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ORIGINAL ARTICLE

R. B. Bajnath · K. Dekker · H. R. De Jonge
J. A. Groot

Chloride secretion induced by phorbol dibutyrate and forskolin in the human colonic carcinoma cell line HT-29Cl.19A is regulated by different mechanisms

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Abstract The human colonic carcinoma cell line HT-29cl.19A responds to the protein kinase C activator PDB (4-β-phorbol 12,13-dibutyrate), as it does to forskolin (an activator of adenylyl cyclase), with a secretory response when the cells are grown on filters and studied at 36 °C. Previously, we showed that when cells were grown on Petri dishes and studied at about 25 °C with the cell-attached patch-clamp technique, forskolin, but not PDB, could activate 8-pS chloride channels (cystic fibrosis transmembrane conductance regulator, CFTR, channels). The present work was carried out to study this discrepancy. Experiments in Ussing chambers, at different temperatures, showed that the responses to PDB and forskolin differ in their temperature sensitivity. This was also found following conventional microelectrode and Ussing chamber studies with nystatin-permeabilized epithelial layers carried out at 25 °C and at 36 °C. Pre-incubation with the microtubular disruptive agents nocodazole or colcemid did not affect the response to PDB or forskolin, suggesting that chloride secretion induced by these agonists in these cells is independent of the microtubular structure. Pre-incubation with brefeldin A strongly inhibited the response to PDB, but the response to forskolin was hardly affected. The differing effect of temperature and brefeldin A on the responses to forskolin and PDB may be due to the activation of two distinct mechanisms by protein kinases A and C.

Key words HT-29cl.19A · Human colon cell line · Temperature · Brefeldin A. Nocodazole · Phorbol ester · PDB · Forskolin · Nystatin · CFTR

R. B. Bajnath (✉) · K. Dekker · J. A. Groot
Institute of Neurobiology, University of Amsterdam, Faculty of Biology, Kruislaan 320, 1098 SM Amsterdam, The Netherlands

H. R. De Jonge
Department of Biochemistry, Erasmus University, Rotterdam, The Netherlands

Introduction

Transepithelial chloride secretion in epithelial cells is critically dependent on the activation of chloride channels in the apical membrane. The most prominent chloride channel in salt-secreting epithelia appears to be the product of the CF-gene called the cystic fibrosis transmembrane conductance regulator (CFTR), which is regulated principally by adenosine 3′-5′-cyclic monophosphate (cAMP).

From studies with CFPAC cells and human airway epithelial cells it has been suggested that activation of CFTR by cAMP-dependent protein kinase (PKA) regulates plasma membrane recycling via inhibition of endocytosis [7, 20] and stimulation of exocytosis. A similar observation has been reported for the T-84 colonic carcinoma cell line, where stimulation with forskolin was found to inhibit the endocytosis of fluid-phase markers [6] and to stimulate vesicle migration to the plasma membrane [21].

Whereas much attention has been paid to the regulation of CFTR by PKA, studies regarding the mechanism by which protein kinase C (PKC) stimulates chloride secretion are very scarce. In previous studies, using the conventional microelectrode technique applied to confluent and filter-grown monolayers of HT-29cl.19A cells studied at 36 °C in an Ussing-type chamber, we found that addition of 4-β-phorbol 12,13-dibutyrate (PDB) or forskolin caused an increase in the chloride conductance of the apical membrane [2,3]. When forskolin and PDB were added after each other, they acted synergistically on apical membrane conductance [4]. With the cell-attached patch-clamp technique performed at 25 °C on Petri-dish-grown cells, we found that forskolin activated the CFTR chloride channel. Interestingly, a larger number of activated CFTR chloride channels could be observed when PDB was added after forskolin or when forskolin was added after PDB [5]. This may explain the potentiating effect of PDB, as observed in the microelectrode studies.

However, when PDB was added alone, no CFTR chloride channel activity could be observed. This was in strong contrast to the PDB-induced effects observed using microelectrodes in Ussing chamber studies. Differences between the microelectrode studies and the patch-clamp studies were such that the latter were performed at about 25 °C and with cells grown in Petri dishes. So, the different results in patch-clamp and microelectrode studies could be due to growth conditions or to the experimental temperature. In the present study we have attempted to discriminate between these possibilities and we have found that the temperature sensitivity of the PDB response between 20 °C and 30 °C was larger than that of the forskolin response, so that at about 25 °C PDB appeared to be almost without effect on the transepithelial potential (V_t) and intracellular potential (V_a).

