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Calcium- and cyclic AMP-dependent chloride secretion in human colonic epithelia

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1 Three stable epithelial cell lines (HCA-7, HCA-7-Col 1 and HCA-7-Col 3) all derived from the same human adenocarcinoma have been cultured on collagen-coated Millipore filters. These epithelial monolayers have been used to record short circuit current (SCC) in response to a variety of secretagogues. Similar monolayers, but grown on plastic dishes, were used for measurements of tissue cyclic AMP.

2 Lysylbradykinin, applied to either side of the monolayers, increased SCC in HCA-7 cells but had little effect on the other two lines. The responses showed rapid desensitization, which could be prevented by cooling to 4°C. Responses to kinin were not significantly attenuated by piroxicam, an inhibitor of cyclo-oxygenase.

3 Other secretagogues, vasoactive intestinal polypeptide (VIP) and carbachol also increased SCC in monolayers. The responses to VIP were greatest in HCA-7-Col 1 monolayers while responses were virtually absent in HCA-7-Col 3. A similar profile was seen with carbachol except that responses of HCA-7 and HCA-7-Col 1 monolayers were more equal. With one exception the responses to VIP and carbachol showed sidedness, acting only from the basolateral side.

4 The effects of the secretagogues were inhibited by piretanide, a loop diuretic, applied basolaterally. It is presumed that SCC responses represent electrogenic chloride secretion.

5 Treatment with forskolin increased SCC in HCA-7 and HCA-7-Col 1 monolayers with little effect in HCA-7-Col 3. Nevertheless cyclic AMP levels were elevated most in HCA-7-Col 3 and least in HCA-7-Col 1 monolayers, in reciprocal relationship to the functional response.

6 A23187 increased SCC when applied to HCA-7 and HCA-7-Col 3 monolayers with little effect on HCA-7-Col 1.

7 The differential responses of the three human cell lines provide unique opportunities to discover the functional responsibilities of entities involved in the chloride secretory process. HCA-7-Col 3 cells which generate high levels of cyclic AMP in response to forskolin but which fail to show a substantial chloride secretory response may be a useful model of some disease conditions.

Introduction

The use of cultured epithelial monolayers is a powerful method for examining epithelial transport processes and effects of agents which modify these (Handler, 1983). In particular there can be no doubt that agents affecting transport must be acting directly on the epithelial cells, rather than indirectly on elements in the lamina propria, such as neurones (Cuthbert & Hickman, 1985) or cells of the immune system (Cuthbert *et al.*, 1983). In a previous paper we showed that kinin caused chloride secretion in monolayers of a human colonic epithelium derived from an adenocarcinoma (Cuthbert *et al.*, 1985b). Here we describe the actions of lysylbradykinin (LBK), vasoactive intestinal polypeptide (VIP), carbachol, A23187 and forskolin on three human cell lines, designated HCA-7, HCA-7-Col 1 and HCA-7-Col 3. We show that the effects of these agents are very different in each epithelial type. From the results it appears that each of the three lines is unique, where the differences may be due to the absence, in particular lines, of one or more

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of the components of the transporting mechanisms. We consider lines with deficiencies may be of importance as models of some disease states, for example cystic fibrosis, and further may be useful for characterizing transport mechanisms.

Methods

(a) Establishment of cell sub-populations from the HCA-7 cell line

HCA-7-Col 1 HCA-7 cells (passage 13) (Kirkland, 1985) were treated with 5 mM sodium butyrate using a protocol modified from that described by Augeron & Laboisse (1984). HCA-7 cells were dissociated with trypsin (3 × crystallized and dialysed; Worthington Biochemicals) 0.05% (w/v) in versene (Glasgow formula). Cells were seeded into 25 cm^2 culture flasks in Dulbecco's Eagles medium (Gibco-Europe Ltd, Paisley, Scotland) with glucose (4500 $mg\,l^{-1}$), 10% foetal calf serum (Gibco), kanamycin (100 $\mu g\,ml^{-1}$; Bristol Laboratories, Langley, Slough), amphotericin B (2.5 $\mu g\,ml^{-1}$; E.R. Squibb, Morton, Cheshire) and sodium pyruvate (110 $mg\,l^{-1}$; BDH Chemicals, Poole, Dorset). At Day 3, cells were transferred to medium containing 5 mM sodium butyrate (Sigma Chem. Co. Ltd., Poole, Dorset) and medium changed every two days. At Day 11 cells from a 25 cm^2 culture flask were dissociated and seeded back into a single 25 cm^2 flask, medium was changed every 2 days until Day 25 when cells were changed to medium without sodium butyrate. Only a few cells survived this treatment, these cells were fed twice weekly and slowly grew into colonies of a few hundred cells. One such colony was selected with a cloning cylinder because of its increased dome forming ability. These cells, designated HCA-7-Col 1, grew as tightly packed monolayers and retained increased doming ability when compared to the parent cell line. HCA-7-Col 1 cells formed domes in subconfluent cultures while in monolayers of the parent cell line, domes were never observed until confluence was reached. HCA-7-Col 1 cells were subcultured weekly at a split ratio of 1:5.

At present it is not possible to state whether sodium butyrate treatment resulted in selection of cells with increased doming ability or whether butyrate treatment had induced permanent changes in cells. The morphological and proliferative characteristics of the HCA-7-Col 1 cells have been retained for 29 passages *in vitro*.

HCA-7-Colony 3 HCA-7 cells (passage 12) were sparsely seeded into a 25 cm^2 culture flask. Cells were fed twice weekly until colonies of a few hundred cells were observed. One such colony, with a different morphology from the parent cell line, was selected with a cloning cylinder and designated HCA-7-Col 3. HCA-7-Col 3 cells grew as monolayers on tissue culture plastic with areas of whorling cells. Domes were not observed in monolayers of HCA-7-Col 3 cells even when confluence was reached. The cells were subcultured weekly at a split ratio of 1:20 compared to 1:5 for the parent cell line. HCA-7-Col 3 cells have retained their morphological and proliferative characteristics for 38 passages *in vitro*.

(b) Preparation of epithelial monolayers on pervious supports

Millipore filters were coated with collagen (0.25% in 0.2% acetic acid) and allowed to dry. A silicone washer with 0.2 cm^2 hole was stuck in the centre of each filter with Silastic 734 RTV adhesive, creating a small well into which cells could be seeded. The units were sterilized by u.v. irradiation.

Monolayers of HCA-7, HCA-7-Col 1 and HCA-7-Col 3 were grown to confluence in Dulbecco's modified Eagles medium (DMEM) with foetal calf serum (10%), kanamycin, 100 $\mu g\,ml^{-1}$ and amphotericin B 1.2 $\mu g\,ml^{-1}$. They were dissociated as given under (a) with trypsin and versene. The cells ($3-6 \times 10^5$ cells in 100 μl) were seeded into the wells of the collagen-coated millipore filters. Four units were floated on 12 ml of medium in 30 cm^2 petri dishes. For biochemical experiments cells were seeded into small petri dishes (10 cm^2) using $5-10 \times 10^5$ cells. Both types of culture were incubated at 37°C under 5% CO_2 and the medium changed every 3 days until used.

