

Electrophysiological Studies of Forskolin-Induced Changes in Ion Transport in the Human Colon Carcinoma Cell Line HT-29 cl.19A: Lack of Evidence for a cAMP-Activated Basolateral K^+ Conductance

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Summary. Forskolin (i.e., cAMP)-modulation of ion transport pathways in filter-grown monolayers of the Cl^- -secreting subclone (19A) of the human colon carcinoma cell line HT29 was studied by combined Ussing chamber and microimpalement experiments.

Changes in electrophysiological parameters provoked by serosal addition of 10^{-5} M forskolin included: (i) a sustained increase in the transepithelial potential difference (3.9 ± 0.4 mV), (ii) a transient decrease in transepithelial resistance with $26 \pm 3 \Omega \cdot cm^2$ from a mean value of $138 \pm 13 \Omega \cdot cm^2$ before forskolin addition, (iii) a depolarization of the cell membrane potential by 24 ± 1 mV from a resting value of -50 ± 1 mV and (iv) a decrease in the fractional resistance of the apical membrane from 0.80 ± 0.02 to 0.22 ± 0.01 . Both, the changes in cell potential and the fractional resistance, persisted for at least 10 min and were dependent on the presence of Cl^- in the medium. Subsequent addition of bumetanide (10^{-4} M), an inhibitor of Na/K/2Cl cotransport, reduced the transepithelial potential, induced a repolarization of the cell potential and provoked a small increase of the transepithelial resistance and fractional apical resistance. Serosal Ba^{2+} (1 mM), a known inhibitor of basolateral K^+ conductance, strongly reduced the electrical effects of forskolin. No evidence was found for a forskolin (cAMP)-induced modulation of basolateral K^+ conductance.

The results suggest that forskolin-induced Cl^- secretion in the HT-29 cl.19A colonic cell line results mainly from a cAMP-provoked increase in the Cl^- conductance of the apical membrane but does not affect K^+ or Cl^- conductance pathways at the basolateral pole of the cell. The sustained potential changes indicate that the capacity of the basolateral transport mechanism for Cl^- and the basal Ba^{2+} -sensitive K^+ conductance are sufficiently large to maintain the Cl^- efflux across the apical membrane. Furthermore, evidence is presented for an anomalous inhibitory action of the putative Cl^- channel blockers NPPB and DPC on basolateral conductance rather than apical Cl^- conductance.

Key Words Cl^- transport · K^+ conductance · forskolin · cAMP · HT-29 cl.19A · electrophysiology

Introduction

Following the introduction of a general transport model accounting for Cl^- secretion by epithelial tis-

sues (Frizzell, Field & Schultz, 1979), a number of studies have provided detailed information about the nature of the transport systems and the signal transduction mechanisms involved.

In airway epithelial cells in particular, the model has been evaluated in depth using electrophysiological methods (Welsh, 1987) and here it is well established that second-messenger-operated Cl^- channels in the apical membrane play a key role in transepithelial Cl^- secretion. These channels provide the gateway for passive efflux of Cl^- from the cell to the lumen. The necessary cellular electrochemical accumulation of Cl^- is maintained by a bumetanide-sensitive Na/K/2Cl cotransport system in the basolateral membrane. The efflux of Cl^- is electrically compensated by an efflux of K^+ through K^+ channels in the basolateral membrane. The channels appear to be regulated by extracellular signals through changes in the level of intracellular signaling molecules, e.g., cAMP and Ca^{2+} .

In intestinal epithelial cells the presence of a similar mechanism is suggested from *in vitro* flux measurements and transepithelial potential and short circuit current studies with isolated intestinal epithelial sheets (Heintze, Stewart & Frizzell, 1983) and with monolayers of the colonic epithelial cell lines Caco-2, T₈₄ and HT-29.

With T₈₄ cells it has been shown that cAMP-linked secretagogues (e.g., VIP) increased the short circuit current, the efflux of Cl^- through conductance pathways and the K^+ efflux through the basolateral membrane, but also the bidirectional transepithelial Cl^- fluxes. (Dharmasathaphorn et al., 1985; McRoberts, Beuerlein & Dharmasathaphorn, 1985).

Microelectrode studies with the Caco-2 cell line likewise suggest that these cells possess the cAMP-dependent apical Cl^- conductance (Grasset, Bernabeu & Pinto, 1985). However, transepithelial poten-

tial changes across these cells are transient and net Cl^- flux is very small, while changes across the apical membrane seem to be steady. This suggests that the Cl^- efflux is limited by Cl^- influx across the basolateral membrane or by an insufficient conductance for compensating ion currents in this cell line. In contrast, sustained transepithelial and intracellular changes, induced by db-cAMP have been reported in a preliminary communication for subclone 19A of the HT-29 colon carcinoma cell line (Augeron et al., 1986).

In contrast to the original HT-29 cells, the 19A subclone is morphologically and functionally uniform (Augeron & Laboisse, 1984) and allowed us to study ion transport modulation in the absence of complicating factors such as secretagogue-provoked nerve reflexes and paracrine cascade mechanisms. Confluent monolayers of the subclone were grown on permeable and transparent polycarbonate filters, and the effects of the cAMP agonist forskolin on the transepithelial and intracellular potential and resistances were evaluated using an equivalent electrical circuit model (Groot & Bakker, 1988) for leaky epithelia. Our findings indicate that the apical Cl^- conductance in these cells is persistently activated in response to forskolin or a cAMP analog and suggest that maintenance of the transcellular current does not require an increase in the basolateral conductance. The proposed secretion model deviates in several aspects from earlier models based on the above-mentioned studies in Caco-2, T₈₄, rabbit colon and rat small intestinal cell types and does not corroborate the previous suggestion that during cAMP-induced Cl^- secretion the basolateral conductance for K^+ is increased (Dharmasathaphorn et al., 1985; McRoberts et al., 1985).

Materials and Methods

CELL CULTURE METHODS

The Ht-29 cl.19A cell line (passage number 8–30) was grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum in 25-cm² culture flasks in a humidified atmosphere of 5% CO_2 /95% air. Confluent monolayers were subcultured after trypsinization and plated on rat tail collagen-coated nucleopore PC filters, 13-mm diameter. (Nucleopore, Pleasanton, CA) glued to Lexan rings (Dharmasathaphorn et al., 1985). The permeable supports were placed on top of a layer of glass beads in 30-mm culture dishes to permit bottom feeding. The cells were plated at about 5×10^5 cells/cm² and used between day 7 and 31 with the medium being replaced every 2 days. About half of the experiments were done with cells grown without antibiotics. No differences were observed with cells grown in the presence of antibiotics (penicillin: 40 mg/liter, ampicillin: 8 mg/liter, streptomycin: 90 mg/liter, Sigma Chemical, St. Louis, MO).

