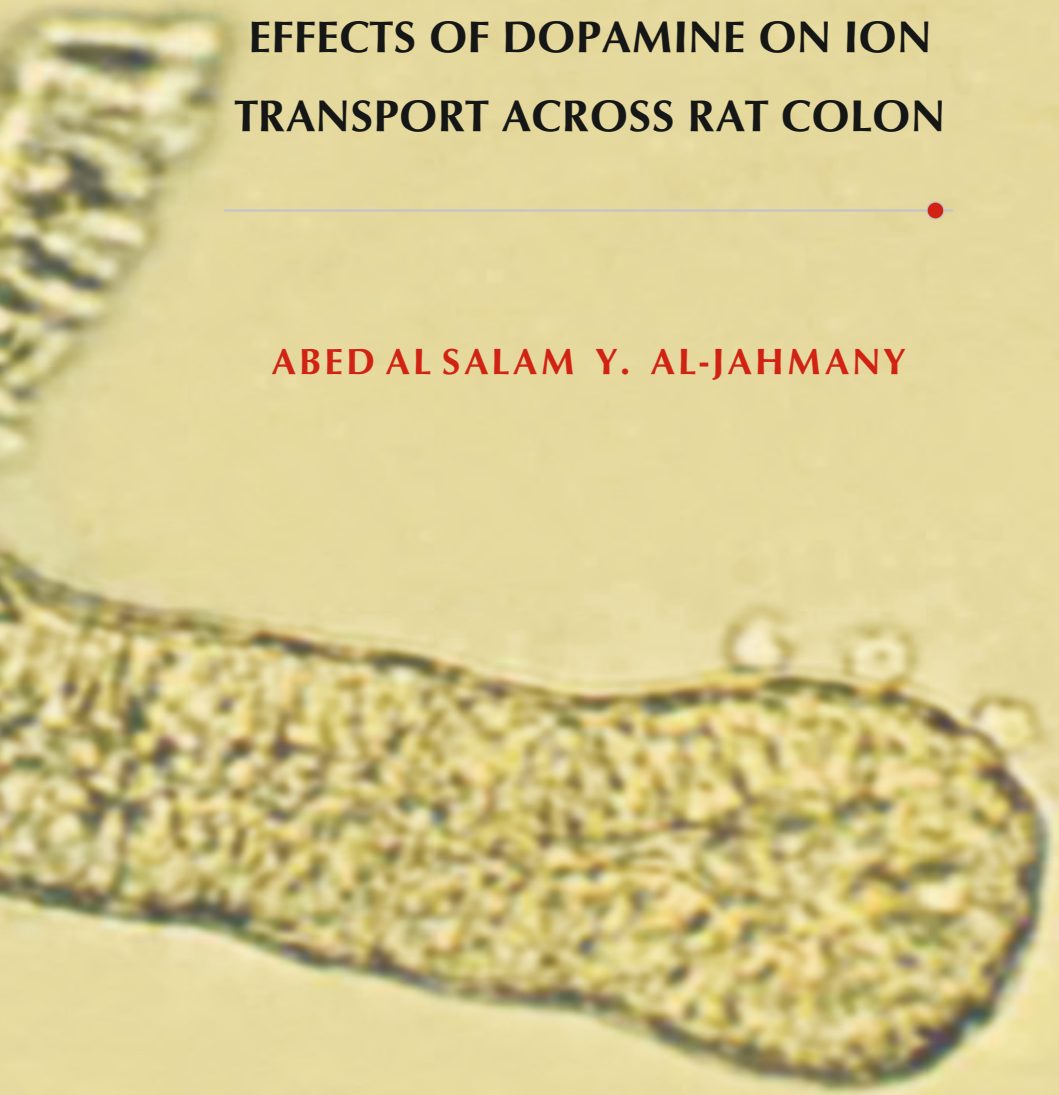
EFFECTS OF DOPAMINE ON ION TRANSPORT ACROSS RAT COLON

ABED AL SALAM Y. AL-JAHMANY



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Effects of dopamine on ion transport across rat colon

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Submitted by Abed Al salam Y. Al-Jahmany

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Contents

Abbreviations:

AM	Acetoxymethylester
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine 3',5'-monophosphate
CFTR	Cystic fibrosis transmembrane regulator
^{36}Cl	Chloride isotope
COMT	Catechol-O-methyltransferase
DMSO	Dimethylsulfoxide
EDTA	Ethylene diamino tetraacetic acid
Fura-2	Ca^{2+} sensitive-fluorescent dye
G-protein	Guanine-nucleotide binding protein
Gt	Tissue conductance
HEPES	N-(2-hydroxyethyl)piperazine-N'-2-ethansulfonic acid
Isc	Short-circuit current
L-DOPA	L-3,4-dihydroxyphenylalanine
MAO	Monoamine oxidase
$NMDG^+$ chloride	N-methyl-D-glucamine chloride
Pd	Potential difference
^{86}Rb	Rubidium isotope
SEM	Standard error of the mean
TEA	Tetraethylammonium chloride
Tris base	Tris(hydroxymethyl)aminomethane
TTX	Tetrodotoxin

1. Introduction

1.1. Function of the colon

The mammalian large intestine plays an important role in the regulation of the volume and the electrolyte composition of the stool. It is well documented that the transport of electrolytes is not homogeneous along the longitudinal axis of the colon (see e.g. Nobles *et al.* 1991); significant segmental differences have been found in many species (Binder & Sandle 1994). Therefore, the colon can functionally not be considered as a single organ, in which electrolyte transport processes are uniformly distributed.

One main function of the colon is the regulation of the balance of electrolytes (Kunzelmann & Mall 2001). Large and small intestine share some similarities in the mechanisms involved in the absorption and secretion of fluid and electrolytes. However, several differences are known characterising the colonic epithelium as an unique epithelium. For example, with the exception of the dog (Robinson *et al.* 1973) and neonatal animals (Binder & Sandle 1994) no absorption of glucose or amino acids takes place in the large intestine of mammals. Furthermore, the colon has developed a tighter epithelium than the small intestine due to differences in the structures of the tight junctions (Powell 1981).

1.2. Ion transporters in colonic epithelia

Because the experiments described in the present study were performed at the rat colon, the dominant mechanisms of ion transport shall be described in the two functionally different segments of this organ, i.e. the proximal and the distal colon (Nobles *et al.* 1991).

One of the main cations absorbed by the colonic epithelium is Na^+ .The predominant mechanism responsible for Na^+ absorption in rat colon is electroneutral transport (Binder & Sandle 1987). This is indicated by the fact that in contrast to other species such as man or rabbit (Clauss *et al.* 1988) low concentrations ($\leq 10^{-5}$ mol·l^{-1}) of the diuretic drug amiloride, which acts as a blocker of Na^+ channels, do neither affect net Na^+ transport nor short-circuit current (Isc), which is an indicator of net charge movement across the mucosa. There is no experimental evidence for the presence of Na^+-conductive channels under control conditions. However, treatment of the animals with corticoid hormones or dietary induction of a hyperaldosteronism by a Na^+-poor diet leads to the substitution of electroneutral Na^+ absorption by electrogenic Na^+ transport via Na^+ channels located in the apical membrane (Halevy *et al.* 1986, Fromm *et al.* 1993).

Electroneutral Na^+ absorption is coupled to Cl^- absorption so that the tissue effectively transports NaCl (Binder & Sandle 1987). Voltage-clamp experiments and experiments with apical membrane vesicles gave evidence for the model that the electroneutral NaCl absorption represents the coupling of two exchangers in the apical membrane, i.e. a Na^+-H^+ and a Cl^--HCO_3^- antiporter (Fig.1.1; for references see Binder & Sandle 1987, Rajendran & Binder 1993). Several observations support this model. For

example, inhibition of the enzyme carbonic anhydrase, responsible for the generation of HCO_3^- necessary for Cl^- absorption via the Cl^--HCO_3^- exchanger, with acetazolamide leads to the inhibition of both net Na^+ and net Cl^- absorption (for references see Binder & Sandle, 1994). A similar inhibition can be reached with a high concentration of amiloride (10^{-3} mol·l^{-1}), which in this concentration range acts as effective inhibitor of Na^+-H^+ exchangers (Bridges *et al.* 1989). In contrast, K^+ absorption seems not to interfere with NaCl transport, because neither the removal of K^+ from the apical side of the tissue nor the addition of bumetanide, a diuretic which inhibits the Na^+-K^+-2Cl^- cotransporter, had any effect on net Na^+ or net Cl^- transport (for references see Binder & Sandle 1994).

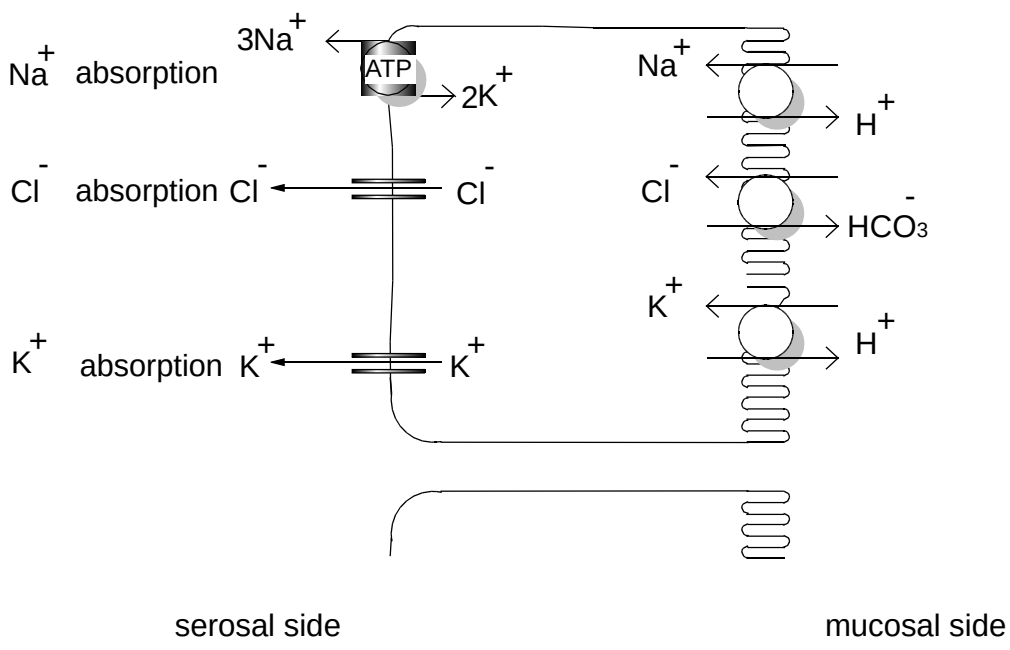

Figure 1.1: Main absorption mechanisms present in rat colon. The figure illustrates the movement of ions from the mucosal (luminal) side to the serosal side. For further details and for comparison between distal and proximal colon, see text.

In the basolateral membrane, several transport systems for Na^+ have been identified. These include a $3Na^+$-$2K^+$ATPase (Na^+-K^+-pump), a Na^+-K^+-$2Cl^-$ cotransporter, electroneutral Na^+-H^+ exchange and an electrogenic Na^+-HCO_3^- cotransporter (for references see Binder & Sandle 1994). Recently, many studies have provided information regarding Na^+ transport processes in the basolateral membrane of rat and rabbit colonocytes. Unfortunately, except for the classical Na^+-K^+-pump and the Na^+-K^+-$2Cl^-$ cotransporter, we have not yet a full information about the other mechanisms whether they are present in all colonocytes or whether there is a gradient along the

longitudinal axis of the colon.

The Na^+-K^+-pump plays a central role in transepithelial Na^+ absorption and transepithelial Cl^- secretion. The extrusion of Na^+ across the basolateral membrane via the Na^+-K^+-pump keeps the intracellular concentration of this cation low (Kaplan 1985). This, in turn, provides the driving force for Na^+ to enter the cell across the apical membrane. Although this model is quite well accepted for many epithelia, its experimental proof is circumstantial for rat epithelia, because in this species the Na^+-K^+-ATPase is quite resistant against typical blockers of this enzyme such as ouabain (Robinson 1970).

In contrast to the primarily active transporter, i.e. the Na^+-K^+-ATPase, the basolateral Na^+-K^+-$2Cl^-$ cotransport works as a secondarily active ion transporter. It uses the Na^+gradient generated by the Na^+-pump in order to accumulate K^+ and Cl^- inside the cell above their electrochemical equilibrium (Russell 2000). This cotransporter is therefore one of the main uptake mechanisms involved in the secretion of KCl in the colon (Fig. 1.2).

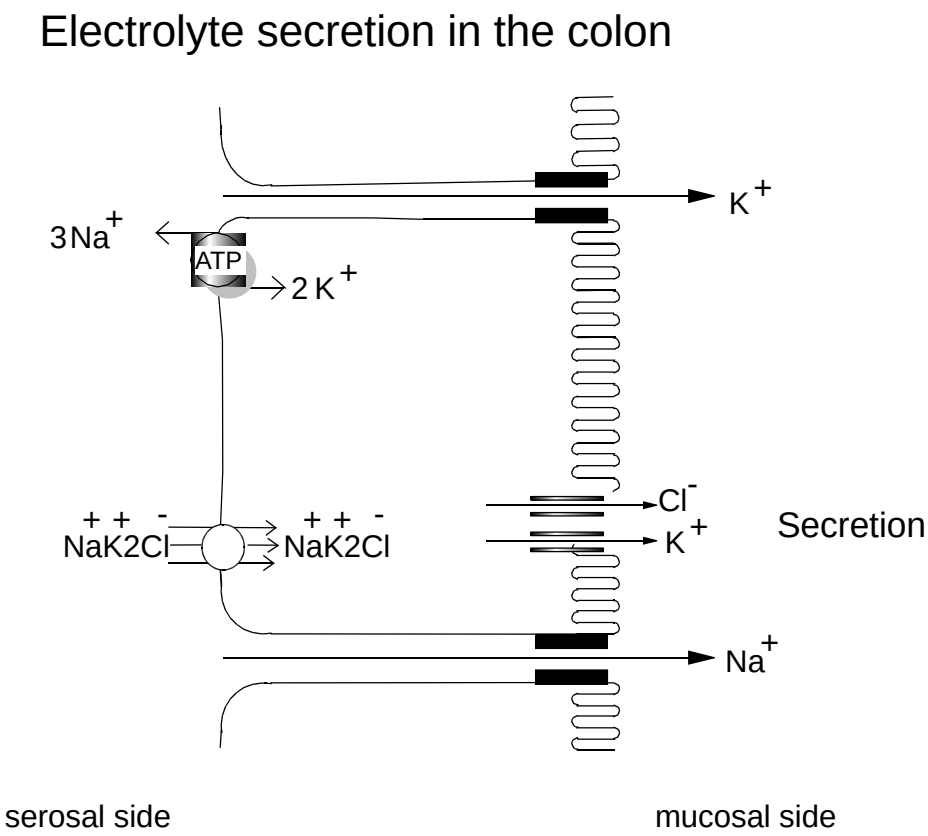

Figure 1.2: Main secretion mechanisms, which occur in rat colon. The figure illustrates the movement of ions from the serosal side (blood side) to the mucosal side (lumen). For further details and for comparison between distal and proximal colon, see text.

Another two additional Na^+ transport mechanisms were found in the basolateral membrane of the rat distal colon, i.e. an electroneutral Na^+-H^+ exchange and a Na^+-anion cotransport (Rajendran *et al.* 1991). In studies with basolateral membrane vesicles, this latter cotransporter has been shown to work in an electrogenic way. As anions both OH^- as well as HCO_3^- ions are accepted, however with HCO_3^- showing a higher affinity.

In the proximal colon, there is net Na^+ transport, while net Cl^- movement is nearly zero (Foster *et al.* 1986). Absorption of Na^+ seems to be mediated by apical Na^+-H^+ exchangers, too.

Cl^- absorption in the colon is, as stated above, an electroneutral process, in which, at least for the distal colon, apical anion antiporters play a central role. Recent studies suggest the existence of two distinct anion-exchange mechanisms, i.e. Cl^--HCO_3^- and Cl^--OH^- exchanger (Rajendran & Binder 1993). Probably the Cl^--HCO_3^- antiport is responsible for Cl^- absorption, whereas the other exchange is associated with intracellular pH regulation. Cl^- ions absorbed leave then the epithelium via basolateral efflux pathways including basolateral Cl^- channels (Diener *et al.* 1992).

In addition to its capacity to absorb Cl^-, upon stimulation by enteric secretomotor neurons, intestinal hormones or paracrine substances, the colonic mucosa is able to secrete Cl^- into the colonic lumen, too (for review, see Diener 1997). For this purpose, Cl^- anions are taken up across the basolateral membrane mainly via the Na^+-K^+-$2Cl^-$ cotransporter (see above). They can then be secreted into the colonic lumen after opening of apical Cl^- channels, which are predominantly formed by CFTR-type (CFTR = cystic fibrosis transmembrane regulator) Cl^- channels (Greger 2000).

A further ion transported by the colonic mucosa is K^+. Indeed, the colon plays an important role in the potassium homeostasis, especially when the renal function is impaired. Studies performed at the rat distal colon measuring $^{42}K^+$ fluxes under

voltage-clamp conditions have proven the presence of active potassium absorption in this colonic segment (Foster *et al.* 1984). This electroneutral absorption is Na^+-independent, because removal of Na^+ ions from both the serosal and the mucosal side did not alter the absorptive K^+ movement. Removal of chloride ions from the mucosal side partially reduced net potassium absorption; however, the role of chloride ions in this process is not totally understood. All these observations are consistent with a K^+-H^+-ATPase in the apical membrane. There are several forms of this enzyme in the colon, an ouabain-sensitive and an oubain-resistant form (Abrahamse *et al.* 1995, Rajendran *et al.* 1998). Efflux of absorbed potassium ions across to the basolateral side occurs through channels. In the proximal colon, the active uptake of potassium across the apical membrane has never been observed (Binder & Sandle 1994).

Beside its ability to absorb K^+, the colonic mucosa can, similar as it is the case for Cl^-, also secrete this ion actively (Foster *et al.* 1983). Both the basolateral Na^+-K^+-ATPase as well as the basolateral Na^+-K^+-$2Cl^-$ cotransporter can transport K^+ ions across the basolateral membrane in order to accumulate this cation in the cytosol. Movement of potassium into the mucosal and/or the serosal bathing solution is determined by the relative conductance of the apical and the basolateral membranes, respectively (Diener *et al.* 1996, Schultheiss & Diener 1997). Thus, the regulation of potassium channels in the apical and the basolateral membrane plays an important role in the secretion and the absorption of K^+ (Binder & Sandle 1994). In addition, the colon is able to transport potassium ions by the paracellular pathway, too (McCabe *et al.* 1986).

1.3. Regulation of intestinal ion transport by dopamine

Ion transport across the intestinal mucosa is under the control of the enteric nervous system, i.e. ganglionated neuronal plexus, especially the submucosal plexus, within the gut wall (Surprenant 1994, Binder & Sandle 1994). One of the transmitters released from enteric neurons is the catecholamine dopamine (Schultzberg *et al.* 1980, Eaker *et al.* 1988, Anlauf *et al.* 2003).

Dopamine binds to specific receptors in order to exert its biological actions. In the central and the peripheral nervous system, dopamine receptors are widely distributed and are involved in the regulation of many functions, for example sodium homeostasis, vascular tone, gastrointestinal motility or hormone secretion (Missale *et al.* 1998). It was assumed that in rodents such as rat and mouse, but obviously not in man, catecholaminergic intrinsic innervation of the gut is only transiently observed during development (for references, see Anlauf *et al.* 2003). However, recent evidence demonstrates the existence of enteric dopaminergic neurons in adult mouse and guinea pig colon (Li *et al*. 2004). Dopamine, however, is not only released from enteric neurons, but in addition the epithelial cells themselves have been demonstrated to be able to synthesize this catecholamine, which acts as a paracrine or autocrine modulator of ion transport (for references, see Vieira-Coelho & Soares-da-Silva 2001). This paracrine mucosal system is probably even more important for the physiological regulation of ion transport by dopamine.

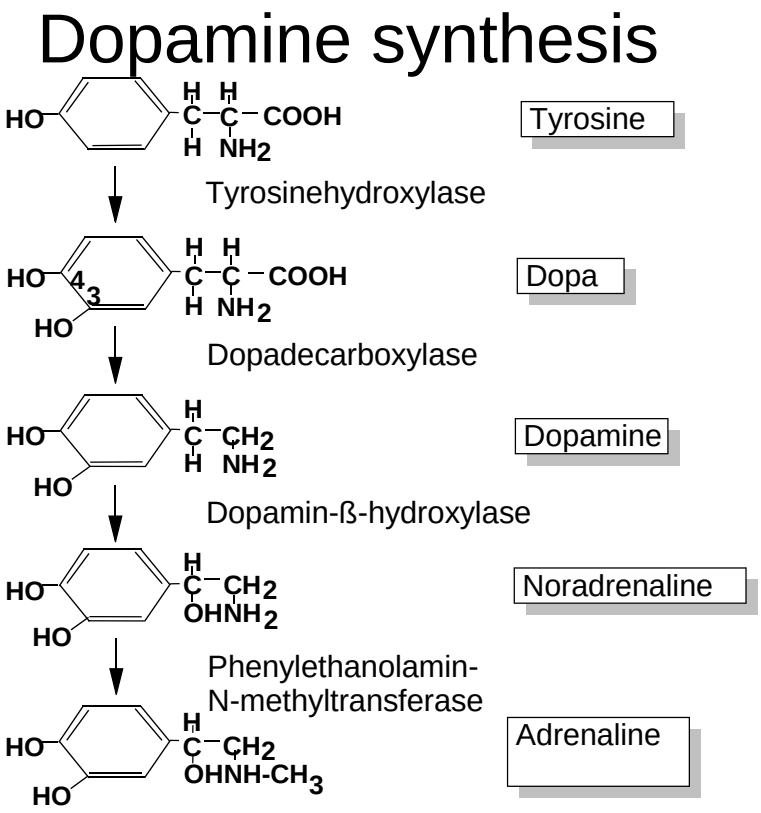

Figure 1.3: Biosynthesis of catecholamines (dopamine, adrenaline and noradrenaline). Given are the steps which proceed their formation, their formulas and essential enzymes.

The principal catecholamines found in the body - dopamine, adrenaline (epinephrine) and noradrenaline (norepinephrine) – are formed by hydroxylation and decarboxylation of the amino acid tyrosine. Most of the tyrosine is of dietary origin, however, some is formed from phenylalanine. By a concentrating mechanism tyrosine is transported into catecholamine–secreting neurons and adrenal medullary cells. Conversion of tyrosine to DOPA (3,4-dihydroxiphenylalanine) and then to dopamine occurs in the cytoplasm of the cell by tyrosine hydroxylase and DOPA-decarboxylase, respectively. Dopamine then enters the granulated vesicles, in which it is converted to noradrenaline. Some

neurons and adrenal medullary cells also contain a cytoplasmic enzyme, which catalyzes noradrenaline to adrenaline. Catecholamines are released by exocytosis from autonomic neurons and adrenal medullary cells. Metabolization of catecholamines into inactive biological products occurs by oxidation and methylation. The enzyme, which is responsible for the former reaction, is monoamine oxidase (MAO), the latter reaction occurs by the catechol–O–methyltransferase (COMT) (Ganong 1999).

Dopamine acts via stimulation of two main classes of G-protein coupled receptors, the D_1-like receptors (D_1-and D_5-subtype) and the D_2-like receptors (D_2-, D_3- and D_4-subtype). Each category shows a very high homology in their transmembrane domains and differs in their mode of coupling to adenylate cyclase, i.e. activation via D_1-like (positive coupling) and inhibition via D_2-like receptors (negative coupling; for review, see Missale *et al.* 1998).

Dopamine has been shown to alter intestinal ion transport. This catecholamine has been found to stimulate active Na^+ and Cl^- absorption in rabbit ileum (Donowitz *et al.* 1982), to increase rat ileal and colonic water absorption (Donowitz *et al*. 1983), and to alter in a age-dependent manner short-circuit current as a measure of net ion transport across rat jejunum (Vieira-Coelho & Soares-da-Silva 1998). Most of these responses were inhibited by the $\alpha 2$–adrenoceptor blocker, yohimbine, suggesting the involvement of adrenergic receptors. However, there is also strong evidence e.g. for the participation of specific dopamine receptors of the D_1-like type in the inhibition of Na^+-K^+-ATPase activity by dopamine in jejunal enterocytes from young rats (Vieira-Coelho & Soares-da-Silva 2000, 2001).

