THE ACTIONS OF SOME VASOACTIVE POLYPEPTIDES AND THEIR ANTAGONISTS ON THE ANOCOCCYGEUS MUSCLE

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1   The action of three polypeptides, bradykinin, substance P and eledoisin known to inhibit vascular smooth muscle has been examined on the anococcygeus muscle of the rat, cat and rabbit.

2   In the atonic rat muscle, bradykinin and substance P had little or no effect on tone but eledoisin produced a sustained dose-related contraction which could be abolished by phentolamine (1 μM) and is, therefore, probably an indirect sympathomimetic effect. On the motor response to field stimulation of adrenergic nerves, bradykinin had no effect whereas both substance P and eledoisin reduced this response. The mechanism of action was further analysed with eledoisin by examining its effect on the response to noradrenaline. Eledoisin did not alter the dose-response curve to noradrenaline and its inhibitory action is likely, therefore, to be presynaptic.

3   In the rat anococcygeus muscle in which the tone was raised by guanethidine or carbachol, bradykinin and substance P reduced this tone whereas eledoisin continued to exert a motor action. Compared with substance P the inhibitory effect of bradykinin appeared at lower concentrations (threshold 0.01 μg/ml), developed more rapidly and the size of the response was greater.

4   The effect of bradykinin on the tonically contracted cat and rabbit anococcygeus muscles was examined in addition to that of the rat. In all three species bradykinin caused inhibition and the magnitude of the response was equal to the maximum effect of inhibitory nerve stimulation. None of the peptides affected the inhibitory response to nerve stimulation itself.

5   The effects of three substances, hesperitin, khellin and apiin, reported in other tissues to antagonize the action of bradykinin were examined both on the inhibitory response to bradykinin and to field stimulation. None of them was able to inhibit either response, although they reduced tone when given by themselves. During these experiments it was found that ethanol antagonized the inhibitory response to field stimulation.

6   The possibility that bradykinin or some related peptide might play a part in the inhibitory response to nerve stimulation in the anococcygeus is discussed.

Introduction

In 1973 Gillespie & McGrath reported that stimulation within the spinal canal of the inhibitory nerves to the anococcygeus muscle in the pithed rat produced not only inhibition of that muscle but also small, consistent reductions in arterial blood pressure. This raised the possibility that the inhibitory transmitter in the anococcygeus might be a vasodilator substance. However, the rat anococcygeus muscle is not relaxed by several naturally occurring vasodilator substances such as acetylcholine, adenosine triphosphate or histamine or by exogenous substances such as isoprenaline. These compounds were either ineffective or caused motor responses in this species (Gilles-

pie & McGrath, 1974). One other group of naturally occurring substances with vasodilator properties are the vasoactive polypeptides, such as bradykinin. It has been suggested that this substance is responsible for the physiological vasodilatation which accompanies secretory activity in the salivary glands (Hilton & Lewis, 1956) and in the pancreas (Hilton & Jones, 1968). Another polypeptide, substance P, has been suggested (Bisset & Lewis, 1962) to play a similar role as vasodilator in the intestine and brain where it occurs in high concentrations (von Euler & Gaddum, 1931).

We have examined the effect of these two substances and of eledoisin, an endecapeptide related to substance P (Erspamer & Anastosi, 1962), on the anococcygeus muscle of three species, the rat, cat and rabbit. Previous work has shown that while the receptor population and response to drugs varies greatly

in these three species all have one feature in common, a motor adrenergic innervation and an inhibitory innervation whose transmitter is unknown (Gillespie, 1972; Gillespie and McGrath, 1974; Creed & Gillespie, 1976). If any of these polypeptides is involved in this neural inhibition then it should relax the muscle of all three species. In addition, we have examined the effect on the response to inhibitory nerve stimulation and to bradykinin of three drugs reported to block the motor responses to bradykinins in the guinea-pig ileum, namely khellin, hesperitin and apiin (Garcia Leme & Walaszek, 1973).

A preliminary account of these experiments has previously been published (Gillespie & McKnight, 1976).

