Br. J. Pharmac. (1984), **82**, 597–607

Mediators of the secretory response to kinins

A.W. Cuthbert, P.V. Halushka,* H.S. Margolius* & J.A. Spayne

Department of Pharmacology, University of Cambridge, Hills Road, Cambridge CB2 2QD and *Departments of Pharmacology and Medicine,* Medical University of South Carolina, Charleston, South Carolina 29403, U.S.A.

1 The output of immunoreactive (i) 6 keto prostaglandin $F_{1\alpha}$ (i6ketoPGF$_{1\alpha}$), iPGE$_2$ and ithromboxane B_2 (iTXB$_2$) from isolated colonic epithelium of the rat into the apical and basolateral bathing solution has been measured. In some instances tissues were also voltage clamped at 0 mV to measure short circuit current (SCC).

2 Kallidin (lysylbradykinin) stimulated the output of all three eicosanoids, specifically from the basolateral face of the tissue. The output was similar whether or not the tissues were short circuited.

3 Both the SCC response and eicosanoid output were dependent upon the concentration of kallidin, but not in a strictly proportional manner, there being relatively more eicosanoid output at submaximal kinin concentrations.

4 Indomethacin, 5 μM, abolished the eicosanoid output, in response to kinin, while some part of the SCC response remained.

5 Calcium removal from the basolateral bathing fluid severely attenuated the SCC response, reduced the output of i6ketoPGF$_{1\alpha}$ to half, but left the output of iPGE$_2$ unchanged. In the presence or absence of calcium it is probable that sufficient PGE material is released to cause part of the SCC changes seen with kinin.

6 Kinin and PGE$_1$ increased the cyclic AMP content of intact epithelia, provided a phosphodiesterase inhibitor was added at the same time.

7 It is proposed that kinin causes an increase in calcium influx at the basolateral pole of the tissue. This calcium is necessary for the production of some eicosanoids and the subsequent generation of cyclic AMP, which then increases apical chloride permeability. In addition, calcium may facilitate entry of chloride through the basolateral face of the cells by activating a cotransport mechanism.

Introduction

The actions of bradykinin and related kinins on their receptors has defied rigorous pharmacological analysis, mainly because of a lack of specific, high affinity antagonists. Nevertheless, two types of receptors are thought to exist, designated B_1 and B_2, based on differing potency ratios of a series of agonists and the actions of some B_1 inhibitors (Regoli & Barabé, 1980). Much earlier, a classification of kinin receptors was suggested based on the ability of aspirin-like drugs to inhibit only some of the actions of kinins (Collier & Shorley, 1960). The recently discovered kinin effects on chloride secretion (Cuthbert & Margolius, 1982; Manning *et al.*, 1982) in intestinal epithelia have yet to be classified. However, it is known some analogues have strikingly different potencies when comparisons are made of their activity on the vasculature compared with that on chloride secretion (Spraggs *et al.*, 1983). The effect on chloride secretion is sensitive to indomethacin, suggesting the involvement of eicosanoid formation in kinin action (Cuthbert & Margolius, 1982). In the preceding paper (Cuthbert *et al.*, 1984) we have studied, in some detail, the dependence upon calcium of the chloride secretory process. Here we concentrate on correlations between biophysical and biochemical events following addition of kinin. On the basis of the results we propose a primary receptor event which leads to calcium influx into the epithelial cells. A variety of consequences are envisaged, of which eicosanoid formation is only one.

Methods

Short-circuit recording

The methods used for recording short-circuit current (SCC) from isolated colonic epithelia were the same as in the preceding paper (Cuthbert *et al.*, 1984). In a few experiments we used Ussing-type chambers with a window area of 2.0 cm^2, rather than the usual 0.6 cm^2. This variant was particularly useful when the bathing fluid was collected for measurement of released eicosanoids.

Immunoassays for eicosanoids

In these experiments tissues were prepared and mounted as for SCC recording in the normal way. Usually, but not invariably, the volume of fluid bathing the apical and basolateral surfaces was reduced to 10 ml from the conventional 20 ml. Samples of bathing fluid (usually 7 ml) were collected separately from both sides of the tissue, after the appropriate time intervals and experimental protocol. The samples were placed in plastic vials, shell frozen in a mixture of solid CO_2 and ethanol and placed in a freeze drier. The dried samples were kept refrigerated until assayed. Immediately before assay the samples were reconstituted by addition of the appropriate volume of distilled water. Measurement of immunoreactive (i) PGE_2, 6 keto$PGF_{1\alpha}$ and TXB_2 were as follows. For i6keto$PGF_{1\alpha}$ and iTXB_2 aliquots (50 and 100 μl) were taken directly from the reconstituted samples and assayed by a previously described radioimmunoassay (Burch *et al.*, 1979; Wise *et al.*, 1980). For iPGE_2 a 5 ml aliquot was acidified with formic acid and extracted with ethylacetate. The ethylacetate was evaporated under nitrogen and the residue chromatographed on silicic acid. The PGE fraction was collected and reconstituted in buffer for radioimmunoassay (Webb *et al.*, 1978). The antibody for PGE_2 was kindly provided by Dr W.B. Campbell, University of Texas, Dallas. All three antibodies used in the assays showed no significant cross reactivity with other eicosanoids. All standard eicosanoids used in the assays were kindly provided by Dr J. Pike, Upjohn Company, Kalamazoo.

Measurement of cyclic AMP content and adenylate cyclase activity

The activity of adenylate cyclase was measured in broken cell preparations scraped from the surface of the colon with a glass microscope slide. Cells were suspended in Krebs-Henseleit solution containing gentamycin, 600 $\mu g\,ml^{-1}$ and trasylol 200 $\mu g\,ml^{-1}$ and homogenized by hand. To a sample of this suspension (250 μl), prewarmed to 30°C, was added 1 ml of a prewarmed reaction mixture containing ATP (0.8 mM), creatine phosphate (20 mM), phosphocreatine kinase (1 $mg\,ml^{-1}$), Tris HCl pH 7.5 (2.5 mM) and bovine serum albumen (4 $mg\,ml^{-1}$). After addition of the drugs under test, incubation was continued for a fixed time at 30°C and the reaction then stopped by placing tubes in a boiling water bath for 5 min. After cooling, the tubes were centrifuged and the supernatant removed and stored frozen until assay. Protein concentrations were measured by the method of Lowry *et al.* (1951).