ELECTROPHYSIOLOGICAL MEASUREMENTS

The filters were mounted horizontally in a small Ussing-type chamber, leaving an oblong area of 0.30 cm². The upper and lower compartments were continuously perfused using roller pumps (Buchler Instruments, Fort Lee, NJ). The solutions were maintained at 36°C and gassed with 5% CO_2 /95% O_2 . The compartments were connected through Ringer/agar bridges to Ag-AgCl electrodes to monitor the transepithelial electrical potential. Open-circuit conditions were used throughout. Usually the upper electrode served as the common ground for transepithelial and intracellular measurements. The offset between the upper and lower electrodes was measured before and after mounting of each filter. Transepithelial electrical resistance was calculated from voltage deflections induced by a current pulse of +10 μA for a duration of 1 sec and a negative pulse of equal strength for the subsequent 1 sec. Pulses of +50 and -50 μA were used for calculation of apical-to-basolateral resistance ratio. Corrections for the resistance of the fluid between the tips of the bridges were made. The current electrodes (Ag-AgCl) were situated in the walls of the upper and lower compartments.

Apical Membrane Potentials

Apical membrane potentials were measured with glass microelectrodes made on a Flaming Brown P-87 micropipette puller (Sutter Instrument, San Rafael, CA) from Kwik-Fil borosilicate glass capillaries of 1-mm o.d. (Clarck, Electromedical Supplies, Reading, UK). The tip resistance of the electrodes, filled with 0.5 M KCl and inserted in the bath solution was 100–200 M Ω ; the tip potential was about -2 mV. Impalements were performed at an angle of about 60° under microscopic control (CK2, inverted-phase contrast microscope, Olympus Optical, Japan) using a Narishige MO-22 hydraulic micromanipulator mounted on a M-152 manipulator (Narishige Scientific Instruments, Japan). The potentials were measured with M-4A electrometer probes (W-P Instruments, New Haven, CT) and recorded on a multipen recorder (Rikadenki Kogyo, Japan). The criteria for successful impalements were: abrupt negative deflection of the potential, establishment of a stable potential usually more negative than the initial potential difference for at least 2 min, constancy in the potential deflections induced by bipolar current injections and return of the microelectrode potential to its initial value (± 2 mV) when the tip was withdrawn from the cell. The effect of added chemicals was studied while the microelectrode was in the cell. This required stable intracellular potential measurements for prolonged periods. Usually it was possible to record for more than 30 min from one cell. However, some recordings were lost or ended on purpose before an experimental situation was created. These recordings of cell potential and fractional resistance were pooled to obtain the basal values for the electrical parameters.

Solutions

The standard solution had the following composition (in mM): NaCl 117.5, KCl 5.7, NaHCO_3 25.0, NaH_2PO_4 1.2, CaCl_2 2.5, MgSO_4 1.2, glucose 27.8 (pH 7.4). Low Cl-Ringer solutions were made by replacing all Cl except for 0.1 mM by gluconate. High KCl or K-gluconate solutions were made by replacing the sodium salt. The effects of ethanol and DMSO as carrier solutions for forskolin and NPPB (5-nitro-2-(3-phenylpropylamino)-benzoate or DPC (diphenylamine-2-carboxylate) was checked in separate

experiments. They were without effect in the concentrations used (0.1% as a maximum).

Forskolin and 8-Br-cAMP were from Sigma Chemical, St. Louis, MO, and bumetanide was a gift from Leo Laboratories, Ballerup, Denmark. NPPB and DPC were a gift from Dr. R. Greger, Freiburg, Germany.

EQUIVALENT ELECTRICAL CIRCUIT ANALYSES

A conventional model (Groot & Bakker, 1988) was used to analyze the time-dependent changes in electrical parameters with the following equations:¹

$$R_t = R_p(R_a + R_b)/(R_a + R_b + R_p) = 1/G_t \quad (1)$$

$$fR_a = R_a/(R_a + R_b) \quad (2)$$

$$G_t = G_p + G_b(1 - fR_a) \quad (3)$$

$$I_{sc} = I_{pc} = \Psi_t \cdot G_p \quad (4)$$

$$E_a = \Psi_a + I_{sc}R_a \quad (5)$$

(If the bath resistance and filter resistance are fully compensated, the short-circuit current would be equal to I_{sc} .)

After forskolin addition, Ψ_a and fR_a changed to a new steady-state value which was maintained as long as forskolin was present. This suggests that R_b may remain constant. By performing frequent measurement of G_t and fR_a we found that Eq. (3) could be solved for G_p and G_b in the first 20 sec after forskolin. Equations (4) and (5) were used to obtain the time-dependent change in I_{sc} and E_a .

Statistics.

All values are presented as means \pm SEM. Statistical significance was evaluated using paired or unpaired *t* tests as appropriate.

Results

BASAL VALUES OF ELECTRICAL PARAMETERS

The monolayers were studied between day 7 and 31 after plating on the collagen-coated filters. The results of combined measurements of transepithelial potential, resistance and intracellular potential are

¹ Abbreviations: Ψ_t : transepithelial potential, basolateral side with respect to apical bath which is grounded, in mV. Ψ_a : transmembrane potential, cell with respect to apical bath, in mV. I_{sc} , I_{pc} : transcellular and paracellular currents, respectively, in $\mu A/cm^2$. R_t , R_a , R_b , R_p : slope resistances of monolayer, apical membrane, basolateral membrane and paracellular pathway respectively, in Ωcm^2 . G_t , G_a , G_b , G_p : electrical slope conductances in mS/cm^2 . E_a , E_b : electromotive forces across the apical and the basolateral membrane, respectively, in mV. fR_a , fR_b : fractional resistance of apical and basolateral membrane, respectively, ($fR_a = R_a/(R_a + R_b)$, $fR_b = 1 - fR_a$).

Table 1. Electrical parameters of filter-grown monolayers of HT-29 cl.19A colon carcinoma cells

	Ψ_t (mV)	Ψ_a (mV)	R_t (Ωcm^2)	fR_a
Control Ringer	1.2 ± 0.2	-46 ± 1	119 ± 8	0.80 ± 0.01

The data shown represent the mean \pm SEM of 57 monolayers.

shown in Table 1. The transepithelial resistance, measured 30 min after mounting the filters in the chamber, varied between 20 and 262 Ωcm^2 (57 monolayers). The spontaneous transepithelial potential varied between -3.2 and $+11$ mV, and the intracellular potential varied between -20 and -71 mV. No systematic differences in intracellular or extracellular electrical parameters were observed by varying the culture periods between 7 and 30 days. Neither did we find a relation between passage number (8–30) and electrical parameters. Surprisingly, the spontaneous potential differences did not correlate with the transepithelial resistances.

