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Activation of ion transport by combined effects of ionomycin, forskolin and phorbol ester on cultured HT-29cl.19A human colonocytes

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Abstract. The differentiated clone 19A of the HT-29 human colon carcinoma cell line was used as a model to study the intracellular electrophysiological effects of interaction of the cAMP, the protein kinase C (PKC) and the Ca^{2+} pathways. (a) A synergistic effect between ionomycin and forskolin was observed. From intracellular responses it was concluded that the synergistic effect is caused by activation of an apical Cl^- conductance by protein kinase A and a basolateral K^+ conductance by Ca^{2+}. (b) A transient synergistic effect of ionomycin and the phorbol ester phorbol dibutyrate (PDB) was found. The decrease of the response appeared to be due to PKC-dependent inactivation of the basolateral K^+ conductance. The synergism is caused by PKC-dependent increase of the apical Cl^- conductance and Ca^{2+}-dependent increase of the basolateral K^+ conductance. (c) The effects of carbachol and PDB were not fully additive presumably because of their convergence on PKC activation. (d) Forskolin and PDB, when added in this order, had a less than additive effect. Results of cell-attached patch-clamp studies, presented in the accompanying paper, showed a synergistic effect of forskolin and PDB on non-rectifying small-conductance Cl^- channels. Assuming that these channels are involved in the transepithelial responses it is suggested that forskolin and PDB induce a modulatory, synergistic increase of the apical Cl^- conductance when both pathways are activated simultaneously. (e) The HT-29cl.19A cells differ from T_{84} cells in that the latter did not respond with an increase of the short-circuit current to addition of phorbol ester. This may be due to a very low expression of PKCα.

Key words: Forskolin – Carbachol – Ionomycin – PDB – Protein kinase C – Cl^- channels – K^+ channels – Intestinal secretion – Cystic fibrosis – T_{84} cell line

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Introduction

The clone 19A of the human colon carcinoma cell line HT-29 consists of homogeneous, permanently differentiated intestinal epithelial cells, which can be grown to confluence on permeable filters and then show many features associated with Cl^--secretory epithelia [2, 3]. These cells can be studied with conventional microelectrode techniques, so that in combination with transepithelial measurements the location of changes in potential and conductance can be deduced [4, 5, 7].

An important aim of studying the regulation of Cl^- secretion in intestinal epithelium by neurotransmitters and hormones is to understand their mutual interactions. A difficulty is that, because of the complexity of the experimental models, it is usually not possible to discriminate between direct effects and effects caused by the production of secondary mediators in the lamina propria. The HT-29cl.19A cells can be used as a model to study the possible interaction of stimulation of Cl^- secretion by different intracellular pathways. We report here the effect of addition of ionomycin followed by forskolin and of these two secretagogues in the reversed order. The effect of application of forskolin followed by ionomycin was compared with the effect of phorbol 12,13-dibutyrate (PDB) followed by ionomycin in order to emphasize the difference between protein kinase A (PKA) and protein kinase C (PKC) activation. Because the effect of ionomycin is partially similar to the effect of carbachol it was of interest to compare effects of PDB followed by ionomycin with PDB followed by carbachol. Further, we studied the effect of forskolin followed by PDB to find possible interactions of stimulation of the PKA and PKC pathways. Finally we investigated a probable cause for the differing susceptibility of HT-29cl.19 cells and T_{84} cells to activation with phorbol esters.

The results suggest the following. (a) The synergistic effect of ionomycin plus forskolin or PDB on changes in

transepithelial potential and the equivalent short-circuit current can be explained by activation of Cl^- conductance in the apical membrane by forskolin or PDB, and K^+ conductance in the basolateral membrane by ionomycin. The latter led to a decrease of the resistance of the basolateral membrane to 35% of its initial value. (b) The increase of intracellular Ca^{2+} by ionomycin may further activate the PDB-dependent pathway. (c) PDB and carbachol may act on the same pool of PKC. (d) The activation of the PKC pathway by PDB modulates the PKA-activated Cl^- conductance and suggests that the PKA and PKC pathways can converge on the same type of Cl^- channel. This is supported by the results of single-channel studies by the patch-clamp technique [8]. (e) It appears that the expression of PKCα, which may be involved in the PDB response [24], is much lower than in HT-29cl.19A cells.

Materials and methods

Cell culture and electrical measurements. Methods for growth and subculturing HT-29cl.19A cells and for the electrophysiological measurements have been previously described [4]. In short, HT-29cl.19A cells (passage numbers 8–22) were grown to confluence on rat-tail-collagen-coated Nuclepore PC filters, 13 mm in diameter (Nuclepore Corp. Pleasanton, USA). Confluent monolayers were mounted horizontally in a small Ussing-type chamber, leaving an oblong area of 0.30 cm^2. The upper (apical) and lower (basolateral) compartments were continuously perfused with solutions maintained at 36°C and gassed with 5% CO_2/95% O_2. The transepithelial potential (V_t) was measured with Ringer/agar bridges, which were connected to Ag/AgCl electrodes. The intracellular potential (V_a) was measured with glass microelectrodes filled with 0.5 mol/l KCl and connected to a high-input-impedance amplifier (W-P-Instruments, New Haven, Conn.). All measurements were performed under open-circuit conditions and the apical solution was used as reference for transepithelial and intracellular measurements. Transepithelial resistance (R_t) was calculated from the change in V_t induced by bipolar current pulses of 1 s duration ($\pm$ 10 μA and $\pm$ 50 μA). Current was passed through electrodes (Ag/AgCl) that were located in the walls of the upper and lower compartments. The fractional resistance of the apical membrane ($fR_a = R_a/(R_a + R_b)$) was calculated from the ratio of the changes in V_a and V_t induced by the current pulses. Only monolayers with transepithelial resistances above 50 Ω cm^2 were used. Corrections for solution resistance and potential offset were made. The microelectrodes had tip resistances between 100 MΩ and 200 MΩ and the tip potentials were about −2 mV. The effect of added chemicals was studied during simultaneous recordings of the intracellular potential and the transepithelial potential. The changes in potential and resistance were analysed by means of an equivalent electrical circuit for leaky epithelia [15].

