The effect of an inhibitory factor from the bovine retractor penis on the gastro-intestinal tract and gall bladder of the guinea-pig

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1 The effect of a smooth muscle inhibitory factor extracted from the bovine retractor penis has been examined on a variety of *in vitro* smooth muscle preparations from the guinea-pig alimentary canal and on the guinea-pig gall bladder.

2 The inhibitory factor caused relaxation of spontaneous and carbachol-induced tone in the taenia coli, the stomach fundal strip and the duodenum and colon. There was little effect on the ileum. Sensitivity was highest in the taenia coli where the response to the inhibitory factor mimicked the response to stimulation of the non-adrenergic, non-cholinergic (NANC) inhibitory nerves.

3 In the taenia coli the inhibitory response to stimulation of the NANC nerves and to ATP was abolished by apamin 5×10^{-8} M, whereas this or higher concentrations had no effect on the response to the inhibitory factor. This makes it unlikely that the latter is the neurotransmitter in these NANC nerves.

4 The inhibitory factor had no effect on the gall bladder. Inhibitory responses to field stimulation were obtained in this tissue but these were insensitive to tetrodotoxin in concentrations greater than those needed to block the motor cholinergic nerves.

Introduction

The rat anococcygeus and the bovine retractor penis (BRP) muscles possess a dense adrenergic motor innervation and an inhibitory non-adrenergic non-cholinergic (NANC) innervation, the mediator of which is unidentified. The response to inhibitory nerve stimulation in these tissues is not mimicked by adenosine 5′-triphosphate (ATP) (Gillespie, 1972; Klinge & Sjöstrand, 1977) but is by an inhibitory extract from the BRP or the rat anococcygeus (Ambache, Killick & Zar, 1975; Gillespie & Martin, 1980; Gillespie, Hunter & Martin, 1981). The guinea-pig taenia coli also possesses an NANC inhibitory innervation, and in this tissue ATP does mimic the effects of inhibitory nerve stimulation and has been postulated to be the neurotransmitter (Burnstock, 1972; 1979). Since the anococcygeus is related to the gastro-intestinal tract the transmitter in the NANC nerves in the two tissues may be the same. If so, the inhibitory extract from the BRP should relax the guinea-pig taenia coli and other regions of the gut which share this innervation. An inhibitory

NANC innervation has also been described in the guinea-pig gall bladder (Davison, Al-Hasani, Crowe & Burnstock, 1978) and we have investigated the effects of the inhibitory extract on this tissue also. The bee venom, apamin, is known to block the inhibitory response to nerve stimulation and to ATP in the guinea-pig taenia coli and we have examined its effect on the response of this tissue to inhibitory extract. Some of these results have already been reported to the Physiological and Pharmacological Societies (Crossley & Gillespie, 1980; Bowman & Gillespie, 1981).

Methods

Bovine retractor penis muscles from the abattoir were minced, extracted with methanol, and partially purified by anion exchange followed by elution with 500 mM sodium chloride solution and lyophilisation (Bowman, Gillespie & Martin, 1979). Before assay the powdered extract was reconstituted in distilled water and activated by acidifying to pH 2.0 with 1 M HCl. After a period of 10 min the activated ex-

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tract was neutralized with 1 M NaOH to a pH of 6.8. This activated extract was then diluted with distilled water to a concentration equivalent to 1 g wet weight per ml. Since the active material in these extracts is unstable the solutions were kept on ice. These extracts contained adenine nucleotides. In routine assays on the BRP, which is insensitive to ATP, this was of no consequence but in tissues such as the guinea-pig taenia coli, which were readily inhibited by ATP and ADP, it was necessary to remove these compounds. This was done by passing them at pH 9 through an alumina column. Adenine nucleotides were largely retained and the inhibitory factor in the extracts mostly passed through the column (Bowman et al., 1979). The removal of adenine nucleotides was checked by spectrophotometric measurements at 260 nM on a Unicam SP8000 spectrophotometer.

