG can be formed via activation of PC-PLC, which hydrolyzes PC into DAG and choline. Figure 1 provides a schematic presentation of the activation of phospholipases after muscarinic receptor activation.

Figure 1. Schematic presentation of the phospholipase pathways involved in muscarinic and histamine receptor activation. For abbreviations see text.

In Chapters 2 and 5 we suggest that chloride secretion induced by muscarinic receptor activation and histaminic receptor activation in HT29cl.19A cells involved activation of PLD. We used propranolol to block the conversion of PA to DAG, via inhibition of PAP. In Chapter 3 we show that application of propranolol resulted in increased PA levels, confirming the inhibitory action of propranolol on PAP in the cell line used. Application of propranolol strongly decreased the secretory responses to carbachol and histamine, implicating an important role for PAP, and thus the PLD pathway in chloride secretion. Given the relative inhibitory effect of propranolol we estimated that the transepithelial secretory response in the HT29cl.19A cells depended on the activation of the PLD pathway for more than 50 percent.

Moreover, in Chapters 3 and 5 we confirm that the activation of the muscarinic and histamine receptor respectively, did indeed result in an activation of PLD, as indicated by measurements of the stable PLD product phosphatidylbutanol (Pbut). The physiological importance of the activation of the PLD pathway in intestinal epithelial cells has not yet been extensively studied. The intermediate PA has been described as a messenger molecule itself, although we are aware of only one study of PA in intestinal epithelium [4]. The effect of PA in the intestinal cell line T84 is a modulation of stimulated chloride secretion. Addition of PA results in an augmented carbachol-secretory response, but the secretory response to vasoactive intestinal peptide (VIP) is inhibited. The authors proposed that the effect of PA on carbachol-induced chloride secretion may be explained by its augmentation of the carbachol-induced increase in intracellular calcium levels. However, they do not yet offer an
explanation for the inhibitory effect. Many functions for PA have been proposed in other tissues, including promotion of mitogenesis in fibroblasts, stimulation of respiratory burst in neutrophils, activation of specific protein kinases and increases in intracellular calcium (for a review see [5]).

Physiological relevance of PLD activation

Although activation of PLC and PLD both result in an increased formation of DAG, which is the major cellular regulator of most isotypes of PKC (Figure 1), there is a difference in the time course for the generation of DAG. Whereas the formation of PA is usually monophasic in time, the formation of DAG shows a biphasic curve. It is believed that the two phases of the increase in the content of DAG consists of initial formation from PIP₂ by PI-PLC, followed by a slower, more sustained formation via activation of PLD and PAP [6, 7]. Moreover, the magnitude of DAG accumulation exceeds the cell content of PIP₂, indicating its origin from another source [8]. The biphasic increase of the DAG concentration may have effects on the pattern of activation of PKC isotypes, which are activated by DAG. Interestingly, Ha and Exton report differential translocation of PKC isozymes in fibroblasts [9]. The first DAG peak was shown to involve a translocation of PKCα, which also required calcium whereas the second phase of the increase of DAG was related to increased, calcium independent, PKCe translocation in these cells. The prolonged accumulation of DAG causes prolonged membrane association of PKCe, but not of PKCα. Thus, they postulated that the second phase of the DAG accumulation results in an increased and sustained activation of PKCe, whereas the first phase of DAG production is responsible for a transient PKCα activation. Therefore, the mechanistic relevance of PLD activation might be in the generation of a prolonged and sustained activation of a specific isotype of PKC, as opposed to the brief activation of other PKC isotypes by the transient increase of calcium and DAG.

An important issue is to pinpoint the targets for the different isotypes of PKC. This knowledge is necessary in order to understand the cellular effects of prolonged versus transient increases of DAG. The differential activation of PKC isotypes may explain different effects in cell lines and tissues of extracellular activators of PKC. It may be that the expression of isotypes of PKC differs in cell lines as we have shown for PKCα.

In conclusion, our finding that extracellular messengers can activate more than one type of phospholipase in intestinal cells may explain the multiple effects often induced by these messengers via activation of differing isotypes of PKC.

