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Biphasic Increase of Apical Cl^- Conductance by Muscarinic Stimulation of HT-29cl.19A Human Colon Carcinoma Cell Line: Evidence for Activation of Different Cl^- Conductances by Carbachol and Forskolin

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Summary. The modulation of ion transport pathways in filter-grown monolayers of the Cl^--secreting subclone (19A) of the human colon carcinoma cell line HT-29 by muscarinic stimulation was studied by combined Ussing chamber and microimpalement experiments.

Basolateral addition of 10^{-4} M carbachol induced a complex poly-phasic change of the cell potential consisting of (i) a fast and short (30-sec) depolarization of 15 ± 1 mV from a resting value of −52 ± 1 mV and an increase of the fractional resistance of the apical membrane (first phase), (ii) a repolarization of 22 ± 1 mV leading to a hyperpolarization of the cell (second phase), (iii) a depolarization of 11 ± 1 mV and a decrease of the fractional resistance of the apical membrane (the third phase), (iv) and sometimes, a hyperpolarization of 6 ± 1 mV and an increase of the fractional resistance of the apical membrane (fourth phase). The transepithelial potential increased with a peak value of 2.4 ± 0.3 mV (basolateral side positive). The transepithelial PD started to increase (serosa positive), coinciding with the start of the second phase of the intracellular potential change, and continued to increase during the third phase. Ion replacements and electrical circuit analyses indicate that the first phase is caused by increase of the Cl^- conductance in the apical *and* basolateral membrane, the second phase by increased K^+ conductance of the basolateral membrane, and the third phase and the fourth phase by increase and decrease, respectively, of an apical Cl^- conductance. The first and second phase of the carbachol effect could be elicited also by ionomycin. They were strongly reduced by EGTA. Phorbol dibutyrate (PDB) induced a sustained depolarization of the cell and a decrease of the apical fractional resistance. The results suggest that two different types of Cl^- channels are involved in the carbachol response: one Ca^{2+} dependent and a second which may be PKC sensitive.

In the presence of a supramaximal concentration of forskolin, carbachol evoked a further increase of the apical Cl^- conductance.

It is concluded that the short-lasting carbachol/Ca^{2+}-dependent Cl^- conductance is different from the forskolin-activated conductance. The increase of the Cl^- conductance in the presence of forskolin by carbachol may be due to activation of different Cl^- channels or to modulation of the PKA-activated Cl^- channels by activated PKC.

Key Words ionomycin · phorbol-ester · basolateral K^+ conductance · Cl^- channel · cystic fibrosis · secretion

Introduction

The subclone 19A of the human colon carcinoma cell line HT-29 expresses mechanisms for cAMP-mediated increase of the apical Cl^- conductance, leading to a sustained depolarization of the cell and a sustained increase of the transepithelial potential in response to cAMP-linked secretagogues (Augeron et al., 1986; Bajnath et al., 1991). A secretory electrical response is also elicited by muscarinic activation but with a different time course and amplitude.

Although the general model for salt secretion is well accepted, several crucial details remain to be elucidated. Especially, the differences in cAMP-related activation of secretion and the secretion induced by muscarinic receptor activation or by an increase of intracellular Ca^{2+} are not well understood. An important question, related to cystic fibrosis research is whether the intracellular messenger pathways linked to cAMP and Ca^{2+} converge on one type of Cl^- channel.

The subclone 19A of the HT-29 human colon carcinoma cell line (Augeron & Laboisse, 1984) lends itself very well to electrophysiological studies and has been advantageously used as a model for colonic epithelial cells in earlier studies (Bajnath et al., 1991). To characterize the main electrophysiological effects of carbachol and related secretagogues like phorbol esters and Ca^{2+} ionophore we applied conventional microelectrode techniques to filter-grown monolayers in Ussing-type chambers. The method allows assigning changes in potential and conductance to either the apical or the basolateral membrane and is indispensable to complete data obtained by other methods like patch clamping (Bajnath et al., 1990) or isotope-efflux measurements (Vaandrager et al., 1991). It was found that the electrophysiological effect of carbachol stimulation dif-

fered strongly from the cAMP effect. Muscarinic stimulation evoked a Ca^{2+}-dependent K^+ conductance in the basolateral membrane and a complex increase of the cellular Cl^- conductance. The results suggest the presence of two different carbachol-related Cl^- conductances, one of which is quickly but transiently activated in both the apical and the basolateral membrane and a second which is slowly activated in the apical membrane. Synergistic effects of forskolin and carbachol and the different latencies of the responses suggest that the secretagogues most likely activate different sets of Cl^- channels. This conclusion is corroborated by ^{36}Cl and ^{125}I efflux studies, showing that the response to Ca^{2+} ionophore and cAMP have different characteristics (Vaandrager et al., 1991) and by "cell-attached, single-channel" patch-clamp studies, showing activation of Cl^- channels by carbachol and cAMP in different patches and with distinct characteristics (Bajnath et al., 1990).

As far as we know this is the first report concerning intracellular electrophysiological effects of muscarinic stimulation of filter-grown confluent monolayers of intestinal cells. The results appear to be consistent with data from the literature obtained from isolated intestinal epithelium. However, it must be stressed that the cell line showed variabilities, probably related to unknown variations in culture conditions. The variability in response to carbachol stimulation was much larger than observed with forskolin. It appears, however, to add insight into the mechanism underlying the response to muscarinic stimulation. Part of this work has been presented as abstracts (Bajnath et al., 1989*a–c*).

Materials and Methods

The cell culture method and electrophysiological techniques have been previously described (Bajnath et al., 1991). In short, HT-29cl.19A colonocytes, passage number 8–22 (kindly obtained from Drs. Laboisse and Augeron, Paris) were grown for 7–21 days on rat tail collagen-coated Nuclepore PC filters, 13-mm diameter (Nuclepore, Pleasanton, CA), glued to Lexan rings.

The filters were mounted horizontally in a small Ussing-type chamber, leaving an oblong area of 0.30 cm^2. The upper and lower compartments were continuously perfused with solutions 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. The upper electrode served as the common ground for transepithelial and intracellular measurements. Transepithelial electrical resistance and the fractional apical resistance ($fR_a = R_a/(R_a + R_b)$ were calculated from voltage deflections induced by positive and negative current pulses with a duration of 1 sec. The current electrodes (Ag-AgCl) were situated in the walls of the upper and lower compartments. It has been checked that with this pulse duration capacitive artifacts do not play a role in the interpretation of changes in the voltage deflections induced by secretagogues (R.B. Bajnath and J.A. Groot, *unpublished observations*). Equivalent short-circuit currents (I_{sc}) were calculated from the transepithelial potential and the transepithelial resistance.

Monolayers with transepithelial resistances lower than 50 Ωcm^2 were not used. All measurements were corrected for solution resistance and potential offset. Glass microelectrodes were filled with 0.5 M KCl. Inserted in the bath solution, the tip resistances were between 100–200 MΩ; the tip potential was about −2 mV. The effect of added chemicals was studied while the microelectrode was positioned in the cell. This required stable intracellular potential measurements for prolonged periods.

Statistics

All values are presented as mean ± SEM. Statistical significance was evaluated using paired and unpaired *t* tests as appropriate.

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, and glucose 27.8 (pH 7.4). Low Cl^- Ringer's solutions were made by replacing all Cl^- except for 0.1 mM by gluconate. Forskolin, carbachol, ionomycin, PDB and atropine were from Sigma Chemical, St. Louis MO; bumetanide was a gift from Leo Laboratories, Ballerup, Denmark.