Figure 1 shows a histogram of the intracellular potentials measured in 249 cells from these monolayers. The values had a normal distribution around a mean of 46.7 ± 0.6 mV (median 47 mV). The impalements could typically be maintained for 30 min or longer.

Six monolayers with transepithelial resistances lower than $50 \Omega \cdot cm^2$ were not used further.

EFFECTS OF FORSKOLIN

The effect of forskolin (10^{-5} M) on the electrical parameters was tested in 21 monolayers; the cAMP analogue, 8-Br-cAMP (10^{-4} M), was studied only in a few experiments. In all experiments the secretagogue was added to the basolateral perfusate while intracellular recordings were made continuously. In most experiments the effect of the washing out could also be followed. In contrast to the fast changes in electrical parameters upon addition, the withdrawal had a rather slow effect. The addition of 8-Br-cAMP or forskolin had fully comparable effects, and the results were pooled.

Figure 2 shows the time course of the change in Ψ_t and Ψ_a from a typical experiment. The intracellular potential, Ψ_a , depolarized and showed no signs of a repolarization in the continued presence of forskolin. The amplitude of the vertical voltage deflection of the intracellular recording, induced by transepithelial current injection, decreased strongly after addition of forskolin. In combination with the relatively small decrease of

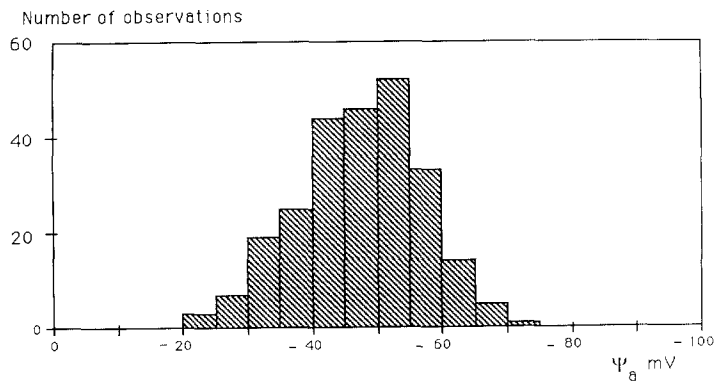


Fig. 1. Histogram of 249 individual apical membrane potentials

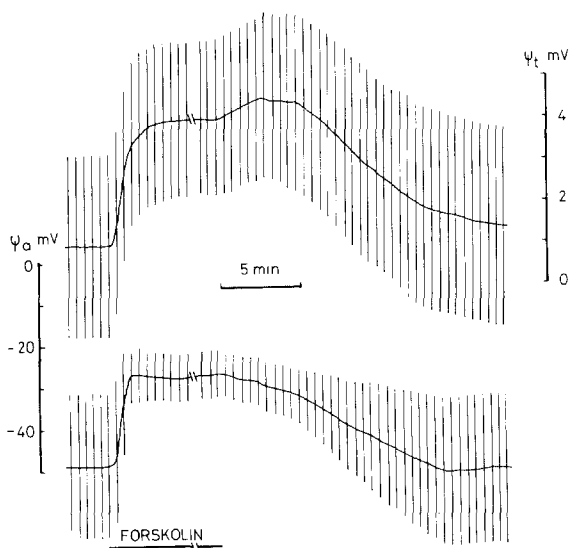


Fig. 2. Redrawn from a recording of transepithelial potential (Ψ_t , upper tracing) and cellular potential (Ψ_a , lower tracing) and voltage deflections induced by transepithelial current injections (10 and 50 μ A, respectively). The bar indicates the presence of forskolin (10^{-5} M) in the lower compartment bathing the basolateral side. The interruption is for 10 min

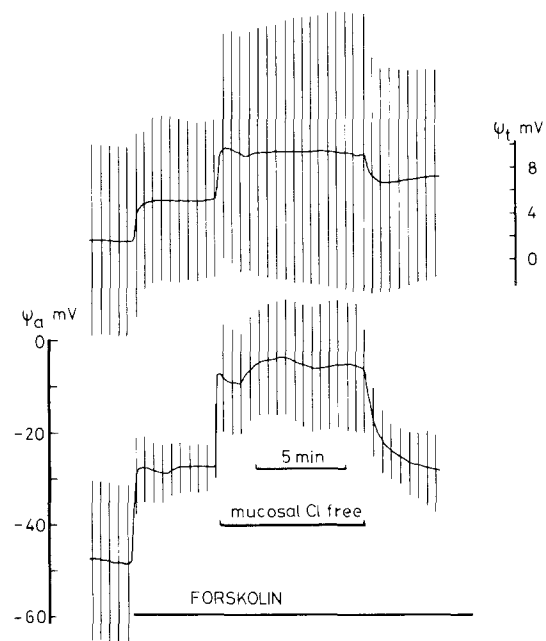


Fig. 3. Changes in transepithelial potential (upper tracing) and intracellular potential (lower tracing) induced by low Cl^- Ringer's at the apical (mucosal) side in the presence of forskolin

the transepithelial voltage deflections in the first 4 min, this indicates that fR_a strongly decreased upon forskolin addition. Similar recordings were obtained in 57 other experiments.

The mean values of the electrical parameters derived just before addition of forskolin and 3 min afterwards are given in Table 2. The cell potential depolarized by 26 mV, and the transepithelial potential became 4.0 mV more positive. The mean value of R_t just before addition of forskolin was slightly higher in this set of observations than the mean resistance of the 58 monolayers reported in Table 1. This is partially because we selected the monolayers with transepithelial resistances higher than $50 \Omega\text{cm}^2$. The acute effect of forskolin was always a decrease of R_t (see Table 1), but in some monolayers, R_t started to

increase after a few minutes, and after washout of forskolin, R_t was higher than before forskolin. This phenomenon also affected the mean value of R_t .

The time course of the potential changes and the changes in the voltage deflections during the first 60 sec after the initiation of the response to forskolin was studied in more detail in 11 of these experiments performed on 5 monolayers. The experiments will be discussed in the section on electrical circuit analyses.