Microtubules are thought to play a role in protein transport and incorporation and withdrawal of proteins from the membrane. By analogy with the postulated effects of PKA mentioned above, it might be that PKC acts by stimulating incorporation of chloride channels into the plasma membrane. Therefore, we studied the influence of substances known to interfere with the microtubules. Although microtubular-disruptive agents, e.g. nocodazole and colcemid, appeared to interfere with the basal secretory state of the cells, we could not find evidence supporting a major role of the microtubules in activation by PDB or forskolin of the CFTR chloride channel in the HT-29cl.19A cell type. In contrast, brefeldin A, a fungal metabolite known to block transport of newly synthesized proteins out of the endoplasmic reticulum, suppressed the effect of PDB on the chloride conductance, but pre-incubation with brefeldin A appeared to be without effect on the characteristic ability of forskolin to increase the chloride conductance.

Materials and methods

Cell culture

HT-29cl.19A cells [2] (passage nos. 20–27) were grown on 25-mm diameter permeable Falcon filters (Becton Dickinson Labware, N.J., USA) to confluency, which was reached 7 days after seeding; cells were used when they were 10–19 days old. In previous studies no age-related differences of the response to forskolin or PDB of these cells were found. Maintenance and subculturing of the cells were carried out as described previously [2].

Microelectrode studies

For microelectrode experiments, confluent monolayers were mounted horizontally in a small Ussing-type chamber, leaving a oblong area of 0.35 cm^2. The upper (apical) and lower (basolateral) compartments were continuously perfused (polystaltic pump, Buchler Instruments, Fort Lee, N.J., USA) with solutions maintained at the desired temperatures (see below) and gassed with 5% CO_2/95% O_2. V_t was measured with Ringer/agar bridges, which were connected to Ag–AgCl electrodes. V_a was measured using glass microelectrodes filled with 0.5 mol/l KCl which were connected to a high input impedance amplifier (World Precision Instruments, New Haven, Conn., USA). All measurements were performed under open-circuit conditions and the apical solution was used as the reference for transepithelial and intracellular measurements. The effect of added chemicals was studied during simultaneous recordings of V_a and V_t.

To calculate resistances, bipolar current pulses of 1 s duration ($\pm 10\,\mu A$ and $\pm 50\,\mu A$) were injected via Ag–AgCl electrodes located in the walls of the upper and lower compartments. From the voltage deflections of V_a and V_t induced by these current pulses, the transepithelial resistance (R_t) and the fractional resistance of the apical membrane ($fR_a = R_a/(R_a+R_b)$ were calculated. Corrections for solution resistance and for the potential offset of the electrodes were made by measuring the potential difference and current-induced voltage deflections between the electrodes before mounting and after removal of the filter. The microelectrodes had tip resistances of between 100 MΩ and 200 MΩ and the tip potentials were about −2 mV.

Temperature

Ringer's solutions were brought to the desired temperatures by a heated waterbath and were maintained at these temperatures during flow through water-jacketed tubes and reservoirs. The temperature of the Ringer's solution in the experimental chamber was continuously monitored using a digital thermometer.

Solutions

Experiments were carried out with a standard Ringer solution of 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). Chloride-free solutions were prepared by replacing all NaCl, except for 0.1 mmol/l, by Na gluconate.

Short-circuit current (I_{sc}) measurements with nystatin-treated monolayers

For measurements of chloride current, monolayers of HT-29cl.19A cells were grown on permeable polycarbonate filters (6.4 mm diameter, Transwell, Costar). HT-29cl.19A cells grown on these filters respond similarly to forskolin or PDB, as compared to cells grown on Falcon filters. The exposed area of the filter was 0.35 cm^2. The basolateral side of the filter was bathed in a buffer solution containing (in mmol/l): NaCl 105, KCl 4.7, $CaCl_2$ 1.3, $MgCl_2$ 1.0, $NaHCO_3$ 20.2, Na_2HPO_4 0.33, 4-(2-hydroxyethyl) 1-piperazine-ethanesulphonic acid (HEPES) 10, glucose 10. The buffer solution at the apical side had the same composition, except for NaCl and KCl which were replaced by equal amounts of Na gluconate and K gluconate, respectively. Before stimulation with PDB or forskolin, 0.36 g/l nystatin was added to the basolateral solution, while V_t was clamped at 0 mV.

Chemicals

All chemicals including forskolin, PDB, colcemid, nocodazole and brefeldin A were purchased from Sigma, St Louis, Mo., USA. Cell culture materials were purchased from GIBCO.

Statistics

The paired or unpaired *t*-test was used to evaluate statistical significance.

Results

The effects of PDB and forskolin on V_t and R_t were compared at different temperatures. Each filter was exposed to only one temperature, and forskolin or PDB (usually followed by PDB or by forskolin, respectively) were applied only once. Figure 1 shows the relative temperature dependency of the PDB- and the forskolin-induced equivalent I_{sc} (eq. I_{sc}). The effect of PDB at 25 °C was only 13% of its effect at 36 °C, whereas the effect of forskolin at 25 °C was about 33% of its effect at 36 °C. The effect of forskolin increased monotonously with temperature. In contrast, between 20 °C and 25 °C the effect of PDB was rather small, but increased rapidly as the temperature was raised to 36 °C. The time needed to reach the maximal change (see Table 1) was shorter for forskolin, except at 20 °C. At this temperature, the time course of the response to both secretagogues was nearly the same, but the effect of forskolin was much larger.

