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# Activation of ion channels by lysylbradykinin in the HCA-7 colony 29 human adenocarcinoma cell line

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1 The patch-clamp technique, both cell attached and inside-out patches, was used to examine the effects of lysylbradykinin (LBK) and A23187 on ion channels in cultured Colony 29 epithelial cells derived from a human adenocarcinoma.

2 LBK and A23187 applied directly to the intact cell stimulated the opening of a number of types of ion channel including $Ca^{2+}$-activated $K^+$channels.

3 By use of inside-out patches, anion channels could be stimulated to open by application of protein kinase A and ATP to the cytosolic surface. $Ca^{2+}$-activated $K^+$ channels were also identified in isolated membrane patches.

4 The results suggest that the anion secretion which is stimulated by LBK is a complex event, involving the activation of a number of different types of ion channel, and that part of the response is the result of hyperpolarization of the cell by activation of $Ca^{2+}$-activated $K^+$ channels. From the data presented in this and the accompanying papers it appears that the $Ca^{2+}$-sensitive $K^+$ channels would be equally effective in either the apical or basolateral membranes.

**Keywords:** Ion channels; lysylbradykinin; A23187; colon

## Introduction

The occurence of electrogenic chloride secretion in response to kinins has been recognised for some years (Gaginella & Kachur, 1989), from early experiments on guinea-pig ileum epithelium (Manning *et al.*, 1982) and rat colon epithelium (Cuthbert & Margolius, 1982). In these tissues the mechanisms through which kinins increase chloride secretion were investigated by the short ciruit current (SCC) technique. Production of eicosanoids, leading to adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation, plus a requirement for calcium ions was found (Cuthbert *et al.*, 1984b,c). It was suggested that the calcium ions were important in the generation of prostaglandins (Cuthbert *et al.*, 1984b), and might also facilitate secretion by activation of a transporter located on the basolateral pole of the cell (Cuthbert *et al.*, 1984c). Subsequent experiments performed in the presence of the cyclo-oxygenase inhibitor, piroxicam, showed kinin-stimulated $Cl^-$ secretion in rat colon can take place in the absence of eicosanoid synthesis (Cuthbert *et al.*, 1984a), thus reinforcing the probability of a fundamental role for calcium in the response. More recently, lysylbradykinin (LBK) has been shown to stimulate $Cl^-$ secretion, again measured using SCC recordings, in a human colonic cell line, HCA-7 (Cuthbert *et al.*, 1987). In later experiments on Colony 29 cells, a mutant derived from HCA-7, it was shown that LBK increased intracellular $Ca^{2+}$ concentration, $Ca_i$, but that the relation between $Ca_i$ and SCC was complex (Pickles & Cuthbert, 1991).

In this study the effects on ion channels of LBK, forskolin, an activator of adenylate cyclase, and the calcium ionophore A23187 have been studied in Colony 29 cells. To do this the patch clamp technique has been used, both in the cell-attached and inside-out patch configurations.

## Methods

### *Cell culture*

HCA-7 Colony 29 cells, derived from a human colonic adenocarcinoma were cultured as described previously (Cuthbert *et al.*, 1985; Pickles & Cuthbert, 1991). Cells were seeded onto 10 $cm^{-2}$ Petri dishes (Cell Cult) and cultured in Dulbecco's Modified Eagle's Medium supplemented with glucose (25 mM) and 10% foetal calf serum-kanamycin (100 $\mu g\ ml^{-1}$) and amphotericin B (2.5 $\mu g\ ml^{-1}$). Cells were grown at 37°C in an atmosphere of 95% $O_2$/5% $CO_2$ as described previously (Cuthbert *et al.*, 1987). All cells were used for experiments 5–10 days after seeding.