Immunoblotting of PKCα. HT-29cl.19A cells and T_{84} cells (passage 43) were plated at a 1 :2.5 surface ratio on tissue-culture-treated polycarbonate filters (Transwell, Costar) and grown for different lengths of time (3–24 days) as described earlier [4]. The preparation of cell lysates in sodium dodecyl sulphate sample buffer, the separation of proteins by sodium dodecyl sulphate/polyacrylamide gel electrophoresis, their transfer to nitrocellulose paper and their incubation with PKCα-specific antiserum were performed as described in a previous paper [24].

Solutions and chemicals. All experiments were carried out with a standard Ringer solution with the following composition (in mmol/l): 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). Forskolin, ionomycin, PDB, and carbachol were purchased from Sigma Chemical Company, St Louis. Rainbow protein molecular mass markers were from Amersham International (UK). Rabbit polyclonal PKCα-specific antibody was kindly donated by Dr. P. J. Parker (I. C. R. F. London). The T_{84} human colon carcinoma cell line was a gift from the late Dr. K. Dharmsathaphorn (University of California, San Diego, School of Medicine, USA).

All results are expressed as means $\pm$ SEM. The Student *t*-test (paired or unpaired) was used to evaluate statistical significance. In the original recorder tracings V_t and V_a are displaced in time because the recorder pens use the full paper-width and usually the tracings cross each other. Therefore the recordings were copied and the tracing aligned in time.

Glossary of symbols used

V_t, V_a, electrical potential difference across the monolayer and the apical cell membrane (both with the apical solution as reference) respectively, in mV.

E_a, E_b, electromotive force across the apical and the basolateral membrane respectively, in mV

R_t, transepithelial resistance in Ω cm^2

R_a, R_b, R_p, electrical resistance of the apical- and the basolateral cell membrane and the paracellular pathway, in Ω cm^2

G_t, G_p, G_c, transepithelial conductance and conductance of the paracellular and the cellular pathway respectively, in mS cm^{-2}

G_a, G_b, conductance of the apical and the basolateral membrane respectively, in mS cm^{-2}

fR_a, fR_b, fractional resistance of apical and basolateral membrane, respectively [$fR_a = R_a/(R_a + R_b)$, $fR_b = 1 - fR_a$].

Results

Forskolin and ionomycin

By sequentially activating the Ca^{2+} pathway and the cAMP pathway in this and in the reversed order we studied the possible cause for the synergistic effect on the transepithelial potential of stimulating the two pathways. Moreover, these experiments could be used to elucidate the location of the Ca^{2+}-activated conductances. We have reported [5] that the application of ionomycin has a biphasic effect on the intracellular potential but a negligible effect on the transepithelial potential. The lack of effect on the transepithelial potential indicates that no transcellular current can flow. This may be because the conductance is increased in both membranes or because the conductance in the non-activated membrane is insufficient to carry the current. The transient depolarization during the first phase of the response to ionomycin (7.5 $\pm$ 2.0 mV, $n = 11$) was dependent on the presence of Cl^-, indicating the activation of a Cl^- conductance. The absence of a transepithelial effect in comparison with the effect of forskolin [4] suggests that the activation is not in the apical membrane, but in both membranes or only in the basolateral membrane.

Table 1 shows that the ionomycin-induced persistent hyperpolarization of V_a is accompanied by an increase fR_a. The decrease of R_t, accompanying the increase of fR_a when ionomycin was added in the presence of forskolin (see Table 2) indicates that the increase of fR_a is mainly due to a decrease of R_b. An explanation for the hyperpolarization may be an increased K^+ conductance

Table 1. Effect of cumulative addition of ionomycin followed by forskolin[a]

Condition	V_t (mV)	V_a (mV)	R_t (Ω cm^2)	fR_a	eq. I_{sc} (μA/cm^2)
Control	0.7 ± 0.2	−51 ± 2	127 ± 20	0.86 ± 0.04	5 ± 2
Ionomycin	0.8 ± 0.3*	−60 ± 2**	124 ± 22*	0.95 ± 0.03**	6 ± 2*
Ionomycin + forskolin	7.7 ± 1.5**	−25 ± 2**	110 ± 18**	0.16 ± 0.01**	70 ± 12**

[a] Values represent the mean and SEM for five experiments. The values with ionomycin were read 5 min after its addition. I_{sc} was calculated from V_t and R_t
* Values do not differ significantly from the values in the previous condition.
** Significantly different from previous condition ($P < 0.01$, paired t-test)

Table 2. Effect of forskolin and ionomycin in the presence of forskolin[a]

Condition	V_t (mV)	V_a (mV)	R_t (Ω cm^2)	fR_a	eq. I_{sc} (μA/cm^2)
Control	1.0 ± 0.1	−48 ± 2	154 ± 18	0.86 ± 0.02	7 ± 1
Forskolin	5.4 ± 0.9	−18 ± 1	138 ± 16	0.12 ± 0.02	39 ± 4
Forskolin + ionomycin	8.1 ± 1.8	−27 ± 3	128 ± 14	0.24 ± 0.04	63 ± 14

[a] Values represent the mean and SEM for ten experiments. All changes are statistically significant ($P < 0.01$, paired t-test)

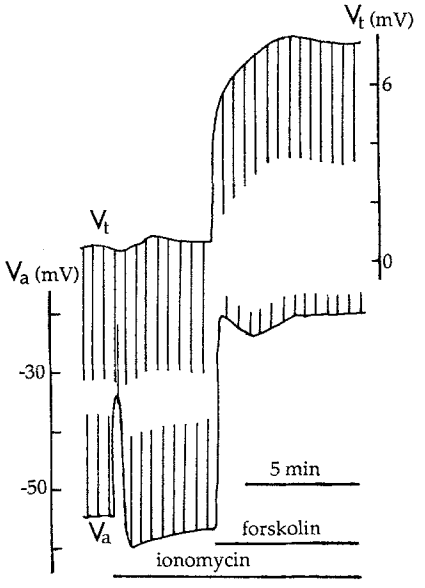

Fig. 1. Changes in intracellular potential (V_a) and of the transepithelial potential (V_t) induced by ionomycin (10 μM) followed by forskolin (10 μM), as indicated by the *horizontal bars*. Note the negligible change in V_t after ionomycin and the large change of V_t after forskolin, which is larger than when forskolin is added alone (compare Fig. 2, Tables 1 and 2). The vertical deflections are from current pulses to calculate the resistances. The voltage excursions have been redrawn in only one direction; however, the pulses were bipolar (± 10 μA for V_t and ± 50 μA for V_a)