(c) Short-circuit current (SCC) in epithelial monolayers

The millipore-washer-cell monolayer complexes were clamped between the two halves of an Ussing chamber. A ring of stainless steel pins in one half chamber penetrated the silicone washer and entered holes in the other half chamber. Thus the cellular monolayer (0.2 cm^2) was held in the centre of a window (area 0.6 cm^2) in a way which avoided edge damage to the tissue. The arrangements for recording transepithelial potential, SCC and transepithelial resistance were as described previously (Cuthbert *et al.*, 1985a).

The monolayers were short-circuited with a W-P dual-voltage clamp (WP Instruments, New Haven, Connecticut) which has provision for compensation for the fluid resistance between the tips of the potential electrodes. Continuous records of SCC were displayed on pen-recorders.

Each side of the tissue was bathed in 20 ml of KHS solution maintained at 37°C by heat exchangers and circulated through the chambers by use of a gas-lift (95% O_2:5% CO_2) which also maintained the pH at 7.4. Drugs were added directly to the bathing fluid.

(d) Estimation of cyclic adenosine monophosphate (cyclic AMP) in epithelial monolayers

Epithelial monolayers were grown in petri dishes (10 cm^2) until confluence was reached. Before use, the medium was removed and replaced with KHS solution, with or without added drug, and returned to the incubator at 37°C for the appropriate period. Following this the KHS solution was removed and boiling Brown's buffer (500 μl) was added. Brown's buffer consisted of (mM): Tris HCl 50, theophylline 5 and mercaptoethanol 6. Cells were dislodged from the plastic surface with a rubber 'policeman' and the suspension transferred to plastic tubes which were stored at −20°C until assayed.

To assay the cyclic AMP in samples, they were thawed and refrozen three times and then centrifuged at 12,000 r.p.m. for 3 min. Aliquots of the supernatant were assayed by a standard radioimmunoassay depending on the displacement of [^{3}H]-cyclic AMP from a cyclic AMP binding protein. A standard curve was constructed for each experiment. Validation of the assay was performed by (a) showing that known amounts of cyclic AMP added to the samples were accounted for quantitatively and (b) confirming that different sized aliquots from the same sample gave internally consistent values.

Finally, a few mg of washed sand was added to the sample and the tissue homogenized, with a glass rod, in the Brown's buffer. Aliquots were taken for protein assay by the method of Lowry *et al.* 1951. The cyclic AMP content is given as pmol mg^{-1} protein to allow for possible differences in cell numbers in each dish.

(e) Drugs and solutions

Krebs-Henseleit solution (KHS) had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 11.1. This solution had a pH of 7.4 when equilibrated with 95% O_2 and 5% CO_2 at 37°C. All chemicals used were of reagent grade and drugs were obtained from the following sources: lysylbradykinin (Sigma), VIP (Sigma), carbachol (Sigma), forskolin (Calbiochem) and A23187 (Sigma). Statistical analyses were carried out using a Student's *t* test.

Results

Responses to lysylbradykinin (LBK) and forskolin

We showed earlier (Cuthbert *et al.*, 1985b) that HCA-7 monolayers responded to LBK when added to either the apical or basolateral bathing solution. In Figure 1 we show a comparison of the effects of LBK and forskolin, an activator of adenylate cyclase (Seamon *et al.*, 1981), on the three types of epithelial monolayers, both agents being used at supramaximally effective concentrations. HCA-7-Col 1 and HCA-7-Col 3 monolayers showed little or no response to LBK, unlike HCA-7 cells. However, both HCA-7 and HCA-7-Col 1 cells showed very large responses to forskolin, while HCA-7-Col 3 monolayers responded only poorly. Typical records upon which the data in Figure 1 are based are shown in Figure 2.

We took the opportunity in these studies to collect information about transepithelial resistance of the three types of cell monolayers grown on collagen-coated millipore filters. Values found were HCA-7, $78.3 \pm 6.8\,\Omega\,cm^2$ ($n = 17$); HCA-7-Col 1, $42.3 \pm 5.4\,\Omega\,cm^2$ ($n = 20$) and HCA-7-Col 3, $117.1 \pm 13.6\,\Omega\,cm^2$ ($n = 12$). The value for HCA-7 epithelia was significantly different from those of the other two types ($P < 0.01$).

To investigate further the mechanism by which kinins cause an increase in SCC, cultures were incubated with a cyclo-oxygenase inhibitor, piroxicam (5 μM) and the responses to apical and basolateral kinin were compared to those measured in non-treated controls. The data from six paired experiments is given in Figure 3. There was a modest reduction in both the apically and basolaterally induced LBK responses, but in neither instance was statistical significance achieved. This result suggests that generation of eicosanoids can only be a minor feature in the genesis of response to kinins.

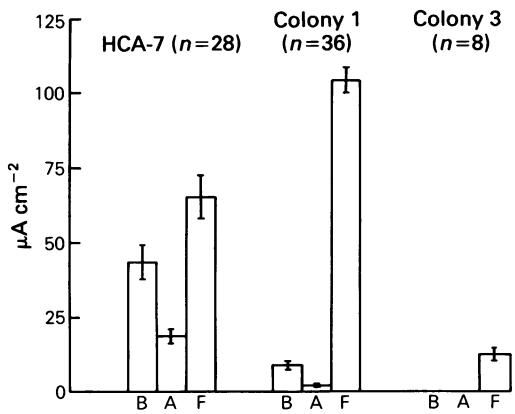

Figure 1 Peak SCC responses ($\mu A\,cm^{-2}$) in epithelial monolayers of HCA-7, HCA-7-Col 1 and HCA-7-Col 3 cells. Each monolayer had an area of 0.2 cm^2. Responses were obtained to lysylbradykinin (LBK, 100 nM) applied separately to the apical and basolateral side of the tissue. These responses were transient and responses to forskolin (10 μM) applied in both bathing solutions were obtained after the responses to kinin had disappeared. B and A refer to basolateral and apical kinin respectively, while F indicates addition of forskolin. Mean values for *n* observations are given; vertical lines indicate s.e.