The cyclic AMP content of colonic epithelium following addition of kallidin or PGE_1 was measured using epithelial discs as follows. Epithelium was dissected from the descending colon as usual and discs of 1.13 cm^2 cut from the tissue with a stainless steel cutter. Discs were incubated in Krebs-Henseleit solution at 30°C together with the appropriate drug additions. At the end of the incubation period discs were quickly removed, blotted and placed in boiling Brown's buffer. Subsequently the tissue was homogenized in the buffer and the whole centrifuged. The cyclic AMP content of this supernatant and also that of the incubation solution were measured.

Both for experiments with homogenates and with epithelial discs cyclic AMP was assayed by a standard radio-immunoassay. Occasional checks were made to show that the amount of cyclic AMP measured varied linearly with the volume of solution assayed and that known amounts of cyclic AMP added to the extracts could be accounted for quantitatively on assay. These methods are essentially similar to those we have used before for epithelial tissues (Cuthbert & Wilson, 1981; Cuthbert & Spayne, 1983).

Solutions

Tissues were bathed in Krebs-Henseleit solution of the composition given in the previous paper (Cuthbert *et al.*, 1984). This solution had a pH of 7.4 when gassed with 95% O_2: 5% CO_2 at 37°C.

Results

Eicosanoids released during kinin action

Indirect evidence for the involvement of eicosanoids in the responses to kinins was obtained previously by use of indomethacin and mepacrine (Cuthbert & Margolius, 1982). Here we have measured the release of eicosanoids directly into the bathing solution in response to challenge with a near maximally effective (1 μM) concentration of kallidin. Release into the serosal and apical bathing solutions was measured separately in paired preparations, one only of which

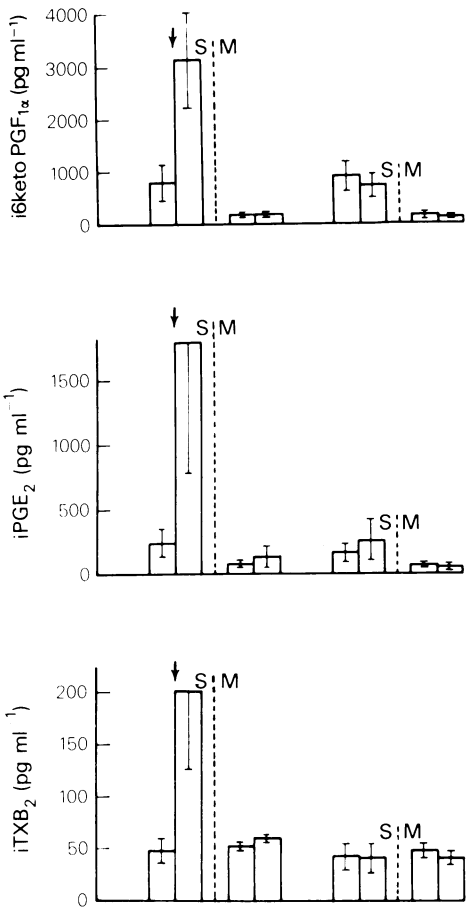

Figure 1 Release of i6ketoPGF$_{1\alpha}$, iPGE$_2$ and iTXB$_2$ from rat colon epithelia. Release into the apical and serosal bathing fluids was measured separately. Collection periods were 20 min in all instances. The results shown at the left of the diagram were from preparations which were challenged with kallidin, 1 μM, on the serosal side after an initial control collection period. The dotted lines separate the values for the serosal (S) and mucosal (M) fluids. Note kinin causes no increased release into the mucosal fluid. Results given on the right side of the figure are for tissues where two control collection periods without kinin challenge were used. They show that spontaneous release remains reasonably constant during the experiment. Paired preparations were obtained from each animal, one being used to examine release with kinin while the other acted as a non-stimulated control. Each preparation was 2.0 cm^2 and the volume of bathing fluid on each face was 10 ml. Total amounts of material released from each cm^2 in 20 min can be obtained by multiplying the values given by 5. Mean values and standard errors are given for six measurements. Preparations were open circuited. Kallidin caused a significant ($P<0.05$, Mann & Witney test) increase in the release of all three eicosanoids.

was challenged with kinin. The results of six experiments are given in Figure 1. Several features of the results are apparent as follows. First there is a low level release of i6ketoPGF$_{1\alpha}$, iPGE$_2$ and iTXB$_2$ in the absence of kinin which is less into the apical than into the serosal bathing solution. In the preparations which were not stimulated by kinin the amount released during the second 20 min period was much the same as in the first. Addition of kinin produced a statistically significant increase in the amount released of all three eicosanoids measured, the extra material being released entirely into the serosal bath. Relatively small extra amounts of iTXB$_2$ were released, while the amounts of i6ketoPGF$_{1\alpha}$, the major metabolite of prostacyclin, were roughly twice those of PGE$_2$. It is interesting that the ratio of serosal to apical release of both i6ketoPGF$_{1\alpha}$ and iPGE$_2$ is clearly greater than one, while that for iTXB$_2$ is near unity.

Relation between kinin concentration and release of i6ketoPGF$_{1\alpha}$

The concentrations of kinin used to elicit the release of prostaglandins in the experiments illustrated in Figure 1 were near maximally effective on SCC and it might be that eicosanoid release was due to mechanisms triggered by full activation of transport processes rather than to a kinin effect *per se*. In these next experiments SCC was monitored throughout so that any relationship between prostaglandin release and response could be discerned. We compared the re-

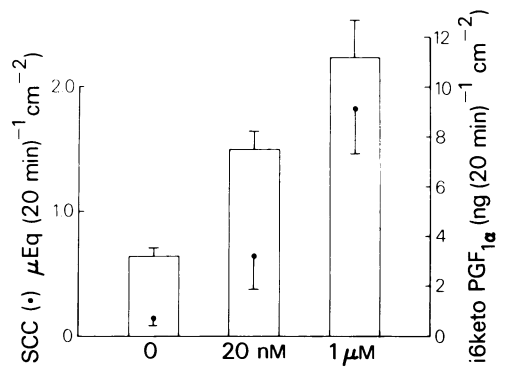

Figure 2 Release of i6ketoPGF$_{1\alpha}$ (ng (20 min)$^{-1}$ cm^{-2}) in response to kallidin, 0, 20 nM and 1 μM. The preparations were short-circuited and responses were calculated from the area under the SCC record. These are given as μEq (20 min)$^{-1}$ cm^{-2}. All values given are means with s.e. for seven experiments. In these experiments tissue area was 0.6 cm^2 and 10 ml of bathing fluid was used on each side of the tissue. The values obtained with kallidin, 20 nM, were significantly ($P<0.001$) different from those obtained either with no kallidin or with kallidin, 1 μM.