We will first discuss the functions of the different PKC isotypes that occur in intestinal tissue.
PKC isotypes in intestinal tissue

PKC comprises a family of protein kinases and was at first sub-divided into three groups depending on their requirements for Ca\(^{2+}\) and DAG: the classical, novel and atypical type [10]. The classical PKCs (\(\alpha\), \(\beta\), and \(\gamma\)) require calcium and DAG for their activation. The novel iso-enzymes (\(\delta\), \(\varepsilon\), \(\eta\) and \(\theta\)) are calcium-independent and require DAG for activation. The atypical (\(\zeta\) and \(\tau\)) isoforms are Ca\(^{2+}\)- and DAG-independent. Recently, a new type was discovered (\(\mu\) and \(\tau\)) that represents a fourth class, topologically related to the PKC family. This class possesses a kinase domain which is related more to calcium/calmodulin-dependent kinases. The classical and novel isoenzymes can be activated by phorbol esters.

The present thesis shows that expression of PKC isotypes in the cell lines HT29cl.19A and T84 cells differed substantially. HT29cl.19A cells expressed considerably more PKC\(\alpha\) than T84 cells did. This may explain the difference in carbachol-induced chloride secretion, as will be discussed below. Little is known about the role of different PKC isoforms in the regulation of ion secretion. In T84 cells, PKC\(\varepsilon\) is recognized to directly or indirectly interact with a basolateral K\(^{+}\) channel and to modulate chloride secretion in T84 cells [11]. In HT29cl.19A cells, PKC\(\alpha\) was found to be involved in the carbachol-induced ion secretion [12].

Other effects of different PKC isoforms on intestinal epithelial functions, apart from the regulation of ion secretion, have been described. In human and rat colonic mucosa members from each group have been identified: the classical \(\alpha\) and \(\beta\), the novel \(\delta\), \(\eta\) and \(\varepsilon\), and the atypical \(\zeta\) have been detected at the protein level [13, 14]. Song et al. recognized that PKC\(\varepsilon\) regulates basolateral endocytosis in T84 cells [15]. PKC\(\varepsilon\) is also pinpointed as the likely mediator of mucin exocytosis in these cells and in HT29/A1 cells [16]. Furthermore, overexpression of PKC\(\varepsilon\) was reported oncogenic in a rat colonic epithelial cell line through its interaction with the ras signaling pathway [17]. Several studies have revealed decreased abundancy of PKC\(\alpha\), \(\beta\), \(\delta\), \(\varepsilon\), \(\zeta\) and/or \(\eta\) in colonic tumors [18, 19, 14, 20, 21]. Overexpression of PKC\(\beta\) and PKC\(\delta\) in intestinal epithelium results in growth suppression [22, 23]. These findings suggest that activation of PKC isoforms in intestinal tissue participate in negative growth-regulatory and/or differentiation-related events.

Synergy between TNF\(\alpha\) and secretagogues acetylcholine and histamine

We demonstrate that TNF\(\alpha\), by induction of \textit{de novo} protein synthesis, could augment the secretory responses to the acetylcholine agonist carbachol and the mast cell mediator histamine in HT29cl.19A cells. In addition, in native tissue from the mouse (distal colon) a similar potentiation of the effect of these secretagogues was demonstrated. From intracellular measurements in HT29cl.19A cells we deduced that the cause of the larger increase of the
short circuit current by carbachol after exposure to TNFα was due to an increased apical Cl⁻ conductance. Earlier results of research with this cell line by Bajnath et al. and Vaandrager et al. suggests that carbachol may increase the transepithelial Cl⁻ flux by activation of Cl⁻ channels in the apical membrane and by activation of K⁺ channels in the basolateral membrane [24, 25]. The latter is ascribed to increased activation of Ca²⁺-regulated K⁺ channels and the former is ascribed to an effect of PKCα. The transient character of the response may be due to an inhibitory effect of PKC on the Ca²⁺-regulated K⁺ channels or to other intracellular inhibitors like inositol 3,4,5,6-tetraphosphate IP₄ [26]. On the basis of patch clamp analyses Bajnath et al. concluded that the activation Cl⁻ channels by PKC could be due to increased incorporation of Cl⁻ channels which are then activated by the basal activity of protein kinase A (PKA) [27]. To elucidate the cause of the increased apical conductance in TNFα-exposed cells by carbachol we followed a procedure of elimination of possible causes.