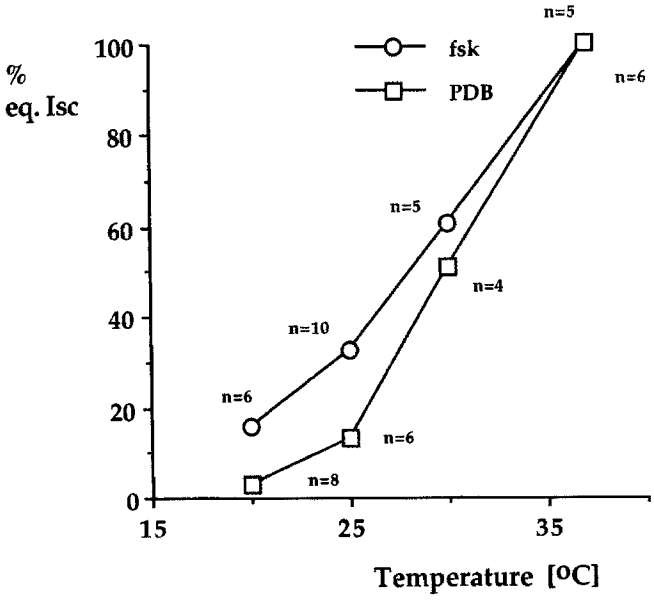

Fig. 1 Plot of relative equivalent short-circuit current (eq. I_{sc}) against temperature (°C) induced by application of 4-β-phorbol 12,13-dibutyrate (*PDB*, 10^{-6} M) and forskolin (*fsk*, 10^{-5}M). Values represent mean ± SEM for the number (n) of experiments as indicated at different temperatures. Values of SEM are smaller than the symbols used. Eq. I_{sc} was calculated from transepithelial potential (V_t) and the transepithelial resistance (R_t). The maximal I_{sc} induced by forskolin or PDB at 36 °C was 31.3 ± 6 μA/cm² and 21.2 ± 5 μA/cm², respectively. These values are not significantly different ($P > 0.1$, unpaired t-test)

Table 1 shows the changes in eq. I_{sc} induced by the secretagogues alone and in combination. PDB addition after forskolin induced a larger change than when added alone. The effect of addition of forskolin after PDB was strongly reduced at 36 °C, due to the described effect of PDB on the basolateral K^+ conductance [3]. At the other temperatures, the effect on eq. I_{sc} of addition of forskolin after PDB was not different to the effect of when it was added alone.

I_{sc} measurements in nystatin-treated monolayers at 25 °C and at 36 °C

I_{sc} is not only dependent on the apical membrane chloride conductance, but also on the basolateral K^+ conductance and the Na/K/Cl-cotransporter and Na/K pump. To test the possibility that the difference in temperature sensitivity of the PDB and forskolin effects are due to differences in basolateral membrane characteristics, we permeabilized the basolateral membrane with nystatin. Monolayers were voltage-clamped at 0 mV and exposed to nystatin on the basolateral side in the presence of a chloride concentration gradient at 25 °C or at 36 °C. Addition of PDB at 25 °C increased I_{sc} by only 1.2 ±0.6 μA/cm² (mean ±SEM, $n = 9$).

Table 1 Comparison of the effects of forskolin and 4-β-phorbol 12,13-dibutyrate (PDB) at different temperatures on the equivalent short-circuit current (change of eq. I_{sc} in μA/cm²). Values were taken at the maximum of the response, which was reached at the time indicated. All values are mean ± SEM. Number of experiments in *parentheses*

Temperature (°C)	Change in eq. I_{sc} (μA/cm²) under the following conditions:			
	Forskolin	PDB	PDB after forskolin	Forskolin after PDB
20	5.0 ± 1.6(6)	2.0 ± 0.7(8)	4 ± 1 (6)a	4.7 ± 1.2 (8)
Time to maximum (min)	38 ± 4	39 ± 4	30 ± 1.4	19 ± 1.8
25	10 ± 2 (10)	2.8 ± 1.2 (6)	15 ± 5 (8)[a]	12 ± 5 (6)
Time to top (min)	5.4 ± 0.4	15 ± 0.6	9.4 ± 0.4	4.0 ± 0.3
30	17 ± 6 (5)	11 ± 1 (4)	nd	nd
Time to top (min)	3.0 ± 0.3	8.0 ± 0.3		
36	31 ± 6 (5)	21 ± 5 (6)	39 ± 10 (5)	13 ± 1 (5)[b]
Time to top (min)	3.0 ± 0.2	6.0 ± 0.5	2.2 ± 0.1	1.7 ± 0.3