lease of i6ketoPGF$_{1\alpha}$ in the resting condition and in the presence of 20 nM and 1 μM kallidin. The lower kinin concentration caused approximately a third of the maximal effect on chloride secretion. The results from 7 experiments of this type are given in Figure 2. Notice that the actual release of i6ketoPGF$_{1\alpha}$ into the serosal bath in response to kallidin, 1 μM was very similar to that shown in Figure 1 (11.0 ng (20 min)$^{-1}$ cm^{-2} compared to 15.0 ng (20 min)$^{-1}$ cm^{-2} in Figure 1). This similarity is despite the different conditions in the two experiments. In experiment 1 the tissue areas were 2.0 cm^2 and the tissues were open circuited, while in this experiment the area was only 0.6 cm^2 and the tissues were short circuited. In this series we see that the lower concentration of kinin caused significantly ($P < 0.001$) less release and less chloride secretion (measured as SCC) than obtained with kinin, 1 μM but that, in turn, the responses to kinin, 20 nM were significantly greater than in the control condition. The two types of response were not however strictly proportional, indeed there was relatively more i6ketoPGF$_{1\alpha}$ released in relation to the SCC response at the lower kinin concentration. This result means that we must question whether kinin action in this tissue is mediated solely through an eicosanoid involvement.

Effect of calcium removal on prostaglandin release by kinins

In the previous paper (Cuthbert *et al.*, 1984) it was shown that calcium removal attenuated the responses to kinin and it was decided to investigate how this manoeuvre would affect prostaglandin release. Two sets of experiments were carried out with similar protocols, one in the open circuited state and one short circuited. In five paired experiments in the open circuited condition one of each pair was bathed in calcium-free solution (containing EGTA). Release of i6ketoPGF$_{1\alpha}$, iPGE$_2$ and iTXB$_2$ were measured during a 20 min control period in both preparations and then for 20 min after stimulation with kallidin, 1 μM. Results for i6ketoPGF$_{1\alpha}$ were as follows. In the controls release after kallidin was increased from 1.2 ± 0.2 ng cm^{-2} (20 min)$^{-1}$ to 14.3 ± 4.8 ng cm^{-2} (20 min)$^{-1}$ while in the absence of calcium spontaneous release was itself increased to 3.8 ± 1.4 ng cm^{-2} (20 min)$^{-1}$ and kinin caused a further increase in release to 9.0 ± 2.3 ng cm^{-1}. In both conditions kinin caused a significant increase in release ($P < 0.05$, Mann & Witney test). The kinin-induced release of i6ketoPGF$_{1\alpha}$ was significantly greater in the controls (13.0 ± 4.6 ng cm^{-2} (20 min)$^{-1}$) than in the absence of calcium (5.2 ± 1.1 ng cm^{-2} (20 min)$^{-1}$, $P < 0.05$, Mann & Witney test), although clearly there was still a substantial amount of release in this latter condition. However, calcium removal produced no significant reduction in the amount of iPGE$_2$ or iTXB$_2$ released by kinin in these experiments. The results are summarized in Figure 3. It was possible that prostaglandin formation by kinin in the absence of calcium might be different under short circuit conditions, furthermore it might be important to correlate prostaglandin release with the SCC response in the absence of calcium. In six further experiments paired tissues were short circuited in the presence or ab-

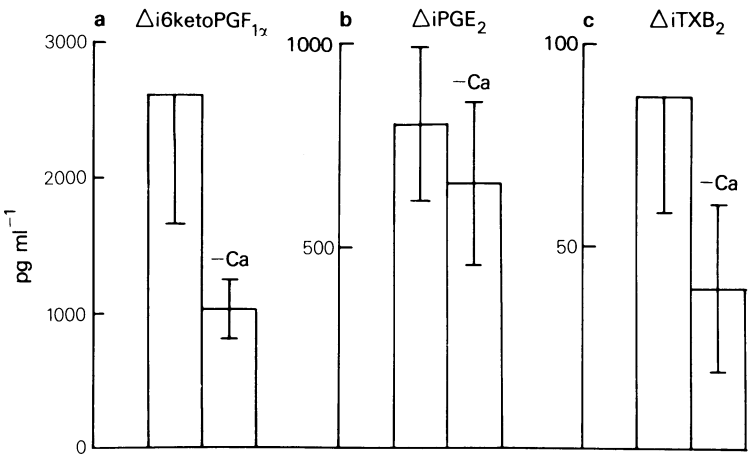

Figure 3 Effect of calcium removal on the release of (a) i6ketoPGF$_{1\alpha}$, (b) iPGE$_2$ and (c) iTXB$_2$ by kallidin, 1 μM. Data from five paired experiments are shown. The increase in the release of each eicosanoid during 20 min following addition of kallidin, in the presence and absence of calcium ions is shown (values are mean with s.e. indicated by vertical lines). Only with i6ketoPGF$_{1\alpha}$ is the change caused by calcium significant ($P < 0.05$, Mann & Witney test). Epithelial area was 2 cm^2 with 10 ml of bathing solution on each side. Tissues were open circuited.

sence of calcium and the release of i6ketoPGF$_{1\alpha}$ and iPGE$_2$ in response to kinin were measured. The results are given in Figure 4. Calcium removal produced the usual, highly significant, reduction in the SCC response to kinin. The release of i6ketoPGF$_{1\alpha}$ in response to kinin was reduced by half, as in the open circuit condition, but this contrasts with the 80% reduction in the SCC response, bringing to mind the previous lack of correlation referred to with respect to Figure 2. The result with iPGE$_2$, again as was found in the open circuit condition, was that calcium removal had virtually no effect on the release of iPGE$_2$. This finding is particularly important as we shall show, later in this paper, that probably sufficient PGE$_2$ is released in response to kinin to generate a substantial part of the SCC responses observed.

Effects of indomethacin on prostaglandin release

Indomethacin (5 μM) was incubated with one each of six pairs of colon preparations so that the effect of this agent on both basal and kallidin-stimulated release of i6ketoPGF$_{1\alpha}$ could be measured. In addition, the tissues were short circuited throughout so that correlations between the effects on transport and production of the metabolite might be compared. Indomethacin had little effect on basal SCC but significantly reduced the effect of kinin (Figure 5). Although the basal output of i6ketoPGF$_{1\alpha}$ fell in the presence of indomethacin the fall was not statistically significant. In the presence of indomethacin, kinin produced no increase at all in the release of i6ketoPGF$_{1\alpha}$, while the output was trebled in the controls. That a SCC response remains in the presence of indomethacin means that kinin may have effects independent of prostaglandin formation, assuming the output of i6ketoPGF$_{1\alpha}$ is typical of other eicosanoids.