Adult guinea-pigs of either sex weighing 350–500 g were stunned and bled. Two to 3 cm lengths of duodenum, ileum (10 cm from the ileo-caecal junction) and pelvic colon were mounted as Magnus preparations in 10 ml organ baths. Similar lengths of taenia coli were mounted as strip preparations in 2 or 10 ml organ baths. The fundus of the stomach was converted into a strip preparation after the method of Vane (1957) and the gall bladder was treated in a similar fashion, after being first converted into a flat sheet by two diametrically opposed cuts, one on each side from its opening up to the fundus. The resulting sheet was then incompletely divided along its midline and pulled out into a single strip. Stomach and gall bladder strips were mounted in 2 and 10 ml organ baths respectively. All preparations were arranged for tension recording by means of a Grass FT03 isometric transducer and displayed on either a Grass Polygraph or a Linseis two-channel recorder. The preparations were initially stretched to produce a resting tension of between 0.5 and 1.0 g. In some instances the preparations were threaded through ring electrodes for field stimulation of their intramural nerves with square wave pulses of 0.5 ms duration, supramaximal voltage and at the frequencies given in the text. One other preparation, the whole gall bladder arranged for intraluminal pressure recording and field stimulation as described by Davison, et al. (1978), was used mounted in a 100 ml organ bath. The bathing solution for all preparations, other than the whole gall bladder, was Krebs physiological saline of the following composition (mM): NaCl 118, KCl 5.0, $CaCl_2$ 2.4, $MgSO_4$ 1.1, $NaHCO_3$ 25, KH_2PO_4 1.2, and dextrose 11. Tyrode solution was used for the whole gall bladder and had the following composition (mM): NaCl 137, KCl 2.7, $CaCl_2$ 2.2, $MgSO_4$ 0.1, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, and dextrose 5.5. The bath temperature was maintained at 37°C and bubbled with 95% O_2 plus 5% CO_2.

Drugs or extracts were added to the baths in vol-umes not exceeding 0.4 ml for the 2 and 10 ml bath and 1.0 ml for he 100 ml bath. Where necessary tone was induced with carbachol. Since the concentration of carbachol was found to affect sensitivity to inhibitory extract, the minimum concentration needed to induce a stable plateau of submaximal tension was used. The following drugs were used and concentrations, with the exception of tetrodotoxin and caerulin, refer to the base: adenosine 5'-triphosphate (Sigma); atropine sulphate (BDH); barium chloride (BDH); carbachol (BDH); guanethidine sulphate (Ciba); lignocaine hydrochloride (Astra); tetrodotoxin (Sigma); apamin (Serva); Caerulin (Sigma).
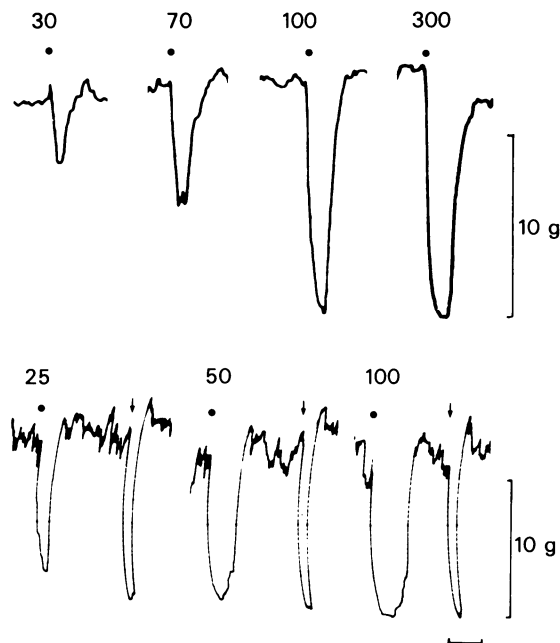

Figure 1 The effect of increasing doses of inhibitory extract in the guinea-pig taenia coli. In the upper records tone has been induced with carbachol 3×10^{-8} M. In the lower records the tone is spontaneous and atropine 2×10^{-7} M and guanethidine 2×10^{-6} M have been added to uncover the inhibitory response to transmural stimulation of the non-adrenergic, non-cholinergic nerves. The figures above the traces are the volumes in μl of extract added to the bath. In the lower records transmural stimulation at 4 Hz was applied at the arrows. The inhibitory factor produces a dose-related relaxation of both carbachol-induced and spontaneous tone and this response mimics that to stimulation of the inhibitory nerves. The preparation was washed at the break in the trace after each addition of extract. Time marker 2 min.