Glossary of Symbols

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.
R_t, R_a, R_b, R_c, R_p: slope resistances of monolayer, apical membrane, basolateral membrane, apical plus basolateral membrane and paracellular pathway, respectively, in Ωcm^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 ($fR_a = R_a/(R_a + R_b)$, $fR_b = 1 - fR_a$).

Theory

The interpretation of the secretagogue-induced response of the intracellular potential and fractional resistance requires the application of the equivalent electrical circuit for leaky epithelia. The following equations are helpful for qualitative interpretation of potential changes:

$$\psi_t = (E_b - E_a)^* R_p/(R_p + R_c) \quad (1)$$

$$\psi_a = E_a + (E_b - E_a)^* fR_a - \psi_t^* fR_a \quad (2)$$

$$fR_a = R_a/(R_a + R_b) = R_a/R_c \quad (3)$$

$$R_t = R_p^* R_c/(R_p + R_c) \quad (4)$$

From Eq. (1): an increase of ψ_t (basolateral side more positive) can occur through (i) a hyperpolarization of E_b, in that case ψ_a would hyperpolarize (*see* Eq. (2)), or (ii) through depolarization

of E_a, in that case ψ_a would depolarize (*see* Eq. (2)), or (iii) through increase of R_p, which would be measured by an increase of R_t (*see* Eq. (4)) and a depolarization of ψ_a (*see* Eq. (2)), or finally (iv) through a decrease of R_t. If this decrease is polarized two cases can be distinguished: (i) decrease of R_a, and thus, decrease of fR_a, and therefore, depolarization of ψ_a (*see* Eq. (2)), (ii) decrease of R_b, and thus, increase of fR_a, and therefore, hyperpolarization of ψ_a.

Combination of measurements of fR_a and R_t can help to localize changes in resistance. Assuming that R_p does not change, from Eqs. (3) and (4) it can be seen that simultaneous increase, or decrease, of fR_a and R_t reflects the increase, or decrease, of R_a, respectively. Opposite changes of fR_a and R_t indicate that the change is in the basolateral membrane. The conclusions about localization rests heavily on the assumption that R_p does not change. As the monolayer appears to have 95% of its transepithelial conductance in the paracellular pathway (Bajnath et al., 1991) small changes in R_p strongly influence R_t. Therefore, inconsistencies between changes in potential and changes in (fractional) resistance can occur due to changes in R_p.

Results

Complex Electrical Response to Carbachol Stimulation

There was a great deal of variability in the pattern and magnitude of extracellular and intracellular potential changes. Two types of responses of the transepithelial potential difference emerge: transient potential changes, in which the transepithelial potential (and the equivalent short-circuit current) fell back to a value less than 50% of its peak value and sustained potential changes in which the transepithelial potential remained within 10% of its peak value in the presence of carbachol. This difference was used in Table 1 to divide the responses into class I and class II. The effects of carbachol were fully reversible in both cases. Figure 1 shows an example of a transient change of the transepithelial potential (ψ_t) and the concomittant change of the intracellular potential (ψ_a). Figure 2 shows an example in which the large transepithelial potential change is sustained. In both examples, the sharp depolarization of the cell potential led only to a very small change in ψ_t, implying that the depolarization occurred in the apical as well as in the basolateral membrane with $\Delta\psi_b$ being slightly larger than $\Delta\psi_a$. The fast depolarization of ψ_a will be called the "first phase" ($\Delta\psi_{a1}$) of the carbachol response. ψ_t started to increase after the depolarization of ψ_a had reached a maximum. At this time point the fractional resistance of the apical membrane was increased. The directions of the changes in the "second phase" of the cell potential ($\Delta\psi_{a2}$) and the transepithelial potential suggest that this phase was caused by a hyperpolarization of the basolateral membrane. The intracellular repolarization reached a maximum which was usually followed by the "third phase" ($\Delta\psi_{a3}$), consisting of a depolarization and a decrease of the fractional resistance of the apical membrane. Importantly, the reversal in the cellular potential trace between the second and third phase occurred while ψ_t continued to increase. Together with the decrease of the fractional resistance this revealed that the third phase was caused by a depolarization and an increase of the conductance of the apical membrane. The third phase was sometimes followed by a repolarization in the direction of the original membrane potential ($\Delta\psi_{a4}$), as shown in Fig. 1. When this change was present, it coincided with the decrease of ψ_t, implying that the source of the fourth phase is also in the apical membrane. In addition during $\Delta\psi_{a4}$ the apical fractional resistance (fR_a) increased.

The effects of carbachol addition to 53 monolayers during 71 cellular recordings are summarized in line 1 of Table 1.

Based on the phenomenological difference in the time course of the increase of the transepithelial potential ($\Delta\psi_t$), all registrations were classified as transient responses (class I) or sustained responses (class II). Statistical analyses (unpaired Student's t test) of the class I and class II responses showed that transient and sustained responses differ only significantly in the last phase of the intracellular potential change ($\Delta\psi_{a4}$ and fR_{a4}). In the group of transient responses, the fractional resistance (fR_a) increased in all experiments (means from 0.65 ± 0.04 to 0.75 ± 0.05) simultaneously with the repolarization in the fourth phase. The changes in potential and fractional resistance during the third and fourth phase in class I responses can be interpreted as a transient increase of the apical conductance leading to a transient depolarization of E_a.

The sustained responses could be further subdivided on the basis of the amplitude of the transepithelial potential change (class IIA and class IIB). These subgroups appear to differ in R_t and in the magnitude of ΔR_t, ΔI_{sc}, $\Delta\psi_{a3}$, and fR_{a3}. The differences between these groups were not correlated with age or passage number.

Effect of Atropine

To check whether carbachol expresses its action via a muscarinic receptor type, monolayers were preincubated with atropine (10^{-6} M). The response to carbachol was totally inhibited by atropine (*not shown*), suggesting that the cells of the HT-29 subclone have muscarinic receptors.

Table 1. Summary of effects of carbachol on potential, resistance and equivalent short-circuit current

	ψ_t	R_t	ψ_a	fR_a	$\Delta\psi_t$ peak	ΔR_t peak	ΔI_{sc} peak
All expt. (n = 71)	1.1 ± 0.1	123 ± 7	−52 ± 1	0.79 ± 0.02	2.4 ± 0.3	0 ± 2	21 ± 3
	Subdivision of all responses based on time course of $\Delta\psi_t$						
Class I Transient responses of $\Delta\psi_t$ (n = 29)	1.1 ± 0.2	113 ± 9	−52 ± 1	0.75 ± 0.03	2.3 ± 0.4	2 ± 4	24 ± 5
Class II Sustained responses of $\Delta\psi_t$ (n = 42)	1.2 ± 0.2	130 ± 11	−52 ± 2	0.81 ± 0.02	2.5 ± 0.5	0 ± 4	19 ± 3
	NS	NS	NS	NS	NS	NS	NS
	Subdivision of sustained responses based on amplitude of $\Delta\psi_t$						
Class IIA Large responses of $\Delta\psi_t$ (n = 21)	1.5 ± 0.3	159 ± 17	−54 ± 2	0.78 ± 0.03	4.4 ± 0.7	−9 ± 6	35 ± 6
Class IIB Responses <1 mV (n = 21)	0.8 ± 0.2	110 ± 11	−50 ± 3	0.83 ± 0.03	0.5 ± 0.1	8 ± 2	4 ± 1
	NS	$P < 0.01$	NS	NS	$P < 0.001$	$P < 0.01$	$P < 0.001$