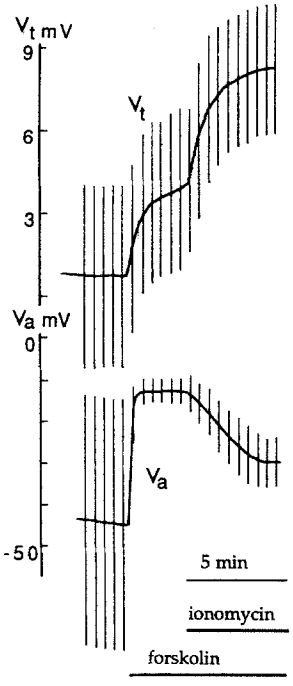

Fig. 2. Changes in potential induced by the cumulative addition of forskolin and ionomycin, as indicated by the *horizontal bars* (concentrations as in Fig. 1). Compare the effect of ionomycin on V_t in this figure with the effect shown in Fig. 1. Vertical deflections are from current pulses (± 10 μA for V_t and ± 50 μA for V_a)

of the basolateral membrane. This would increase the driving force for Cl^- efflux if a Cl^- channel was activated. As expected from an increased basolateral K^+ conductance, when forskolin was added after ionomycin (Fig. 1), the increase of V_t and the equivalent short-circuit current was larger than that without preincubation with ionomycin (compare Tables 1 and 2).

The question whether ionomycin activates Cl^- and K^+ channels in both membranes or only in the basolateral membrane has been studied by analysing the effect of addition of ionomycin after forskolin. If ionomycin, during the first phase, increased only the basolateral conductance for Cl^- we would expect that the transcellular current would decrease transiently because then part of the Cl^- could flow through the basolateral membrane. Figure 2 illustrates that the transepithelial potential did not show a sign of decrease but started to increase simultaneously with the hyperpolarization of V_a. Therefore we conclude that ionomycin induced an increase of the Cl^- conductance in both membranes. The first phase of the

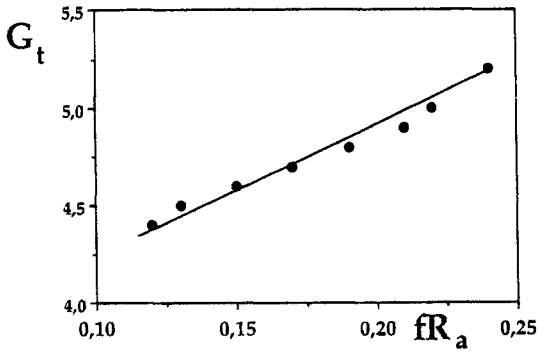

Fig. 3. Plot of G_t in mS/cm² versus fR_a after addition of ionomycin in the presence of forskolin. The addition of ionomycin after forskolin induced an increase of G_t and fR_a. Points are taken at 30-s intervals after addition of ionomycin. The relation appears to be linear, suggesting that G_p and G_a (= $1/R_a$) remain constant under these conditions and during this period (3.5 min). Example of four similar experiments

ionomycin response could not be observed in the intracellular tracing. This is similar to findings with carbachol [5] and may be because forskolin already brought the potential near to the Cl^- equilibrium potential [4].

Whether ionomycin increased the K^+ conductance in both or only the basolateral membrane was studied from the relation between G_t and fR_a during activation by ionomycin in the presence of forskolin.

From equivalent circuit analyses [4], because $G_t = G_p + G_c$, it can be deduced that

$$G_t = G_p + G_a \cdot fR_a = G_p + G_b \cdot fR_b.$$

If a secretagogue selectively alters only one of the resistive barriers of the cellular pathway a linear relation between G_t and fR_a or fR_b will exist. An estimation can then be made of the conductance of the non-changing membrane and of the conductance of the paracellular resistance; e. g. if G_a and G_p are constant a plot of G_t versus fR_a yields a slope of G_a and an intercept of G_p. We plotted the values of G_t versus fR_a at 30-s intervals after the addition of ionomycin in the presence of forskolin. The linear relation between G_t and fR_a (Fig. 3) suggests that, under these conditions, G_p and G_a remained constant and that only R_b was decreased by ionomycin. Because this is simultaneous with a hyperpolarization, it appears that the increase in fR_a induced by ionomycin (see Tables 1 and 2) can be ascribed to the activation of K^+ channels in the basolateral membrane.

Because from these plots G_a and G_p can be estimated, G_b can be calculated at each point. From four similar experiments the time course of the decrease of R_b was calculated. Figure 4 shows that ionomycin reduced R_b to about 35% of its original value with a half-time of 40 s.

Table 2 shows further that, when the conductance of the apical membrane had been previously increased by activation of the PKA pathway, cumulative addition of ionomycin induced a further increase of V_t and an equivalent short-circuit current of 24 μA/cm². This is in sharp contrast with the insignificant effect of ionomycin on the transepithelial potential when added alone.