The basal electrophysiological parameters of the monolayer were not changed by TNFα exposure, indicating that TNFα did not affect the targets of the regulatory mechanism for secretion. In other words, it was unlikely that the cytokine had increased the number of spontaneously active Cl⁻ channels or K⁺ channels or had activated PKA. Also, application of forskolin to TNFα exposed cells in order to activate PKA did not have a different effect than in cells without prior exposure to TNFα. Activation of secretion by ionomycin, which induces activation of basolateral K⁺ channels and thereby increases the driving force for Cl⁻ secretion, was not affected by exposure to TNFα. TNFα may have caused an increase in the number of Cl⁻ channels. Following activation of PKC, these channels could be incorporated in the membrane. Alternatively, TNFα may have increased the pool of activable PKC. Both possibilities were excluded by studying the effect of the phorbol ester PDB. Exposure to TNFα did not affect the normal response to PDB. We then addressed the question whether PKC was involved in the activation of the short circuit current by carbachol and by carbachol after exposure to TNFα by using the PKC inhibitor bisindolylmaleimide I (GF109203X). This showed that, at the concentration used, the response to carbachol could be reduced by about 70 per cent. Interestingly, the reduction of the response after exposure to TNFα was more or less similar, namely 64 percent. GF109203X is a competitive inhibitor with respect to ATP [28]. Assuming that the ATP concentration in the cells had not changed drastically, the inhibition by a similar percentage suggests that the turnover of PKC after exposure to TNFα may be increased. Because the response to PDB had not changed, we excluded upregulation of PKC. Possibly, the turnover of PKC was increased because of an increased amount of DAG. These findings led us to postulate that the increased effect of carbachol was upstream from PKC, in other words between carbachol and PKC. The possibility that TNFα acted by inducing a larger amount of muscarinic receptors was unlikely, since TNFα also augmented the response to histamine. Thus, it could be that exposure to TNFα had increased the efficiency of activation of one of the phospholipases. We reasoned that because the first
Phase of the response to carbachol or to histamine (which is dependent on the Ca\(^{2+}\) peak induced by IP\(_3\) from PI-PLC action) was not affected by TNF\(\alpha\); it was less likely that TNF\(\alpha\) increased the activation of PI-PLC by the extracellular messengers. To confirm this, we studied the effect of the putative PI-PLC inhibitor U73122. The compound did not show inhibitory effects on the short circuit current induced by carbachol. Moreover, the negative control compound U73143 showed an anomalous prolonged depolarization after addition of carbachol, which is similar to the effect of U73122. On the basis of these findings we concluded that we could not use the compound as a tool to study the role of PI-PLC in these cells.

Given the inhibitory action of propranolol, we deduce that the PLD pathway was part of the reaction pathway between the muscarinic receptor and PKC. The finding that propranolol and cycloheximide fully suppressed the effect of exposure to TNF\(\alpha\) supports the hypothesis that the cytokine, by induction of de novo protein synthesis, increased the activity of the PLD pathway. To test this we performed phospholipid analysis by thin layer chromatography after growing cells in \(^{32}\)P-containing medium. In the presence of a primary alcohol, 1-butanol, PLD could catalyze the transphosphatidylation to the stable compound phosphatidylbutanol, Pbut [29]. The increased level of this lipid after exposure to carbachol or histamine lent further evidence to the hypothesis that both secretagogues activated the PLD pathway. However, the increase in Pbut was not larger after exposure of the cells to TNF\(\alpha\), whereas the level of PA was consistently lower. These findings, together with preliminary observations that the level of DAG is 20 percent higher in cells exposed to TNF\(\alpha\) and carbachol than in cells exposed to carbachol alone, suggest that the primary effect of TNF\(\alpha\) was on the conversion of PA to DAG by PAP.