Carbachol was added to the basolateral perfusate at 0.1 mM while the microelectrode was in the cell. The left four columns show the control values, mean and SE of the mean. Carbachol-induced changes in transepithelial potential, resistance and equivalent short circuit current are from the peak values. The change in intracellular potential ($\Delta\psi_a$) and apical fractional resistance (fR_a) are given for the four

Low Cl^- Ringer's

The observed depolarization of the cell potential may reflect increased chloride or sodium conductance or decreased potassium conductance. To help deciding between these possibilities the extracellular Cl^- concentration was lowered to 0.1 mM and replaced by gluconate. After preincubation for approximately 20 min in this solution the carbachol-induced equivalent short-circuit current was reduced to 5 ± 2 $\mu A/cm^2$ (*see* Table 2) or 25% of the control value (Table 1). The intracellular recordings showed only a hyperpolarization and an increase of the fractional resistance of the apical membrane (*see* Fig. 3 which is typical for three other measurements; the effects of the increase in K^+ are discussed in a following section). After reintroduction of Cl^-, the effect of carbachol was normal, indicating that a 30-min period in Cl^--free Ringer's had no irreversible effects.

The involvement of the Cl^- conductance was further tested by replacement of Cl^- in the apical bathing solution during the third phase of a sustained response to carbachol. The low Cl^- Ringer's depolarized the membrane potential with 28 ± 2 (n = 8) mV. Without secretagogues, low Cl^- in the apical bath induced a small depolarization of only 5.9 ± 0.4 (n = 7) mV (Bajnath et al., 1991). The larger depolarization in the presence of carbachol indicates that during the third phase the apical Cl^- conductance was much larger than without carbachol ($P < 0.001$). The depolarization is also shown in Fig. 5 where, as a part of another experiment, low Cl^- Ringer's was applied apically in the presence of serosal Ba^{2+} (5 mM), a K^+ conductance blocker, plus carbachol. Note that in Fig. 5, no corrections were made for the liquid junction potential across the tip of the Ringer-agar bridge in the apical solution (−9.5 mV). Low Cl^- in the apical solution could induce a large depolarization of ψ_a during a sustained response as long as carbachol was present. This indicates that the increase of the Cl^- conductance in the third phase of this type of response is long lasting. In contrast, apical Cl^- replacement in the steady-state phase of a transient carbachol response induced a much smaller depolarization, suggesting that the Cl^- conductance was only transiently increased.

Bumetanide

Evidence for involvement of Cl^- conductance in the first and third phase was also obtained in experiments where bumetanide was used. Figure 4 shows the response on carbachol addition after 30-min preincubation of 10^{-4} M bumetanide. The first phase

Table 1. Continued

$\Delta\psi_{a1}$	$\Delta\psi_{a2}$	$\Delta\psi_{a3}$	$\Delta\psi_{a4}$	fR_{a1}	fR_{a2}	fR_{a3}	fR_{a4}
15 ± 1	−22 ± 1	11 ± 1	−6 ± 1	0.85 ± 0.02	0.84 ± 0.02	0.64 ± 0.03	0.73 ± 0.04
18 ± 2	−23 ± 2	8 ± 1	−11 ± 2.0	0.79 ± 0.04	0.80 ± 0.04	0.65 ± 0.04	0.75 ± 0.05
14 ± 2 NS	−21 ± 2 NS	13 ± 2 NS	−2 ± 1 $P < 0.01$	0.87 ± 0.03 NS	0.86 ± 0.03 NS	0.63 ± 0.04 NS	0.63 ± 0.02 $P < 0.05$
15 ± 2	−19 ± 2	18 ± 3	−2 ± 1	0.88 ± 0.01	0.84 ± 0.02	0.56 ± 0.05	0.56 ± 0.05
13 ± 3 NS	−22 ± 3 NS	7 ± 1 $P < 0.001$	0 ± 1 NS	0.87 ± 0.04 NS	0.88 ± 0.02 NS	0.73 ± 0.04 $P < 0.01$	0.73 ± 0.04

phases as indicated in Fig. 1. Positive values for $\Delta\psi_a$ are depolarizations; negative values denote hyperpolarizations. The subdivision of the total of 71 observations (line 1) is discussed in the text.

was absent, which is compatible with Cl^- being in equilibrium after inhibition of the NaK2Cl carrier. The second phase, consisting of the hyperpolarization and the increase of fractional resistance, coincides with the small transepithelial potential change. However, while ψ_t continued to increase, fR_a decreased and ψ_a depolarized. Thus, the third phase can be observed, apparently because the membrane potential has moved away from the Cl^- equilibrium potential.

The subsequent decline of ψ_t occurred simultaneously with the hyperpolarization of ψ_a while fR_a remained essentially constant. This indicates that the origin of this change was in the hyperpolarization of E_a, presumably because of reduction in intracellular Cl^-.

Basolateral K^+ Conductance

Figure 3 shows that the response of ψ_a to carbachol after incubation in low Cl^- Ringer's consists of only a hyperpolarization and an increase of the fractional resistance. Apparently, in the absence of Cl^- the second phase of the carbachol response is maintained. It is concluded from the directions of the changes of ψ_a and ψ_t and from the increase of the fractional resistance that the hyperpolarization is due to an increase of the basolateral membrane conductance, presumably caused by an increase of a carbachol-activated K^+ conductance in the basolateral membrane. Figure 3 also shows that a step change in the basolateral K^+ concentration from 5.7 to 46 mM induced a larger depolarization in the presence of carbachol. This is to be expected from an increased K^+ conductance of the basolateral membrane. The carbachol-induced hyperpolarization after incubation with bumetanide also indicates that the secretagogue activates a basolateral K^+ conductance.

Effects of Ba^{2+}

The participation of a Ba^{2+}-sensitive basolateral K^+ conductance in the secretory response to forskolin in this subclone has been documented (Bajnath et al., 1991). Table 2 and Fig. 5*a* show that the response of ψ_t to carbachol was also reduced after preincubation with Ba^{2+}. The smaller depolarization of ψ_a in the first phase should be expected, because of the smaller difference between cell potential and Cl^- equilibrium potential. The small repolarization in the second phase may be caused by closing of these Cl^- channels but the increase of the fractional resistance during the repolarization and the direction of the, albeit small, $\Delta\psi_t$ argues against this possibility and suggests that carbachol