Results

Since the inhibitory extract used in these experiments was impure it was important to know whether any effect observed was due to the same inhibitory factor we assayed on the BRP or to some other component of the powder. An obvious example is the presence of ATP. We tried to control this problem first by removing known contaminants such as ATP and, secondly, by examining the effect of acid activation and of boiling. Only those effects which were not present before acid activation and which disappeared after boiling were considered as due to the same material that relaxed the BRP.

Effects of inhibitory extract on the taenia coli

After removing ATP the inhibitory extracts still caused dose-related relaxation of both spontaneous and carbachol-induced tone in the guinea-pig taenia coli (Figure 1). This effect of the extract was seen only after activation by acid and was largely, though not completely, abolished by placing it for 2 min in a boiling water bath. Spontaneous activity in the taenia usually consisted of large rhythmic contractions superimposed on a relatively low level of maintained tone. This was unsuited to the measurement of inhibitory activity. Maintained tone could be induced by carbachol and this drug was routinely used. However, the concentration of carbachol influenced the magnitude of the inhibitory response as Figure 2b illustrates. An increase in carbachol concentration from 3×10^{-8} to 10^{-7} M caused a four to five fold shift to the right in the dose-response curve. For this reason the minimum concentration of carbachol necessary to sustain tone, usually 3×10^{-8} M, was used in all other experiments. Under these circumstances the sensitivity of the taenia to the inhibitory factor in these extracts was similar to that of the BRP with spontaneous tone (Figure 2a).
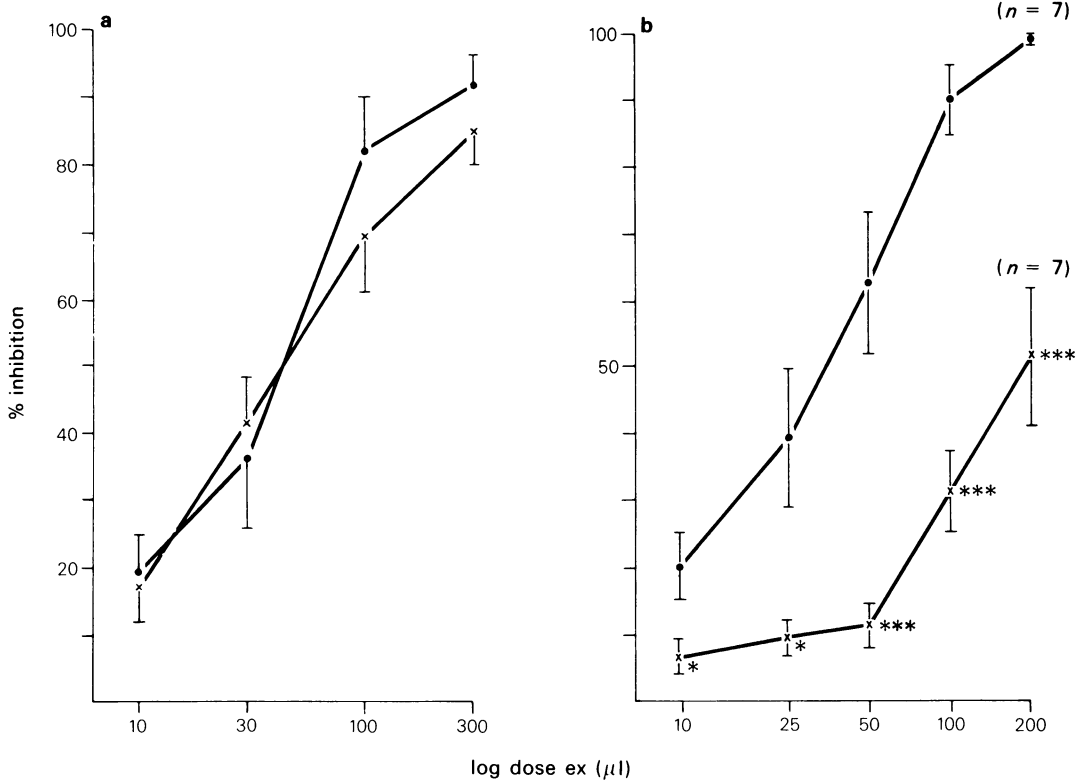

