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Modulation of chloride, potassium and bicarbonate transport by muscarinic receptors in a human adenocarcinoma cell line

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1 Short-circuit current (I_{SC}) responses to carbachol (CCh) were investigated in Colony 1 epithelia, a subpopulation of the HCA-7 adenocarcinoma cell line. In Krebs-Henseleit (KH) buffer, CCh responses consisted of three I_{SC} components: an unusual rapid decrease (the 10 s spike) followed by an upward spike at 30 s and a slower transient increase (the 2 min peak). This response was not potentiated by forskolin; rather, CCh inhibited cyclic AMP-stimulated I_{SC}.

2 In HCO_3^- free buffer, the decrease in forskolin-elevated I_{SC} after CCh was reduced, although the interactions between CCh and forskolin remained at best additive rather than synergistic. When Cl^- anions were replaced by gluconate, both Ca^{2+}- and cyclic AMP-mediated electrogenic responses were significantly inhibited.

3 Basolateral Ba^{2+} (1–10 mM) and 293B (10 μM) selectively inhibited forskolin stimulation of I_{SC}, without altering the effects of CCh. Under Ba^{2+}- or 293B-treated conditions, CCh responses were potentiated by pretreatment with forskolin.

4 Basolateral charybdotoxin (50 nM) significantly increased the size of the 10 s spike of CCh responses in both KH and HCO_3^- free medium, without affecting the 2 min peak. The enhanced 10 s spike was inhibited by prior addition of 5 mM apical Ba^{2+}. Charybdotoxin did not affect forskolin responses.

5 In epithelial layers prestimulated with forskolin, the muscarinic antagonists atropine and 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP, both at 100 nM) abolished subsequent 10 μM CCh responses. Following addition of *p*-fluoro hexahydro-sila-difenidol (*p*F-HHSiD, 10 μM) or pirenzepine (1 μM), qualitative changes in the CCh response time-profile also indicated a rightward shift of the agonist concentration-response curve; however, 1 μM gallamine had no effect. These results suggest that a single M_3-like receptor subtype mediates the secretory response to CCh.

6 It is concluded that CCh and forskolin activate discrete populations of basolateral K^+ channels gated by either Ca^{2+} or cyclic AMP, but that the Cl^- permeability of the apical membrane may limit their combined effects on electrogenic Cl^- secretion. In addition, CCh activates a Ba^{2+}-sensitive apical K^+ conductance leading to electrogenic K^+ transport. Both agents may also modulate HCO_3^- secretion through a mechanism at least partially dependent on carbonic anhydrase.

Keywords: Epithelial transport; carbachol; potassium channel

Introduction

Acetylcholine (ACh) is a major enteric neurotransmitter involved in the regulation of intestinal ion transport. Immunohistochemical studies have demonstrated the presence of choline acetyltransferase-like immunoreactivity in the submucous plexi of rat and guinea-pig small intestine (Bornstein & Furness, 1988) and in the majority of submucous neurones in both human small and large bowel (Potter *et al*., 1996); a subpopulation of cholinergic neurones thus identified projects directly to the epithelium. ACh is released from nerve terminals in close proximity with the colonic mucosa in response to depolarizing stimuli (rat; Wu *et al*., 1982), and after electric field stimulation (guinea-pig; Javed & Cooke, 1992). In the latter study, ACh release was closely correlated with an increase in short-circuit current (I_{SC}) attributable to the secretion of Cl^- anions. Secretory responses to ACh or metabolically stable cholinomimetics such as carbachol (CCh) have also been observed in *in vitro* preparations of small or large intestinal mucosae from a number of other species, including porcine distal jejunum (Chandan *et al*., 1991a) and colon (Traynor *et al*., 1991), rat distal colon (Strabel & Diener, 1995) and human ileum (Isaacs *et al*., 1976). The effects of CCh are mediated both indirectly, through stimulation of transmitter release from submucosal neurones, and *via* a direct action on epithelial muscarinic receptors (Chandan *et al*., 1991b; O'Malley *et al*., 1995).

The mechanism by which epithelial cells secrete Cl^- anions has been extensively studied in a number of human adenocarcinoma cell lines, in which the absence of modulation by neuronal or immune effects is assured (for a review, see Binder & Sandle, 1994). It is now generally accepted that Cl^- ions enter the cell through a basolateral $Na^+/K^+/2Cl^-$ cotransporter, inhibitable by loop diuretics such as piretanide, and leave through Cl^- channels in the apical membrane. This electrogenic transport requires the activation of basolateral K^+ channels, which serve to recycle K^+ ions across the basolateral membrane and to hyperpolarize the cell, creating a favourable electrochemical gradient for apical Cl^- exit. Regulation by second messengers occurs at two principal levels; the basolateral K^+ conductances are sensitive to intracellular Ca^{2+} and cyclic AMP concentrations, and the cystic fibrosis transmembrane conductance regulator (CFTR), an apical Cl^- channel critically involved in intestinal Cl^-

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secretion, is also activated indirectly by cyclic AMP (Morris *et al*., 1994). Ca^{2+}-gated Cl^- channels have also been identified (Bajnath *et al*., 1992), although the extent to which these are restricted to the apical domain is controversial (Morris *et al*., 1994). In two human colonic cell lines, T84 and HT-29, CCh stimulates phosphoinositide hydrolysis and thereby increases intracellular Ca^{2+}, effects mediated by the M_3 muscarinic receptor (Dickinson *et al*., 1992; Kopp *et al*., 1989). Although such Ca^{2+} elevations are sustained in T84 epithelia, the associated Cl^- secretory response is short-lived (Dharmsathaphorn *et al*., 1989) and may be limited by the release of additional inhibitory second messengers (e.g. arachidonic acid; Devor & Frizzell, 1998). On the other hand, the transient nature of Ca^{2+} increases measured in the HCA-7 human adenocarcinoma cell line may be sufficient to account for the timecourses of Cl^- secretion in response to either lysylbradykinin or histamine (MacVinish *et al*., 1993).

The interaction between Ca^{2+}- and cyclic AMP-elevating agonists and the subsequent regulation of intestinal secretion is physiologically relevant for a number of reasons. In the guinea-pig small intestine, cholinergic secretomotor neurones also contain a number of co-released peptides which influence epithelial cyclic AMP levels *via* specific receptor populations, e.g. neuropeptide Y and calcitonin gene related peptide (Bornstein & Furness, 1988). Vasoactive intestinal peptide (VIP) is also a prominent submucous neurotransmitter, providing an extensive innervation of the epithelial layer, for example in rat jejunum (Ekblad *et al*., 1987) and human colon (Domoto *et al*., 1990). Its release results in a potent secretory response mediated by epithelial cyclic AMP accumulation (Schwartz *et al*., 1974). Thus the epithelial actions of ACh *in vivo* are invariably dependent on a background of other agents which stimulate or inhibit cyclic AMP formation. Indeed the presence of endogenous neurotransmitters and eicosanoids is necessary for efficient induction of Cl^- secretion by CCh in the rat distal colon (Strabel & Diener, 1995). Cellular investigations have generally revealed a synergistic interaction between cyclic AMP- and Ca^{2+}-mediated secretory agonists, including those in T84 (Dharmsathaphorn & Pandol, 1986), HT-29 cl. 16E (Merlin *et al*., 1995) and HCA-7 (MacVinish *et al*., 1993) adenocarcinoma cell lines. In HT-29 and T84 cells this synergism relies on the cooperative activation of discrete populations of basolateral K^+ channels governed by Ca^{2+} and cyclic AMP. In contrast, preliminary studies with a subpopulation of the HCA-7 cell line, called Colony 1, have shown some unusual features in electrogenic CCh responses (Holliday *et al*., 1997); in particular, forskolin-stimulated increases in I_{SC} are inhibited by CCh, rather than potentiated. In this study we therefore pursue the mechanisms underlying the changes in I_{SC} associated with CCh and forskolin, using charybdotoxin, Ba^{2+} ions and the chromanol derivative 293B to discriminate between different Ca^{2+}- and cyclic AMP-gated K^+ channel populations. We show that the absence of potentiation arises from both a limitation in the rate of Cl^- secretion across the apical membrane and the additional involvement of Ca^{2+}-stimulated transepithelial K^+ and HCO_3^- transport.