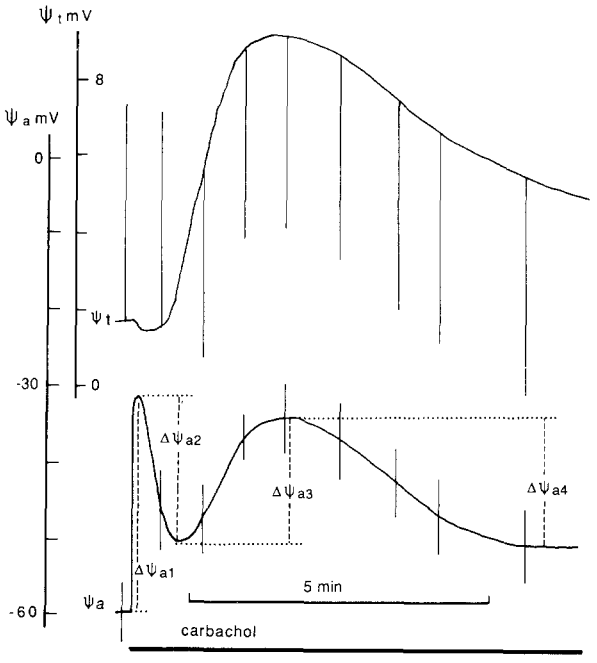

Fig. 1. Carbachol-induced effects on electrical parameters. The figure is an example of a very large but transient transepithelial potential (ψ_t) change. The bar indicates the presence of carbachol (0.1 mM) in the basolateral perfusate. The increase of ψ_t is preceded by a very small decrease concomitant with the fast depolarization of the intracellular potential (ψ_a). The change of the intracellular potential consists of four phases, indicated by $\Delta\psi_{a1}$, $\Delta\psi_{a2}$, $\Delta\psi_{a3}$ and $\Delta\psi_{a4}$. $\Delta\psi_{a2}$ and $\Delta\psi_{a3}$ coincide with the increase of ψ_t, and $\Delta\psi_{a4}$ coincides with the ensuing decrease of ψ_t. At 10 min after carbachol addition $\Delta\psi_t$ was reduced to 40% of its peak value. Because of the transient transepithelial potential change these type of responses were pooled as class I responses in Table 1. The transepithelial and the intracellular voltage deflections are induced by symmetrical transepithelial current injections of +10 and −10 μA. The voltage deflections on the transepithelial potential tracing were redrawn in only one direction; the deflections were symmetrical. Note that the time bar of 5 min in this figure is longer than in the following figures.

in the presence of Ba^{2+} can transiently activate a K^+ conductance. (This hypothesis was tested in experiments as shown in Fig. 5*b* which will be discussed in the next paragraph). The third phase was not present in one of these experiments; in the other four experiments the small repolarization was followed by a depolarization while ψ_t continued to increase, suggesting the activation of the apical Cl^- conductance. To verify this, we lowered the apical Cl^- concentration to 0.1 mM (gluconate replacement). As shown in Fig. 5*a*, this induced a depolarization, which after correction for the liquid junction potential amounts to 21 mV.

To verify whether carbachol in the presence of Ba^{2+} could activate a basolateral K^+ conductance forskolin was added after Ba^{2+} to bring Cl^- in equilibrium. Figure 5*b* shows that forskolin could not induce an increase of ψ_t. However, addition of carbachol transiently increased ψ_t and fR_a and hyperpolarized ψ_a. Thus, it appears that carbachol can activate an extra K^+ conductance, which, when activated, becomes sensitive to inhibition by Ba^{2+}.

Effects of EGTA

Carbachol was found to increase the intracellular Ca^{2+} activity in this subclone (N. van den Berghe et al., *submitted*). The effect of lowering of the extracellular Ca^{2+} activity on the electrophysiological response to carbachol was studied by application of EGTA (4 mM), thereby reducing the Ca^{2+} activity in the medium to 10^{-7} M.

The addition of EGTA to both bathing solutions rapidly decreased the cell potential by 17 mV, suggesting a decrease of the K^+ conductance (*see* Fig. 6 and Table 2). Subsequent addition of carbachol induced, as a first phase, only a small depolarization of 6 ± 2 mV. The repolarization was not accompanied by the usual increase of the fractional resistance, suggesting that it only represented the closure of the Cl^- conductance. The third phase appeared to be more or less normal, but $\Delta\psi_t$ was only 0.1 ± 0.1 mV, compatible with a decreased K^+ conductance. After washout of EGTA the intracellular carbachol response became normal but because the resistance of the monolayer did not recover to its pre-EGTA value, $\Delta\psi_t$ remained low. These results suggest that extracellular Ca^{2+} is partially necessary for the response or that EGTA has a direct inhibitory effect on the processes underlying these potential changes.

Ionomycin

The suggestion from the previous section is that Ca^{2+} may be involved in the first and second phase of the carbachol response. This was further studied by increasing the intracellular Ca^{2+} activity by adding the Ca^{2+} ionophore, ionomycin, to the basolateral solution (10^{-6} M). The ionophore induced only a very small change of ψ_t (*see* Fig. 7 and Table 3). The change in ψ_a mimicked the first and second phase of the carbachol response, although the magnitude of the first phase was significantly smaller. After preincubation in Ringer's with 0.1 mM Cl^- the response to ionomycin consisted of a hyperpolarization only, indicating that the depolarization was due to a Cl^- efflux (*not shown*). A depolarization in the third phase together with a decrease of fR_a and R_t was observed in only 1

Table 2. Effect of carbachol on intracellular and transepithelial potentials in low Cl^- Ringer or in the presence of Ba^{2+} or EGTA

Experimental condition	Control		Experimental condition		Carbachol					
	ψ_t	ψ_a	ψ_t	ψ_a	$\Delta\psi_t$	ΔI_{sc}	$\Delta\psi_{a1}$	$\Delta\psi_{a2}$	$\Delta\psi_{a3}$	n
Cl^- free	1.2 ± 0.5	−56 ± 4	1.1 ± 0.7	−62 ± 4	1.2 ± 0.5	5 ± 2	3 ± 2	−13 ± 3	0 ± 0	4
Ba^{2+}	1.0 ± 0.4	−52 ± 4	0.6 ± 0.3	−34 ± 2	0.8 ± 0.7	3 ± 3	6 ± 2	−5 ± 1	7 ± 1	5
EGTA	1.2 ± 0.1	−51 ± 2	0.6 ± 0.1	−34 ± 3	0.1 ± 0.1	—	6 ± 2	−9 ± 2	11 ± 2	10

Potentials and potential changes are in mV; I_{sc} is in $\mu A/cm^2$; Carbachol = 0.1 mM; Cl^- was 0.1 mM; Ba^{2+} = 5 mM at the basolateral side. EGTA was at both sides at 4 mM. No equivalent short-circuit current was calculated because R_t decreased due to EGTA.

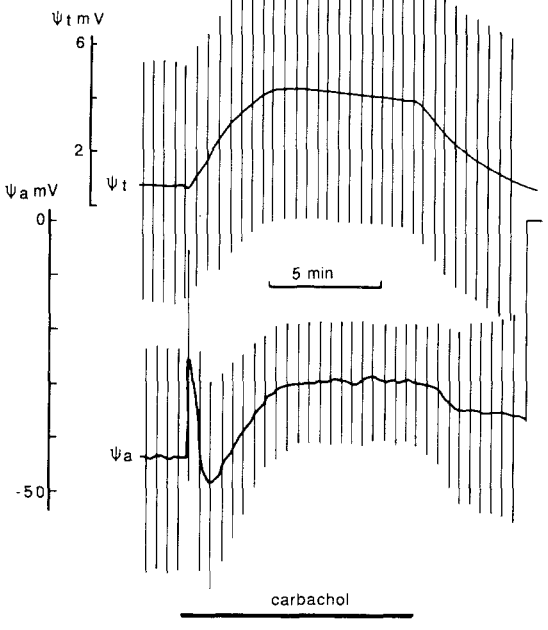

Fig. 2. Example of a carbachol-induced *sustained* transepithelial potential change (ψ_t). The difference from Fig. 1 is that during the presence of carbachol ψ_t did not decrease after its peak value and that the intracellular potential change has a triphasic character; no $\Delta\psi_{a4}$ could be observed. Because of the sustained character of $\Delta\psi_t$ the response was classified as a class II response (Table 1). The voltage deflections in the transepithelial potential and the intracellular potential are induced by bipolar transepithelial current injections of + 10 and − 10 μA and + 50 and − 50 μA, respectively. The microelectrode went out of the cell during the washout of carbachol.

