Br. J. Pharmacol. (1993), **108**, 469–478
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Kinin-induced chloride permeability changes in colony 29 epithelia estimated from $^{125}I^-$ efflux and MEQ fluorescence

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1 The changes in apical Cl^- permeability of Colony 29 human colonic epithelial monolayers were estimated from the rate constant of $^{125}I^-$ efflux from tissues loaded with the isotope.

2 Forskolin was used to increase intracellular concentrations of adenosine 3:5′ cyclic-monophosphate (cyclic AMP), and A23187 to increase intracellular free Ca^{2+} (Ca_i). Both treatments increased the rate constant for $^{125}I^-$ efflux, indicating an increase in apical Cl^- permeability.

3 Lysylbradykinin (LBK) also increased the rate constant for $^{125}I^-$ efflux, sometimes biphasically. Chelation of intracellular Ca^{2+} with BAPTA or prevention of prostaglandin formation with piroxicam, attenuated but did not eliminate the effect of LBK. It is concluded that LBK affects $^{125}I^-$ efflux through the agency of both cyclic AMP and Ca^{2+}.

4 Ba^{2+} attenuated the effect of LBK and A23187 on $^{125}I^-$ efflux, but had no effect on the action of forskolin. It is concluded that Ca^{2+} has a major effect on K^+ channels, the resulting hyperpolarization increasing the driving force for $^{125}I^-$ efflux. A secondary effect on Ca^{2+}-sensitive Cl^- channels is possible. By contrast, cyclic AMP exerts it major effect on apical Cl^- channels.

5 Using a Cl^- sensitive fluorescent dye, MEQ, the intracellular chloride concentration, Cl_i was estimated to be around 30 mM, which was increased to around 50 mM by forskolin, suggesting cyclic AMP could activate the Na-K-2Cl co-transporter.

6 MEQ fluorescence was used to estimate Cl^- influx and efflux rates of epithelial cells. These were increased three fold by forskolin and dibutyryl cyclic AMP and two fold by LBK and histamine.

7 It is concluded that LBK increases electrogenic chloride secretion in Colony 29 epithelia through the generation of second messengers cyclic AMP and Ca^{2+}, each of which may act on both apical and basolateral membranes.

Keywords: Lysylbradykinin; forskolin; A23187; Ca^{2+}; $^{125}I^-$ efflux; epithelia; chloride secretion

Introduction

Data presented in the preceding paper (MacVinish *et al.*, 1993) indicated that lysylbradykinin affects transepithelial chloride secretion by more than one mechanism. In, for example, Colony 29 epithelia it was concluded that efflux of Cl^- across the apical surface was the result of a cyclic AMP-induced activation of chloride channels together with an increase in the electrical gradient for Cl^- efflux, brought about by hyperpolarization consequent on activation of Ca^{2+}-sensitive K^+ channels. The evidence in this earlier paper was rather indirect and based upon transepithelial chloride transport measured as short circuit current.

In this study we have used two methods to examine the Cl^- permeability of the apical membranes of Colony 29 epithelial monolayers. In the first method cells are loaded with $^{125}I^-$ and the washout of the isotope measured. Addition of drugs affecting anion efflux then alters the washout pattern of the radioisotope. $^{125}I^-$ is an ideal marker for Cl^- since it is a poor substrate for the Na-K-2Cl cotransport pathway (O'Grady *et al.*, 1987) or anion exchange mechanisms (Dalmark & Wieth, 1972) while relative conductive permeabilities of I^- and Cl^- rarely vary by more than two fold (Halm *et al.*, 1988). In the third paper of this series (Henderson & Cuthbert, 1993) we have used patch clamping to look for anion channels, yet that technique has its drawbacks. First, relevant anion channels may be too small to resolve by patching and furthermore, run down is not an uncommon phenomenon in isolated patches. The I^- efflux technique measures the collective action of all types of chloride channels including those in which kinetic characteristics are modified by agonists. The I^- efflux technique is similar to that used previously (Venglarik *et al.*, 1990; Clancy *et al.*, 1990; Henderson *et al.*, 1992).

The second method was to use a fluorescence indicator, MEQ, which is sensitive to Cl^- concentration (Biwersi & Verkman, 1991). This technique allows both estimation of intracellular chloride concentration (Cl_i) , and the flux rate for Cl^- movement into and out of the cells. The two methods are essentially complementary and we have confirmed that LBK affects electrogenic chloride secretion by more than one mechanism.

Methods

Cell culture

Colony 29 cells were grown in 6-well (35 mm diameter) plates (Cell Cult, Sterilin) or on the plastic strips in Leighton tubes (Costar). They were maintained in Dulbecco's Modified Eagle's Medium (Gibco, Europe) supplemented with glucose (25 mmol l^{-1}) and 10% foetal calf serum (Gibco), kanamycin (100 $\mu g\ ml^{-1}$) (Bristol Laboratories), and amphotericin B (2.5 $\mu g\ ml^{-1}$) (E.R. Squibb). Cells were grown in an atmosphere of 5% CO_2 at 37°C. T84 cells, another human colonic epithelial cell line, were cultured exactly as for Colony 29. They were used only for verification of intracellular Cl^- measurements by comparison with published values.

^{125}Iodide efflux

The cells were cultured as above for 5–6 days until they were 50–70% confluent. To load with $^{125}I^-$, cells were exposed to

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buffer containing 2.5 μCi ml^{-1} $^{125}I^-$ for 90 min at 37°C. After this, cells were washed three times with 'efflux buffer' (see below). Efflux of $^{125}I^-$ was then measured every 30 s by rapidly removing efflux buffer (3 ml) and replacing it. This procedure was continued for 10 min with buffer maintained at 37°C. At convenient times, efflux buffer containing drugs was substituted for plain buffer. At 10 min, 3 ml 0.1 N HNO_3 was added and left for 30 min to extract residual isotope. All samples were counted for γ-irradiation. The data were processed as described in detail previously (Henderson et al., 1992). In some experiments BAPTA-AM (1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid ester; Molecular Probes, Oregon) was used to chelate intracellular Ca^{2+}. To do this, cells were preincubated with acetylmethoxy ester (5 μM) at the same time as the cells were loaded with $^{125}I^-$. Esterase action liberated BAPTA which was trapped inside the cells.

The efflux buffer used had the following composition (mM): NaCl 135, $CaCl_2$ 1.2, $MgCl_2$ 1.2, KH_2PO_4 0.6, K_2HPO_4 2.4, glucose 10 and HEPES 10 (pH 7.2). In experiments where Ba^{2+} was used, the phosphates were omitted and replaced with KCl, 3 mM, and the HEPES concentration was increased to 15 mM.