1.4. Questions addressed in this study:

In contrast to the action of other catecholamines such as norepinephrine or epinephrine, which induce a K^+ secretion in the colon of different species (Smith & McCabe 1986, Rechkemmer *et al.* 1996, Hörger *et al.* 1998), only few data are available concerning the regulation of ion transport in colonic tissue by dopamine. Therefore, in the present study I investigated dopamine-induced changes in ion transport across rat colon in vitro with special emphasis on possible actions on K^+ secretion.

The following questions should be addressed:

- Does dopamine modulate K^+ transport in rat distal and proximal colon ?
- Which receptors are involved in this response ?
- Does dopamine act directly at the epithelium or is there evidence for subepithelial action sites ?
- Which transporters are responsible for the changes in K^+ transport induced by dopamine ?
- Does dopamine affect Cl^- secretion ?

2. Material and Methods

2.1. Solutions

The Ussing chamber experiments were carried out in a Parsons solution (Parsons & Paterson 1965) with the following composition (Table 2.1):

Table 2.1: Parsons solution

Substance	**Final concentration ($mmol \cdot l^{-1}$)**	**For 1 litre solution**
NaCl	107	6.253 g
$NaHCO_3$	25	2.100 g
NaH_2PO_4 x H_2O	0.2	0.028 g
Na_2HPO_4 x 12 H_2O	1.8	0.645 g
KCl	4.5	0.336 g
Glucose	12.2	2.198 g
$MgSO_4$ x 7 H_2O	1.0	1 ml from a 1 $mol \cdot l^{-1}$ stock solution (12.325 g $MgSO_4$ x 7 H_2O in 50 ml aqua dest.)
$CaCl_2$ x 2 H_2O	1.25	1 ml from a 1.25 $mol \cdot l^{-1}$ stock solution (9.19 g $CaCl_2$ x 2 H_2O in 50 ml aqua dest.).

The solution was gassed with carbogen (5% CO_2 / 95% O_2, v/v). When the pH had fallen below a value of 7.8 during the carbogen gassing, $CaCl_2$ was added in order to avoid precipitation of Ca^{2+} in form of $CaCO_3$. Then the pH was adjusted by addition of HCl (1 $mol \cdot l^{-1}$) or $NaHCO_3$ (1 $mol \cdot l^{-1}$) until a final pH of 7.4.

In the experiments with ^{86}Rb, KCl in the Parsons solution was equimolarly substituted by RbCl (4.5 $mmol \cdot l^{-1}$). In some experiments, a mucosa-to-serosa K^+ gradient was applied. Therefore, the KCl concentration in the mucosal buffer solution was increased

to 13.5 $mmol \cdot l^{-1}$ while reducing the NaCl concentration to 98 $mmol \cdot l^{-1}$ in order to maintain isoosmolarity. In another set of experiments, the basolateral membrane was depolarized by a high K^+ concentration. In this buffer, NaCl was totally replaced by KCl reaching a final concentration of 111.5 $mmol \cdot l^{-1}$ KCl solution on the basolateral side.

In other experiments, the apical membrane was permeabilzed by an ionophore, nystatin, and the apical compartment of the Ussing chamber was filled with a Na^+-free Parsons solution of the following composition (Table 2.2):

Table 2.2: Na^+-free Parsons solution

Substance	**Final concentration ($mmol \cdot l^{-1}$)**	**For 1 litre solution**
NMDG-Base	107	20.886 g
Choline bicarbonate	25	4412 µl from a 80 % stock solution, w/v
NaH_2PO_4 x H_2O	0.2	0.028 g
Na_2HPO_4 x 12 H_2O	1.8	0.645 g
Glucose	12.2	2.198 g
$MgSO_4$ x 7 H_2O	1.0	1 ml from a 1 $mol \cdot l^{-1}$ stock solution (12.325 g $MgSO_4$ x 7 H_2O in 50 ml aqua dest.)
HCl	107	107 ml from 1 $mol \cdot l^{-1}$ stock solution
$CaCl_2$ x 2 H_2O	1.25	1 ml from a 1.25 $mol \cdot l^{-1}$ stock solution (9.19 g $CaCl_2$ x 2 H_2O in 50 ml aqua dest.)

For the isolation of intact colonic crypts, the following buffer was used (Table 2.3).

Table 2.3: Crypt isolation buffer

Substance	**Final concentration ($mmol \cdot l^{-1}$)**	**For 1 litre solution**
NaCl	107	6.253 g
$NaHCO_3$	25	2.100 g
KCl	4.5	0.336 g
Glucose	12.2	2.198 g
Na_2HPO_4 x $12H_2O$	1.8	0.645 g
NaH_2PO_4 x H_2O	0.2	0.028 g
EDTA	10	0.372 g

In this buffer, the Ca^{2+} chelator EDTA (ethylene diamino tetraacetic acid) served to remove extracellular Ca^{2+} and Mg^{2+} in order to open the tight junctions. The pH of this buffer was adjusted to 7.4 with HCl (1 $mol \cdot l^{-1}$) or tris base (tris(hydroxymethyl)aminomethane; 1 $mol \cdot l^{-1}$) during gassing with carbogen (5% CO_2 / 95% O_2, v/v). In general, 100 ml of this buffer solution were sufficient for successful crypt isolation.

Isolated crypts were stored in a high K^+ Tyrode buffer solution (Böhme *et al.* 1991) with the following composition (Table 2.4). The benefit from this solution is to prolongate the life span of the isolated crypts (DelCastillo 1987).

Table 2.4: High K^+ Tyrode solution

Substance	**Final concentration ($mmol \cdot l^{-1}$)**	**For 1 litre solution**
K gluconate	100	23.425 g
KCl	30	2.237 g
NaCl	20	1.169 g
HEPES	10	2.381 g
$MgCl_2$ x 6 H_2O	1	1 ml from a 1 $mol \cdot l^{-1}$ stock solution (10.166 g $MgCl_2$ x 6 H_2O in 50 ml aqua dest.)
$CaCl_2$ x 2 H_2O	1.25	1 ml from a 1.25 $mol \cdot l^{-1}$ stock solution (9.19 g $CaCl_2$ x 2 H_2O in 50 ml aqua dest.)
Glucose	12.2	2.198 g
Na pyruvate	5	0.553 g
Bovine serum albumin (BSA)	1 $g \cdot l^{-1}$	1 g

The pH of this solution was adjusted to 7.4 by the aid of KOH (1 $mol \cdot l^{-1}$) or HCl (1 $mol \cdot l^{-1}$). In order to protect the pH electrode, the bovine serum albumin (BSA) was added after the pH calibration. Generally, 100 ml of this buffer were sufficient for one experiment.

For the superfusion of the crypts during the fura-2 experiments, a standard Tyrode solution was used with the following composition (Table 2.5).

Table 2.5: Standard Tyrode solution

Substance	**Final concentration ($mmol \cdot l^{-1}$)**	**For 1 litre solution**
NaCl	140	8.182 g
KCl	5.4	0.403 g
HEPES	10	2.383 g
Glucose	12.2	2.198 g
$MgCl_2$ x 6 H_2O	1	1 ml from a 1 $mol \cdot l^{-1}$ stock solution (10.166 g $MgCl_2$ x 6 H_2O in 50 ml aqua dest.)
$CaCl_2$ x 2 H_2O	1.25	1 ml from a 1.25 $mol \cdot l^{-1}$ stock solution (9.19 g $CaCl_2$ x 2 H_2O in 50 ml aqua dest.)

The pH of this solution was adjusted to 7.4 with NaOH (1 $mol \cdot l^{-1}$) or HCl (1 $mol \cdot l^{-1}$).

2.2. Tissue preparation

For the Ussing chamber experiments, Wistar rats from both sexes were used with a weight of 180 – 220 g. For the imaging experiments, Wistar rats with a weight of only 120 - 160 g could be used, since older animals with a higher body weight gave only a poor yield of intact crypts.

The animals had free access to water and standard diet (diet no. C1000, Altromin, Lange, Germany) until the day of the experiment. Animals were stunned by a blow on the head followed by cervical displacement and killed by exsanguination (approved by

Regierungspräsidium Gießen, Gießen, Germany).

The colon (from the pelvic ring to the caecum) was removed by the aid of scissors, starting from the distal segment in a proximal direction. The colon was placed in normal, ice-cold Parsons solution gassed with carbogen. Then the inside of the intestinal segment was cleaned by infusion of ice-cold Parsons solution with the aid of a syringe connected with a tube ending with a pipette tip.

The colon was placed on a plastic rod with a diameter of 5 mm. With the aid of the blunt side of a scalpel, a circular dissection was made at the end of the distal part of the colon. This dissection was located only in the outer muscular layer, not through the mucosa-submucosa layer. Starting from the distal end, the serosa and the muscularis propria were stripped away by hand to obtain a mucosa-submucosa preparation of the colon (Andres *et al.* 1985). The appearance of palm-like foldings was used to distinguish between the proximal and distal colon (Lindström *et al.* 1979). During the preparation, the gut was continuously rinsed with the ice-cooled buffer in order to prevent drying.

2.3. Crypt isolation

The stripped mucosa-submuocosa preparation was used as starting preparation for the isolation of intact crypts. The stripping removes the diffusion barrier for the Ca^{2+} chelator, EDTA, which has to reach the tight junctions of the epithelium. With the plastic rod still in the interior of the colon, a longitudinal cut was made across the mucosa-submucosa preparation with a sharp scalpel. The resulting segment was fixed by a cyanacrylate glue over a plastic holder with an oval hole in the middle (Fig. 2.1).

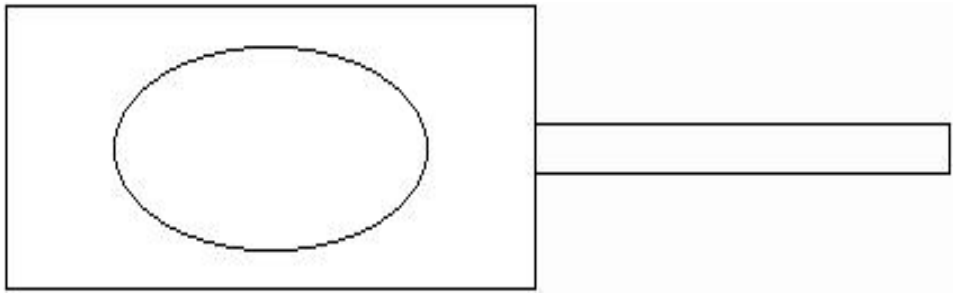

Figure 2.1: The picture illustrates the Lucite holder, which is used for fixation of the colon during crypt isolation. This holder had a length of about 8 cm, in the middle the oval hole over which the colon is glued.

Fixed in this way, the colon was exposed to the isolation buffer at 37°C and gassed with carbogen for an incubation time between 6 and 8 min. The shorter incubation times were used for tissues from younger, the longer incubation times for tissues from older animals.

Then the holder was vibrated strongly by a mixing device (Chemap Vibromixer, A1-Biotech, Martinsried, Germany) for 30 s in order to isolate intact crypts, which appear as turbidity in the solution during the vibrating time. The vibration procedure took place in intracellular-like high K^+ Tyrode solution (Table 2.4). Using this solution as a storage for the crypts causes a prolongation for the enterocytes life span as mentioned above (Del Castillo 1987, Schultheiss *et al.* 2002). Subsequently, the integrity of the crypts was checked under a light microscope (Figure 2.2).

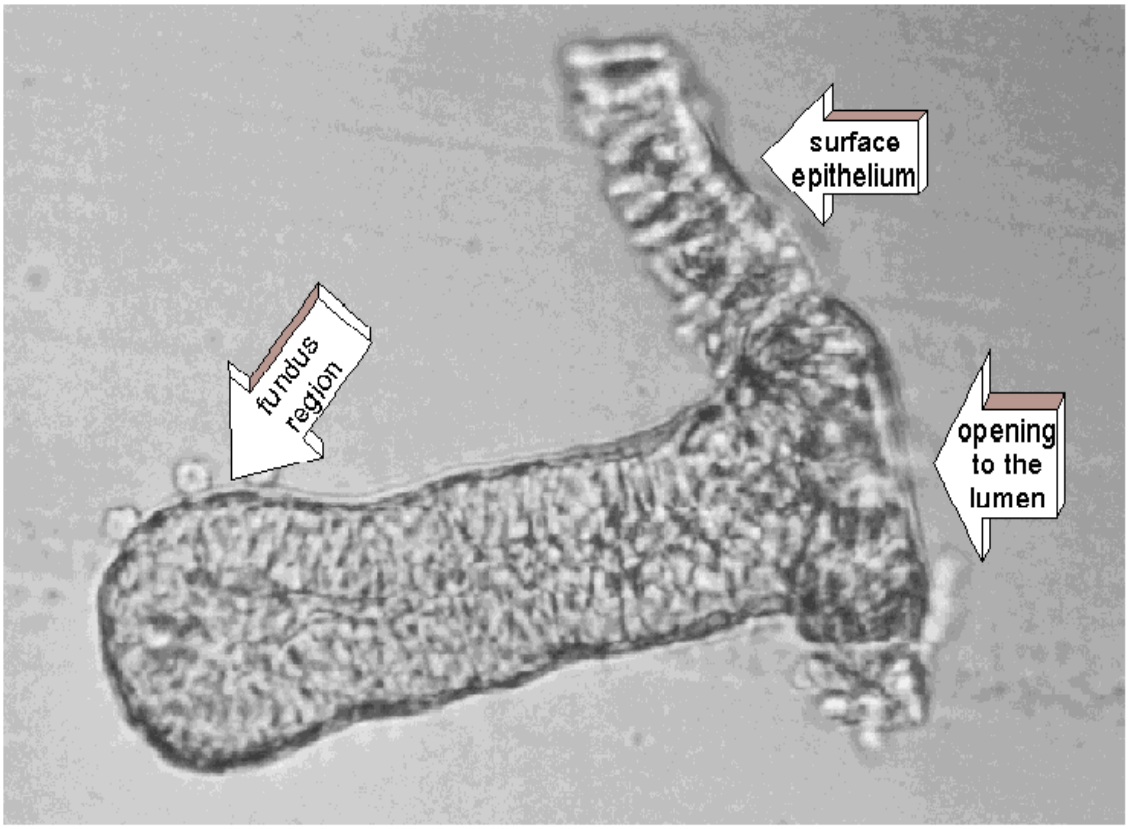

Figure 2.2: Light microscopical picture of an isolated crypt from rat colon.

Selected crypts were transferred by the aid of a pipette to glass slides covered with poly-L-lysine. In order to cover the slides, they were heated to about 50° C and an aqueous poly-L-lysine solution (molecular weight > 300 kDa; concentration 0.1 $g \cdot l^{-1}$; Biochrom, Berlin, Germany) was distributed over them with the aid of a pipette tip until dryness of the surface. The poly-L-lysine serves as adhesive material for the crypts in order to avoid movement of the crypts during the fura-2 experiments (Schultheiss *et al.* 2002).

The glass slides were transferred to a four well-chamber. All procedures until this step were carried out at room temperature. Unused crypts were kept at about 4° C in the refrigerator to increase their life time and to minimize the production of the mucous.

The visual inspection of the crypts just before the fixation on poly-L-lysine and during the fura-2 experiments is helpful for successful experiments with this preparation. For example, the integrity of the crypts might be affected by a too long incubation period in the EDTA-containing isolation buffer or the usage over a prolonged period (i.e. longer than 6 h). The rounding of the basal pole of the crypts is the main characteristics of this phenomenon. The reason for this damage is the loss of the tight junctions, which separate basolateral and apical membrane components (Ziomek *et al.* 1980). The consecutive increase in cell volume leads to the activation of volume-dependent K^+ and Cl^- channels (Diener *et al.* 1992) and thereby modifies the properties of the cells. Therefore, crypts with rounded cell poles were not used for the experiments. This aging of the crypts developed faster at 37°C. Consequently, all experiments with isolated crypts were performed at room temperature.

2.4. Measurement of short-circuit current in Ussing chamber experiments

Principle of the the method

The Ussing chamber technique is a method to quantify electrogenic ion transport across epithelia. This method was developed by the Danish physiologist Hans H. Ussing in the fifties of the last century (Ussing & Zerahn 1951). This chamber provides the tissue with suitable conditions (Fig. 2.3). For example, rat colonic epithelium can be kept in an Ussing chamber for at least 6 h without any visible cellular damage under the electrone microscope (Diener *et al.* 1989).

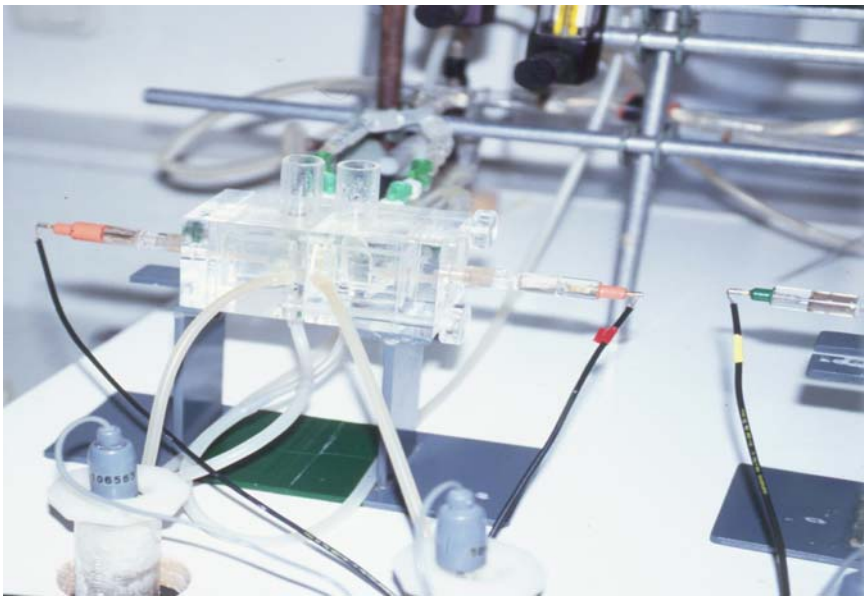

Figure 2.3: Photograph of the modified Ussing chamber.

The chamber consisted of two halves, which could be gathered by screws. Small pins around a central opening served to fix the tissue in the middle of the chamber (Fig. 2.4). The chamber looked like a 'sandwich': two halves with the epithelium fixed in the middle.

The chamber had two electrodes for current injection and two for voltage measurement. The epithelium was bathed with an incubation solution (Parsons solution) at both sides of the epithelium (the serosal side and the mucosal side). The temperature of the solution was kept at 37°C by a water circulation through the outer parts of the chamber.

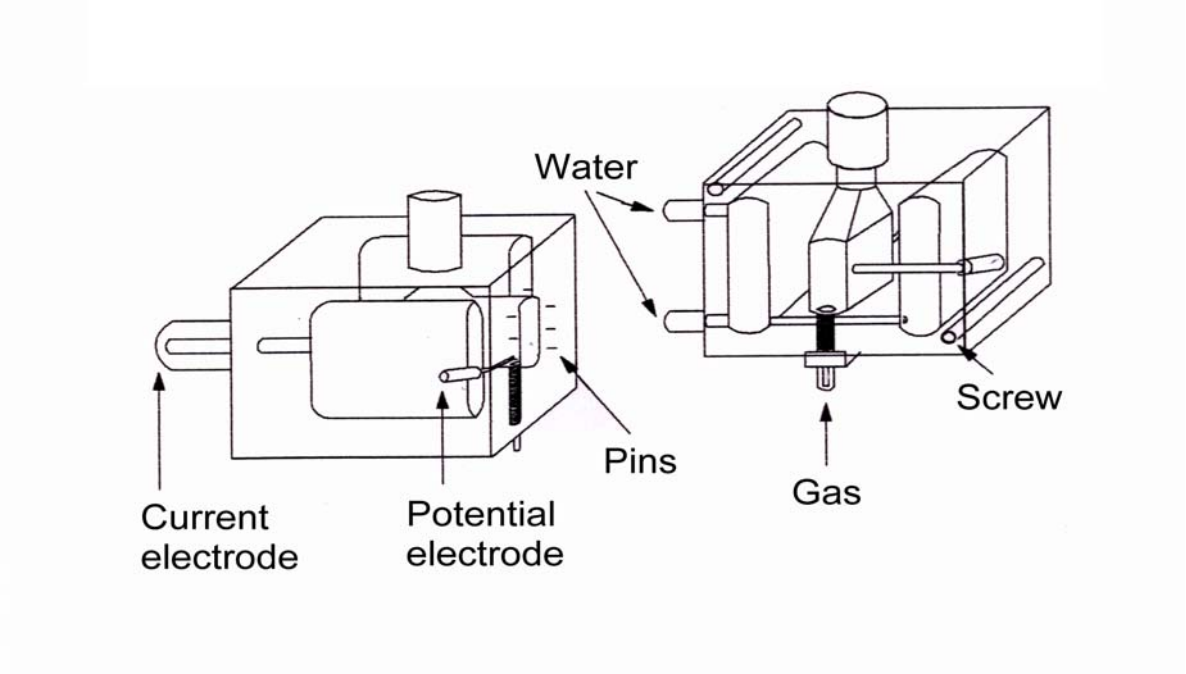

Figure 2.4: Schematic drawing of the modified Ussing chamber.

An opening in the upper part of each half chamber was used in order to add fresh buffer or to wash it out. Another opening at the lower part of each half chamber was used as entrance of the carbogen gas. This gas is necessary to supply the tissue with a suitable amount of oxygen and to keep the pH of the HCO_3^--containing buffer at 7.4.

After mounting the tissue in the chamber, the mucosa will spontaneously transport ions across the epithelial layer. The basal ion movement across the rat colonic epithelium is dominated by a spontaneous secretion of anions, mainly of Cl^- ions (Binder & Sandle 1994). The consequence is the generation of a transepithelial potential difference (Pd; Fig. 2.5).

The Ussing chamber technique can be used to discriminate between active and

passive transport of ions. It is possible to distinguish between these mechanisms by adding the same solution to both sides of the epithelium. Thus, the possibility of a chemical driving force affecting ion transport is excluded.

By two identical electrodes connected to a voltmeter, the potential difference between both sides of the epithelium can be measured. Under voltage-clamp conditions, the electrical driving force, which affects passive ion movement across the epithelium, is removed by clamping the potential difference to zero. To do this, an adjustable battery is used, which is connected by two current electrodes with the epithelial preparation. A feedback device serves to continuously adjust the applied current so that the potential difference is hold constant at 0 mV.

In the rat colon, ion transport is dominated by a spontanous transcellular secretion of Cl^- ions (Fig. 2.5), which causes a potential difference across the epithelium (serosal side positive) under open-circuit conditions, i.e. without voltage-clamping. This potential will in vivo be used to drive the passive flux of a counter ion, in this case Na^+, across the paracellular pathway. When the potential difference is clamped to zero, a current, the so-called short-circuit current (Isc) is applied, which just nullifies the transepithelial ion current, i.e. the transcellular Cl^- current. This Isc can therefore be used as measure for active ion transport. A positive Isc means net absorption of cations or secretion of anions. Vice versa, a negative Isc means net absorption of anions or secretion of cations.

A further advantage of the Ussing method is that it is possible to compare the amplitude of the current with the flux of different ion species, if this technique is combined with radio tracer methods (see below).

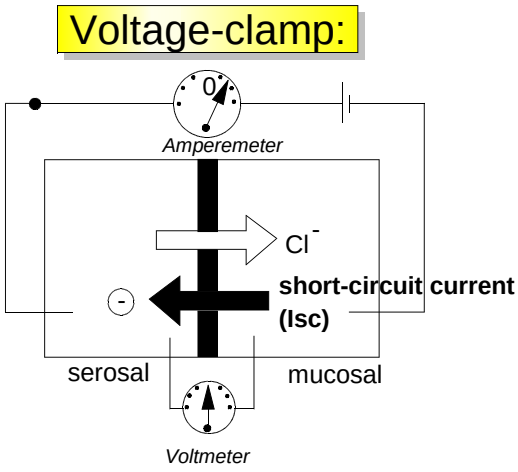

Figure 2.5: Measurement of the short-circuit current (Isc) under voltage-clamp conditions in the Ussing chamber. This figure shows how the potential difference is clamped to zero (voltage-clamp) by a feedback control device. The necessary current, which has to be applied to reach a potential difference of 0 mV, is called short-circuit current (Isc).