Methods

Cell culture

Colony 1 epithelia were kindly provided by Dr S. Kirkland (Imperial College, London, U.K.). This cell line was initially isolated by seeding HCA-7 cells at low density and using cloning cylinders to trypsinize the resulting individual colonies (Marsh *et al*., 1993). They were maintained in 25 cm^2 flasks (Falcon, Beckton Dickinson, Oxford, U.K.) containing Dulbecco's Modified Eagle's medium (DMEM; Gibco, Paisley, U.K.) supplemented with glucose (25 mM), foetal calf serum (10%; ICN Biomedicals, Oxford, U.K.) and the antibiotics kanamycin (100 mg ml^{-1}; ICN) and amphotericin B (1.2 mg ml^{-1}; ICN). Cells were incubated at 37°C in a humidified atmosphere of 95% O_2 / 5% CO_2. Once confluent, epithelial layers (passages 17–22) were trypsinized (0.5% w/v in versene; Worthington, Lorne Laboratories, Reading, U.K.) and seeded onto collagen-coated filters (Millipore, Hertfordshire, U.K.) as previously described by Cuthbert *et al*. (1987). Growth was restricted to a central well of 0.2 cm^2 area by the use of a Sylgard washer.

Short-circuit current (I_{SC}) measurement

After 6–8 days, confluent Colony 1 cells on filters were mounted in Ussing chambers and bathed in one of the following solutions (composition in mM): Krebs-Henseleit (KH; NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, glucose 11.1; pH 7.4), HCO_3^- free (for experiments requiring the use of Ba^{2+} ions; HEPES 10, NaCl 140, KCl 5.4, $CaCl_2$ 1.2, $MgCl_2$ 1, glucose 11.1; pH 7.4) or Cl^- free buffers (Na gluconate, 118, K gluconate 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaSO_4$ 2.5, glucose 11.1; pH 7.4). These solutions (15 ml in each reservoir) were aerated with 95% O_2 / 5% CO_2 (KH and Cl^- free buffers) or 100% O_2 (HCO_3^- free). Epithelial layers were voltage-clamped at zero potential (DVC-1000, World Precision Instruments, Florida, U.S.A.) and the resulting I_{SC} recorded continuously on chart recorders. Drugs were added to the basolateral reservoir (unless otherwise stated) and the peak changes in I_{SC} were measured and calculated as μA cm^{-2}. Concentration-response curves were constructed from single additions of the desired agent, to eliminate difficulties associated with the rapid desensitization of Colony 1 cells to many agonists, and EC_{50} values were calculated (with 95% confidence limits) using the curve-fitting program Graphpad Prism (v. 2.01, Graphpad Software Inc., California, U.S.A.). Cells were pretreated with K^+ channel blockers for 10 min before stimulation with either forskolin or CCh; in experiments where charybdotoxin was used, 0.01% bovine serum albumin (BSA) was added to both basolateral and apical reservoirs prior to its addition. Results are expressed as the means $\pm$ 1 s.e.mean. of n observations. Statistical analysis of two data groups was carried out using the Student's unpaired t-test; for multiple comparisons one way analysis of variance was performed followed by Bonferroni's corrected t-test. A significance level of $P<0.05$ was adopted throughout.

Materials

293B (*trans*-6-cyano-4-[N-ethylsulphonyl-N-methylamino]-3-hydroxy-2,2-dimethyl-chromane) was the kind gift of Prof R. Greger (Physiologisches Institut der Albert-Ludwigs-Universität, Freiburg, Germany). Piretanide was a gift from Hoechst Marion Roussel (Swindon, U.K.). Other drugs were purchased from the following companies: forskolin, CCh, acetazolamide and atropine (Sigma, Poole, U.K.), 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), *p*-fluoro hexahydrosila-difenidol HCl (*p*F-HHSiD), gallamine and pirenzepine (RBI, St. Albans, U.K.) and charybdotoxin (Alamone Labs, Jerusalem, Israel). Agents were made up as aqueous stock solutions with the following exceptions: 293B (dimethyl

sulphoxide), forskolin and *p*F-HHSiD (95% ethanol) and charybdotoxin (prepared in 0.1% BSA, 100 mM NaCl, 10 mM Tris and 1 mM EDTA; pH 7.5). Non-aqueous solvents were diluted to a concentration of 0.1% v/v on addition of drugs to the reservoir. $BaCl_2$ was dissolved at the required concentration in HCO_3^- free solution.

Results

Responses to CCh in untreated and forskolin-stimulated cells

Krebs-Henseleit Colony 1 cells exhibited basal resistances of $39.3 \pm 0.6\ \Omega\ cm^2$ and basal I_{SC} levels of $10.8 \pm 0.4\ \mu A\ cm^{-2}$ ($n = 520$). Addition of 10 μM CCh to untreated epithelial layers resulted in a rapid downward spike at 10 s, followed by a transient increase in I_{SC} (maximum at 2 min) which returned to basal levels within 15 min (Figure 1A and Table 1). EC_{50} values for these two phases were 6.5 μM (3.1 – 13.7 μM) and 3.6 μM (1.1 – 11.1 μM; $n = 4 – 14$) respectively (Figure 1B). A shoulder in the I_{SC} increases after 10 μM CCh was distinguished as a separate spike component at 30 s in 4 out of 14 cases and became more clearly defined after higher agonist concentrations (100 μM response: $+10.9 \pm 2.7\ \mu A\ cm^{-2}$, $n = 4$; approximate EC_{50} 19.9 μM (0.8 – 49.0 μM; $n = 4$); Figure 1A).

Forskolin, a direct activator of adenylyl cyclase, produced sustained elevations in I_{SC} (EC_{50} 517 nM; Holliday *et al*., 1997), the response after a maximal concentration (10 μM) being $+64.0 \pm 2.7\ \mu A\ cm^{-2}$ ($n = 76$). Following forskolin pretreatment, stimulation with 10 μM CCh resulted in a rapid reduction in I_{SC} (Figure 2), the maximal decrease at 30 s being greatest after 1 μM forskolin ($-21.8 \pm 4.3\ \mu A\ cm^{-2}$, $n = 5$); subsequently, I_{SC} recovered to a peak at 2 – 3 min after agonist addition. Only in cells treated with a low forskolin concentration (100 nM or 300 nM) did this peak response represent an overall increase above the initial elevated I_{SC} levels (Figure 2). 10 s upward ($+4.4 \pm 1.6\ \mu A\ cm^{-2}$, $n = 5$) or downward spikes ($-5.8 \pm 1.7\ \mu A\ cm^{-2}$, $n = 5$; revealed on an extended time scale in the inset in Figure 3) were also often observed, akin to the CCh responses in otherwise unstimulated cells.