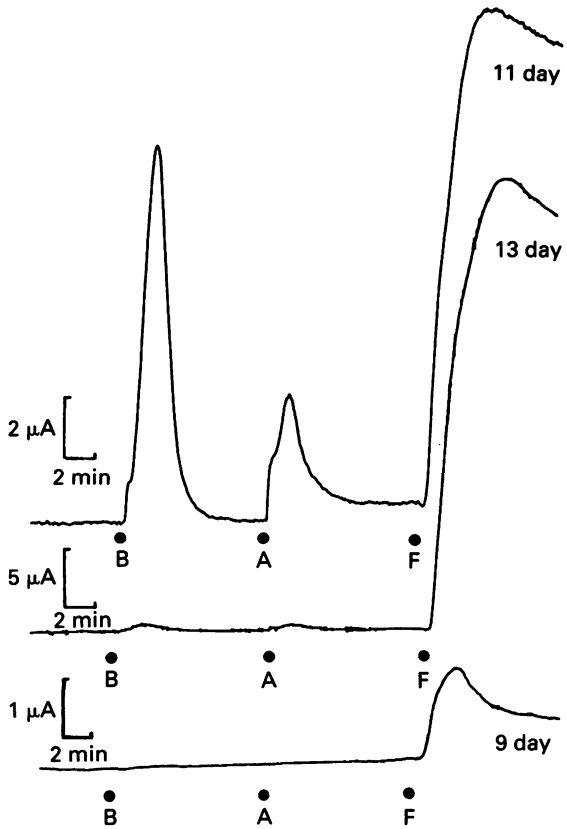

Figure 2 Typical SCC records from confluent monolayers (0.2 cm²) of HCA-7 cells (upper trace), HCA-7-Col 1 cells (middle trace) and HCA-7-Col 3 cells (lower trace). Each was treated with lysylbradykinin, 100 nM on the basolateral side (B) and then on the apical side (A). Finally monolayers were treated on both sides with forskolin, 10 μM (F). Note the difference in calibration scales. We detected no significant differences in the responses of monolayers cultured for different times, provided they were confluent.

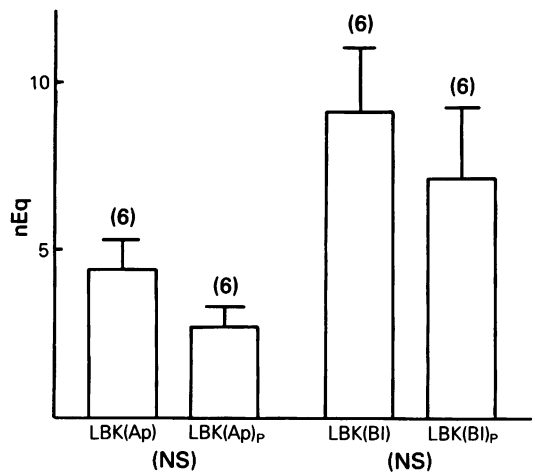

Figure 3 Effects of piroxicam (5 μM) on responses to lysylbradykinin (LBK) in HCA-7 monolayers. Paired monolayers were used either as controls or were treated with piroxicam on both sides for 30 min. Responses to LBK (100 nM) were obtained by apical (Ap) and basolateral (Bl) application. Responses are given as nEq obtained by integrating the SCC versus time curves. The subscript P indicates tissues were treated with piroxicam.

In the experiments with piroxican we were obliged to use separate cultures for the test and control responses. The reason is that the responses to LBK show extreme desensitization and repeated stimulation of a single monolayer with LBK fails to produce an equivalent response even when the time interval is as great as 3 h.

Figure 4a gives an example of how the response to LBK recovers following removal of the peptide. It can be seen that recovery is less than 50% of the initial response after 3 h and barely discernible after 45 min. Desensitization to LBK was more complete at higher concentrations of the peptide. This is confirmed by the set of results given in Figure 4b. HCA-7 monolayers were exposed, in turn, to LBK at a concentration of 10 nM and 100 nM or, alternatively, in the reverse order. Even at 10 nM LBK there is some desensitization to the response to 100 nM, while 100 nM LBK completely obliterates any subsequent response to the peptide at 10 nM.

We have discovered one procedure that attenuates desensitization to LBK, namely cooling. Figure 5 illustrates the data from a series of experiments in which the actions of LBK, both apical and basolateral and the effects of forskolin were measured at three different temperatures on HCA-7 monolayers. Exposure to kinin at 37°C not unexpectedly severely attenuated the subsequent responses compared to controls. The monolayers desensitized to LBK could show a full secretory response as evidenced by the response to forskolin, which was not significantly different from controls. The situation was rather similar when the measurements were carried out at 20°C, although the severity of the desensitization was less. However, when the preliminary exposure to kinin was at 4°C there was no attenuation of the response to LBK applied apically while the response to basolaterally applied LBK was reduced, but not significantly so. However, pre-exposure to KHS at 4°C reduced the response to forskolin by an equivalent amount whether or not monolayers had also been exposed to LBK.

The responses of HCA-7, HCA-7-Col 1 and HCA-

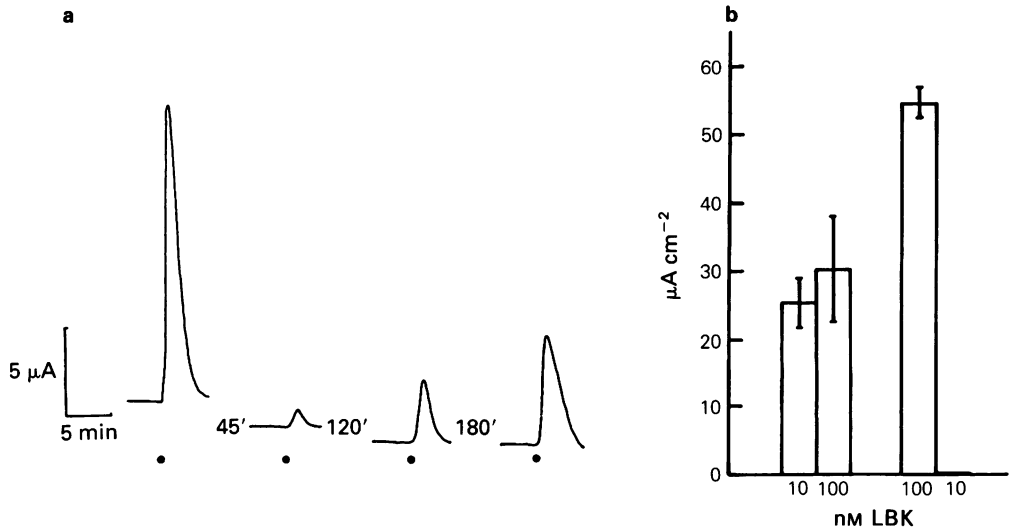

Figure 4 Desensitization to lysylbradykinin (LBK) (basolateral) in HCA-7 monolayers. (a) Four responses to LBK (100 nM) in the same monolayer with washing between each exposure. The intervals between the responses (in minutes) are shown. (b) Responses to LBK in HCA-7 monolayers. Each was exposed twice to LBK with washing in between. One set received the peptide at concentrations of 10 nM followed by 100 nM 30 min later (left) while in the other the peptide was given in the reverse order (right). Mean responses are given for six preparations with s.e. shown by vertical lines.