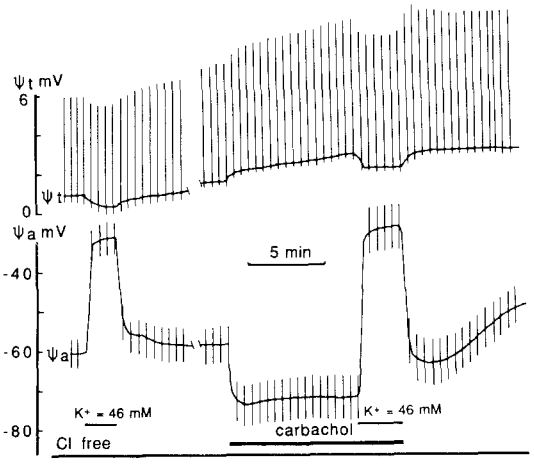

Fig. 3. Redrawing of the response of the intracellular potential (ψ_a) and transepithelial potential (ψ_t) to carbachol (0.1 mM) in low Cl^- Ringer's (0.1 mM) and to high K^- solutions (46 mM) in the basolateral perfusate in the absence and in the presence of carbachol. Cl^- was replaced by gluconate 20 min before the addition of carbachol. The first phase and the third phase of the carbachol response in low Cl^- Ringer's are completely absent, but the hyperpolarization is present. Transepithelial bipolar current injections were + 5 and − 5 μA (ψ_t) and + 50 and − 50 μA (ψ_a). The voltage deflections on the transepithelial potential tracing were redrawn in only one direction; they were symmetrical. After a 30-min period in control Ringer's the addition of carbachol induced a response like in Fig. 1 or 2, illustrating that the effect of low Cl^- is reversible.

out of 9 experiments. It is therefore concluded that, in general, an increase of the intracellular Ca^{2+} activity is not involved in the activation of the slow apical Cl^- conductance during the third phase of the carbachol response in these cells.

Phorbol 12,13-dibutyrate

Muscarinic agonists are thought to stimulate specific phospholipases, leading to the generation of diacylglycerol (DAG), the physiological activator of protein kinase C. The phorbol ester PDB acts as direct activator of this kinase and may therefore be used as a tool to differentiate between Ca^{2+}- and DAG-related phenomena. PDB was used at 10^{-7} and 10^{-6} M. The effect of the lower concentration had a slower onset but the same general effect. Addition to the basolateral solution evoked a depolarization of the cell potential and an increase of ψ_t with a lag time of about 2 min. The addition of PDB to the apical bathing solution reduced the lag time considerably. In 3 out of 20 experiments the addition of PDB had no effect on ψ_t within a period of 10 min. Table 4 shows the results of 13 other experiments in which ψ_a could

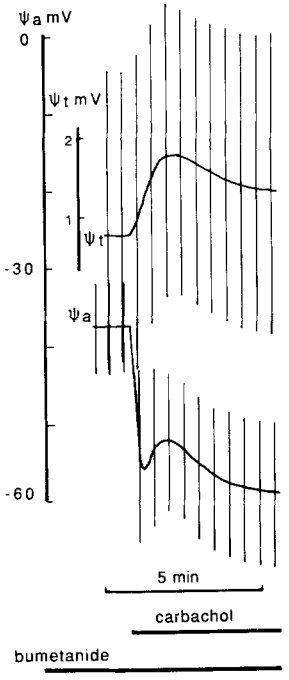

Fig. 4. Redrawing of the changes in transepithelial potential (ψ_t) and intracellular potential (ψ_a) induced by carbachol (0.1 mM), after 30-min preincubation with bumetanide (0.1 mM), perfused basolaterally. Transepithelial current injections were +5 and −5 μA (ψ_t) and +50 and −50 μA (ψ_a).

be recorded throughout. The change of ψ_a was monophasic and of the same amplitude as $\Delta\psi_{a3}$ in the large, sustained, carbachol responses (class IIA in Table 1). These results suggest that the slow Cl^- conductance in the apical membrane may be activated by PKC.

The effects of PDB are the subject of another communication (R.B. Bajnath et al., *submitted*) and will not be discussed in detail in the present study.

Synergistic Effects of Forskolin and Carbachol

To examine the question whether the slow Cl^- conductance, activated in the third phase of the carbachol response, and the cAMP-activated conductance may be the same, the effect of carbachol addition was studied after activation of the cAMP pathway. Figure 8 shows ψ_a and ψ_t during the consecutive stimulation by a supramaximal concentration of forskolin (10^{-4} M) and carbachol (10^{-4} M). The forskolin stimulation brought the cell potential near the Cl^- equilibrium potential so that it could be expected that the first phase of the carbachol response could not be observed. The activation of the basolateral K^+ conductance was evident from the hyperpolarization of the cell potential, the increase of the apical fractional resistance and the increase of ψ_t.

The quintessence of the tracing is in the subsequent depolarization of the cell potential and the decrease of the fractional resistance while the transepithelial potential continued to increase and the transepithelial resistance decreased. The time point of the maximum hyperpolarization of ψ_a coincided with 77 ± 4% of the maximal $\Delta\psi_t$(n = 19). This indicates that a substantial part of the increase of ψ_t has its origin in the depolarization of E_a. Evidently, in addition to the cAMP-activated conductance an extra conductance was recruited by carbachol. Table 5 shows that the addition of carbachol in the presence of forskolin induced a significantly larger increase of ψ_t as compared with Table 1. Similarily, the increase of the equivalent short-circuit currents induced by carbachol in the presence of forskolin (58 ± 8 $\mu A/cm^2$) is significantly larger than when added alone (21 ± 3 $\mu A/cm^2$, Table 1 for n = 71). The peak value of the short-circuit current after cumulative addition of forskolin and carbachol is nearly 150% of the sum of the currents induced by individual addition.

Discussion

An advantage of the technique used in the present study is that the results are obtained in an intact and polarized epithelial monolayer so that the localization of the carbachol-induced changes can be determined. A drawback is that characterization of the conductance changes can only be based on: (i) the direction of the potential change in combination with an educated guess of the equilibrium potentials of the relevant ions, (ii) the increase or decrease of the transepithelial and apparent fractional resistance which suffer from uncertainties as discussed in Materials and Methods and (iii) on the effect of ion substitutions. The voltage dependence of the changes cannot be studied and the time dependence is more difficult to assert than in voltage-clamp studies.