HCO_3^- free Unstimulated epithelia in HCO_3^- free solution exhibited basal resistances ($30.4 \pm 0.8\ \Omega\ cm^2$, $n = 102$), I_{SC} ($14.3 \pm 1.6\ \mu A\ cm^{-2}$, $n = 53$) and responses to 10 μM CCh that were similar to those in KH buffer (Figure 3A; Table 1). The 10 s downward spike after CCh was observed less often (four out of nine responses), while the 30 s upward spike was more

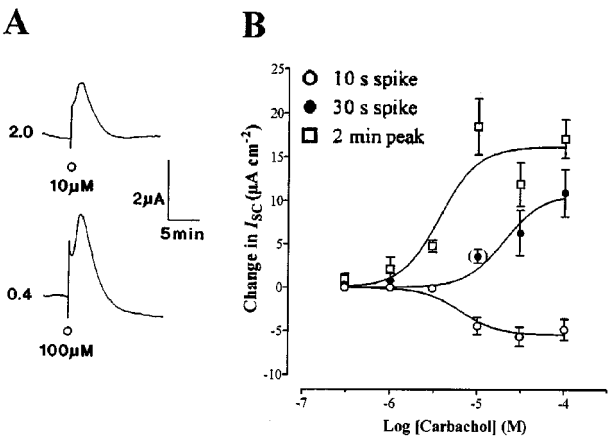

Figure 1 Responses to CCh in Colony 1 epithelial layers. (A) shows representative traces in which Colony 1 cells in KH buffer were stimulated with 10 μM (upper trace) or 100 μM (lower trace) CCh, with initial baseline currents (in μA) given to the left of each example. In (B) single additions of increasing CCh concentration (0.3 – 100 μM; $n = 4 – 14$) are represented as a series of concentration response relationships showing the 10 s downward spike, the 30 s spike and the 2 min transient increase in I_{SC}. A separate 30 s component could not be distinguished after 3 μM CCh and was observed in only a minority of cases (4 out of 14, bracketed value) after 10 μM agonist. EC_{50} values are quoted in the text.

Table 1 Summary of the effects of different solutions, charybdotoxin and apical Ba^{2+} on CCh responses

Pretreatment	*Solution*	*Responses to 10 μM CCh (μA cm^{-2})*		
		10 s spike	*30 s spike*	*2 min peak*
None	KH	-4.4 ± 1.1 (14)	$+3.6 \pm 0.8$ [4]	$+18.5 \pm 3.2$ (14)
ChT (50 nM, bl)	KH	-13.0 ± 2.0 (3)	–	$+11.3 \pm 5.4$ (3)
ChT (50 nM, ap/bl)	KH	-9.7 ± 1.8 (3)	–	$+7.2 \pm 3.9$ (3)
None	HCO_3^- free	-3.0 ± 1.6 [4]	$+3.2 \pm 1.1$ (9)	$+9.5 \pm 1.8$ (9)
ChT (50 nM, bl)	HCO_3^- free	-9.1 ± 1.9 (4)	–	$+10.4 \pm 2.7$ (4)
Ba^{2+} (5 mM, ap)	HCO_3^- free	0.0 ± 0.0 (4)	$+4.8 \pm 0.4$ (4)	$+3.3 \pm 0.8$ (4)
ChT (bl)/Ba^{2+} (ap)	HCO_3^- free	-3.4 ± 0.5 (4)	–	$+4.4 \pm 1.5$ (4)
None	Cl^- free	0.0 ± 0.0 (5)	–	$+1.5 \pm 0.2$ (5)

Colony 1 cells were bathed in KH solution or buffers lacking either HCO_3^- or Cl^- anions, and pretreated with charybdotoxin (ChT) and Ba^{2+} added apically (ap) or basolaterally (bl) as indicated. Peak changes in I_{SC} are given for each measurable phase of the subsequent 10 μM CCh response; in a number of cases the 30 s spike was absent. Values in parenthesis indicate either the total number of observations (rounded brackets) or the number of times a particular component could be distinguished [square brackets]. Significant differences are presented elsewhere in the text or in Figures 7 and 8.

prominent and easily distinguished from the slower transient elevation in I_{SC}. Forskolin-induced increases in I_{SC} (10 μM; $+49.2\pm8.3$ μA cm^{-2}, $n=8$) were smaller than but not significantly different from those observed in KH buffer. After 30 min prestimulation with 10 μM forskolin, 10 μM CCh elicited a rapid 30 s spike ($+8.2\pm1.0$ μA cm^{-2}), followed by a transient increase (peak at 2 min: $+1.9\pm1.8$ μA cm^{-2}; $P<0.05$ compared to unstimulated control responses) and a sustained decrease in I_{SC} (-4.2 ± 1.3 μA cm^{-2}; $n=8$; Figure 3B).

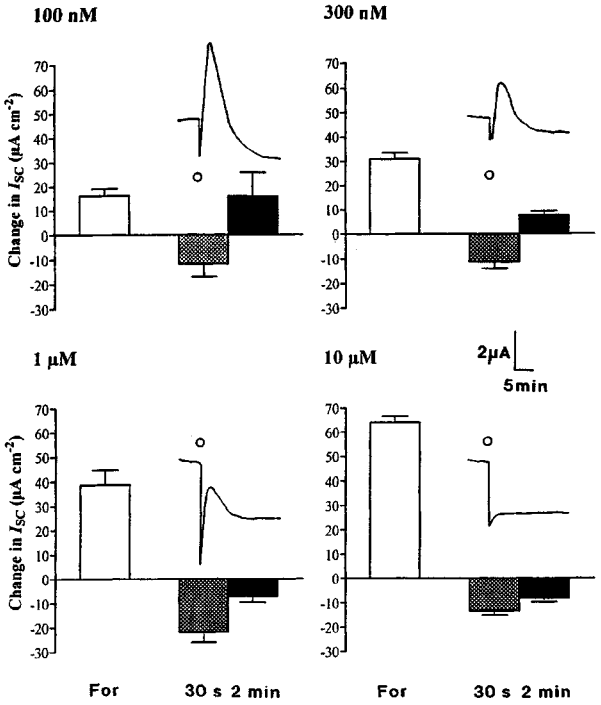

Figure 2 The effect of forskolin stimulation on CCh-induced changes in I_{SC}. Histograms show the increases in I_{SC} after four different concentrations of forskolin (For) in epithelia bathed in KH solution (100 nM – 10 μM; $n=4-76$; open bars) and the responses to 10 μM CCh added 30 min subsequently, summarized as the 30 s peak decrease in I_{SC} (hatched bars) and the maximum at 2 min after agonist addition relative to initial levels (solid bars). Example traces are given as an inset to each histogram, with CCh added at the time indicated.

Cl^- free In Cl^- free solution, all components of the response to CCh (10 μM) in unstimulated epithelial layers were significantly reduced ($P<0.05$, Table 1 and Figure 3A). Responses to 10 μM forskolin were also inhibited ($+10.0\pm1.6$ μA cm^{-2}, $n=5$; $P<0.01$ compared to responses in KH solution), and the residual increase in I_{SC} was insensitive to the loop diuretic piretanide (200 μM). After 10 μM forskolin, CCh decreased I_{SC} (-1.1 ± 0.4 μA cm^{-2} at 2 min, $n=5$, Figure 3B).

The effects of K^+ channel inhibitors on CCh and forskolin responses

293B The chromanol derivative 293B has been used in previous studies as a specific inhibitor of basolateral cyclic AMP-gated K^+ channels in a variety of epithelial cell types (Busch & Suessbrich, 1997). Consistent with this action, basolateral pretreatment with 10 μM 293B (in HCO_3^- free solution) did not alter the Ca^{2+}-mediated I_{SC} changes following 10 μM CCh addition (Figure 4), but the sustained increases in I_{SC} after 10 μM forskolin were significantly attenuated (Figure 5A; $P<0.01$ compared to controls). In the presence of 293B, the responses to 10 μM CCh added 30 min after forskolin were strikingly potentiated (Figure 5B); the 30 s spike component was increased 10 fold compared to control CCh responses after forskolin ($+81.3\pm14.3$ μA cm^{-2}, $n=5$; $P<0.001$) and in the two cases where it could be distinguished, the peak at 2 min was also greater ($+50.8$ μA cm^{-2}).

