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Neuropeptide Y inhibits the protein kinase C-stimulated Cl^- secretion in the human colonic cell line HT29cl.19A cell line via multiple sites

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Abstract

Neuropeptide Y is known to exert inhibitory effects on ion secretion in the intestine by reducing the activity of adenylyl cyclase. In the human intestinal epithelial cell line HT29cl.19A, it has been previously shown that neuropeptide Y inhibits the electrophysiological phenomena related to Cl^- secretion, when induced by elevation of cAMP via forskolin. Moreover, the secretion induced via elevation of intracellular calcium levels via muscarinic activation can be inhibited by neuropeptide Y. Part of these inhibitions appeared to be due to lowered calcium activity in the epithelial cells, thereby reducing the basolateral K^+ conductance. The phorbol ester 4-phorbol-12,13-dibutyrate (PDB) can induce secretion in this cell line via activation of protein kinase C as well; however, the effect of neuropeptide Y on this pathway has not yet been studied. In the present experiments, it is shown that neuropeptide Y inhibits the PDB-induced secretion at two sides: one located in the apical membrane and another in the basolateral membrane. It is shown that the latter effect can, at least partially, be explained via a direct effect of neuropeptide Y on the K^+ conductance. This was concluded from the observation that neuropeptide Y could also reduce basolateral K^+ conductance when intracellular calcium was dramatically increased by ionomycin. The observed inhibitory effects suggest that neuropeptide Y is a very powerful antisecretory peptide in human intestinal epithelial cells.

Keywords: Neuropeptide Y; Protein kinase C; Cl^- secretion; K^+ conductance; Electrophysiology; Epithelia

1. Introduction

Neuropeptide Y is a 36-amino-acid peptide and a member of the pancreatic polypeptide family (Tatemoto et al., 1982). Neuropeptide Y is widely distributed throughout the central and peripheral nervous system, and has been reported to be one of the most abundant peptides in the mammalian brain (Adrian et al., 1983; Lundberg et al., 1984). The peptide is involved in many important physiological activities, including effects on psychomotoric activity, food intake, modulation of neurotransmitter release and vasoconstriction (Flood et al., 1989; Matsuda et al., 1993; Potter, 1988; Waeber et al., 1988; Walker et al., 1988). In the intestine, neuropeptide Y has been localized in subpopulations of myenteric and submucosal enteric neurons, which directly innervate the epithelial cells of the intestinal mucosa in different species, including human (Furness et al., 1983; Koch et al., 1988; Sundler et al., 1983). Neuropeptide Y may play an important role in the regulation of ion transport in intestinal epithelia. In different species, including human, it has been shown that neuropeptide Y is a potent inhibitor of intestinal Cl^- secretion. In vivo and in vitro studies showed that both the stimulated as well as the resting Cl^- secretion is inhibited by neuropeptide Y (Brown et al., 1990; Cox and Cuthbert, 1988; Holzer-Petsche et al., 1991; Saria and Beubler, 1985; Strabel and Diener, 1995).

Neuropeptide Y receptors have been identified in a wide variety of tissues and can be divided into five subtypes (Y_1, Y_2, Y_3, Y_4 and Y_5) on the basis of pharmacological criteria (Blomqvist and Herzog, 1997; Hoyle, 1999). These receptor subtypes are members of the G-protein-coupled receptor superfamily; the G protein involved may be in the form of G_i or G_o (Ewald et al., 1988; Feth et al., 1991). No fixed coupling between receptor type and intracellular messenger system has been described. Second messenger coupling of a Y-receptor seems to be cell