Methods

Anococcygeus muscles from male rats (200–300 g) and rabbits (2–3 kg) of either sex were removed as previously described (Gillespie, 1972). Cat anococcygeus muscles were obtained from animals primarily used for experiments by other workers on the isolated perfused spleen. For their purposes the cats had previously been anaesthetized with a mixture of halothane and N_2O in O_2 and given (intravenously) 70 μg of prostaglandin E_1 and 20,000 iu of heparin. The anococcygeus muscles were removed as described by Gillespie & McGrath (1974).

Preparations from all three species were drawn through bipolar Ag/AgCl electrodes similar to those described by Burn & Rand (1960) and set up in 10 ml organ baths containing Krebs-Ringer at 37°C through which 5% CO_2 in O_2 was bubbled. Tension was measured with Grass FTO3C isometric transducers and displayed on a Grass Polygraph. Field stimulation of intramural nerves was carried out with 1 ms pulses of supramaximal voltage delivered at the frequencies indicated in the text, either for a fixed time period (10 s) or with a fixed number of pulses (100).

Drugs used were apiin (ICN Pharmaceuticals Inc.), bradykinin triacetate (Sigma), carbachol (carbomyl choline chloride, Sigma), eledoisin trifluoroacetate (Sigma), guanethidine sulphate (CIBA), hesperitin (Sigma), histamine acid phosphate (BDH), khellin (Sigma), (+)-lysergic acid diethylamide (LSD, Sandoz), (−)-noradrenaline bitartrate (Koch-Light), phentolamine mesylate (CIBA). Substance P was generously supplied by Dr S. Leeman, Harvard Medical School, Boston, Mass. With the exception of the peptides, all doses refer to the base. Concentrations of substance P are given as $\mu g/ml$ and for comparison the other peptides have been similarly expressed. Since the molecular weight of bradykinin and eledoisin is close to 1000 these concentrations are approximately molar.

Results

Effects on the atonic rat anococcygeus muscle

In vitro, the rat anococcygeus lacks both tone and rhythmic activity and responds to field stimulation with contraction. Bradykinin (0.01 to 100 $\mu g/ml$) had either no effect on its tone or sometimes produced a small transient rise in tone. The motor response to field stimulation was virtually unaltered (Figure 1). With substance P (0.01 to 10 $\mu g/ml$), no increase in tone was ever seen but at concentrations higher than 1 $\mu g/ml$ a reduction in the motor response to field stimulation was observed at all frequencies used (Figure 1). Eledoisin (0.01 to 100 $\mu g/ml$) consistently produced dose-related contractures slow to develop but well-maintained. Against this background of increased tone it was to be expected that the amplitude of the motor responses would be reduced but the reduction observed was more than could be accounted for simply by the rising baseline and the absolute tension achieved at each frequency was reduced (Figure 1). The rat anococcygeus is particularly sensitive to the action of indirect sympathomimetics and it was possible that this was the mechanism of the motor effects of eledoisin. This was tested by examining the effect of phentolamine on the motor response. As Figure 2 shows, the addition of phentolamine 1 μM to the bath abolished entirely the motor response to eledoisin suggesting it was indeed an indirect sympathomimetic effect.