Basolateral Ba^{2+} The effects of basolateral Ba^{2+} ions (in HCO_3^- free buffer) were essentially identical to those observed

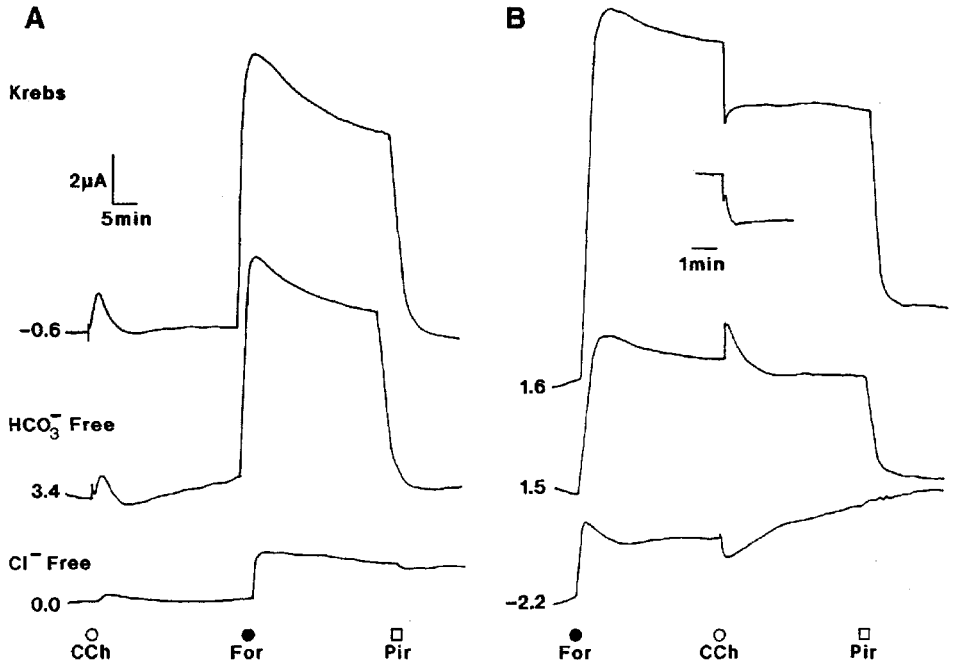

Figure 3 CCh and forskolin responses in Krebs, HCO_3^-- and Cl^--free buffers. (A) The effects of CCh (10 μM), forskolin (10 μM; For) and piretanide (200 μM; Pir) at 30 min intervals are shown in representative traces from cells bathed in KH, HCO_3^- free or Cl^- substituted solutions. In (B), the order of agonist addition is reversed, and an inset to the uppermost trace (KH buffer) displays the changes in I_{SC} after CCh on an extended time-scale to highlight the additional spike component present in the response. Basal I_{SC} is given in μA to the left of each trace.

after treatment with 293B. CCh responses in otherwise unstimulated cells were unaffected (Figure 4); at higher Ba^{2+} concentrations (5 and 10 mM), the 30 s spike component was only observed in a minority of cases, but a 'shoulder' in the majority of the responses suggested it was still present. Forskolin-induced I_{SC} increases were in contrast substantially inhibited (significantly so after 5 mM and 10 mM Ba^{2+}; Figures 5A and 6), while subsequent CCh responses were potentiated (e.g. 2 min peak after 5 mM Ba^{2+}: $+48.3 \pm 6.7$ μA cm^{-2}, $n=6$; $P<0.001$ compared to controls; Figures 5B, 6); under these conditions the 30 s spike was rarely distinguished.

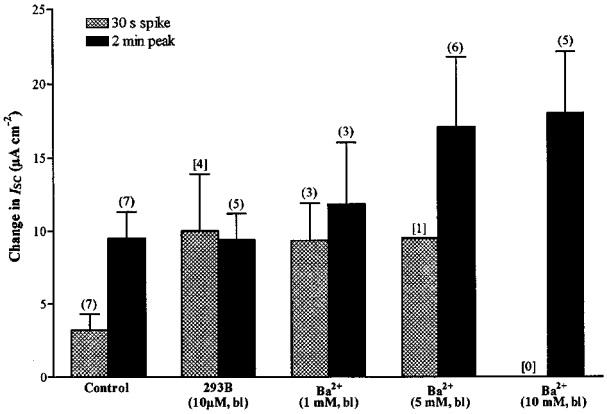

Figure 4 The effect of 293B and Ba^{2+} on CCh stimulation. Epithelial layers in HCO_3^- free buffer were pretreated for 10 min with basolateral 293B (10 μM) or Ba^{2+} (as $BaCl_2$, 1–10 mM) followed by 10 μM CCh. The 30 s spike, where clearly defined, and the 2 min peak increase in I_{SC} are illustrated; the 10 s spike was rarely observed (not shown). *N* values (rounded brackets) or the number of times a component was observed (square brackets) are given above each bar.

Charybdotoxin and apical Ba^{2+} Charybdotoxin (50 nM, basolateral), a specific inhibitor of Ca^{2+}-gated K^+ channels in T84 cells (Devor & Frizzell, 1993), brought about a profound qualitative change in CCh responses in KH buffer (Figure 7). The initial 10 s spike was significantly larger (Table 1, $P<0.01$ compared to controls) and prolonged; the 30 s spike was apparently absent, while the increase in I_{SC} at 2–3 min was unchanged (Table 1, $P=0.35$ compared to controls). Pretreatment with apical as well as basolateral charybdotoxin (both 50 nM) had little or no additional effect on 10 μM CCh responses (Figure 7, Table 1), although the changes in I_{SC} observed for both the 10 s spike and the 2 min peak were marginally smaller (though not significantly) than those observed in cells treated with basolateral charybdotoxin alone.

In HCO_3^- free buffer, the response to CCh in cells pretreated with charybdotoxin (50 nM, basolateral) also consisted of an augmented 10 s spike, no 30 s upward spike and a transient increase in I_{SC} (Figure 8, Table 1). Apical Ba^{2+} (5 mM) in combination with basolateral charybdotoxin

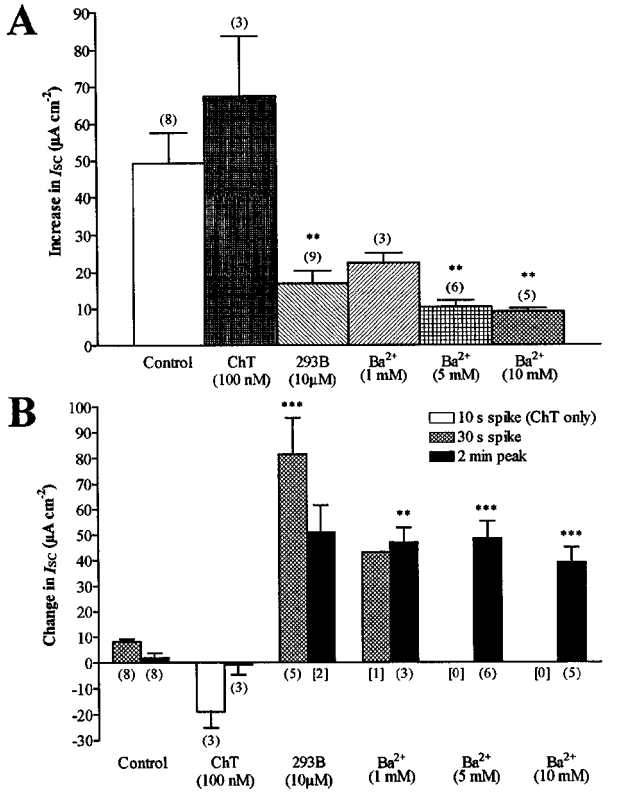

Figure 5 Inhibition of forskolin-induced I_{SC} increases by K^+ channel blockade and the subsequent effects on CCh stimulation. (A) illustrates the effect on 10 μM forskolin responses of 10 min pretreatment with basolateral charybdotoxin (ChT, 100 nM), 293B (10 μM) or Ba^{2+} (1–10 mM). (B) shows the responses to 10 μM CCh added 30 min after forskolin in the same experiments, with each bar representing the 10 s spike (charybdotoxin treated cells only), 30 s spike and 2 min peak. The total number of observations or the frequency of individual components are indicated in round and square brackets respectively; $^{**}P<0.01$ and $^{***}P<0.001$ compared to controls.