MEQ fluorescence

Synthesis of 6-methoxy-N-ethyl-1,2-dihydroquinoline (DiH-MEQ) Synthesis was carried out as described by Biwersi & Verkman (1991). The unstable lipophilic product was stored in small aliquots under N_2 in ampoules at − 70°C. DiH-MEQ is insensitive to [Cl^-] but is readily oxidized to MEQ which is [Cl^-]-sensitive. Oxidation of DiH-MEQ by bubbling with O_2 in a phosphate buffer (pH 7.4) gave a compound with an emission spectrum identical to that published. Fluorescence quenching by Cl^- using peak excitation (350 nm) and peak emission (435 nm) wavelengths for MEQ showed that the Stern-Volmer plot was linear over the concentration range 0–50 mM Cl^- with a Stern-Volmer constant, K_{Cl} of 154 M^{-1} and where

$$\frac{F_o}{F} = 1 + K_{Cl}[Cl] \quad (1)$$

with F_o being the fluorescence intensity in the absence of Cl^-, while F is the fluorescence intensity in the presence of Cl. The value of K_{Cl} is close to the value of 145 M^{-1} recorded by Biwersi & Verkman (1991).

Loading Colony 29 monolayers with DiH-MEQ DiH-MEQ was dissolved in a few μl (5–10) of NaOH (pH 9.0) and the volume adjusted to give a solution of around 400 μM in a standard salt solution (solution 1, Table 1). Colony 29 monolayers on plastic slips were exposed to this solution for 10 min at 37°C. Afterwards the monolayers were washed in solution 1 and incubated for a further 15 min at 37°C to allow for oxidation of DiH-MEQ and distribution in the cells.

Table 1 Composition of solutions: concentrations are in mM

	Solution number					
	1	2	3	4	5	6
NaCl	137			25		
$NaNO_3$		137				25
Na isethionate			137			
Na gluconate					25	
KCl	5.4			120		
KNO_3		5.4				120
K acetate			5.4			
K gluconate					120	
$CaCl_2$	1.0			2.0		
$Ca(NO_3)_2$		1.0				4.0
$CaSO_4$			3.0			
Ca gluconate					4.0	
$MgSO_4$	0.3	0.3	0.3	2.0	2.0	2.0
KH_2PO_4	0.4	0.4	0.4	0.4	0.4	0.4
HEPES	10	10	10	10	10	10
Glucose	11	11	11	11	11	11

All solutions are adjusted to pH 7.4

Recording of MEQ fluorescence The plastic strips bearing MEQ loaded Colony 29 cells were cut into two and each held in a vertical plastic block which fitted into a cuvette so that emitted fluorescence could be recorded as described by Pickles & Cuthbert (1991). Cuvettes were mounted in a LS-5B Perkin-Elmer spectrofluorimeter interfaced with a 640 Kbyte IBM compatible PC with a Perkin Elmer Software Package. Monolayers were excited at 350 nm and emission measured at 435 nm. Values of the emission intensity were recorded every 2.5 s and captured on disc, for playback and analysis.

Procedures for calibration and solution changing The cuvette and contents were maintained at 37°C by a heat exchanger and the cuvette could be either perfused with solutions at 37°C (flow rate 8.5 ml min^{-1}) or the solutions changed within 3 s by hand-held syringes connected by tubing to the cuvette for either solution addition or withdrawal.

To calibrate MEQ fluorescence within cells the double ionophore technique was used (Chao et al., 1990). Using high potassium containing solutions (mixtures of solutions 4 and 5 for Colony 29 or mixtures of solutions 4 and 6 for T84 cells) containing nigericin, 5 μM, and tributyltin, 8 μM, the value of F_o/F was measured for a number of Cl^- concentrations. Finally the perfusing solution was changed to potassium thiocyanate solution (170 mM) containing valinomycin, 5 μM, to quench completely fluorescence due to MEQ and define the basal fluorescence unconnected with MEQ (autofluorescence). In the conditions used for calibration, the high K^+ solutions depolarize the cells, nigericin acts as a K^+/H^+ exchanger and clamps the pH and tributyltin acts as a Cl^-/OH^- exchanger to abolish the chloride gradient. In this situation the intracellular and extracellular Cl^- concentrations are equal. Treatment with nigericin and tributyltin increases cellular permeability and dye leakage so experiments were completed in 4 min and calibration at only two Cl^- concentrations was attempted with each preparation.

Measurement of Cl^- efflux rates Flux rates, both influx or efflux, were obtained from the rate of change of fluorescence on changing from zero chloride (solution 2) to high Cl^- (solution 1) or *vice versa*, and finally exposing to KSCN solution with valinomycin. Influx or efflux was calculated as J_{Cl} from

$$J_{Cl} = \frac{F_o}{K_{Cl}\,F^2}\,\frac{dF}{dt} \quad (2)$$

where F and F_o have the same meaning as before, K_{Cl} is the Stern-Volmer constant from the intracellular calibration curve and dF/dt is the initial rate of change of fluoresence. In the experiments in which ionophores were not used the experiments lasted about 15–20 min without great inaccuracy due to MEQ loss. The rate of loss in MEQ monolayers perfused with solution 1 was 25 ± 3% ($n = 4$) per hour.

Drugs and materials

6-Methoxyquinoline, iodoethane, sodium borohydride and diphenylamine carboxylate were from Lancaster Synthesis. Nigericin, tributyltin, valinomycin and 4-bromo A23187 were from Sigma and concentrated stock solutions were made up in ethanol or 95% ethanol. Forskolin was from Calbiochem and again stock solutions were made in 95% ethanol. All other drugs were from Sigma and were dissolved in the appropriate solutions 1 to 6 (Table 1). Hoe 140 was a gift from Dr B. Schölkens of Hoechst AG.

Results are given as mean ± s.e.mean.

Results

Responses to second messengers

As most of this paper is concerned with the effects of LBK on epithelial responses in cultured monolayers and in particular the role of second messengers, typical responses to manoeuvres which increase the concentration of cyclic AMP and Ca^{2+} within cells were investigated. Both forskolin, to increase cyclic AMP levels, and A23187, to increase Ca_i, produced a rapid increase in the rate constant for $^{125}I^-$ efflux underlying a presumed increase in apical chloride permeability (Figure 1). Although the responses appeared rapidly they were not maintained at the initial peak values, but fell to a plateau value which was usually greater than the values before agents were added. The high values of the rate constants seen in the first minute or so of the 10 min experimental period represent washout of residual loading solution. It was preferable not to extend the washing period since the radioactivity released becomes progressively less throughout the experiment. Even with the current format standard errors became somewhat larger in the last minute or two, a consequence of the reducing pool of $^{125}I^-$ left in the tissue.

As with forskolin and A23187, LBK also produced an increase in the $^{125}I^-$ efflux rate constant, as shown in Figure 2a, but the effect was smaller. Figure 2a shows that a secondary increase in rate constant occurs at 6.5 min, a result more clearly understood by examining the individual experiments making up Figure 2a and shown in Figure 2b. Following addition of LBK there was a synchronized rapid increase in rate constants in all monolayers. There was, however, a further secondary increase in rate constants which occurred at variable times after the first peak and which all but disappeared when the data were consolidated (Figure 2a). Nevertheless, this result may indicate that LBK affects apical anion efflux by more than one mechanism, the second being less synchronized than the first.