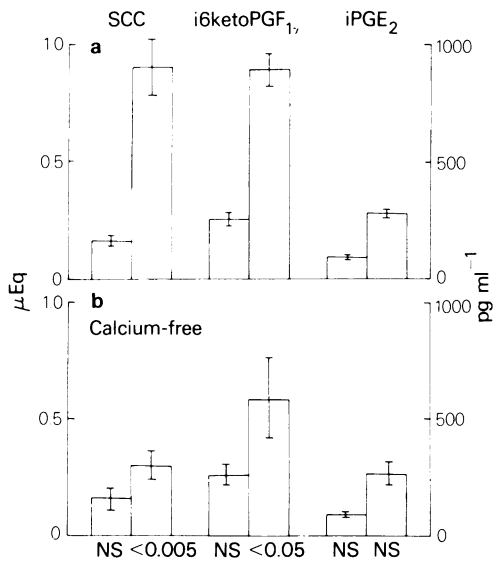

Figure 4 Effect of calcium removal on the release of i6ketoPGF$_{1\alpha}$ and iPGE$_2$ by kallidin, 1 μM, in six paired short circuited preparations. Values on the left of each pair of columns represent the control situation while those on the right are after addition of kallidin. The SCC responses (in μEq) and the release of eicosanoids (pg ml^{-1}) are for periods of 20 min for preparations of 0.6 cm^2 bathed on each side by 10 ml of Krebs-Henseleit solution. The results for the calcium-free situation are shown in (b). These values have been compared with those obtained in the presence of calcium. Calcium removal significantly inhibited the SCC response to kallidin ($P<0.005$, Student's t test) and significantly reduced the output of i6ketoPGF$_{1\alpha}$ ($P<0.05$, Mann & Witney test).

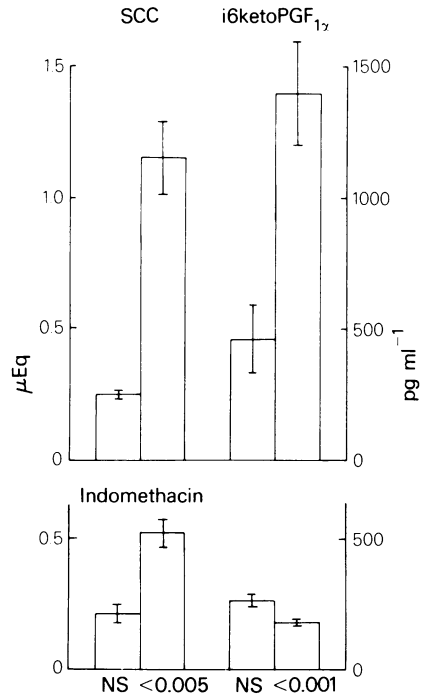

Figure 5 Effect of indomethacin on the output of i6ketoPGF$_{1\alpha}$ in six paired preparations in response to kallidin, 1 μM. The experimental design and conditions were similar to those used for Figure 4. Again SCC responses (in μEq) and i6ketoPGF$_{1\alpha}$ output (in pg ml^{-1}) are for tissues of 0.6 cm^2, during 20 min, with 10 ml fluid bathing each side of the tissue and under short circuit conditions. Values in the presence of indomethacin (5 μM) are compared with those under control conditions. Indomethacin caused a significant ($P<0.005$, Student's t test) reduction in the SCC response to kallidin and caused a significant ($P<0.001$, Student's t test) reduction in the output of i6ketoPGF$_{1\alpha}$.

Possible involvement of cyclic AMP in the responses to kinins

In the previous paper (Cuthbert *et al.*, 1984) we showed that the actions of both kinins and PGE_2 on SCC in rat colon were potentiated by inhibiting phosphodiesterase with isobutylmethylxanthine (IBMX). Determination of cyclic AMP content of tissues following addition of kinins was made to obtain direct evidence for or against the involvement of adenylate cyclase. Initially, a variety of alternative procedures using broken cell preparations was tried. Kinin associated increases in cyclic AMP production were not seen consistently, although forskolin was able to generate cyclic AMP under these conditions. In this situation it became obvious that it was necessary to inhibit phosphodiesterase in order to measure cyclic AMP accumulation. The influence of a phosphodiesterase inhibitor and of the presence and absence of calcium on the generation of cyclic AMP by forskolin in a broken cell preparation is illustrated in Figure 6. Cyclic AMP accumulation is barely detectable in the absence of IBMX, but when phosphodiesterase was inhibited, accumulation of cyclic AMP was greater when calcium ions were also present. Kallidin was also used in this experiment (data not shown) but produced no consistent effect on cyclic AMP accumulation.

It is possible that cyclic AMP accumulation in response to kinin may only occur in intact cells and to this end our attention turned to epithelial discs. Figure 7 gives results from three separate experiments where we have measured both cyclic AMP release into the medium as well as that accumulating in the tissue. In the absence of IBMX (Figure 7a) no

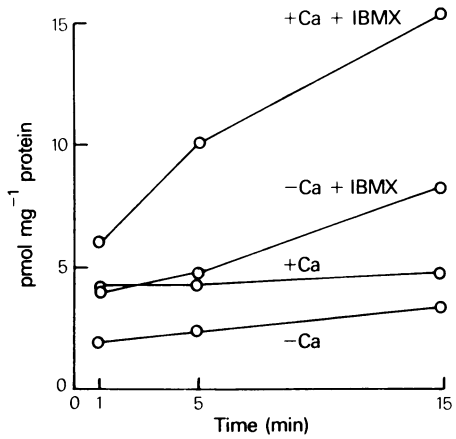

Figure 6 Effect of forskolin (10 μM) on the generation of cyclic AMP by a broken cell preparation. Cyclic AMP formation with time was measured either in the absence or presence of Ca (2.5 mM) or in the absence or presence of isobutylmethylxanthine (IBMX, 1.0 mM).