7-Col 3 monolayers to forskolin were inhibited by piretanide and frusemide, inhibitors of Na-K-2Cl cotransport (data not shown). This result, together with the increase in inward flowing current caused by forskolin is indicative that the SCC responses in these epithelia result from electrogenic anion secretion, most probably chloride (Cuthbert *et al.*, 1985b). We considered whether the ability of forskolin to generate these responses was in proportion to the activation of adenylate cyclase and the accumulation of cyclic AMP within the tissue. The surprising result was that they were not so related as illustrated in Figure 6. Basal levels in the three types of epithelia were low (23.8 ± 4.0 pmol mg^{-1} protein) but were significantly increased above control by exposure to forskolin. In HCA-7 cells values were less at 15 min than at 5 min after exposure to forskolin, while the reverse was true of HCA-7-Col 1 and HCA-7-Col 3. HCA-7-Col 1 cells which showed the largest SCC responses generated the smallest increase in cyclic nucleotide. Most surprisingly HCA-7-Col 3 which showed very small, non-maintained SCC responses to forskolin showed the greatest increases in cyclic AMP, significantly greater than those of the other two types ($P < 0.005$). It would appear that processes subsequent to cyclic AMP generation are deficient in HCA-7-Col 3 cells.

The effects of LBK (100 nM) and VIP (10 nM) applied apically to epithelial monolayers grown in dishes were investigated in a number of experiments. No significant effect of these agents on cyclic AMP content was recorded at either 5 or 15 min exposure.

Changes in SCC caused by vasoactive intestinal peptide

All three cell lines responded to VIP, Figure 7b shows the effects of 10 nM VIP, a maximally effective concentration, when applied in either the apical or basolateral bathing fluid. Unlike the responses to kinin the responses to VIP were maintained. Responses in HCA-7 and HCA-7-Col 3 monolayers were sided, that is, significant responses were obtained only when the peptide was added to the basolateral side. The responses of HCA-7-Col 1 monolayers were unusually large, almost equalling the responses to forskolin; further, they were not sided, responses being obtained with apical or basolateral application. However we are not convinced that VIP receptors are indeed located in both membrane domains of the epithelium. The reasoning behind this view can be appreciated from the illustration given in Figure 7a. First, SCC responses to VIP applied to the basolateral face were not further potentiated when VIP was subsequently added to the apical bathing fluid. However, basolaterally applied VIP caused an additional SCC effect when applied following apical VIP. Secondly, piretanide, which usually acts basolaterally in chloride secreting epithelia, inhibited the effects of VIP when added apically but there was additional inhibition with

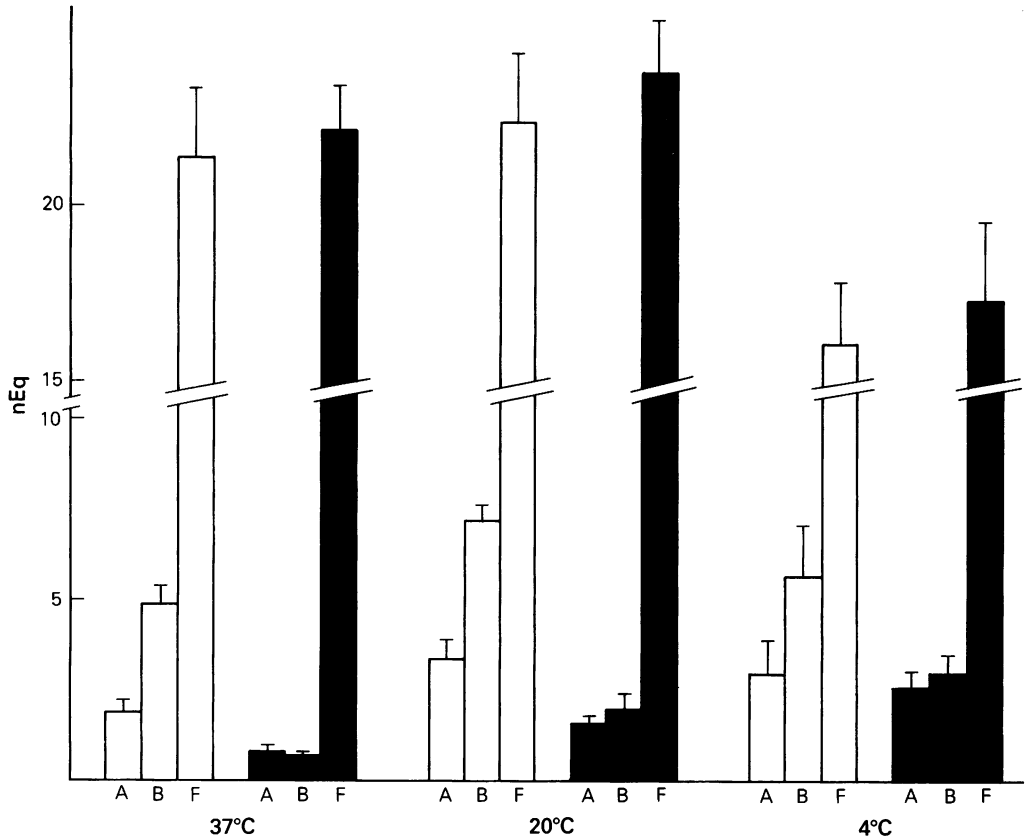

Figure 5 Effect of temperature on desensitization to lysylbradykinin (LBK) in HCA-7 monolayers. All monolayers were dipped into KHS solution at either 37°C, 20°C or 4°C for 5 min without (open columns) or containing LBK, 100 nM (shaded columns). Subsequently monolayers were washed in KHS solution and mounted for SCC recording at 37°C and responses were recorded 30 min later. Reponses (in nEq) to apical (A) and basolateral (B) kinin and to forskolin (F) (10 μM applied both sides) are shown. The control responses to kinin (open columns) were not significantly different irrespective of the initial preincubation temperature. However, the responses were significantly less than control values in tissues pre-exposed to kinin at either 37°C or 20°C ($P < 0.05$ or less). The responses to kinin in tissues pre-exposed at 4°C were not significantly different from controls. Although responses to forskolin were reduced in tissues exposed to KHS at 4°C the values were not different from those measured at 37°C. Mean values for six measurements are given with s.e. shown by vertical lines. The forskolin responses were for an 8 min exposure to the drug.

subsequent basolateral addition. On the other hand, if piretanide was added initially to the basolateral side there was no further inhibition when the blocker was added apically. These data can be unified if it is assumed that VIP receptors and the piretanide-sensitive cotransport mechanism are located exclusively on the basolateral side, but that both agents can penetrate to the basolateral domain from the apical side. In several experiments HCA-7-Col 1 monolayers were mounted with no potassium in the apical bathing solution. In this configuration apically applied piretanide still inhibited transport although no potassium was available for the cotransport mechanism on the apical side.