**Phosphatidic acid phosphatase and its regulation**

As mentioned above, TNF\(\alpha\) is thought to act on the expression of PAP, thereby generating increased levels of DAG, causing subsequent PKC activation. A few research groups are presently studying the structure and functions of the PAP enzyme. Two classes of PAP enzymes can be distinguished. PAP-1 is Mg\(^{2+}\)-dependent and sensitive to inhibition by sulphydryl-reagents including N-ethylmaleimide [30, 31]. PAP-1 is found in the cytosol of the cells and is able to translocate to the endoplasmatic reticulum or mitochondria. A second and distinct PAP was identified in the plasma membranes of rat liver [30]. PAP-2 has attracted attention as the enzyme participating in the control of signal transduction mediated by PLD [6]. It does not require Mg\(^{2+}\) and is insensitive to inhibition with N-ethylmaleimide [32, 33]. At least three subtypes from PAP-2 have been identified up to now: PAP-2a, PAP-2b and PAP-2c [34]. PAP is identified as an integral plasma membrane glycoprotein proposed to contain four intracellular segments, including both N- and C-terminal regions, six transmembrane regions, and three extracellular loops [35]. It has a receptor like appearance.
This type of PAP can convert extracellular applied PA to intracellular DAG. Relatively little information is available concerning PAP regulation. Brindley et al. reviewed that PAP activity could be increased or decreased in several cell types, but the mechanisms remain uncertain [37]. Jiang and co-workers found evidence for a role for PKC in the regulation of PAP. They propose that PKCε may serve to either allosterically modify PAP or to localize PAP to appropriate membrane locations where PA substrate is localized [38]. These findings indicate that PKC may modulate the activity of PAP, besides affecting PLD activity (see below). Other reports propose coordination between PLD and PAP. Sciorra et al. showed that PLD and PAP are sequentially activated in specific membrane domains [39]. Martin et al. recognized a coordinated decrease in activity of PAP and PLD in oncogenically transformed fibroblasts as compared to controls [40].

Comparison of the HT29cl.19A and T84 cells

In T84 cells, the exposure to TNFα did not change the basal electrophysiological parameters and it did not increase the carbachol induced short circuit current (Chapter 4). This was in accordance with the idea that PKC plays no role in Cl− secretion in T84 cells. We base this conclusion on the following observations: 1) Activation of PKC with the phorbol esters TPA [41] and PDB (this thesis and [42]) did not affect basal ion secretion in T84 cells. 2) The PKC inhibitor GF109203X had no effect, corroborating observations by Lindeman and Chase with another PKC inhibitor [43]. In addition, we confirmed that T84 cells express little PKCα, considerably less than do HT29cl.19A cells. Others have shown that application of carbachol did not result in a translocation of PKCα [15]. Based on these findings, we hypothesize that the lack of the potentiating effect of TNFα on the carbachol response in T84 cells was due to an insufficient expression of PKCα in this cell line. Thus, these cells did not express the constituents to show the possible effects of exposure to TNFα on the PLD route.

The T84 cell line is used in a large number of laboratories as the model for colonocytes. Because the cells appear to develop less synchronically than the HT29 clone and because it is much more difficult to obtain stable microelectrode readings from these cells [44], T84 cells have not been extensively used in our laboratory. For a comparison with the HT29 clone we had to perform microelectrode analysis of the response to carbachol. An important difference between the two cell lines is that in T84 cells the resting membrane potential appeared to be depolarized and the effect of carbachol was primarily activation of K+ channels in the basolateral membrane. The small increase in short circuit current could therefore be interpreted as a consequence of the increased driving force for Cl− exit through already open Cl− channels in the apical membrane.

By means of thin layer chromatographic analysis of T84 cells we observed that, as was the case in HT29cl.19A cells, the formation of Pbut was increased after stimulating the
muscarinic receptor with carbachol. This indicates that in these cells carbachol activated PLD as well. Moreover, after exposure to TNFα, a consistently higher level of Pbut was observed. Thus, in HT29cl.19A cells, TNFα exposure seemed to increase the activation of PAP, whereas in T84 cells, TNFα seemed to increase PLD activity. It is possible that TNFα affected different proteins in the two cell lines. But another explanation for the differences in these cell lines may be offered in analogy with the observed difference in PKC expression in the cell lines. In Chapter 3 we stated that in HT29cl.19A cells the carbachol-induced increase in Pbut was not diminished by the PKC inhibitor GF109203X. Preliminary results in T84 cells showed that the increase in Pbut after carbachol was attenuated by the PKC inhibitor. This suggests that the regulation of PLD activity by PKC may be different in the two cell lines.