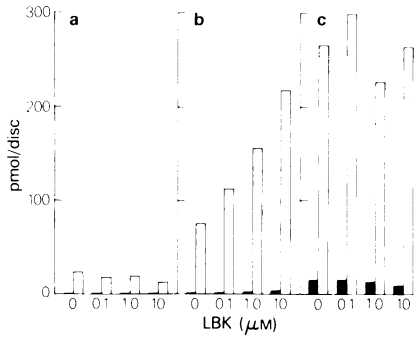

Figure 7 Effect of kallidin (LBK) on cyclic AMP formation in discs of intact epithelium. Each disc was 1.13 cm^2 in area. The cyclic AMP in the tissue (open columns) and that released into the bathing fluid (closed columns) were measured separately. Each sample was measured at two different dilutions and the mean value taken. In (a) no isobutylmethylxanthine (IBMX) was present in the incubation mixture while in (c) the tissue was preincubated with IBMX, 5 mM for 30 min. In (b) the tissue discs were exposed simultaneously to kinin and IBMX. In all instances incubation was for 10 min at 37°C.

cyclic AMP production in response to kinin was seen while in tissues preincubated with the inhibitor (Figure 7c), large and rather variable accumulations occurred, beyond which no further accumulation due to kinin could be detected. However, if kallidin and IBMX were added simultaneously (Figure 7b), there was a concentration-dependent increase in cyclic AMP production. Very little cyclic AMP apparently escaped into the medium but in comparable experiments with forskolin it was found that concentrations in the medium were 7 times those detected with kinin. Using an entirely comparable protocol, a concentration-dependent increase in cyclic AMP concentration was detected following PGE_1 (Figure 8). The concentrations of both PGE_1 and kallidin which produced approximately half-maximal increases in cyclic AMP production were supramaximal with respect to their effects on SCC in isolated colonic epithelia.

Possible correlations between mediators and SCC responses

Although the colonic epithelium responds to cyclic AMP poorly and to dibutyryl cyclic AMP well (Cuthbert & Spayne, 1983), the concentrations of the latter required to cause SCC responses, comparable to those given by kallidin, are in the millimolar range. From the results given above it would appear that the cell membrane prevents easy access to the cell interior and the relative insensitivity to the nucleotide in relation to the amounts produced following kinin is not surprising.

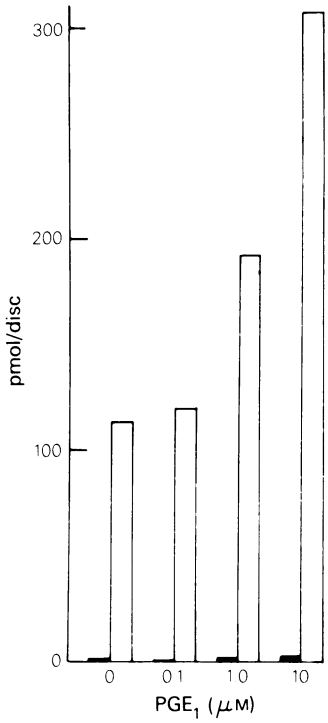

Figure 8 Effect of prostaglandin E_1 (PGE_1) on cyclic AMP formation in discs of intact epithelium. Each disc was 1.13 cm^2 in area. The cyclic AMP in the tissue (open columns) and that released into the bathing fluid (closed columns) were measured separately. Each sample was measured at two different dilutions and the mean taken. The tissues were exposed simultaneously to PGE_1 and isobutylmethylxanthine. Incubation was for 10 min at 37°C.

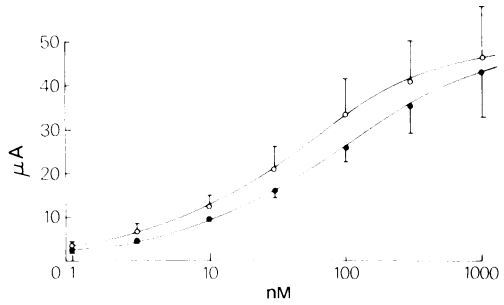

Figure 9 Concentration-response curves for prostaglandin E_1 (○) and prostaglandin E_2 (●) measured on rat colon epithelia from cumulative responses. Paired preparations were obtained from five animals and one preparation was used for each of the two prostaglandins. As responses to prostaglandins show maintained plateaus the peak heights of the responses have been measured. Each preparation had an area of 0.6 cm^2.

SCC responses to PGE_1 and PGE_2 were very comparable, as shown by the concentration-response curves (Figure 9), and the half-maximally effective concentrations of each were approximately 40 nM and 80 nM respectively. It is presumed that these responses did indeed represent chloride secretion since they were sensitive to piretanide. The $iPGE_2$ released by near maximally effective concentrations of kinins (Figure 1) produced a concentration of approx. 5 nM in the serosal bathing fluid and undoubtedly higher concentrations in the environs of the epithelium. It seems entirely reasonable, therefore, from the data given in Figure 9 to suggest that sufficient PGE_2 was released to produce effects on transport.

Even larger amounts of PGI_2 metabolites ($6ketoPGF_{1\alpha}$) are released into the bathing fluid (approximately 7.5 nM) yet PGI_2 (prostacyclin) proved to be rather ineffective on SCC. Responses were small and transient, lasting a minute or so. Typically a SCC response to PGI_2 was equivalent to only 0.008 μEq at a concentration of 10 μM. This

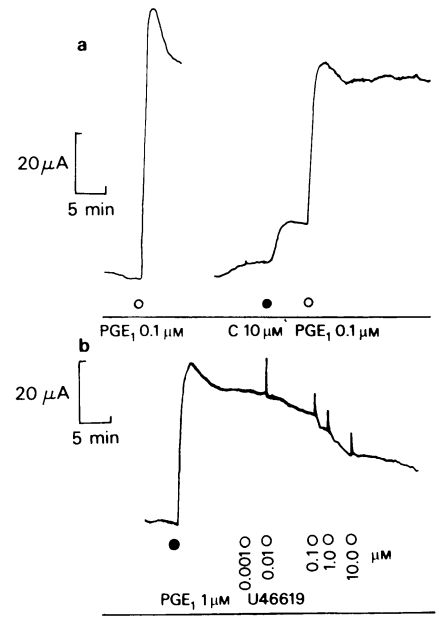

Figure 10 (a) Compares the responses of prostaglandin E_1 (PGE_1) with that to carbacyclin (C). Note carbacyclin has a relatively minor action which does not prevent a subsequent response to PGE_1. (b) Effects of the thromboxane-mimetic U46619 on SCC in rat colon during the plateau of a response to PGE_1. Four of the five additions of U46619 gave a transient increase in SCC followed by a decline in SCC (compare with the maintained plateau following PGE_1 in a). Both preparations had an area of 0.6 cm^2. Horizontal lines indicate zero SCC.

value is to be compared with mean values of approx. 0.3 μEq for kallidin (1 μM) (Table 1, Cuthbert *et al.*, 1984). The undoubted instability of PGI_2 is a complicating factor in making these comparisons, so some experiments were done with carbacyclin, a stable analogue of PGI_2. Figure 10a shows a typical response to carbacyclin, 10 μM in comparison with responses to PGE_1. Lack of material prevented a proper comparison being made but it is not difficult to assert that this analogue had at least one hundred fold less activity than PGE_1.