The ability of these three peptides to reduce the motor response to field stimulation varied. Bradykinin had little effect whereas substance P, and particularly eledoisin, had a considerable effect. The site of the inhibition of the motor response, whether pre-or post-junctional, was investigated by comparing the effect on the response to adrenergic nerve stimulation with the effect on the response to noradrenaline. Only bradykinin and eledoisin were used because of limitations in the quantity of substance P available. The results for the effect on the response to noradrenaline are shown in Figure 3. Bradykinin 1 $\mu g/ml$ produced a small but significant depression of the response at two points on the dose-response curve and eledoisin 1 $\mu g/ml$ a small elevation at one point but the central straight section of the curves were unaltered. There was no significant change in the maximum response to noradrenaline. This dose of eledoisin nevertheless consistently reduced the motor response to field stimulation of the adrenergic nerves. Since eledoisin had the greatest inhibitory effect on the motor response to field stimulation and consistently reduced it at the concentrations used in these experiments on the dose-response curve, it seems unlikely that the effect on the nerve response is postsynaptic.
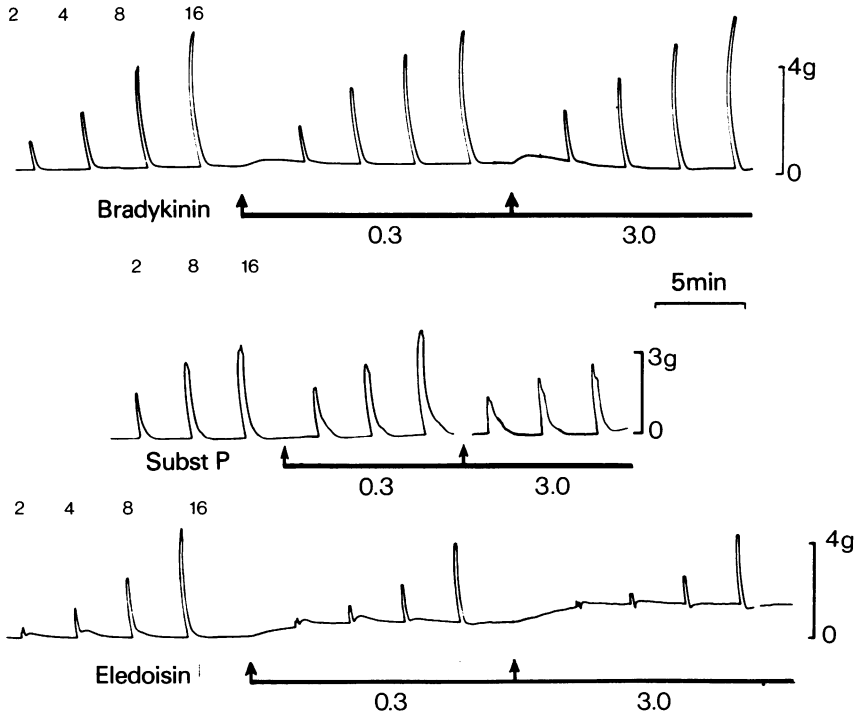
Figure 1 The effect of bradykinin, substance P and eledoisin on the rat anococcygeus muscle and its motor response to field stimulation. The muscle was stimulated for 10 s periods at the frequency shown above the first set of responses in each trace. Bradykinin, substance P and eledoisin were added at the arrows; the doses are given as μg/ml. Bradykinin and substance P had little or no effect on tone, whereas eledoisin produced a slow but maintained contraction. Bradykinin had no effect on the motor response to field stimulation, but both substance P and eledoisin reduced this response.

Effects on tonically contracted muscles

Unlike that of the rat, the anococcygeus muscles of the cat and rabbit usually possess intrinsic tone and
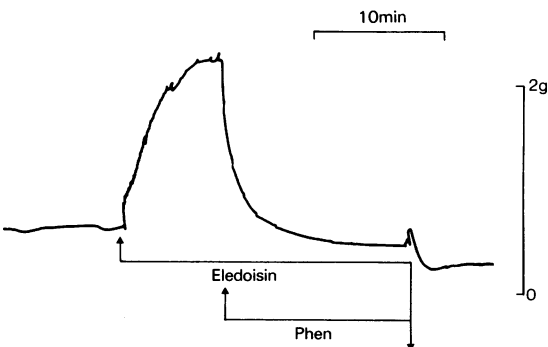

Figure 2 The motor response of the rat anococcygeus to eledoisin 10 μg/ml was abolished by phentolamine (Phen) 1 μM.

rhythmic activity. This, however, is too inconstant to serve as a baseline for the examination of a drug or nerve action and tone was, therefore, induced in the rat either with guanethidine 30 μM or carbachol 10 μM, in the rabbit with histamine 1 μM and in the cat with LSD 10 μM. Where tone was induced with carbachol or histamine, phentolamine 1 μM was also added to block any indirect sympathomimetic effects of the peptides. This was not possible with guanethidine or LSD as it is the indirect sympathomimetic action of these drugs which is responsible for the raised tone. In the rat no difference was observed between the results with guanethidine and carbachol and in any event only eledoisin possessed a significant indirect sympathomimetic action.

