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The involvement of afferent nerve terminals in the stimulation of ion transport by bradykinin in rat isolated colon

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1 The actions of bradykinin (Bk) were investigated on rat colon epithelium preparations that had been stripped of the muscle layers. The electrogenic ion flux was monitored by measuring changes in the short circuit current (SCC) produced by addition of drugs. Bk, administered to the basolateral side, but not apical side, of the epithelium evoked an increase in SCC which was separable into two distinct components, both of which were mediated mainly by chloride efflux.

2 The early component was robust, reproducible and exhibited clear concentration-dependency with an EC_{50} of 6.2 nM. The second phase of the response exhibited a much slower time course than the first phase and diminished amplitude with repeated applications of Bk.

3 In preparations of unstripped epithelium, bradykinin (Bk) evoked mainly a slow neurogenic response which was attenuated or abolished by tetrodotoxin (TTX). When the epithelium was stripped off, TTX had little effect either on the baseline SCC or on responses to Bk.

4 Perfusion with zero calcium solution did not affect the early phase but abolished the late phase of the Bk response. Verapamil (20 μM), but not nifedipine (20 μM), also attenuated the later phase of the response.

5 Capsaicin (2 μM) administered to the basolateral, but not the apical, side produced an increase in SCC. Following desensitization to capsaicin the second phase of the response to Bk was abolished with little effect on the initial response to Bk.

6 The data suggest that Bk increases the efflux of chloride ions across the colonic epithelium in at least two ways: (a) by an action on the epithelial cells and (b) by an action on neuronal elements within the epithelium. This latter effect of Bk is due to stimulation of capsaicin-sensitive nerve terminals within the mucosa of the colon epithelium causing the release of a mediator which is responsible for the second phase of the response to Bk.

Introduction

Electrolyte transport in the descending colon has been studied on preparations of epithelium *in vitro* either with or without the submucosal plexuses (Andres *et al.*, 1985; Rangachari & McWade, 1986; Bridges *et al.*, 1986). As neurones within the submucosal plexus respond to a variety of neuroactive substances it is difficult in such a preparation to distinguish between indirect actions of agents acting on these neurones and direct actions on the epithelium (Keast *et al.*, 1985).

In preparations of colonic epithelium devoid of submucosal layers, kinins, including bradykinin (Bk), have been shown to produce a net secretion of chloride ions from the basolateral to apical side measured as an increase in the short circuit current (SCC) recorded under voltage clamp conditions (Cuthbert & Margolius, 1982; Manning *et al.*, 1982). While using an identical preparation of colonic epithelium to investigate the actions of Bk, we consistently observed a biphasic response of the SCC to Bk. The first phase of the response appeared to correspond to the well-documented increase in chloride transport (Cuthbert & Margolius, 1982; Manning *et al.*, 1982). We have investigated the second phase of the response to Bk and show that this may be due to an effect on capsaicin-sensitive nerve terminals within the epithelium. A preliminary account of this work has been published (Dray *et al.*, 1987).

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Methods

Male Sprague Dawley rats (200–400 g) were killed by cervical dislocation. The proximal descending colon was quickly removed and placed in Krebs-Henseleit solution (equilibrated with 95% O_2:5% CO_2) of the following composition (mM): NaCl 118, KCl 4.3, $CaCl_2$ 2.6, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 11.7.

The colon was cut along the mesenteric border, pinned onto a bed of Sylgard in a petri dish, apical surface down, and then covered with Krebs solution, at room temperature, which was continuously oxygenated throughout the dissection. The underlying muscle coats and submucosal layers containing the nerve plexuses were then carefully stripped off until all that could be seen under the dissecting microscope was the apical epithelium with presumably the lamina propria still present. A few unstripped preparations were also used to provide a comparison with the stripped epithelial preparation.

Two adjacent sections of tissue with an area of 0.6 cm^2 were clamped in perspex Ussing-type chambers and bathed on both sides by 10 ml of continuously oxygenated Krebs solution. The fluid on each side was recirculated via a peristaltic pump at a flow rate of approximately 12 ml min^{-1}, care being taken to ensure this was the same on each side. The reservoirs of fluid were heated in a thermostatically controlled water bath to give a temperature of 37°C in the chambers.