PKC plays a major role in the regulation of PLD as demonstrated by the activation of PLD by phorbol esters [45, 46, 47, 48]. Downregulation of classical and novel PKC isotypes blocks the ability of many agonist to stimulate PLD [3]. Moreover, alterations of the expression of PKC isoymes in cells results in altered PLD activity. Overexpression of PKCα and PKCβ1 show enhanced PLD responses to several agonists [49, 50]. By contrast, depletion of PKCα by transfection with antisense cDNA decreases the activation of PLD by agonists in MDCK cells [51]. These findings implicate an important role for the classical PKC isoforms in regulation of PLD activity. PKC is thought to modulate the PLD activity via two distinct mechanisms (reviewed in [45, 46, 47]). The first mechanism is thought to involve phosphorylation and to require ATP. Phosphorylation of PLD itself or some associated regulatory component has been reported. The second mechanism is phosphorylation-independent. Certain PKC isoforms do not require ATP for their regulation of PLD activity. There is no protein phosphorylation and they are thought to occur via protein-protein interactions. Additionally, small G-proteins like Arf and Rho are able to activate the activity of PLD individually, but also can act synergistically with PKC in activating PLD, however, their mechanism of action has not yet been identified [47, 45]. At this moment, the mechanisms of PLD activation in intestinal cell lines is not known.

The levels of phospholipids differed in the two cell lines. In Table 1 the amounts of the phospholipids Pbut and PA (as percentage of total labeled phospholipids) are presented for the two cell lines in the unstimulated and stimulated condition. The basal level of Pbut in HT29cl/19A cells was about 2.4 times larger than in T84 cells (0.55 vs. 0.25). Assuming that the relative amount of 32P-labeled substrate for PLD was not different, this might indicate that in HT29cl.19A cells the PLD activity was 2.4 times larger. The table also shows that the increase in PLD activity by carbachol was about 2.5 times larger in HT29cl.19A cells than in T84 cells (0.10 vs. 0.04). Furthermore, the increases of the PA levels were about twice as large in HT29cl.19A cells than in T84 cells (3.13 vs. 1.87). The difference between the two cell lines in Pbut and PA levels were compatible with preliminary Western blots, which
showed a larger amount of PLD in HT29cl.19A cells as compared to T84 cells. Thus, the expression and activity of PLD in HT29cl.19A cells appeared to be larger than in T84 cells.

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Table 1. Amounts of $^{32}$P-labeled phospholipids (phosphatidylbutanol, Pbut, and phosphatidic acid, PA) as percentage of total labeled phospholipids.

Comparison with intestinal tissue

It is interesting that the results obtained in the HT29cl.19A cell line could be extrapolated to native tissue. In Chapters 4 and 5 we show that TNFα also potentiated the response to carbachol and histamine in mouse distal colon. As this potentiation was observed in the presence of indomethacin to prevent prostaglandin synthesis and TTX to prevent neuronal activity, we assume that we have demonstrated synergy of TNFα and carbachol and histamine at the level of the epithelial cell. Whether TNFα acts via a similar mechanism of action still remains to be investigated. Preliminary results pointed out that propranolol was able to diminish the carbachol-induced ion secretion as well. It must be noted, however, that propranolol has additional effects on native tissue, which preclude a final conclusion at this moment.

Role of TNFα in inflammatory diarrhea

In contrast to our finding that TNFα increased the secretory responses to acetylcholine and histamine, in inflamed tissue from patients suffering inflammatory bowel disease (IBD) it has been found that secretory responses are diminished [52, 53, 54, 55, 56, 57]. These observations were made in tissue which had been in an inflammatory state for a prolonged period. The diminished secretory responses are ascribed to desensitization for these secretagogues [55].

In models consisting of T84 cells in co-culture with immune-activated peripheral blood mononuclear cells (PBM), the secretory responses to carbachol and forskolin are diminished and the barrier function is also decreased [58, 59]. This effect is ascribed to joint action of
interferon γ and TNFα, because antibodies to these cytokines can prevent or strongly reduce their disabling effects. The measurements were taken at 24 hours or more after stimulation of the PMN with inflammatory agents and no data are presented for earlier secretory activity. However, Zareie et al. showed that co-culturing T84 cells with activated monocytes during 48 hours, results in an increase of basal ion secretion. The secretory response to forskolin is also diminished, which may be due to the increase in basal secretory state of the epithelium [60]. They suggest that TNFα autocrinely affects the monocytes, thereby causing the release of other monocyte-derived mediators involved in the altered epithelial function.