In the guanethidine-treated rat anococcygeus muscle none of the three polypeptides had any effect on the inhibitory response to field stimulation although all three produced dose-related changes in tone (Figure 4). Eledoisin produced a slow and well-maintained additional contracture but both bradykinin and substance P produced inhibition with a
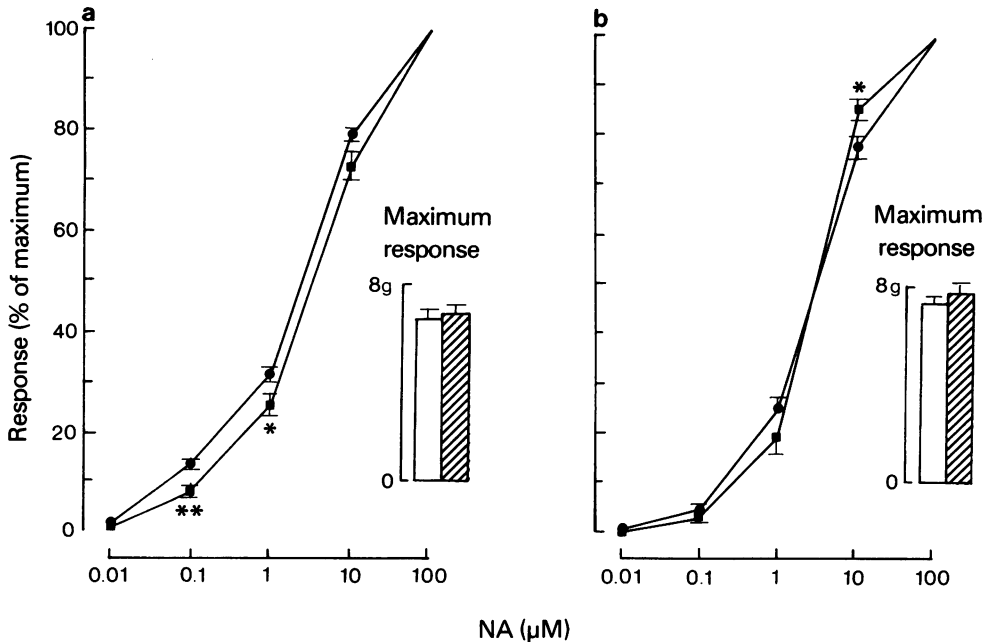
Figure 3 The effect of bradykinin or eledoisin 1 µg/ml on the dose-response curve and maximum response to noradrenaline (NA): (●) are control responses in the absence of peptides and (■) the responses in the presence of bradykinin (a) or eledoisin (b). The difference between control and test responses at each concentration of noradrenaline was analysed by Students' paired t test and statistical differences are indicated by asterisks: $*P < 0.05$; $**P < 0.01$. The insert histograms show the means of the mean of the maximum responses, open columns control, hatched columns in the presence of bradykinin or eledoisin. Vertical lines show s.e. mean. The number of experiments in each test group was 4 and in the controls 8.

threshold for bradykinin of about 0.01 µg/ml and for substance P of 3 µg/ml. The inhibitory responses obtained with substance P were small and slow to develop in comparison with bradykinin (Figure 4). Bradykinin seemed the more interesting compound and further experiments were, therefore, carried out to see whether it would inhibit the tonically contracted muscle from all three species and whether the magnitude and time course of the maximum response obtained was comparable to the inhibitory response to field stimulation. An example of the results is shown in Figure 5. In all three species bradykinin consistently produced dose-related inhibitions comparable in magnitude to the responses to inhibitory nerve stimulation. The dose-response curves for each species are shown in Figure 6 from which it can be seen that a greater percentage inhibition of tone was obtained in the cat than in the rabbit, which in turn was greater than the rat. This roughly mirrors the relative effectiveness of inhibitory nerve stimulation in the three species.