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type-specific, depending on the specific repertoire of G proteins and effector systems present in the given cell type (Ballantyne et al., 1993; Herzog et al., 1992). However, neuropeptide Y effects on two second messenger systems have frequently been described, namely, inhibition of adenylyl cyclase and increase as well as decrease of intracellular calcium levels (Cox et al., 1988; Lundberg et al., 1988; Michel et al., 1992; Motulsky and Michel, 1988). In the butyrate-treated human colonic tumour cell line HT29, a type Y_1 receptor has been recently cloned (Mannon et al., 1994). In these cells, the receptor was negatively coupled to cAMP via inhibition of adenylyl cyclase. Clone 19A of the HT-29 cell line represents a valuable model to study the electrophysiology of intestinal epithelial secretion (Bajnath et al., 1991). It was found that a variety of intracellular pathways can induce Cl^- secretion in these cells, including cAMP via activation of protein kinase A, activation of protein kinase C and increased intracellular calcium levels. Recently, it has been shown that neuropeptide Y inhibits cAMP and calcium-mediated Cl^- secretion in the HT29cl.19A cells by lowering the levels of both intracellular messengers (Bouritius et al., 1998). The stimulation of Cl^- secretion by activation of protein kinase C is thought to be due to a synergism between protein kinase C and protein kinase A and, therefore, dependent on the prevailing cAMP activity (Bajnath et al., 1993, 1995). Thus, one would postulate to find a reduced effect of the phorbol ester PDB after incubation with neuropeptide Y. This has been tested in the present experiments. From the observed electrophysiological effects, we concluded that the PDB response was partially reduced because of a reduced K^+ conductance in the basolateral membrane. To test the possibility that neuropeptide Y exerts a direct effect on the K^+ conductance, we increased intracellular calcium levels drastically using the calcium ionophore ionomycin to overrule effects of neuropeptide Y on calcium levels. The results from previous studies (Bouritius et al., 1998) and the present experiments show that neuropeptide Y affects Cl^- secretion via at least three mechanisms.

2. Materials and methods

2.1. Cell culture

HT29cl.19A cells were grown and subcultured as described (Bajnath et al., 1991). The cells were grown to confluence on permeable filters (Falcon, 25 mm diameter). Confluence was reached 7 days after seeding. Cells were used between 13 and 26 days after seeding and between passages 12 and 29.

2.2. Electrophysiological experiments

Filters, containing confluent monolayers, were mounted horizontally in a small Ussing chamber, leaving an oblong area of 0.35 cm^2. The apical and basolateral compartments were continuously perfused with a Ringer's buffer at a temperature of 37°C and gassed with 5% CO_2/95% O_2. 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, mannitol 28. The transepithelial potential (V_t) was measured with Ringer/agar bridges, which were connected to Ag–AgCl electrodes. The intracellular potential (V_a) was measured using a glass microelectrode filled with 0.5 M KCl and connected to a high-input-impedance amplifier. The impedance of the glass microelectrode varied between 100 and 200 MΩ, and the tip potentials between −2 and −5 mV. All measurements were performed under open circuit conditions and the apical solution was used as a reference for V_t and V_a measurements. Transepithelial resistance (R_t) and the fractional resistance of the apical membrane [$f R_a = R_a/(R_a + R_b)$] were calculated from voltage deflections induced by bipolar current pulses of 1 s (10 and 50 μA). The current electrodes were situated in the walls of the apical and basolateral compartments. The equivalent short-circuit current (I_{sc}) was calculated from V_t and R_t. The measurements were corrected for the potential offset, the solution resistance and the resistance of the filter without cells.

In experiments performed in the presence of neuropeptide Y, the cells were preincubated during 20 min and neuropeptide Y remained present in the serosal solution during the experiment.

2.3. Materials

Neuropeptide Y, PDB, ionomycin and adenosine were purchased from Sigma (St. Louis, MO). The final concentration of the carrier solution dimethyl-sulfoxide (DMSO) (for PDB and ionomycin) was 0.01% (v/v). This concentration was without electrophysiological effect. Cell culture materials were obtained from Gibco.

2.4. Statistics

All data are presented as means ± S.E. Statistical significance was evaluated using the unpaired Student *t*-test.