Performance of the experiments

The mucosa-submucosa preparation was fixed in the modified Ussing chamber and bathed at both sides with standard Parsons solution (Andres *et al.* 1985). The volume of each half chamber was 3.5 ml. Two distal and two proximal segments of the colon of each rat were prepared. These two segments strongly differ in their transport properties (Nobles *et al.* 1991). In general, two tissues (1 proximal, 1 distal) served to measure the control response evoked by dopamine and the other were treated with

putative antagonists before dopamine was applied. If the antagonist had to be administered in a solvent, the control tissue was pretreated with the solvent, too.

The tissue was incubated in 37°C and short-circuited by a computer controlled voltage-clamp device (Ing. Büro für Mess-und Datentechnik K. Mußler, Aachen,Germany). The exposed surface of the tissue amounted to 1 cm^2. Short-circuit current (Isc) was recorded every 6 s and stored on the hard disk of a computer. Tissue conductance (Gt) was measured every minute. Measurement of conductance took place by applying a current pulse (± 50 µA, duration 200 ms) under open-circuit conditions and measurement of the induced voltage deviation. From this, using Ohm's law Gt was calculated according to:

$Gt = \Delta I / \Delta V$

with ΔV is the voltage change induced by the current pulse (ΔI).

Dopamine was applied in increasing concentrations to the same tissue, a washing step separated the individual administrations. Between the individual administrations, the serosal compartment was washed three times with 5x the chamber volume and a stabilisation of baseline Isc was waited for until the next concentration was applied. The baseline in electrical parameters was determined as the mean over the 3 min immediately prior to administration of the drug. Changes in Isc induced by dopamine or other drugs are given as change (Δ Isc) against this baseline.

2.5. Measurement of basolateral ionic currents (nystatin experiments)

In order to specify the role which the basolateral membrane plays in the dopamine-induced changes in ion transport, an ionophore, nystatin, was used (Schultheiss and

Diener 1997). Nystatin is an antifungal polyene, i.e. a member of a group of drugs, which includes also natamycin and amphotericin B (Fig. 2.6). Its action occurs by the binding with cholesterol in the cell membrane, leading to pore formation in the cell membrane (Zhang *et al.* 2002). When nystatin is added to the mucosal compartment of an Ussing chamber, the drug incorporates in the apical membrane of the epithelium and thereby causes a bypassing of this membrane by the formation of intramembranous pores. Consequently, all currents measured in the presence of apical nystatin are determined by ion transport processes across the basolateral membrane.

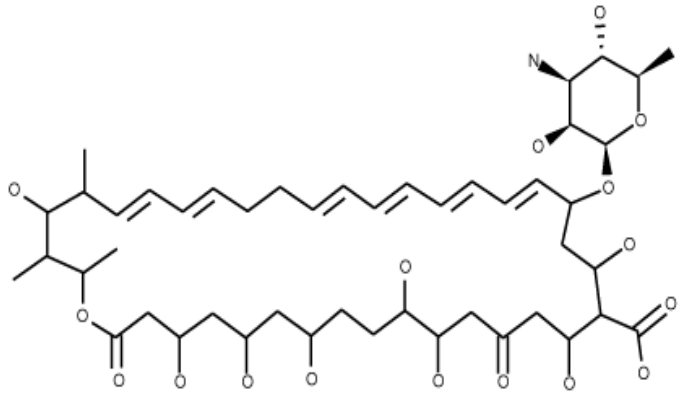

Figure 2.6: Structure of nystatin (from www.sigmaaldrich.com).

The permeabilization of the apical membrane was performed by addition of nystatin (100 $\mu g \cdot ml^{-1}$ at the mucosal side) dissolved in dimethylsulphoxide (DMSO; final concentration 2 $ml \cdot l^{-1}$). The nystatin was kept under light protection until usage. The solution was ultrasonified immediately before use. For driving K^+ currents across the basolateral membrane, a K^+ gradient of 3 : 1 was administered (13.5 $mmol \cdot l^{-1}$ K^+ at

the mucosal and 4.5 $mmol \cdot l^{-1}$ K^+ at the serosal side). Some experiments were in addition performed under Na^+-free solution with a solution, in which NaCl was replaced by N-methyl-D-glucamine ($NMDG^+$) chloride.

2.6. Measurement of apical ionic currents (basolateral depolarization)

Neither nystatin nor other ionophores are able to permeabilize the basolateral membrane in order to measure the ionic current across the apical membrane. The reason for this failure is the presence of subepithelial tissue (such as connective tissue or smooth muscle cells), which prevents these ionophores from reaching the basolateral membrane in a sufficient concentration. Therefore, the technique of basolateral depolarization was used to selectively measure currents across the apical membrane (Fuchs *et al.* 1977). The basolateral membrane was depolarized by a high K^+ bathing solution, i.e. a 111.5 $mmol \cdot l^{-1}$ KCl solution was used on the basolateral side. This manoeuvre electrically eliminates the basolateral membrane with its high K^+ conductance, thus all currents measured under these conditions are determined by apical ion conductances (Schultheiss & Diener 1997).

2.7. Unidirectional fluxes

Measurement of the unidirectional transport of different ion species was performed by addition of radioactive tracers such as $^{86}Rb^+$ to one side, e.g. the mucosal side, and measuring the appearance of the tracer on the unlabelled side, e.g. the serosal side. In independent experiments, the flux of the tracer from the mucosal to the serosal side (Jms) and the flux from the serosal to the mucosal side (Jsm) was measured. From

these values, net transport (Jnet) of the ion was calculated according to:

Jnet = Jms - Jsm.

A positive Jnet means net absorption of the ion, a negative Jnet means net secretion.

For the measurement of unidirectional fluxes, the mucosa-submucosa preparation from both colonic segments, i.e. the distal and the proximal colon, were fixed in the modified Ussing chamber (see above). For the experiments, in which the transport of $^{86}Rb^{+}$ as a marker for K^{+} transport (Foster *et al.* 1983) was measured, KCl in the Parsons solution was replaced by RbCl.

At the beginning of the experiment, a volume of 3.7 ml was added to the side of the chamber, which should be labelled with the tracer ('hot side'), whereas the unlabelled side ('cold side') contained only 3.5 ml Parsons solution. After an equilibration period of about 1h, in which the Isc reached stable values, 2 µCi $^{86}Rb^{+}$ were added to the hot side. After 30 min, in which the isotope fluxes reached a steady state, a sample (2 x 100 µl) was taken from the hot side, which served as standard to calculate the amount of $^{86}Rb^{+}$ in a sample from the measured radioactivity. After taking this sample, i.e. during the rest of the experiment, the volumes of the serosal and mucosal compartment of the chamber were identical avoiding that a pressure gradient might affect passive ion transport.

Unidirectional fluxes were then measured over two sequential 20 min periods, a control period followed by the measurement of fluxes in the presence of dopamine ($5 \cdot 10^{-4}$ $mol \cdot l^{-1}$). Samples (2 x 250 µl) were therefore taken at the beginning and the end of each period. The volume removed was replaced by unlabelled Parsons solution. An

adequate compensation for this replacement volume was made during calculation of the fluxes. At the end of the experiment, a sample (2 x 100 µl) was taken from the labelled side in order to check, whether the radioactivity of the hot side had not decreased during the experiment.

At the end of the experiment, the scintillation fluid (Rotizint® eco plus, Carl Roth GmbH, Karlsruhe) was added. The radioactivity of $^{86}Rb^{+}$ was then measured in a ß-counter (TRI-CARB® 2000CA Liquid Scintillation Analyzer, Packard, Frankfurt).

2.8. Efflux experiments

In order to measure the efflux of an isotope to the serosal and the mucosal compartment, the following procedure was used. The colon was mounted in an Ussing chamber and bathed with 2.5 ml of Parson solution on each side. After an equilibrating period of 30 min, radioactivity (2 µCi $^{86}Rb^{+}$) was added on both sides of the tissue.
This incubation lasted for 90 min in order to allow a sufficient uptake of the $^{86}Rb^{+}$ into the tissue. The loading period was stopped by washing the tissue three times with unlabelled Parsons solution. During this procedure, the whole volume of the chamber was washed with 20 ml Parsons solution on both sides. The washing step was repeated twice at 5-minutes intervals.
In order to determine the efflux of the isotope, 2 × 0.5 ml aliquots were taken from the mucosal and the serosal compartment at 6-minutes intervals. After 2 control periods, i.e. after 12 min, dopamine ($5 \cdot 10^{-4}$ mol·l^{-1}) was added to the serosal compartment. Then again 3 efflux period were measured. All the aliquots were replaced by unlabelled buffer solution, an appropriate correction of this replacement was performed during

calculation of the data.

At the end of the experiment, the tissue was removed from the chamber and blotted on a filter paper. This step took 1 - 2 minutes. After that the tissue was dissolved in 1 ml 0.1 $mol \cdot l^{-1}$ HNO_3 for 20 hours at 70°C (Venglarik *et al.* 1990). In order to neutralize HNO_3, subsequently 0.1 ml 1 $mol \cdot l^{-1}$ NaOH was added. Then the amount of radioactivity in the tissue was determined in liquid scintillation counter (Ribeiro *et al.* 2001). Efflux was expressed as efflux in % of the actual amount of radioactivity in the tissue per time.

With the same protocol, the efflux of $^{36}Cl^-$ was determined in an independent series of experiments. For the loading of the tissue, 1 µCi of $^{36}Cl^-$ was added to both the serosal and the mucosal compartment.

2.9. Fura-2 experiments

Calcium as a second messenger plays an important role in the cell. This ion has proven to be a key regulator of many cellular processes. In order to measure relative changes in the intracellular Ca^{2+} concentration, the Ca^{2+}-sensitive fluorescent dye, fura-2, was used. This dye acts as chelator, that means it is a highly charged molecule, which can bind intracellular Ca^{2+}. Binding of Ca^{2+} causes a shift in the excitation optimum of this dye (Fig. 2.7). After binding of the divalent cation, the wavelength, at which the fluoresence of the dye can maximally be induced, is shifted to shorter wavelengths.

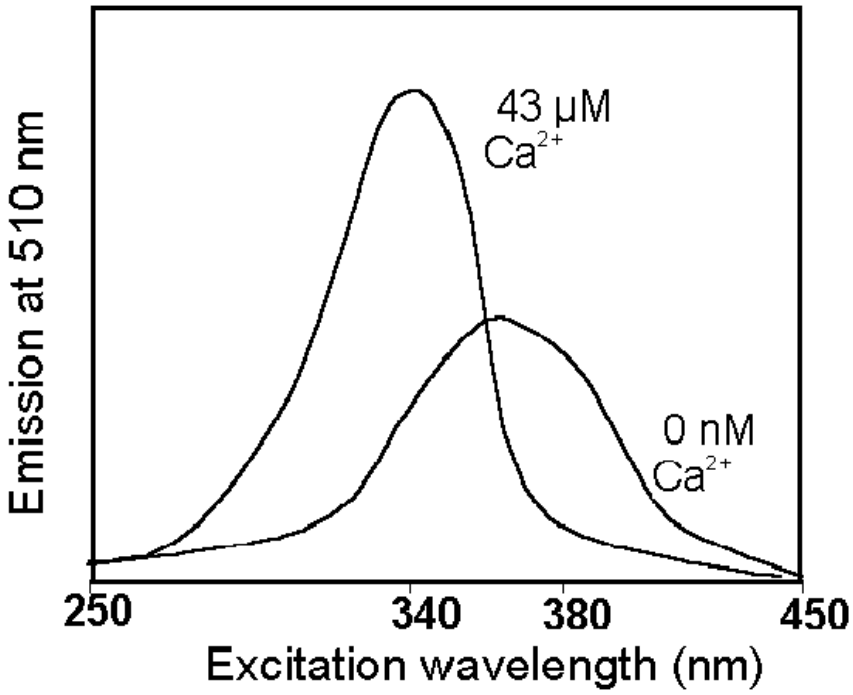

Figure 2.7: Fluorescence excitation spectra of fura-2 at different concentrations of Ca^{2+} . Emission was measured at 510 nm. The excitation spectrum shifts to a shorter wavelength when the Ca^{2+} concentration is increased.

Cells were loaded with the uncharged, lipophilic form of fura-2, the fura-2-acetoxymethylester (fura-2/AM; Fig. 2.8). This ester is cleaved inside the cell by esterases releasing the free fura-2 acid, which can no more passively leak out of the cell due to its charge.

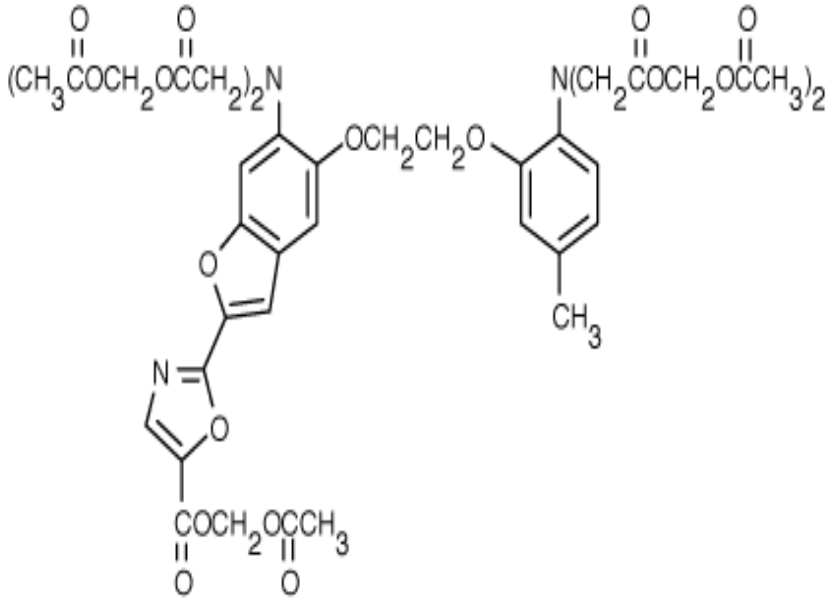

Figure 2.8: Structure of Fura-2/AM (from www.probes.com).

The crypts were incubated with 2.5 $\mu mol \cdot l^{-1}$ fura-2/AM in Tyrode solution together with the detergent Pluronic® (0.05 $g \cdot l^{-1}$). For a sufficient penetration of the dye into the crypt cells, one hour incubation period was needed. Because of the high sensitivity of the fura-2 against light, this incubation step had to be performed in darkness.

By using a pipette, the fura-2/AM was carefully replaced with standard Tyrode solution. This step was repeated two times.

With the aid of a needle and a forceps, the cover slip with the attached crypts was removed from the incubating chambers and put on a filter paper. The cover slip was fixed to the bottom of the experimental chamber with a drop of silicon oil. The volume of the chamber amounted to 3 ml. The chamber was transferred to the stage of an inverted microscope (Olympus IX-50) equipped with an epifluorescence set-up and an image analysis system (Till Photonics, Martinsried, Germany).

Subsequently, a suitable field with an intact crypt was chosen for the measurement. The loading of the crypt with fura-2 was controlled by exciting shortly the crypts with UV light (340 nm). Within the image analysis system, several regions of a crypt were selected and marked. Each region of interest had the size of about one cell.

Two wave lengths, 340 nm and 380 nm, were chosen for excitation. So, during the experiment, the crypt was alternatively exposed to two distinct wave lengths: of 340 nm and 380 nm at a 510 nm emission wavelength. The light pulses had a duration of 20 ms and were applied every 5 s.

By an infusion set, the preparation was superfused hydrostatically throughout the experiment with the Tyrode solution. Perfusion rate was about 1 $ml \cdot min^{-1}$.

Calibration of the fura-2 ratio signal is essential for the calculation of the intracellular Ca^{2+} concentration. A calibration was done with a set of extracellular buffer solutions containing a wide range of different Ca^{2+} concentrations and measuring the fura-2 ratio in the respective solutions. Nine solutions (Molecular Probes, Leiden, The Netherlands) with free fura-2 acid and free Ca^{2+} concentrations ranging from 0 to 39.8 $\mu mol \cdot l^{-1}$ were used for the calibration procedure.

The ratio values measured were used to calibrate the fura-2 ratio signals with the Grynkiewics equation (Grynkiewics *et al.* 1985) for determination of the intracellular concentration of the Ca^{2+}.

The Grynkiewics- equation

$$[Ca^{2+}]_i = \frac{K_D \times ß \times (R - R_{min})}{(R_{max} - R)}$$

Figure 2.9: The Grynkiewics-equation.

$[Ca^{2+}]_i$ = intracellular concentration of Ca^{2+}.

ß = fluorescence during excitation at 380 nm at 0 µmol·l^{-1} Ca^{2+} divided by the fluorescence during excitation at 380 nm at 39.8 µmol·l^{-1} Ca^{2+} (= maximal Ca^{2+} binding).

R = measured ratio.

Rmin, Rmax: ratio at 0 and 39.8 µmol·l^{-1} Ca^{2+}, respectively.

K_D = Dissociation constant.

2.10. Chemicals

Fura-2-acetoxymethylester (fura-2/AM; Molecular Probes, Leiden, The Netherlands), nystatin (Calbiochem, Bad Soden, Germany), and phentolamine (Aldrich, Steinheim, Germany) were dissolved in dimethylsulfoxide (DMSO; final maximal concentration 2.5 ml·l^{-1}). Pluronic® (BASF, Weyandotte, USA) was dissolved in DMSO as a 200 g·l^{-1} stock solution (final maximal DMSO concentration 2.5 ml·l^{-1}). Atenolol (gift from Zeneca, Plankstadt, Germany), prazosin hydrochloride (gift from Pfizer, Karlsruhe, Germany), propranolol (Alfa Aesar, Karlsruhe, FRG), and tetraethylammonium chloride (TEA) were dissolved in aqueous stock solutions. Bumetanide and indomethacin were dissolved in ethanol (final maximal concentration 2.5 ml·l^{-1}). TTX was dissolved as a

stock solution in citrate buffer (20 $mmol \cdot l^{-1}$). If not indicated differently, drugs were from Sigma, Deisenhofen, Germany. Radiochemicals ($^{36}Cl^-$, $^{86}Rb^+$) were obtained from Perkin Elmer Life Sciences, Köln, Germany.

2.11. Statistics

Values are given as means ± standard error of the mean (SEM). Comparing the means between several groups needed first an analysis of variances. If this analysis indicated significant difference between the groups, further comparison was performed by Student's *t* test or Mann-Whitney U-test. The decision, which test had to be used, was taken by an *F*-test. Both paired and unpaired two-tailed Student's t-tests were applied as appropriate. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Concentration-dependence

After about one hour stabilization in the Ussing chamber, the mucosa-submucosa preparations of the distal colon exhibited a spontaneous baseline short-circuit current (Isc) of 1.8 ± 0.08 $\mu Eq \cdot h^{-1} \cdot cm^{-2}$ at a tissue conductance (Gt) of 14.1 ± 0.03 $mS \cdot cm^{-2}$ (n = 126). The corresponding values for the proximal colon were Isc: 1.7 ± 0.07 $\mu Eq \cdot h^{-1} \cdot cm^{-2}$ and Gt: 23.5 ± 1.3 $mS \cdot cm^{-2}$ (n = 107). Dopamine, when administered to the serosal side, induced a fast change in Isc without a pronounced change in Gt. Such an increase in Isc (defined as an increase of at least 0.05 $\mu Eq \cdot h^{-1} \cdot cm^{-2}$ above the baseline just prior to administration of dopamine) was observed only in about 60 % of the tissues (73 out of 126) for the distal colon and 78 % (83 out of 107) for the proximal colon (Fig. 3.1).

Dopamine, when given at the mucosal side, had no effect (n = 8, data not shown). The action of dopamine was not enhanced, if both monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT), the two main enzymes responsible for dopamine degradation (for reference see Martel *et al.* 1996), were inhibited by a combination of pargyline ($5 \cdot 10^{-4}$ $mol \cdot l^{-1}$) and U-0521 (3,4-dihydroxy-2-methyl-propiophenone $1.2 \cdot 10^{-6}$ $mol \cdot l^{-1}$; n = 8; data not shown).

This initial and inconsistent increase in Isc (1. phase) was followed by a long lasting decrease (2. phase; Fig. 3.1). This phase, defined as decrease of at least 0.05 $\mu Eq \cdot h^{-1} \cdot cm^{-2}$ below the baseline just prior to administration of dopamine, was noticed in almost all the tissues, i.e. 98 % (124 out of 126) for the distal and 99 % (106 out of 107) for the proximal part of the colon, respectively.

The concentration dependence of the 1. phase of the Isc response was not quantified due to its inconsistent nature. The negative current induced by dopamine, however, exhibited a clear concentration dependence in both segments. The proximal part of the colon was more sensitive for dopamine (Fig. 3.1). A maximal response was observed at a concentration of $5 \cdot 10^{-4}$ $mol \cdot l^{-1}$, this concentration was used for all further experiments.

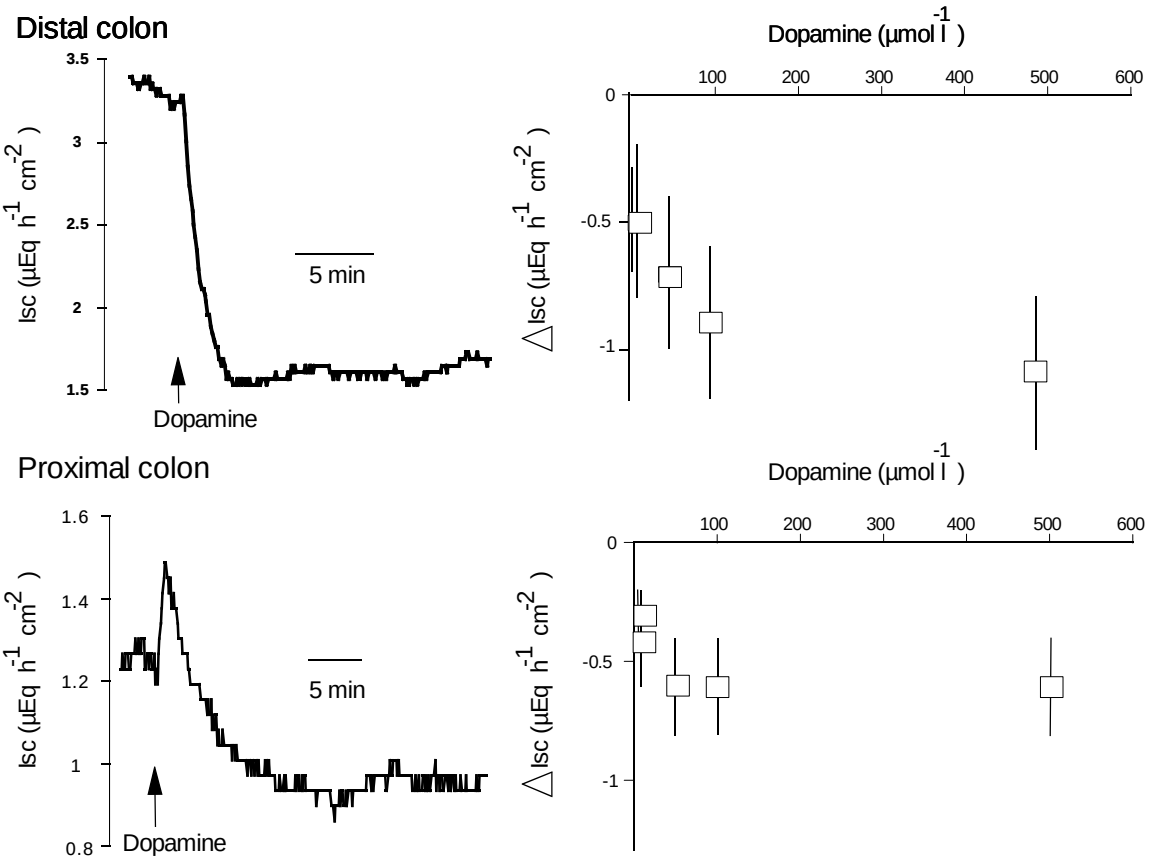

Figure 3.1: Concentration-dependent induction of a negative Isc by dopamine ($5 \cdot 10^{-6}$ - $5 \cdot 10^{-4}$ mol·l^{-1} at the serosal side) in rat distal colon (upper right side) and proximal colon (lower right side). Dopamine was applied in increasing concentrations to the same tissue, a washing step (see Methods) separated the individual administrations. Values are given as difference to the baseline in Isc just prior administration of dopamine (∆ Isc) and are means (symbols) ± S.E.M, n =5 - 6. In the left side of the picture the original records depict the time course of the negative Isc induced by dopamine ($5 \cdot 10^{-4}$ mol·l^{-1} at the serosal side) and the inconsistent short-lasting increase (peak), which preceded this response.