[a] Different from effect of PDB alone $P < 0.05$,
[b] Different from effect of forskolin alone $P < 0.05$

Consecutive stimulation with forskolin increased I_{sc} by 54.3 ±4.1 μA/cm² (mean ±SEM, $n = 9$), indicating a chloride flux from the basolateral to the apical side. Forskolin, when added alone, increased I_{sc} by 23.2 ± 4.1 μA/cm² ($n = 6$). In two experiments at 36 °C, PDB increased I_{sc} by 45 μA/cm² and by 37 μA/cm², respectively. Application of forskolin alone increased I_{sc} by 87 ± 5 μA/cm² ($n = 7$) at 36 °C. The PDB- or forskolin-induced I_{sc} was not inhibited by bumetanide or barium at the basolateral side, indicating that nystatin effectively permeabilized the basolateral membranes. Thus, even when effects via the basolateral membrane were ruled out, PDB appeared to have a negligible effect at 25 °C, but when forskolin was applied after PDB, the increase of the apical chloride conductance was potentiated as in patch-clamp experiments carried out at 25 °C. The nystatin experiments suggest that the absence of synergism of forskolin application after that of PDB on the eq. I_{sc} at 20 °C and 25 °C, as shown in Table 1, may be due to the limited conductance of the basolateral membrane.

Microelectrode experiments at 25 °C or 36 °C

Figure 2A,B shows a comparison between the effects of PDB and forskolin when added separately and when added consecutively on V_a and fR_a at 25 °C and at 36 °C. At 25 °C, PDB had no significant ($P > 0.1$) effect on V_a (Fig. 2A) or fR_a (Fig. 2B), but at 36 °C PDB induced a depolarization of V_a and a decrease of fR_a of a magnitude comparable to results reported previously [3]. In contrast, forskolin induced a depolarization of V_a of similar magnitude at both temperatures (Fig. 2A). The decrease of fR_a at 36 °C was significantly ($P < 0.001$) larger than at 25 °C. PDB addition after forskolin decreased fR_a in every experiment at both temperatures. Addition of forskolin after PDB induced a further depolarization of V_a and a further decrease of fR_a.

The different temperature sensitivity of the responses to PDB and to forskolin suggests that these agonists exert their effects via activation of different mechanisms. One possibility may be that PKC acts primarily by stimulating the transport and incorporation of channel proteins in the apical membrane and that these channels are opened by spontaneously active PKA. Both membrane incorporation and the number of spontaneously active PKA molecules will be less at lower temperatures and it may be that the smaller amount of endogeneously activated PKA and the smaller number of channels decrease the chance of interaction and activation. To investigate the possible involvement of protein transport as a part of the PDB effect, experiments were carried out with nocodazole and brefeldin A. These agents are known to interfere with membrane protein recruitment processes in some, but not all, cell types [8, 17, 18].

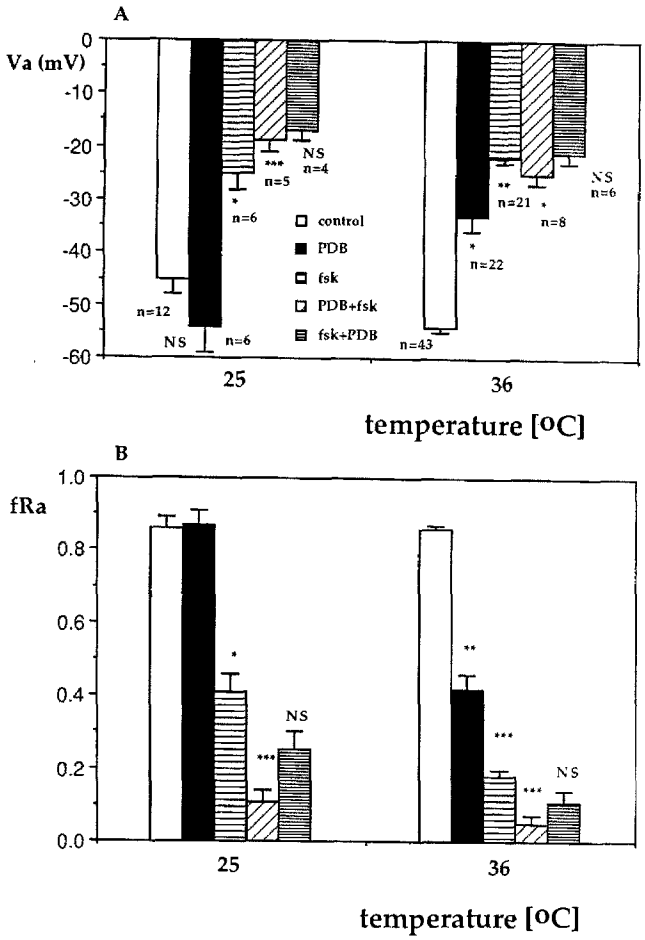

Fig. 2 Membrane potential (V_a) (**A**) and fractional resistance of the apical membrane (fR_a) (**B**) showing the effects of (10^{-6} M) PDB or (10^{-5} M) forskolin separately and when added sequentially at two different temperatures. $*P < 0.01$, $**P < 0.001$, $***P < 0.0001$. Values represent mean ± SEM for (n) experiments