Activation of Cl^- Conductances by Stimulation of the Muscarinic Receptor

The arguments that lead to the conclusion that a transient Cl^- conductance is responsible for the *first phase* of the carbachol response can be summarized as follows: (i) In bilateral Cl^--free Ringer's this phase was abolished. Thus, it is not plausible that the depolarization was caused by influx of Na^+. An

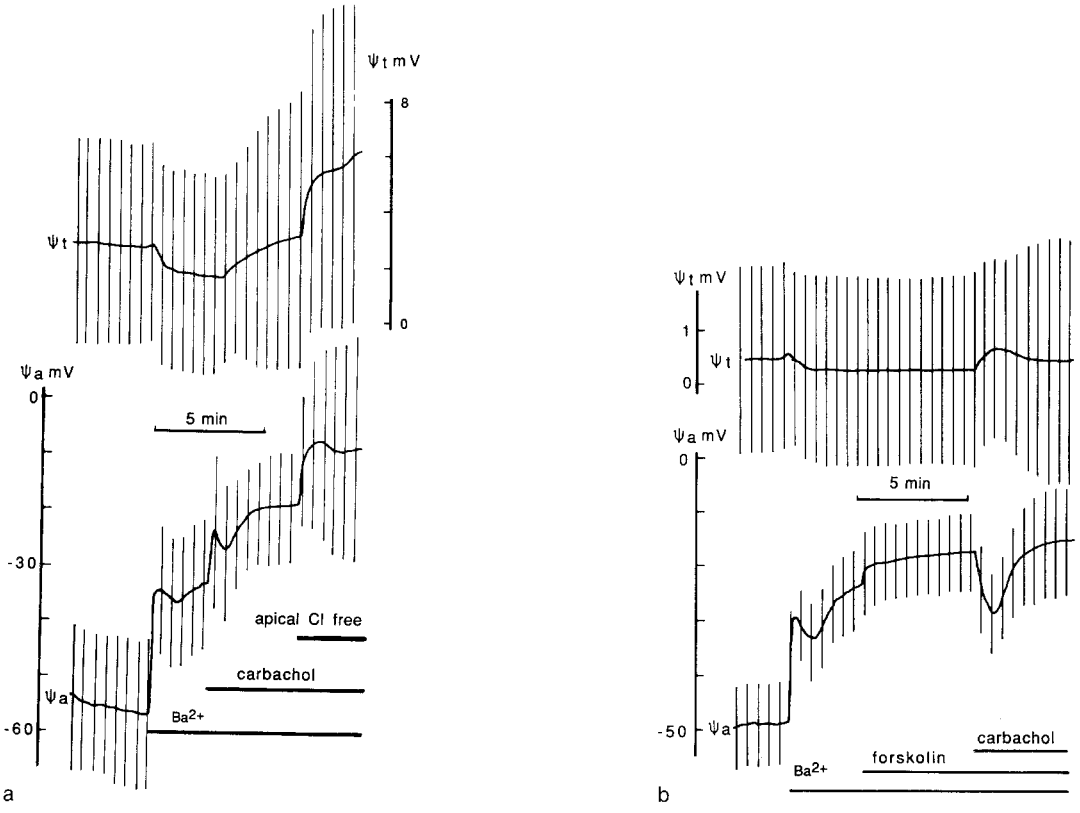

Fig. 5. (*a*) Redrawing of a recording of the transepithelial potential (ψ_t) and intracellular potential (ψ_a) during cumulative basolateral addition of Ba^{2+} (5 mM) and carbachol (0.1 mM) and replacement of control Ringer's by low Cl^- (0.1 mM) Ringer's in the apical perfusate. Voltage deflections are from transepithelial current injections of +10 and −10 μA (ψ_t) and +50 and −50 μA (ψ_a). (*b*) Redrawing of a recording of the transepithelial potential (ψ_t) and intracellular potential (ψ_a) during cumulative addition of Ba^{2+} (5 mM), forskolin (0.01 mM) and carbachol (0.1 mM) to the basolateral solution. The first phase of the carbachol response was refractory. The change of ψ_t and the hyperpolarization of ψ_a followed the same time course in opposite directions, indicating that the decrease of ψ_t is caused by the depolarization of E_b, or in other words, that the second phase of the carbachol response is transient and no third phase occurred. The voltage deflections of the transepithelial voltage trace (ψ_t) were induced by current injections of +10 and −10 μA. Voltage deflections of ψ_a were induced by current pulses of +50 and −50 μA.

influx of K^+ would be against its electrochemical driving force, and a redistribution of K^+ by electroneutral transport is not likely because of the rapidity of the response. (ii) After preincubation with bumetanide, which, presumably, led to equilibration of Cl^-, the first phase is absent. Similarly, the first phase was reduced or not observed after depolarizing the cell membrane potential near to the Cl^- equilibrium potential by Ba^{2+}, EGTA or by forskolin.

The large intracellular depolarization, without a significant transepithelial change, indicates that the increase of the Cl^- conductance must take place in both the apical and in the basolateral membrane. This conclusion is supported by the results of experiments using the isotope efflux technique (Vaandrager et al., 1991). Carbachol and Ca^{2+} ionophore activated a bidirectional increase of ^{125}I efflux from HT-29cl.19A cells grown on permeable supports, but forskolin only increased the efflux through the apical membrane. Although the fast and transient depolarization induced by carbachol appears to be common to the original HT-29 cell line (Ziss, Fromm & Hegel, 1988), as far as we know, the observation that the conductance increase took place in both membranes is unique. Application of the whole-cell voltage-clamp technique on T_{84} cells (Cliff & Frizzell, 1990) revealed a short-lasting Ca^{2+}-dependent Cl^- conductance, but this technique cannot discriminate between apical and basolateral membranes, and with transepithelial measurements, the symmetrical depolarization of the cell cannot be detected.

Evidence for the activation of an apical Cl^- conductance in the *third phase* of the carbachol response is based on the observations that this phase is also linked to the presence of Cl^- and that reduc-

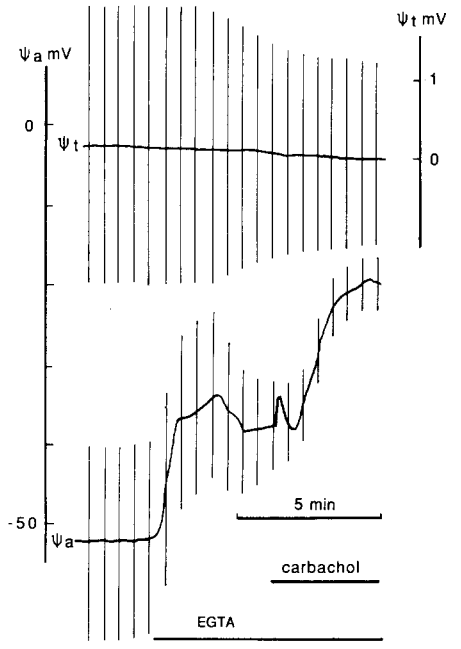

Fig. 6. Redrawing of a recording showing the depolarization of the intracellular potential induced by the addition of EGTA (4 mM) in both compartments and the inhibition of the carbachol response. The tracing of ψ_a shows the first and the third phase. ψ_t does not change, presumably because of the blockage of the basolateral K^+ conductance. After a lag time EGTA reduced the transepithelial resistance. The current injections were +20 and −20 μA (ψ_t) and +100 and −100 μA (ψ_a).