The trans-epithelial potential was held at zero, and the SCC continuously recorded with a W.P.I. dual voltage clamp (model DVC-1000) as described by Cuthbert & Margolius (1982). The electrodes consisted of physiological saline-agar bridges connected to the voltage clamp by Ag-AgCl electrodes. The current required to maintain zero potential difference after compensation for fluid resistance, the short circuit current (SCC), was then recorded on a pen recorder. In addition, at intervals of 15 s the tissue was clamped for 2 s at +2 mV in order to calculate conductance.

Drugs were added to the appropriate fluid reservoir(s) and rapidly equilibrated to the final concentration in the tissue containing chamber. Fluid on either side of the tissue could be rapidly exchanged and each tissue was washed between each drug application. In later experiments the tissue in the second chamber acted as a matched control and received the identical dose of agonist as the first chamber, but no other treatment. The procedure controlled for desensitization or 'fade' of the agonist response.

The drugs used were; bradykinin,Lys-bradykinin (kallidin) (synthesized at Sandoz), capsaicin, ethylene glycol *bis*-(2 aminoethyl)tetra acetic acid (EGTA, prepared as the sodium salt), verapamil, nifedipine, tetrodotoxin (TTX); all from Sigma.

Statistical analysis was performed by means of the *t* test with appropriate tests for normality of distribution.

Results

Stripped epithelial preparation

A response to Bk was seen only when it was applied to the basolateral side of the epithelium. This response to Bk was biphasic consisting of a rapid rise in SCC which attained maximum within 30–40 s of Bk reaching the tissue and a second slower increase in SCC taking 2–3 min to reach a peak amplitude (Figure 2). The first phase of the response, which showed little evidence of desentization if the interval between successive applications of Bk was 20 min or more (see below) reached a maximum amplitude (25 ± 1.2 μA 0.6 cm^{-2}; $n = 31$) with 100–200 nM Bk, the EC_{50} being 6.2 ± 0.77 nM (mean ± s.e.) (see Figure 1). Although the second phase of the response also showed a dose-dependency, desensitization (see below) made it difficult to obtain a dose-response curve.

When the tissue was first challenged with Bk the two phases of the response were not clearly discern-

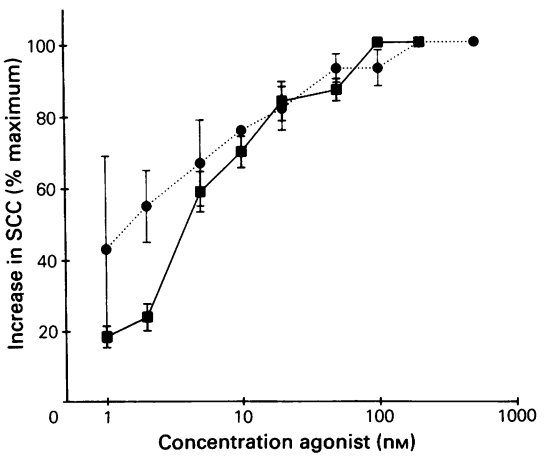

Figure 1 Concentration-response curves of the first phase of the response to bradykinin (■) and kallidin (●). The results are expressed as a percentage of the maximum response (mean values are shown with s.e. indicated by vertical lines). The value of *n* varies from 2 to 5 for kallidin and between 5 and 14 for bradykinin. The variable *n* values reflect the fact that although each individual concentration-response curve was taken to maximum, the number of lower concentrations tested varied.