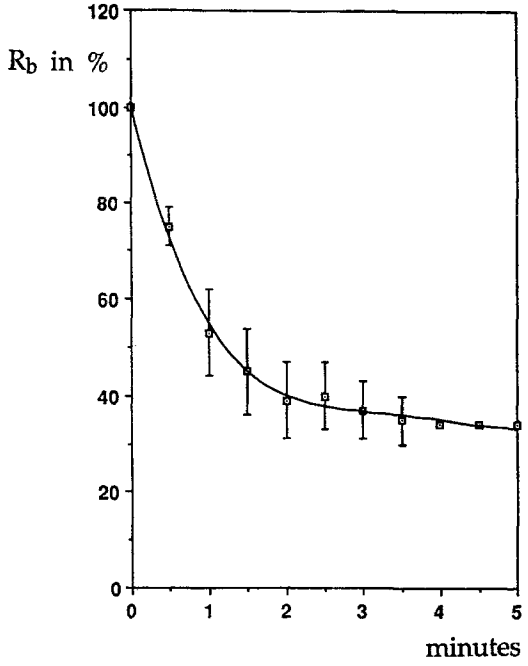

Fig. 4. Time course of the change in the basolateral membrane resistance (R_b) induced by ionomycin in the presence of forskolin. The value of R_b was calculated from fR_a, knowing the value of R_a, which was determined from the slope of graphs as shown in Fig. 3. The points are means ± SEM from four experiments except for the last three points, which are from one experiment

PDB followed by ionomycin

Because PDB and forskolin differ in their effects, in that PDB increases the apical Cl^- conductance less efficiently and inhibits the basal and the ionomycin-induced K^+ conductance in the basolateral membrane [7, 23], it was of interest to compare the effect of ionomycin after PDB with its effect after forskolin. Ionomycin was added at the summit of the PDB-induced increase of V_t. Figure 5 A and Table 3 show the complex response of V_a and V_t. As when it was added first, ionomycin induced a transient depolarization of V_a (6.5 ± 2.6 mV), but this time V_t decreased by 0.9 ± 0.3 mV (n = 8). The ensuing increase of V_t appeared to coincide with the repolarization of V_a and increase of fR_a. From the time course of the following depolarization of V_a and changes in V_t one can conclude that the depolarization of V_a must be caused by depolarization of E_a and a slower depolarization of E_b. The depolarization of E_a suggests that the high Ca^{2+} concentration may induce a further activation of PKC or a further increase of the PKC-activated Cl^- conductance. The depolarization of E_b and concomitant decrease of V_t was anticipated from the already known inhibitory effect of PDB on ionomycin-activated K^+ conductance [7].

PDB followed by carbachol

Table 4 and Fig. 5 B show the effect of pre-incubation with PDB on the carbachol response. Carbachol was added at the top of the PDB-induced response of V_t. Like

Table 3. Effect of phorbol dibutyrate (PDB) and of ionomycin in the presence of PDB[a]

Condition	V_t (mV)	V_a (mV)	R_t (Ω cm^2)	fR_a	eq. I_{sc} (μA/cm^2)
Control	1.0 ± 0.4	−49 ± 3	159 ± 26	0.89 ± 0.03	6 ± 2
PDB	4.2 ± 0.7	−32 ± 3	156 ± 24	0.53 ± 0.06	28 ± 3
PDB + ionomycin at					
V_a = min	3.3 ± 0.6	−26 ± 2	155 ± 24	0.70 ± 0.04	22 ± 3
V_a = max	5.5 ± 1.0	−45 ± 5	152 ± 24	0.78 ± 0.04	38 ± 5
V_t = max	6.5 ± 1.1	−41 ± 3	157 ± 25	0.64 ± 0.05	42 ± 6
t = 9 min	5.1 ± 1.0	−30 ± 3	156 ± 25	0.49 ± 0.06	33 ± 5

[a] Values represent the mean and SEM for eight monolayers. Ionomycin (1 μM) was added to the apical perfusate at the peak of the change in V_t induced by PDB (1 μM), reached at 6 ± 0.3 min. The values are those after addition of ionomycin at the top of the ionomycin-induced depolarization (V_a = min), at the top of the ionomycin-induced hyperpolarization (V_a = max), at the summit of the change in V_t (V_t = max) and 9 ± 0.2 min after addition of ionomycin. All changes are statistically significant ($P < 0.01$, paired t-test) except for ΔR_t

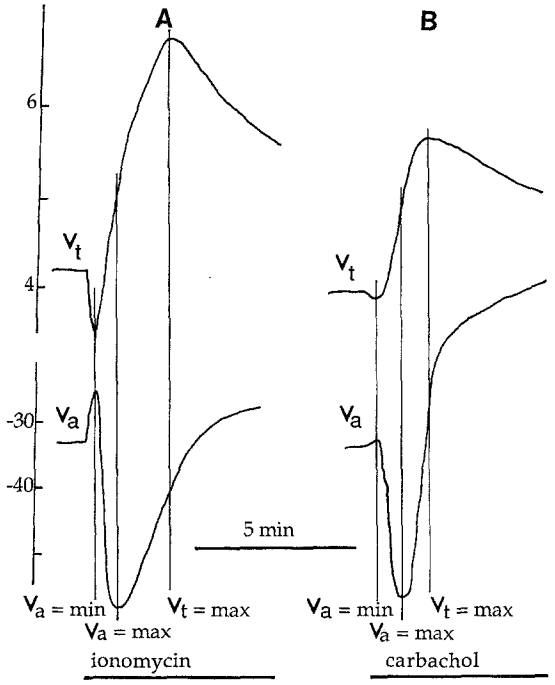

Fig. 5. A The tracings show the complex time course of the changes in V_a and V_t induced by ionomycin (1 μM), added to the basolateral side at the summit of the phorbol-dibutyrate(PDB)-induced increase of V_t. PDB (1 μM) was applied to the apical side. The *vertical lines* indicate (from *left* to *right*) V_a = min, V_a = max and V_t = max. Mean values at these times are given in Table 3. The decrease of V_t and concomitant depolarization of V_a is followed by an increase of V_t and V_a but note that at the maximum of V_a the tracing of V_t continues to increase while V_a is again depolarized. The ensuing decrease of V_t occurs while V_a is still depolarizing. Voltage deflections induced by current injection were left out. **B** Tracings of an experiment showing the changes in V_t and V_a induced by carbachol (0.1 mM basolateral side) added after PDB (1 μM, apical side). Table 4 gives the means at the times indicated by the *vertical lines*

ionomycin, carbachol induced a small depolarization of V_a concomitant with a negative deflection of V_t, which in the case of carbachol was much smaller than with ionomycin. The subsequent changes of V_t and V_a were similar to the changes induced by ionomycin. Thus, carbachol also appeared to activate PKC or PKC activated channels further and PDB also inactivated the carbachol-stimulated basolateral K^+ conductance.