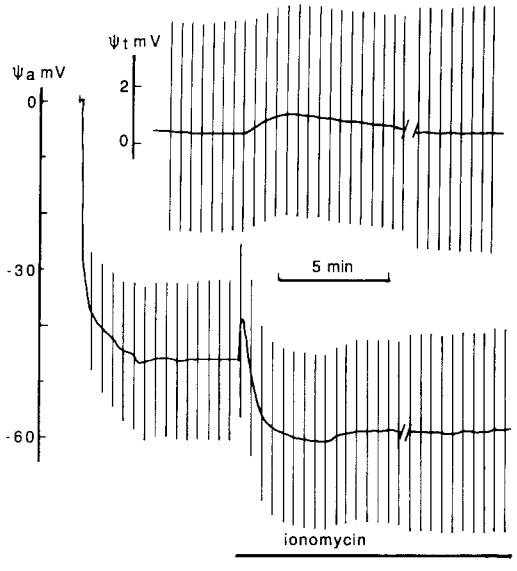

Fig. 7. Redrawing of recordings of the changes in intracellular potential (ψ_a) and the transepithelial potential (ψ_t) induced by basolateral addition of ionomycin (1 μM) for a total period of 25 min. The microelectrode tracing shows the biphasic effect of ionomycin. Voltage deflections are induced by bipolar transepithelial current injections of +20 and −20 μA (ψ_t) and +100 and −100 μA (ψ_a).

tion of the Cl^- concentration in the apical bath led to a large depolarization.

The third phase and the decrease of fR_a was totally absent in 15% of the responses (belonging to class IIB of Table 1), although the first and second phases were normal. These responses were also characterized by very little activation of I_{sc}. It is concluded that the refractoriness of the apical Cl^- conductance is the underlying cause for the diminished transepithelial response. Presumably, the Cl^- channels involved in the third phase were not expressed or could not be activated. This difference in activation or expression of the Cl^- conductances in the first and third phases also suggests that they may be different entities. Further support for the notion that carbachol may activate two different types of Cl^- channels can also be drawn from the different effects of EGTA, ionomycin and PDB on the fast and transient depolarization (Ca^{2+} dependent) and on the slow depolarization (PKC dependent).

The slow depolarization appears to be the determining factor for the magnitude of the carbachol-induced increase of the transepithelial potential and short-circuit current. This can also be reasoned from the response to ionomycin. The very small $\Delta\psi_t$ induced by ionomycin is not unique for this clone. It has also been observed in the T_{84} cell line (Wong et al., 1989) and in mammalian colon, provided that prostaglandin formation was inhibited by indomethacin (Smith & McCabe, 1984).

Activation of Basolateral K^+ Conductance by Muscarinic Stimulation

The *second phase* in the intracellular carbachol response appears to be caused by the increase of a basolateral K^+ conductance. This is most likely because a hyperpolarization together with a decrease of the membrane resistance can only be explained by increased K^+ conductance. The activation of this K^+ conductance appears to be Ca^{2+} dependent and long lasting. It occurs even after preincubation with Ba^{2+}. However, the activated conductance is susceptible to inhibition by Ba^{2+} as shown by the transient behavior in the presence of Ba^{2+} (*see* Fig. 5*b*).

Thus the complex response of HT-29cl.19A cells to carbachol appears to consist of a two-component depolarizing Cl^- current separated by a hyperpolarizing K^+ current. This behavior is reminiscent to the muscarinic response in *Xenopus* oocytes (Lupu-Meiri, Shapira & Oron, 1989) but differs in its regulation in that in the oocytes both Cl^- currents are activated by Ca^{2+}. In the HT-29 clone the slow Cl^- current appears to be dependent on the activation of PKC. Assuming that this may be extrapolated to

Table 3. Effect of ionomycin on intracellular and transepithelial potentials and fractional resistance

ψ_t	$\Delta\psi_t$	ψ_a	$\Delta\psi_{a1}$	$\Delta\psi_{a2}$	fR_a	fR_{a1}	fR_{a2}
1.3 ± 0.2	0.5 ± 0.2	−53 ± 2	8 ± 1	−15 ± 3	0.79 ± 0.02	0.87 ± 0.02	0.85 ± 0.02

Potentials and potential changes are in mV; a positive change of ψ_a is a depolarization. Ionomycin was added to the basolateral side at 1 μM. $n = 9$.

Table 4. Effect of phorbol 12,13-dibutyrate on potential and resistance

ψ_t	$\Delta\psi_t$	ψ_a	$\Delta\psi_a$	R_t	ΔR_t	fR_a	ΔfR_a (PDB)
0.4 ± 0.1	2.5 ± 0.6	−58 ± 2	24 ± 4	134 ± 16	4 ± 4	0.81 ± 0.03	0.40 ± 0.06

Potentials and potential changes are in mV; R_t is in Ωcm^2. PDB was added at 1 μM to the basolateral side or to the apical side. $n = 13$.

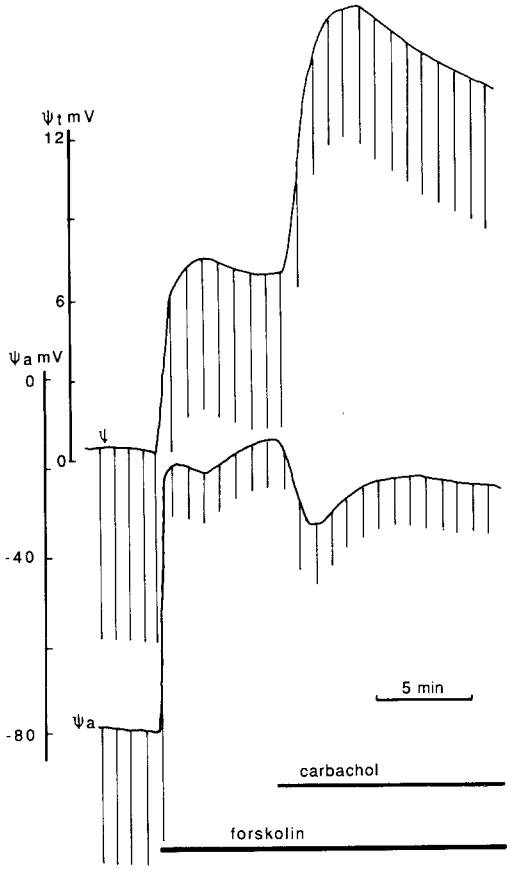

Fig. 8. Example of the effect of cumulative addition of a supramaximal concentrations of forskolin (0.1 mM) followed by carbachol (0.1 mM). The increase of ψ_t induced by carbachol coincides with the hyperpolarization and the subsequent depolarization, indicating that the basolateral and the apical membranes are involved. The voltage deflections were redrawn in only one direction; they were symmetrical. The transepithelial current injections were +10 and −10 μA (ψ_t) and +50 and −50 μA (ψ_a).

the human intestinal epithelium, the suggestion from this study is that the carbachol-provoked increase in ψ_t and I_{sc} in intestine is most likely a consequence of the activation of basolateral K^+ channels by a Ca^{2+}-related mechanism and the opening of PKC-activated apical Cl^- channels. This would imply that the abnormal response to carbachol in cystic fibrosis-affected intestines (de Jonge, Bijman & Sinaasappel, 1987; Berschneider et al., 1988; Taylor et al., 1989) reflects a defect in the PKC-activated, rather than the Ca^{2+}-activated Cl^- channel. Such a model would bring the intestine more in line with airway and sweat gland epithelium in which both PKA and PKC but not Ca^{2+}-regulated apical Cl^- channels appear to be defective in cystic fibrosis (Quinton, 1990).