### *Patch-clamp experiments*

Experimental arrangements were essentially as described previously (Henderson & Cuthbert, 1991; Henderson *et al.*, 1992; Sheth *et al.*, 1992). Experiments were conducted using the cell-attached and inside-out mode of the patch-clamp technique (Hamill *et al.*, 1981). Cells were studied at 400 × magnification using a Nikon Diaphot microscope equipped with Hoffman Modulation Contrast optics. Recordings of single ion channel activity were made with an Axopatch 200 amplifier (Axon Instruments, Foster City, CA, U.S.A.), filtered at 1500 Hz with an 8 pole Bessell filter (Frequency Devices Model No. 902, Lyons Instruments, Hoddesdon, England) and recorded on digital audio tape (DAT) using a Sony DTC-1000 DAT recorder. Single channel events were viewed during the course of recording on a Gould 400 digital oscilloscpe (Gould, Hainault, England). Patch pipettes were made from 1.2 mm o.d. filamented capillary tubing (GC120F-10; Clark Electromedical Instruments, Pangbourne, England) to give a resistance of 10–25 MΩ. Pipettes mounted on the amplifier head-stage were positioned on cells by use of a Narishige MO-303 low-drift hydraulic micromanipulator (Narishige Instruments, Tokyo, Japan), the head-stage of which was attached to the microscope by a Narishige MN-3 micromanipulator, for coarse positioning. A further MN-3 micromanipulator was attached to the opposite side of the microscope stage and held a puffer-pipette for administration of drugs to the cell surface. Pressure was applied to the back of the puffer-pipette from a WPI PV-800 pneumatic picopump (World Precision Instruments, Sarsota, Florida, U.S.A.). Drugs applied from the puffer-pipette were dissolved in the solution which was currently bathing the cell. Single channel data were analysed on IBM AT-compatible computers using the pClamp system (Version 5.5.1; Axon

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Instruments, Inc.), in combination with a Tecmar Labmaster A/D D/A board. Current amplitudes were determined from single-point histograms and single channel conductances derived from current-voltage relationships obtained by recording amplitude histograms at a number of different holding potential ($V_h$) values. In contrast to previous studies (Henderson & Cuthbert, 1991; Henderson *et al.*, 1992) the cells were not treated with trypsin to aid seal formation. This was to avoid the possibility of the trypsin damaging receptors or ion channels on the surface of the cell membrane. Data are presented as mean ± s.e. of mean.

### *Solutions for patch-clamp experiments*

The compositions of the solutions used in the experiments are shown in Table 1. The pipette solution is referred to as 'solution P', the bath solution as 'solution B'. Occasionally solution P was used in the bath to depolarize the cells. When ATP was added to solutions total $Ca^{2+}$ and $Mg^{2+}$ were adjusted to allow for complex formation. Solution G contained gluconate substituting for $Cl^-$. In this case total calcium was elevated by ten fold to allow for chelation by gluconate.

### *Chemicals*

All chemicals used were of the highest commerical grade. LBK, A23187, ATP, dibutyryl cyclic AMP and protein kinase A (PKA) were obtained from Sigma, Poole, England. Forskolin was obtained from Calbiochem (Novabiochem, Nottingham, England). Dulbecco's Modified Eagle's Medium and foetal calf serum were from Gibco Europe, Paisley, Scotland. Kanamycin was from Bristol Laboratories, Langley Slough, England and amphotericin B from E.R. Squibb, Morton, Cheshire, England.

## Results

### *Patch-clamp experiments*

In these experiments the aim was to demonstrate single channel events which could be responsible for the secretory activity of the tissue as a whole. Cell-attached and inside-out preparations (Hamill *et al.*, 1981) were used. The strategy of the cell-attached experiments was to make a seal on the cell membrane and to check for any channel activity by applying various holding potentials ($V_h$, typically from −40 to +40 mV). If no channel activity was evident then LBK (1 μM), A23187 (10 μM) or forskolin (100 μM) was applied to the cell by the picopump with a 100 ms pressure puff. These concentrations are higher than those used for steady state responses measured by other techniques in the accompanying papers. Rapid diffusion of the compounds from the application site served to dilute them to below effective concentrations in a few seconds.