In HCA-7 monolayers, apical piretanide did not affect the responses to VIP applied to the basolateral side. When both agents were added basolaterally piretanide inhibited the response to VIP.

Differential effects of A23187 and forskolin

Both intracellular calcium and cyclic AMP have been considered to be stimuli for electrogenic chloride secretion (see Cuthbert, 1985; Donowitz & Welsh,

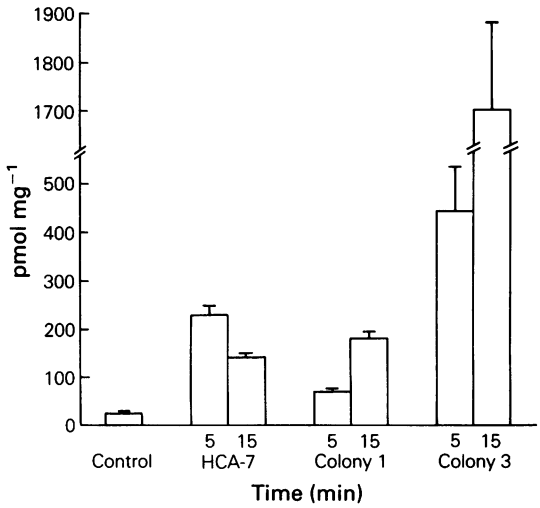

Figure 6 Effect of forskolin on cyclic AMP accumulation in epithelial monolayers. Cells were grown to confluence in plastic dishes and exposed to forskolin, 10 μM on the apical side for either 5 or 15 min. Mean values (as pmol mg^{-1} protein) are given for 5 observations, except for controls, vertical lines show s.e. We could detect no difference in the basal levels of cyclic AMP without forskolin in the three cell lines. The control values have been pooled and represent 14 measurements. In a further 6 controls the values were so low they could not be estimated. Thus the control value shown represents an upper limit for the resting state.

1986). We compared the SCC responses to A23187 and forskolin, arguing that these agents would increase intracellular calcium and cyclic AMP respectively. The data from these experiments are given in Figure 8. We found it necessary to add A23187 to the apical medium, and consider the reason for this is that this lipid-soluble agent partitions into the millipore filter when added to the basolateral side. HCA-7-Col 1 cells which responded most to forskolin were the least responsive to the calcium ionophore. On the other hand HCA-7-Col 3 responded as well as HCA-7 to A23187 yet very poorly to forskolin, in spite of the massive increase in cyclic AMP production shown earlier.

Both forskolin and A23187 gave sustained increases in SCC in epithelial monolayers which could be

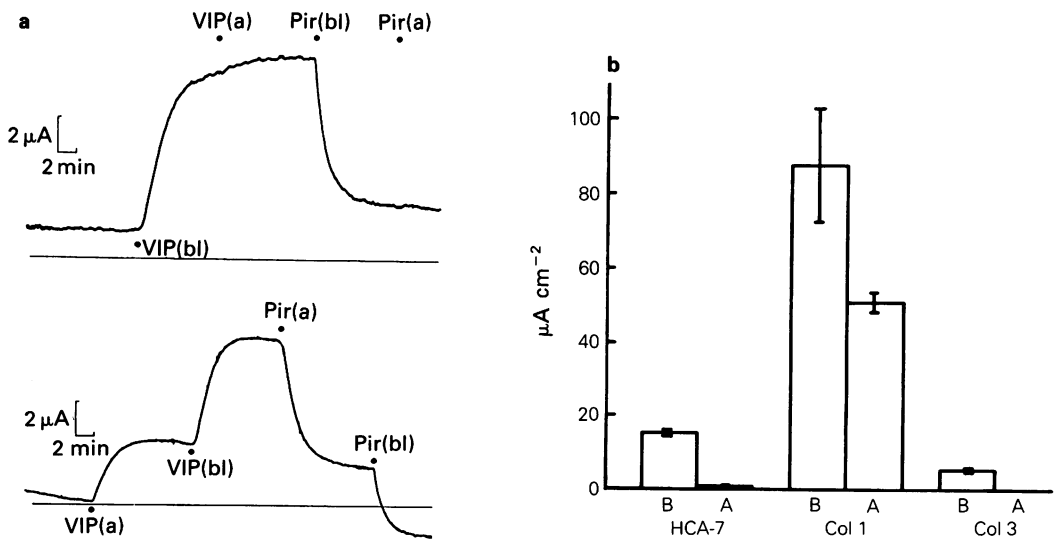

Figure 7 Effect of vasoactive intestinal peptide (VIP, 10 nM) on SCC in epithelial monolayers. (a) Effect of VIP on SCC in two HCA-7-Col 1 monolayers. In one, VIP was applied basolaterally (bl) then apically (a) followed by pirentanide (0.2 mM) applied basolaterally then apically (upper trace). In the other the order was reversed. Note that apical responses are seen only when the addition precedes addition to the basolateral side. (b) Responses, in μA cm^{-2}, in HCA-7, HCA-7-Col 1 and HCA-7-Col 3 monolayers to VIP applied apically and basolaterally. Mean values are shown with s.e. indicated by vertical lines. Number of observations varied between 4 and 11.

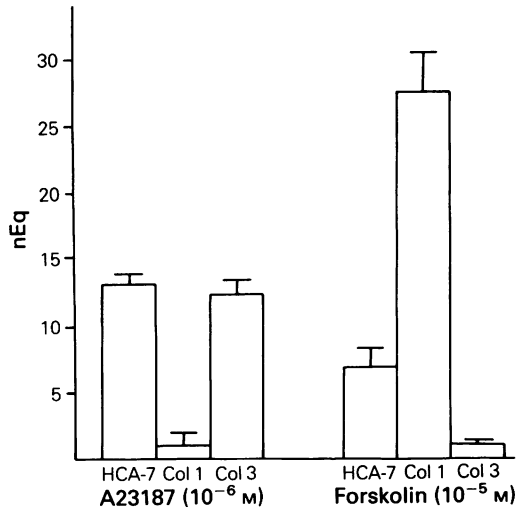

Figure 8 Responses in epithelial monolayers to A23187 (1 μM) apically applied and forskolin (10 μM), applied apically and basolaterally. Responses are given as nEq obtained by integrating the SCC versus time curves for 8 min following addition of the drugs. Values are for means for 6 separate observations with s.e. indicated by vertical lines.

inhibited by basolateral addition of piretanide. These results emphasize the very different transporting characteristics of the three cell lines, all derived from a single human adenocarcinoma.