3. Results

3.1. Effect of neuropeptide Y on the PDB-induced secretion

Previous studies have shown that the phorbol ester 4-phorbol 12,13-dibutyrate (PDB) activates apical Cl^- conductance and, with a much slower time course, reduced the basolateral K^+ conductance via protein kinase C in HT29cl.19A cells (Bajnath et al., 1992b). Fig. 1 shows a typical response, representing seven identical experiments consisting of application of 1 μM PDB to the mucosal side

of the cells. Initially, V_t and I_{sc} increased concomitantly with depolarisation of V_a and a decrease in fR_a. This indicates an enhancement of the Cl^- conductance in the apical membrane. After reaching its maximal value (indicated by the thin line *a*, see Fig. 1), V_t and I_{sc} started to decline slowly, although V_a further depolarised, indicating an inhibition of basolateral K^+ conductance. This reduced the driving force for Cl^- efflux and, therefore, decreased V_t and I_{sc}. These data corroborated earlier observations in these cells (Bajnath et al., 1992b). In the presence of neuropeptide Y, the PDB-induced increase in V_t and I_{sc} was reduced significantly; however, neuropeptide Y had no measurable effect on PDB-induced changes in V_a and fR_a. The effects of neuropeptide Y on PDB-induced electrical parameters are summarised in Table 1. A decrease of V_t and I_{sc}, without a measurable change in V_a and fR_a, requires that PDB-induced changes in both E_a and E_b (the electromotive forces across the apical and the basolateral membrane) change in such a way that their difference becomes smaller. This requires a reduced depolarisation of E_a and a depolarisation of E_b. The depolarising effect of neuropeptide Y on E_b may be due to a decrease of the K^+ conductance because of a reduction in intracellular calcium levels; however, it could also be that neuropeptide Y had a direct effect on the K^+ conductance. This possibility has been tested using ionomycin.

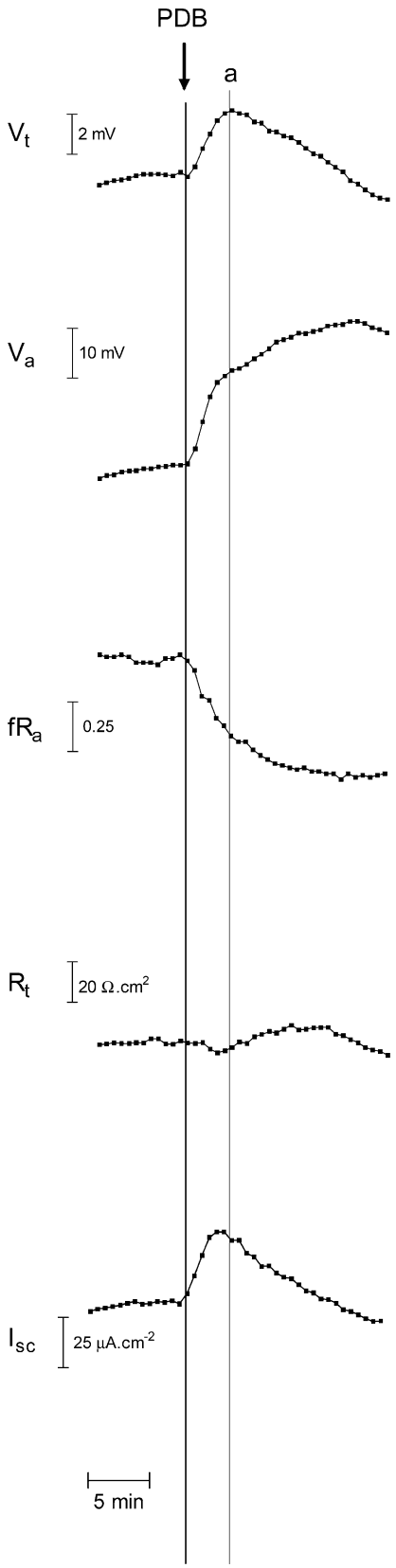

Fig. 1. Typical response, representing seven experiments showing the effects of application of 1 μM PDB to the apical side of the cells. V_t: transepithelial potential, V_a: membrane potential across the apical membrane, R_t: transepithelial resistance, fR_a: fractional resistance of the apical membrane, I_{sc}: short circuit current calculated from V_t and R_t., $t = a$, indicates the time point where the V_t and I_{sc} had reached their maximum values. In the presence of neuropeptide Y, the transients were qualitatively not different. For quantitative differences at $t = a$, see Table 1.