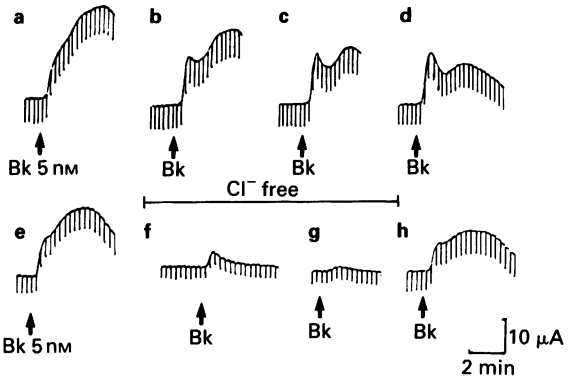

Figure 2 (a–d) Short circuit current (SCC) records showing examples of the biphasic effect of bradykinin (Bk). The early phase of the effect was consistent but the later phase showed 'fade' or desensitization. Bk (5 nM) was applied, at 20 min intervals, to the tissue at the time marked by the arrows. Note that upon first exposure to Bk the two phases of the response are not clearly separated in time. (e–f) Responses from another preparation showing chloride-dependency of the responses. Bk (5 nM) was applied as above but after the response shown in (e) the perfusate on the basolateral side of the preparation was changed to a chloride-free solution (f and g); (h) shows part recovery of the Bk response after washing in normal Krebs-Henseleit solution. The downward deflections in this figure, and all subsequent figures, are the current responses to a voltage step of +2 mV for 2 s repeated every 15 s. Calibration; 10 μM, 2 min.

ible, though careful examination of the record often revealed an inflection on the rising phase of the response near the peak of the early phase which corresponded to the onset of the late phase (Figure 2). During the first challenge with Bk, at any given concentration, the late phase of the response was invariably larger than the early phase. However, following repeated administration of Bk at intervals of 20 min at 5 nM, a concentration close to the EC_{50}, the late phase of the response became clearly distinguishable but progressively declined in amplitude until after 3–4 doses of Bk it was reduced by up to 80%. This decline was accelerated by repeated doses of Bk at higher concentrations. By contrast the early phase of the response was robust, showing little desensitization to repeated doses of Bk. Similar effects were observed with kallidin with an EC_{50} of 4.1 ± 2.6 nM (mean ± s.e.) for the first phase of the response. To reduce desensitization, drug tissue contact time was minimized by washing the tissue soon after the late phase of the response had reached a plateau. For this reason the full time course of the late phase of the Bk response was not evaluated.

In 12 preparations the increase in conductance during the first phase of the response to 5 nM Bk was measured giving a mean increase of 0.75 ± 0.08 mS cm^{-2} and this was maintained during the second phase.

To assess the effects of drugs on the phases of the response to Bk, desensitization of the second phase of the response was controlled for by running a control tissue in parallel with the test tissue (see Methods). The ratio of the peak amplitudes of the second phase of two consecutive responses (S_1 and S_2) was calculated for the control tissue. In the experimental tissue the test drug was applied for 15 min before the second response and a similar ratio calculated. These ratios were then compared statistically.

In two preparations, chloride was replaced by substituting NaCl with sodium gluconate and KCl with potassium gluconate on the basolateral side of the tissue. This had little or no effect on basal SCC as can be seen in Figure 2 just prior to the addition of the drugs. Both response phases to Bk were abolished or greatly attenuated when chloride was removed in this manner (see Figure 2).

In 3/8 preparations TTX (0.1–2 μM) applied to both basolateral and apical sides reduced the second phase by up to 25% but did not abolish this phase of the response (see Figure 3). In the other five prep-

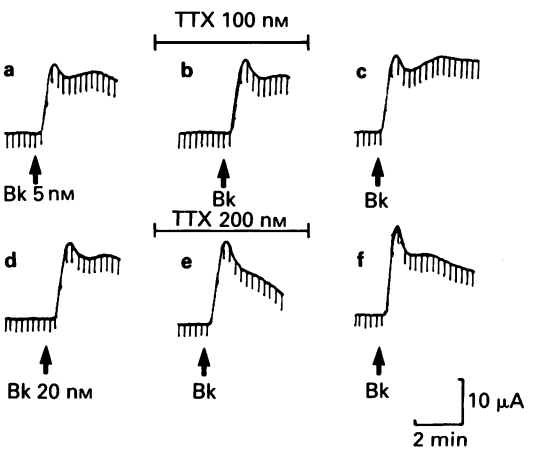

Figure 3 Biphasic response to bradykinin (Bk) showing partial antagonism of the second phase of the response by tetrodotoxin (TTX): (a–f) are sequential records from the same preparation. In (a), (b) and (c), Bk (5 nM) was applied at the point marked by the arrows; (b) was taken after the tissue had been bathed with 100 nM TTX on both apical and basolateral sides for 15 min. Responses shown in (d) and (e) were obtained later from the same tissue with a similar protocol of drug administration but the dose of Bk was 20 nM and the TTX concentration in (e) was 200 nM; (c) and (f) are Bk responses after 20 min wash with normal Krebs-Henseleit solution. Calibration; 10 μA, 2 min.

arations, TTX had no discernible effect on the second phase of the Bk response. The first phase of the response was unaffected by TTX. TTX had no effect on the baseline SCC in any of the preparations.