PDB after forskolin

From results of earlier experiments it can be predicted that exposure of the HT-29cl.19A cells to phorbol ester and forskolin would have different effects depending on the order of addition. We showed [7] that forskolin, when added after PDB, induced a small and transient increase of V_t although V_a and fR_a decreased further. The transient nature was due to the inactivation of the basolateral K^+ conductance by activated PKC. Thus, the cumulative effect of activation of the PKC pathway by PDB after forskolin should have a larger effect on V_t than when forskolin is added after PDB. Figure 6 and Table 5 show the effect of the consecutive addition of PDB after a supramaximal dose of forskolin (0.1 mM). PDB further increased the equivalent short-circuit current by 42% but did not change V_a and had only a small effect on fR_a (fR_a decreased by 0.024 ± 0.005). The increase of V_t was transient. During the decrease of V_t, a further decrease of V_a and fR_a was observed, illustrating the inactivation of the basolateral K^+ conductance by PDB. It took about 12 min to bring V_t back to the pre-PDB value. The decrease continued slowly, following the same time course as the decline in V_t after PDB alone [7].

The interesting observation in these experiments is the increase of V_t accompanied by a small but statistically significant decrease of fR_a while V_a remained constant. From the Goldman, Hodgkin and Katz equation it can be argued that if the apical Cl^- permeability was further increased by PKC, this would hardly be visible as a change in V_a because the permeability is already very high owing to the PKA-activated Cl^- conductance. However, if V_a is moved away from E_{Cl} artificially, a further increase of the apical Cl^- conductance might be revealed by a depolarization of V_a. We displaced V_a by addition of ionomycin after forskolin and added PDB on top of forskolin and ionomycin. As shown in Fig. 7 and Table 5 B, PDB induced a depolarization of V_a, a decrease of fR_a and a transient increase of V_t. This corroborates the idea that PDB can increase the apical Cl^- conductance even after pre-stimulation with a supramaximal concentration of forskolin.

We have shown that during the first 20 s after addition of forskolin, G_a is the only changing conductance in the equivalent circuit, G_p and G_b remain constant [4].

Table 4. Effect of PDB and of carbachol in the presence of PDB[a]

Condition	V_t (mV)	V_a (mV)	R_t (Ω cm^2)	fR_a	eq. I_{sc} (μA/cm^2)
Control	0.5 ± 0.2	−58 ± 3	97 ± 10	0.80 ± 0.05	5 ± 1
PDB	3.3 ± 0.7	−35 ± 5	103 ± 10	0.35 ± 0.06	32 ± 5
PDB + carbachol at					
V_a = min	3.2 ± 0.7	−33 ± 5	102 ± 10	0.36 ± 0.07	31 ± 5
V_a = max	4.9 ± 1.1	−44 ± 7	102 ± 10*	0.48 ± 0.08	48 ± 9
V_t = max	5.3 ± 1.1	−36 ± 4	97 ± 10	0.36 ± 0.07	55 ± 8
t = 8 min	3.4 ± 0.6	−27 ± 3	108 ± 12	0.22 ± 0.05	31 ± 5

[a] Values represent the means and SEM for ten experiments. Carbachol (0.1 mM) was added to the basolateral perfusate at the peak of the change in V_t induced by PDB (1 μM), reached at 7.6 ± 0.6 min. The values are those after addition of carbachol at the top of the carbachol-induced depolarization (V_a = min), at the top of the carbachol-induced hyperpolarization (V_a = max), at the summit of the change in V_t (V_t = max) and 8 ± 0.2 min after addition of carbachol. All changes are statistically significant ($P < 0.01$, paired t-test) except for the initial carbachol-induced changes (line 3) and the value marked *

Table 5. Effect of addition of PDB after incubation with forskolin or after forskolin plus ionomycin[a]

Condition	V_t (mV)	V_a (mV)	R_t (Ω cm^2)	fR_a	eq. I_{sc} (μA/cm^2)
A					
Control	0.5 ± 0.1	−57 ± 3	147 ± 23	0.85 ± 0.03	3 ± 1
Forskolin	5 ± 1	−22 ± 3	135 ± 22	0.11 ± 0.03	37 ± 7
Forskolin + PDB	7.4 ± 1.1	−22 ± 3*	140 ± 21*	0.09 ± 0.02	53 ± 8
Forskolin + PDB (t = 12 min)	4.8 ± 1.1	−20 ± 3	156 ± 24	0.08 ± 0.02*	31 ± 6
B					
Forskolin + ionomycin	8.1 ± 1.8	−27 ± 3	128 ± 14	0.24 ± 0.04	63 ± 14
Forskolin + ionomycin + PDB	9.8 ± 1.8	−23 ± 2	130 ± 12*	0.15 ± 0.03	73 ± 13
(t = 13 min)	6.2 ± 1.2	−19 ± 2	147 ± 19	0.11 ± 0.04	40 ± 5

[a] Values represent means ± SEM of six experiments in A and ten in B. The values in B are derived from the same set of experiments as shown in Table 2. All changes are statistically significant ($P < 0.01$, paired t-test) except for the changes marked *

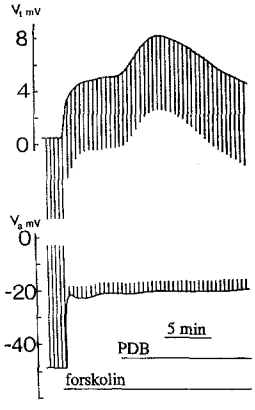

Fig. 6. Potential changes induced by cumulative addition of forskolin (0.1 mM, basolateral side) and PDB (1 μM, apical side). Note the transient increase of V_t while V_a remains constant. Deflections of the potential were redrawn in only one direction. The current pulses used to induce these excursions were bipolar (± 10 μA for V_t and ± 50 μA for V_a)