3.2. Direct versus indirect actions of dopamine

Secretagogues can modify ion transport across the epithelium by direct and/or indirect effects, e.g. by an action at enteric neurons releasing neurotransmitters or by an effect at subepithelial cells, releasing e.g. prostaglandins, and thereby indirectly cause a change in epithelial ion transport. In order to distinguish between these possibilities, tissues were pretreated either with the neurotoxin, tetrodotoxin (for references see Catterall 1980), or with the cyclooxygenase inhibitor, indomethacin (for references see Shen 1979).

Tetrodotoxin and indomethacin themselves induced a decrease in Isc (see Table 3.1 for the action of these and all other inhibitors on baseline Isc). In the presence of tetrodotoxin (10^{-6} mol·l^{-1} on the serosal side), the negative current induced by dopamine was significantly reduced by about 75 % in the distal colon (Fig. 3.2, Table 3.2a, 3.2b). In the proximal colon, the negative current was reduced insignificantly by about 50 % by this neurotoxin (Fig. 3.2, Table 3.2a, 3.2b). In contrast, pretreatment with indomethacin (10^{-6} mol·l^{-1} on the mucosal and the serosal side) nearly suppressed the negative current evoked by dopamine in both colonic segments (Fig. 3.2, Table 3.2a, 3.2b), suggesting a predominant - but not exclusive (see below) - subepithelial site of action of this catecholamine.

Table 3.1: Effects of putative inhibitors on baseline Isc in rat colon

	Δ Isc (μEq·h^{-1}·cm^{-2})		
Inhibitor	Distal colon	Proximal colon	n
Atenolol	-0.1 ± 0.1	-0.1±0.1	7 - 9
Bumetanide	-0.8 ± 0.1*	-1.2±0.2*	6 -9
ICI-118851	-0.6 ± 0.2 *	-0.4±0.1*	7 -9
Indomethacin	-1.0 ± 0.2*	-1.1±0.2*	6 -7
L-741,626	-0.9 ± 0.2*	0.2±0.1*	8 -9
L-745,870	-0.5 ± 0.1*	0.1±0.0*	8 -9
Quinine	-0.7 ± 0.3*	-0.7±0.2*	7 - 9
Phentolamine	-0.8 ± 0.4*	-0.4±0.1*	6 - 7
Prazosin	-0.2 ± 0.1	-0.3±0.1*	6 - 7
Propranolol	-0.6 ± 0.1*	-0.3±0.2	7
SCH 23390	0.8 ± 0.2*	0.8±0.2*	8 - 9
Tetraethylammonium	-0.2 ± 0.2	-0.2±0.1	8 - 9
Tetrapentylammonium	-0.7 ± 0.2*	-0.3±0.1*	7 - 9
Tetrodotoxin	-0.8 ± 0.3	-0.8±0.2*	6
Yohimbine	-1.2 ± 0.3*	-0.7±0.1*	8 -11

Effect of putative inhibitors on Isc in rat distal and proximal colon. Concentrations of the inhibitors were: atenolol (10^{-4} mol·l^{-1} on the serosal side), bumetanide (10^{-4} mol·l^{-1} on the serosal side), ICI-118851 (10^{-5} mol·l^{-1} on the serosal side), indomethacin (10^{-6} mol·l^{-1} on the mucosal and the serosal side), L741,626 (10^{-5} mol·l^{-1} on the serosal side), L745,870 (10^{-5} mol·l^{-1} on the serosal side), phentolamine (10^{-4} mol·l^{-1} on the serosal side), prazosin (10^{-6} mol·l^{-1} on the serosal side), propranolol (5.10^{-6} mol·l^{-1} on the serosal side), quinine (10^{-3} mol·l^{-1} on the mucosal side), SCH 23390 (10^{-5} mol·l^{-1} on the serosal side), tetraethylammonium ($5 \cdot 10^{-3}$ mol·l^{-1} on the mucosal side), tetrapentylammonium (10^{-4} mol·l^{-1} on the mucosal side), tetrodotoxin (10^{-6} mol·l^{-1} on the serosal side), yohimbine (10^{-5} mol·l^{-1} on the serosal side). Values are given as difference to the baseline prior administration of the inhibitor (Δ Isc) and are means ± S.E.M.* $P < 0.05$ versus baseline prior administration of the inhibitor.

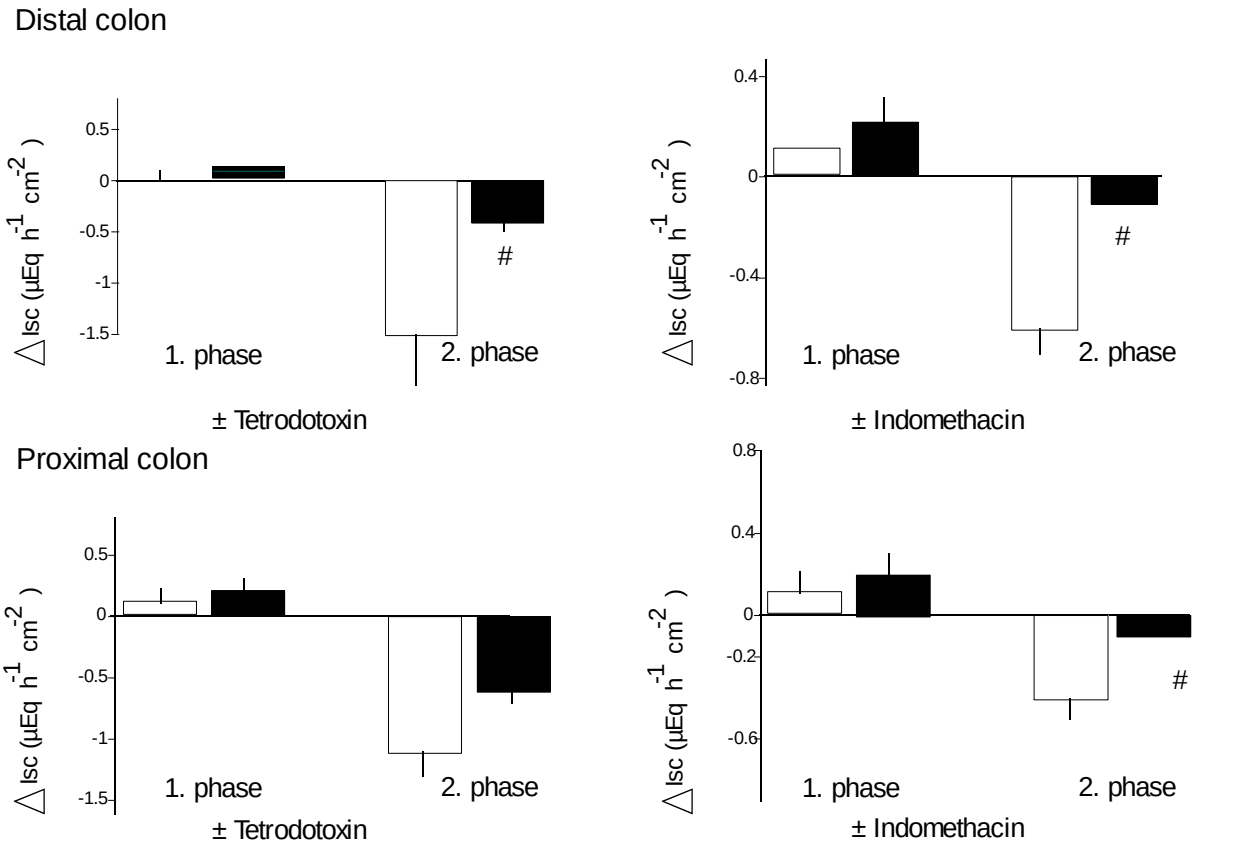

Figure 3.2: Effect of dopamine ($5 \cdot 10^{-4}$ mol l^{-1} at the serosal side) under control conditions (white bars) and in the presence of tetrodotoxin (10^{-6} mol·l^{-1} at the serosal side, black bars in the left half of the figure) or indomethacin (10^{-6} mol·l^{-1} at the mucosal and the serosal side, black bars in the right half of the figure) in the distal (upper part of the figure) and proximal (lower part of the figure) rat colon. Values are given as difference to the baseline prior administration of dopamine (Δ Isc) and are means ± S.E.M, n = 6 – 7 **,** # $P < 0.05$ versus same phase under control conditions.

Table 3.2a,b: Effect of putative inhibitors and anion substitution on the Isc response induced by dopamine

a)

	Δ Isc (μEq·h^{-1}·cm^{-2})				
	Distal colon				
Inhibitor	Dopamine under control conditions		Dopamine in the presence of the inhibitor		
	1. phase	2. phase	1. phase	2. phase	n
Bumetanide	0.0 ± 0.1	-1.0 ± 0.1*	0.2 ± 0.1	-0.4±0.1*#	9
Cl^--free buffer	0.2 ± 0.1	-0.7 ± 0.1*	0.3 ± 0.1*	-0.4±0.1*#	7
Indomethacin	0.1 ± 0.0	-0.6 ± 0.1*	0.2 ± 0.1	-0.1±0.0*#	6
Quinine	0.1 ± 0.1	-0.5 ± 0.2*	0.3±0.1*#	0.1 ± 0.1#	8 -9
TEA	0.2 ± 0.1	-1.0 ± 0.3*	0.4 ± 0.2	-1.0 ± 0.2*	6 -10
TPeA	0.1 ± 0.1	-1.0 ± 0.2*	0.3 ± 0.1*	-1.0 ± 0.3*	7 -8
Tetrodotoxin	0.0 ± 0.1	-1.5 ± 0.5*	0.1 ± 0.0	-0.4±0.1*#	6

b)

	Δ Isc (μEq·h^{-1}·cm^{-2})				
	Proximal colon				
Inhibitor	Dopamine under control conditions		Dopamine in the presence of the inhibitor		
	1.phase	2. phase	1.phase	2. phase	n
Bumetanide	0.4±0.2	-0.7±0.2*	0.2±0.0*	-0.5±0.2*	6
Cl^--free buffer	0.2±0.1*	-0.5±0.1*	0.1±0.0	-0.5±0.2*	7
Indomethacin	0.1±0.1	-0.4±0.1*	0.2±0.1*	-0.1±0.0*#	6 -8
Quinine	0.2±0.1*	-0.4±0.1*	0.1±0.0*	-0.1±0.0*#	6 -8
TEA	0.2±0.1	-0.6±0.0*	0.2±0.2*	-0.5±0.1*	7 -10
TPeA	0.1±0.1*	-0.7±0.1*	0.2±0.1*	-0.8±0.2*	6 -8
Tetrodotoxin	0.1±0.1	-1.1±0.2*	0.2±0.1	-0.6±0.1*	6

Effect of putative inhibitors and anion substitution on the Isc evoked by dopamine (5 · 10^{-4} mol·l^{-1} on the serosal side) in rat distal (a) and proximal colon (b). Concentrations of the inhibitors were: bumetanide (10^{-4} mol·l^{-1} on the serosal side), indomethacin (10^{-6} mol·l^{-1} on the mucosal and the serosal side), quinine (10^{-3} mol·l^{-1} on the

mucosal side), tetraethylammonium (TEA; $5 \cdot 10^{-3}$ mol·l^{-1} on the mucosal side); tetrapentylammonium (TPeA; 10^{-4} mol·l^{-1} on the mucosal side), tetrodotoxin (10^{-6} mol·l^{-1} on the serosal side). Anion substitution was performed both in the mucosal and the serosal compartment; for these experiments, the control experiments were performed in Cl^--containing buffer. Values are given as difference to the baseline just prior administration of dopamine (Δ Isc) and are means ± S.E.M.* $P < 0.05$ versus baseline prior administration of the inhibitor, # $P < 0.05$ versus same phase in the absence of any inhibitors.

3.3. Involvement of adrenoceptors

It is known that the effects of dopamine on ion transport are mediated by stimulation of α_2-adrenoceptors in rat jejunum (Vieira-Coelho & Soares-da-Silva 1998). Therefore, it was tested, whether inhibitors of adrenoceptors might interfere with the Isc response to dopamine in rat colon. All blockers of adrenoceptors were used in concentrations, which had been tested for inhibition of the action of epinephrine and norepinephrine in this tissue (Hörger *et al.* 1998, Schultheiss & Diener 2000). The nonselective α-adrenoceptor blocker, phentolamine (10^{-4} mol·l^{-1} on the serosal side; for references see Bylund *et al.* 1994), significantly reduced the negative Isc induced by dopamine by about 50 % in both colonic segments (Table 3.3a, 3.3b, Fig. 3.3).

This effect in the distal colon was partially mimicked by the α_2-adrenoceptor blocker yohimbine (10^{-5} mol·l^{-1} on the serosal side; Table 3.3a, 3.3b , Fig. 3.3; for references of the inhibitor see Bylund *et al.* 1994), although this inhibition failed to reach statistical

significance. In the proximal part of the colon, yohimbine abolished the second phase completely. In contrast, the α_1-adrenoceptor antagonist prazosin (10^{-6} mol·l^{-1} on the serosal side; for references see Bylund *et al.* 1994), was completely ineffective in both colonic segments (Table 3.3a, 3.3b).

In the presence of the non-selective ß-adrenoceptor blocker propranolol ($5 \cdot 10^{-6}$ mol·l^{-1} on the serosal side; for references see Bylund *et al.* 1994), a partial reduction of the negative Isc evoked by dopamine was observed in the distal colon, while in the proximal colon there was no effect of this substance (Table 3.3a, 3.3b, Fig. 3.4). This inhibition, however, was not mimicked by either the $ß_1$-selective antagonist, atenolol (10^{-4} mol·l^{-1} on the serosal side; for references see Bylund *et al.* 1994), nor by the $ß_2$-selective antagonist, ICI-118851 (10^{-5} mol·l^{-1} on the serosal side; Table 3.3a, 3.3b; for references see Bylund *et al.* 1994). In contrast, atenolol even enhanced significantly the first phase of the dopamine response in the distal colon (Fig. 3.5).

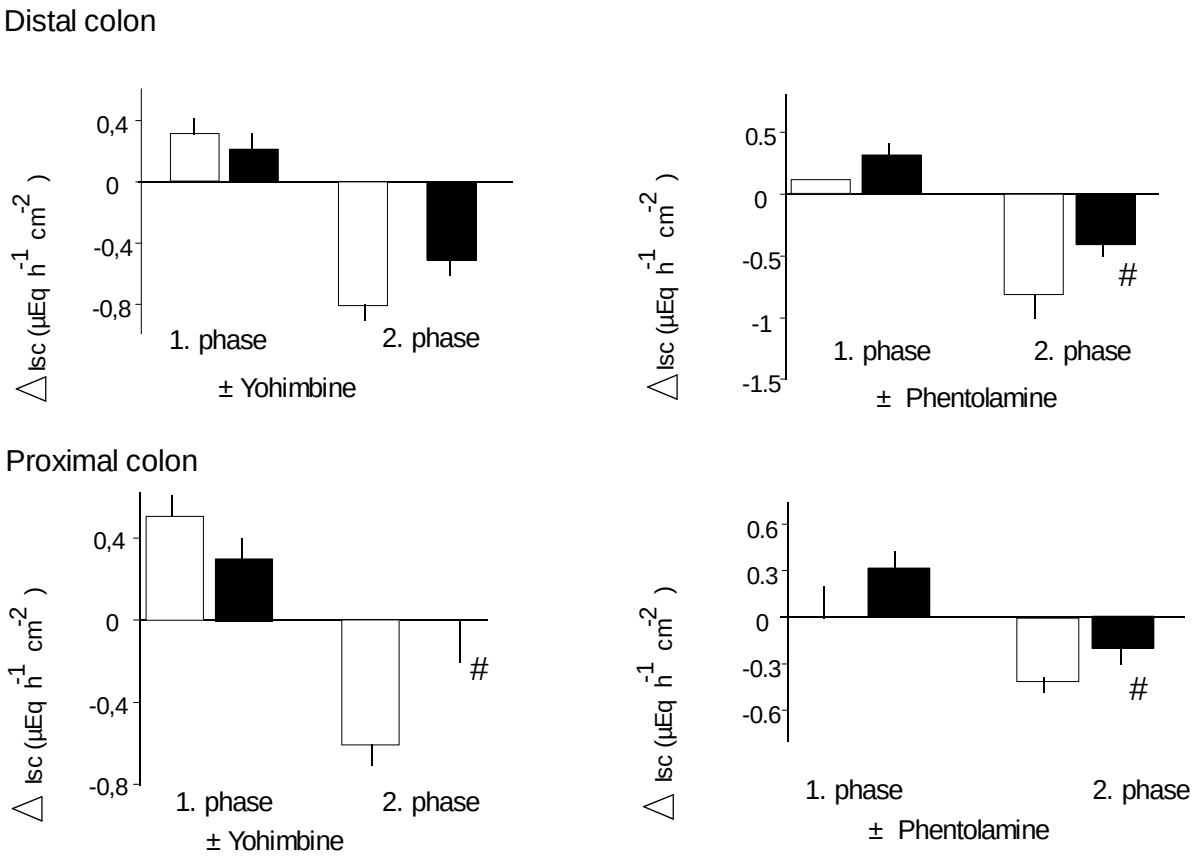

Figure 3.3: Effect of dopamine ($5 \cdot 10^{-4}$ mol·l^{-1} at the serosal side) under control conditions (white bars) and in the presence of phentolamine (10^{-4} mol·l^{-1} on the serosal side, black bars in the right side of the figure) or yohimbine (10^{-5} mol·l^{-1} on the serosal side, black bars in the left side of the figure) in the distal (upper part of figure) and the proximal (lower part of figure) rat colon. Values are given as difference to the baseline prior administration of dopamine (Δ Isc) and are means ± S.E.M, n = 6 - 12, # $P < 0.05$ versus same phase under control conditions.

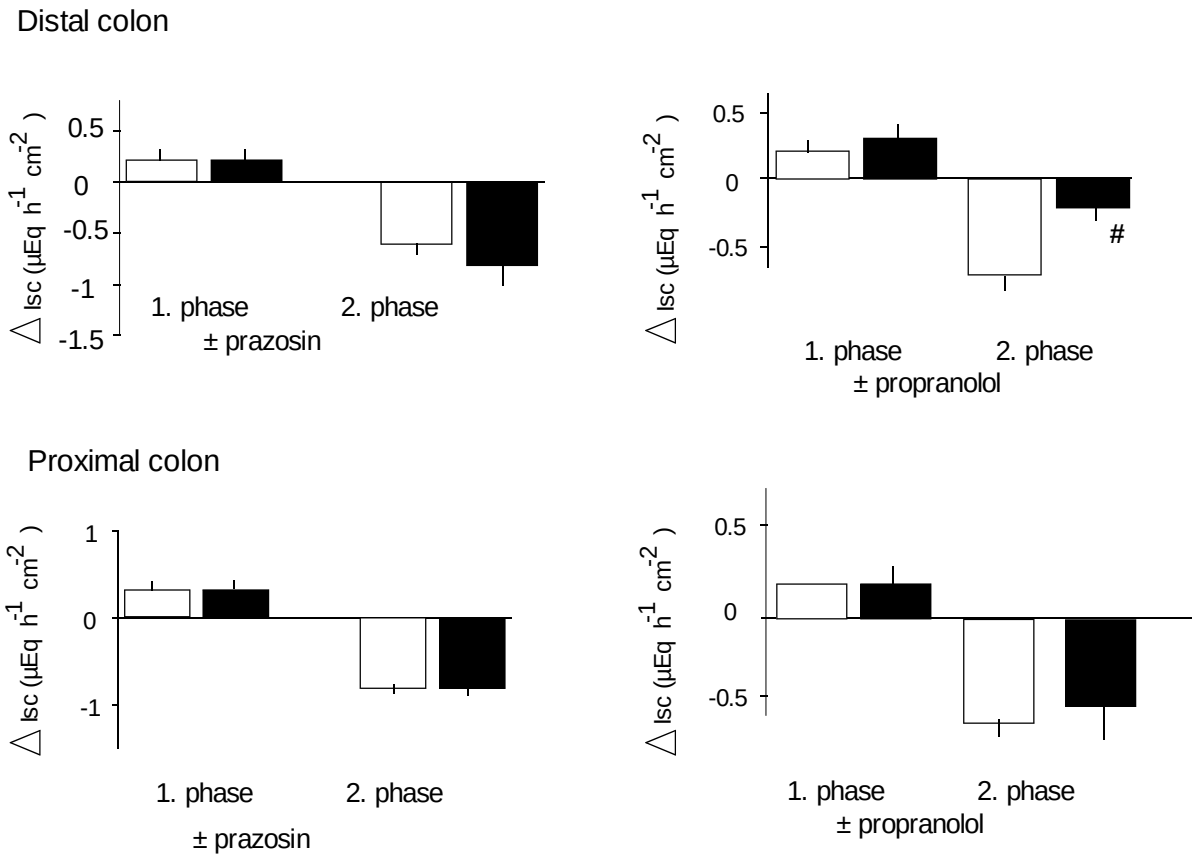

Figure 3.4: Effect of dopamine ($5 \cdot 10^{-4}$ mol·l^{-1} at the serosal side) under control conditions (white bars) and in the presence of propranolol ($5 \cdot 10^{-6}$ mol·l^{-1} on the serosal side, black bars in the right side of the figure) or prazosin (10^{-6} mol·l^{-1} on the serosal side, black bars in the left side of the figure) in the distal (upper half of the figure) and the proximal (lower half of the figure) rat colon. Values are given as difference to the baseline prior administration of dopamine (Δ Isc) and are means± S.E.M, n = 6 - 7, # $P < 0.05$ versus same phase under control conditions.

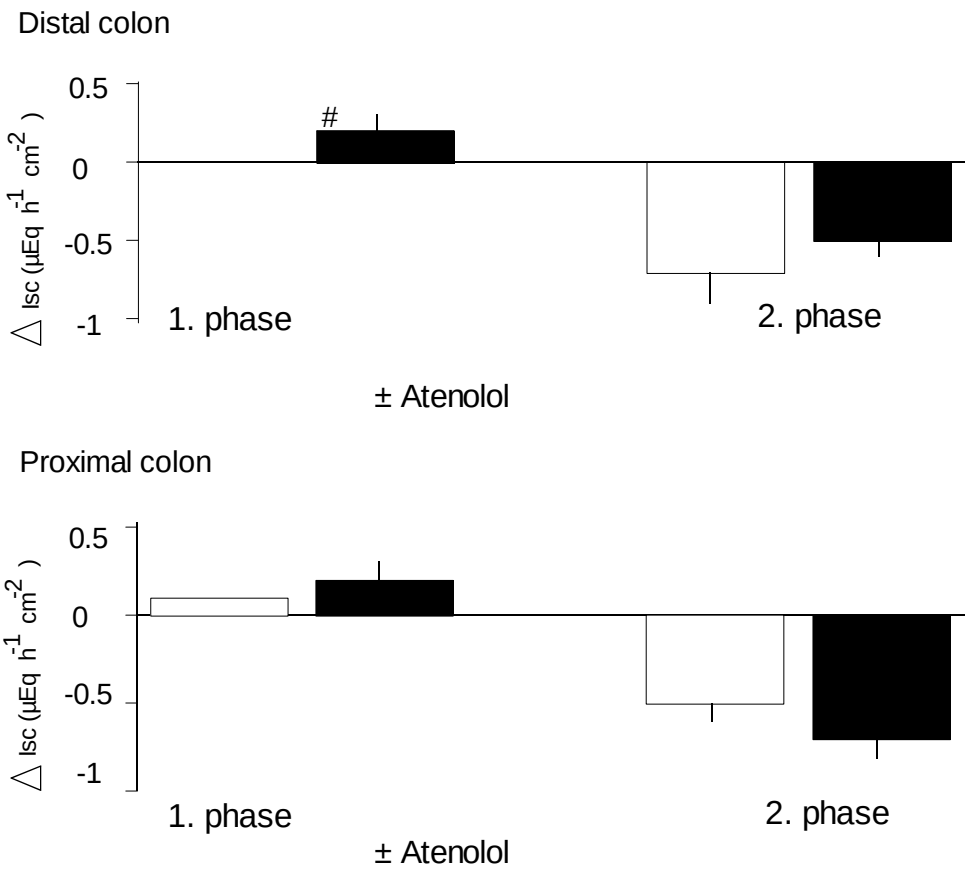

Figure 3.5: Effect of dopamine ($5 \cdot 10^{-4}$ mol·l^{-1} at the serosal side) on Isc in rat colon under control conditions (white bars) and in the presence of atenolol (10^{-4} mol·l^{-1} on the serosal side, black bars). Values are given as difference to the baseline prior administration of dopamine (Δ Isc) and are means ± S.E.M, n = 5 - 9, # $P < 0.05$ versus same phase under control conditions.