Comparison with T_{84} Cells

The intracellular effects of carbachol in this cell line differ from the model based on extracellular measurements and flux experiments in T_{84} cells (Dharmsathaphorn & Pandol, 1986) and whole-cell voltage-clamp studies (Devor, Simasko & Duffey, 1990) which suggests that the sole effect of carbachol was the increase of the basolateral K^+ conductance. This model not only conflicts with the present results but also with the results of recent whole-cell voltage-clamp experiments in T_{84} cells (Cliff & Frizzell, 1990). In a recent review, McRoberts and Barrett (1989) suggested that the apparent absence of carbachol-activated Cl^- channels in the earlier work could be related to the time elapsed after plating, and thus, the degree of confluency; the carbachol-activated Cl^- channels may be lost in fully confluent monolayers. This appears not to be the case in the HT-29 clone. In addition, in

Table 5. Effect of consecutive addition of forskolin and carbachol on transepithelial potential and resistance and comparison with the effect of singular addition

ψ_t	R_t	$\Delta\psi_{t(fsk)}$	$\Delta R_{t(fsk)}$	$\Delta I_{sc(fsk)}$	Effect of carbachol addition in the presence of forskolin			$\Delta I_{sc(fsk+carb)}$	n
					$\Delta\psi_{t(carb)}$	$\Delta R_{t(carb)}$	$\Delta I_{sc(carb)}$		
1.0 ± 0.2	141 ± 15	3.5 ± 0.4	−19 ± 3	30 ± 4	6.0 ± 0.6	−11 ± 2	58 ±8	88 ± 9	19
Singular additions									
1.3 ± 0.2	138 ± 13	3.9 ± 0.4	−25 ± 3	37 ± 5	(from Bajnath et al., 1991)				58
1.5 ± 0.3	124 ± 7	—	—	—	2.4 ± 0.3	0 ± 2	21 ± 3	(from Table 1)	71

Potentials and potential changes are in mV; R_t is in Ωcm^2; I_{sc} is in $\mu A/cm^2$. Forskolin and carbachol were added at 0.1 mM to the basolateral side.

experiments with the T_{84} cells, grown to confluency on the same support as the HT-29 clone, a complex, multiphasic, behavior was observed in intracellular recordings of the carbachol effect (Bajnath et al., 1989*a*). Depolarizations and hyperpolarizations were observed, which could be assigned to increased apical and basolateral conductances, respectively. However, the sequence and the magnitude of these changes was highly variable, preventing the analyses of a sufficient number of similar recordings. In 2 out of 15 recordings the same profile as in the HT-29 clone was observed. Interestingly, Stewart and Turnberg (1989) recently reported that the application of acetylcholine to rat ileal mucosa elicited multiphasic intracellular responses that differed from the response to PGE_2.

Transient Transepithelial Response *Versus* Sustained Responses

The results summarized in Table 1 show that the transient character of some of the transepithelial responses to carbachol correlates with the appearance of a fourth phase (repolarization) of the intracellular response. Because the repolarization of ψ_a coincides with the decrease of ψ_t and the increase of fR_a, it is plausible that it reflects the closure of the slowly activated Cl^- conductance (presumably the PKC-activated Cl^- conductance) in the apical membrane. In sustained transepithelial responses, in only 3 out of the 44 responses a repolarization after $\Delta\psi_{a3}$ was observed. However, this was accompanied by a decrease of fR_a and not by a decrease of ψ_t, suggesting that another mechanism than closure of the apical Cl^- channels was involved. An intriguing question is: what determines the different behavior (transient *versus* sustained opening) of the slowly activated Cl^- conductance. Preliminary results suggest that the batch of calf serum may contain a determining factor. It appears that the transient response resembles the transepithelial change of isolated intestine upon carbachol addition. However, it may be that part of this response is due to the release of endogenous extracellular messengers (Diener et al., 1989; Chandan et al., 1991).

Synergistic Effect of cAMP and Carbachol

The large and sustained response of ψ_t to carbachol is characterized by a lasting increase of basolateral K^+ conductance and apical Cl^- conductance. The magnitude of the equivalent short-circuit current (35 ± 6 $\mu A/cm^2$) is not significantly different from the equivalent current induced by forskolin (37 ± 5 $\mu A/cm^2$, Bajnath et al., 1991), although the forskolin response occurred without an increase of the K^+ conductance (Bajnath et al., 1991). One interpretation may be that in the carbachol response the Cl^- conductance is current limiting while with forskolin the K^+ conductance limits the transcellular current flow.

The significantly larger effects of carbachol in the presence of forskolin, as compared to its single effect, results in a more than an additive effect on transepithelial potential and equivalent short-circuit current of cAMP plus carbachol. This finding fully agrees with similar observations in the T_{84} cells (Cartwright et al., 1985). Part of the explanation for the synergistic effect is that carbachol increased the basolateral K^+ conductance, which may be current limiting in the forskolin response. In addition, from intracellular recordings, it can also be seen that in the third phase of the carbachol response a further increase of the apical Cl^- conductance occurred. Thus, it appears that the synergistic effect is caused by the activation of the basolateral K^+ conductance and the extra Cl^- conductance in the apical membrane.

Do Carbachol and Forskolin Activate the Same Cl^- Channels?

Related to cystic fibrosis, an important question is whether the two types of carbachol-activated Cl^- conductance and the forskolin-activated Cl^- conductance are expressions of the same or of different Cl^- channels. In a previous section we concluded that the two carbachol-activated Cl^- conductances differ in the intracellular messengers involved in their regulation and in their kinetics and localization. Because forskolin never induced a short-lasting intracellular depolarization we suggest that the Ca^{2+}-linked, transiently activated Cl^- conductance and the cAMP-linked Cl^- conductance are due to different channels. Further evidences for this suggestion are: (i) the $^{125}I/^{36}Cl$ selectivities of the channels activated by A23187 or by forskolin are different (Vaandrager et al., 1991), (ii) in whole-cell voltage-clamp experiments in T_{84} cells it was observed that the transient activation of Cl^- current with Ca^{2+} ionophore adds to the sustained current induced by forskolin (Cliff & Frizzell, 1990).

The question whether the cAMP-linked Cl^- channel is the same or different from the channel that is activated during the third phase of the carbachol response (presumably PKC activated) is difficult to answer. The increase of conductance, discussed in the previous section, may occur from (i) the modulation of the PKA-activated channels by PKC (Bajnath et al., 1992) or (ii) it may be that PKA and PKC activate different channels. Arguments against the first proposition are: (i) The activity of forskolin-activated Cl^- channels in "on cell" patches was not changed by the addition of carbachol (Bajnath et al., 1990). (ii) The activation of Cl^- channels with open activities lasting for more than 10 min in "on cell" patches by carbachol was observed, although a previous addition of forskolin was without effect on the same patch. Moreover, the forskolin-activated and carbachol-activated channels differ in conductivity (Bajnath et al., 1990). (iii) Biochemical evidence, obtained in the T_{84} cells (Cohn, 1990), indicates that carbachol and phorbol esters can stimulate the phosphorylation of substrates that were not susceptible to the effect of forskolin. If it would turn out that the product of the defective gene is the Cl^- channel itself (Anderson et al., 1991; Kartner et al., 1991), the abnormal response to forskolin and carbachol in cystic fibrosis-affected human intestine would argue against different PKA- and PKC-activated Cl^- channels.

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Note Added in Proof

In a recent paper (Anderson, M.P., Welsh, M.J. 1991 *Proc. Natl. Acad. Sci. USA* **88:**6003–6007) it has been stated that HT-29cl.19A cells do not have Ca^{2+}-stimulated Cl^- channels in the apical membrane. The authors used nystatin (0.36 mg/ml) to make the basolateral membrane permeable. We found that this manoeuvre blocked the first phase of the carbachol and ionomycin responses but not the depolarization induced by forskolin or PDB.