To investigate the possible role of thromboxanes in the responses to kinin, a thromboxane synthetase inhibitor, benzylimidazole, was used. No difference was recorded in the responses to kallidin, 1 μM in the presence and absence of benzylimidazole, 30 μM, in six experiments on paired tissues (0.21 ± 0.05 μEq $0.6\,cm^{-2}$ in control versus 0.26 ± 0.03 μEq in the preparations treated with benzylimidazole). Using U46619, a thromboxane-mimetic, only small, very transient effects on SCC were observed. However applied during the plateau phase of a response to PGE_1 the compound again produced transient increases in SCC but subsequently the plateau was lowered (Figure 10b).

Finally the effects of the leukotrienes LTC_4 and LTD_4 (100 nM) on SCC in the colon were examined. In agreement with the findings of Musch *et al.* (1982) on rabbit colon, each of these produced very small 'blips' in the SCC record (< 5 μA). No effect was seen when the leukotrienes were given during the plateau of the response to PGE_1. BW 755-C (30 μM), a mixed inhibitor of the cyclo-oxygenase and lipoxygenase pathways, was less effective than indomethacin (5 μM) at inhibiting responses to kallidin. Piroxicam (5 μM) a pure cyclo-oxygenase inhibitor was as effective as indomethacin in inhibiting the response to kinin.

An important and hitherto unknown action of kinins was discovered during the experiments in which release of prostaglandin metabolites was being measured in the presence of indomethacin. Previously (Cuthbert & Margolius, 1982) it was found using submaximal concentrations of kallidin that the responses were completely suppressed in the presence of indomethacin (5 μM). However, when using near maximally effective concentrations of kallidin there was a substantial but transient response which remained in the presence of indomethacin. The responses showed a peak, followed by a small hump; the former, but not the latter, remained after the addition of piretanide. Similar transient responses remained after calcium removal from the bathing fluid which became further attenuated on repeated exposure to the kinin, yet they were never completely eliminated (Figure 11). Regrettably these responses were too transient to examine by means of standard flux measurements so we are unable to assert which ions carry current during these transient responses to kallidin.

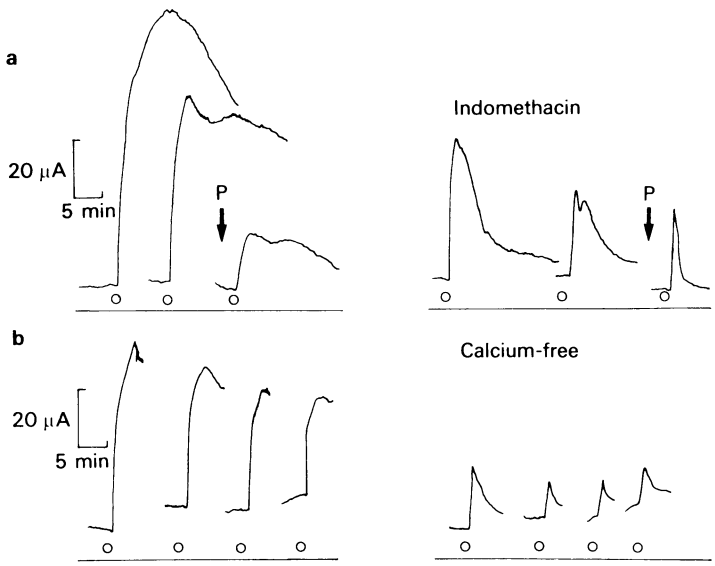

Figure 11 Open circles show responses to kallidin, 1 μM. The kinin was washed away after each response. In (a) responses were in normal Krebs-Henseleit solution (left) and in the presence of indomethacin, 5 μM. At P piretanide, 0.1 mM, was added to the serosal bathing fluid. Both preparations were from the same animal (area $0.6\,cm^2$). Horizontal lines indicate zero SCC. In (b) paired preparations were bathed either in normal solution (left) or in the absence of calcium (right). Horizontal lines indicate zero SCC. Area of epithelium, $0.6\,cm^2$.

Discussion

Circumstantial evidence to suggest that the effects of kinins on chloride secretion are dependent on eicosanoid production were obtained previously (Cuthbert & Margolius, 1982; Manning *et al.*, 1982). In essence this consisted of the attenuation of the kinin effect by inhibitors of the cyclo-oxygenase and phospholipase A_2 systems. The known calcium dependence of phospholipase A_2 (Blackwell & Flower, 1983), plus the calcium dependence of the kinin effect on chloride secretion, led directly to the hypothesis presented in the accompanying paper, in which a crucial role for extracellular calcium was considered to be at the proximal end of the kinin-induced cascade.

In this study we have shown that three eicosanoids, at least, are generated in the colon in response to kinin, viz prostacyclin, PGE_2 and TXB_2. It seems likely, from a quantitative viewpoint, that sufficient PGE_2 is found to account for a substantial fraction of the kinin-induced SCC response. In our hands both prostacyclin, and its stable analogue carbacyclin, produced only minor effects on SCC, although we are aware of a brief report claiming that prostacyclin does cause chloride secretion in the colon (Goerg *et al.*, 1983). The thromboxane-mimetic compound, U46619, had an inhibitory, albeit weak, effect on SCC in the presence of PGE_1 and it is possible that thromboxane released following kinin serves as a feedback inhibitor to the actions of PGE_2. Relatively more TXB_2 was released into the mucosal bathing fluid than with the other two eicosanoids measured, perhaps indicating that this material is generated at a different locus in the epithelial cell. Kinin is effective only when applied to the basolateral or serosal surface of the epithelium and the extra release of eicosanoids also occurs at this side. Although bradykinin receptors have been detected in the lamina propria (Manning *et al.*, 1982) there is no incontrovertible evidence that the eicosanoids are actually generated by the transporting epithelial cells themselves. Perhaps the strongest evidence to involve the epithelial cells is that cultures of collecting tubule cells can release eicosanoids when challenged with kinin (Grenier *et al.*, 1981). Our results, for PGE_2, have been confirmed in rabbit ileum and colon, where kinin was found to induce a five fold increase in release (Musch *et al.*, 1983).