Table 6. Estimates of the resistance of the apical membrane[a]

Condition	R_a (Ω cm^2)	n	ΔG_a (mS/cm^2)
1. Control	2774 ± 350	11	–
2. Forskolin	280 ± 50	11	3.2
3. PDB	820 ± 156	6	0.9
4. PDB + forskolin	41 ± 12	6	24

[a] Resistance was calculated from the linear relation of G_t and (1 − fR_a) yielding a value for G_p and G_b so that R_b can be estimated at t = 0 and t = 20 s after addition of forskolin without PDB (lines 1 and 2) and after incubation with PDB (lines 3 and 4). Values for R_a differ significantly from control values ($P < 0.01$, paired and unpaired t-test). R_a in the presence of PDB is significantly larger than with forskolin (lines 3 and 2, $P < 0.01$, unpaired t-test). R_a in the presence of PDB plus forskolin (line 4) is significantly smaller than with forskolin (line 2, $P < 0.01$, unpaired t-test) or PDB alone (line 3, $P < 0.01$, paired t-test). ΔG_a was calculated as $1000/R_{a(exp)} - 1000/R_{a(contr)}$

We measured G_t and (1 − fR_a) at intervals of 5 s after the addition of forskolin (see [4]), and from a plot of G_t versus (1 − fR_a) after forskolin and after PDB plus forskolin the values of G_p and G_b can be found, so that the values of R_a before and after forskolin can be calculated. Table 6 shows the values of R_a after forskolin addition ($R_{a(fsk)}$), after addition of PDB but before forskolin ($R_{a(PDB)}$) and after the combined action of PDB and forskolin. If PDB and forskolin activate parallel Cl^- conductances one would expect that the value of R_a after the combined action of the drugs can be predicted by the equivalent resistance replacing $R_{a(fsk)}$ and $R_{a(PDB)}$ i. e. 210 Ω cm^2. Table 6 shows that the combined activation

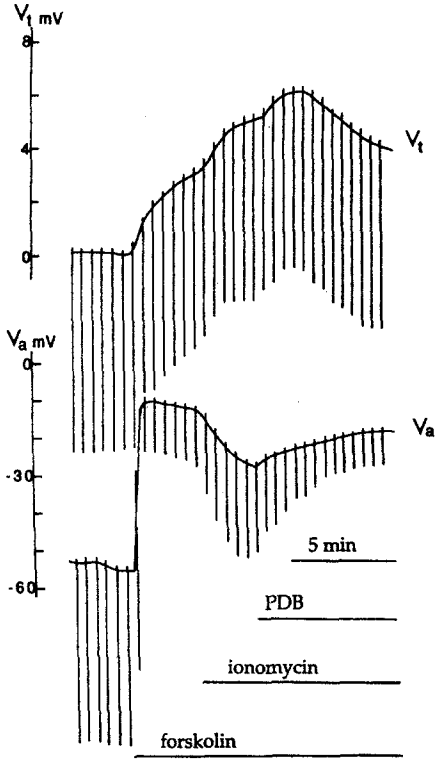

Fig. 7. Potential changes induced by cumulative addition of forskolin (0.1 mM, basolateral side), ionomycin (1 μM, basolateral side) and of PDB (1 μM, apical side). Note that the transient change of V_t is now accompanied by a depolarization of V_a. Current pulses were symmetrical (± 10 μA for V_t and ± 50 μA for V_a), the voltage deflections were redrawn in only one direction

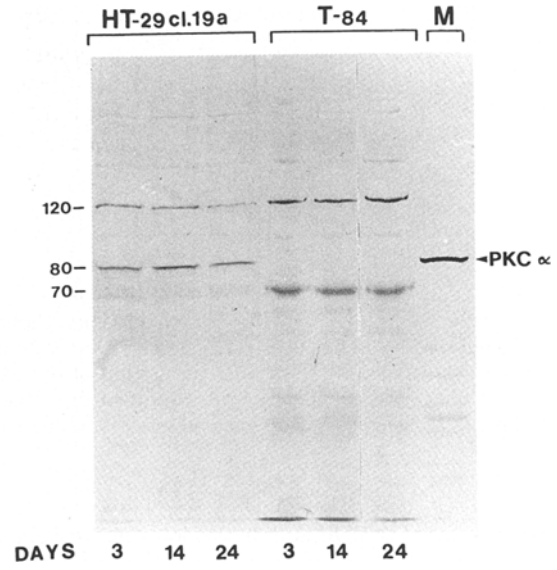

Fig. 8. Immunoblot showing different expression levels of protein kinase Cα (*PKCα*) in HT-29cl.19A and T_{84} colonocytes. HT-29cl.19A and T_{84} cells were grown for 3, 14 and 24 days as described in Materials and methods. Samples of cell homogenates (15 μg protein) were separated by SDS/PAGE, transferred to nitrocellulose paper and incubated with PKCα-specific antiserum (1 : 500) as described previously [24]. *Lane M* contains a purified PKC mixture from human brain, together with Rainbow marker proteins to calibrate the molecular mass (in kDa) of the major immunoreactive bands (not shown). The identification of the 80-kDa band as PKCα was confirmed earlier by its blockade by the antigenic peptide and by its selective down-regulation in phorbol-ester-treated HT-29cl.19A cells (see [24] Fig. 3 A). Immunostaining of the 70-kDa and 120-kDa bands was not blocked by the antigenic peptide (results not shown)

of the PKA and PKC pathways reduced R_a to a much lower value. This suggests that the PKA and PKC pathways can converge on one type of Cl^- channel.

Comparison of HT-29cl.19A cells and T84 cells

An intriguing difference between the HT-29cl.19A cell line and the T_{84} cell line is that the latter did not respond to PDB with an increase of the transepithelial potential [26] (unpublished observations). We have explored the possibility whether this might be because an isotype of PKC, namely PKCα that appeared to be involved in the PDB response [24], was not expressed in T_{84} cells. As shown in Fig. 8, immunoblots of homogenates of HT-29cl.19A and T_{84} cells, taken after they had been grown for 3, 14 and 24 days, show large differences in expression of PKCα, suggesting that in the clone of T_{84} cells that we used, the level of expression of PKCα was indeed very low.