Effect of nocodazole at 36 °C

Table 2 shows that after incubation with nocodazole for 1–7 h (4 ±0.3 h, mean ±SEM, $n = 11$) one could observe a significant increase of V_t, a depolarization of V_a and decrease of fR_a with respect to control values. As a consequence, eq. I_{sc} was significantly increased. A plot of V_t against V_a of nocodazole-treated monolayers (not shown) yielded a linear relationship with a slope which was different ($P < 0.001$) from zero, whereas no relationship between V_t and V_a was found in untreated monolayers. This suggests that the larger V_t after nocodazole treatment is related to the depolarization of V_a. Similar effects were also found when monolayers were pre-incubated with colcemid, another frequently used microtubule-disruptive agent (results not shown).

Table 3 shows a summary of the changes induced by PDB or forskolin in control conditions and after incubation with nocodazole. The PDB- or forskolin-induced changes were usually smaller than without pre-treatment with nocodazole, but the membrane

Table 2 Electrical parameters of the cells under control conditions or after incubation with nocodazole or brefeldin A. (V_t transepithelial potential, V_a apical membrane potential, R_t transepithelial resistance, fR_a fractional apical membrane resistance, N number of experiments).Values shown are mean ± SEM. Monolayers were treated with either 33 μM nocodazole for 1–7 h or with 1–10 μg/ml brefeldin A for 1–8 h. During experiments the same concentrations of the drugs were present in the apical and the basolateral perfusate

Condition	Parameter					
	V_t (mV)	V_a (mV)	R_t (Ωcm^2)	fR_a	eq. I_{sc} ($\mu A/cm^2$)	N
Control	0.8 ± 0.1	−54 ± 1	150 ± 9	0.86 ± 0.01	6 ± 1	49
Nocodazole	2.5 ± 0.5**	−48 ± 3*	157 ± 13	0.62 ± 0.06**	17 ± 4**	11
Brefeldin A	0.6 ± 0.1	−55 ± 2	171 ± 14	0.82 ± 0.02	4 ± 1	17

* $P < 0.01$, ** $P < 0.001$, Unpaired t-test

Table 3 Comparison of changes in V_a, fR_a and I_{sc} induced by forskolin (*fsk*) (10^{-5} M) or PDB (10^{-6} M) under control conditions and after pre-incubation with 33 μM nocodazole for 1–7 h. Values shown are mean ± SEM. Changes induced by PDB or fsk without or with nocodazole pre-incubation were not significantly different from each other; $P > 0.1$, unpaired t-test. Data with respect to nocodazole pre-incubation are from Table 2

Condition	Parameter						
	V_a (mV)	ΔV_a (mV)	fR_a	ΔfR_a	eq. I_{sc} ($\mu A/cm^2$)	Δeq. I_{sc} ($\mu A/cm^2$)	N
Control	−55 ± 2		0.85 ± 0.01		5 ± 1		13
Fsk	−25 ± 2	30 ± 3	0.23 ± 0.03	0.62 ± 0.02	34 ± 3	29 ± 3	11
PDB	−32 ± 3	23 ± 4	0.41 ± 0.01	0.44 ± 0.01	24 ± 2	19 ± 2	4
Nocodazole (1–7 h pre-incubation)	−48 ± 3[a]*	7 ± 4	0.62 ± 0.06[a]**	0.23 ± 0.06	17 ± 4[a]**	12 ± 4	11
Nocodazole + Fsk	−26 ± 5	22 ± 6	0.18 ± 0.04	0.44 ± 0.07	36 ± 3	19 ± 5	5
Nocodazole + PDB	−31 ± 2	17 ± 4	0.30 ± 0.05	0.32 ± 0.08	35 ± 6	18 ± 7	6

* $P < 0.01$, ** $P < 0.001$ unpaired t-test
[a] Comparison with control values

potential, the fractional resistance and eq. I_{sc} were not significantly different.

Effect of brefeldin A at 36 °C

Incubation for 1–8 h (4 ± 0.7 h, mean ± SEM, $n = 17$) of monolayers with brefeldin A (1–10 μg/ml) had no effect on basal electrical parameters (see Table 2). The effects of forskolin on V_a, fR_a or I_{sc} after pre-incubations with brefeldin A (Fig. 3A–C) were not significantly affected as compared with controls. In contrast, all PDB-induced changes were significantly smaller, although the responses were qualitatively unaltered. Pre-incubation with brefeldin A strongly suppressed the PDB-induced increase of I_{sc} after treatment with forskolin (Fig. 4).