Table 1
Effect of neuropeptide Y on the changes in electrical parameters induced by PDB

	V_t (mV)	R_t (Ω cm^2)	I_{sc} (μA/cm^2)	V_a (mV)	fR_a
Control					
Baseline	4.8±0.5	133±14	39±5	−49±2	0.72±0.02
PDB (t = a)	7.5±0.7	119±12	66±7	−28±2	0.34±0.02
ΔPDB	2.7±0.2	−14±3	27±2	20±2	−0.38±0.04
NPY preincubation					
Baseline	2.0±0.5[a]	109±11	22±5[a]	−47±3	0.66±0.02
PDB (t = a)	3.9±0.5[a]	98±10	40±5[a]	−28±2	0.34±0.03
ΔPDB	1.8±0.3[a]	−11±3	19±3[a]	19±2	−0.31±0.02

Values are means±S.E.; $n = 7$ monolayers. The electrical parameters given for $t = a$ are the values at the time the V_t and the I_{sc} has reached their maximal value after addition of 1 μM PDB to the mucosal side of the cells (see Fig. 1). After this moment, V_a continued to depolarise see Fig. 1.

[a] $P < 0.05$ with respect to control values.

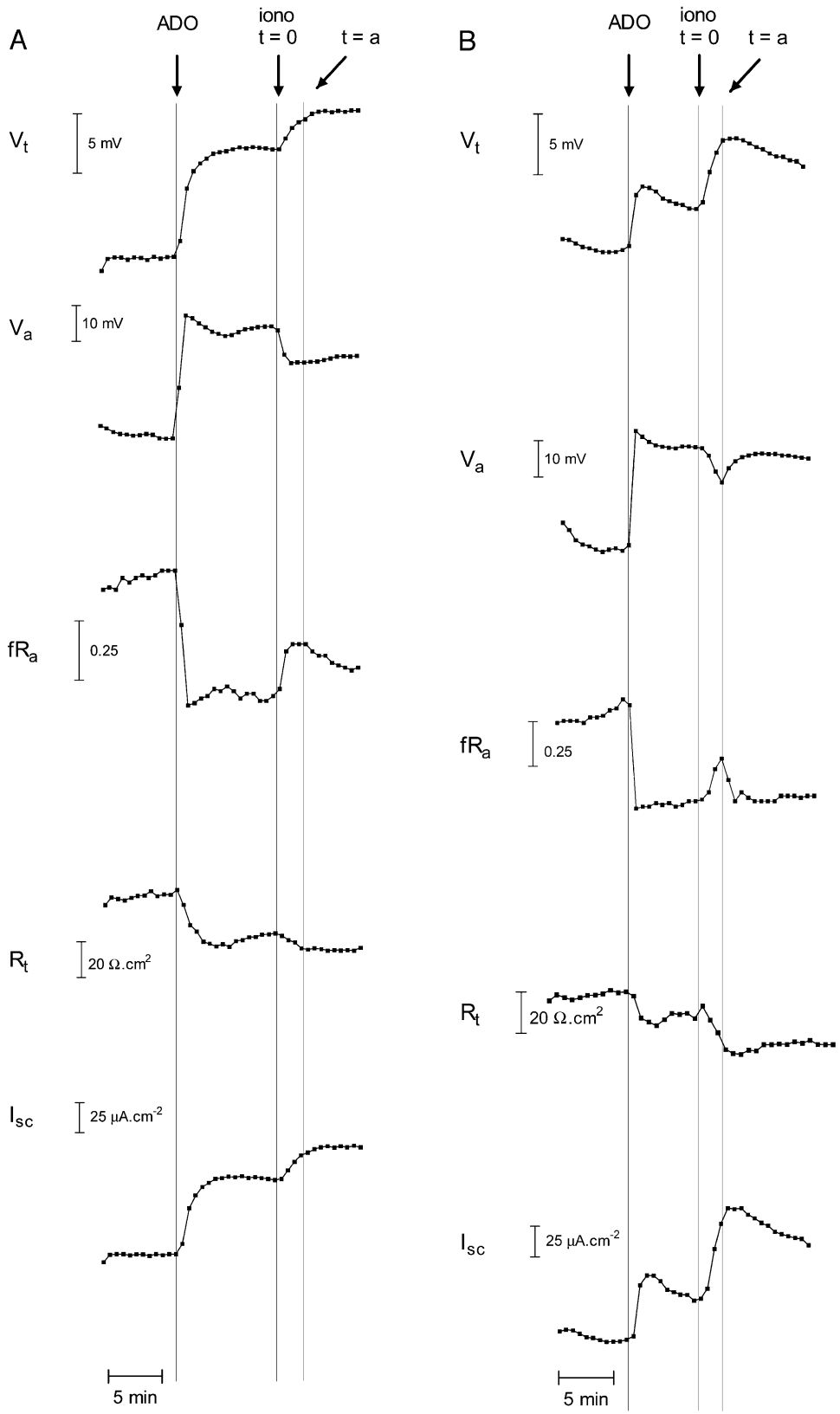

Fig. 2. (A) Typical electrophysiological response after subsequent addition of adenosine (ADO; serosal) and ionomycin (iono; mucosal) to HT29cl.19A cells. For explication of symbols see Fig. 1. (B) Typical response of subsequent addition of adenosine (ADO) and ionomycin (iono) to HT29cl.19A cells in the presence of neuropeptide Y (added at $t = -20$).

Table 2
Effect of neuropeptide Y on changes in electrical parameters induced by ionomycin