Diphenylamine carboxylate (DPC), a chloride channel blocker (Distefano *et al.*, 1985), and a B_2 kinin receptor antagonist, Hoe 140 (Hock *et al.*, 1991) reduced the increase in $^{125}I^-$ efflux in Colony 29 monolayers caused by LBK as shown in Figure 3. Notice there is a late onset effect of LBK after Hoe 140, which is discussed later. Therefore it seems reasonable to assume that LBK, acting at a B_2 receptor, increases $^{125}I^-$ efflux through chloride channels which are

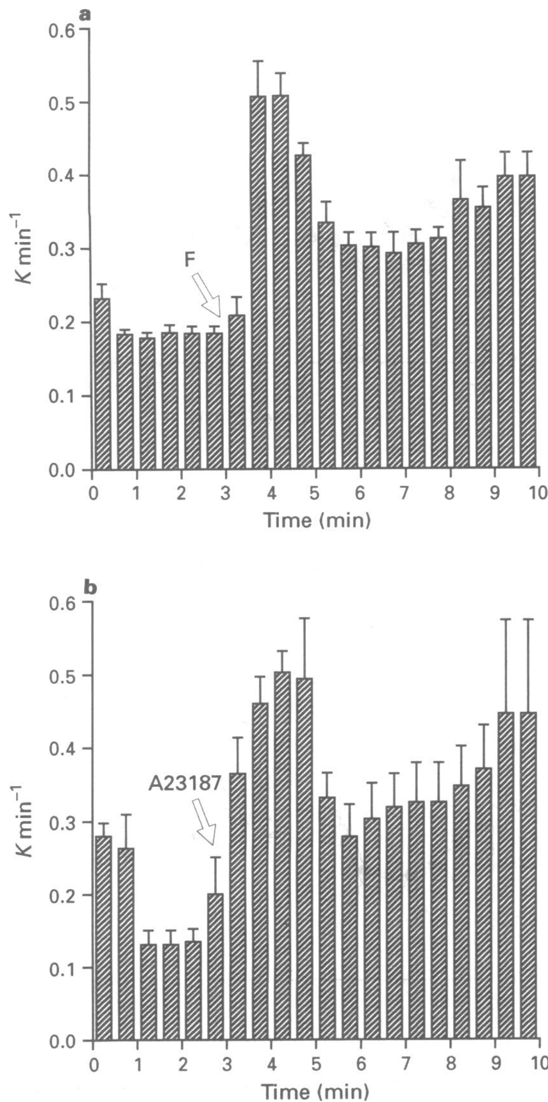

Figure 1 Effect of forskolin (F) (10 μM) (a), and A23187 (1 μM) (b) on the rate constant for $^{125}I^-$ efflux from Colony 29 epithelia. Each column shows the mean ± s.e.mean (vertical bars) for six measurements. Drugs were applied at the arrows.

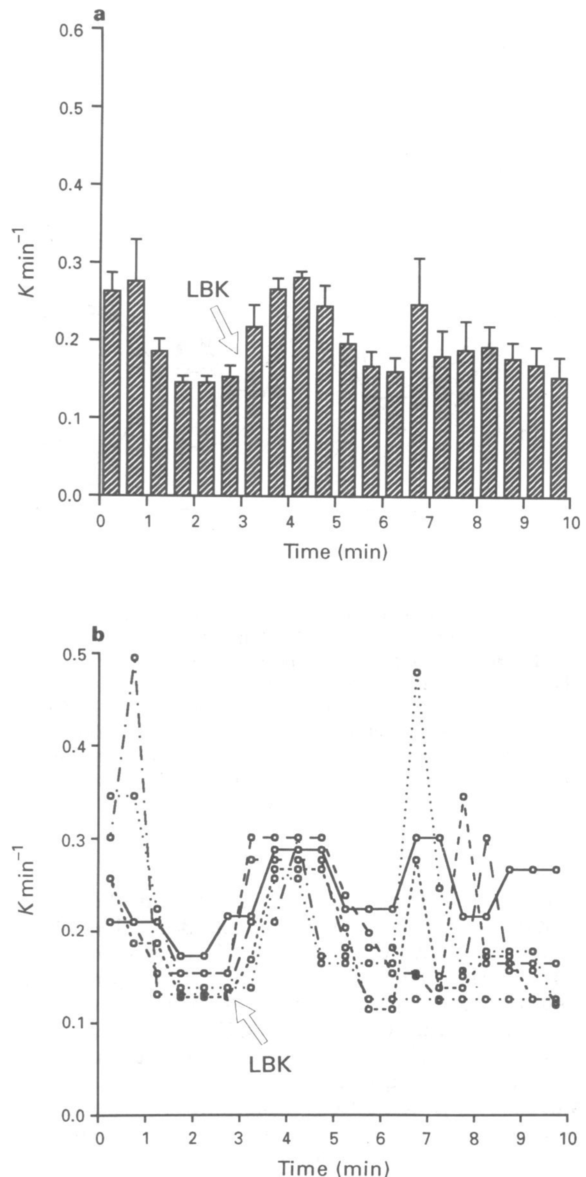

Figure 2 Effect of lysylbradykinin (LBK, 0.1 μM), applied at arrow, on the rate constant for $^{125}I^-$ efflux from Colony 29 epithelia. Each column shows the mean ± s.e.mean (vertical bars) for six measurements. In (b) the individual experiments contributing to the averaged data in (a) are shown.

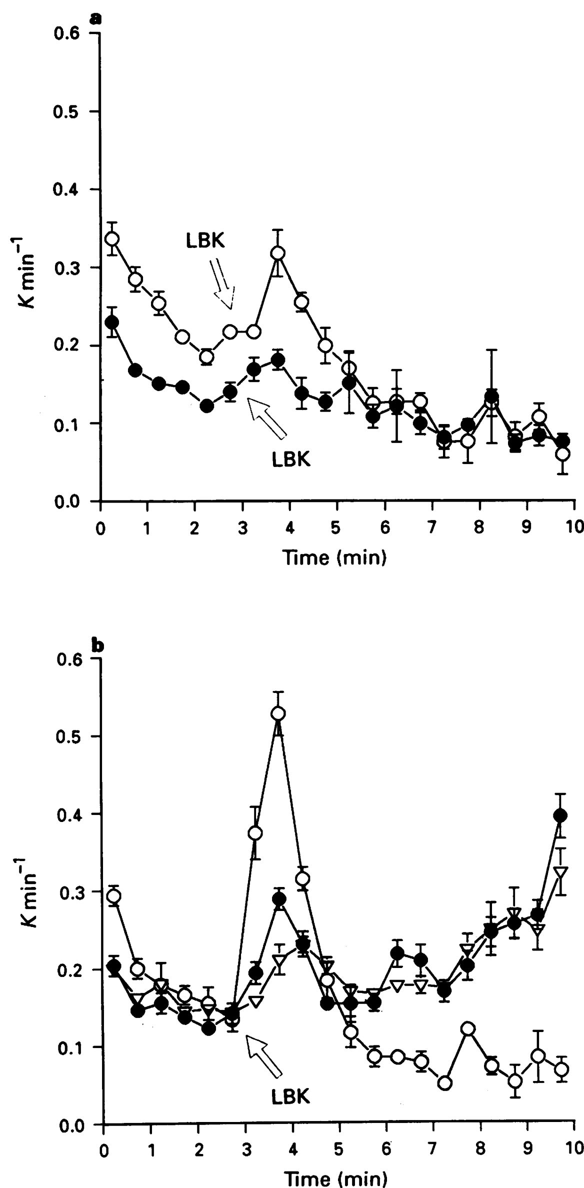

Figure 3 (a) Effects of lysylbradykinin (LBK, 0.1 μM), applied at arrows, on the rate constant for $^{125}I^-$ efflux: (○) are control observations while (●) are for epithelia exposed to diphenylamine carboxylate (DPC) 30 μM. The maximum increase caused by LBK was 0.10 min^{-1} in the control and 0.02 min^{-1} in the presence of DPC. (b) As for (a) with control values shown by (○); (●) and (Δ) representing tissues pre-exposed to Hoe 140, 3 nM and 10 nM respectively for 30 min. All values are the means ± s.e.mean (vertical bars) for six observations with Colony 29 epithelia.