Table 3.3a, b: Effect of adrenergic inhibitors on the Isc response induced by dopamine

a)

Δ Isc ($\mu Eq \cdot h^{-1} \cdot cm^{-2}$)					
Distal colon					
Inhibitor	Dopamine under control condition		Dopamine in the presence of the inhibitor		
	1. phase	2. phase	1. phase	2. phase	n
Phentolamine	0.1 ±0.0*	-0.8 ±0.2*	0.3 ± 0.1*	-0.4 ± 0.1*#	7
Prazosin	0.2 ± 0.1	-0.6 ±0.1*	0.2 ± 0.1	-0.8 ± 0.2*	5 -7
Yohimbine	0.2 ±0.1*	-0.8 ±0.1*	0.3 ± 0.1*	-0.5 ± 0.1*	11 -12
Propranolol	0.2 ±0.1*	-0.7 ±0.1*	0.3 ± 0.1*	-0.2 ±0.1*#	7
Atenolol	0.0 ± 0.0	-0.7 ±0.2*	0.2±0.1*#	-0.5 ± 0.1*	8 -9
ICI-118851	0.2 ± 0.1	-0.8 ±0.1*	0.1 ± 0.0*	-0.7 ± 0.1*	8 -9

b)

Δ Isc (μEq·h^{-1}·cm^{-2})					
Proximal colon					
Inhibitor	Dopamine under control condition		Dopamine in the presence of the inhibitor		
	1. phase	2. phase	1. hase	2. phase	n
Phentolamine	0.0±0.2	-0.4±0.1*	0.3±0.1*	-0.2±0.1*#	7
Prazosin	0.3±0.1*	-0.8±0.1*	0.3±0.1*	-0.8±0.1*	6 -8
Yohimbine	0.3±0.1*	-0.6±0.1*	0.5±0.1*	0.0±0.2#	6 -8
Propranolol	0.2±0.0*	-0.6±0.1*	0.2±0.1	-0.5±0.2*	5 -8
Atenolol	0.1±0.0	-0.5±0.1*	0.2±0.1*	-0.7±0.1*	5 -8
ICI-118851	0.4±0.1*	-0.9±0.1*	0.2±0.1	-0.6±0.1*	6 -8

Effect of adrenergic receptor blockers on the Isc evoked by dopamine (5.10^{-4} mol·l^{-1} on the serosal side) in rat distal (a) and proximal colon (b). Concentrations of the inhibitors were: Atenolol (10^{-4} mol·l^{-1} on the serosal side), ICI-118851 (10^{-5} mol·l^{-1} on the serosal side), prazosin (10^{-6} mol·l^{-1} on the serosal side), phentolamine (10^{-4} mol·l^{-1} on the serosal side), propranolol (5.10^{-6} mol·l^{-1} on the serosal side), yohimbine (10^{-5} mol·l^{-1} on the serosal side). Values are given as difference to the baseline just prior administration of dopamine (Δ Isc) and are means ± S.E.M.* $P < 0.05$ versus baseline prior administration of dopamine, # $P < 0.05$ versus same phase under control conditions.

3.4. *Involvement of dopamine receptors*

The experiments described above revealed a partial resistance of the dopamine response against adrenoceptor antagonists. This suggests that in part the action of this catecholamine in rat colon might be mediated by specific dopamine receptors. Commercially available inhibitors of different subtypes of dopamine receptors were tested for their ability to interfere with the dopamine response in order to test this hypothesis.

The D_1-receptor antagonist, SCH 23390 (10^{-5} mol·l^{-1} on the serosal side; for references of this inhibitor see Missale *et al.* 1998), proved to be completely ineffective in both colonic segments (Table 3.4a, 3.4b, Fig. 3.6). In contrast, both the D_2-receptor antagonist, L-741,626 (10^{-5} mol·l^{-1} on the serosal side; for reference see Kulagowski *et al.* 1996, Pillai et al. 1998), and the D_4-receptor antagonist, L-745,870 (10^{-5} mol·l^{-1} on the serosal side; for references see Rowley *et al.* 1996), inhibited the negative current induced by dopamine in both colonic segments (Table 3.4a, 3.4b, Fig. 3.7).

Table 3.4a,b : Effect of dopamine receptor blockers on the Isc response induced by dopamine

a)

	Δ Isc (μEq·h^{-1}·cm^{-2})				
	Distal colon				
Inhibitor	Dopamine without inhibitor		Dopamine with inhibitor		
	1. phase	2. phase	1. phase	2. phase	n
SCH23390	0.2 ± 0.1	-1.0±0.2*	0.0 ± 0.0	-1.0±0.2*	8 -9
L-741,626	0.2 ± 0.2	-0.8±0.2*	0.3±0.1*	-0.2±0.1#	9
L-745,870	0.1±0.0*	-0.8±0.1*	0.3±0.1*	-0.1±0.1 #	8

b)

	Δ Isc (μEq·h^{-1}·cm^{-2})				
	Proximal colon				
Inhibitor	Dopamine without inhibitor		Dopamine with inhibitor		
	1. phase	2. phase	1. phase	2. phase	n
SCH23390	0.2±0.1*	-0.4±0.1*	0.1±0.1	-0.5±0.1*	8 -9
L-741,626	0.2±0.1*	-0.5±0.1*	0.3±0.2	-0.2±0.0*	7-10
L-745,870	0.7±0.3	-0.5±0.1*	0.2±0.0*	-0.3±0.1*#	8 -9

Effect of dopamine receptor blockers on the Isc evoked by dopamine (5.10^{-4} mol·l^{-1} on the serosal side) in rat distal (a) and proximal (b) colon. Concentrations of the inhibitors were: L741,626 (D_2-antagonist, 10^{-5} mol·l^{-1} on the serosal side), L745,870 (D_4-antagonist, 10^{-5} mol·l^{-1} on the serosal side), SCH 23390 (D_1-antagonist, 10^{-5} mol·l^{-1}

on the serosal side). Values are given as difference to the baseline just prior administration of dopamine (Δ Isc) and are means ± S.E.M.* P < 0.05 versus baseline prior administration of dopamine, # P < 0.05 versus same phase under control conditions.

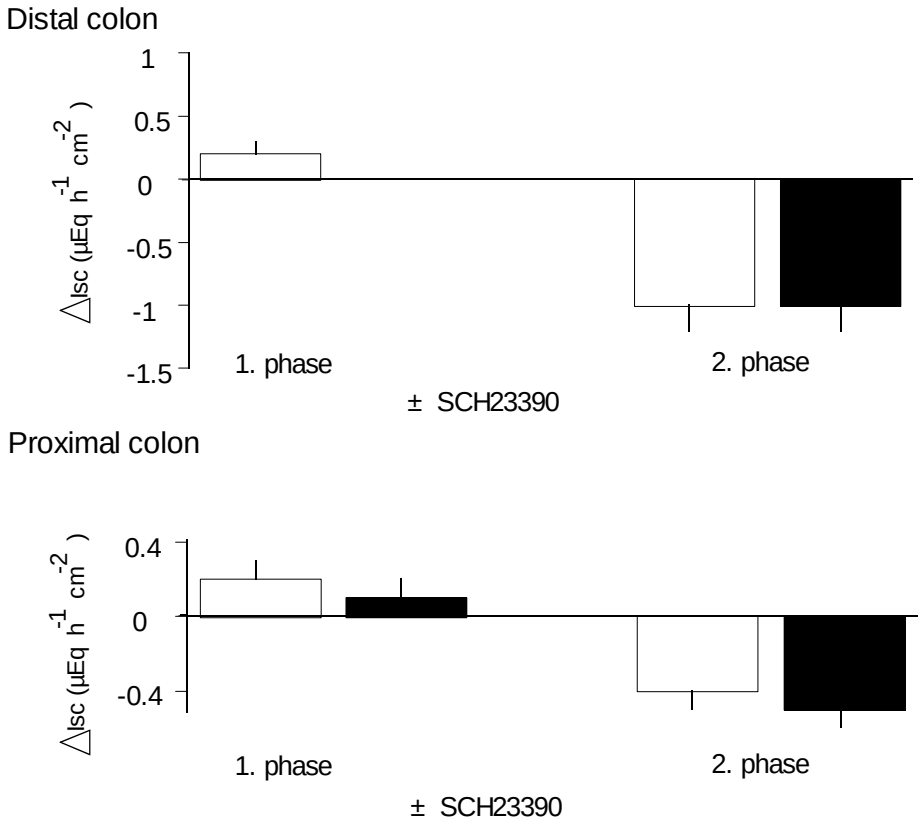

Figure 3.6: Effect of dopamine ($5 \cdot 10^{-4}$ mol·l^{-1} at the serosal side white bar) on Isc under control conditions (white bars) and in the presence of SCH 23390 (10^{-5} mol·l^{-1} on the serosal side, black bars) in distal (upper part of the figure) and proximal (lower part of the figure) rat colon. Values are given as difference to the baseline prior administration of dopamine (Δ Isc) and are means ± S.E.M, n = 8 - 9, # P < 0.05 versus same phase under control condition.

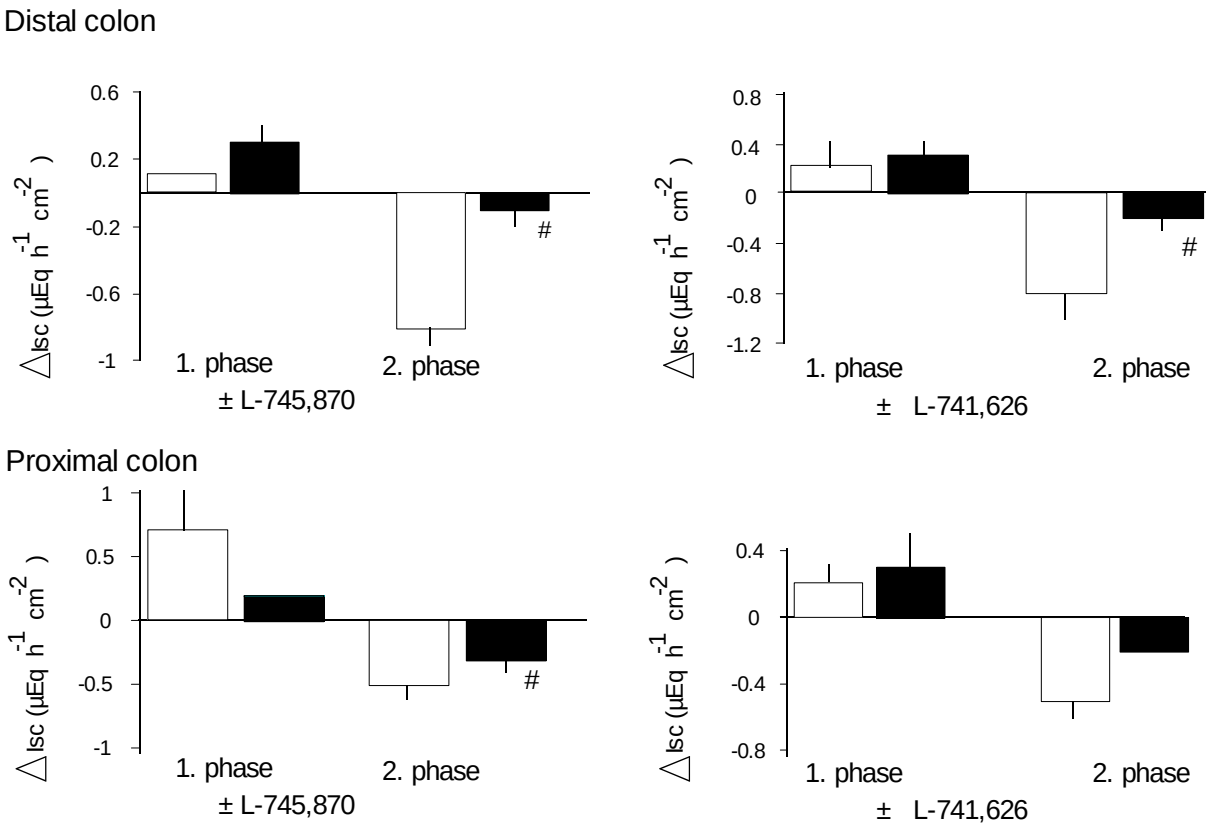

Figure 3.7: Effect of dopamine ($5 \cdot 10^{-4}$ mol·l^{-1} at the serosal side, white bars) on Isc in the distal (upper part of the figure) and the proximal colon (lower part of the figure) under control conditions (white bars), and in the presence of L-745,870 (10^{-5} mol·l^{-1} on the serosal side, black bars in the left half of the figure) or L-741,626 (10^{-5} mol·l^{-1} on the serosal side, black bars in the right half of the figure). Values are given as difference to the baseline prior administration of dopamine (Δ Isc) and are means ± S.E.M, n = 8 - 10 , # P < 0.05 versus same phase under control condition.

Based on these inhibitor experiments, two agonists at dopamine receptors, the D_1-agonist SK&F 38393 and the D_2-agonist quinpirole, were tested for their ability to mimic the action of dopamine (for references for the agonists, see Missale *et al.* 1998). SK&F 38393 concentration-dependently induced a negative Isc, when the drug was administered to the serosal bath (Figs. 3.8, 3.9). No transient increase in Isc was induced by this agonist in both colonic segments at any concentration used (Table 3.5). In contrast, quinpirole at concentrations $\geq 10^{-4}$ mol·l^{-1} induced a negative Isc, which at concentrations $\geq 2.5 \cdot 10^{-4}$ mol·l^{-1} was preceded by a transient increase in Isc (Figs. 3.8 -3.10, Table 3.5).

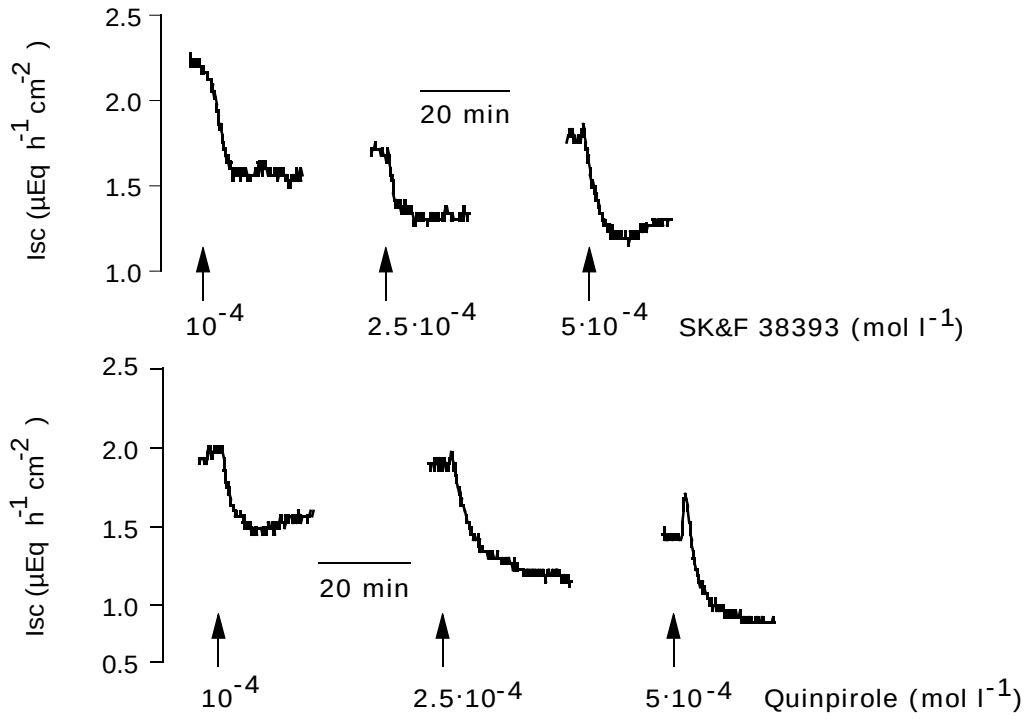

Figure 3.8: Effect of different concentrations of the D_1-agonist SK&F 38393 (administered at the serosal side; arrows in the upper part of the figure), or of the D_2-agonist quinpirole (administered at the serosal side; arrows in the lower part of the figure) on Isc in the distal part of the colon. The original tracings are representative for 8 - 9 experiments with each agonist; for statistics, see Table 3.5.

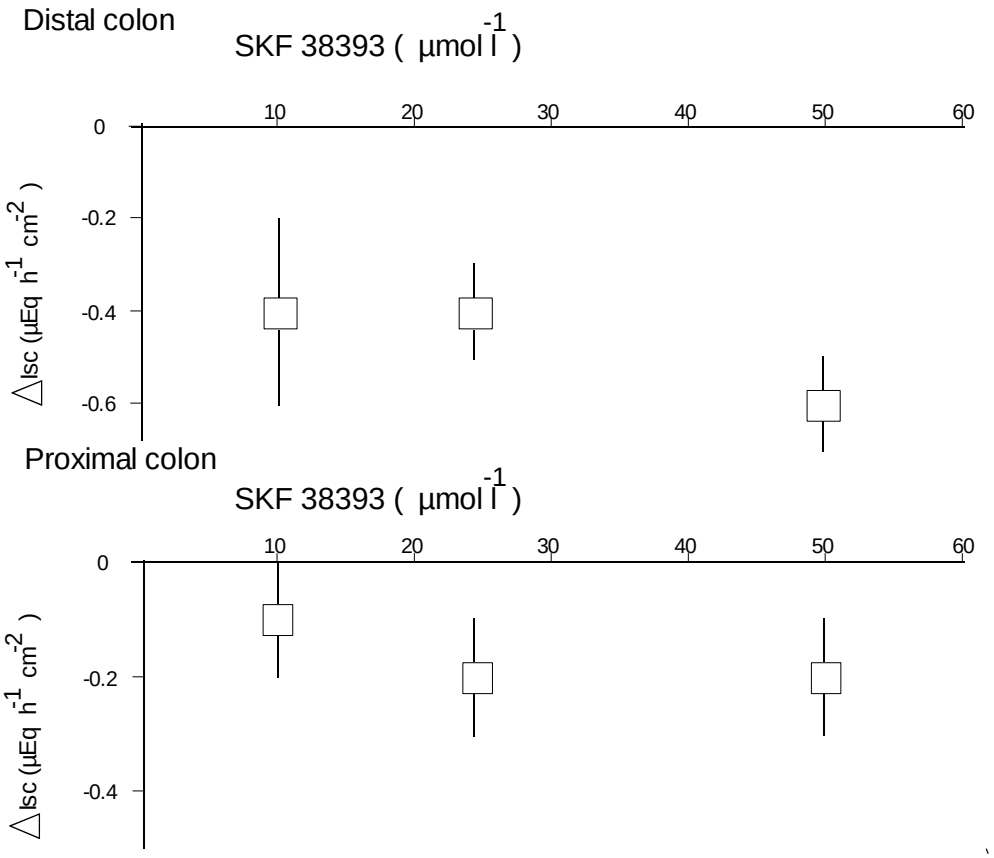

Figure 3.9: Effect of different concentrations of the D_1-agonist SK&F 38393 (administered at the serosal side) on the Isc in the distal (upper part of the figure) and proximal (lower part of the figure) rat colon. Values are given as difference to the baseline prior administration of SKF38393 (Δ Isc) and are means (symbols) ± S.E.M (error bars), n = 8 - 9.

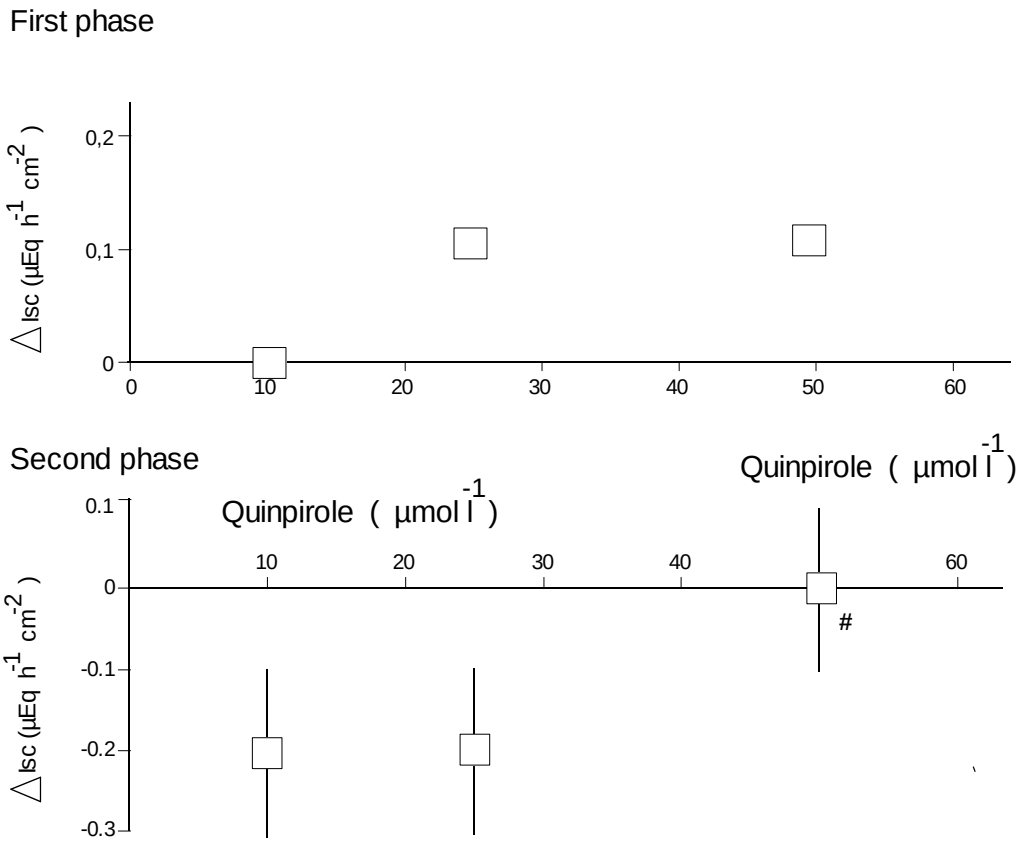

Figure 3.10: Effect of different concentrations of the D_1-agonist quinpirole (administered at the serosal side) on Isc in the proximal part of the colon first phase (upper part of the figure) and second phase (lower part of the figure). Values are given as difference to the baseline prior administration of quinpirole (Δ Isc) and are means ± S.E.M. n = 8 - 9, # P < 0.05 versus lower concentrations.

Table 3.5: Effect of dopaminergic receptor agonists on Isc

	Δ Isc ($\mu Eq \cdot h^{-1} \cdot cm^{-2}$)				
		Distal colon		Proximal colon	
Agonist	Concen-tration ($mol \cdot l^{-1}$)	1. phase	2. phase	1. phase	2. phase
SK&F 38393	10^{-4}	-	-0.4± 0.2*	-	-0.1 ± 0.1
	$2.5 \cdot 10^{-4}$	-	-0.4± 0.1*	-	-0.2 ± 0.1
	$5 \cdot 10^{-4}$	-	-0.6± 0.1*	-	-0.2 ± 0.1*
Quinpirole	10^{-4}	-	-0.7± 0.1*	-	-0.2 ± 0.1*
	$2.5 \cdot 10^{-4}$	0.1 ± 0.0*	-0.6± 0.1*	0.1 ± 0.0	-0.2 ± 0.1*
	$5 \cdot 10^{-4}$	0.2 ± 0.0*	-0.4± 0.1*	0.1 ± 0.0*	0.0 ± 0.1*

Table 3.5: All agonists were administered on the serosal side, a washing step separated the subsequent administrations of different concentrations of each agonist. Values are given as difference to the baseline just prior administration of dopamine (Δ Isc) and are means ± S.E.M.* $P < 0.05$ versus baseline, $n = 8 - 9$.