	V_t (mV)	R_t (Ω cm^2)	I_{sc} (μA/cm^2)	V_a (mV)	fR_a
Control					
Before ionomycin ($t = 0$)	6.0 ± 0.7	165 ± 32	50 ± 11	-18 ± 1	0.13 ± 0.02
After ionomycin (t = a)	8.4 ± 0.7	153 ± 27	69 ± 12	-29 ± 1	0.34 ± 0.01
Δ Ionomycin	2.4 ± 0.5	-12 ± 7	19 ± 6	-10 ± 1	0.21 ± 0.02
6 min after ($t = 6$)	9.1 ± 0.8	155 ± 26	73 ± 14	-28 ± 1	0.30 ± 0.04
Δ Ionomycin $t = 6$ vs. $t = 0$	3.0 ± 0.7	-10 ± 7	23 ± 8	-10 ± 1	0.17 ± 0.02
NPY preincubation					
Before ionomycin $t = 0$	5.2 ± 0.7	151 ± 19	39 ± 8	-17 ± 2	0.10 ± 0.01
After ionomycin (t = a)	8.3 ± 1.0	140 ± 18	68 ± 13	-25 ± 3	0.29 ± 0.04
Δ Ionomycin	3.1 ± 0.5	-11 ± 3	29 ± 7	-8 ± 2	0.19 ± 0.03
6 min after ($t = 6$)	8.3 ± 1.0	142 ± 17	64 ± 13	-21 ± 2[a]	0.18 ± 0.03[a]
Δ Ionomycin $t = 6$ vs. $t = 0$	3.2 ± 0.5	-9 ± 4	26 ± 5	-5 ± 1[a]	0.08 ± 0.02[a]

Values are means $\pm$ S.E.; $n = 8$ or 9 monolayers. The electrical parameters measured at $t = 0$ are measured at the moment just prior to addition of ionomycin (see Fig. 2). The change in electrophysiological parameters induced by ionomycin (Δ ionomycin) were measured at t = a (see Fig. 2A and B). At $t = 6$, parameters were measured and compared to the parameters measured at $t = 0$ to show neuropeptide Y-dependent changes in V_a and fR_a.
[a] $P < 0.05$ with respect to control values.