Figure 2   Dose-response curves for relaxation by the inhibitory factor in the bovine retractor penis (●) of and the guinea-pig taenia coli (x). Inhibition was measured against spontaneous tone in the retractor penis and against tone induced by carbachol for the taenia: the concentration of carbachol in (a) was 3×10^{-8} M and in (b), 3×10^{-7} M. With the lower concentration of carbachol the sensitivity of the taenia is similar to that of the retractor penis, but a 10 fold increase in carbachol concentration significantly depresses sensitivity. In (b) the comparison between responses at each dose of extract is by Student's *t* test and asterisks indicate the degree of significance of the difference. The number of observations in (a) was 6.
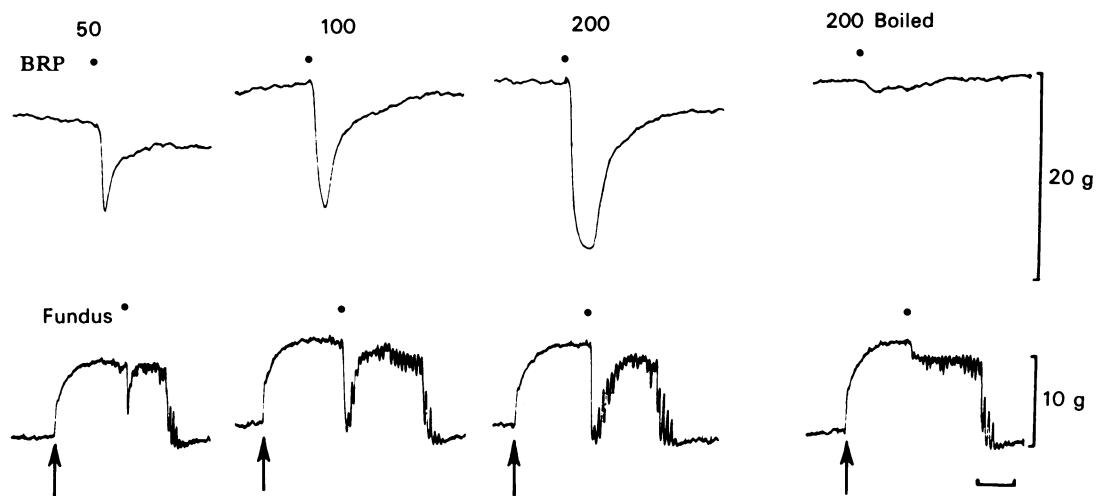
Figure 3   A simultaneous comparison of the effect of inhibitory factor on the bovine retractor penis (BRP) and the guinea-pig fundal srip. Tone in the BRP is spontaneous and in the fundal strip is induced by carbachol 10^{-7} M added at the arrows. The doses of inhibitory factor in μl added to the bath are shown above each response. The inhibitory factor produces a graded relaxation in both preparations and activity is abolished by a 2 min exposure to a boiling water bath. Time 2 min.
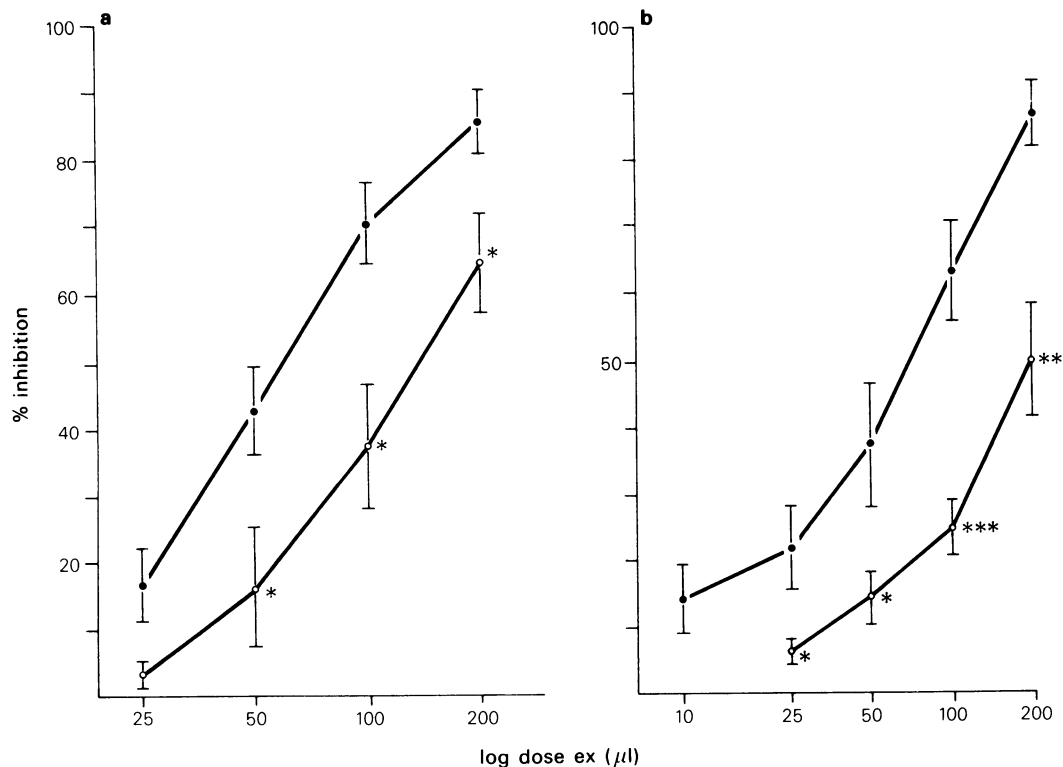
Figure 4   Dose-response curves for relaxation by the inhibitory factor in the bovine retractor penis (●) and the guinea-pig fundal stomach strip (O), Tone in the retractor penis was induced by carbachol 10^{-7}M in (a) and 3×10^{-7}M in (b). The number of experiments is 8 throughout. Comparisons are by Student's t test. The stomach is less sensitive to inhibition than the retractor penis with the low concentration of carbachol, and increasing the carbachol concentration depresses sensitivity further.