In 5 preparations the basolateral side was perfused with calcium-free Krebs-Henseleit solution containing 100 μM EGTA. As can be seen in Figure 4, this severely reduced the second phase of the response with little or no effect on the first component. Table 1 shows the peak amplitude response ratios obtained with matched control tissues, there being a significant reduction in the test ratios when compared to the control ratios. A small (39%) but significant reduction in the second response was seen with verapamil (up to 20 μM) and the ratios of the responses are shown in Table 1. In contrast, nifedipine at 20 μM had no significant effect on the second phase although a small (15%) but significant reduction of the first response was observed.

Capsaicin (2 μM) was added to the perfusate on the basolateral side of the tissue in three preparations. There was a monophasic increase in SCC with capsaicin (see Figure 5) and subsequent capsaicin applications (up to 10 μM) produced no further responses suggesting rapid desensitization of the effect. Only the second phase of the Bk response was significantly reduced during the after capsaicin treatment (see Figure 5c and f). The ratios of the two responses are shown in Table 1. There was little or no recovery of the second phase of the Bk response following capsaicin desensitization.

In four preparations, indomethacin (5 μM) was added to the basolateral side 15 min before Bk. Both phases of the response to 5 nM Bk were reduced by

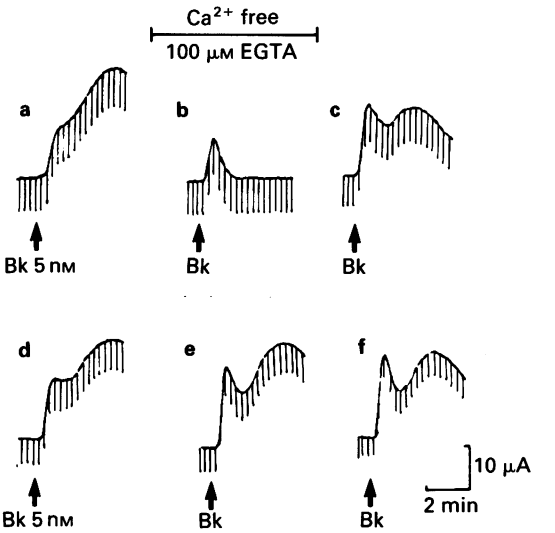

Figure 4 Biphasic responses to 5 nM bradykinin (Bk) in paired preparations illustrating the calcium-dependency of the second phase of the response: (a), (b) and (c) are records from one preparation showing the response to 5 nM Bk. In (a) the control response to 5 nM Bk is shown and (b) was obtained after the perfusate on the basolateral side was changed to Krebs-Henseleit containing zero calcium plus 100 μM EGTA for 15 min. The second phase of the response was abolished. The recovery of the second phase after a 20 min wash in normal Krebs-Henseleit solution is shown in (c); (d), (e) and (f) are records showing the responses to Bk (5 nM) in the second preparation obtained at the same time as (a), (b) and (c) respectively, but with normal calcium throughout. Calibration; 10 μA, 2 min.