**Table 1** Solutions used for patch-clamp experiments (in mmol $l^{-1}$)

| | *NaCl* | *KCl* | *K-gluconate* | *Na-HEPES*[1] | *EGTA*[2] |
|---|---|---|---|---|---|
| Solution P | – | 145 | – | 10 | 1 |
| Solution B | 140 | 5 | – | 10 | 1 |
| Solution G | – | 5 | 140 | 10 | 1 |

$MgCl_2$ was added to give a free $Mg^{2+}$ concentration of 2 mmol $l^{-1}$ and $CaCl_2$ to give a free $Ca^{2+}$ concentration of 1 μmol $l^{-1}$ (calculated from equations given by Fabiato & Fabiato, 1979).
[1]*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid adjusted to pH 7.4 with NaOH.
[2]1,2-Di(2-aminoethoxy)ethane-*N,N,N',N'*-tetra-acetic acid.

### *Effect of lysylbradykinin and A23187*

Figure 1a shows that effect of application of LBK to a cell whilst a recording was made in the cell-attached configuration. The cell was bathed in solution B and was therefore at its normal resting potential (given that the experiment is performed at room temperature). The patch was first held at +17 mV relative to the cell membrane potential and then returned to the initial potential for application of the LBK. The peptide resulted in the opening of three channels, two of the same type, with a current amplitude (determined from single-point amplitude histograms) of approximately 1.45 pA, the other smaller one with an amplitude of 0.4 pA. All were carrying inward current.

If LBK is applied whilst the cell is bathed in solution B it is difficult to make any deductions regarding the specificity of any ion channels which might be activated. The cell is at an unknown potential, and it is not possible to be certain of the intracellular activities of $K^+$, $Na^+$ or $Cl^-$. the former of these constraints can be addressed by stimulating the cell while it is bathed in solution P, the high $K^+$ concentration depolarizing the cell membrane potential. Figure 1b shows the effect of application of LBK under these circumstances. The patch was held first at 0 mV, then at +38 mV and finally at −38 mV. The steps in voltage discount the possibility of there being channels activated directly by voltage changes. Addition of LBK opened two channels of approximately 1.6 pA in amplitude. LBK was applied to Colony 29 cells in the cell-attached mode on 11 occasions in all and channel openings were elicited in 8 of these. The responses shown in Figure 1 are typical of these experiments. On two occasions it was possible to construct current-voltage relationships for channels in the cell-attached mode, immediately after stimulation with LBK and with solution P in the bath, i.e. with the cells depolarized. The reversal potentials on these two occasions were +16 mV and +10 mV which suggests the channels are $K^+$-selective. If the channels were perfectly $K^+$ selective and the cells were completely depolarized intracel-

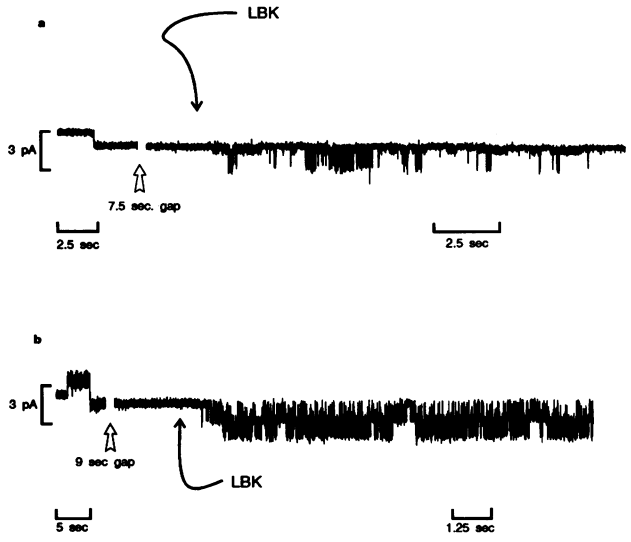


**Figure 1** (a) Direct effect of lysylbradykinin (LBK) on channel activity in cell-attached patch on intact cell. The patch is first held at +17 mV with respect to the membrane potential and then returned to the resting value for application of the LBK. The change in base-line with the change in $V_h$ is due to leak current between the membrane and the pipette. No channel activity was visible during the 7.5 s gap and the recording is thus cut at this point. (b) Effect of LBK on cell-attached patch with solution P bathing the cells (i.e. membrane potential = 0). The patch is first held at 0 mV, then at +38 mV and finally at −38 mV when LBK is applied. Note that in this figure and in Figure 2 the time scale is slightly expanded following the gap to clarify the single channel record.

lular $K^+$ concentrations of 77 mM and 97 mM respectively are predicted.