3.5. Ionic nature of the Isc response

In order to find out the ionic nature of the Isc evoked by dopamine, two series of experiments were performed, i.e. transport inhibitor experiments and anion replacement experiments. Bumetanide (10^{-4} mol·l^{-1} on the serosal side; for references see Russell 2000) was used as an inhibitor of the basolateral Na^+-K^+-2 Cl^- -cotransporter responsible for the uptake of K^+ and Cl^- to be secreted. The effect of this inhibitor was an inhibition of the negative Isc induced by dopamine. This inhibition amounted to 60 % in the distal part of the colon. The first phase of the dopamine response was unaffected. Bumetanide had no significant effect in the proximal part, neither on the first phase nor the second one (Table 3.2a, 3.2b, Fig. 3.11; for the effect of bumetanide and all other inhibitors used on baseline Isc, see Table 3.1).

In the second series of experiments, when Cl^- in the buffer solution was replaced by the impermeant anion, gluconate, a similar partial inhibition was observed in the distal part of the colon only (Table 3.2a, 3.2b, Fig. 3.12). In the third series of the experiments, when both Cl^- and HCO_3^- were substituted (using HEPES as buffer), both the first as well as the second phase of the dopamine responses were completely abolished in both colonic segments (n = 7, Fig. 3.13).

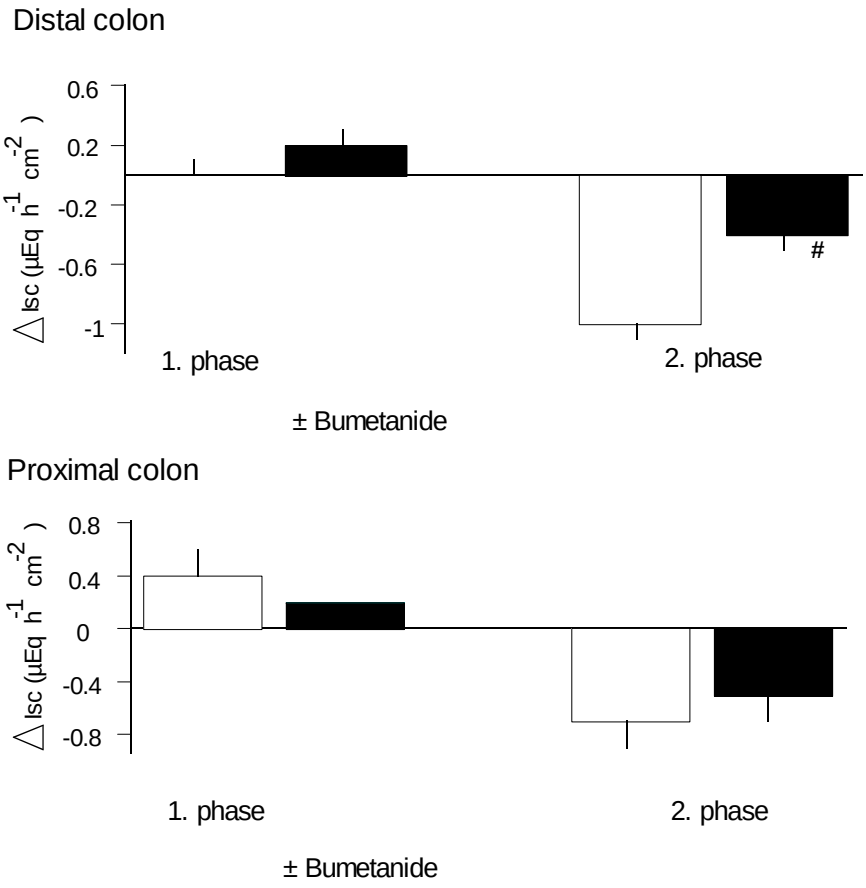

Figure 3.11: Effect of dopamine ($5 \cdot 10^{-4}$ mol·l^{-1} at the serosal side) under control conditions (white bars) and in the presence of bumetanide (10^{-4} mol·l^{-1} on the serosal side, black bars) on Isc in rat distal (upper part of the figure) and proximal (lower part of the figure) colon. Values are given as difference to the baseline prior administration of dopamine (Δ Isc) and are means ± S.E.M, n = 6 - 9, # $P < 0.05$ versus same phase under control conditions.

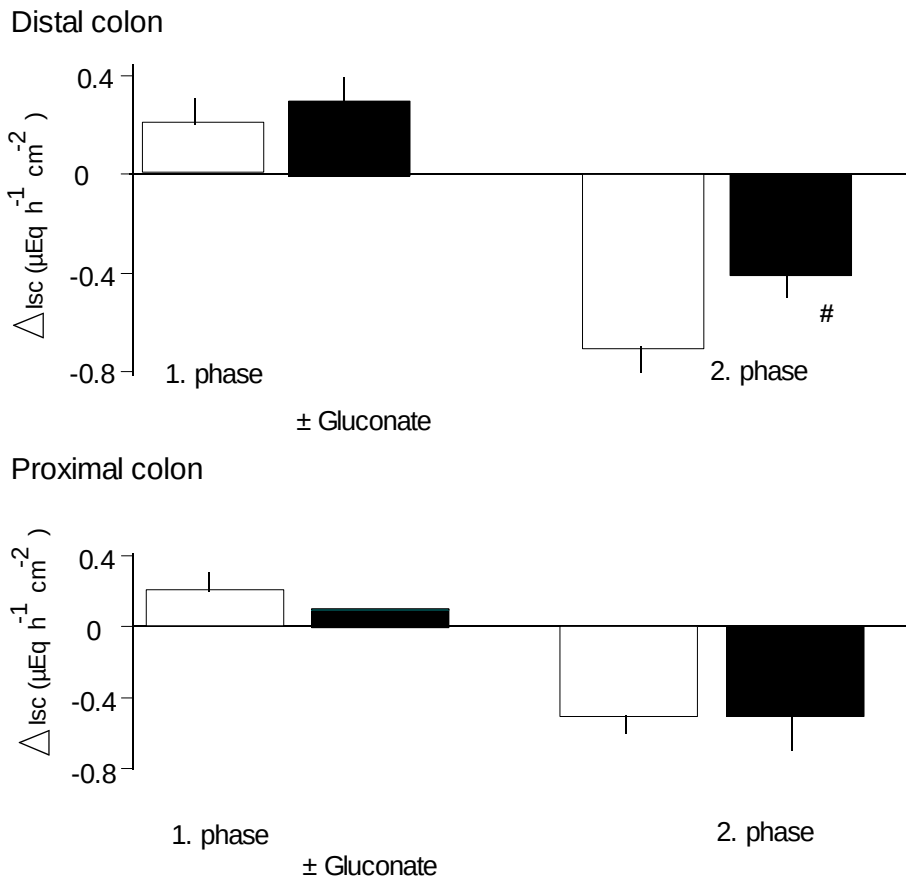

Figure 3.12: Effect of dopamine ($5 \cdot 10^{-4}$ mol·l^{-1} at the serosal side) in the presence (white bars) and absence (black bars) of chloride in the rat distal (upper part of the figure) and proximal (lower part of the figure) colon. Chloride ions were isoosmolarly replaced by gluconate in the buffer. Values are given as difference to the baseline prior administration of dopamine (Δ Isc) and are means ± S.E.M , n = 7**,** # P < 0.05 versus same phase under control conditions.

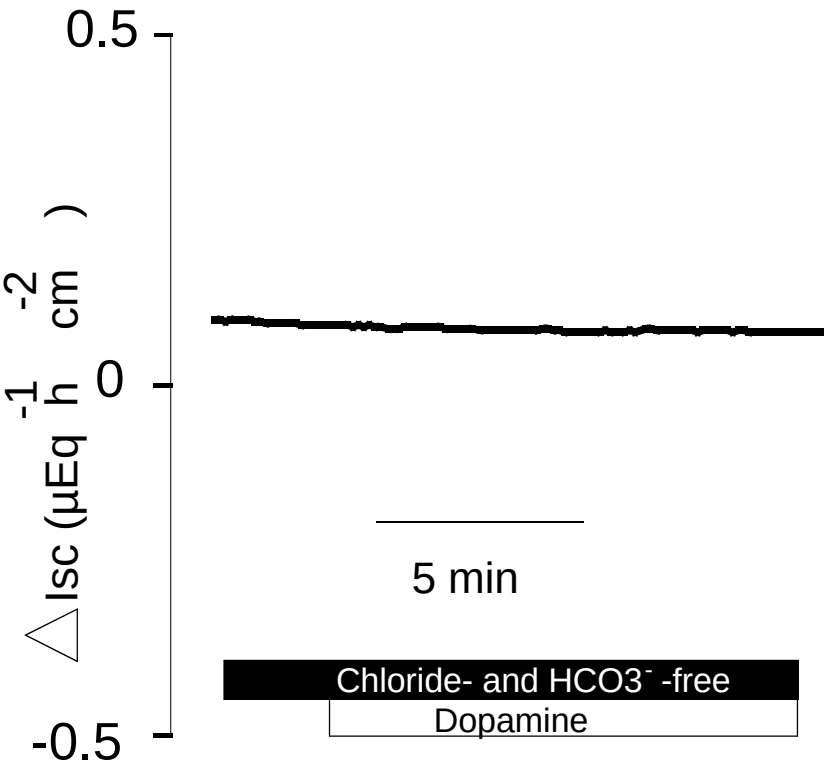

Figure 3.13: Missing effect of dopamine ($5 \cdot 10^{-4}$ mol·l^{-1} at the serosal side; white bar) on Isc, when the catecholamine was administered in Cl^-- and HCO_3^--free Tyrode solution (black bar). Typical tracing for 7 experiments with each colonic segment. Because in none of the tissues tested a response could be observed, the effects of dopamine in Cl^-- and HCO_3^--free buffer were not quantified.

3.6. The first phase: a chloride secretion

Based on these results, further experiments were performed to find out whether a transient activation of apical Cl^- channels might be responsible for the first phase of the dopamine response. The basolateral depolarization technique was used by administration of a KCl buffer (111.5 $mmol \cdot l^{-1}$ KCl) at the serosal side, a manoeuvre, which electrically eliminates and bypasses the basolateral membrane (Schultheiss & Diener 1997). By application of a chloride gradient (107 $mmol \cdot l^{-1}$ K gluconate/4.5 $mmol \cdot l^{-1}$ KCl at the mucosal side), a chloride current was driven across the apical membrane.

Under these conditions, dopamine ($5 \cdot 10^{-4}$ $mol \cdot l^{-1}$) induced in the distal and the proximal colon a transient positive current of 0.3 ± 0.1 $\mu Eq \cdot h^{-1} \cdot cm^{-2}$ ($P < 0.05$ versus baseline, n = 5 – 8, Fig. 3.14) [1] . This effect was concomitant with a significant increase in Gt only in the proximal part which amounted to 1.8 ± 0.6 $mS \cdot cm^{-2}$. This observation is in accordance with the assumption that the catecholamine transiently stimulates an apical Cl^- conductance leading to a Cl^- current driven by the applied concentration gradient.

1 In contrast to the dopamine response in intact tissue, this increase in Isc was consistently observed in all basolaterally depolarized preparations. The reason for this discrepancy is probably the fact that in intact tissue the simultaneously induced K^+ secretion, which will lead to a negative current, is partially covering the anion secretion.

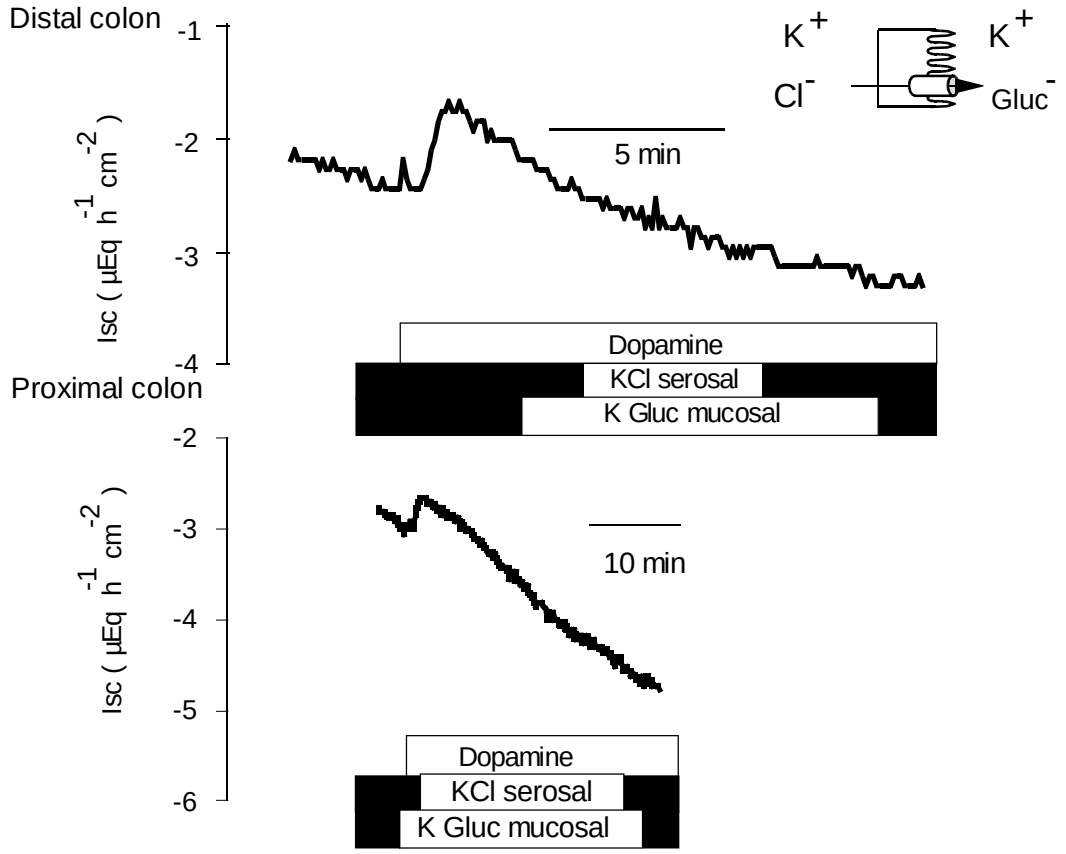

Figure 3.14: Action of dopamine ($5 \cdot 10^{-4}$ mol·l^{-1} at the serosal side; white bar) under conditions, in which the basolateral membrane was depolarized by a high K^+ concentration (111.5 mmol·l^{-1} KCl solution at the serosal side; upper black bar) in the presence of a serosally to mucosally directed Cl^- gradient (107 mmol·l^{-1} KGluc /4.5 mmol·l^{-1} KCl solution at the mucosal side; lower black bar). The schematic drawing summarizes the experimental conditions. The line tracing is typical for 5 - 8 experiments with similar results for distal and proximal colon respectively; for statistics, see the text.

The experiments under conditions, in which the basolateral membrane was depolarized by a high K^+ concentration, suggest that the first phase of the dopamine response is caused by the secretion of Cl^-. In order to confirm this assumption, efflux of $^{36}Cl^-$ was measured. Under basal conditions, in the distal colon the serosal efflux of Cl^- exceeded the mucosal efflux by a factor of about 1.5. In contrast, in the proximal part the mucosal efflux exceeded the serosal efflux approximately by the same factor. Dopamine induced different actions in the two colonic segments. In the distal colon, there was an increase of the serosal efflux accompanied by a slow increase in the mucosal efflux. Therefore, because the serosal efflux values were about two times higher than the mucosal values, absorption of the chloride will be favored during the prolonged presence of dopamine.

The transient secretory response, which leads to the transient increase in Isc (see above), was not observed with this protocol, probably because it was too short lasting to be found in the 6 minutes efflux interval necessary for this experiments technique. In contrast, in the proximal colon the effect of dopamine was inconsistent because the serosal and the mucosal efflux curves were undulating.

Taken together, the redistribution of cellular Cl^- conductance observed at least in the distal colon in favor of a serosal efflux pathway would favor net Cl^- absorption. However, none of these changes reached statistical significance due to a large variability (Fig. 3.15).

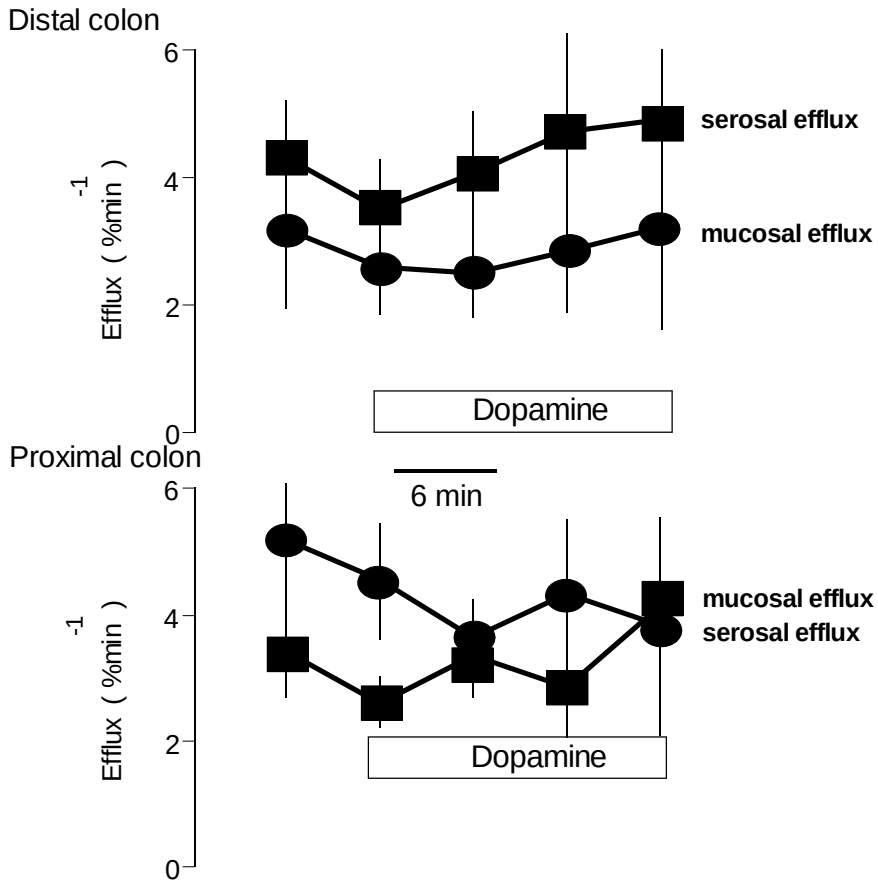

Figure 3.15: Effect of dopamine ($5 \cdot 10^{-4}$ mol·l^{-1} at the serosal side, white bar) on the efflux of $^{36}Cl^-$ into the mucosal (closed circles) and the serosal side (closed squares). Values are means (symbols) ± S.E.M (error bars), n = 10.

3.7. Measurement of basolateral K^+-currents

In order to investigate the regulation of basolateral electrogenic transport pathways for K^+ in rat colon, the apical membrane was permeabilized with the ionophore nystatin (100 µg·ml^{-1} at the mucosal side, for references see Diener *et al.* 1996). For measuring K^+ currents across the basolateral membrane, a K^+ gradient of 3 : 1 was administered (13.5 mmol·l^{-1} at the mucosal and 4.5 mmol·l^{-1} at the serosal side). Dopamine had no effect on Isc in the distal colon under these conditions (Fig. 3.16).

In contrast, dopamine under the same conditions tested showed a significant effect in the proximal colon. After administration of dopamine ($5 \cdot 10^{-4}$ mol·l^{-1} at the serosal side), the catecholamine induced a positive current of 1.5 ± 0.5 µEq·h^{-1}cm^{-2} in about 80 % of the tissue (8 of 11 tissues; Fig. 3.16). This response was followed by a consistent decrease in Isc of -0.8 ± 0.3 µEq·h^{-1}cm^{-2}.

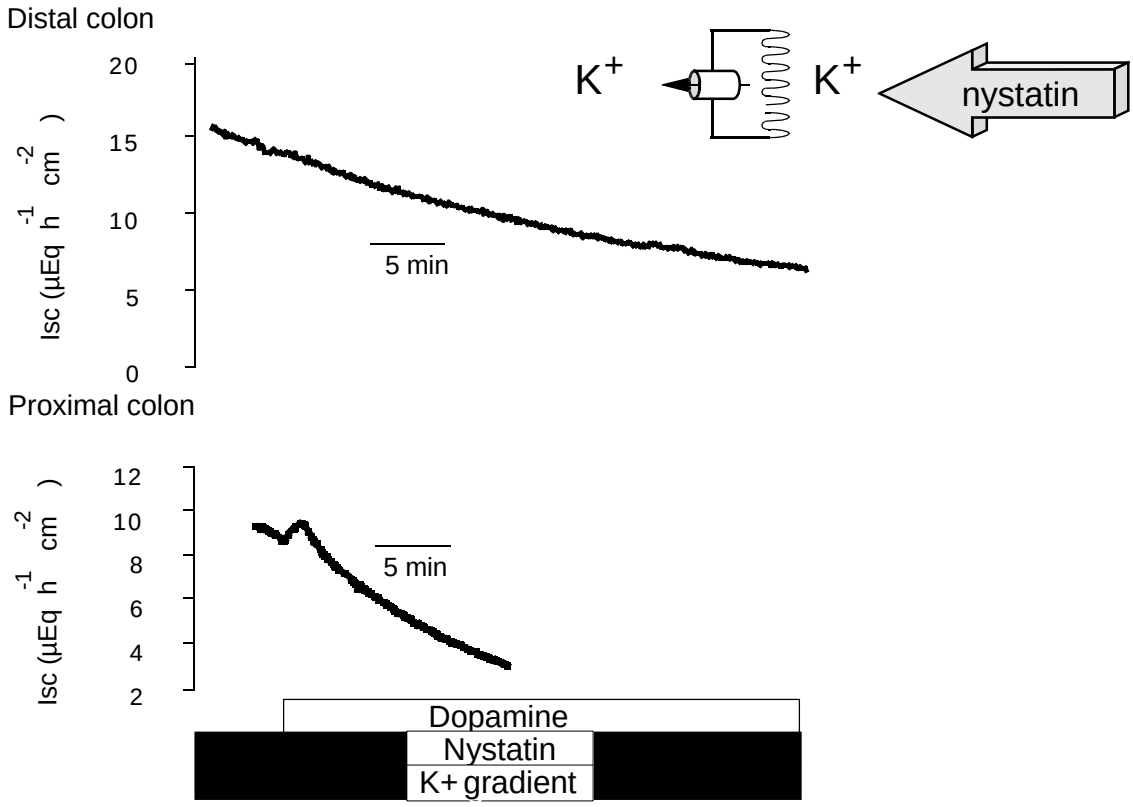

Figure 3.16: Effect of dopamine ($5 \cdot 10^{-4}$ mol·l^{-1} at the serosal side; white bar) on Isc in a nystatin-permeabilized colon in the presence of Na^+ and in the presence of K^+ gradient (13.5 mmol·l^{-1} at the mucosal and 4.5 mmol·l^{-1} at the serosal side). The original tracings are representative for 11 experiments with the distal (upper part of the figure) and 8 out of 11 experiments with the proximal colon (lower part of the figure).

As the voltage difference across the basolateral membrane is 0 mV and there is no chloride gradient, this effect in the proximal colon must be carried by a cation transport either via the basolateral Na^+-K^+-pump or via basolateral K^+ channels. In order to differentiate between these two pathways, the permeabilization of the apical membrane was performed under Na^+-free conditions. Under these conditions, dopamine induced an increase in Isc, which amounted to 0.6 ± 0.1 $\mu Eq \cdot h^{-1} cm^{-2}$ followed by a decrease in Isc by -0.9 ± 0.2 $\mu Eq \cdot h^{-1} cm^{-2}$ (Fig. 3.17). Because the baseline Isc in the absence of dopamine was not stable, the current was extrapolated by linear regression; effects of dopamine are given as difference to this extrapolated Isc. The first phase was observed in about 63 % (10 out of 16 tissues) and the second phase was observed in about 88 % (14 out of 16 tissues) of the Isc. The Gt was not affected during the administration of dopamine (Fig. 3.18). Under these conditions, i.e. in the absence of Na^+, dopamine can only act at basolateral K^+ channels.