Prolonged incubations (22–24 h) of monolayers with brefeldin A (1 μg/ml) further reduced the effect of PDB on V_a (Fig. 5), but, in addition, strongly reduced the R_t of the monolayer, making calculations of fR_a and I_{sc} rather uncertain. In two experiments carried out on separate filters incubated for 21 h with brefeldin A, forskolin induced a depolarization of V_a by 35 mV from a membrane potential of −47 mV and by 31 mV from a membrane potential of −43 mV, respectively. We tested whether the depolarization of the apical membrane induced by forskolin after this long treatment with brefeldin A was due to the opening of chloride channels by lowering the chloride concentration at the apical side from 127 mmol/l to 0.1 mmol/l. This induced a further depolarization of V_a by 32 mV and by 38 mV, respectively (after correction by −9.5 mV for the liquid junction potential across the apical salt bridge). These changes are of similar magnitude to those without brefeldin A treatment [3]. This differs from the absence of effect of apical chloride replacement in the presence of PDB after brefeldin A pre-incubation (Fig. 5). Figure 5 also shows that subsequent addition of forskolin reversed V_a to +27 mV. The absence of an effect of apical chloride replacement after PDB and brefeldin A treatment contrasts with the effect when the cells were not treated with brefeldin A. In two experiments, PDB depolarized V_a by 21 mV from a membrane potential of 51 mV and by 20 mV from a membrane potential of 65 mV. Subsequent lowering of the apical chloride concentration further depolarized V_a by 15 mV and by 13 mV (corrected for the liquid junction potential). From these experiments it can be deduced that the PDB-induced depolarization after brefeldin A treatment was not due to activation of apical chloride channels. The cause of the PDB-induced depolarization after prolonged brefeldin A incubation remains to be investigated.

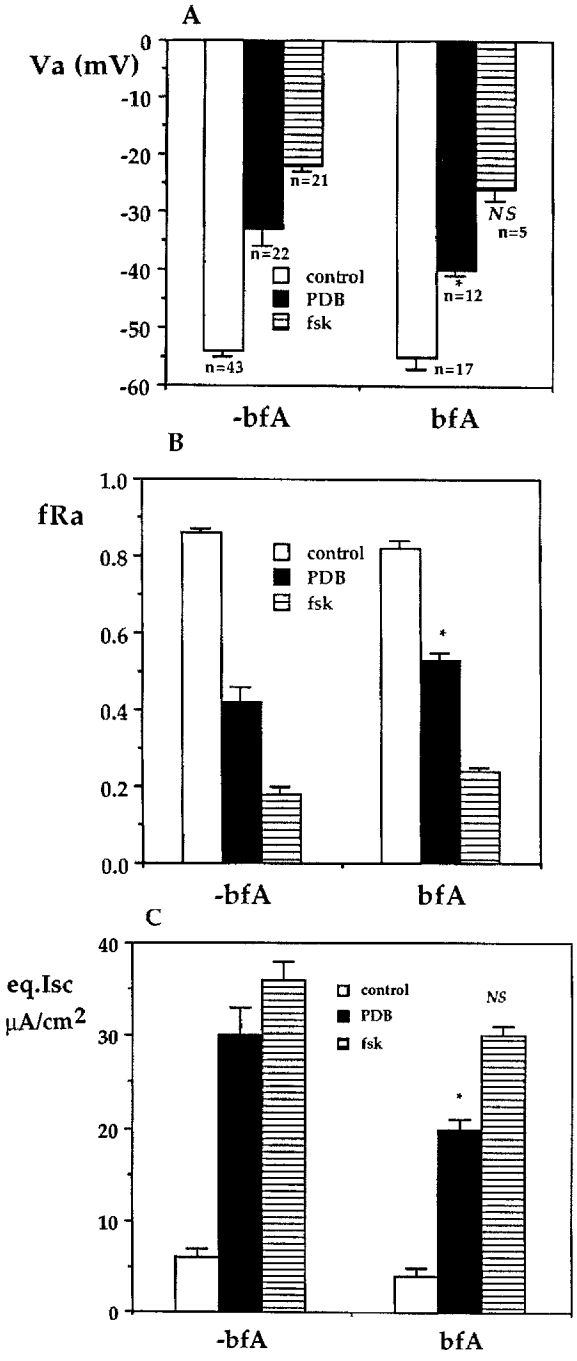

Fig. 3 Effect of pre-incubation with brefeldin A (*bfA*, 1 μg/ml) on the (PDB-and the forskolin-induced changes of V_a (**A**) f*Ra* (**B**) and eq. I_{sc} (**C**) $^*P < 0.02$, NS = not significantly different from values without brefeldin A pre-incubation. Values represent mean ± SEM for (*n*) experiments