The effects of A23187 in cell-attached patch-clamp experiments were determined in similar experiments using puffs of a solution containing A23187, 10 μM. In Figure 2a is represented a cell bathed in solution B and thus at its normal membrane potential. Application of A23187 led to channel activation and, as in the LBK experiments, currents were inward under these conditions. The mean amplitude of the channel shown here was 2.00 pA.

Figure 2b shows a representative result obtained when the experiment was repeated with the cell bathed in solution P. The patch was first held at +32 mV then the $V_h$ was stepped to −32 mV, back to 0 mV and then again to −32 mV at which time the A23187 was applied. In common with the LBK experiments, the lack of induction of channel activity during the voltage steps suggests that it is unlikely that there are channels simply activated by changes in voltage in this patch, at least in the range of $V_h$s examined. Rapid channel activation occurred and again inward currents were evident following application of A23187. The mean current amplitude for this experiment was 1.36 pA.

These results with A23187 are taken from 14 similar experiments of which 12 patches showed A23187 induced channel activity. Many attempts were made to observe channel activity following puffs of forskolin (100 μM). None were seen although forskolin is a powerful secretagogue. It may be that this highly lipophilic drug is taken up into lipid compartments when applied in a few microlitres at high concentration, without significantly affecting cellular adenylate cyclase.

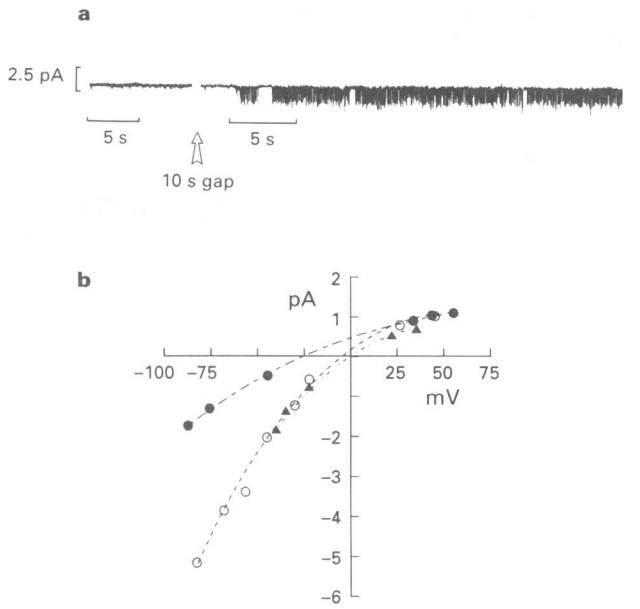


**Figure 3** (a) Effect of exposure of inside-out patch to protein kinase A (PKA, 20 units $ml^{-1}$) and ATP (1 mM), further details in the text. (b) The current-voltage relationship for the channel shown above with symmetrical solution P (▲) and for a channel from a cell pre-stimulated with dibutyryl cyclic AMP (100 μM) bathed in symmetrical solution P (○); (●) show the result with solution G in the bath.