In this thesis we report on the more direct action of TNFα on epithelium. Our hypothesis is that prolonged exposure to TNFα in intestinal epithelium may result in prolonged PKC activation, due to the upregulation of the PLD pathway. This may result in downregulation of secretory mechanisms. Prolonged activation of PKC is thought to inhibit basolateral K⁺ conductance [61, 62, 63, 64, 41], which may subsequently result in an inability to reveal a secretory response to acetylcholine or histamine.

We were unable to provide evidence for the effect of TNFα on the barrier function. TNFα did not induce cell death of the epithelial cells (Chapter 2). This might be due to the rather low concentration of the cytokine in our experiments and because of the lack of interferon γ (IFNγ) in our incubation medium. Inflamed tissue from humans and animal models are reported to have a decreased barrier function [65, 57, 66, 67, 68, 69]. In epithelial cell lines too, TNFα decreases the epithelial resistance and increases the permeability for macromolecules [70, 71, 72]. Recently, Mankertz et al. reported that TNFα affects the barrier by a downregulation of the expression of the tight junction protein occludin [73]. However, these experiments were performed after long exposure to the cytokine and the concentration of TNFα was relatively high, or the measurements were performed in the presence of IFNγ, which is believed to induce TNFα receptors, thereby increasing the susceptibility to the cytokine [74]. Possibly, due to the upregulation of the PLD pathway by TNFα, prolonged PKC activation results in decreased barrier function, since it has been shown that massive stimulation of PKC by phorbol esters causes a decrease in permeability with a slow time course [75, 76].

Future directions

The results presented here lead to more questions concerning the effect of TNFα on epithelial ion secretion. We would like to know via which receptor TNFα exerts its effect on the epithelium and which intracellular pathway couples to this receptor in increasing the synthesis of a protein involved in the PLD pathway. We do not know whether TNFα directly affects the de novo synthesis of PAP, or whether an intermediate activator is involved. Western blot analysis with PAP antibodies could provide us with more direct evidence for the
involvement of the expression of PAP. Unfortunately, commercial antibodies against PAP are not available yet. Measurements of PAP activity could also help to disclose more information about the mechanism involved. Measurements of activity of PAP have been described [77, 78]. However, activity measurements do not tell whether there is more PAP protein or whether there is an increased activity of some stimulating protein. Therefore, we are planning to combine Western blot analyses with activity measurements for PAP. We will follow a similar approach for PLD.

We hypothesize that the chloride secretion after exposure to TNFα in HT29cl.19A cells is increased via DAG-activated PKCα. This requires further confirmation by direct analyses of DAG in the cells. Furthermore, we want to find out whether other DAG-activated PKC-isotypes are also activated.

PKC translocation assays, together with activity assays, could provide more evidence for the involvement of the different isotypes. Inhibition of specific PKC isoforms using different combinations of isotype-specific inhibitors [79], antisense oligonucleotides to specific PKC isoforms [80] or modulation of activities of individual isoforms by an inducible expression system [81], will yield more information. Furthermore, it is of great importance to evaluate the expression and activation of different PKC isoforms in native tissue and to study the effect of TNFα. Recently, Chang et al. provided evidence for alterations of PKC isoforms in intestinal tissues of inflamed origin compared to control intestinal tissue [82]. This finding indicates that a differential expression exists in inflamed tissue compared to control tissue. Possibly, TNFα contributes to this differential expression pattern of PKC isotypes.

To evaluate the mechanism of action of TNFα in mouse distal colon, more electrophysiological experiments need to be performed. The role of PKC and PLD activation in the carbachol and histamine response in native tissue need to be investigated in more detail, as well as the phospholipids involved in these responses. Measurements with native tissue are more difficult to interpret since a mixture of cells, including nerve cells, immunocytes and fibroblasts are present in the preparation. They may all react to the drugs applied by secreting messengers, which activate the epithelial cells. It also appeared to be more difficult to keep the intestinal tissue viable during the electrophysiological experiments. This may be a crucial factor in the phospholipid labeling studies.