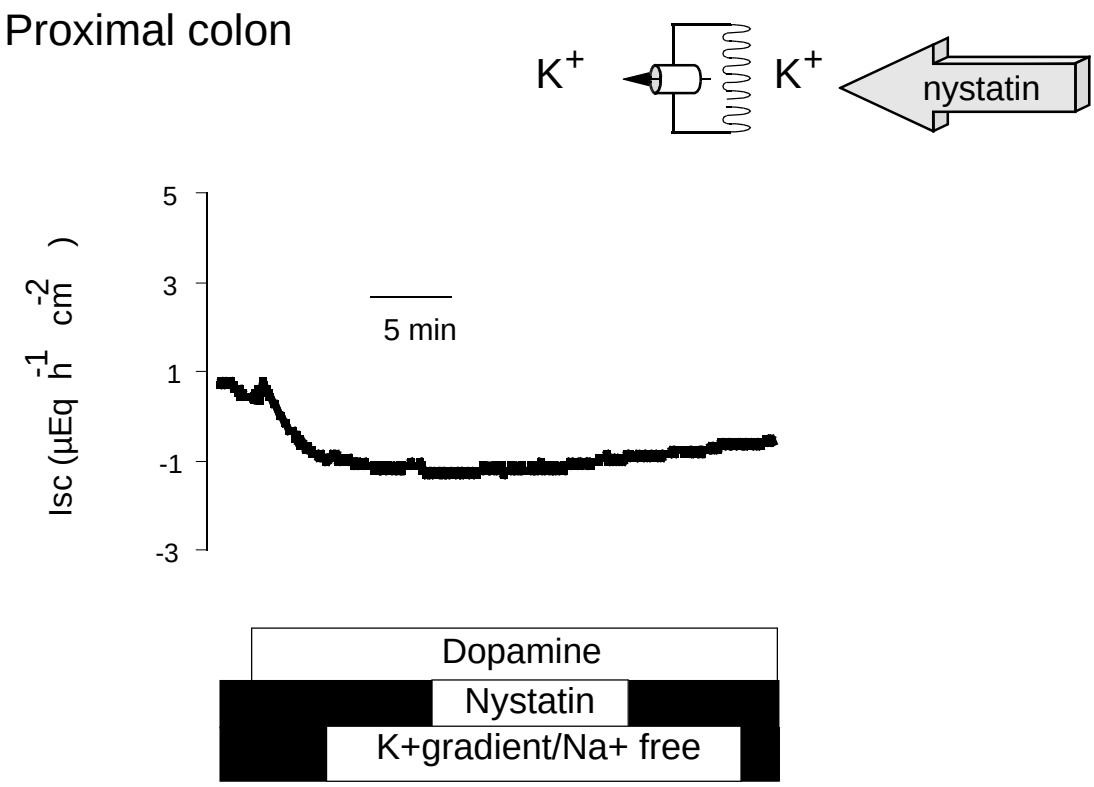

Figure 3.17: Effect of dopamine ($5 \cdot 10^{-4}$ mol·l^{-1} at the serosal side; white bar) on Isc in a nystatin-permeabilized proximal colon in the absence of Na^+ and in the presence of K^+ gradient (13.5 mmol·l^{-1} at the mucosal and 4.5 mmol·l^{-1} at the serosal side). The original tracing is representative for 16 experiments.

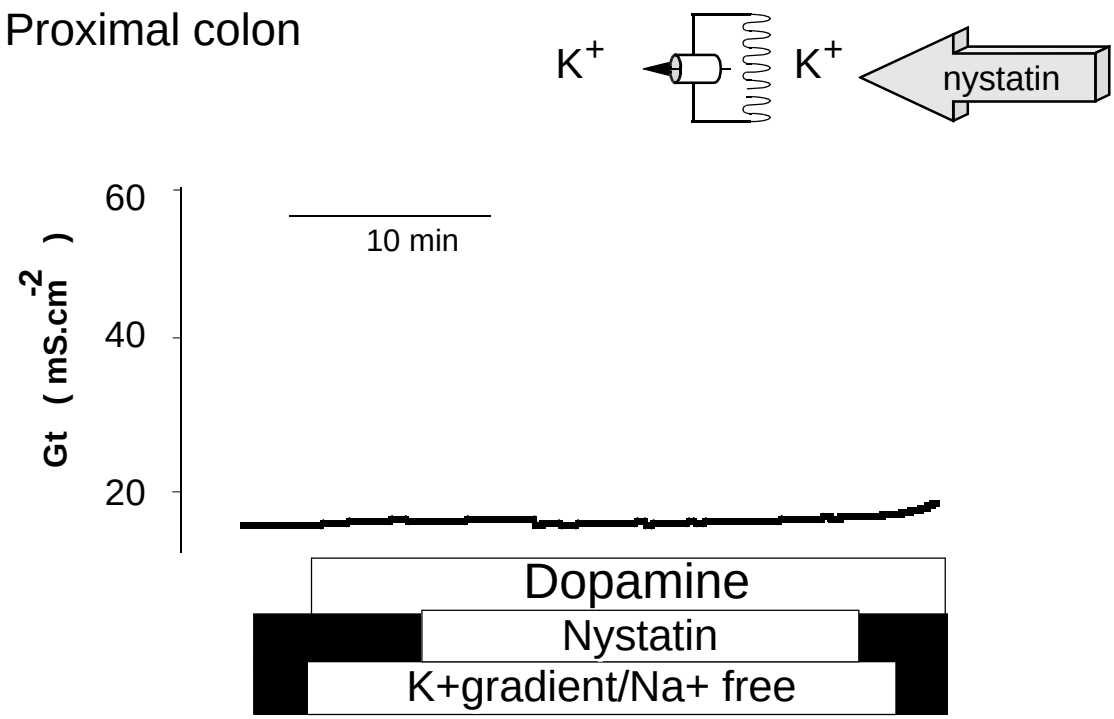

Figure 3.18: Effect of dopamine ($5 \cdot 10^{-4}$ mol·l^{-1} at the serosal side; white bar) on Gt in a nystatin-permeabilized proximal colon in the absence of Na^+ and in the presence of K^+ gradient (13.5 mmol·l^{-1} at the mucosal and 4.5 mmol·l^{-1} at the serosal side). The original tracing is representative for 16 experiments.

3.8. *The second phase: a potassium secretion*

As catecholamines such as epinephrine or norepinephrine are known to induce a K^+ secretion (Rechkemmer *et al.* 1996, Hörger *et al.* 1998, Schultheiss & Diener 2000) which will induce a negative Isc, the interference of K^+ channel blockers with the Isc evoked by dopamine was tested. Two K^+ channel blockers, tetraethylammonium ($5 \cdot 10^{-3}$ mol·l^{-1} on the mucosal side) and tetrapentylammonium (10^{-4} mol·l^{-1} on the mucosal side; for reference see Cook & Quast 1990), did not affect the action of a subsequent dopamine administration neither in the proximal nor in the distal part of the colon (Table 3.2a, 3.2b, Fig. 3.19). However, the K^+ channel blocker quinine (10^{-3} mol·l^{-1} on the mucosal side; for reference see Cook & Quast 1990) suppressed significantly the negative Isc evoked by the catecholamine in the proximal part and even reversed it into an increase in current for the distal part of the colon. In addition, the K^+ channel blocker significantly enhanced the first phase of the dopamine action in the distal colon (Fig. 3.20, Table 3.2a, 3.2b). This observation together with the known Cl^- dependence of basolateral K^+ uptake via the Na^+-K^+-2Cl^- cotransporters in the distal colon suggest that a K^+ secretion contributes to the negative current induced by dopamine.

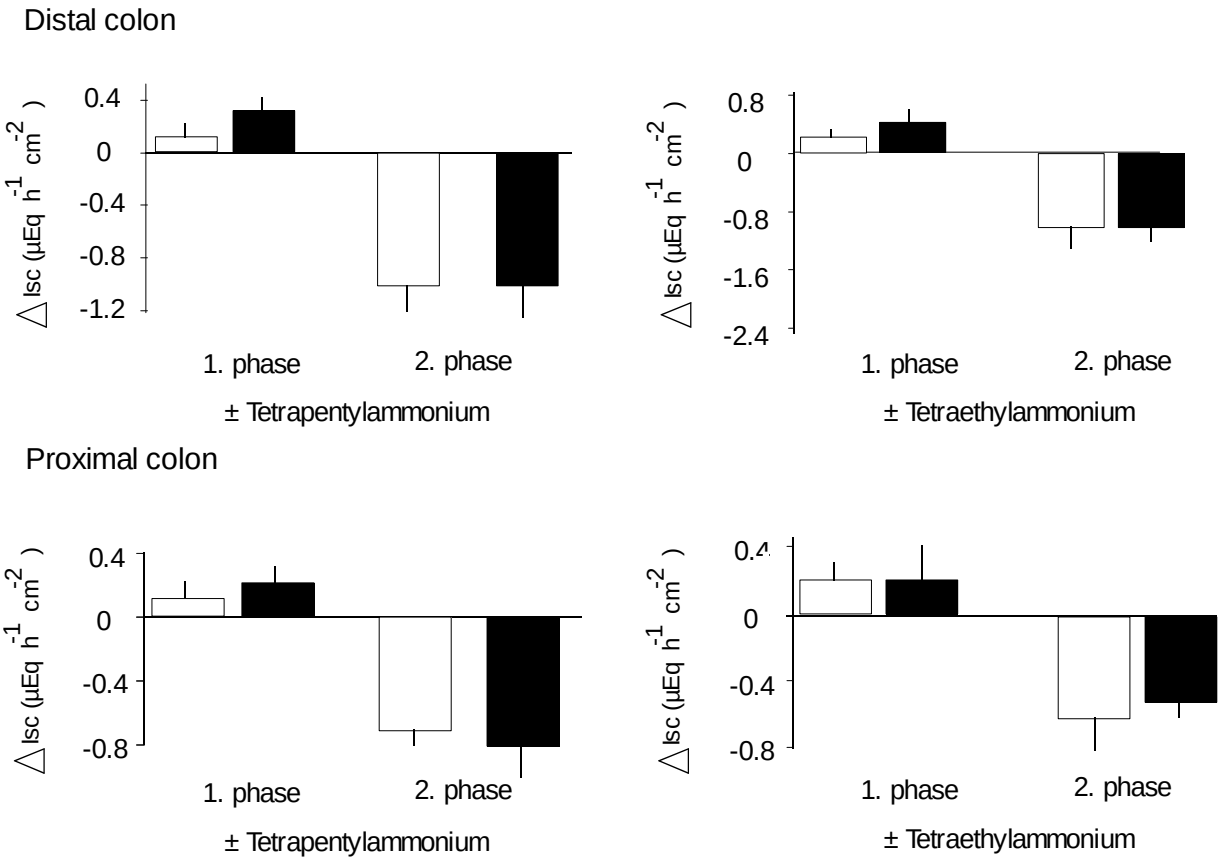

Figure 3.19: Effect of dopamine ($5 \cdot 10^{-4}$ mol·l^{-1} at the serosal side) on Isc in rat colon under control conditions (white bars) and in the presence of tetrapentylammonium (10^{-4} mol· l^{-1} on the mucosal side, black bars in the left half of the figure) or tetraethylammonium ($5 \cdot 10^{-3}$ mol·l^{-1} on the mucosal side, black bars in the right half of the figure). Values are given as difference to the baseline prior administration of dopamine (Δ Isc) and are means ± S.E.M, n = 6 - 10.

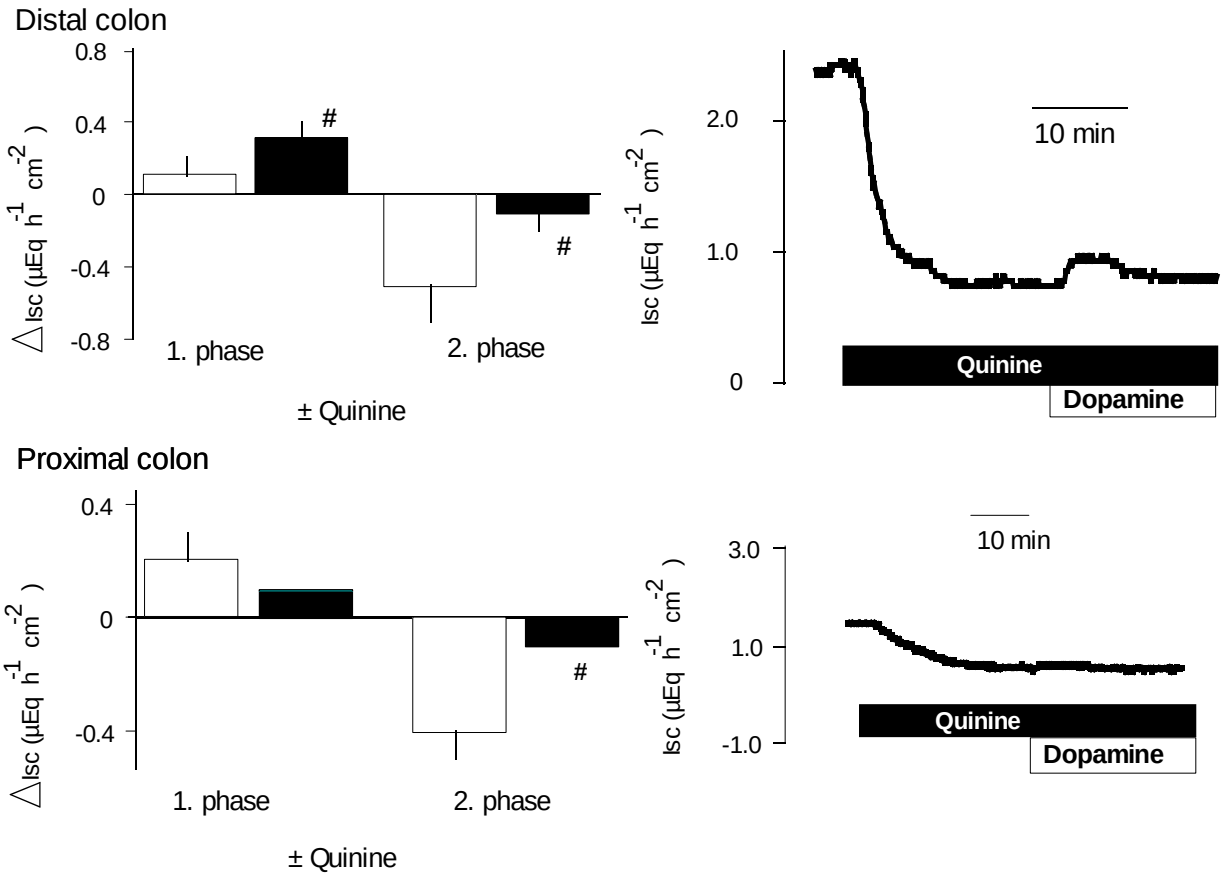

Figure 3.20: Effect of dopamine ($5 \cdot 10^{-4}$ mol·l^{-1} at the serosal side) under control conditions (white bars) and in the presence of quinine (10^{-3} mol·l^{-1} on the mucosal side; black bars) in the rat distal (upper part of the figure) and proximal (lower part of the figure) colon. Values are given as difference to the baseline prior administration of dopamine (Δ Isc) and are means ± S.E.M. The right side of the figure shows typical original records, n =6 - 9, # $P < 0.05$ versus same phase under control conditions.

3.9. *Characterization of K^+ channels involved in the dopamine response*

The above experiments and the inhibitor data, especially the reversal by quinine of the negative Isc induced by dopamine into a positive current in the distal colon, suggest the induction of K^+ secretion by dopamine. In order to confirm this assumption, two series of experiments were performed: unidirectional flux experiments and measurement of the efflux of $^{86}Rb^+$, a marker for K^+ (for reference see Foster *et al.* 1983, Hörger *et al.* 1998).

For the measurement of unidirectional fluxes, two different protocols were used. In both protocols, baseline transport of $^{86}Rb^+$ was measured in a 20 min control period (from t0 to t20). In the first series of experiments, dopamine was added at t25 and 5 min later, i.e. at t30, a new 20-min period started to measure possible changes in $^{86}Rb^+$ transport induced by dopamine. However, no catecholamine-induced changes in $^{86}Rb^+$ transport were observed with this protocol (n = 8, data not shown).

Therefore, in a second protocol a longer pre-incubation period with dopamine was used, i.e. dopamine was added at t30 and the fluxes were measured between t50 and t70, i.e. in a 20 min period starting 20 min after administration of the catecholamine. With this protocol, it revealed that dopamine induced an insignificant reduction in the absorptive flux, Jms, of $^{86}Rb^+$ in the distal colon, but stimulated significantly the secretory flux, Jsm, in the proximal colon (Table 3.6a, 3.6b). Both results are compatible with the conclusion that dopamine stimulates potassium secretion.

Table 3.6a,b

a)

	Distal colon				
	Jms	*Jsm*	*Jnet*	*Δ Isc*	*Gt*
Control t0-t20	0.69±0.1	0.69±0.1	0.0±0.1	2.7±0.3	12.7±0.5
Dopamine t50-t70	0.60±0.1	0.67±0.1	-0.7±0.1	4.5±04	12.9±0.5

b)

	Proximal colon				
	Jms	*Jsm*	*Jnet*	*Δ Isc*	*Gt*
Control t0-t20	0.5±0.1	0.7±0.1	-0.2±0.1	1.5±0.3	21.9±1.8
Dopamine t50-t70	0.6±0.1	0.9±0.1*	-0.3±0.1	3.4±0.9	26.3±2.2

Table 3.6a, b: Effect of dopamine ($5 \cdot 10^{-4}$ mol·l^{-1} at the serosal side) on the unidirectional $^{86}Rb^{+}$ fluxes in distal (a) and proximal (b) colon. The results were given as mean ± S.E.M, n = 18. * $P < 0.05$ versus control period. Fluxes and Isc are given in µEq·h^{-1}·cm^{-2}, Gt is given in mS·cm^{-2}, t indicates the time in min.

In addition, this long-time protocol revealed an additional action of the catacholamine previously not observed with the shorter time protocols, i.e. the decrease in Isc evoked by dopamine was followed by a secondary compensatory increase in Isc, while Gt remained unchanged in distal part (Fig. 3.21). A similar response was observed in the proximal colon, too. However, Gt increased also (Fig. 3.21).

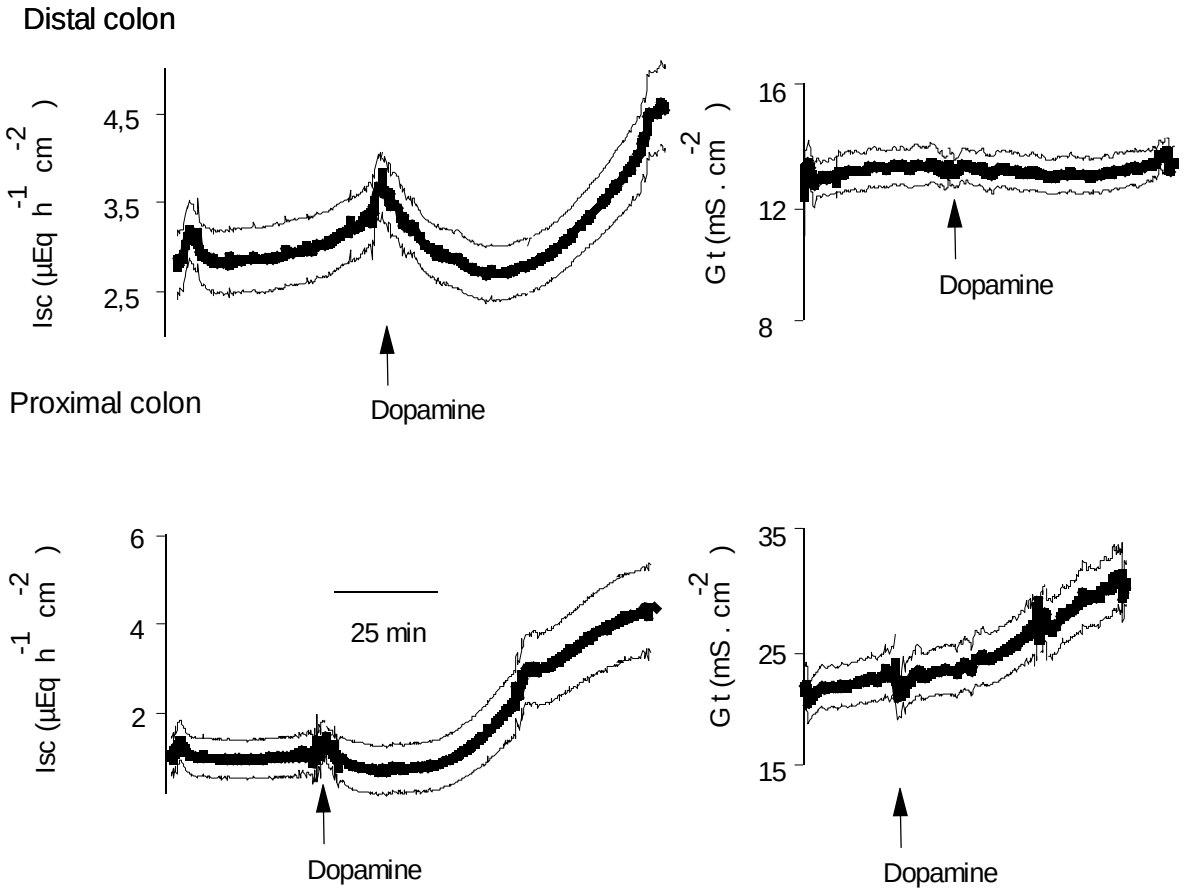

Figure 3.21: Long-term effects of dopamine ($5 \cdot 10^{-4}$ mol·l^{-1} at the serosal side) on Isc (left side of the figure) and Gt (right side of the figure) during the measurement of unidirectional $^{86}Rb^{+}$ fluxes in the distal (upper half of the figure) and proximal (lower half of the figure) colon. Values are mean (thick line) ± S.E.M (thin lines), n = 8 - 10. For statistics see table 3.6a, 3.6b.

The second series of experiments with $^{86}Rb^{+}$ was measuring the efflux of this isotope. Under basal conditions, the serosal efflux of $^{86}Rb^{+}$ exceeded the mucosal efflux by a factor of about 2.5 in both colonic segments. In the presence of dopamine, two simultaneous actions took place: a decrease in the serosal and an increase in the mucosal efflux. These actions were observed in both colonic segments (Fig. 3.22). Although only the first part of the response reached statistical significance in the distal colon, both the change in the apical as well as that in the basolateral efflux were significant in the proximal colon. This redistribution of cellular K^{+} conductance in favor of the apical conductance would favor net K^{+} secretion across the tissue.

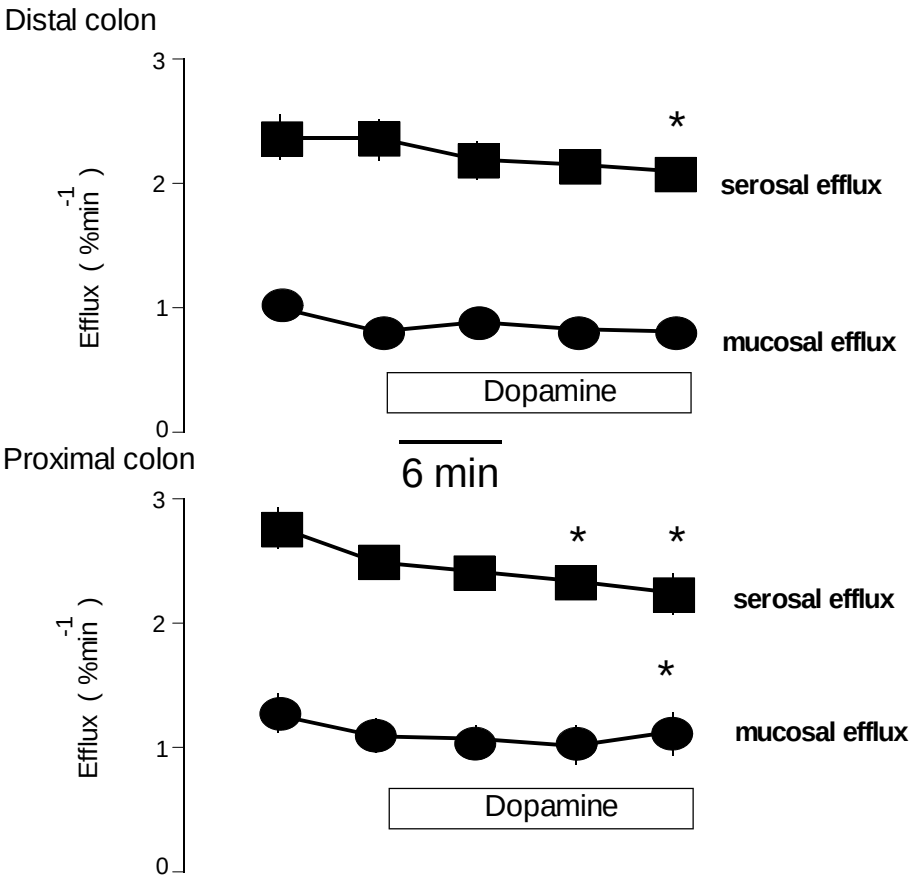

Figure 3.22: Effect of dopamine ($5 \cdot 10^{-4}$ mol·l^{-1} at the serosal side, white bar) on the mucosal (closed circles) and the serosal (closed squares) efflux of Rb^+ in the distal (upper half of the figure) and the proximal colon (lower half of the figure). Values are means (symbols) ± S.E.M (error bars), n = 10 - 11, * P < 0.05 versus last control period.