In a number of experiments we have tried to correlate the biophysical effects of kinin with the release of prostaglandin metabolites. The overall impression is that the hypothesis, that all the effects of kinin on SCC are due to eicosanoid formation, cannot be sustained. We can cite (a) the lack of proportionality between i6ketoPGF$_{1\alpha}$ release and the SCC response at different kinin concentrations (Figure 2), (b) the complete abolition of i6ketoPGF$_{1\alpha}$ release in response to kinin in the presence of indomethacin (5 μM) (Figure 5) and (c) the effects of calcium removal. It is this last finding which provides the most compelling evidence against the hypothesis. Calcium removal had no effect on the release of PGE_2 by kinin, under both open and closed circuit conditions, even though the SCC responses are inhibited by 70–80%. Yet we have argued earlier that sufficient PGE_2 is released to affect SCC under conditions where calcium is present. Although prostacyclin has only minor effects on SCC its release is significantly altered by removing external calcium, but even so the decrease is only by 50%, substantially less than the effect on SCC. We find these results are not unique to the colon. For example, the relaxant activity of kinin on coronary vessels persists after indomethacin has been used to block prostacyclin release (Schrör *et al.*, 1979). Also PGE release in the perfused rabbit ear following kinin infusion was not inhibited by calcium-free conditions (Juan, 1979). It would appear that kinin has calcium-dependent effects that are not dependent on eicosanoid formation. A possible scheme for the chloride secreting cell is depicted in Figure 12 and, while only circumstantial evidence is available in support, it provides a framework for further investigation. Kinin reacts with receptors in the basolateral aspect of the cell which result in the activation of a calcium channel. The blocking effects of verapamil (Cuthbert *et al.*, 1984) and Co^{2+} (Manning *et al.*, 1982) give partial

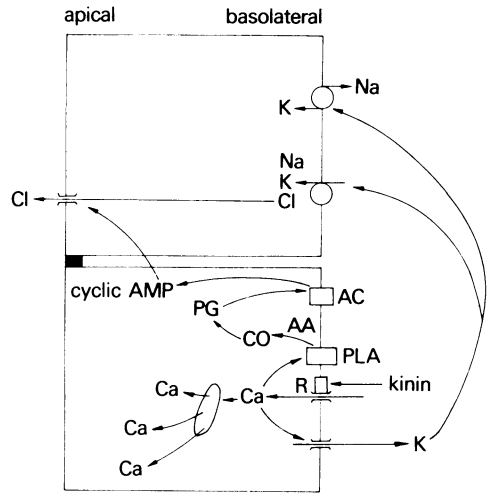

Figure 12 Hypothetical model of chloride secretion in response to kinin in an epithelial cell. All the processes are envisaged as occurring in a single cell but are shown in two adjacent cells for reasons of clarity. See text for details. Abbreviations used are adenylate cyclase AC; arachidonic acid AA; phospholipase PLA; receptor R; cyclo-oxygenase CO and prostaglandin PG.

support to this. The calcium entering the cell might then have multiple effects such as (a) calcium-stimulated calcium release from intracellular sites, (b) phospholipase activation and (c) a calcium activated increase in potassium permeability. We have no evidence for (a) but the reduction in i6ketoPGF$_{1\alpha}$ formation under calcium-free conditions may be evidence for (b). The reason why iPGE$_2$ generation is unaffected is unknown but possibly it is derived by a different route (see Bareis *et al.*, 1983) or at a different cellular location. For example it is known that the phospholipid composition of epithelial cells is different in the apical and basolateral domains (Viso & Cuthbert, 1979). Calcium activated increases in potassium permeability have proved to be rather common (see Marty, 1981) and furthermore have been demonstrated in epithelial cells (Maruyama *et al.*, 1983). A recent microelectrode study of a chloride secreting epithelium (Welsh, 1983) has confirmed the model put forward originally by Frizzell *et al.* (1979). Chloride is accumulated in the cell across the basolateral border by a non-electrogenic cotransport process, powered by the sodium gradient, and exits across the apical surface by a passive mechanism. Further, after chloride secretion is stimulated the intracellular chloride activity does not change, indicating that both the entry and exit processes to and from the cell have been activated. Although originally the cotransport system was considered to involve only Na and Cl, more recently it has been shown to be a Na-K-Cl cotransporter (Chipperfield, 1980; Greger & Schlatter, 1981) and external K has been shown to stimulate NaCl cotransport in a marine teleost (Musch *et al.*, 1982). Thus a calcium-activated increase in potassium permeability would facilitate the entry process for chloride. Whether or not this results in an increase in electrogenic chloride secretion will depend on both the electrochemical gradient across the luminal membrane and its chloride permeability. Concomitant events which increase the permeability of the apical membrane to chloride will then facilitate transepithelial chloride transport. Without these latter it would be expected that the intracellular chloride activity would rise and the increase in chloride secretion be modest. Alternatively, if the apical chloride permeability were increased but without facilitation of the basolateral entry process then again only a modest increase in secretion might be expected. These proposals are similar to those made by Maruyama *et al.* (1983) for secretory processes in the salivary glands. It is of considerable interest that bradykinin induces a calcium-dependent increase in potassium permeability in aortic endothelial cells (Gordon & Martin, 1983).

The model (Figure 12) shows that the chloride permeability of the apical face is controlled by cyclic AMP. Supporting evidence for this comes from studies of chloride secretion in tracheal epithelium (Smith *et al.*, 1982; Welsh *et al.*, 1982). Kinin has been shown to increase cyclic AMP content of guinea-pig lung (Stoner *et al.*, 1973) and human fibroblasts (Bareis *et al.*, 1983) by a prostaglandin-dependent, indomethacin-sensitive process. Similarly we have shown that cyclic AMP content of the colon can be increased following kinin, provided phosphodiesterase is inhibited.

In summary, it is proposed that calcium is important in relation to kinin effects on chloride secretion, both for the generation of eicosanoids and subsequent activation of adenylate cyclase and also for facilitation of chloride entry to the transport mechanism. In the absence of external calcium, sufficient release of internal calcium may occur to allow some eicosanoid generation. When eicosanoid generation is prevented by indomethacin, in the presence of calcium increased chloride entry through the basolateral pole of the tissue may still occur. In both of these situations only a partial kinin response will occur, a full response requiring both the ability to form eicosanoids plus the presence of external calcium to trigger other essential events.

J.A.S. was in receipt of a SERC Case Award with the Wellcome Laboratories. Laboratory costs were partly defrayed by support from an NIH grants, HL 17705-08 and HL 29566.

References

BAREIS, D.L., MANGANIELLO, V.C., HIRATA, F., VAUGHAN, M. & AXELROD, J. (1983). Bradykinin stimulates phospholipid methylation, calcium influx, prostaglandin formation and cAMP accumulation in human fibroblasts. *Proc. natn. Acad. Sci.*, U.S.A., **80,** 2514–2518.

BLACKWELL, G.J. & FLOWER, R.J. (1983). Inhibition of phospholipase. *Br. med. Bull.*, **39,** 260–264.