In order to evaluate the role of the PLD/PKC pathway in inflamed tissue from patients suffering IBD, analysis of the involvement of the PLD pathway in human biopsies should be performed. The activity of the PLD pathway in biopsies from these patients should be analyzed, and related to electrophysiological studies.
Role of NPY as an anti-secretory peptide

In the second part of this thesis we present results concerning the effects of NPY on ion secretion in HT29cl.19A cells. It has been shown that NPY is a potent inhibitor of intestinal chloride secretion in native tissue [83-89]. The mechanisms of the inhibition were not studied in detail. We showed that NPY inhibited chloride secretion induced by several secretagogues, acting via different second messengers. The secretory response to the cAMP stimulus forskolin was inhibited by NPY. The increase in $I_{sc}$ was reduced by over 50 percent and the cellular depolarization induced by forskolin returned to basal values after the addition of NPY. This phenomenon was not surprising as NPY is known to activate G$_i$-protein and thereby reduces the activity of adenylate cyclase. Additionally, we showned that NPY could reduce ion secretion induced by carbachol and this effect appeared to be largely due to a diminished rise of the intracellular calcium levels (Chapter 6). NPY also reduced the basal $I_{sc}$ and the transepithelial resistance. The intracellular Ca$^{2+}$ level was also decreased by NPY. By contrast, the intracellular potential $V_a$ and the fractional resistance $fR_a$ were not affected. The latter intracellular parameters reflected the combined characteristics of the apical and the basolateral membranes. Therefore, the observed changes in extracellular parameters required that the electromotive force of the apical membrane increased (hyperpolarized) and the electromotive force of the basolateral membrane reduced (depolarized). At the apical side this could be an effect of reduced cAMP and, therefore, reduced activation of PKA sensitive Cl$^-$ channels in the apical membrane. The decreased intracellular Ca$^{2+}$ level may have reduced the activity of Ca$^{2+}$-dependent basolateral K$^+$ channels, a finding also reported by Xiong and Cheung [90]. An alternative explanation could be that by reducing cAMP, NPY decreased activity of cAMP-activated K$^+$ channels. Such K$^+$ channels have been described by Greger's group in rat and rabbit colon cells as very small, highly abundant channels that could only be studied by noise analyses [91]. They have a slower activation than the apical CFTR channels and, therefore, may show up as a repolarization in the cell potential change induced by cAMP. By means of time analyses of changes in fractional resistance induced by the increase of cAMP by forskolin, Bajnath et al. concluded that this clone of HT29 cells does not express cAMP-activated K$^+$ conductance in the basolateral membrane [92]. Furthermore, NPY may affect the K$^+$ conductance directly, and not via the lowering of the intracellular Ca$^{2+}$ level.

The aim of the study presented in Chapter 7 was to test this possibility. We reasoned that the occurrence of an effect of NPY on basolateral K$^+$ conductance in the presence of ionomycin, which increased intracellular Ca$^{2+}$ level drastically, would indicate that NPY could have a Ca$^{2+}$ independent effect on the K$^+$ conductance. We actually observed this by comparing ionomycin induced changes in $V_a$ and $fR_a$ in the absence and presence of NPY.
We therefore concluded that NPY could have an effect on the \( \text{Ca}^{2+} \)-activated \( \text{K}^+ \) channels that do not require the lowering of intracellular \( \text{Ca}^{2+} \).

We also looked for a possible interaction of NPY with phorbol ester-stimulated \( \text{Cl}^- \) secretion. The response of the monolayer to the phorbol ester PDB in the presence of NPY showed that NPY also reduced the secretory response elicited by activation of PKC. This may be explained in the context of our view of the action of PKC. Bajnath et al. suggested that the activation of \( \text{Cl}^- \) channels via PDB-activated PKC occurs via increased incorporation of \( \text{Cl}^- \) channels in the apical membrane. These channels are then activated by the current PKA activity [61]. By reducing cAMP, NPY would reduce PKA activity and, therefore, the effect of PKC activation. An intriguing observation was that, although the exposure to NPY reduced the extracellular effect of PDB, the intracellular effect (maximal depolarization of \( V_a \) and decrease of \( fR_a \)) was not affected significantly. We expected to find a reduced depolarization and reduced decrease of \( fR_a \). Again, an inhibition of transepithelial current requires that the difference between \( E_a \) and \( E_b \) is smaller. A reduced depolarization of \( E_a \) and a concomitant depolarization of \( E_b \) could explain an unchanged value for \( V_a \) and \( fR_a \). The smaller depolarization of \( E_a \) was predicted. The depolarization of \( E_b \), however, was not expected and may be explained by an increased inactivation of the basolateral \( \text{K}^+ \) channels in the presence of NPY.