Table 1 Table of ratios, S_2/S_1, of the amplitude of each phase of two consecutive responses, where S_1 is the amplitude of the first response and S_2 is the amplitude of the respective phase of the succeeding response

	Ratio of S_2/S_1 (n)	
Tissue treatment	*1st phase*	*2nd phase*
Control	1.38 ± 0.22 (5)	0.79 ± 0.1 (5)
Calcium-free + EGTA 100 μM	1.04 ± 0.07 (5)	0.07 ± 0.04 (5)**
Control	0.92 ± 0.06 (6)	0.70 ± 0.09 (6)
Verapamil 20 μM	0.85 ± 0.11 (6)	0.43 ± 0.09 (6)*
Control	0.97 ± 0.15 (5)	0.49 ± 0.08 (5)
Nifedipine 20 μM	0.83 ± 0.12 (5)*	0.58 ± 0.12 (5)
Control	1.07 ± 0.15 (3)	0.89 ± 0.04 (3)
Capsaicin 2 μM	1.05 ± 0.14 (3)	0.30 ± 0.05 (3)**

Values are mean ± s.e. mean.
In the test responses, the appropriate drug was added to the tissue after the first challenge to bradykinin and 15 min before the second challenge. The control responses are those from the untreated, matched preparation (see Results).
* $P < 0.05$; ** $P < 0.01$; Student's t test.

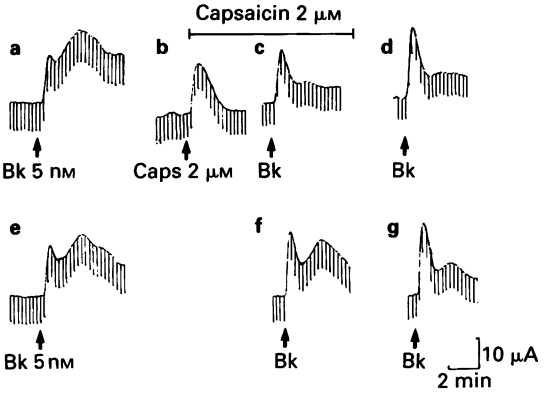

Figure 5 Records from two, paired preparations (a, b, c, d) and (e, f, g) respectively. The responses to bradykinin (Bk, 5 nM) shown in (a), (c), (d) and (e), (f), (g) were obtained at 30 min intervals. Fifteen minutes before the response in (c) was obtained capsaicin (Caps, 2 μM) was added to the perfusate on the basolateral side. This produced a monophasic increase in short circuit current; shown in (b). This response declined whilst capsaicin was maintained in the perfusate and then Bk was administered, (c). The second phase of the Bk response was almost abolished. Following this the tissue was washed for 30 min and challenged again with Bk, (d). No recovery of the second phase of the response was observed; (e), (f) and (g) are the control responses in the second preparation receiving 5 nM Bk at the same time as in (a), (c) and (d) respectively. Calibration; 10 μA, 2 min.

90% or more in the presence of indomethacin. However, maximal concentrations of Bk (100 nM) still produced a monophasic SCC response which appeared to correspond in time course to the first phase of the response to Bk described above. Increasing the indomethacin concentration to 100 μM failed to reduce further this response to 100 nM Bk.

Unstripped epithelial preparations

Five experiments were performed with the unstripped preparations of colon epithelium. Compared with the stripped preparation, the responses to Bk were slower to develop and were more prolonged (Figure 6) and it was not possible to distinguish two components of the response. TTX (100 nM) caused a decrease in baseline SCC in 3/5 preparations and attenuated or abolished the response to Bk in all cases, with a mean reduction in the Bk response of 84% (see Figure 6). The example shown in Figure 6 also shows small irregular fluctuations in baseline SCC, particularly during exposure to Bk; these were also reduced by TTX.

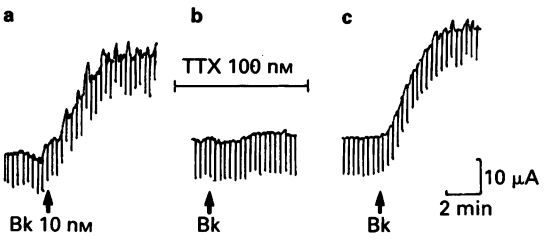

Figure 6 Records from an unstripped colon preparation showing the response to bradykinin 10 nM and the effect of tetrodotoxin (TTX, 100 nM). TTX was added to both sides of the preparation after the response shown in (a) was obtained and 15 min before (b) was obtained; (c) shows recovery of the response to Bk after a 20 min wash in normal Krebs-Henseleit solution. Calibration; 10 μA, 2 min.