sensitive to DPC. However, this does not mean the action of the peptide is directly on the channel. Both Hoe 140 and DPC inhibit the SCC responses to LBK, an expected result if the $^{125}I^-$ effluxes report upon apical chloride permeability. Hoe 140 was effective at nM concentrations here and had a K_i of around 5 nM against LBK with SCC measurements (Cuthbert *et al.*, 1992). DPC was much less effective against LBK on $^{125}I^-$ efflux. This agent acts on the apical side of Colony 29 epithelia and is non-selective, blocking Cl^- exit activated by secretagogues. For example, DPC (30 μM) significantly reduced the effect of ATP (30 μM) on Colony 29 monolayers from $14.6 \pm 0.9\ \mu A\ cm^{-2}$ (mean ± s.e.mean, $n = 18$) to $8.0 \pm 0.8\ \mu A\ cm^{-2}$ (mean ± s.e.mean, $n = 6$). These values were significantly different at $P<0.01$.

Further experiments were performed to examine the mechanism by which LBK brings about the change in $^{125}I^-$ efflux. First, is stimulation of anion efflux related to the known increase in intracellular Ca^{2+} (Pickles & Cuthbert, 1991)? This was investigated by use of BAPTA as an intracellular Ca^{2+} chelator. Inclusion of BAPTA inside the cells reduced the efflux rate significantly ($P<0.001$) during the first minute after addition of LBK. Thereafter, the delayed peak followed the time course of the declining phase of the uninhibited response. This is to be compared with the effects of forskolin which gave identical changes in maximal efflux rate constants whether BAPTA was present or not (Figure 4a,b). Thus it appears that BAPTA can influence the response to LBK but not that to forskolin, suggesting part of the LBK response in $^{125}I^-$ efflux is calcium-dependent.

The role of prostaglandin formation

It was shown in the accompanying paper (MacVinish *et al.*, 1993) that SCC responses to LBK in Colony 29 monolayers are attenuated but not abolished by piroxicam, a cyclo-

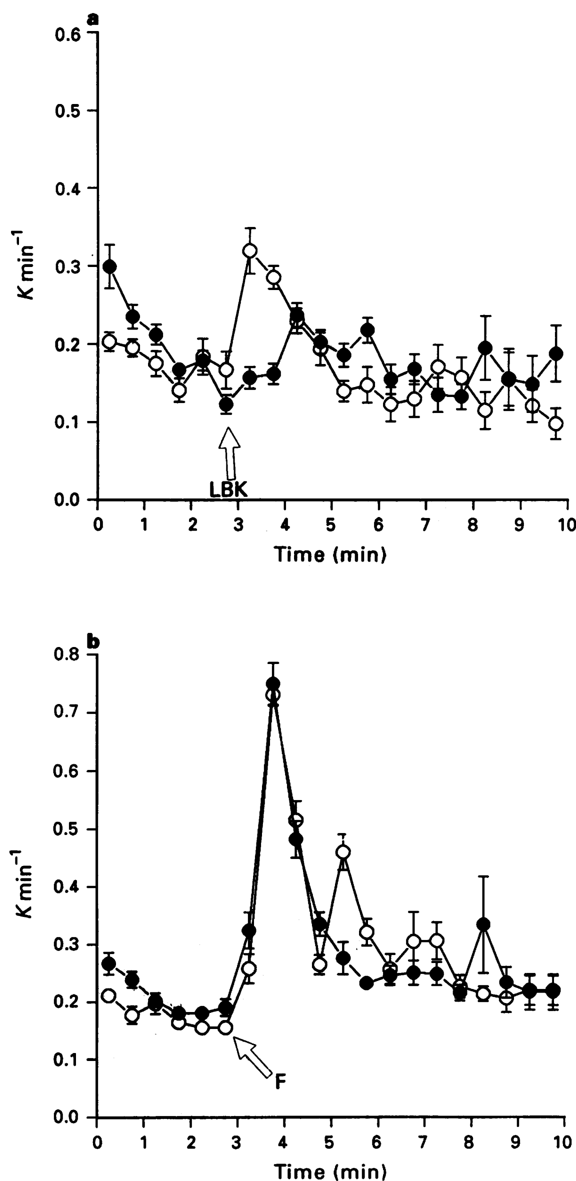

Figure 4 (a) Effect of lysylbradykinin (LBK, 0.1 μM) on the rate constant for $^{125}I^-$ efflux: (○) show control values, while (●) are with tissues containing the intracellular Ca^{2+} chelator, BAPTA. (b) Effect of forskolin (F) (10 μM) on $^{125}I^-$ efflux: (○) are controls and (●) are from tissues containing BAPTA. All measurements are the means ± s.e.mean (vertical bars) for 6 measurements with Colony 29 epithelia.

oxygenase inhibitor. Using $^{125}I^-$ efflux it was shown (Figure 5a) that piroxicam also attenuates the response to LBK. In this example the secondary increase in $^{125}I^-$ efflux in response to LBK is well demonstrated in the controls, but is not so in piroxicam-treated monolayers. It seems likely from these results that prostaglandins are responsible for part of the anion efflux response caused by bradykinin, but additionally there is a non-prostaglandin-dependent component. To obtain data about the first of these, it was necessary to show prostaglandin E_2 (PGE_2) could increase $^{125}I^-$ efflux (Figure 5b). Furthermore the opportunity was taken to examine if the response to LBK could be potentiated in the presence of both piroxicam and PGE_2, as indeed SCC responses were shown to be in the accompanying paper (MacVinish *et al.*, 1993).

In the piroxicam-treated tissues the effects of LBK were only slightly, but not significantly, increased by pre-exposure to PGE_2 (Figure 5c), even though under these conditions SCC responses would have been potentiated. In HCA-7 epithelia where piroxicam has no effect on SCC responses to LBK, the responses to the peptide can be potentiated by forskolin. A further experiment was therefore carried out, but on this occasion with HCA-7 monolayers. As in Figure 5c the $^{125}I^-$ efflux response to LBK was marginally, but not significantly increased by pre-incubation with an agent to increase cyclic AMP content (Figure 5d). Thus in the circumstances which cause potentiation of the transporting responses to LBK there was no significant effect on the increase in efflux rate constant caused by LBK.