### *Identification of channel types*

Stimulation of channel opening by administration of exogenous agents to the intact cells only gives limited information regarding the identity of channel types involved. In order to clarify the identity and possible mechanisms of regulation of channels, recordings were made from excised inside-out patches. Figure 3a shows the effect of exposure of the intracellular surface of a previously silent patch to solution P, which in addition to its normal constituents contained $Ca^{2+}$ (1 μM), PKA (20 units $ml^{-1}$) and ATP (1 mM). After 3 min of incubation, channel activity was elicited in the patch, showing predominantly the activity of a single channel type. The current-voltage relationship for this channel is shown in Figure 3b (triangles). Also shown in Figure 3b are the current-voltage relationships for a similar channel which was observed in an inside-out patch from another cell which had been pre-incubated for 10 min before seal formation with dibutyryl cyclic AMP, 100 μM. Relationships are shown for this channel when solution P was in the bath (open circles), and when solution G was present in the bath. Note the current-voltage relationship for the channel activated by PKA/ATP was identical to the one opened by prior exposure of the cell to dibutyryl cyclic AMP. With the latter and with solution G, the reversal potential ($E_{rev}$) was moved to approximately −25 mV, with outwards current flow at 0 mV. Given the prevailing ion gradients, this corresponds to inward flow of anions. Since the relationship for the PKA/ATP-induced channel overlies that of the dibutyryl cyclic AMP pre-incubated channel (Fig. 3b), the current-voltage relationship showing inward rectification, inward conductance (at −50 mV) is approximately 50 pS, outward conductance (at +50 mV) is approximately 24 pS, it is likely this is a chloride channel.

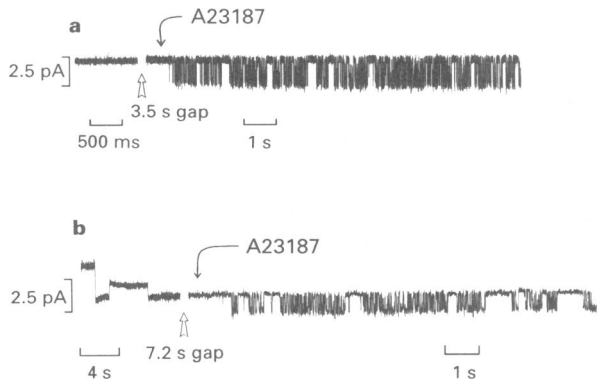


**Figure 2** (a) Effect of A23187 on cell-attached patch with low $K^+$ (solution B) bathing solution. The applied $V_h$ is 0 mV. (b) Effect of A23187 with solution P in the bath. The patch is first held at +32 mV and stepped successively at −32 mV, 0 mV and finally −32 mV, when the ionophore was applied.

Most of the channels seen in isolated patches did not have the characteristics of anion channels. Cation channels were also found which demonstrated calcium sensitivity. Figure 4a shows the single channel recordings for an inside-out patch at a $V_h$ of −11 mV, and Figure 4b the single point current amplitude histogram at a $V_h$ of −48 mV is shown in the absence of bath $Ca^{2+}$, and with $Ca^{2+}$, 1 μM, present. No channel openings were discernible in the absence of $Ca^{2+}$, but channel activity was stimulated in its presence. These channels are therefore sensitive to intra-cellular $Ca^{2+}$, furthermore as illustrated in Figure 5, they are selective for $K^+$.

Figure 5 shows the current-voltage relationships obtained from two patches, one from an unstimulated cell, and the other from a cell in which channels had been pre-activated by stimulation with A23187. In symmetrical solution P the channels showed inward rectification with an inward conductance of 19.0 pS and an outward conductance of 12.5 pS at a holding potential of ±40 mV. Replacement of solution P in the bath with solution B (for the patch from the unstimulated cell) produced a shift in the curve, suggesting $K^+$-selectivity. It was not possible to elicit outward currents under these circumstances. Similar channels were seen on other occasions at a $V_h$ of ±40 mV, the mean inward conductance was 18.9 ± 0.6 pS ($n = 4$) (mean ± s.e.mean and number of observations) and the mean outward conductance was 12.7 ± 0.2 pS ($n = 4$).