Discussion

A number of kinins have been shown to produce an increase in short circuit current of the descending colon epithelium from rat and guinea-pig (Cuthbert & Margolius, 1982; Manning *et al.*, 1982; Cuthbert *et al.*, 1984a, b). This effect is predominantly due to an increase in chloride transport from the basolateral to the apical side (Cuthbert & Margolius, 1982; Manning *et al.*, 1982). However, in these papers only monophasic responses were illustrated during cumulative dose administrations of a kinin (Cuthbert & Margolius, 1982). In our hands, using identical tissue preparations and recording methods, both Bk and kallidin produced an increase in SCC but the response was clearly biphasic, with both components apparently being mediated by an increase in chloride ion transport. The early phase of the response was relatively robust but the later phase declined following repeated administrations of Bk. At present it is not clear whether the phase two Bk response was mediated by an action on different receptors and whether the decline of the second phase was due to a receptor desensitization or possibly depletion of a secondary mediator from a releasable pool.

The reduction of submaximal responses to Bk by indomethacin suggests that both phases of the response involve the production of prostaglandins. Sensitivity of the Bk SCC response to indomethacin in this preparation has been described previously (Cuthbert & Margolius, 1982; Manning *et al.*, 1982). However, in later studies, measuring the release of prostaglandins and their metabolites, Cuthbert and his colleagues concluded that eicosanoid synthesis was not essential for a response to kinins (Cuthbert *et al.*, 1984b, c).

The second phase of the response to Bk has not been described in other studies although Cuthbert *et al.* (1984a) refer in their study to a plateau phase that

occurred after the first kinin response. The significance of this observation was not discussed further. A more detailed study of a two phase response to electrical stimulation and high concentrations of 5-hydroxytryptamine was described by Keast *et al.* (1985). The conclusion was that nerve fibres of presumed submucosal origin were being stimulated. Other experiments have also shown that submucosal nerves influence ion exchange in the colon. Thus studies in dog and rat have revealed that blockade of nerve conduction with TTX produced a dramatic effect on the baseline SCC (Andres *et al.*, 1985; Rangachari & McWade, 1986) as well as inhibiting spontaneous fluctuations. Confirming this, we also observed a fall in baseline SCC in our unstripped epithelium with TTX, in most cases, though of a more modest nature than described by the above authors.

In addition, low concentrations of TTX virtually abolished the response to Bk in the unstripped, intact colon where the submucosal and myenteric plexuses would certainly be present. This would be consistent with a Bk-induced stimulation of ganglion neurones, the generation of TTX-sensitive action potentials and release of neuroactive substances. The direct stimulation of Bk of nerve terminals causing calcium-dependent release of an active substance would not be expected to be TTX-sensitive.

By contrast, TTX had no effect on the baseline SCC in the stripped epithelium which suggested that there was no TTX-sensitive background spontaneous neural activity and in turn this suggests that the associated submucosal nerve plexuses had indeed, been largely if not completely removed. In addition, the response to Bk was largely resistant to TTX, despite the fact that concentrations of TTX (100 nM–2 μM) were far in excess of those used in previous studies to block neuronal conduction in the submucosal ganglia (Andres *et al.*, 1985; Rangachari & McWade, 1986). The small reduction of the Bk response seen with TTX could be explained by a conduction block in axon collaterals following activation of their terminals by Bk. However, without histological verification there is still the possibility that some of the associated nerve plexuses such as Meissners may still be present.

These data, therefore suggest that the second phase of the response to Bk was due to activation of specific nerve terminals within the mucosa. This hypothesis was further supported by studies where calcium was removed from the perfusion solution. This practically abolished the second phase of the Bk response. In addition, verapamil also produced a significant reduction in this phase of the response. The calcium-dependency of the response strongly suggested an indirect action of Bk causing the release of an active substance(s) from nerve terminals.

The lack of effect of zero calcium upon the first response to Bk would, at first, seem to be contrary to the conclusions reached by Cuthbert *et al.* (1984a). However, in that study calcium was removed by EGTA added to the preparation during the plateau phase of a response to kinin. Our observations suggest that at the relatively high kinin concentrations used by Cuthbert *et al.* (1984a, b) the second phase of the response would be predominant and moreover, in a non-desensitized tissue the biphasic effect of Bk would not be easily discriminated. It is therefore likely that the attenuation of the kinin response in low calcium solution observed by Cuthbert *et al.* (1984a, b) was due predominantly to a reduction of a second phase of the Bk response.