3.2. Effect of neuropeptide Y on the ionomycin response

To study the possible direct effect of neuropeptide Y on the K^+ conductance, we increased the intracellular calcium concentration drastically by adding the calcium ionophore ionomycin to the apical side of the cells, to overrule effects of neuropeptide Y on intracellular calcium levels.

Ionomycin induces a transient depolarisation followed by a hyperpolarisation of rather variable magnitude (Bajnath et al., 1992a). To make the hyperpolarisation of the ionomycin response less variable, we increased the conductance of the apical membrane for chloride. This increases the driving force for K^+ efflux and, therefore, increases the transepithelial potential changes and I_{sc}. We used 0.1 mM adenosine at the basolateral side to enhance the apical Cl^- conductance. Basolateral application of adenosine increases the apical chloride conductance via an activation of the phospholipase A2 pathway in these cells (Bouritius et al., 1999). It has been shown previously that its effect was not affected by neuropeptide Y (Bouritius et al., 1998). Fig. 2A shows a registration of a control experiment, representing eight experiments. Application of 0.1 mM adenosine to the basolateral side of the cells resulted in an increase of V_t and I_{sc}, a depolarisation of V_a and a decrease of fR_a. After adenosine reached its maximal effect, 1 μM ionomycin was added to the apical side of the cells (indicated with the line $t = 0$). This increased V_t and I_{sc}, concomitant with a sustained repolarisation and increase in fR_a, caused by enhancement of calcium-dependent K^+ conductance (indicated with line $t = a$). Fig. 2B shows a typical recording of the effect of ionomycin in the presence of neuropeptide Y representing nine monolayers. Preincubation with 1 μM neuropeptide Y did not affect the adenosine response, as expected, and also did not affect the ionomycin response immediately (at $t = a$). However, the repolarisation and increase in fR_a were now transient, returning to preionomycin levels. Table 2 shows a summary of the electrical parameters and the changes induced by ionomycin at the time of its maximal effect ($t = a$) and 6 min after its addition. At this time V_a and fR_a were significantly decreased compared to control values, indicating reduction of the basolateral K^+ conductance. The transepithelial parameters declined as well, but with a slower rate, and their time course was not followed long enough in all experiments.

4. Discussion

The peptide neuropeptide Y is known to play an important role in regulating ion secretion in the intestine. Ion secretion is activated by different second messenger systems. Increasing cAMP levels via activation of adenylyl cyclase and increasing intracellular calcium levels lead to an enhancement of the Cl^- secretion by an increase of the apical Cl^- conductance and of the basolateral K^+ conductance, respectively. It has already been shown that neuropeptide Y inhibits the cAMP- and calcium-induced Cl^- secretion in the human intestinal cell line HT29cl.19A. In the present study, we investigated the effect of neuropeptide Y on the protein kinase C-induced secretion in this model. The PDB-induced increase in I_{sc} is significantly reduced by neuropeptide Y. Protein kinase C is thought to induce the Cl^- secretion by increasing the incorporation of Cl^- channels in the apical membrane, which then are activated by basal protein kinase A activity (Bajnath et al., 1993, 1995). Decreased activity of protein kinase A, caused

by inhibition of adenylyl cyclase as reported earlier (Bouritius et al., 1998), could have caused the smaller increase of the apical Cl^- conductance and thereby a decreased ion secretion when PDB was applied after preincubation with neuropeptide Y. However, if only the cAMP-dependent Cl^- conductance was involved in the inhibitory action of neuropeptide Y, one would expect an effect on the intracellular potential and the fractional resistance; however, these parameters remained unchanged.