INVOLVEMENT OF Cl^- IN THE RESPONSE TO FORSKOLIN

The prevalent idea is that the depolarization of the cell potential is driven by an electrogenic efflux of

Table 2. Electrical parameters before and 3 min after addition of forskolin

	Control				Forskolin							
	Ψ_t mV	Ψ_a mV	R_t Ωcm^2	fR_a	Ψ_t mV	Ψ_a mV	R_a Ωcm^2	fR_a	$\Delta\Psi_t$ mV	$\Delta\Psi_a$ mV	ΔR_t Ωcm^2	ΔfR_a
Mean	1.3	-50.1	138	0.80	5.2	-23.9	111	0.22	3.9	26.1	-25.6	0.58
SEM	0.2	1.0	13	0.02	0.4	0.7	12	0.01	0.4	0.9	2.9	0.01
<i>P</i>					<0.001	<0.001	<0.1	<0.001	<0.001	<0.001	<0.001	<0.001

The data shown represent the mean \pm SEM of 58 experiments in 21 monolayers. *P* values are with respect to control values or to zero in case of Δ .

Table 3. Effect of forskolin in control and low Cl^- Ringer

	- Forskolin				+ Forskolin			
	Ψ_t mV	Ψ_a mV	R_t Ωcm^2	fR_a	$\Delta\Psi_t$ mV	$\Delta\Psi_a$ mV	ΔR_t Ωcm^2	ΔfR_a
Control	1.1 \pm 0.2	-53 \pm 1	123 \pm 17	0.85 \pm 0.01	2.2 \pm 0.1	25 \pm 2	22 \pm 2	0.58 \pm 0.04
Low Cl^- *	0.5 \pm 0.2	-57 \pm 2	172 \pm 23	0.93 \pm 0.07	-0.1 \pm 0.05	1.0 \pm 0.5	0 \pm 0	0.01 \pm 0.01

The data shown represent the mean \pm SEM of five experiments.

*Changes induced by low Cl^- occurred in all five experiments in the same direction.

Table 4. Changes in potential induced by low Cl^- Ringer's in the apical compartment in the absence and presence of forskolin (10^{-5} M), $n = 7$

	$\Delta\Psi_t$ (mV)	$\Delta\Psi_a$ (mV)
- Forskolin	7.3 \pm 0.5	5.9 \pm 0.4
+ Forskolin	17.1 \pm 2.4	34.5 \pm 1.5
<i>P</i>	<0.001	<0.001

Data shown are means \pm SEM for seven experiments.

Cl^- through cAMP-activated Cl^- channels in the apical membrane. To study the participation of Cl^- in the forskolin response we perfused the monolayers with a saline in which all Cl^- except for 0.1 mM was replaced by gluconate. During the incubation in the low Cl^- solution the serosal perfusate was changed for a short period to a high K^+ gluconate saline ($\text{K} = 57$ mM) to probe the cell potential change induced by K^+ . Approximately 40 min after Cl^- replacement forskolin was added.

Table 3 shows that under these conditions forskolin had no effect on the transepithelial or intracellular potential. Also, the fall in the fractional resistance of the apical membrane was totally absent in the low Cl^- Ringer. These results strongly suggest that the forskolin response requires the presence of Cl^- and suggest that the electrophysiological changes are related to Cl^- -specific conductance increase in the apical membrane.

Table 5. Effect of NPPB (10^{-5} M) in the presence of forskolin

	Ψ_t mV	Ψ_a mV	R_t Ωcm^2	fR_a
Control	0.5 \pm 0.1	-48 \pm 7	135 \pm 12	0.80 \pm 0.02
+ Forskolin	3.5 \pm 0.3	-25 \pm 4	119 \pm 12	0.26 \pm 0.07
+ Forskolin + NPPB	1.0 \pm 0.5	-14 \pm 5	147 \pm 19	0.10 \pm 0.01

The data shown represent the mean \pm SEM of three experiments. Values after forskolin were taken 5 min after addition. Values after NPPB were taken 2 min after addition.

If the depolarization is caused by the opening of Cl^- channels in the apical membrane, one would expect that a change in the Cl^- gradient should have a larger effect in stimulated as compared to resting conditions. This was tested by rapid changes of the apical perfusate from the normal Ringer's to the low Cl^- Ringer's, in the absence or in the presence of forskolin in the basolateral perfusate.

Table 4 shows that the effects of apical Cl^- substitution on potentials are significantly larger in the presence of forskolin.

Figure 3 shows the recording of a typical response of the potentials on apical Cl^- replacement by gluconate in the presence of forskolin. Correction of this recording for liquid junction potentials across the salt bridge in the apical solution (-9.5 mV) indicates that the intracellular potential actually becomes positive after Cl^- replacement. A positive

Table 6. Effect of bumetanide in the presence of forskolin

	Ψ_i (mV)	Ψ_a (mV)	R_i (Ωcm^2)	fR_a
Control	1.2 ± 0.3	-44.8 ± 1.5	215 ± 10	0.66 ± 0.06
+ forskolin	4.7 ± 0.7	-22.2 ± 1.8	184 ± 6	0.16 ± 0.05
Δ	3.5 ± 0.5	22.6 ± 1.5	-30 ± 4	-0.50 ± 0.10
+ forskolin + bumetanide	1.8 ± 0.6	-38.6 ± 4.0	194 ± 4	0.26 ± 0.06
Δ	-2.9 ± 0.4	-16.4 ± 3.7	9 ± 4	0.10 ± 0.04

The data shown represent the mean \pm SEM of five experiments. Values after forskolin were taken 5 min after addition. Values after bumetanide were taken 30 min after addition.

potential is to be expected with a large Cl^- conductance in the apical membrane and an outwardly directed chemical potential for Cl^- .

PUTATIVE Cl^- -CHANNEL BLOCKERS

The involvement of Cl^- channels is often documented by their susceptibility to putative Cl^- channel blockers like DPC and NPPB (Wangemann et al., 1986). The addition of these blockers to the apical solution in concentrations up to 10^{-6} M were without measurable electrophysiological effects in the clone. At 10^{-5} M and higher we observed a decrease of the transepithelial potential and a depolarization of the cell potential. Together with the decrease in fR_a and increase in R_i this indicates that the drugs had a pronounced effect on the basolateral membrane of the cells rather than on the apical Cl^- conductance (see Table 5). Moreover, low Cl^- Ringer in the apical compartment in the presence of NPPB and forskolin induced depolarizations not different from those reported in Table 4. These results contrast with published observations of Cl^- channel inhibition in excised patches of the apical membrane of HT-29 cells (Hayslett et al., 1987), as well as with similar observations on excised patches of the clone 19A in our own laboratory (*unpublished results*).

EFFECT OF BUMETANIDE

The depolarization of the apical membrane upon opening of a Cl^- channel requires that the intracellular electrochemical potential of Cl^- is higher than in the apical saline. In a number of epithelia the Cl^- accumulation is maintained by a Na^+ - and K^+ -dependent Cl^- transport system which is bumetanide sensitive. The presence of this transport system in HT-29 cl.19A cells can be inferred from preliminary reports showing that bumetanide inhibits the db-cAMP induced transepithelial potential change and Rb^+ transport and that bumetanide can be specifi-

cally bound by these cells (Augeron et al., 1986; Augeron, Popowicz & Simmons, 1987).