Involvement of calcium-sensitive potassium channels

Calcium-sensitive potassium channels are present in epithelia, including Colony 29 monolayers (Henderson & Cuthbert, 1993). Experiments were designed to examine the hypothesis that opening of these channels influences the efflux of $^{125}I^-$ from preloaded cells. The first approach was to use Ba^{2+} ions to block K^+ channels and to examine the effects of LBK. Figure 6a shows that 1 mM Ba^{2+} significantly reduced the effect of LBK on $^{125}I^-$ efflux, but there remained a small

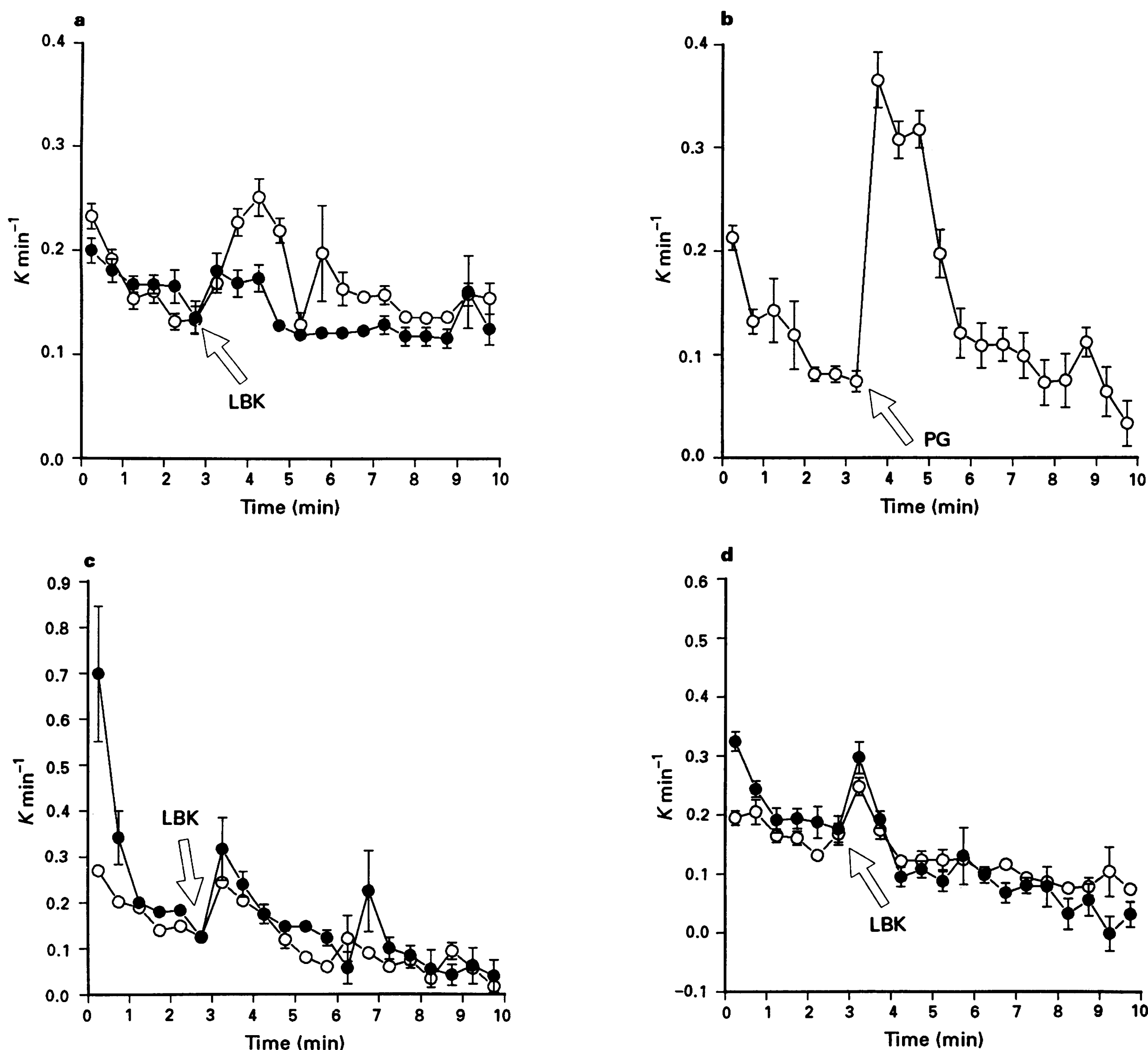

Figure 5 Parts (a–c) illustrate data obtained with Colony 29 epithelia, while (d) are data obtained with HCA-7 monolayers. (a) Effects of lysylbradykinin (LBK, 0.1 μM) in the absence (○), and presence (●) of piroxicam 10 μM. (b) Effect of prostaglandin E_2 (PG) (10 μM) on $^{125}I^-$ efflux. (c) Effect of LBK (0.1 μM) in piroxicam (10 μM)-pretreated tissues in the absence (○), and presence (●) of prostaglandin E_2 (10 μM). (d) Effect of LBK (0.1 μM) on HCA-7 cells in the absence (○), and presence (●) of forskolin, 10 μM. Each value shows the mean ± s.e.mean (vertical bars) for six observations in (b–d) and for 12 observations in (a). Drugs were added at the times indicated by the arrows.