The effects of bradykinin antagonists

In our first experiments hesperitin and khellin were used, dissolved in ethanol and diluted with 0.9% w/v NaCl solution (saline). Such solutions gave a maximum alcohol concentration in the organ bath of 1% at a drug concentration of 100 µg/ml. In this solvent both hesperitin and khellin in concentrations of 10 µg/ml reduced guanethidine-induced tone in the rat anococcygeus and reduced the response to inhibitory nerve stimulation (Figure 7). Increasing the concentration to 100 µg/ml increased the block of the inhibitory nerve response and at the same time caused contraction of the muscle. Unfortunately both the block of the inhibitory response to field stimulation and the contraction were reproduced when the appropriate controls with alcohol alone were carried out and are, therefore, an effect of ethanol. Both hesperitin and khellin were found to be almost ten times more soluble in acetone than in alcohol so that at the maximum drug concentration tested of 100 µg/ml the bath
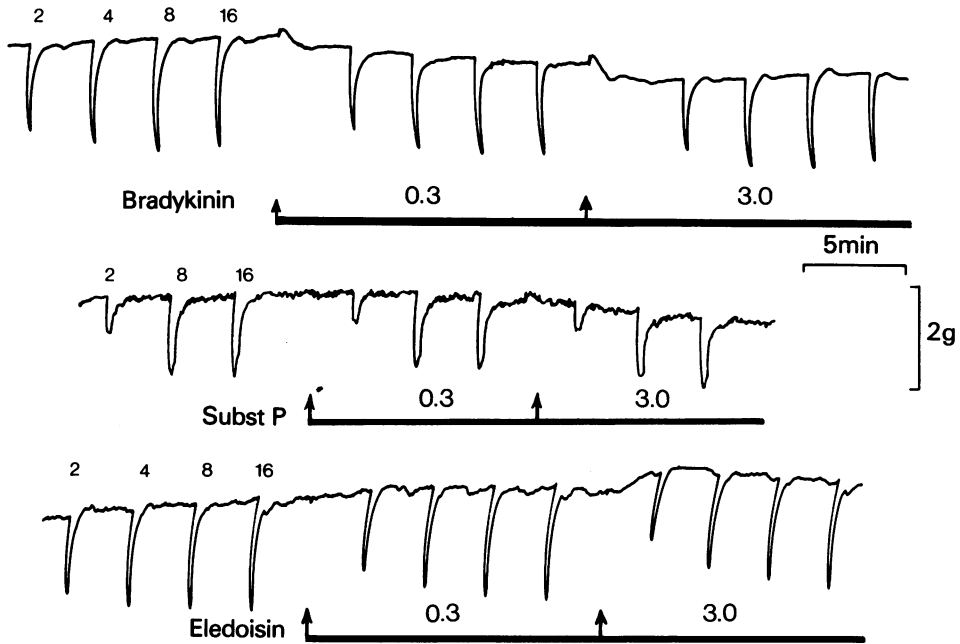
Figure 4 The effect of bradykinin, substance P and eledoisin in the concentrations shown (µg/ml) on the guanethidine-induced tone and inhibitory response to field stimulation of the rat anococcygeus muscle. The frequencies of stimulation are shown above the first set of responses in each record. Bradykinin and substance P inhibited tone, whereas eledoisin caused a further increase in tone.
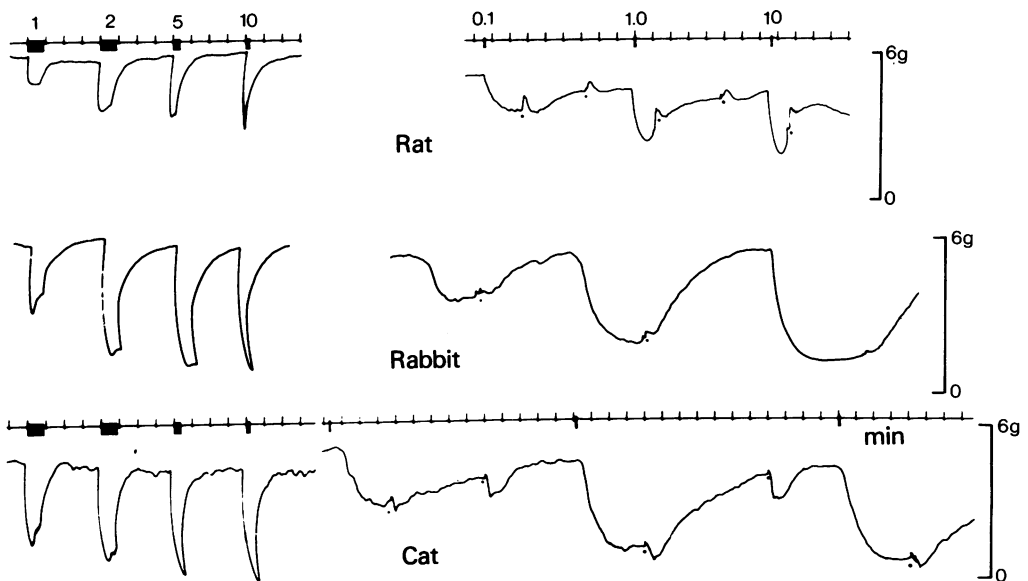
Figure 5 A comparison of the inhibitory effect of bradykinin in the concentration (µg/ml) shown with the inhibitory response to field stimulation in the rat, rabbit and cat anococcygeus. In these experiments the muscles were stimulated with a fixed number of 100 pulses at the frequencies shown. In each experiment bradykinin caused inhibition and in each at 10 µg/ml the inhibitory effect was comparable in magnitude with the maximum response to inhibitory nerve stimulation.