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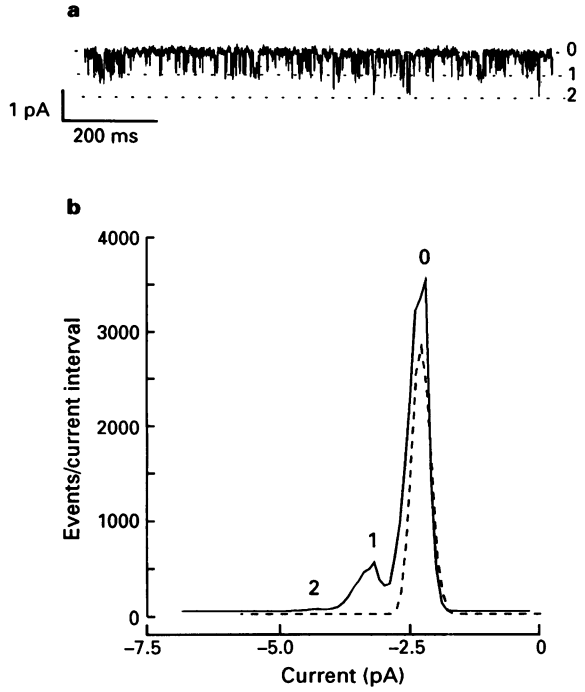


**Figure 4** (a) single channel recording from a patch made at $V_h$ of − 11 mV, symmetrical solution P, with $Ca^{2+}$, 1 μM, in the bath. Two channels are evident and the levels for no channels, one channel or two channels being open are shown by dotted lines and labels. (b) Single point current amplitude histogram for the same channel in the presence of $Ca^{2+}$, 1 μM, (solid line) and in the absence of $Ca^{2+}$ from the bath (dashed line). The $V_h$ was −48 mV. The relative areas under each peak reflect the total time the patch remains in the corresponding conductance state, i.e. 0, 1 or 2 channels open.

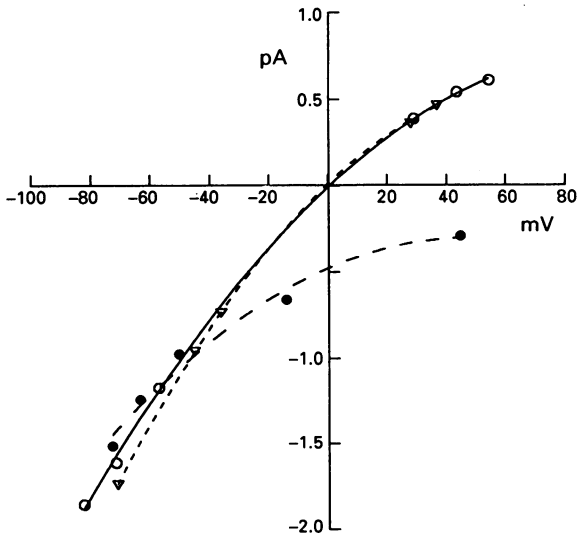


**Figure 5** Current-voltage relationship for single $Ca^{2+}$-activated $K^+$ channels in inside-out patches: (○) show the relationship for a channel with symmetrical solution P, which was silent in the cell attached mode and in the absence of calcium, but activated by adding calcium (1 μM) to the bath; (●) show the result obtained when the bath solution P is replaced with solution B; (△) show the relationship again with symmetrical solution P, determined from a channel which had been activated in the cell-attached state by treatment of the cell with A23187.

## Discussion

In the accompanying papers it was argued that LBK affects chloride secretion in Colony 29 monolayers by more than one mechanism. Indirect evidence for the involvement of cyclic AMP and $Ca^{2+}$ was obtained in the first (MacVinish *et al.*, 1993a), while in the second, direct evidence for an increase in chloride permeability was obtained (MacVinish *et al.*, 1993b). Furthermore, it was argued that part of the increased anion efflux resulted from an increase in the electrical gradient, following activation of $Ca^{2+}$-sensitive K-channels. The object of this study was to demonstrate the ion channels in Colony 29 cells that are activated by LBK, specifically to see if the data underpin the conclusions from the less direct approaches.