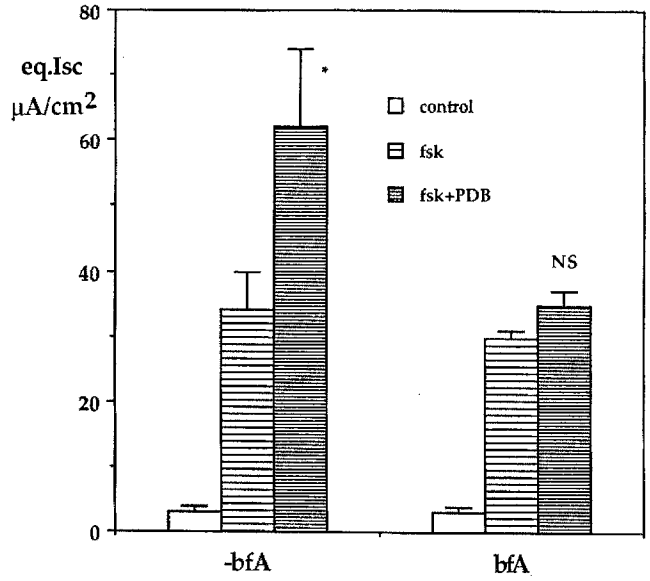

Fig. 4 Inhibitory effect of brefeldin A (*bfA*, 1 μg/ml) pre-incubation (1–7 h) on the increase of eq. I_{sc} evoked by PDB when added after stimulation with forskolin. $^*P < 0.05$, NS = not significantly different from values in the presence of forskolin alone. Values represent mean ± SEM for 5 experiments in the absence of brefeldin A and for 4 after pre-incubation with brefeldin A

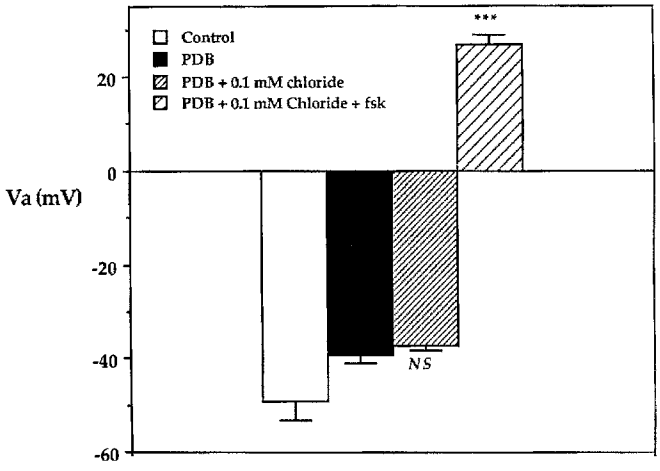

Fig. 5 Membrane potentials after prolonged (22–24 h) incubation with brefeldin A without (control) and after addition of 10^{-6} M PDB. The *third bar* shows the lack of effect of lowering the chloride concentration to 0.1 mmol/l in the apical compartment (chloride replaced by gluconate) in the presence of PDB and the large depolarization (*fourth bar*) after addition of 10^{-5} M forskolin. $^{***}P < 0.0001$, NS = not significantly different from values in the presence of control solution. Values represent mean ± SEM for 6 experiments

Discussion

The present work was carried out as an attempt to find an explanation for the discrepancy of the PDB response between microelectrode studies at 36 °C with filter-grown monolayers and patch-clamp studies with cells grown in Petri dishes and studied at 25 °C. The main findings are that forskolin- and PDB-induced chloride secretion show different temperature sensitivity and a different sensitivity to brefeldin A.

Effect of temperature

Eq. I_{sc} at 25 °C or 36 °C with nystatin in the basolateral compartment showed that forskolin can increase

I_{sc} at both temperatures. Nystatin selectively eliminates the contribution of the basolateral membrane to the transcellular resistance, therefore the measured current across the monolayer reflects the chloride current flowing through activated apical chloride channels, driven by the experimental chloride concentration gradient, from the serosal to the mucosal side [1]. In contrast, PDB failed to increase the current at 25 °C, but potentiated the forskolin-induced I_{sc}. As expected from microelectrode studies, at 36 °C PDB increased I_{sc} in the nystatin experiments as well. The almost complete absence of chloride current at 25 °C after stimulation with PDB and the large increase of the current in the presence of PDB plus forskolin fully agree with our earlier findings from cell-attached patch-clamp studies and indicate that, at the lower temperature, application of PDB does not lead to activation of chloride channels in filter-grown cells. This is similar for cells grown in Petri dishes and indicates that the absence of chloride channel activation by PDB in the patch-clamp studies was not due to different growth conditions. This is in agreement with the results of the microelectrode studies performed at 25 °C (Fig. 2A,B). It is hard to explain the changes in eq. I_{sc} measured at 20–25 °C (Fig. 1, Table 1) as being due to PDB-induced chloride currents, because PDB did not change V_a at 25 °C and had hardly any effect on the chloride conductance in the nystatin experiments. The nature of this current remains to be studied.