Effects of carbachol

Carbachol, 10 μM, produced transient increases in SCC in HCA-7 and HCA-7-Col 1 (Figure 9). The effects were rather variable in amplitude and were apparent only when the agonist was added to the basolateral side. In contrast HCA-7-Col 3 showed very small responses, again from the basolateral side only. In the same preparations which were stimulated transiently by carbachol, forskolin was able to produce a maintained increase in SCC.

Structural features

All three lines grown on plastic dishes form confluent monolayers with tight junctions at the apical borders. The features of HCA-7 monolayers have been described by Kirkland (1985). Only HCA-7 and HCA-7-Col 1 monolayers can show dome formation, an anatomical indicator of vectorial fluid transport in the apical to basolateral direction, when grown on plastic. The ability to form domes is greater in HCA-7-Col 1 than the parent line, while domes were never seen with HCA-7-Col 3 monolayers. The morphological features of the three cell lines will be given elsewhere but we show here a comparison of HCA-7-Col 1 monolayers grown on plastic and upon collagen-coated Millipore filters (Figure 10). In the former situation, simple monolayers were formed while when grown on collagen a multilayered preparation is generated which has, nevertheless, the usual apical surface structures with microvilli and tight junctions. However below the surface small acini are sometimes found with apical microvilli directed to the lumen. It is possible that the lack of sidedness to VIP in HCA-7-Col 1 monolayers is connected with these usual structures. Our failure to measure any elevation of cyclic AMP when VIP was added to the apical surface of monolayers grown on plastic argues that conventional monolayer structures show the appropriate sidedness.

Discussion

The three cell lines used in this study were all isolated from cultures of a single colonic carcinoma. The cell lines retained their morphological, proliferative and pharmacological characteristics during 20–30 passages *in vitro*. Such heterogeneity in colon carcinoma cultures has previously been described (Dexter *et al.*, 1979; Brattain *et al.*, 1981). It is not known whether the three types arose by independent mutation from an original monoclonal population and were selected out by the isolation procedures or, alternatively, arose from permanent changes caused by the treatment used for selection. However, the stable variants demonstrated here provide a unique opportunity for further studies in which crucial roles for various components of the transport mechanism can be worked out. While this work was in progress three sublines for another epithelial cell line derived from pig kidney (LLC-PK_1) were described which varied in their response to calcitonin (Wohlwend *et al.*, 1986).

The discussion of these results can be divided into two main parts. First the properties of kinin receptors in relation to electrogenic chloride secretion in epithelia and secondly the components involved in this secretion. From the latter standpoint we must consider the presumed basolateral cotransport mechanism(s) and the chloride channel in the apical face as well as the second messengers, such as Ca^{2+} and cyclic AMP, which may be involved. In this first paper we do not pretend to have done more than to indicate the major differences between the three lines and recognize that each cell type will require a major study before the transporting characteristics are defined in terms of the component parts.

Both HCA-7 and HCA-7-Col 1 cells have functional kinin receptors on their apical and basolateral surfaces while they are absent from HCA-7-Col 3 cells.

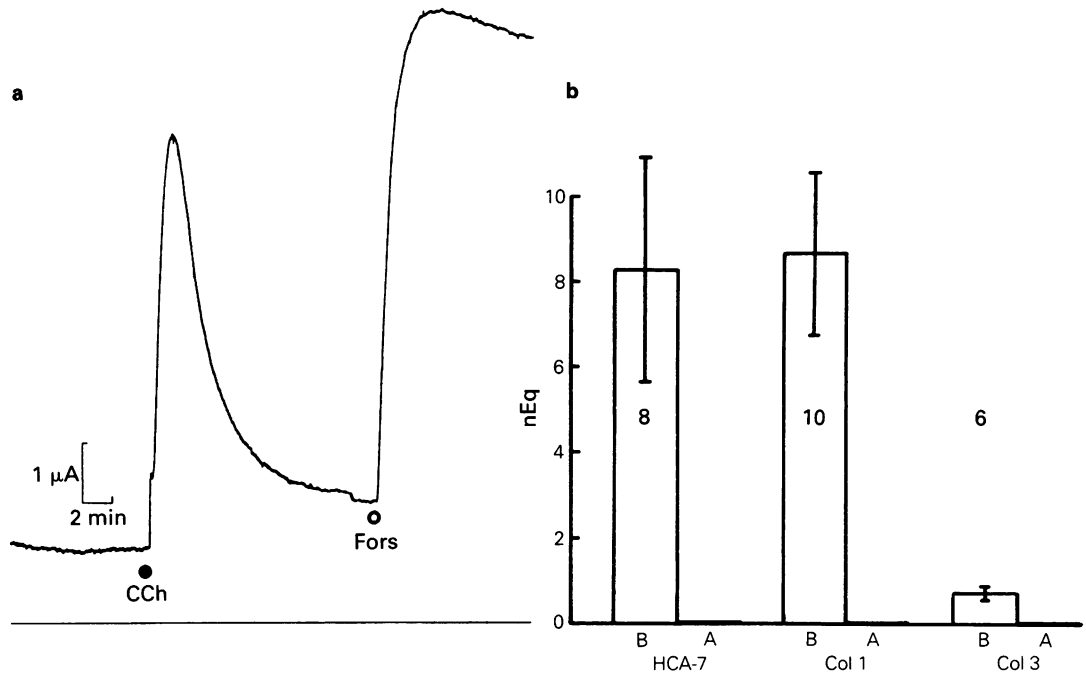

Figure 9 (a) Effects of carbachol (CCh, 10 μM) applied basolaterally and forskolin (10 μM) applied apically on SCC in an HCA-7 monolayer. (b) Responses to carbachol (10 μM) applied basolaterally (B) and apically (A) in HCA-7, HCA-7-Col 1 and HCA-7-Col 3 monolayers. Current versus time traces were integrated over a period of 8 min following addition of the drug. Number of observations are shown over each pair of histograms.

This does not mean, of course, that kinin receptors are necessarily absent; they may be present but uncoupled and this question can only be answered by a binding study. Although we are contemplating this, there are likely to be difficulties because of the severe desensitization we have encountered. Again it is not known whether desensitization is because of a failure in coupling or if the receptors themselves become desensitized or internalized. There is, however, evidence that human fibroblasts have characteristics close to those reported by us in epithelial cells. In fibroblasts, pre-exposure to kinin reduces the binding of the tritiated peptide without changing its affinity and also inhibits subsequent kinin-induced eicosanoid formation. The loss of binding sites can be prevented by cooling to 4°C (Roscher *et al.*, 1984), a manoeuvre which we have shown reduces desensitization.