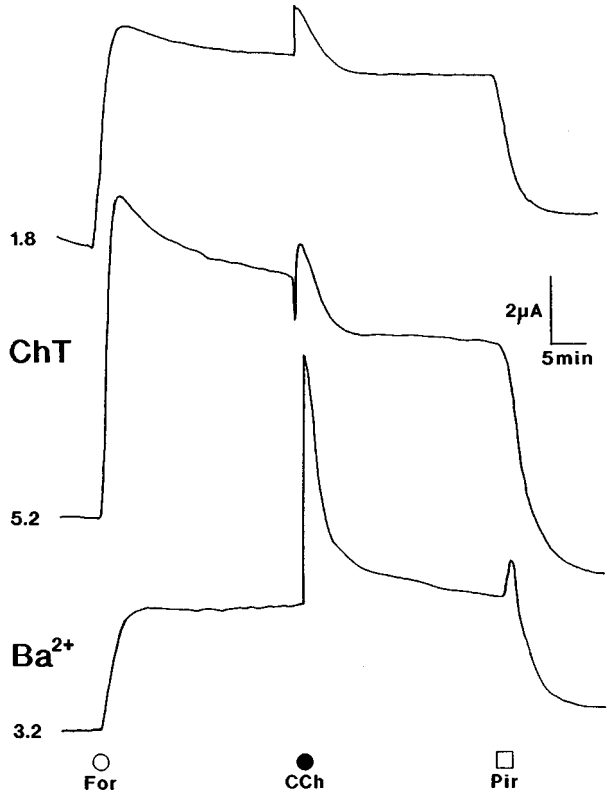

Figure 6 Forskolin and CCh responses in the presence of charybdotoxin or Ba^{2+}. Example experiments from Colony 1 epithelia in HCO_3^- free buffer show the effect of 10 min pretreatment with either 50 nM charybdotoxin (ChT) or 1 mM Ba^{2+} compared to untreated cells (upper trace). Agents were added at the following concentrations: forskolin (10 μM; For), CCh (10 μM) and piretanide (200 μM; Pir). Initial I_{SC} levels are indicated on the left (in μA) of each representative trace.

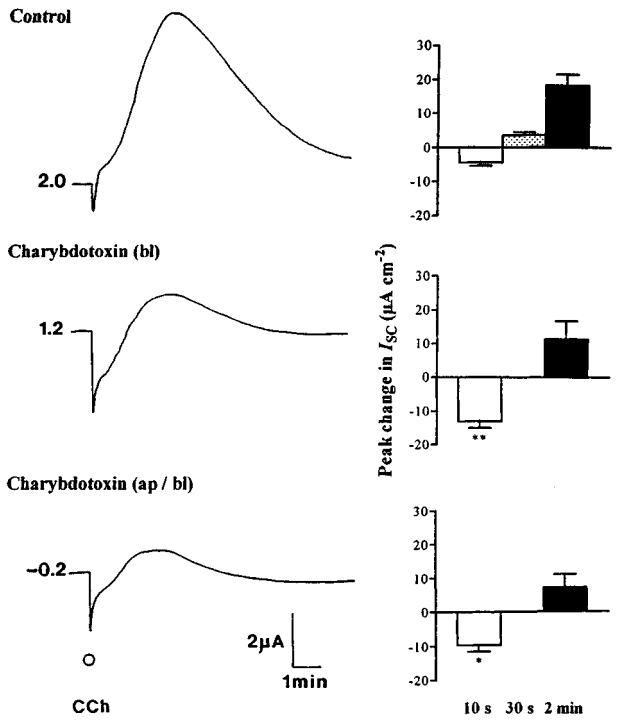

Figure 7 Alteration of CCh responses by charybdotoxin. Representative traces illustrate the CCh-induced changes in I_{SC} from the basal levels indicated, in control cells in KH solution or in those incubated with basolateral (bl) or both apical (ap) and basolateral charybdotoxin (50 nM) for 10 min. To the right of each trace, the histograms show the size of the 10 s spike (open bars), 30 s spike (shaded bars) or the 2 min increase in I_{SC} (solid bars). Significant differences between control values ($n=14$) and those in charybdotoxin-pretreated cells (both $n=3$) are indicated by $^{*}P<0.05$ and $^{**}P<0.01$.

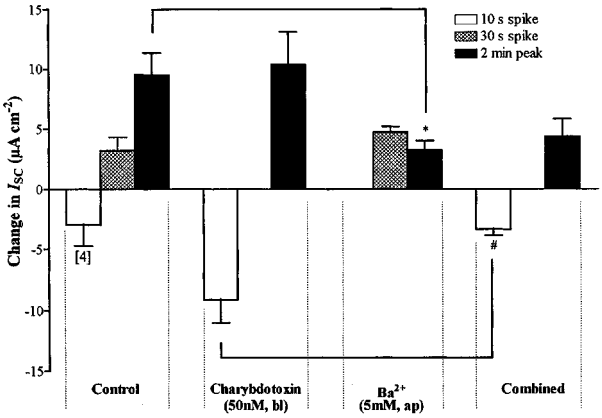

Figure 8 Inhibition of CCh-stimulated changes in I_{SC} by apical Ba^{2+} and charybdotoxin. The histogram shows the separate phases of 10 μM CCh responses in untreated cells in HCO_3^- free solution (control, $n=9$) or those exposed to 50 nM basolateral (bl) charybdotoxin ($n=4$), 5 mM apical (ap) Ba^{2+} ($n=4$) or both treatments ($n=4$) for 10 min. Note that the 10 s spike component of the control group was present in only 4 out of 9 observations, and that the 30 s spike was absent in charybdotoxin-treated cells. Significant differences are indicated as follows: $^{*}P<0.05$ compared to control cells; $\#P<0.05$ compared to pretreatment with charybdotoxin only.

substantially reduced both these phases of the CCh response (e.g. 10 s spike; $P<0.05$ compared to cells inhibited with charybdotoxin only; Figure 8). When added alone, apical Ba^{2+} did not alter the size of the 30 s spike after 10 μM CCh, but did reduce the 2 min peak compared to control responses in untreated cells ($P<0.05$; Table 1). 10 μM forskolin responses were unaffected by a higher concentration of charybdotoxin (100 nM; Figure 5A); however, subsequent changes in I_{SC} after CCh were altered in a manner similar to that observed in unstimulated cells (Figures 5B and 6), consisting of a rapid decrease (-19.0 ± 6.4 μA cm^{-2}), followed by a recovery to approximately initial I_{SC} levels (-1.0 ± 3.8 μA cm^{-2}) and a sustained decrease in I_{SC} (-9.2 ± 1.9 μA cm^{-2}, $n=3$).

Effect of Ba^{2+} and 293B on piretanide responses

Addition of the loop diuretic piretanide after forskolin and CCh stimulation reduced I_{SC} to approximately basal levels. In both control and charybdotoxin treated epithelial layers, these decreases were monophasic (e.g. -35.9 ± 8.5 μA cm^{-2} in control cells, $n=8$; Figure 6). However, piretanide responses were consistently biphasic in nature following Ba^{2+} or 293B pretreatment. In 293B inhibited cells, piretanide addition gave a transient increase in I_{SC} of $+18.4\pm10.6$ μA cm^{-2} ($n=5$) followed by a sustained decrease (-13.8 ± 2.5 μA cm^{-2}); equivalent measurements in epithelia exposed to 5 mM Ba^{2+} were $+4.2\pm1.7$ μA cm^{-2} and -6.4 ± 0.9 μA cm^{-2} ($n=6$) respectively (Figure 6, lowest trace).