This unusual observation can be explained by considering the equivalent electrical circuit for leaky epithelia (Groot and Bakker, 1988). Changes in the transepithelial potential are the consequence of changes in transcellular current flowing back through the paracellular resistance. Because in leaky epithelia the transepithelial resistance is a good monitor for the paracellular resistance, a constancy of resistance at a changing transepithelial potential indicates a change in the transcellular current. With a small inaccurateness, the transcellular current can be calculated from the transepithelial potential and resistance by Ohm's law.

The transepithelial current is the reason that the membrane potential across the apical or the basolateral membrane deviates from its electromotive force, emf (determined by the Nernstian diffusion potentials for the ions and their relative conductance according to the Hodgkin–Horowitz equation), by an amount equal to the product of the membrane resistance and the transcellular current.

However, the difference between the emf of the apical and basolateral membrane is the driving force for the transcellular current. Thus, in formula:

$$I_{sc} = \frac{E_a - E_b}{R_a + R_b + R_l}.$$

This can be represented in a graph to aid the visual analyses of changes in V_a, V_t and fR_a $[= R_a/(R_a + R_b)]$ measured with the extracellular and intracellular electrodes. The slope of the line connecting E_a and E_b indicates the strengths of the current.

In the case of neuropeptide Y, we found that I_{sc} decreased without a change in V_a and fR_a. This can occur by a concomitant decrease in apical Cl^- conductance (leading to an increase (hyperpolarisation) of E_a and R_a) and a decrease of the basolateral K^+ conductance (leading to decrease (depolarisation) of E_b and increase of R_b). This is shown in Fig. 3 with the displacement from the control (con) to the neuropeptide Y situation (dark continuous line to the thin continuous line). Under basal conditions (con), the addition of PDB leads to a depolarisation (decrease) of V_a, a decrease of fR_a and increase of I_{sc}. This situation is depicted in Fig. 3 with the change from the dark continuous line to the dash–dot line. In the presence of neuropeptide Y, the application of PDB leads to a smaller increase