The effects of forskolin and subsequently bumetanide are shown in Table 6. Bumetanide (10^{-4} M) caused a slow repolarization of the cell potential to nearly the prestimulated value. Concomitantly, a small increase of the fractional resistance occurred.

POTASSIUM PERMEABILITY

The observations so far fit well to a model for secretory epithelia postulating a Cl^- -accumulating system in the basolateral membrane and Cl^- channels in the apical membrane. As shown in several secreting epithelia, however, secretagogues may additionally induce an increase of the basolateral K^+ conductance resulting in a repolarization of the cell potential and maintenance of the driving force for Cl^- excretion through the apical membrane. In the present experiments, however, no repolarization could be observed after the forskolin-induced depolarization. In addition, the fractional resistance stayed at the low level reached after forskolin. These observations suggest that in these cells the potassium conductance is not further increased by cAMP. However it may be that R_b changed as fast as R_a , or in other words, that the change in potassium conductance is hidden by simultaneous but more dramatic changes in the apical Cl^- conductance. In such a case the effect of opening of K^+ channels on Ψ_a would be easier to observe in the absence of Cl^- and would become manifest by a hyperpolarization and an increase of the apical fractional resistance. However, as shown in Fig. 4 and in the experiments already reported in Table 3, no hyperpolarization and no increase of fractional resistance was observed. In addition, the depolarization induced by the increase of the basolateral K^+ concentration was not larger in the presence of forskolin. These observations suggest that the sustained transepithelial current induced by forskolin can be maintained by the resting ionic conductance of the cells.

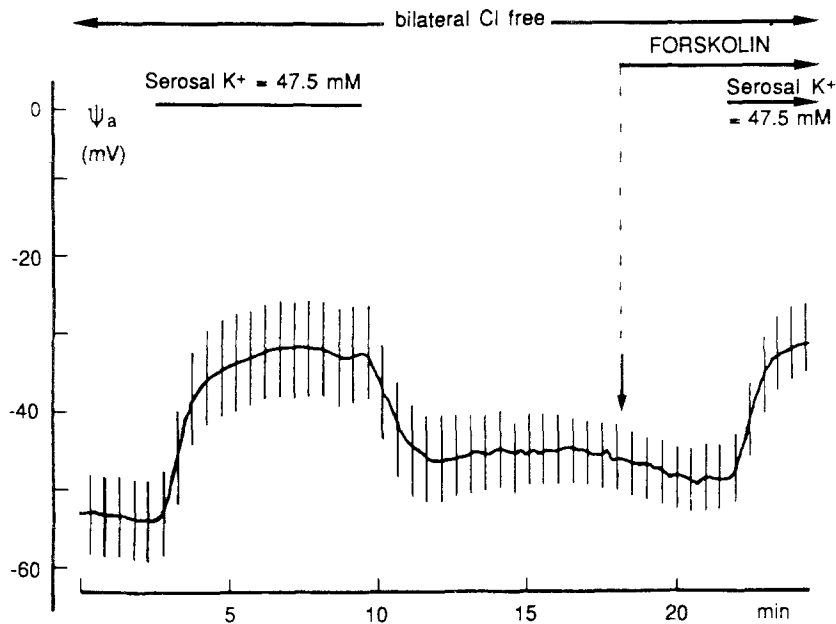


Fig. 4. Depolarizations induced by high K^+ solutions in the basolateral (serosal) compartment in the absence and presence of forskolin (10^{-5} M), and lack of effect of forskolin on the apical membrane potential in low Cl^- Ringer's. This experiment is representative of four other experiments

Prime candidate for this conductance is the Ba^{2+} -sensitive K^+ conductance in the basolateral membrane present in a number of Cl^- -secreting epithelia. In two experiments we studied the effect of forskolin after preincubation of the monolayer for 10 min with 1 mM Ba^{2+} at the basolateral side. Ba^{2+} alone induced a depolarization of the cell potential from -56 to -30 mV and a small decrease of the apical fractional resistance from 0.69 to 0.60. The change in G_t was statistically insignificant, and Ψ_t decreased by 0.5 mV. The subsequent addition of forskolin further decreased the apical fractional resistance to 0.27, whereas the cell potential showed a small further depolarization of 5 mV to -25 mV, and the transepithelial potential increased by only 0.5 mV.

In two other experiments the order of addition of Ba^{2+} and forskolin was reversed. In the presence of forskolin, Ba^{2+} decreased the transepithelial potential to 0.3 mV above its pre-forskolin level, whereas the intracellular potential further depolarized with 5 mV from -24 mV in the presence of forskolin to -19 mV in the combined presence of forskolin and Ba^{2+} . These results corroborate the possibility that a basolateral Ba^{2+} -sensitive K^+ conductance is necessary for the maintenance of the cAMP-induced transepithelial current.

The apparent insensitivity of the basolateral conductance to cAMP also appears from conventional equivalent electrical circuit analyses and opens the possibility to calculate the parameters of the circuit.

CIRCUIT ANALYSES

Current pulses with a duration of 200 msec were injected every second so that the change in G_t and fR_a could be analyzed with a greater time resolution than shown in Fig. 2. The graphs in Fig. 5 show that the changes in Ψ_a and fR_a are at a maximum at about 20 sec after the onset of the response to forskolin, whereas the maximum of Ψ_t and G_t was reached 30–40 sec later.

The transepithelial conductance can be considered as two conductances in parallel, i.e., the transcellular conductance and the paracellular conductance. The transcellular conductance can be considered as the apical membrane conductance in series with the basolateral membrane conductance. In the course of this study we found that forskolin apparently had no effect on the basolateral membrane conductance. If in addition, the paracellular conductance does not change in the first period of the action of forskolin, then, a plot of G_t versus $(1 - fR_a) = fR_b$ would give a straight line with a slope of G_b and an intercept of the ordinate representing G_p . In 10 out of 11 experiments we found a linear relation G_t and fR_b in the first period after the onset of the effect of forskolin. In one recording, not used further, the increase of G_t took place after a time lag in which most of the change of the fractional resistance had already occurred.