Role of carbonic anhydrase

When Colony 1 cells were bathed in HCO_3^- free rather than KH solution, differences in the time-profile of the CCh responses were observed, particularly after first stimulating the cells with forskolin. To examine the extent to which these differences might originate from an inhibition of HCO_3^- transport, epithelia bathed in KH buffer were treated with the carbonic anhydrase inhibitor acetazolamide (450 μM, added both apically and basolaterally). In unstimulated cells, acetazolamide produced a small decrease in I_{SC} (-0.7 ± 0.1 μA cm^{-2}, $n=10$) which was also transient. Following forskolin (10 μM; $+64.0\pm2.7$ μA cm^{-2}, $n=76$), a sustained reduction in I_{SC} of -4.2 ± 0.7 μA cm^{-2} ($n=4$) was observed which accounted for approximately 5% of the forskolin response, the remainder of which was inhibited by piretanide (-66.3 ± 8.8 μA cm^{-2}, $n=4$). Figure 9 shows the effect of acetazolamide pretreatment on 10 μM CCh responses in cells previously treated with forskolin (300 nM), compared with control traces from cells in KH or HCO_3^- free solution. In epithelia treated with acetazolamide, CCh addition resulted in a biphasic response consisting of a 30 s spike ($+3.8\pm1.5$ μA cm^{-2}) and a transient increase in I_{SC} ($+6.8\pm0.7$ μA cm^{-2}, $n=3$); this time-profile was essentially the same as that obtained in HCO_3^- free solution (30 s spike: $+6.6\pm1.1$ μA cm^{-2}; 2 min peak: $+9.0\pm1.5$ μA cm^{-2}, $n=4$).

Action of muscarinic antagonists

To determine whether the separate components of the CCh response following forskolin were mediated by different receptors, the effects of a number of muscarinic antagonists were investigated (Figure 10). Pretreatment for 10 min with either the non-selective antagonist atropine (100 nM) or 4-DAMP (100 nM; M_3 and M_1 selective), dramatically reduced the changes in I_{SC} associated with CCh, while 1 μM gallamine (M_2 selective) and 1 μM *p*F-HHSiD (M_3 selective) were without effect. Following addition of *p*F-HHSiD (10 μM) or

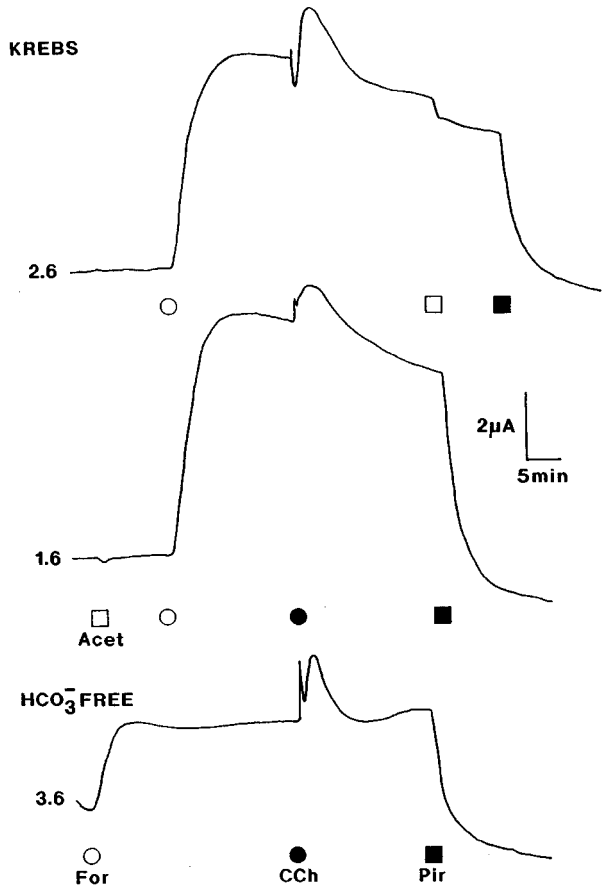

Figure 9 The effect of acetazolamide pretreatment. Example traces show the effect of acetazolamide addition (450 μM, added apically and basolaterally; Acet) on forskolin (300 nM; For) and CCh responses (10 μM) in KH compared with those from otherwise untreated cells bathed in either KH, or HCO_3^- free buffer (lowest trace only). Piretanide (200 μM, Pir) was added at the end of each experiment. Basal I_{SC} (in μA) in each recording is indicated on the left.

pirenzepine (1 μM, M_1 selective), the time-profile of the CCh response was significantly altered, the major component being a small transient increase in I_{SC} at 2 min. Identical responses were obtained when forskolin treated cells were stimulated with a 10 fold lower concentration of CCh (1 μM, Figure 10).

Discussion

CCh responses ± forskolin

Responses to CCh in Colony 1 epithelial layers consisted of transient increases in I_{SC}, which were attributable to the net electrogenic secretion of Cl^- anions and were in general typical of other colonic carcinoma cell lines, such as T84 (Dharmsathaphorn & Pandol, 1986) and the parent cell line HCA-7 (Cuthbert *et al.*, 1987; MacVinish *et al.*, 1993). In otherwise unstimulated cells, this transient elevation in I_{SC} was preceded by two components. In common with HCA-7 responses and those of other subpopulations (MacVinish *et al.*, 1993), there was an upward spike with a maximum at 30 s after agonist addition; however a very rapid initial reduction in I_{SC}, peaking at 10 s, was also observed and is so far unique to Colony 1 cells. Secondly, interactions between CCh and forskolin in these epithelia do not appear to have synergistic actions on transepithelial ion transport (Holliday *et al.*, 1997), in marked contrast to most other epithelial cell lines studied. In KH solution, forskolin-stimulated epithelial layers showed a marked initial decrease in I_{SC} after CCh followed by a recovery, the extent of which depended on the prior forskolin concentration.

K^+ channel inhibition of forskolin responses

Basolateral 293B (10 μM) did not significantly affect CCh responses but substantially inhibited the increases in I_{SC} after forskolin, a selective blockade which is in agreement with previous studies. 293B inhibits forskolin stimulated secretion in rat colonic crypts (Warth *et al.*, 1996), and recently it has

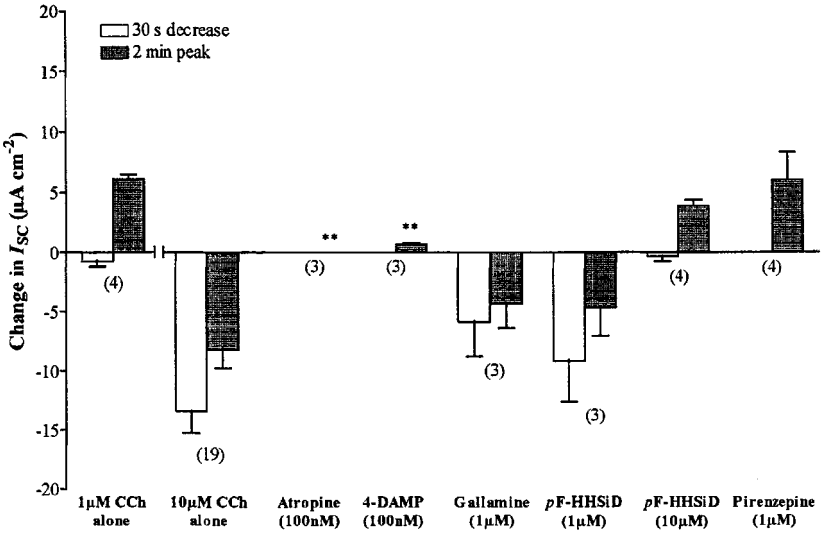

Figure 10 Inhibition of CCh responses by muscarinic antagonists. CCh (10 μM) was added to epithelia bathed in KH solution and prestimulated for 30 min with forskolin (10 μM), with the exception of a separate data group where 1 μM CCh controls are included solely for comparison. Antagonists were applied at the concentrations indicated 10 min prior to the addition of 10 μM CCh, and the subsequent peak changes in I_{SC} in each case are represented by open (initial 30 s decrease) and shaded bars (2 min peak). Responses were compared either with control increases in I_{SC} after 1 μM CCh (atropine, 4-DAMP, *p*F-HHSiD (10 μM) and pirenzepine), or with control decreases in I_{SC} after 10 μM CCh (gallamine, 1 μM *p*F-HHSiD). Significant differences are highlighted by asterisks (**$P<0.01$); numbers in parenthesis denote *n* values.