Discussion

Ionomycin induces a transient increase of the Cl^- conductance in the apical and basolateral membrane

Comparison of the initial effect of ionomycin (depolarization of V_a) with the effect of forskolin, which predominantly activates Cl^- channels in the apical membrane, makes it less probable that ionomycin increased only the apical Cl^- conductance. On the other hand, an exclusive effect of ionomycin on basolateral Cl^- channels would reduce transiently the forskolin-induced serosa-to-mucosa Cl^- flux and therefore the transcellular current. But this was not found when ionomycin was added after forskolin. Radioisotope efflux studies from filter-grown cells showed that the Ca^{2+} ionophore increased the Cl^- efflux to both compartments [22]. We therefore conclude that the ionophore increased the Cl^- conductance in both membranes. But if the Cl^- flux leaving the cell through the apical membrane were equal to the flux leaving the cell to the basolateral side, then, because of the polarised uptake of Cl^-, a net transepithelial flux of Cl^- would result, which should approximate half the basolateral uptake into the cells. As with forskolin, the Cl^- efflux should be compensated by a potassium efflux through the basolateral membrane leading to a basolateral-positive potential change. Because this was not found, it is concluded that the Cl^- efflux to the basolateral side must be larger than that to the apical side. This conclusion is in line with the small serosa-negative deflection of V_t usually observed with carbachol or ionomycin.

We suggest that the ionophore- and carbachol-induced rapid increase of the conductance as observed in

whole-cell patch-clamp studies in T_{84} and another HT-29 clone [10, 14, 18] is equivalent to the rapid depolarization in the HT-29cl.19A cells. Recently it has been shown that in T_{84} cells [29] and in airway cells [25] the multifunctional Ca/calmodulin-dependent kinase II mediates the Ca-dependent stimulation of the Cl^- conductance.

The synergism between forskolin and ionomycin is based on the increase of the basolateral K^+ conductance

In an earlier study [4] we have shown that the cAMP-induced sustained increase of the transepithelial potential and short-circuit current was limited by the basolateral conductance. We estimated that the maximum short-circuit current that could be carried by the basolateral efflux of K^+ is about 30 μA cm^{-2} (1.1 μmol cm^{-2} h^{-1}), at $R_t = 130\,\Omega\ cm^2$, corresponding to a ΔV_t of about 4 mV. In the present study we showed that ionomycin, in the second phase of its effect (hyperpolarization of V_a) can decrease the resistance of the basolateral membrane by about 65%. Although the driving force for K^+ efflux is simultaneously reduced by about 10 mV, this reduction will be much smaller than 65%, (assuming E_K to be about −90 mV [18]) so that a much larger current can flow through the basolateral membrane. The Ca^{2+}-dependent increase of the K^+ conductance, and the hyperpolarization induced thereby, which enlarged the driving force for Cl^- efflux across the apical membrane, could well be the explanation for the observed synergism between cAMP- and Ca^{2+}-related secretagogues on transepithelial potential and short-circuit current in monolayers of T_{84} cells [9, 12], isolated and stripped guinea-pig ileum [11], and colon [30] and MDCK cells [21].

Synergism between PDB and ionomycin is transient

The response of the cells to ionomycin after incubation with PDB differed from the response after incubation with forskolin in three respects: (a) after the repolarization of V_a, ionomycin induced a further depolarization of E_a, which may be due to a further activation of PKC. (b) After PDB the first phase of the ionomycin effect appeared to be larger in the basolateral membrane so that a pronounced decrease of V_t could be observed. We speculate that this is due to the PDB-induced decrease of the basolateral K^+ conductance which makes E_b more susceptible to changes in the Cl^- conductance. (c) The ionomycin-induced increase of V_t was transient after incubation with PDB. This would be the consequence of the inhibition of the ionomycin-activated basolateral K^+ conductance by PDB [7].

PDB and carbachol have a less than additive effect

The response to carbachol after incubation with PDB did not differ from the response to ionomycin after PDB. However, without preincubation with PDB, their effects did differ in that the ionomycin effect lacks the third-phase depolarization [5]. Thus, after PDB, the third phase of the carbachol response seems to be absent. This may be because PDB has already activated the pathway that is normally activated by carbachol in its third phase. In other words, we postulate that the PDB effect on the apical membrane and the third phase of the carbachol response are mediated by the same PKC pool. Support for this hypothesis comes from comparison of the effect of carbachol after forskolin and after PDB. In experiments in which carbachol was added after forskolin we found rather large effects of carbachol on the increase of V_t (6.0 ± 0.6 mV [5]). In contrast, after PDB, the response of V_t to carbachol was only 2 mV or about 33% of its reponse after forskolin (see Table 4). The smaller effect is not primarily due to the inactivation of the basolateral K^+ conductance because ionomycin after PDB induced the same increase of V_t as after forskolin (see Tables 2 and 3) and carbachol itself can induce the activation of Ca^{2+}-dependent basolateral K^+ conductance. Further support for the hypothesis is that recent biochemical studies showed that a particular PKC isotype, PKCα plays a crucial role in PDB and carbachol-triggered Cl^- secretion in HT-29cl.19A cells [24].

Do PKA and PKC activate the same Cl^- channels?

Table 5 A and B shows that PDB in the presence of forskolin, or forskolin plus ionomycin, induced smaller increases of the short-circuit currents then when added alone (22 ± 3 μA/cm^2 [7]). Thus PDB plus forskolin had a less than additive effect. Among other explanations, this may be due to a rate-limiting effect of the K^+ conductance in the basolateral membrane (in the experiments in Table 5 A) or to metabolic constraints (in the experiments in Table 5 B). Thus, the results of short-circuit measurements can not be used to reject the possibility that the activation of Cl^- channels by PKA and PKC can be additive or even synergistic.