concentration of acetone was only 0.1% which controls showed to have no effect on the muscle responses. The experiments were, therefore, repeated with hesperitin, khellin and also apiin dissolved in acetone and diluted in saline. The effects of all three were qualitatively similar and attention was concentrated on hesperetin.

Hesperitin, 1 to 100 μg/ml, produced dose-related falls in tone in both rat and rabbit anococcygeus muscles. At 100 μg/ml tone was virtually abolished so that when the effects of hesperetin on the inhibitory responses to field stimulation and bradykinin were examined a dose of 10 μg/ml which did not completely abolish tone was used. The results of such an experiment are illustrated in Figure 8. Hesperitin in this dose produced approximately 20% inhibition of histamine-induced tone in the rabbit muscle but without reducing the response to inhibitory nerve stimulation which indeed was potentiated at low frequencies of stimulation. There was no corresponding consistent potentiation of the response to bradykinin but equally no evidence that the inhibitory effects were reduced.
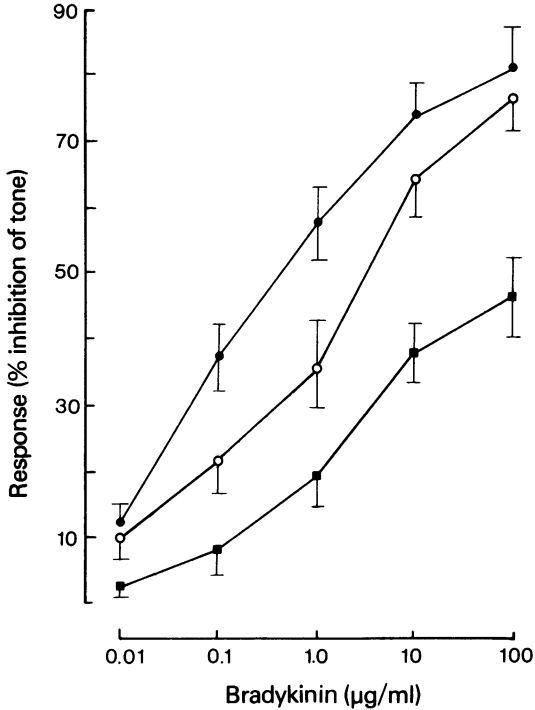
Figure 6 Dose-response curves for the inhibitory effect of bradykinin on guanethidine-induced tone in the rat, rabbit and cat anococcygeus muscle. Mean values are given; vertical lines show s.e. mean; n = 4 for cat ($\bullet$); 8 for rabbit ($\bigcirc$) and 6 for rat ($\blacksquare$).