been shown to specifically target cyclic AMP-gated I_{Ks} channels formed by the KvLQT1 and I_{sK} subunits (Busch & Suessbrich, 1997; Loussouarn *et al*., 1997). The presence of Ba^{2+} (1–10 mM) in the basolateral reservoir also selectively attenuated cyclic AMP-induced Cl^- secretion, as previously observed in T84 monolayers (Dharmsathaphorn & Pandol, 1986) and HT-29 cl. 16E cells (Merlin *et al*., 1995), and consistent with the Ba^{2+} sensitivity of the I_{Ks} current (Busch & Suessbrich, 1997). Although Ba^{2+} has also been reported to inhibit certain Ca^{2+}-gated K^+ channels (Bleich *et al*., 1996), the complementary actions of both 293B and Ba^{2+} provide strong evidence that Colony 1 cells express at least two different basolateral K^+ conductances (Figure 11), of which one is unaffected by either agent and sufficient to enable Ca^{2+}-mediated Cl^- secretion.

Effects of charybdotoxin on CCh responses

Charybdotoxin, a potent inhibitor of epithelial Ca^{2+}-activated K^+ channels identified in basolateral membranes of T84 cells (Ba^{2+}-insensitive; Devor & Frizzell, 1993) and colonic crypts (Ba^{2+}-sensitive; Bleich *et al*., 1996), did not affect the cyclic AMP-mediated increases in I_{SC} after addition of forskolin. However striking differences in the time-profile of the CCh responses were observed, in charybdotoxin pretreated cells bathed in either KH or HCO_3^- free buffer. These consisted of a larger and more prolonged initial reduction in I_{SC} compared to control responses, and the apparent absence of the 30 s spike component. The transient increase in I_{SC}, with a maximum at 2–3 min after agonist addition, was slightly smaller than control responses but not significantly so. The changes in I_{SC} therefore could reflect an initial net electrogenic cationic secretion followed by net anionic secretion. It is clear that the initial decreases in I_{SC} are not dependent on the presence of HCO_3^- anions. Although the responses are less readily observed in control cells bathed in HCO_3^- free buffer, they are augmented in an identical manner when cells are exposed to basolateral charybdotoxin. In addition, all the components associated with the CCh response in otherwise unstimulated Colony 1 cells are critically dependent on the presence of extracellular Cl^- ions, complete inhibition is achieved with Cl^- replacement by gluconate. This experimental evidence leads to a model for secretion in Colony 1 cells in which an increase in the intracellular Ca^{2+} ion concentration leads to the activation of both basolateral and apical K^+ conductances (Figure 11). The basolateral Ca^{2+}-gated K^+ channels, responsible for the 30 s spike component of the CCh response, are inhibited by charybdotoxin. However the opening of apical K^+ channels results in electrogenic K^+ secretion, corresponding to the initial decrease in I_{SC}. This process relies on the entry of K^+ through the basolateral cotransporter and is therefore dependent on extracellular Cl^-. Crucially, the hyperpolarization afforded by the activation of an apical K^+ conductance is sufficient to drive electrogenic Cl^- secretion, and because of the stoichiometry of the $Na^+/K^+/2Cl^-$ cotransporter, a net anionic secretion is predicted overall (observed in charybdotoxin-treated cells as the slower transient rise in I_{SC}). The presence of apical K^+ channels may also partially account for the residual increase in I_{SC} observed after forskolin in 293B or Ba^{2+} treated cells, since subsequent piretanide responses were consistently biphasic, a transient elevation in I_{SC} preceding an overall decrease. These effects of piretanide (not seen in control or charybdotoxin-inhibited epithelia) may represent a reduction in K^+ transport before the reduction in Cl^- secretion.

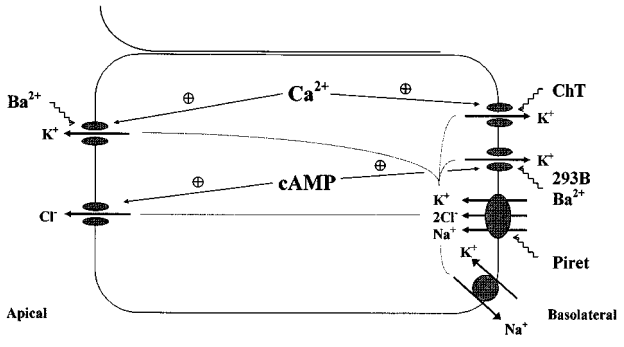

Figure 11 Proposed model for epithelial K^+ and Cl^- secretion in Colony 1 epithelia. The diagram indicates the apical and basolateral membrane conductances that are at present sufficient to explain the observed changes in I_{SC} after either carbachol or forskolin in HCO_3^- free buffer, and the effects on these responses of the range of K^+ channel blockers tested. Elevation of cyclic AMP levels activates basolateral Ba^{2+}- and 293B-sensitive K^+ channels, creating a favourable electrochemical gradient for Cl^- exit through an apical cyclic AMP-gated Cl^- conductance. Na^+ ions, which enter via the $Na^+/K^+/2Cl^-$ cotransporter inhibited by piretanide (Piret), recycle across the basolateral membrane through the Na^+/K^+ ATPase. Ca^{2+}-mediated agonists increase electrogenic Cl^- secretion by opening a separate basolateral K^+ conductance inhibited by charybdotoxin (ChT), but also activate a population of apical K^+ channels that may at least be partially sensitive to Ba^{2+}, allowing electrogenic K^+ secretion. The existence of these apical channels underlies the distinctive biphasic responses to carbachol that are observed after basolateral blockade by charybdotoxin. In Krebs solution, increases in both the cyclic AMP and Ca^{2+} ion concentration may also modulate bicarbonate transport (not shown), as described in the text.

Effects of apical Ba^{2+}

The exchange of drugs that may occur between apical and basolateral bathing solutions presents potential problems for further characterization of the apical K^+ conductance in Colony 1 epithelial layers. However addition of both apical and basolateral charybdotoxin did not significantly change the time-profile or size of CCh responses from those observed after basolateral application alone, thus suggesting that the apical K^+ channels are charybdotoxin-insensitive (Figure 11). In contrast to the insensitivity of Colony 1 CCh responses to basolateral Ba^{2+}, apical addition of $BaCl_2$ (5 mM, HCO_3^- free) did not alter the size of the 30 s spike but it did significantly reduce the subsequent increase in I_{SC}. Although this reduced response might partially result from an elevated apical Cl^- concentration, apical Ba^{2+} also significantly inhibited the initial decrease in I_{SC} induced by CCh when this was revealed by charybdotoxin pretreatment. It might be expected that inhibition of an apical K^+ conductance would lead to an increase in the size of the I_{SC} responses through a reduction in K^+ secretion, but this change is dependent on the time-course over which such secretion occurs. In addition, apical K^+ channels might make a significant contribution to the overall driving force for Cl^- transport, depending on their density. These findings suggest that apical K^+ channels in Colony 1 cells resemble those recently characterized in the rat distal colon (Butterfield *et al*., 1997), which were inhibited by Ba^{2+} ions and were sensitive to the Ca^{2+} ion concentration in the 'cytosolic' patch solution. The selective actions of charybdotoxin and Ba^{2+} distinguish apical and basolateral K^+ channels from each other and suggest that this distribution may be physiologically relevant. The existence of distinct conductances precludes the possibility that basolateral channels are aberrantly targeted to the apical

membrane as a consequence of the Colony 1 carcinoma phenotype.