BURCH, R.M., KNAPP, D.R. & HALUSHKA, P.V. (1979). Vasopressin stimulates thromboxane synthesis in toad urinary bladder. Effects of imidazole. *J. Pharmac. exp. Ther.*, **210,** 344–348.

CHIPPERFIELD, A.R. (1980). An effect of chloride on (Na + K) co-transport in human red blood cells. *Nature*, **286,** 281–282.

COLLIER, H.O.J. & SHORLEY, P.G. (1960). Analgesic antipyretic drugs as antagonists of bradykinin. *Br. J. Pharmac. Chemother.*, **15,** 601–610.

CUTHBERT, A.W., HALUSHKA, P.V., MARGOLIUS, H.S. & SPAYNE, J.A. (1984). Role of calcium ions in kinin-induced chloride secretion. *Br. J. Pharmac.*, **82,** 587–595.

CUTHBERT, A.W. & MARGOLIUS, H.S. (1982). Kinins stimulate net chloride secretion by the rat colon. *Br. J. Pharmac.*, **75,** 587–598.

CUTHBERT, A.W. & SPAYNE, J.A. (1983). Conversion of sodium channels to a form sensitive to cyclic AMP by component(s) from red cells. *Br. J. Pharmac.*, **79,** 783–797.

CUTHBERT, A.W. & WILSON, S.A. (1981). Mechanisms for the effects of acetylcholine on sodium transport in frog skin. *J. memb. Biol.*, **59,** 65–75.

FRIZZELL, R.A., FIELD, M. & SCHULTZ, S.G. (1979). Sodium-coupled chloride transport in epithelial tissues. *Am. J. Physiol.*, **236,** F1–F8.

GOERG, K.J., WANITSCHKE, R. & ROTH, R. (1983). The secretagogue effect of prostacyclin on the short circuited mucosa of the rat colon. *Falk Symposium* (No. 36), *Intestinal Absorption and Secretion*, p. 19.

GORDON, J.L. & MARTIN, W. (1983). Endothelium-dependent relaxation of the pig aorta: relationship to stimulation of ^{86}Rb efflux from isolated endothelial cells. *Br. J. Pharmac.*, **79,** 531–541.

GREGER, R. & SCHLATTER, E. (1981). Presence of luminal K^+, a prerequisite for active NaCl transport in the cortical thick ascending limb of Henle's loop in rabbit kidney. *Pflüg. Arch. ges. Physiol.*, **392,** 92–94.

GRENIER, F.C., ROLLINS, T.E. & SMITH, W.L. (1981). Kinin-induced prostaglandin synthesis by renal papillary collecting tubule cells in culture. *Am. J. Physiol.*, **241,** F94–F104.

JUAN, H. (1979). Role of calcium in prostaglandin E release induced by bradykinin and the ionophore A23187. *Naunyn Schmiedebergs Arch. Pharmac.*, **307,** 177–183.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin-phenol reagent. *J. biol. Chem.*, **193,** 265–275.

MANNING, D., SNYDER, S.H., KACHUR, J.F., MILLER, R.J. & FIELD, M. (1982). Bradykinin receptor mediate Cl^- secretion in the intestine. *Nature*, **299,** 256–259.

MARTY, A. (1981). Ca-dependent K channels with large unitary conductance in chromaffin cell membranes. *Nature*, **291,** 497–500.

MARUYAMA, Y., GALLACHER, D.V. & PETERSEN, O.H. (1983). Voltage and Ca^{2+}-activated K^+ channel in baso-lateral acinar cell membranes of mammalian salivary glands. *Nature*, **302,** 827–829.

MUSCH, M.W., KACHUR, J.F., MILLER, R.J., FIELD, M. & STOFF, J.S. (1983). Bradykinin-stimulated electrolyte secretion in rabbit and guinea-pig intestine. Involvement of arachidonic acid metabolites. *J. clin. Invest.*, **71,** 1073–1083.

MUSCH, M.W., MILLER, R.J., FIELD, M. & SIEGEL, M.I. (1982). Stimulation of colonic secretion by lipoxygenase metabolites of arachidonic acid. *Science*, **217,** 1255–1256.

MUSCH, M.W., ORELLANA, S.A., KIMBERG, L.S., FIELD, M., HALM, D.R., KRASNY, E.J. & FRIZZELL, R.A. (1982). Na^+-K^+-Cl^- cotransport in the intestine of a marine teleost. *Nature*, **300,** 351–353.

REGOLI, D. & BARABE, J. (1980). Pharmacology of bradykinin and related kinins. *Pharmac. Rev.*, **32,** 1–46.

SCHRÖR, K., METZ, U. & KREBS, R. (1979). The bradykinin-induced coronary vasodilation. Evidence for an additional prostacyclin-independent mechanism. *Naunyn Schmiedebergs Arch. Pharmac.*, **307,** 213–221.

SMITH, P.L., WELSH, M.J., STOFF, J.S. & FRIZZELL, R.A. (1982). Chloride secretion by canine tracheal epithelium: I. Role of intracellular cAMP levels. *J. memb. Biol.*, **70,** 217–226.

SPRAGGS, C.F., MARGOLIUS, H.S. & CUTHBERT, A.W. (1983). Selective activity of cyclokinins. *J. Pharm. Pharmac.*, **35,** 266–268.

STONER, J., MANGANIELLO, V.C. & VAUGHAN, M. (1973). Effects of bradykinin and indomethacin on cyclic GMP and cyclic AMP in lung slices. *Proc. natn. Acad. Sci.*, U.S.A., **70,** 3830–3833.

VISO, F. & CUTHBERT, A.W. (1979). Asymmetry of attack of phospholipase C on the two sides of an epithelial membrane. *Comp. Biochem. Physiol.*, **63A,** 467–470.

WEBB, J.G., SAELENS, D.A. & HALUSHKA, P.V. (1978). Biosynthesis of prostaglandin E by rat superior cervical ganglia. *J. Neurochem.*, **31,** 13–19.

WELSH, M.J. (1983). Intracellular chloride activities in canine tracheal epithelium. *J. clin. Invest.*, **71,** 1392–1401.

WELSH, M.J., SMITH, P.L. & FRIZZELL, R.A. (1982). Chloride secretion by canine tracheal epithelium: II. The cellular electrical potential profile. *J. memb. Biol.*, **70,** 227–238.

WISE, W.C., COOK, J.A., ELLER, T. & HALUSHKA, P.V. (1980). Ibuprofen improves survival from endotoxic shock in the rat. *J. Pharmac. exp. Ther.*, **215,** 160–164.

(Received August 3, 1983.
Revised October 12, 1983.)