We know from other drugs which cause K^+ secretion such as carbachol (Heinke *et al.* 1999) that they induce a K^+ secretion via an increase in the intracellular Ca^{2+} concentration. Therefore, a final set of experiments was performed to see whether the changes in K^+ transport are mediated by an increase in the intracellular Ca^{2+} concentration or not. For this purpose crypts were loaded with the Ca^{2+}-sensitive dye, fura-2. However, in none of 7 tested crypts, dopamine ($5 \cdot 10^{-4}$ mol·l^{-1}) did induce any change in the fura-2 ratio signal (Fig. 3.23).

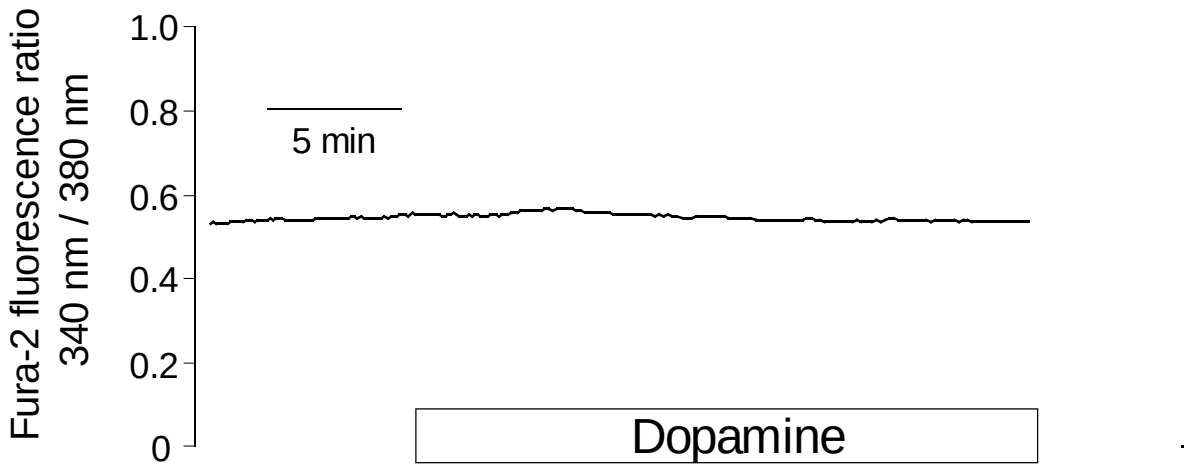

Figure 3.23: Missing effect of dopamine ($5 \cdot 10^{-4}$ mol·l^{-1}, white bar) on the fura-2 signal (emission at an excitation with 380 nm/emission at an excitation with 340 nm). The original tracing is representative for 7 experiments with similar results.

4. Discussion

4.1. Transient induction of anion secretion by dopamine

The aim of this study was to characterize the effects of the catecholamine dopamine on ion transport across rat colon in vitro. Serosal administration of dopamine ($5 \cdot 10^{-4}$ $mol \cdot l^{-1}$) evoked a biphasic change in short-circuit current (Isc) across the proximal and the distal colon of the rat without a pronounced change in tissue conductance (Gt).

The first phase of the current response consisted of a transient increase in Isc (Fig. 3.1). This action was observed only inconsistently in about 60 % of the preparations from the distal and 78 % in the preparations from the proximal colon. This response is assumed to represent the secretion of anions, predominantly of Cl^-, for several reasons. Substitution of Cl^- in the bathing medium by the impermeable anion gluconate reduced the first phase of the dopamine response at least in the proximal colon, although this inhibition did not reach statistical significance due to the high variability of this part of the catecholamine action, whereas substitution of both Cl^- and HCO_3^- in the buffer, completely suppressed it in both colonic segments (Fig. 3.13). A stimulation of Cl^- channels in the apical membrane could be shown by experiments which were performed at basolaterally depolarized epithelia. In these tissues, the basolateral membrane is electrically eliminated by a high K^+ concentration at the serosal side (Fuchs *et al.* 1977); if then a Cl^- concentration gradient is applied from the serosal to the mucosal compartment, any increase in the Cl^- conductance of the apical membrane must lead to an increase in Isc (Schultheiss *et al*. 2003). This was indeed observed, when dopamine was administered (Fig. 3.14). In contrast to the standard experiments without basolateral depolarization, under these experimental conditions all tissues exposed to dopamine responded with a stimulation of a Cl^- current. This is

probably due to the fact that under control conditions the simultaneously induced K^+ secretion leads to the induction of a negative Isc, which in a part of the tissues covers completely the increase in Isc as should be expected by the transient stimulation of anion secretion.

The direct experimental proof for the hypothesis that dopamine exposure leads to a change in the Cl^- permeability of the cells was unsuccessful. In this experimental series tissues were loaded with the radioisotope, $^{36}Cl^-$, and time-dependent efflux of the isotope into the serosal and the mucosal compartment were measured in the absence and presence of dopamine. There was the tendency for a stimulation of basolateral Cl^- efflux, i.e. efflux into the serosal compartment in the prolonged presence of dopamine, an action which would favor net Cl^- absorption in the late phase of the action of this catecholamine (Fig. 3.15). However, the expected increase in the efflux across the apical membrane into the mucosal compartment could not be observed. The most probable reason for this failure is the insufficient time resolution of the efflux experiments. For technical reasons and due to the limited loading of the tissue with the isotope, the interval between the individual efflux period lasted 6 min in order to accumulate sufficient radioactivity in the mucosal and the serosal compartment. This time resolution is probably too slow to prove the anion secretion observed in the electrophysiological experiments with this experimental design.

Nevertheless, the clear anion dependence and the electrophysiological evidence from the basolateral depolarization experiments seem to justify the statement that dopamine has the ability to transiently stimulate colonic anion secretion. The induction of this anion secretion is only inconsistently observed, a property that dopamine shares with the other two physiologically relevant catecholamines, i.e. epinephrine (Hörger *et al.*

1998) and norepinephrine (Schultheiss & Diener 2000). Both of them stimulate a transient increase in Isc due to a transient induction of Cl^- secretion (Hörger *et al.* 1998, Schultheiss & Diener 2000).

4.2. Stimulation of potassium secretion

This initial, transient anion secretion was followed by a long-lasting decrease in Isc, which could consistently be observed in 98 % (124 out of 126) of the preparations from the distal and in 99 % (106 out of 107) of the preparations from the proximal part of the colon, respectively.

This response is assumed to represent the secretion of cations, predominantly of K^+, for several reasons. Bumetanide, the blocker of the basolateral Na^+-K^+-$2Cl^-$ cotransporter responsible for uptake of K^+ to be secreted (Russell 2000, Kunzelmann & Mall 2001), inhibited the 2. phase of the dopamine response, although this inhibition failed to reach statistical significance in the proximal colon (Fig. 3.11). This failure might be interpreted by the hypothesis that in this colonic segment, the basolateral Na^+-K^+-ATPase, which is the second basolateral transporter capable of intracellular accumulation of K^+ (Foster *et al.* 1984), might be more important for transepithelial K^+ secretion than the Na^+-K^+-2 Cl^- cotransporter. The second evidence supporting the assumption of a K^+ secretion during the second phase of the dopamine response are the data obtained with quinine. Quinine is an effective blocker of K^+ channels in the apical membrane of rat colonic epithelium (Hörger *et al.* 1998). In the presence of quinine, the long-lasting decrease in Isc evoked by dopamine was completely suppressed. The current response was even reversed into a long-lasting increase in Isc (Fig. 3.20). This can easily be explained by the assumption that the initial Cl^-

secretion induced by dopamine (see above), which leads to an increase in Isc, is covered under control conditions by a parallel K^+ secretion, which leads to a decrease in Isc. If this latter response is inhibited by blockade of apical K^+ channels, the electrical effect of the first response, i.e. the Cl^- secretion, is unmasked. Other potassium channels blockers such as tetrapentylammonium and tetraethylammonium had no effect.

Radioisotope experiments were performed in order to measure the action of dopamine on K^+ transport more directly. In these experiments, K^+ was substituted by $^{86}Rb^+$. The reason for this is that the transport of Rb^+ is very similar to that of K^+ (Pandiyan *et al.* 1992). It has, however, a much higher half-time (19 d) compared to $^{42}K^+$, which has only a half-time of 12.4 h (Ellis 2000), what makes it quite difficult to work with this isotope.

Under control conditions, efflux of Rb^+ into the serosal compartment was about two times larger than the efflux into the mucosal compartment (Fig. 3.22). This is caused by the higher K^+ permeability of the basolateral membrane compared to the apical membrane (Diener *et al.* 1996). Administration of dopamine caused a redistribution of cellular K^+ conductance in favour of the apical K^+ conductance (Fig. 3.22). Although like in the case of the efflux experiments with $^{36}Cl^-$ not all of these results reached statistical significance, such a redistribution effect would favour transepithelial K^+ secretion by stimulating mucosal and reducing serosal efflux of this cation. Another K^+ secretagogue, forskolin, an activator of the adenylate cyclase (Seamon & Daly 1981), had a similar action, i.e. a reduction of the basolateral versus apical $^{86}Rb^+$ efflux ratio (Diener *et al.* 1996). As the amplitude of the action of forskolin on Isc is about 10 times larger than that of dopamine (see e.g. Bridges *et al.* 1983), it is not surprising that the

effects of this secretagogue on $^{86}Rb^+$ efflux are more pronounced than that of dopamine.

The run-down of the basolateral K^+ conductance induced by dopamine, which would then indirectly favour apical extrusion of this cation, could be directly observed in tissues, in which the apical membrane was permeabilized. To this purpose, nystatin, an ionophore (Schultheis & Diener 1997), was administered to the apical compartment of the Ussing chamber. Under these conditions, dopamine - after a transient and short stimulation - inhibited K^+ currents across the basolateral membrane (Fig. 3.16). It is not clear, why this response could only be observed in the proximal colonic segments. Theoretically, the measured current could be caused by a K^+ flux across K^+ channels, driven by the applied K^+ concentration gradient, or by the Na^+-K^+-pump, stimulated by the increase in the cytoplasmic Na^+ concentration after permeabilization of the apical membrane (Diener & Schultheiss 1996). However, also in the absence of Na^+, i.e. after inhibition of the pump current, dopamine evoked the same action on the current across the basolateral membrane demonstrating that the Na^+-K^+-ATPase cannot be involved in this response. (Fig. 3.17).

Another evidence that this catecholamine induces secretion of K^+ is given by the unidirectional Rb^+ flux experiments. Those experiments revealed that dopamine stimulated significantly the secretory flux (Jsm) in the proximal colon and induced an (insignificant) reduction in the absorptive flux (Jms) of Rb^+ in the distal colon (Table 3.6a, 3.6b). Both results are compatible with the conclusion that dopamine stimulates a modest potassium secretion. Because of the long time needed for this protocol, a new, until now unknown dopaminergic effect on Isc was observed. After the decrease in Isc in the second phase of the dopamine action, a compensatory increase of Isc was

measured. During this phase, the current even increased to values above the former control period (Fig. 3.21). The reason for this compensatory increase in Isc is not known and needs further investigation.

The ability to induce K^+ secretion is a property that dopamine shares with the other two physiologically relevant catecholamines, i.e. epinephrine (Hörger *et al.* 1998) and norepinephrine (Schultheiss & Diener 2000). Quinine, the blocker of apical K^+ channels, inhibited not only the K^+ secretion induced by dopamine but also that stimulated by epinephrine (Hörger *et al.* 1998), suggesting a common final secretory pathway in the apical membrane, by which catecholamines induce colonic K^+ secretion.

A direct proof for the activation of (apical) K^+ channels by dopamine has recently been obtained by our group in patch-clamp experiments at isolated colonic crypts (Al-Jahmany *et al.* 2004). In these exeriments dopamine induced a hyperpolarization of the membrane due to a stimulation of cellular K^+ conductance. The localization of the stimulated channel in the apical or the basolateral membrane cannot be determined in whole-cell patch-clamp experiments, because in this type of measurement currents across both membranes are measured. However, as in intact mucosa dopamine decrease Isc and induces a K^+ secretion (see above), it can be concluded that the channels stimulated by dopamine must be located in the apical membrane. Since the adrenergic agonist epinephrine was ineffective in patch-clamp experiments at colonic crypts (Dr. G. Schultheiss, personal communication), this epithelial effect of dopamine is very likely mediated by dopaminergic receptors and not by adrenergic receptors, which can be stimulated by dopamine, too (see below).

The predominant second messenger regulating the opening of potassium channels in rat colonic epithelium is Ca^{2+} (see e.g. Böhme *et al.* 1991, Heinke *et al.* 1999). An increase of the intracellular concentration of Ca^{2+} leads to an increase in the cellular K^+ permeability via stimulation of Ca^{2+}-dependent K^+ channels. Therefore, I assumed that the stimulation of K^+ secretion by dopamine might be associated with a change in the intracellular Ca^{2+} concentration. However, dopamine did not affect the intracellular Ca^{2+} concentration as judged in experiments on crypts loaded with the Ca^{2+}-sensitive fluorescent dye, fura-2 (Fig. 3.23). Consequently, an other mechanism, yet to be identified, must be responsible for the K^+ channel stimulation by dopamine, observed in the present study.

4.3. Subepithelial action sites

Ion transport across the intestinal wall can be modulated by direct actions of hormones, neurotransmitters or paracrine substances at the epithelial cells themselves or by indirect actions of these messenger substances acting on subepithelial cells involved in the regulation of ion transport across the epithelium. Typical examples for indirect actions of secretagogues on colonic ion transport are e.g. mediators of inflammation such as bradykinin or prostaglandin I_2 (prostacyclin), which indirectly evoke a Cl^- secretion by stimulation of secretomotor neurons within the submucosal plexus (Diener *et al.* 1988a, 1988b).

Subepithelial action sites participate indeed in the mediation of the dopamine effect in rat colon, too. For example, inhibition of the release of neurotransmitters by tetrodotoxin, a blocker of neuronal voltage-dependent Na^+ channels (Catterall 1980), inhibited the change in Isc induced by dopamine (Table 3.2a, 3.2b). This suggests that

in part exogenously administered dopamine acts via stimulation of submucosal neurons present in the submucosa-mucosa preparations, which had been used in the present experiments. Electrophysiological studies with microelectrodes demonstrate that dopamine can indeed induce a hyperpolarization of submucosal neurons (Hirst & Silinsky 1975). Consequently, submucosal neurons must be equipped with receptors for this catecholamine.

Eicosanoids, especially prostaglandins, are produced in the subepithelial tissue by cyclooxygenases (Craven & DeRubertis 1983, McCarn *et al.* 2003). They play a prominent role in the paracrine regulation of intestinal ion transport (Diener *et al.* 1988a, 1988b). Inhibition of the production of prostaglandins (and thromboxanes) by indomethacin, an inhibitor of cyclooxygenase(s) (for references, see Vane *et al.* 1998), inhibits the action of dopamine (Table 3.2a, 3.2b). This suggests that a part of the action of this catecholamine is an indirect one mediated by and/or dependent on the release of neurotransmitters or prostaglandins. The location of the indomethacin-sensitive step might be in submucosal plexus, too, as these neurons themselves are tonically stimulated by prostaglandins (Diener *et al.* 1988b).

The indirect, i.e. subepithelial actions of the three physiologically relevant catecholamines differ from each other in rat colon. The negative Isc (i.e. the K^+ secretion) induced by norepinephrine is inhibited by tetrodotoxin and indomethacin (Schultheiss & Diener 2000), whereas that stimulated by epinephrine is only inhibited by tetrodotoxin, but resistant against indomethacin (Hörger *et al.* 1998). This observations, again, show that there are similarities, but also profound differences with the K^+ secretion induced by different types of catecholamines in rat colon.

4.4. Involvement of adrenergic receptors

Dopamine is known to stimulate adrenergic receptors (Burks 1994). Also for the actions of dopamine on intestinal ion transport, an involvement of adrenoceptors is well documented. For example, in rabbit ileum stimulation of Na^+ and Cl^- absorption (Donowitz *et al.* 1982) as well as the stimulation of water absorption by dopamine in rat ileum and colon (Donowitz *et al.* 1983) were strongly inhibited by the α_2-receptor blocker, yohimbine (for references for the adrenoceptors used, see Bylund *et al.* 1994). A sensitivity against yohimbine and the non-selective adrenoceptor antagonist, phentolamine, was also observed for the action of dopamine on rat jejunal electrolyte transport (Vieira-Coelho & Soares-da-Silva 1998). In accordance with these observations at other intestinal segments, the negative Isc induced by dopamine was reduced by phentolamine and yohimbine. However, the effect of these blockers was more pronounced in the proximal colon than in the distal colon (Table 3.3a, 3.3b). The non-selective ß-adrenoceptor blocker, propranolol, did not affect the first phase of the dopamine response in both colonic segments. In contrast, the second phase was nearly suppressed in the distal colon, but not in the proximal colon. This inhibition, however, was not mimicked by the $ß_1$-selective antagonist, atenolol, nor by the $ß_2$-selective antagonist, ICI-118851, leaving doubt at the specificity of this inhibition (Table 3.3a, 3.3b). Consequently, it seems reasonable to assume that the stimulation of α-adrenoceptors is involved in the action of dopamine on ion transport across rat colon.

4.5. Involvement of dopaminergic receptors

In addition to adrenergic receptors, specific dopaminegic receptors seem to be involved in the action of dopamine in the rat colon. When SCH 23390 (a D_1-receptor antagonist) and L-741,626 (a D_2-receptor antagonist) or L-745,870 (a D_4-receptor antagonist) were used to antagonise D_1- and D_2-like receptors respectively (for references to these inhibitors see Kulagowski *et al.* 1996, Missale *et al.* 1998, Pillai *et al.* 1998), only D_2-like receptor antagonists were able to inhibit the action of dopamine. Antagonists at D_1-like receptor were ineffective in both colonic segments (Table 3.4a,, 3.4b).

In contrast, a concentration-dependent fall in Isc could be induced by both the D_1-like agonist SK&F 38393 as well as the D_2-like agonist quinpirol (Fig. 3.8, Table 3.5). At first glance, this seems to contrast the results obtained in the inhibitor experiments. However, only the D_2-agonist quinpirol mimicked the action of native dopamine completely. Only this agonist could evoke both a transient increase in Isc followed by a sustained decrease (Fig. 3.8), which was not the case with the D_1-like agonist.

This pharmacological profile distinguishes the action of dopamine in rat colon from that observed in the jejunum of young (20 day old) rats. In this tissue, administration of dopamine in the presence of α-adrenoceptor blocker (phentolamine) led to an inhibition of electrolyte absorption (Vieira-Coelho & Soares-da-Silva 2000, 2001). The mechanism consists in an inhibition of Na^+-K^+-ATPase of the enterocytes. This action has been shown to be mediated by stimulation of D_1-like receptors. Failure of dopamine to inhibit of Na^+-K^+-ATPase in adult rat was most probably due to absence of regulation of the enzyme by cholera toxin-sensitive G protein (Vieira-Coelho & Soares-da-Silva 2000, 2001). The difference in the dopamine receptors found in this

study and my experiments may be caused by differences in individual gut segments, i.e. colon versus small intestine.

Surprisingly, D_2-like receptor antagonists nearly suppressed the action of dopamine in rat colon (Table 3.4a, 3.4b), and α-adrenoceptor blockers such as phentolamine and yohimbine reduced it by about 50 % (Table 3.3a, 3.3b). In other words, the sum of the inhibition evoked by both types of receptor blockers exceeded 100 %. In other tissues using yohimbine and haloperidol as adrenergic and dopaminergic receptors blockers, respectively, a similar 'over-additive' action of adrenergic and dopaminergic receptor blockers has been shown, too (Donowitz *et al.* 1982, 1983). One might suppose that non-selective actions of the inhibitors on these types of G-protein coupled receptors are the reason for this phenomenon. Although the relative contribution of adrenergic and dopaminergic receptors to the dopamine response is not known, it seems reasonable to conclude that the actions of dopamine on colonic K^+ secretion are - like that in other intestinal segments - mediated by both types of receptors.

4.6. Biological significance

The actions of dopamine on colonic ion transport are observed at quite high concentrations. Although the proximal colon showed in general a higher sensitivity towards this catecholamine compared to the distal colon (Fig. 3.1), the concentrations used here are much higher than the plasma concentration of dopamine. This has been found to be in the range of $4 \cdot 10^{-9}$ mol·l^{-1} in the rat (Garris *et al.* 1994). This low sensitivity is not an artefact due to a rapid degradation of dopamine in the experimental setup used. Inhibition of both monoamine oxidase (MAO) and catechol-O-methyl

transferase (COMT), the two main enzymes responsible for dopamine degradation, did not enhance the sensitivity against dopamine (n = 8; data not shown).

Consequently, it seems unlikely that dopamine circulating as a hormone might affect colonic salt transport. However, dopamine can also act as paracrine modulator of ion transport. This has been shown in several epithelial systems. Among these is the ascending limb of Henle's loop of the kidney, in which dopamine inhibits NaCl absorption via stimulation of D_1-like receptors (Grider *et al.* 2003). In the small intestine dopamine apparently is involved in the physiological adaptation of Na^+ transport at a high Na^+-diet in young rats. Under these feeding conditions, the dopamine content in the mucosa is upregulated; an effect, which leads to the inhibition of the Na^+-K^+-ATPase activity in the jejunal enterocytes (Vieira-Coelho *et al.* 1998). Dopamine is also produced within the rat colonic mucosa (Magro *et al.* 2004); thus paracrine actions of dopamine in this intestinal tissue might well be possible, too.

Furthermore, the tissue content of dopamine has been shown to vary under pathophysiological conditions. For example, the concentration of dopamine within the rat colonic wall is reduced during experimentally induced colitis (Magro *et al.* 2004) suggesting a possible involvement in the pathogenesis of inflammatory bowel diseases. The current experiments revealed that dopamine - in addition to its known effects on Na^+ and Cl^- transport - has the ability to alter colonic K^+ transport. The physiological conditions, under which this regulation is exerted, reveals further investigation.

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6. Summary

Dopamine ($5 \cdot 10^{-6}$ – 5.10^{-4} mol·l^{-1}) when added serosally induced a concentration-dependent decrease in short-circuit current (Isc) across rat distal and proximal colon. This response was preceded by a transient and inconsistent increase in Isc. A part of the catecholamine action is mediated by subepithelial actions sites as it was indicated by the inhibiton of dopamine effects by the cyclooxygenase inhibitor, indomethacin (10^{-6} mol·l^{-1}), and the neuronal blocker, tetrodotoxin (10^{-6} mol·l^{-1}). The positive Isc evoked by dopamine was due to chloride secretion as indicated when both Cl^- and HCO_3^- were substituted (using HEPES as buffer) and when of the basolateral membrane was depolarizing by high potassium concentration.

The negative Isc evoked by dopamine was due to potassium secretion. This was demonstrated by unidirectional flux experiments. $^{86}Rb^+$ efflux experiments revealed a redistribution of cellular K^+ efflux in favour of the apical K^+ conductance in the presence of dopamine. The negative Isc evoked by dopamine was inhibited by the blocker of apical K^+ channels, quinine (10^{-3} mol·l^{-1}), indicating that a stimulation of K^+ secretion underlies the measured current. Both the α-adrenoceptor blocker phentolamine (10^{-4} mol·l^{-1}) and as well as inhibitors of D_2-like receptors such as L-741,626 (10^{-5} mol·l^{-1}) and L-745,870 (10^{-5} mol·l^{-1}) inhibited the dopamine response. All these observations indicate similarities between dopamine and the other catecholamines derivatives in their effect on ions transport in rat colon.

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