Discussion

These experiments demonstrate that some vasoactive polypeptides can lower the tone of the anococcygeus muscle and that bradykinin can do so in the muscle from three different species which otherwise differ greatly in their receptor sensitivity, Bradykinin is the most interesting, not only for this reason, but also because it was effective in low concentrations, pro-
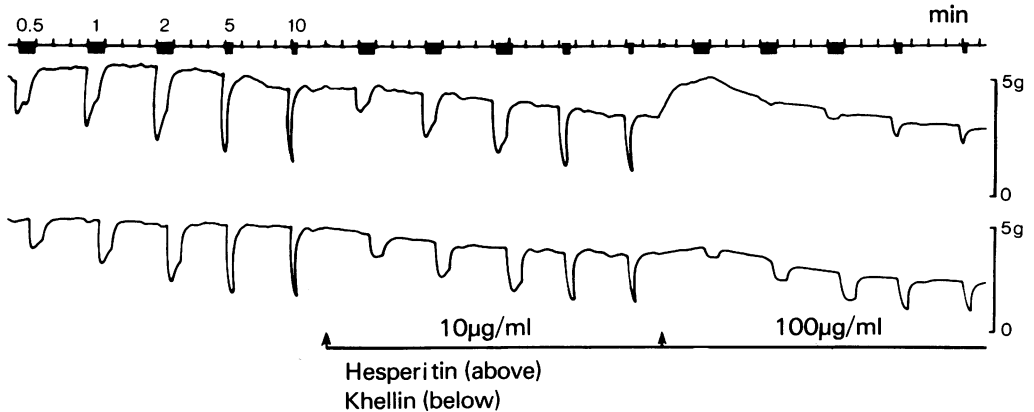

Hesperitin (above)
Khellin (below)

Figure 7 The effect of the bradykinin antagonists, hesperitin and khellin dissolved in ethanol, on the tone and inhibitory response to field stimulation of two preparations of rat anococcygeus muscles from the same rat. Tone had previously been raised by the addition of guanethidine 30 μM. Both hesperitin and khellin reduced the response to field stimulation and at the higher concentration of 100 μg/ml caused a rise in tone.
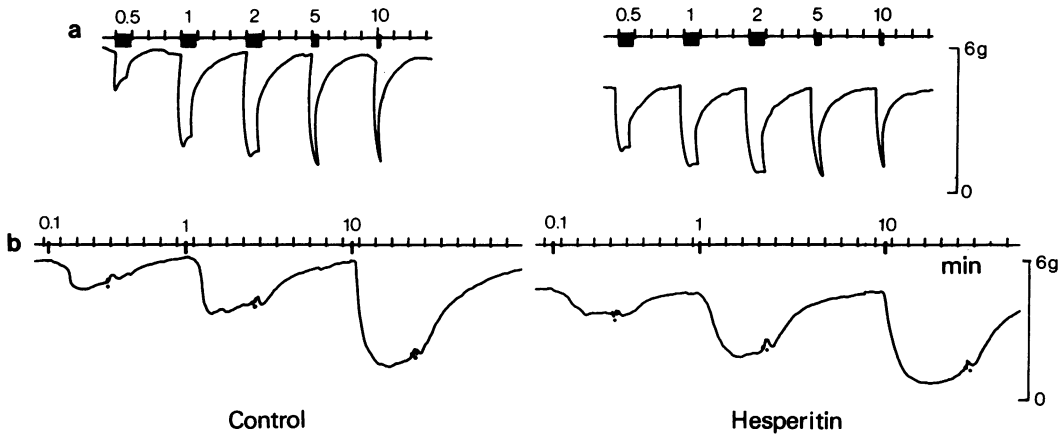
Figure 8 The effect of hesperitin 10 μg/ml on the inhibitory response of the rat anococcygeus to field stimulation (a) and to bradykinin (b) in the concentrations (μg/ml) shown. In this experiment the muscle was stimulated with a fixed number of 100 pulses at the frequencies shown. Hesperitin lowered tone but potentiated the inhibitory response to field stimulation at low frequencies. there was little or no effect on the response to bradykinin. Tone in this experiment was raised with guanethidine 30 μM.