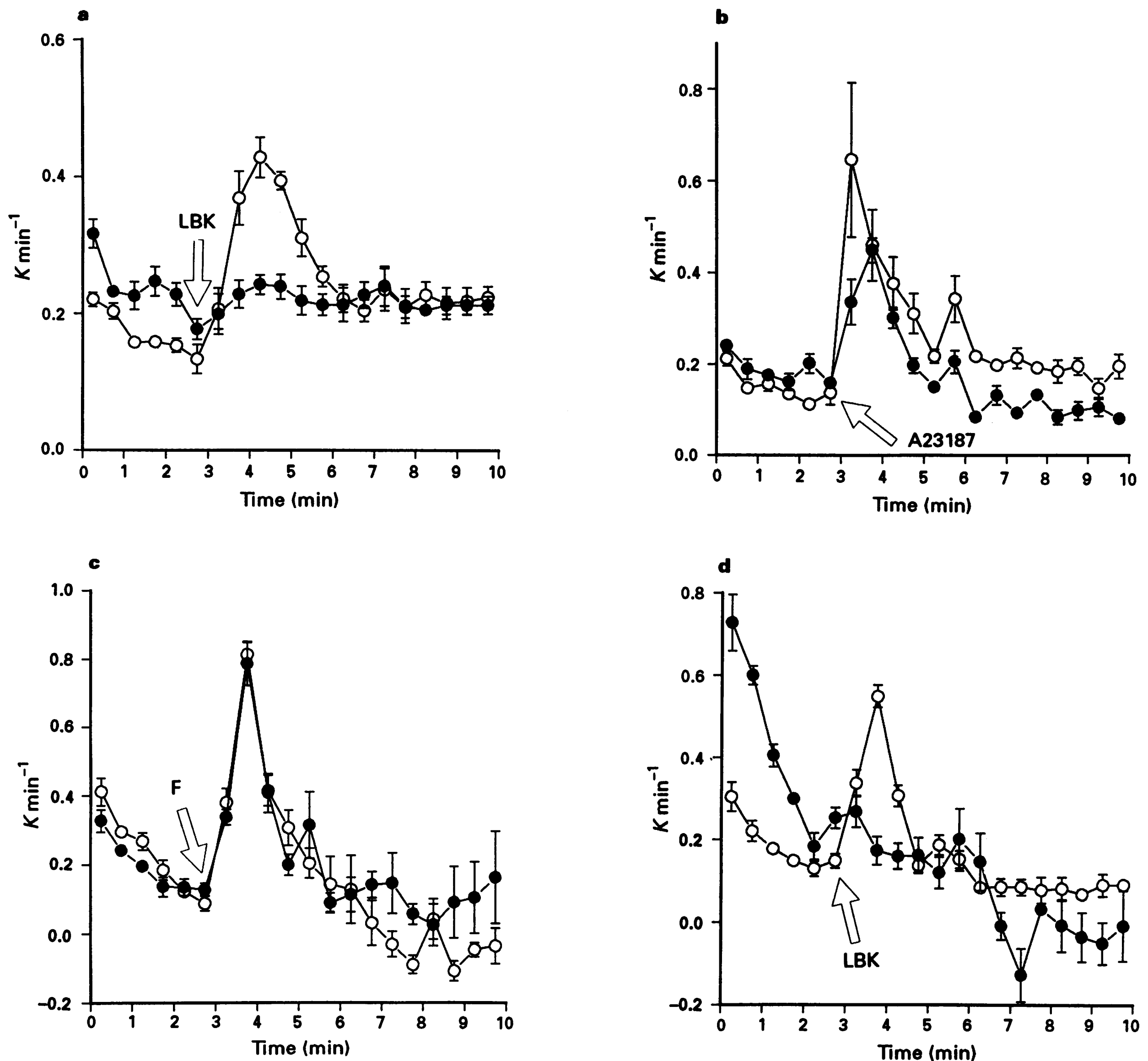

Figure 6 Effect of lysylbradykinin (LBK, 0.1 μM) (a); A23187, 1 μM (b); forskolin (F), 10 μM (c), and LBK, 0.1 μM (d), on $^{125}I^-$ efflux in Colony 29 monolayers. The control results are given by the open symbols. In (a), (b) and (c) Ba^{2+}, 1 mM was present in the test preparations (●), while in (d) test tissues were exposed to high K^+ (145 mM). All values are the means ± s.e. mean (vertical bars) for six observations. Drugs were added as indicated.

increase in the presence of alkaline earth metal ion. Specifically, the peak response to LBK (0.1 μM) in the absence (0.43 ± 0.03 min^{-1}) of Ba^{2+} was significantly greater ($P<0.001$) than the peak response in its presence (0.24 ± 0.01 min^{-1}). Nevertheless the rate constant for efflux in the presence of Ba^{2+} rose from 0.18 ± 0.01 min^{-1} to 0.24 ± 0.01 min^{-1} when LBK was added. These values are significantly different ($P<0.05$) indicating a residual effect of the peptide.

To investigate whether the Ba^{2+}effect was a result of some action other than blocking K^+ channels, such as interfering with the action of LBK at its receptor, the effect of this ion on A23187 effects on $^{125}I^-$ efflux was investigated. In this paradigm the intracellular Ca^{2+} was raised directly by the ionophore without involving surface receptors. While Ba^{2+} delayed and diminished the peak response to A23187 (Figure 6b) significant differences from control ($P<0.05$ to $P<0.01$) only appeared 90 s after addition of the ionophore, indicating that high Ca^{2+} concentrations may have actions other than on K^+ channels. Finally, in Figure 6c, it is shown that the action of forskolin was not affected by Ba^{2+}.

In a final attempt to examine the role of K channels the effects of LBK on $^{125}I^-$ efflux were investigated under high K^+ conditions. It is expected that high K^+ outside the cells would depolarize them and make it impossible to activate Ca^{2+}-sensitive K^+ channels to cause hyperpolarization. This protocol disturbed the normal pattern of $^{125}I^-$ efflux but, nevertheless, there was only a modest increase in efflux after LBK compared to controls (Figure 6d).

Measurements of intracellular [Cl⁻] from MEQ fluorescence

Using the double ionophore technique a Stern-Volmer plot for the relationship between F_o/F and $[Cl^-]$ was constructed for Colony 29 monolayers loaded with MEQ. This is shown in Figure 7, and the value of K_{Cl} estimated as 14.4 M^{-1}, from equation 1. To estimate the intracellular Cl^- concentration, MEQ loaded Colony 29 monolayers were exposed first to Cl^--containing and then to zero Cl^- solutions (solutions 1 then 2 or 3), and finally to $KSCN^-$ in the presence of valinomycin. F_o/F ratios were calculated for 12 separate

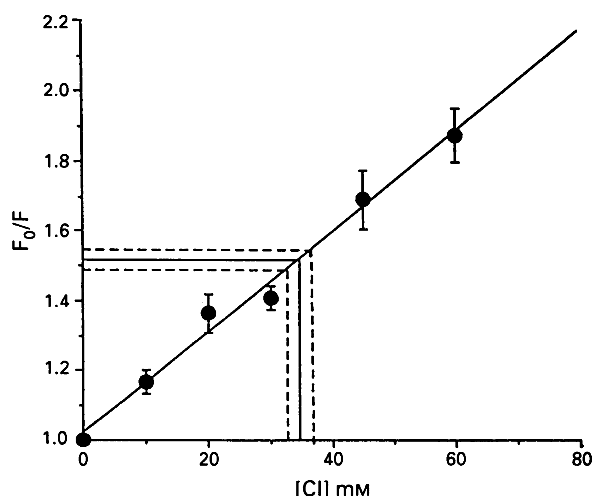

Figure 7 Stern-Volmer plot obtained at a range of Cl^- concentrations in MEQ-loaded Colony 29 monolayers using the double ionophore technique. F_o/F represents the fluorescence ratio as defined in Methods. The F_o/F ratio for 12 untreated monolayers was 1.5 ± 0.02 (mean ± s.e.mean) which translates to a value of Cl_i of 34.1 ± 1.4 mM.

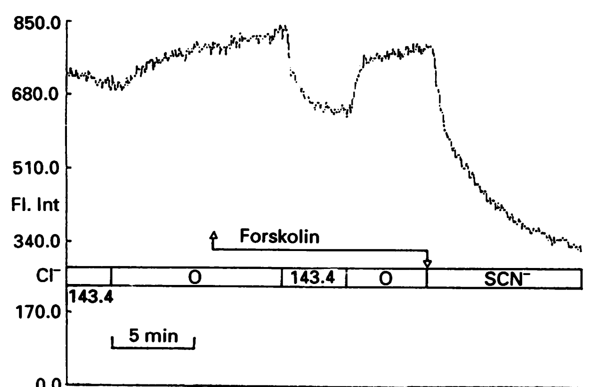

Figure 8 Fluorescence intensity (Fl. Int) from a MEQ-loaded Colony 29 monolayer against time. Measurements were made every 2 s. The Cl^- concentration of the bathing solution (in mM) is as indicated. Finally KSCN (170 mM) plus 10 μM valinomycin was added. Forskolin was present for the period indicated.

monolayers and gave a value of 1.5 ± 0.02. By extrapolation from the Stern-Volmer plot the intracellular Cl^- concentration is 34.1 ± 1.4 mM (i.e. 25.6 mM Cl^- activity). For comparison, Cl^- concentration in T84 epithelial monolayers was also measured and found to be 32.8 mM (24.6 mM activity).