Activation of the eq. I_{sc} by forskolin shows a more or less linear temperature dependency, with still appreciable activation at lower temperatures. Similarly, in T-84 cells, the mechanism appears not to be very temperature sensitive, as Denning et al. [9] have shown appreciable activation even at 5 °C.

Effect of cytoskeletal disruptive agents

To test a possible role of vesicle insertion in the mechanism of CFTR activation in HT-29cl.19A cells, the cytoskeletal architecture was perturbed by the microtubular agonists nocodazole or colcemid. It has been reported that, by depolymerizing the microtubuli, the incorporation of vesicles in the apical membrane can be inhibitied in some, but not all, cells [17, 18].

In the HT-29cl.19A cells these agents appeared to have an effect on the apical membrane conductance. The decrease of fR_a, the depolarization of V_a and the increase of V_t and of eq. I_{sc} after treatment with nocodazole can be interpreted as a consequence of a larger number of basally active chloride channels in the apical membrane. This hypothesis and the possible reason for increased chloride channel activity have not yet been studied.

In contrast to our observations that the electrophysiological effects of secretagogues were not inhibited, Fuller et al. [11] reported that, in T-84 cells, pre-incubation with nocodazole or colchicine partially inhibited the forskolin-induced [$^{125}I^-$] efflux. The T-84 cells were used after 3–5 days culture on plastic plates; it may be that this made the cells less polarized than the HT-29cl.19A cells used in the present study. As it appears that the inhibition of targeted transport by nocodazole is more effective in less polarized cells [12, 22], the degree of polarization may account for the differing sensitivity.

Experiments with brefeldin A

Brefeldin A has been reported to block the vesicular transport of proteins between the endoplasmic reticulum and the Golgi apparatus [14, 15], thus preventing the transport of newly synthesized proteins and membrane transporters [8] to the plasma membrane.

In this study we focused on the difference between the PDB- and the forskolin-induced effects. We grouped the brefeldin A experiments together because all incubation periods were present in the forskolin as well as in the PDB experiments. Brefeldin A reduced the effect of PDB when added alone and after forskolin. This inhibition required a pre-incubation of at least 1 h and the inhibition became more pronounced after prolonged incubations, leading to a total absence of chloride channel activation by PDB. In contrast, brefeldin A had no significant effect on the forskolin-evoked depolarizations. We conclude that, in this laboratory, the activation of apical chloride channels by forskolin was not strongly affected by prolonged (22–24 h) or short-term (1–7 h) incubations with brefeldin A. This is in contrast with a recent publication [16], in which it was shown that a 12-h incubation with brefeldin A inhibits the cAMP-induced I_{sc} in the same clone. In our laboratory, prolonged incubation with brefeldin A strongly reduced the monolayer R_t, making it impossible to measure I_{sc}. However, even then, forskolin was still able to induce a large depolarization of V_a.

Our results suggest that cAMP-induced activation of chloride secretion need not be fully dependent on the translocation of chloride channels from a cytoplasmic pool into the plasma membrane. This postulate is in agreement with results from immunofluorescence observation by Prince et al. [19] and Morris et al. [16]. These authors showed that the CFTR chloride channel was already present in the plasma membrane of T-84 cells and HT-29cl.19A cells under resting conditions and that stimulation with cAMP agonists did not further increase the amount of fluorescence in T-84 cells. Very recently, Huflejt et al. [13] showed that forskolin hardly increased GAG (Glycosaminoglycan) secretion in T-84 cells, suggesting that exocytosis is not the most important part in the sequence of forskolin-induced chloride secretion.

To explain our finding that PDB can activate chloride channels at higher but not at lower temperatures,

we postulate that activation of PKC induces an increase in the number of channels in the membrane. This will increase the probability that these channels are activated by endogenously active PKA. If this postulate is correct, the effect of PDB would be dependent on the endogeneous activity of PKA, which is dependent on the temperature and of course on the cytoplasmic level of cAMP. Such a mechanism may explain findings reported in literature of increased secretion without increased cAMP levels [10]. The synergistic effect of PDB and forskolin on the apical membrane conductance ([4, 5] this report) may be explained by postulating that: (1) PKC can increase the number of chloride channels in the membrane; and (2) the increased number of channels in the membrane can be activated by the much more active PKA. This will lead to a much larger apical membrane conductance than following treatment with forskolin or PDB alone. The reason for the lack of chloride current when PDB was added alone at the lower temperature may be that the endogenous PKA activity at these temperatures is too low to activate the channels in the apical membrane.

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