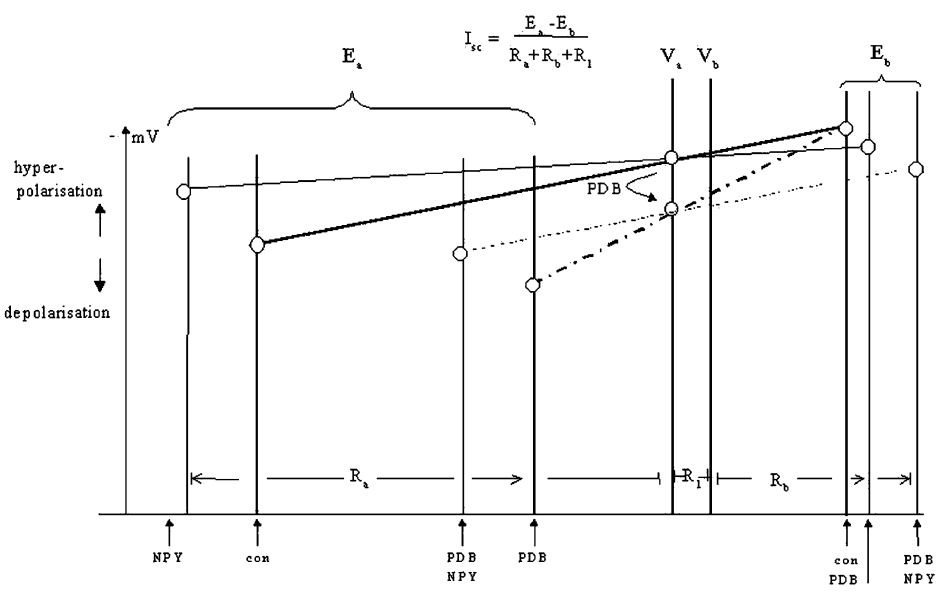

Fig. 3. Geometrical presentation of the current flowing through R_a, R_b and R_l due to the difference in E_a and E_b. The difference of V_a and V_b is the transepithelial potential. The slopes of the lines connecting E_a and E_b indicate the magnitude of the current. See text for further explanation. Four situations are depicted: Control, that is the basal situation without neuropeptide Y or PDB is indicated by the dark continuous lines. Neuropeptide Y: thin continuous lines. PDB: dark lines connected by the dark dashed dot line. PDB in the presence of neuropeptide Y: thin lines connected with the thin dashed dot line. The curved arrow indicates the depolarisation of V_a. R_a: apical membrane resistance, R_b: basolateral membrane resistance, R_l: resistance of the paracellular pathway, E_a and E_b: electromotive forces of the apical and basolateral membrane, respectively. V_a and V_b: potentials of the apical and basolateral membrane potentials.

of I_{sc} (less steep slope), however, with no change in V_a and fR_a. This situation is shown with the thin dash–dot line, which indicates that the electrophysiological characteristics of both membranes change.

In the light of the hypothesis that increases the number of Cl^- channels in the apical membrane, which then are activated by the existing activity of protein kinase A (Bajnath et al., 1992b), the simplest explanation for the inhibitory effect of neuropeptide Y on the protein kinase C-induced change of E_a is via reduced protein kinase A activity because of a lower cAMP concentration. The depolarisation of E_b in the presence of neuropeptide Y may be due to an ongoing effect of neuropeptide Y on the resting K^+ conductance, as observed in the absence of any secretagogues or to an increased rate of the inactivation of the K^+ conductance. The decrease in basolateral K^+ conductance may be due to a reduction in intracellular calcium levels. In previous experiments with the HT29cl.19A, we showed that neuropeptide Y decreased basal intracellular calcium levels as well as the carbachol-induced increase of calcium (Bouritius et al., 1998). It could be that neuropeptide Y also exerts a direct effect on the K^+ conductance. Such an effect has been reported by Xiong and Cheung (1994) in smooth muscle cells in the rat tail artery. To answer this question, we added the calcium ionophore ionomycin to the cells, thereby increasing the calcium levels drastically to overrule effects of neuropeptide Y on intracellular calcium levels. In the absence of neuropeptide Y, this resulted in a sustained hyperpolarisation concomitant with an increase in the fR_a, indicating an enhancement of the calcium-dependent K^+ conductance. However, in the presence of neuropeptide Y, these changes in V_a and fR_a were only transient, suggesting a direct inhibition of the calcium-activated K^+ conductance by neuropeptide Y.

To summarise, we have observed in the HT29cl.19A cells that neuropeptide Y inhibits the protein kinase C-induced Cl^- secretion by a double inhibitive action, namely, via the apical Cl^- conductance and the basolateral K^+ conductance. The reduction of the K^+ conductance in the presence of neuropeptide Y not only occurs because of the decrease in calcium concentration (Bouritius et al., 1998), but also via a direct effect on the K^+ conductance. The K^+ conductance consists of a number of K^+ channels, which can be discriminated by pharmacological criteria (Lomax et al., 1996; Suessbrich et al., 1996). From the present studies, it cannot be concluded which channel(s) are susceptible to neuropeptide Y. To answer this question, patchclamp or noise analysis experiments need to be performed (Greger, 2000). Interestingly, another antisecretory peptide, somatostatin, appeared to have similar effects as neuropeptide Y, inhibiting Cl^- secretion via G-proteins acting on adenylyl cyclase and intracellular calcium (Diener and Gartmann, 1994; Warhurst et al., 1993) as well as directly affecting the basolateral K^+ conductance (Lomax et al., 1995).

Our results support those of earlier studies showing that neuropeptide Y is a very powerful antisecretory neuropeptide because it can reduce secretion via at least three mechanisms: reduction of cAMP, decrease of the intracellular calcium activity and an inhibition of the basolateral K^+ conductance directly. A disturbance in the levels of neuropeptide Y could, therefore, result in a disturbed water balance in the intestine and, thus, may play a pathophysiological role in intestinal disorders, e.g. related to aging (Sweet et al., 1996).

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