A similar technique was used to measure Cl^- flux by measuring the rate of change of fluorescence when Cl^- containing solutions were added to or removed from the monolayers. However, influx and efflux measurements needed to be performed in different monolayers, in order to limit the experimental protocol to 20 min or less. This precaution was to avoid complexities due to significant dye leakage. An example of an experiment to examine the effects of forskolin on Cl^- efflux is given in Figure 8. By changing from a chloride-containing to a chloride-free solution, the fluorescence signal relaxes to a new and higher steady state. The solution changes are then repeated after forskolin is added to the tissue. In this way the rate of loss of Cl^- from the cells in presence and absence of forskolin can be estimated. Chloride influx can be similarly estimated by measuring the rate of change of fluorescence change in moving from Cl^--free to Cl^--containing solutions.

The initial rate of change of fluorescence was converted to either influx or efflux rate by use of equation 2, and the data are summarized in Table 2. The method also allows estimation of F_o/F values, both in the presence and absence of forskolin. It is seen that forskolin significantly increased both the efflux and influx rates for chloride in Colony 29 monolayers. Furthermore, there was a significant increase in the F_o/F ratio caused by forskolin, corresponding to an average increase in $[Cl_i]$ from 33 mM to 51 mM.

Other experiments with dibutyryl cyclic AMP, LBK, histamine and 4-Br-A23187 were carried out in a similar manner and the data are summarized in Table 3. Significant increases were measured with the cyclic AMP analogue, but with LBK and histamine the increases, while significant, were less than double the basal values. The calcium ionophore, by contrast, produced no change in influx or efflux rates.

Table 2 Effects of forskolin, 10 μM, on the influx and efflux rates for Cl^- estimated from the change in MEQ fluorescence and on the values of F_o/F obtained from steady state fluorescence measurements

	Efflux rate (mM s^{-1})		F_o/F	
	Control	*Forskolin*	*Control*	*Forskolin*
1	0.19	1.08	1.38	1.68
2	0.24	0.77	1.30	1.60
3	0.35	0.81	1.47	1.61
Mean ± s.e.	0.26 ± 0.05	0.89 ± 0.10		
	$P<0.05$			
	Influx rate (mM s^{-1})		F_o/F	
	Control	*Forskolin*	*Control*	*Forskolin*
1	0.42	1.61	1.58	1.80
2	0.53	1.07	1.62	1.73
3	0.54	1.74	1.60	1.77
4	0.62	2.20	1.48	2.00
5	0.65	1.91	1.48	1.87
Mean ± s.e.	0.55 ± 0.04	1.71 ± 0.19	1.49 ± 0.04	1.76 ± 0.05
			32.6 ± 2.7 mM	51.3 ± 3.5 mM
	$P<0.005$			
			$P<0.001$	

The mean F_o/F values for all eight experiments were converted to $[Cl_i]$ using equation 1.

Table 3 Effects of drugs on efflux and influx rates estimated from MEQ fluorescence

	Efflux rate (mM s^{-1})	n	P	*Influx rate* (mM s^{-1})	n	P
Control	0.26 ± 0.05	3		0.55 ± 0.04	5	
Forskolin, 10 μM	0.89 ± 0.10	3	<0.05	1.71 ± 0.19	5	<0.005
Control	0.13 ± 0.02	3		0.16 ± 0.04	3	
Dibutyryl cyclic AMP (0.5 mM) plus IBMX (0.1 mM)	0.44 ± 0.02	3	<0.05	0.50 ± 0.11	3	<0.05
Control	0.16 ± 0.04	3		0.31 ± 0.09	4	
LBK, 1 μM	0.22 ± 0.03	3	<0.05P	0.54 ± 0.16	4	<0.05P
Control	0.14 ± 0.02	5		0.19 ± 0.04	8	
Histamine, 100 μM	0.24 ± 0.04	5	<0.05P	0.33 ± 0.06	8	<0.01P
Control	0.20 ± 0.07	3		0.22 ± 0.04	3	
4-Bromo A23187, 3 μM	0.19 ± 0.04	3	NS	0.22 ± 0.03	3	NS

Flux rates were calculated from the initial rate of change of fluorescence using equation 2. Student's *t* test was used to test for significance, Pindicating where it was necessary to use a paired test to obtain significance. Number of observations is indicated by *n*. All values are the mean ± s.e.mean.

Discussion

Unlike the first paper in this series (MacVinish *et al.*, 1993) we have used mainly Colony 29 epithelia in this study, as in this line LBK is considered to affect transepithelial transport by more than one mechanism. The intracellular mediators, cyclic AMP and Ca^{2+}, clearly increase the rate constant for $^{125}I^-$ efflux as shown in Figure 1, but a survey of other figures, for example Figure 3b and Figure 6, shows that these mediators do not necessarily cause a maintained increase in rate constant. At first this may seem unexpected for it implies, for example, that Cl^- channels cease to be sensitive to cyclic AMP. However, it is to be remembered while the $^{125}I^-$ concentration in the cell is falling, the Cl^- content can be replenished. In this circumstance the rate constant for $^{125}I^-$ will only remain constant if the Cl^- concentration is unchanged. Evidence from MEQ fluorscence indicates that the Cl^- concentration actually increases with forskolin so that the specific activity of the $^{125}I^-$ as a marker for Cl^- is depressed further than by a single exponential decline. The conclusion is therefore that while a change in rate constant for $^{125}I^-$ efflux is a useful indicator of a change in chloride permeability, or the driving force for efflux or both, that precise quantitative interpretation is not possible. This would be even more true for agents such as LBK where the action undergoes rapid desensitization. It is shown in Figure 2 that LBK may cause two peaks of increased $^{125}I^-$ efflux, perhaps indicating two separate actions, temporally discrete. The absence of the second phase in the averaged data does not mean the second phase is absent, but simply asynchronous. Again, the method does not allow precise analysis of this and we have relied upon inhibition of the mean overall effect of LBK to unravel mechanisms. From the present data we can conclude that LBK, acting on B_2 receptors, increases anion efflux through DPC-sensitive anion channels. Furthermore the data with monolayers containing BAPTA (Figure 4a) or monolayers pretreated with piroxicam (Figure 5a) allow the conclusion that the well-documented Ca_i signal generated by LBK (Pickles & Cuthbert, 1991) plus prostaglandin formation (Figure 5c) both contribute to the effect of the peptide on anion permeability. Supporting evidence is that PGE_2 itself increases the rate constant for $^{125}I^-$ efflux (Figure 5b) and that there is apparently no Ca^{2+} requirement for the effect of forskolin on anion permeability (Figure 4b).