In Fig. 6 the mean transepithelial conductance, relative to its value before stimulation of the monolayer, is plotted against the mean value of fR_b for

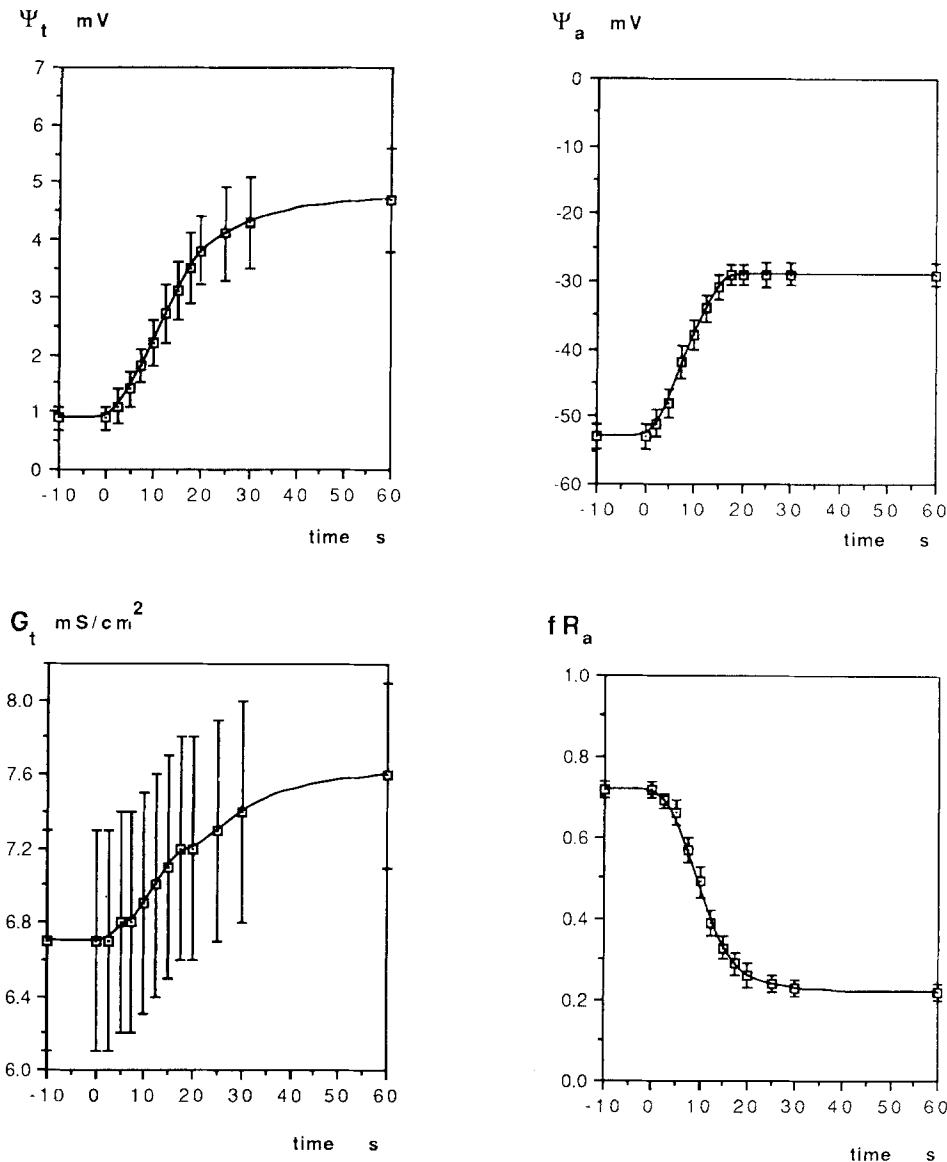


Fig. 5. Effect of forskolin on the potential difference across the apical membrane (Ψ_a) (in mV) and across the monolayer (Ψ_t) (in mV), the conductance of the monolayer (G_t) and the fractional resistance of the apical membrane (fR_a). Values were obtained from 11 cells from 5 monolayers. The bars represent SEM from 11 experiments

a period of 60 sec after the onset of the forskolin response. The graph shows that after 20 sec the change of fR_b is virtually at a maximum while G_t increased further. The mean value of the linear regressions through the points measured in the first 20 sec had a correlation coefficient of 0.96 ± 0.01 . From the slopes and intersections G_b and G_p were estimated during the first 20 sec after forskolin. G_b was found to be 1.13 ± 0.09 mS/cm² and $G_p = 6.26 \pm 0.65$ mS/cm² or 95% of the transepithelial conductance before forskolin addition.

Using the calculated values of G_b and G_p in the first 20 sec, the time course of the change in the

apical membrane resistance (R_a) in each experiment could be calculated. In Fig. 7 the mean values of R_a are plotted against time. From the essentially stable value of fR_a after 20 sec and the absence of effect of forskolin on fR_a in low Cl⁻ Ringer's (see Table 3), it can be concluded that from this time point on G_c may be considered constant, and therefore, the further increase in G_t must result from a change in the paracellular conductance. On this basis values for G_p from 25 sec onwards were calculated. From the values of G_p and the transepithelial potentials, the paracellular current, $I_{pc}(=I_{tc}$ Eq. (4)), was calculated in the 10 individual experiments (Fig. 8). E_a was

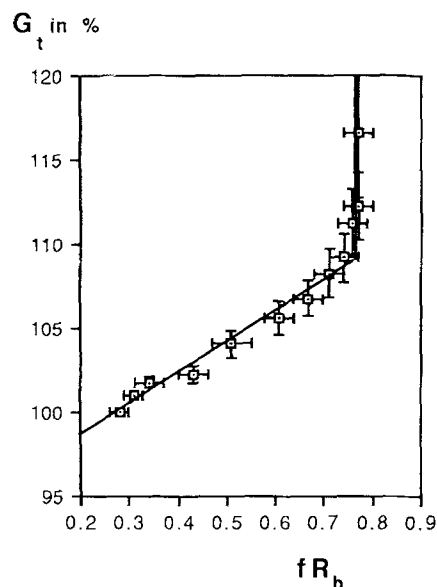


Fig. 6. Relation between relative transepithelial conductance (G_t) and fractional resistance of the basolateral membrane (fR_b) during 60 sec after forskolin addition. Mean values and SEM are from 10 experiments

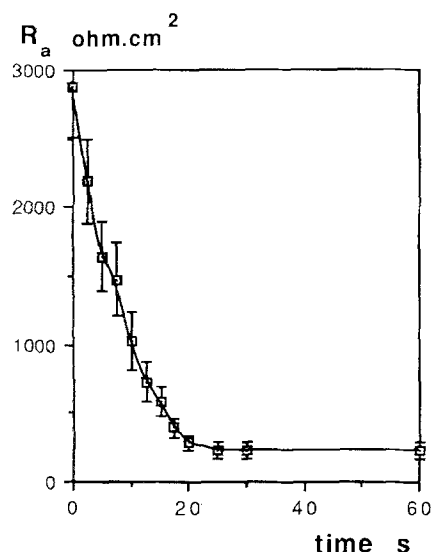


Fig. 7. Effect of forskolin on the apical membrane resistance (R_a). Mean and SEM values are from 10 experiments

calculated according to Eq. (5). In three experiments these calculations gave ambiguous results in that E_a at time zero was only -18 mV, and thereafter, would oscillate around this value. The mean values of the seven other experiments are given in Fig. 9. It is suggested that addition of forskolin depolarizes E_a from -45 ± 4 mV to -25 ± 2 mV at $t = 20$ sec and to -22 ± 2 mV at $t = 60$ sec.