We have not measured eicosanoid formation in monolayers of adenocarcinoma cells, however our results with piroxicam suggest that prostaglandins are relatively unimportant for the effects of kinins on transepithelial transport, although these lines do respond to PGE_2 (data not shown). In the mammalian colon epithelium we have shown that the effects of kinins on chloride secretion are, in part, dependent on prostaglandin formation (Cuthbert *et al.*, 1984a) but that chloride secretion can be triggered without prostaglandin involvement (Cuthbert *et al.*, 1984b). Recently others (Hoult & Phillips, 1986) have suggested, for rat isolated colon, that prostaglandins are generated by interaction of kinins with elements in the lamina propria which then secondarily affect epithelial transport. It is abundantly clear from this and an earlier publication (Cuthbert *et al.*, 1985b) that kinins can have a direct action on transport in epithelial cells in the complete absence of other cell types. There was no proportionality between the responses to kinin and those due to activation of adenylate cyclase with forskolin. However, the ability of forskolin to stimulate SCC was itself unrelated to the ability to generate cyclic AMP in the tissue. We could not detect a significant change in cyclic AMP following challenge with kinin on the apical side. All of this foregoing evidence makes it unlikely that kinin effects are mediated by cyclic AMP in these pure epithelial systems. We chose to compare the actions of kinins with those of VIP and carbachol, the latter two being secretagogues thought to use cyclic AMP and Ca^{2+} respectively as secondary signals. Additionally, by using forskolin and A23187 we had independent

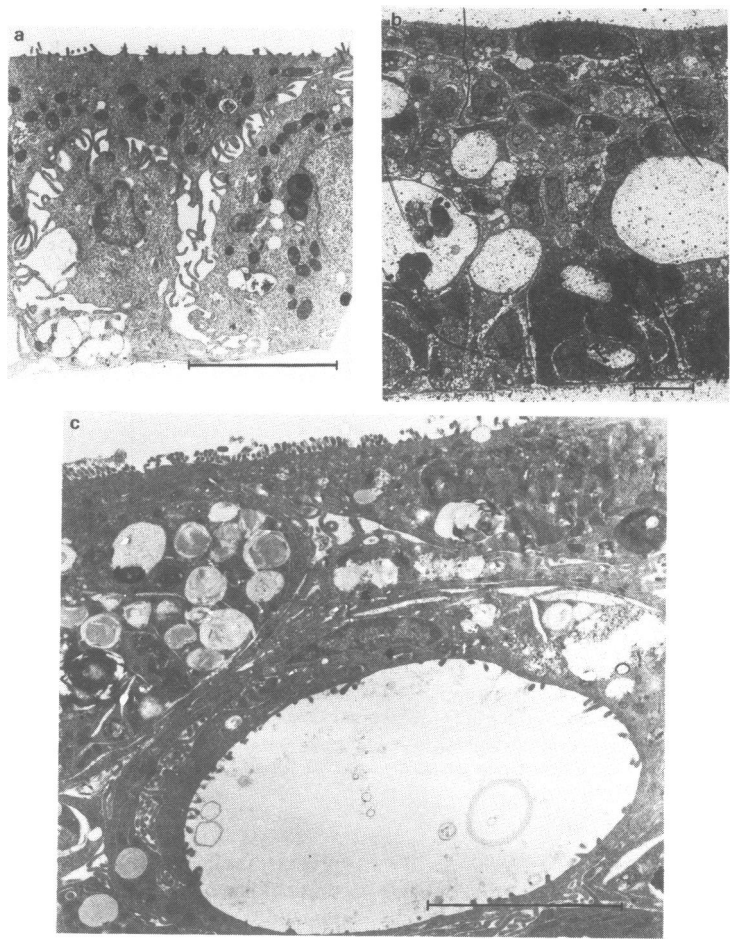

Figure 10 Electron micrographs of HCA-7-Col 1 monolayers. (a) Cultured on plastic showing one cell wide layer with apical microvilli. (b) Cultured in collagen-coated Millipore filter. A complex mutilayered epithelium is formed again showing apical microvilli, but also exhibiting intraepithelial acini. (c) High power version of (b) showing prominent apical microvilli but also microvilli directed into the lumen of the acinus. In all three micrographs the apical surface is uppermost. In (a) and (b) the innermost basolateral face is also shown. Calibrations are 5 μm.

means of raising intracellular cyclic AMP and Ca^{2+}.

Unlike the kinin responses, those to VIP were maintained while the peptide was present. Furthermore the pattern of activation was different, HCA-7-Col 1 being the most responsive. The receptors appear to be sided in HCA-7 and HCA-7-Col 3 while the results with HCA-7-Col 1 are ambiguous. They are best explained by proposing that both the peptide and piretanide can penetrate these monolayers (Figure 7). As VIP stimulates adenylate cyclase in mammalian colonic epithelia (Dharmsathaphorn *et al.*, 1985) it is, on first sight, surprising that adenylate cyclase was not stimulated by VIP, applied apically, to monolayers cultured upon plates. However, we demonstrate an unsual morphology shown by HCA-7-Col 1 cells when grown on collagen supports, while a more conventional monolayer structure was apparent when they were grown on dishes. We suggest therefore that the ambiguous results with HCA-7-Col 1 cells does have a morphological basis.

The questions about sidedness with VIP do not arise with kinins as it is possible, following complete desensitization to either apical or basolateral kinin, to demonstrate an action on the contralateral side (Cuthbert *et al.*, 1985b). With VIP, however, a maximal basolateral response precludes further stimulation

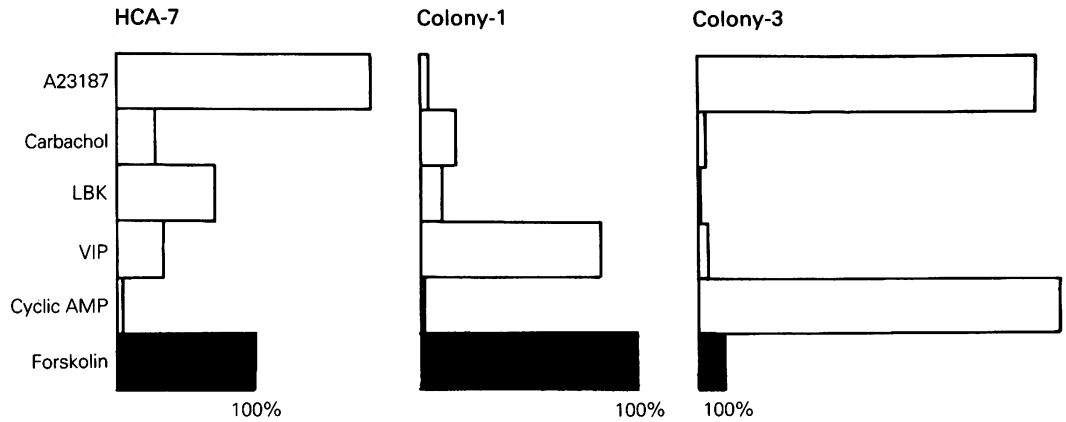

Figure 11 Comparative activity of A23187, carbachol, lysylbradykinin (LBK), vasoactive intestinal peptide (VIP) and forskolin in HCA-7, HCA-7-Col 1 and HCA-7-Col 3 monolayers. The responses to forskolin are taken as 100% for each cell type but are drawn in proportion to the responses obtained. The responses to the other agonists are given in relation to the relevant forskolin control. The cyclic AMP content, measured after treatment with forskolin, is given as the ratio of content divided by the SCC responses. Responses to basolateral addition only of LBK, VIP and carbachol are represented.

from the apical side in HCA-7-Col 1, while HCA-7 and HCA-7-Col 3 respond only to basolaterally applied VIP.