It was shown in the first paper (MacVinish *et al.*, 1993) that increasing cyclic AMP content in HCA-7 cells dramatically potentiates the response to LBK and that a similar phenomenon can be demonstrated in Colony 29 cells if steps are taken to prevent LBK itself increasing cyclic AMP, i.e. by reducing eicosanoid formation with piroxicam. It was also shown that the extent of the Ca_i increase following LBK is not changed by elevated cyclic AMP levels. In this study it was shown that elevation of cyclic AMP concentration in HCA-7 or Colony 29 monolayers does not significantly affect the size of the $^{125}I^-$ efflux response to LBK. Intuitively this seems contrary to expectations. However as shown in Figure 5c,d the efflux rate constant for $^{125}I^-$ has returned to a low value at the time LBK is added. As discussed earlier this does not mean that the apical Cl^- channels are no longer affected by the elevated cyclic AMP but that a new steady state has been achieved, probably with an elevated Cl^- concentration in the cell. It is then reasonable to expect that the resulting increment in rate constant induced by the Ca_i elevating component of the action of LBK will be constant, as is found. This again speaks for the independence of two distinct mechanisms of LBK action which in Colony 29 epithelia is exerted both on membrane permeability for Cl^- and on the driving force for anion efflux.

Using Ba^{2+}, a general blocker of many different sorts of K^+ channels (Kolb, 1990), there was a severe but incomplete inhibition of increased I^- efflux following LBK, although we have not proven this residue is due to cyclic AMP formation. Furthermore the interplay of increased chloride permeability and increased driving force makes it unpredictable what effect chloride permeability alone would have. It is striking that Ba^{2+} has less inhibitory effect on the action of A23187 (Figure 6b) than might be expected and raises the possibility that Cl^- channels sensitive to Ca^{2+} may occur in the apical membrane (Cliff & Frizzell, 1990). However in the following paper (Henderson & Cuthbert, 1993) we have been unable to detect Ca^{2+} sensitive anion channels or indeed anion channels which are responsible for chloride secretion. The single channel basis of epithelial chloride conductance has proved elusive in many studies. Channel characterization is usually done on inside-out patches yet the picture emerging is often in conflict with whole cell current studies or gross transport measurements. However, many Cl^- channels have very small conductances (Gray *et al.*, 1989; Marunaka & Eaton, 1990) which may be swamped if larger channels are also present in the patch. In addition chloride channels in patches may show run down or Ca^{2+}-dependent regulating proteins may be lost. Only rarely have calcium-dependent chloride currents in the whole cell configuration been reasonably well attributed to very small unitary conductances (Marty *et al.*, 1984; Evans & Marty, 1986). In summary, it seems likely that the Ba^{2+} resistant part of the A23187 response may represent a Ca^{2+} effect on calcium-sensitive chloride channels. Ba^{2+} had no effect on the cyclic AMP-dependent increase in Cl^- permeability (Figure 6c), while depolarization with high potassium solution virtually abolished the response to LBK (Figure 6d).

We have used MEQ fluorescence to provide corroborative evidence for the $^{125}I^-$ efflux data. Because MEQ has no isosbestic point, only a single wavelength technique can be

used. Consequently we have carried out a number of checks on the validity of the method. The intracellular Stern-Volmer constant, K_{Cl}, was 14.4 M^{-1}, in the centre of the range found by others, of 12–18 M^{-1} (Verkman, 1990). The value is much lower than that obtained in phosphate buffer (154 M^{-1}), proably because of interaction with fixed anions within the cells. In Colony 29 monolayers, the value of intracellular Cl^- concentration was 34.1 mM and T84 monolayers, 32.8 mM. These values are comparable to those reported for other epithelial cells; rabbit proximal tubule, 27.5 mM (Krapf *et al.*, 1988); canine tracheal cells, 43 mM (Chao *et al.*, 1990) and T84 cells, 36 mM (Biwersi & Verkman, 1991). The value for Colony 29 cells is some 10 mM above the expected concentration for passive distribution, assuming a membrane potential of −45 mV. Presumably this increase represents activity of the Na-K-Cl cotransporter, which is secondarily powered by the sodium pump. In a further series of experiments the value of Cl_i was 32.6 mM but rose to 51.3 mM in the presence of forskolin (Table 2). This is in spite of the increase apical Cl^- permeability created by cyclic AMP. This provides indirect evidence that cyclic AMP, and hence LBK, can stimulate the cotransporter, as shown for other tissues (Pewitt *et al.*, 1990; Paulais & Turner, 1992).

Table 2 also shows that the Cl^- influx and efflux is increased by forskolin. However, it cannot be assumed that these fluxes are entirely through conductive Cl^- channels. Chloride can enter and leave cells on exchangers and the Na-K-2Cl cotransporter is responsible for Cl^- uptake across the basolateral surface. Ideally it would have been preferable to block the cotransporter with a loop diuretic. Unfortunately, in our hands frusemide severely reduced the fluorescence signal, probably by absorbing at the excitation wavelength and piretanide increased the autofluorescence. It is noticeable that the mean efflux rate in 17 control measurements was 0.15 ± 0.013 mM s^{-1} while the mean influx rate in 23 control measurements was 0.28 ± 0.02 mM s^{-1}, these values being significantly different ($P < 0.001$). Both of these must contain a component which represents the conductive flux, plus a component due to the cotransporter when influx is measured. The efflux rate therefore is a truer indication of the rate of Cl^- exchange for NO_3^- through the conductive pathway. Both forskolin and dibutyryl cyclic AMP cause a three fold change in both efflux and influx rates measured from MEQ fluorescence, confirming an effect on apical Cl^- channels. With LBK and histamine significant effects were again observed but the changes were less than two fold. No effect was found with A23187, a finding not in keeping with earlier arguments made for a Ca^{2+}-sensitive Cl^- channel. No explanation for this discrepancy is available.

Finally, a comment is made about the late onset of the effect of LBK on $^{125}I^-$ efflux in the presence of Hoe 140 (Figure 3b). Rapid desensitization occurs when LBK is applied to cultured epithelia. It was demonstrated that cooling can prevent desensitization (Cuthbert *et al.*, 1987) and others showed desensitization is accompanied by receptor internalization (Roscher *et al.*, 1984; Wolsing & Rosenbaum, 1991). Recently, Roscher *et al.* (1991) showed that when Hoe 140 binds to its receptors internalisation is prevented. The late onset effect of LBK may result from freeing of surface receptors when Hoe 140 dissociates, exposing them to activation by the peptide, at a time when all receptors would normally be desensitized. Note that relatively high concentrations of LBK were used (0.1 μM) compared to Hoe 140 (10 nM).

In summary, this study has shown that cyclic AMP and Ca^{2+} increase the rate constant for $^{125}I^-$ efflux indicating an efflux on apical Cl^- permeability or on the electrical driving force for efflux. The Ca^{2+} effect is due to an action on K^+ channels, but some evidence supports a direct action on apical Cl^- channels. Additionally cyclic AMP may have a distinct stimulatory effect on the cotransporter. By separately eliminating the Ca^{2+} component and the prostaglandin generating components of the actions of LBK, it is concluded the peptide uses both second messengers to generate its effects on transepithelial Cl^- secretion.

We are grateful for support from the Wellcome Trust. T.R. was supported by the Cambridge-Thai Foundation.

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(Received July 23, 1992
Revised September 7, 1992
Accepted October 2, 1992)