Since nifedipine did not reduce the second phase of the Bk response, it is unlikely that nifedipine-sensitive voltage-dependent calcium channels are involved in this action of Bk. The reduction of the first response by nifedipine should be interpreted with caution. The time course of the second phase overlaps that of the initial response to Bk. Any non-specific reduction of each response phase would be interpreted as an apparent significant reduction in the first response.

Capsaicin was used to provide further evidence for the neurogenic component of the Bk effect. Capsaicin produces selective stimulation of afferent C-fibres which results in the release of transmitter from their terminals. In addition, prolonged application is accompanied by desensitization and loss of function of sensory nerves (cf. Buck & Burks, 1986). Capsaicin itself evoked an increase in SCC followed by a desensitization during which the second phase of the Bk response was abolished. These observations suggested that the capsaicin-evoked ion flux was due to stimulation and release of a mediator from afferent nerve terminals. When the function of these nerve terminals was lost the late phase of the Bk response was also lost. This sequence of events provides evidence that Bk was acting on afferent nerve terminals within the mucosa.

It is not likely that capsaicin is acting as a Bk antagonist because the first component of the response was not affected by capsaicin. There is also the possibility that capsaicin can affect prostaglandin formation but as indomethacin reduced both response phases while capsaicin reduced only one, this is not likely. In addition, inhibition of prostaglandin synthesis by capsaicin has only been found with much higher concentrations than 2 μM (Dewhirst 1980).

Though the susceptibility to capsaicin suggests that nerve terminals in the colon epithelium are C-fibre afferents, other evidence is more equivocal. There is extensive literature describing the nature and origin of nerve fibres within the small intestine

in the guinea-pig and rat (cf. Furness & Costa, 1980; Gershon, 1981). Morphological and immunohistochemical evidence supports the presence of fibres and terminals within the mucosa of the small intestine (Furness & Costa, 1980; Gershon, 1981). Some of the terminals lie very close to the crypts (Gaginella, 1984) but these fibres appear to be predominantly efferent. Capsaicin-sensitive afferent fibres in this region have not been well documented although circumstantial evidence supports their existence (cf. Buck & Burks, 1980). For example, following capsaicin treatment in neonatal cats and rats, degenerating fibres are found in the mucosa of the small intestine. These fibres have been identified as primary sensory afferent fibres (Hoyes *et al.*, 1981; Feher & Vajda, 1982). Furthermore, the application of Bk and capsaicin to the small intestine can initiate smooth muscle reflexes (Rybicki *et al.*, 1983) and neonatal treatment with capsaicin in rats can prevent Bk-induced inhibition of gastric motility stretch reflex (Cervero & McRitchie, 1982). There are, therefore, presumably afferent terminals sensitive to these compounds in the wall of the intestine. The localization of Bk receptors to the mucosa, particularly near the villi and crypts (Manning *et al.*, 1982) is consistent with this, although most of these receptors are probably on the epithelial cells themselves.

Capsaicin-sensitive primary afferent nerve fibres contain a number of neuropeptides including substance P, neurokinin A, neuropeptide K and calcitonin gene-related peptide (Hua *et al.*, 1986; Sternini *et al.*, 1986; Furness *et al.*, 1985). It is possible that one or more of these substances (McCulloch & Cooke, 1986) may be involved in mediating the acute effect of capsaicin and the late response to Bk. Further work is required to confirm and extend this hypothesis, but these data also suggest the intriguing possibility that primary sensory nerve fibres may have a physiological role in regulating intestinal ion exchange.

In conclusion, we have shown that the actions of Bk on the isolated epithelium of the colon is not solely an action on the epithelial cells but that there is another action of this compound upon neuronal elements within the epithelium. The evidence suggests that this, secondary, response to Bk is due to the release of a substance (or substances) from capsaicin-sensitive afferent nerve terminals lying within the mucosa.

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