It was surprising that carbachol showed sidedness in HCA-7-Col 1, the cell line which we have demonstrated has an abnormal morphology grown on collagen-coated millipore filters. Responses to VIP in HCA-7-Col 1 were considerably greater (5 ×) (Figure 10) than those to carbachol so it is possible that apical responses to carbachol were too small to be detected by our methods, or alternatively carbachol is unable to penetrate the monolayer from the apical side. In the other two lines we had no evidence of a carbachol effect from the apical side.

Finally the three systems we have used varied considerably in their response to A23187, HCA-7-Col 1 being relatively insensitive compared to either HCA-7 or HCA-7-Col 3. Indeed it was striking that HCA-7-Col 1 showed the greatest response to forskolin and the weakest to A23187. The ratio of the response to A23187 to forskolin can be calculated from Figure 8 and gives the values 1.3 (HCA-7), 0.04 (HCA-7-Col 1) and 11.1 (HCA-7-Col 3). It might be considered that the value of this ratio is indicative of whether Ca^{2+} or cyclic AMP signals are more important in triggering secretion, high values being associated with calcium dependence and low values with cyclic AMP dependence.

To help summarise our findings with the three cell lines we have constructed Figure 11 illustrating the various patterns of sensitivity. It is important to remember that all the SCC responses we have recorded are in the same direction and all are sensitive to piretanide, providing strong evidence that, throughout, we are dealing with electrogenic chloride secretion. All three lines therefore have characteristics of crypt cells and thus far we have been unable to find evidence for electrogenic sodium absorption, even after culturing the cells with aldosterone (data not shown). To construct Figure 11 we have based our comparisons upon the actions of forskolin, calling its activity 100% but representing its activity in the three lines in proportion. The actions of the other three agonists are given relative to the effects of forskolin in the appropriate controls. Where responses to agonists were maintained we have sometimes integrated the areas under the current v. time curves, otherwise we have used SCC increases. When cyclic AMP content was measured we have divided this (in nmol mg^{-1} protein) by the forskolin response (in $\mu A\ cm^{-2}$). These units are obscure but provide a way of gauging the relative effectiveness of the nucleotide to stimulate secretion.

Our stated reason for attempting this study was to identify an epithelium with a major defect in its transporting capability and to use this to identify the lesion at the molecular level, believing this may give insight into some disease states. It appears that HCA-7-Col 3 offers these prospects.

Chloride secreting epithelia move chloride uphill across the basolateral surface using a NA-K-2Cl cotransport mechanism, sensitive to loop diuretics, and release the anion across the apical surface, downhill through chloride channels (see for example

Frizzell et al., 1979). There is evidence that apical chloride channels are activated by a cyclic AMP-dependent mechanism (Welsh et al., 1982; Mandel et al., 1986a) while the cotransport mechanism is crucially dependent upon the supply of K^+ leaking from the cells. Additionally, the hyperpolarization caused by the increased basolateral K^+ permeability favours the gradient for the exit of Cl across the apical face. Increased basolateral K^+ permeability is triggered by raised Ca^{2+}; (Welsh & McCann, 1983; Dharmasathaphorm & Pondol, 1986) operating Ca-sensitive K channels, but there is also evidence that there are cyclic AMP-sensitive K channels (Mandel et al., 1986b). To complicate, even further, the intracellular control of chloride secretion there are indications, recently reviewed by Donowitz & Welsh (1986) for cyclic AMP-Ca^{2+} interactions. It is therefore a complex matter to decide for a given epithelium which barrier will be rate limiting following a particular stimulus. In an earlier study with rat colon (Cuthbert et al., 1984b) we suggested that kinin both increases Ca_i and raises intracellular cyclic AMP by a prostaglandin-dependent adenylate cyclase mechanism, which may account for the very powerful secreting capability of this secretagogue.

Finally, to turn to the preliminary conclusions which can be drawn from our current data. Both HCA-7 and HCA-7-Col 1 appear to be particularly sensitive to stimuli, such as forskolin and VIP, which generate cyclic AMP and in which the cyclic AMP-forskolin response ratio is some 70 times less than in HCA-7-Col 3. In total contrast, HCA-7-Col 3 shows no appreciable SCC response to any stimulus, except A23187, even though the ability to generate the cyclic nucleotide is present. It may have been anticipated that Colony 3 cells would respond to carbachol. The failure to see this may be because the calcium signal is transient or the muscarinic receptor reserve is too low. Indeed it is true for all three lines that we have not shown the relative abundance of receptors for either LBK, carbachol or VIP and therefore arguments made in relation to the absence of a response are weak while those in relation to the presence of a response are valid.

The failure to be appropriately triggered by cyclic AMP, as with HCA-7-Col 3, is of considerable interest following two recent reports indicating that in epithelia from cystic fibrosis patients there is a failure downstream from cyclic AMP generation, even though chloride channels and the cotransport mechanism are present (Welsh & Liedtke, 1986; Frizzell et al., 1986). In HCA-7-Col 3, presumably both channels and cotransport mechanisms are present as this line can secrete chloride in response to A23187. The inability to respond to cyclic AMP might result from a failure to trigger chloride channels or cyclic AMP-dependent K^+ channels. At this stage the evidence slightly favours the latter as the presumed operation of calcium-dependent K^+ channels does, with A23187, produce secretion. However, the situation is likely to be far more complex than can be adduced from the present arguments. For example, if the response to A23187 depends only upon the operation of Ca^{2+}-dependent K^+ channels then the conductance of the apical barrier, together with the resultant hyperpolarization of the cell, must operate in a way to make the apical membrane non-rate limiting.

Turning to HCA-7-Col 1 epithelia it is shown that they respond most efficiently to both forskolin and VIP. The possibility that these cells lack calcium-dependent K^+ channels and therefore do not respond to Ca^{2+} signals remains to be investigated.

In respect of the parent HCA-7 cells, it is probable that they are a mixture of cell types. Different cell morphologies are observed in early passages of HCA-7 cells. In addition, the dome forming ability of this line is slowly lost between passages 25 and 30 suggesting overgrowth by a faster proliferating cell type lacking dome forming ability. Detailed studies of all three cell lines are needed to clarify the possibilities discussed above. We have, however, demonstrated the very different properties of three variants as a first stage of this process.

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