In summary, we postulated that NPY inhibits the cAMP-induced secretion and the carbachol-induced secretion via inhibition of adenylate cyclase and a decrease in intracellular calcium levels, which might affect the basolateral \( \text{K}^+ \) conductance. Furthermore, NPY inhibits the calcium-dependent basolateral \( \text{K}^+ \) conductance in a calcium-independent way. This makes NPY a strong anti-secretory messenger. This is in agreement with evidence from the literature. In small and large intestine of several species NPY has been found to decrease basal as well as cAMP-mediated ion secretion [83-89]. Cox and Cuthbert also demonstrated a decrease in \( \text{Ca}^{2+} \)-stimulated ion secretion in rat jejunum by NPY via a decrease in intracellular \( \text{Ca}^{2+} \) levels [83]. To our knowledge no further evidence has been presented for a role for NPY on the basolateral potassium conductance in intestinal epithelium. Unfortunately, we were unable to study the action of NPY on the basolateral potassium conductance further because the cell line lost its responsiveness to NPY. Presumably, due to changes in serum conditions, the cells lost the expression of NPY receptors or an intracellular target for activated receptors. We were unable to restore the previous conditions of the cell line using other sera or earlier passage numbers.

Another neuropeptide, somatostatin, has also been recognized as a potent anti-secretory peptide. It is capable of inhibiting basal, cAMP-induced, and calcium-induced ion secretion in intestinal epithelium [93, 94, 95, 96]. The mechanism by which somatostatin inhibits ion secretion involves inhibition at multiple G-protein-dependent sites with inhibition of cAMP production. In addition, somatostatin has been recognized to inactivate basolateral potassium channels [97, 98]. Thus, the mechanism of action of somatostatin seems to resemble that of
NPY. These potent anti-secretory peptides are increasingly recognized as therapeutic agents in the treatment of secretory disorders. For example, Sandostatin, a somatostatin analogue is used to cure diarrhea [99, 100]. A better understanding of the signaling processes involved may provide a basis for the development of new anti-secretory drugs.

The neuropeptide NPY is considered a pro-absorptive and anti-secretory peptide [84, 101, 102]. It is found in high concentrations in the enteric neurons of the myenteric and submucosal ganglia. Many of these neurons directly innervate the epithelial cells of the intestinal mucosa. NPY colocalizes with another important neurotransmitter vasoactive intestinal peptide (VIP) in the mucosa and submucosa [103, 104]. The presence of the neuropeptides within intrinsic mucosal nerves suggests a physiological role for these peptides in regulating epithelial ion secretion as secretory and anti-secretory messengers. The peptide PYY, which has a similar action as NPY, is found in endocrine cells in the epithelium. We propose that a change in the ratio of NPY(PYY)/VIP might result in a changed absorption/secretion balance, thereby changing ion secretion, which in turn may result in constipation or diarrhea.

Several authors have shown decreased levels of VIP in descending and sigmoid colon of patients with idiopathic constipation [105, 106, 107]. Not much is known about the levels of NPY in colon from patients with idiopathic constipation. In the studies by Koch et al. [106] and Milner et al. [105], the ratio between VIP and NPY is slightly decreased in constipated patients. Sjolund et al. [108] showed that in the descending colon of constipated patients, NPY immunoreactivity is also increased in the myenteric plexus, while the density of VIP nerve fibers is reduced in the mucosa/submucosa. The frequency of PYY-containing cells in the ascending colon is also significantly increased.

Constipation often occurs in the elderly but the mechanism underlying this type of constipation remains uncertain (idiopathic constipation) [109]. It may be related to a change in the ratio of the regulating peptides. An age-associated increase in numbers of NPY containing enterochromaffin cells, has been described [110]. Sweet et al. determined the number of NPY-containing cells in young (2 months) and older (22-30 months) old rats [111]. They found that the number of NPY-containing cells is significantly increased in older rats. Thus, an increased NPY/VIP ratio may contribute to the constipation observed in the elderly.

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