In fact, a comparison of the conductances of the apical membrane in the presence of forskolin or PDB or PDB plus forskolin (Table 6) shows that PDB plus forskolin can induce a larger decrease of the apical resistance than expected from their individual effects. This can only be explained if the PKA and PKC pathways can converge on the same type of channels and increase their total conductance above the level reached by PKA or PKC alone. The results of patch-clamp studies presented as an abstract [6] and in the accompanying paper [8] suggest that, indeed, PKA and PKC can act synergistically on the same Cl^- channel. However, our results can not exclude the possibility that PKA and PKC can also activate different parallel Cl^- channels.

A schematic summary of the postulated activation and inhibition of channels is presented in Fig. 9.

The relevance of studies with cell lines

The difficulty with the results of experiments with cell lines is that one can not be sure that the phenomena

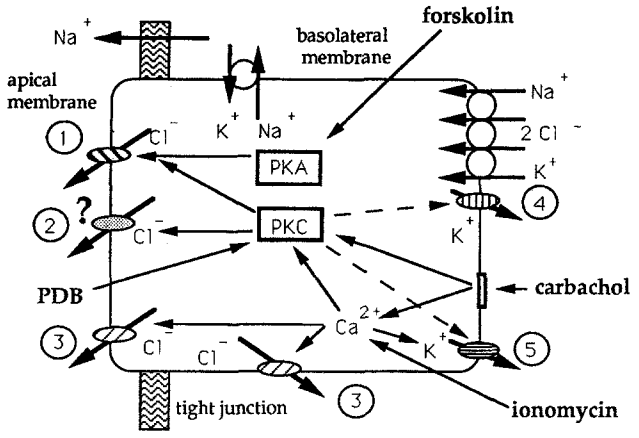

Fig. 9. Schematic summary of postulated activation and inhibition of Cl^- and K^+ channels in HT-29cl.19A cells. Different filling of ovals indicates different types of ion channels. *Thick arrows*, direction of ion movement; *thin and plain arrows*, activation; *dashed arrows*, inhibition; *arrow pointing to arrowhead*, synergistic modulation. *Numbers in circles*: *1*, cAMP-dependent Cl^- channel; *2*, putative Cl^- channel activated by PKC (the presence of such a channel can not be excluded yet); *3*, Ca^{2+}-dependent transient Cl^- channel; *4*, spontaneously active basal K^+ channel; *5*, Ca^{2+}-dependent K^+ channel. *PKA*, cAMP-dependent protein kinase (cAMP being increased by direct activation of adenylyl cyclase by forskolin); *PKC*, protein kinase C (activated by PDB or by increase of diacylglycerol, induced by carbachol). *Ca^{2+}* indicates the Ca^{2+} pathway, which presumably uses multifunctional Ca/calmodulin kinase II [25, 29] (activity of Ca^{2+} increased by the Ca^{2+} ionophore ionomycin or via carbachol)

occurring are of relevance for the behaviour of the original tissue. For instance, in the HT-29cl.19A cells secretion can be induced by addition of ionomycin and phorbol esters; the latter had no effect on a T_{84} clone that we and others [26] have used. In isolated intestine, the effects of phorbol esters and Ca^{2+} ionophores may be largely due to generation of arachidonic acid metabolites in the lamina propria [20], suggesting a difference between the HT-29cl.19A cell line and intestine. However, recently it has been shown that PDB could increase the short-circuit current in isolated human small intestine (but not in the colon) in the presence of inhibitors of the arachidonic-acid-degradative pathways (De Jonge et al., in preparation). The refractoriness of T_{84} cells to phorbol esters is not a uniform finding. For instance, recently [16] it has been shown that in T_{84} cells the phorbol ester, phorbol myristate acetate, had no effect on the short-circuit current but that it could potentiate the effect of Ca^{2+}-dependent- but not cAMP-dependent secretagogues when PMA was added 10 min or less before the secretagogue. This may be of relevance to the observation that the I_{sc} induced by Ca^{2+} ionophores in T_{84} cells is very small [13, 27, 28] and that the ionophores have a much smaller $\Delta I_{sc}/\Delta Ca_i^{2+}$ ratio than carbachol. This suggests that the carbachol-induced effect in T_{84} cells is triggered by other mechanisms (presumably some form of PKC) in addition to increased intracellular Ca^{2+}. In other clones of the T_{84} cells, similar to the HT-29cl.19A cells, phorbol esters increased the Cl^- current even in the absence of Ca^{2+} ionophores and their effect was apparently PKC-mediated [17]. We postulate that a possible clue to the different responses to phorbol esters observed between T_{84} cells and HT-29cl.19A cells and probably among clones of T_{84} cells grown in different laboratories [16, 17, 26] may be our observation that the expression level of PKCα can differ drastically between HT-29cl.19A cells and a PDB-nonresponsive T_{84} cell clone (Fig. 8).

The results of this study may be placed in the perspective of the recent findings on the ion-channel defect involved in the genetic disease cystic fibrosis. An interesting difference between intestine and airway epithelia is that in cystic fibrosis intestine the response to carbachol is strongly attenuated, while it is still present in airway epithelium [19]. The present study and earlier work [5] suggest that the Ca^{2+}-activated Cl^- channels in intestine can not be involved in transepithelial net Cl^- transport, and that PKC may activate the same Cl^- channel as PKA, which is defective in cystic fibrosis. Thus the two intracellular pathways that are activated by carbachol can not lead to activation of the secretion of Cl^- in the cystic fibrosis intestine. In addition, recently it has been reported that the Cl^- conductance in the apical membranes of nystatin-treated airway cells, but not T_{84} cells and HT-29cl.19A cells, can be increased by ionophores [1]. We observed, however, that the nystatin treatment, as used by these authors, inhibits the rapid depolarization of V_a by carbachol and ionomycin in the HT-29cl.19A cells (Bajnath and Groot, unpublished observations). The dissimilar susceptibility of the Cl^- conductance in airway cells and intestinal cells to nystatin treatment suggests that the Ca-dependent Cl^- channels in these cells may be different.

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