duced inhibition with a time course not unlike that of inhibitory nerve stimulation and the maximum response was comparable to that produced by nerve stimulation. Substance P though inhibitory in its action was less effective, slower in action and with a smaller maximum effect. In contrast to these two polypeptides, eledoisin, though reputed to have similar actions to substance P (Sicuteri, Fanciuletti, Franchi & Michelacci, 1967), consistently produced a rise in tone. This would appear to be an indirect sympathomimetic action since it was completely abolished by a low dose of phentolamine. Eledoisin also reduced the response to the motor effect caused by adrenergic nerve stimulation by a presynaptic action. It has previously been reported that other drugs such as LSD, which exert an indirect sympathomimetic action, also act as guanethidine-like neurone blockers in this tissue (Gillespie & McGrath, 1975) and eledoisin may be one other such example.

If any one of these polypeptides is involved in the inhibitory response to nerve stimulation, then bradykinin or a substance related to it would seem the likeliest candidate. The source of the bradykinin is, however, difficult to explain. In various tissues bradykinin has been postulated as a physiological mediator of neuronal vasodilatation; the salivary glands and pancreas have already been mentioned. Closer to the present anatomical site, Hultén (1969) has proposed that a kinin similar to bradykinin is responsible for the atropine-resistant vasodilatation in the colon in response to pelvic nerve stimulation. For the salivary gland and pancreas it was suggested that parasympathetic stimulation releases from the effector cell an enzyme (a kininogenase) which acts on a substrate

(kininogen) derived from the plasma but present in the extracellular fluid, to produce a kinin which is an active vasodilator. Hultén's original suggestion was in agreement with this theory: since it concentrated on the mucosal vasodilatation and attributed this to the release by pelvic nerve stimulation of an enzyme from the secretory cells of the mucosa which acted on a plasma protein to produce the vasodilating kinin. However, in recent papers the suggested intervention of a kinin has been extended to the colon contraction produced by pelvic nerve stimulation (Fasth & Hultén, 1973) and the receptive relaxation of the stomach produced by vagal stimulation (Fasth, Hultén, Jahnberg & Martinson, 1975) though the mechanism of kinin formation is not detailed.

A similar, indeed greater, problem exists in the anococcygeus muscle which consists almost entirely of smooth muscle cells with a little connective tissue and which was suspended in saline and not perfused with blood. There is, therefore, neither a suitable effector cell to serve as a source of enzyme nor a plasma protein to provide a substrate for such an enzyme yet the inhibitory response continues for hours undiminished. It may be that some fixed protein of the basement membrane, a prominent feature of smooth muscle cells in this tissue (Gillespie & Lüllmann-Rauch, 1974), may act as substrate, activated by some change in the environment produced by nerve stimulation. Our experiments have shown that of the various potential inhibitory transmitters we have investigated on this tissue, bradykinin was the only one which was able to cause inhibition in all three species so that it or a polypeptide related to it may in some way be involved in these responses.

The experiments with the bradykinin antagonists were disappointing in so far as they not only failed to inhibit the inhibitory response to field stimulation but also the inhibitory response to bradykinin itself. It may be that the bradykinin receptor in the anococcygeus differs from that in the guinea-pig ileum or just possibly the bradykinin, in producing inhibition in the anococcygeus muscle, does so by releasing inhibitory transmitter from the inhibitory nerves. This latter possibility was not tested in the present experiments. If the inhibitory effect of bradykinin is by a direct action on kinin receptors on the effector cells then the observation that these antagonists are also inhibitory may imply that in this tissue they act as partial agonists.

The observation that ethanol produced a dose-related reduction in the response of the anococcygeus muscle to inhibitory nerve stimulation is under further study. Preliminary experiments suggests the effect is specific to this response and does not extend to other inhibitory stimuli.

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