Apical Cl^- permeability as a determinant of Ca^{2+} - and cyclic AMP-mediated secretion

The lack of potentiation observed between CCh and forskolin does not result from the absence of separate basolateral K^+ conductances activated by Ca^{2+} and cyclic AMP and since a net anionic secretion is predicted even in the presence of apical K^+ transport processes, activation of apical K^+ channels can also not be a factor. Indirect elevation of cyclic AMP levels by CCh through eicosanoid formation (as occurs in the HCA-7 Colony 3 and Colony 29 subpopulations; MacVinish *et al*., 1993) does not occur in Colony 1 cells, since control CCh responses are not inhibited by either 293B or Ba^{2+}. Crosstalk at the intracellular level between the cyclic AMP and Ca^{2+} messenger systems might influence the resulting secretory response, and indeed attenuation of Ca^{2+} influx by elevated cyclic AMP levels has been observed in HT-29 cells (Fischer *et al*., 1996). However this interaction between cyclic AMP and Ca^{2+} ions is apparently outweighed in this cell line by their cooperative activation of membrane conductances. We therefore favour a third mechanism which is consistent with our current data and requires that under forskolin-stimulated conditions the rate of Cl^- secretion is determined by the permeability of the apical membrane to Cl^-, rather than K^+ transport processes. Under these conditions additional activation of Ca^{2+}-gated K^+ channels by CCh would have little or no effect on anion secretion and therefore on the changes in I_{SC} attributable to Cl^- movement. This assumption implies that Ca^{2+}-activated Cl^- channels are not selectively targeted to the apical membrane in Colony 1 cells, so that the predominant effect of increased intracellular Ca^{2+} is on K^+ transport and not apical Cl^- permeability. Consistent with this view is the observation, in HCO_3^- free buffer, that the slower transient rise in I_{SC} induced by CCh is significantly reduced in size if Colony 1 cells are first prestimulated with 10 μM forskolin. Studies on HT-29 cells also suggest that polarization of HT-29 epithelia does not restrict the distribution of Ca^{2+}-gated Cl^- channels (Morris *et al*., 1994), and modulation of Cl^- secretion by CCh in both T84 cells (Dharmsathaphorn & Pandol, 1986) and the rat distal colon (Strabel & Diener, 1995) arises principally through its activation of K^+ conductances. Potentiation of CCh-induced Cl^- secretion by forskolin only occurs in the presence of either Ba^{2+} or 293B, both of which reduce basolateral K^+ transport under cyclic AMP-stimulated conditions. Entry of Cl^- across the basolateral membrane therefore becomes the rate limiting step, and may be accelerated through the effects of CCh on Ca^{2+}-activated K^+ channels.

Electrogenic responses in HCO_3^- free buffer

Investigations with Ba^{2+} required the use of HCO_3^- free buffer as the bathing solution for much of the work on K^+ channel inhibitors in Colony 1 cells. However it is clear that CCh responses are qualitatively different in the absence of HCO_3^- anions after prestimulation with forskolin. Acetazolamide, an inhibitor of carbonic anhydrase, had no sustained effect on the basal I_{SC} of cells bathed in KH solution but did reduce forskolin responses by approximately 5%. Strikingly, the reductions in forskolin-stimulated I_{SC} induced by CCh were also eliminated, such that they became essentially identical to those in HCO_3^- free solution. It is possible that changes in intracellular pH (which may result from both these treatments) could partially account for the differences observed, for example through influences on K^+ conductances (Klaerke, 1997). However the profiles of CCh responses following basolateral charybdotoxin pretreatment suggest that apical Ca^{2+}-gated K^+ channels may still be activated in HCO_3^- free buffer. Alternatively both CCh and forskolin may modulate HCO_3^- transport across Colony 1 epithelial layers, through a mechanism at least partially dependent on carbonic anhydrase. Involvement of the intracellular isoform of this enzyme in HCO_3^- secretion has been previously demonstrated in the rat colon (Feldman, 1994), in guinea-pig duodenum (Muallem *et al*., 1994), in rat epididymal epithelia (Chan *et al*., 1996) and in airway epithelial cells (Smith & Welsh, 1992). Surprisingly, the decreases in I_{SC} after CCh in forskolin-stimulated cells which are sensitive to acetazolamide may represent a reduction in HCO_3^- secretion (or an increase in its absorption). HCO_3^- secretion occurs in colonic epithelia in concert with Na^+ and Cl^- absorption, *via* a carbonic anhydrase dependent mechanism (for a review, see Binder & Sandle, 1994) and in this context, it is interesting to note that flux studies in porcine distal colon have shown that CCh, but not the cyclic AMP-mediated agonist VIP, inhibits Na^+ and Cl^- absorption (Traynor *et al*., 1991). Such a mechanism, if present in Colony 1 cells would explain the reduction in HCO_3^- secretion after CCh and also its dependence on the presence of Cl^- ions, because of the requirement for apical Cl^- HCO_3^- exchange (Feldman & Stephenson, 1990). One limitation is that this transport process is predicted to be electroneutral overall, and therefore it is clear that an elucidation of the exact pathways for HCO_3^- transport in Colony 1 epithelia will require further study.

Muscarinic antagonists

4-DAMP (100 nM), an antagonist with high affinity for human M_1 and M_3 receptors (Eglen *et al*., 1996), and the non-selective muscarinic antagonist atropine both abolished 10 μM CCh responses in Colony 1 cells prestimulated with forskolin. A higher concentration of gallamine (1 μM, M_2 selective) was without effect, while pirenzepine (1 μM, M_1 selective) altered the profile of the CCh response. The latter action reflects the change in time-course that occurs with increasing agonist concentration, and not a selective inhibition of a component of the CCh-induced changes in I_{SC}. The increased potency of 4-DAMP relative to pirenzepine is indicative of the presence of a single M_3-like receptor in Colony 1 cells. Interestingly *p*F-HHSiD, a reported M_3 muscarinic antagonist, did not affect CCh responses except at high concentration (10 μM), but this apparent low affinity does not necessarily indicate an atypical muscarinic receptor subtype. Other functional studies with colonic smooth muscle have shown that *p*F-HHSiD has a low affinity for human M_3 receptors; in circular smooth muscle this antagonist was in fact less potent than pirenzepine (Kerr *et al*., 1995). So far therefore there is no evidence to suggest that the receptors in Colony 1 cells differ from the M_3 subtype characterized in rat colonic epithelia (O'Malley *et al*., 1995) and the human adenocarcinoma cell lines T84 (Dickinson *et al*., 1992) and HT-29 (Kopp *et al*., 1989). Activation of this single receptor population is therefore solely responsible for the complex electrogenic CCh responses recorded.

Colony 1 epithelial layers therefore possess distinct charybdotoxin-sensitive and 293B-inhibitable classes of basolateral K^+ channels which participate in secretory responses; however in contrast to other cell lines, potentiation between Ca^{2+}- and cyclic AMP-elevating agonists is limited by apical permeability to Cl^-. Apical membranes may also contain a Ba^{2+}-sensitive K^+ conductance, the activation of which

underlies many of the distinctive features of the M_3-like receptor response to CCh. Thus Colony 1 cells may prove a useful model in the study of the cellular mechanisms of potassium adaptation in epithelia (Butterfield *et al*., 1997).

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