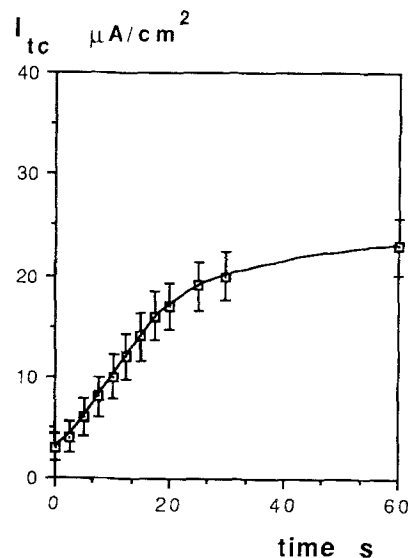


Fig. 8. Mean values and SEM for transcellular current calculated according to Eq. (4); ($n = 7$)

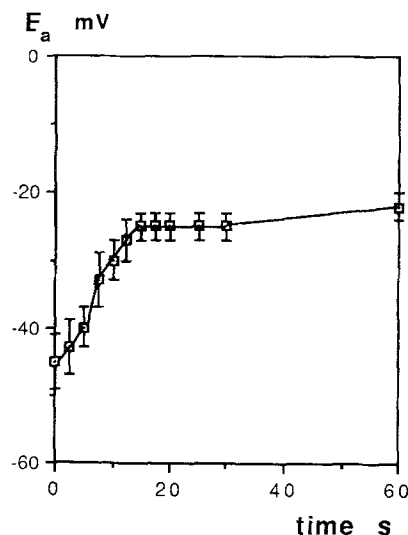


Fig. 9. Mean values and SEM of the electromotive force at the apical membrane calculated from Eq. (5); ($n = 7$)

Discussion

BASAL VALUES OF ELECTRICAL PARAMETERS

The monolayers formed by the HT-29 cl.19A human colon carcinoma cell line displayed transepithelial resistances that were generally lower than reported for T₈₄ cells (Dharmasathaphorn et al., 1985). However, the mean value of $119 \Omega\text{cm}^2$ is in perfect agreement with the value of $114 \Omega\text{cm}^2$ reported ear-

lier for filter-grown cells of this subclone (Augeron et al., 1986). We observed a rather broad range of transepithelial resistances. Microscopic inspection of the monolayers did not reveal consistent morphological differences between monolayers with very high and very low resistances. After 7 days of culture R_t did not correlate with age, neither did we observe a correlation between R_t and the passage number. We presume that variations in R_t may be due to differences in development of tight junctions, but we have not studied this assumption. A similar range of R_t values has been found in Caco-2 cells (Grasset et al., 1984). Moreover, the fractional resistances found in the present study are in perfect agreement with those measured earlier in the HT-29 clone (Augeron et al., 1986) and in domes of the Caco-2 cells (Grasset et al., 1985). The mean intracellular potential was somewhat lower than observed in the study of Augeron et al. (1986) and the transepithelial potential difference was slightly larger.

In a few monolayers, rather large transepithelial potential differences or serosa negative potentials were found while R_t , Ψ_a and fR_a were in the normal range. The origin of these variations in Ψ_t was not further investigated. These monolayers were not used in the study of the forskolin response.

EFFECTS OF FORSKOLIN ON THE ELECTRICAL PARAMETERS

A consistent finding in this cell line was the large and sustained depolarization and sharp decrease in fR_a in response to the serosal addition of forskolin. These effects of forskolin were observed only in the presence of Cl^- . Moreover, the apical membrane potential was much more susceptible to a decrease of the Cl^- concentration in the apical bath after addition of forskolin. This indicates that forskolin induced an increase of the Cl^- conductance of the apical membrane and that the Cl^- equilibrium potential is less negative than the cell potential. This requires that Cl^- is electrochemically accumulated in the cells. The effects of bumetanide further suggest that Cl^- may be accumulated via a Na/K/2Cl co-transport system. The sustained depolarization and reduction of fR_a demonstrate that the conductance increase of the apical membrane is the predominant electrophysiological effect of forskolin. In our laboratory similar results have been obtained with T₈₄ cells (Bajnath et al., 1989; R.B. Bajnath et al., *in preparation*). However, McRoberts et al. (1985) found that secretagogues induced an increase of the Rb^+ efflux and have suggested that cAMP may additionally open potassium channels in the basolateral membranes of T₈₄ cells. We have tested the possibil-

ity of a simultaneous increase in the apical and basolateral membrane conductances by depleting the cells from Cl^- and adding forskolin afterwards. If forskolin also affected the basolateral K^+ conductance we would expect a hyperpolarization of Ψ_a and an increase of fR_a . But neither of these parameters showed a significant change under these circumstances. A difficulty is that the incubation in Cl^- -free solution may have altered the cellular conditions in such a way that an extra K^+ conductance cannot be activated. However, Ca^{2+} -related secretagogues (carbachol, ionomycin) induced a hyperpolarization and an increase of fR_a under Cl^- -free conditions. Moreover, in Cl^- -free solutions, a basolateral increase of K^+ induced a larger depolarization after stimulation with carbachol (R.B. Bajnath et al., *submitted*). This is in contrast with the smaller depolarization observed after forskolin. (The smaller depolarization as observed in Fig. 4 is a consequence of the forskolin-induced change in circuit parameters and does not indicate a decrease in basolateral K^+ conductance.) Based on the responses to the Ca^{2+} -related secretagogues in Cl^- -free solutions, we conclude that it is not likely that in the low Cl^- Ringer K^+ channel activation is inhibited.

In addition, analyses of G_t versus fR_b showed a linear relation during the first 20 sec, suggesting a decrease of R_a only.

We postulate therefore that within the time scale of our experiments (up to 30 min after forskolin addition), cAMP failed to open K^+ channels in the basolateral membrane of the HT-29 cl.19A cells. In view of the similarity with the response of T₈₄ cells we suggest that in this cell line as well, cAMP had no direct effect on the basolateral membrane conductance. This suggestion is in full accordance with recent observations (Cliff & Frizzell, 1990). These authors showed that in T₈₄ cells, voltage clamped at E_{Cl} , forskolin did not change current, and they concluded that cAMP did not affect the K^+ current. As an explanation for the increased Rb^+ efflux we propose that this may be caused by the increased driving force generated by the depolarization of the apical membrane and the coupling of this change to the basolateral membrane due to the internal short circuiting in the monolayer via the low resistance paracellular pathway.