In all the experiments performed in this study the channel openings seen in response to LBK and A23187 in the cell attached configuration always carried an inward current. This was so whether the cells were in their normal polarized state (solution B in bath) or when they were depolarized (solution P in both). It is likely therefore that the currents seen in the cell attached mode in the presence of secretagogues were caused by $K^+$ ions, as indeed indicated by the current voltage relations shown in Figure 5. However, as shown in Figure 1a, stimulation with LBK can result in the opening of more than one type of channel. This smaller channel is not necessarily $K^+$-selective and more likely is a non-selective cation channel. The sodium permeability of these channels was masked in these studies by the lack of sodium in the pipette. Small non-selective cation channels with a conductance of 12–13 pS have been seen in isolated patches of Colony 29 cells and the smaller channel in Figure 1a may fall into this group (Sheth *et al.*, 1992).

Epithelial cells always grow with their basolateral surfaces apposed to the substrate and with their apical surfaces uppermost and accessible to the patch pipettes. It is likely, therefore, that the channels induced to open by LBK or A23187, and $Ca^{2+}$-activated $K^+$ channels recorded in the inside-out configuration are located on the apical membrane of the colon (for review see Dawson, 1991). It is also possible that the channels which were observed in the present study were from the lateral aspect of the cell since it was easier to form seals on cells at the edge of a group of sub-confluent cells, rather than in the middle of such a group. Current fluctuation analysis has revealed a basolateral $K^+$ conductance which could be blocked by $Ba^{2+}$ in rabbit colon (Wills & Zweifach, 1987) and a high-conductance $Ca^{2+}$ activated $K^+$ channel has been isolated from the basolateral membrane of rabbit colon (Turnheim *et al.*, 1989; Salomao *et al.*, 1992). No such high-conductance channels however were seen in the present study.

It is the convention in patch-clamp recording that direction of current flow, and of rectification is always referred with respect to cations. Therefore a current which appears on a current-voltage plot to be carried by cations from the pipette into the cell could also be carried by anions out of the cell. In experiments using the $Cl^-$-sensitive fluorescent dye MEQ, at 37°C, the intracellular $Cl^-$ concentration of Colony 29 cells has been shown to be approximately 35 mM (MacVinish *et al.*, 1993b) which is slightly above the equilibrium value predicted by the Nernst equation (given a membrane potential of = −45 mV the equilibrium $Cl^-$ concentration would be = 25 mM). Under the prevailing conditions produced by stimulation by LBK and A23187, $Cl^-$ currents would appear as outward currents (upward-directed openings on the current records), corresponding to flow of $Cl^-$ ions into the cell. It is perhaps surprising that no single channel currents which could be attributable to anion flow were seen in cell attached patches when the cells were stimulated. The experiments conducted using PKA suggest that anion channels can be stimulated to open in response to second messenger pathways involving cyclic AMP, and indeed that has been documented for a number of anion channels in secretory epithelia includ-

ing the T84 colon cell line (Halm *et al.*, 1988). No direct connection can be made, however, between the PKA-induced anion channels seen in the present study and the forskolin response seen in the efflux experiments (MacVinish *et al.*, 1993b) other than that the forskolin would tend to produce an elevation in cyclic AMP levels and thus put in train events leading to activation of these channels. It was unfortunate that application of forskolin to cell-attached preparations did not yield any results. The reasons for this are unclear, but it maybe that it is not possible to achieve an adequate concentration of the drug at the relevant site because of partitioning into lipophilic sites. However, the single channel $Cl^-$ currents induced in these circumstances might be of such a small amplitude and opening and closings of such high frequency that they fail to be resolved. It would be necessary to study whole cell currents to resolve this. However, the partial inhibition by piroxicam of the effects of LBK on $^{125}I$-efflux (MacVinish *et al.*, 1993b) indicates that not all of the peptide effect can be ascribed to the $Ca^{2+}$ elevating effect of LBK and that part of the effect must reside with an action via the prostaglandin/cylic AMP system.

In conclusion, it is shown that $Ca^{2+}$ requiring agonists LBK and A23187 affect $Ca^{2+}$-sensitive $K^+$ channels in intact cells. The resulting hyperpolarization increases the gradient for anion efflux, shown in the accompanying papers either as increased $^{125}I^-$ efflux or as an increase in transepithelial chloride transport. While these $K^+$ channels may exist on either apical or basolateral membranes to achieve this effect it is more likely that those demonstrated here are of apical origin.

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