Although continuous impalements during db-cAMP application have not been performed in the Caco-2 cell line (Grasset et al., 1985) their results also suggest a sustained depolarized state of Ψ_a and sustained decrease of fR_a .

COMPARISON WITH INTESTINAL EPITHELIUM

These observations in cell lines contrast with the effect of db-cAMP and of PGE_2 in rabbit colon (Welsh et al., 1982; Horvath et al., 1986) and of PGE_2

in rat small intestine (Stewart & Turnberg, 1989). In these tissues small and transient depolarizations were found. Because the transepithelial potential change was stable, one would expect that the repolarization of the cell potential is caused by a hyperpolarization of the basolateral membrane, presumably due to increased potassium conductance. However, in colon and small intestine fR_a did not recover from its decrease.

It may be that the colon carcinoma cell lines have lost their ability to regulate the K^+ conductance in the basolateral membrane by cAMP or, alternatively, that this conductance is already maximally activated in the nonstimulated state. In contrast, we found positive evidence for activation of a basolateral K^+ conductance with carbachol, a Ca^{2+} -mobilizing secretagogue (Bajnath et al., 1989).

On the other hand, it cannot be excluded that the transient depolarization in small and large intestinal cells is partially mediated by secretagogue-induced release of neurotransmitters or paracrine signaling molecules (Groot et al., 1987).

In canine trachea, addition of epinephrine or PGE_2 induced a transient depolarization of Ψ_a and a transient decrease of fR_a , suggesting that these cAMP-related secretagogues affected the apical membrane as well as the basolateral membrane by increasing their conductances for Cl^- and K^+ , respectively (Welsh, Smith & Frizzell, 1983). However, recently it has been shown that cAMP can release Ca^{2+} from its intracellular stores in tracheal epithelial cells (McCann, Bhalla & Welsh, 1989) and that this is sufficient to activate K^+ channels (McCann et al., 1990). In the HT-29 cl.19A cells, however, we could not detect a cAMP-induced increase of intracellular Ca^{2+} with fura-2 or indo 1 as intracellular Ca^{2+} probes (*unpublished results*).

REQUIREMENTS FOR A SUSTAINED ELECTROPHYSIOLOGICAL EFFECT OF FORSKOLIN

The functional importance of the increase of the K^+ conductance in the basolateral membrane is thought to be the maintenance of an electrochemical gradient for Cl^- efflux through the apical membrane. An increase of the basolateral K^+ conductance may also be required to maintain the cellular homeostasis of K^+ because of a larger influx through a Na/K/2Cl cotransport system and the Na/K pump. In the HT-29 cl.19A cells, however, the driving force for K^+ efflux is increased as long as forskolin is present, and the basolateral conductance of about 1 mS/cm² allows for an ohmic current of about 30 μA at the basolateral membrane potential of about -30 mV. This current is at the upper range of transcellular currents calculated from the present experiments.

Can this current be carried by K^+ ? Assuming that the intracellular K^+ activity is not decreased, its driving force across the basolateral membrane in the presence of forskolin is augmented with about 30 mV ($\Delta\Psi_a + \Delta\Psi_t$). If the basolateral conductance reflects largely a K^+ conductance the current can indeed be carried by K^+ and it can provide for a sustained ion flux of about 1 $\mu mol/cm \cdot hr$. Flux measurements in the HT-29 cl.19A cells remain to be done, but the predicted value is in agreement with net Cl^- fluxes found in the T₈₄ cells (Dharmasathaphorn et al., 1985).

From the estimates of R_a and I_{tc} in the presence of forskolin it can be calculated that the electrochemical driving force required for the maintenance of the Cl^- efflux is about 5 mV. Thus, the equilibrium potential for Cl^- (E_{Cl}) should be about 5 mV less negative than Ψ_a during forskolin-induced Cl^- secretion, or about -20 mV. This value is nearly the same as the value calculated for E_a (-22 mV), suggesting that E_a is strongly dominated by E_{Cl} .

With an extracellular Cl^- concentration of 128.2 mM (activity = 93.5 mM) this value of E_{Cl} would require that the intracellular activity is about 40 mM and can be maintained at that level in the presence of forskolin. The Cl^- activity is in the range of activities reported for mammalian airway cells (Shorofsky, Field & Fozzard, 1983; Willumsen, Davis & Boucher, 1989). To our knowledge no intracellular Cl^- activities have been measured by direct techniques in the colon carcinoma cell lines. In the Caco-2 cells estimates of concentrations have been made recently, suggesting a resting concentration of 42 mM (Burnham & Fondacaro, 1989).

The sustained character of the responses in the HT-29 cl.19A and T₈₄ cells suggests that the Cl^- cotransporter has a capacity that is sufficiently large to maintain the transcellular Cl^- flux. The capacity can be estimated from bumetanide-sensitive K^+ influx studies in the HT-29 cl.19A cells and amounts to about 30 nmol/10⁶ cells \cdot min (Augeron et al., 1987). Assuming a cell density of 5×10^5 cells/cm² the influx would be equivalent to a sustained transcellular current of 24 $\mu A/cm^2$. This value falls within the range measured in the present experiments.

Cl CHANNEL BLOCKERS

Further research is clearly needed to explain the anomalous effects of the putative Cl^- channel blockers on HT-29 colonocytes (lack of effect on Cl^- conductance in intact cells and inhibitory action in excised membrane patches). The possibility that the NPPB- and DPC-sensitive Cl^- channel in the excised patch is not related to the cAMP-activated

Cl^- conductance in the intact cell deserves special attention in view of its important consequences for the interpretation of the Cl^- channel defect in cystic fibrosis patients (Kunzelmann, Pavenstadt & Greger, 1989). The possibility that NPPB blocks K^+ channels, and therefore, had similar effects as Ba^{2+} in inhibiting Cl^- transport is also raised by the results of work of Diener and Rummel (1989) on rat colon.

PARACELLULAR PATHWAY

The results also suggest that in addition to the large increase of the conductance of the apical membrane, an increase of the conductance of the paracellular pathway may occur as well. The paracellular conductance consists of the conductance of the tight junctions in series with the (presumably much larger) conductance of the lateral intercellular space. An increase of the tight junction conductance is in accordance with the VIP-induced increase of *both* unidirectional Cl^- fluxes in T_{84} cells (Dharmasathaphorn et al., 1985) and the observation in fish intestine that cAMP increased the Cl^- conductance of the paracellular pathway (Bakker & Groot, 1989).

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