Effect of inhibitory extract on the stomach fundic strip

In this preparation carbachol-induced tone was also inhibited by the extract in a dose-related fashion as Figure 3 illustrates. This tissue was less sensitive to carbachol and for this reason 3×10^{-7} M was used to induce tone. Partly, perhaps, for this reason the sensitivity of the fundic strip to the extract was less than that of the BRP (Figure 4a) and just as in the taenia coli increasing the concentration of carbachol caused a shift to the right in the dose-response curve (Figure 4b).

Effect of extract on other intestinal preparations

The guinea-pig duodenum and colon were less sensitive to the inhibitory factor than either the taenia coli or fundic strip, but doses of extract of 100 µl and 200 µl produced inhibition of carbachol-induced tone in both; this effect required acid activation before it could be observed and was largely abolished by boiling. Examples of such inhibitory effects are illustrated in Figure 5.

The mid-ileum was found to be the least sensitive of all the tissues examined. In six out of the seven preparations examined the extract was without effect, though the same extracts assayed simultaneously on the BRP produced powerful inhibition at these doses. In the seventh preparation, illustrated in Figure 5, 200 µl of extract produced approximately 40% inhibition of tone. These experiments suggest a low sensitivity in this tissue. The use of semi-purified extracts limits the quantity which can be added to the bath before non-specific effects appear and, therefore, limits the dose-range that can be examined.
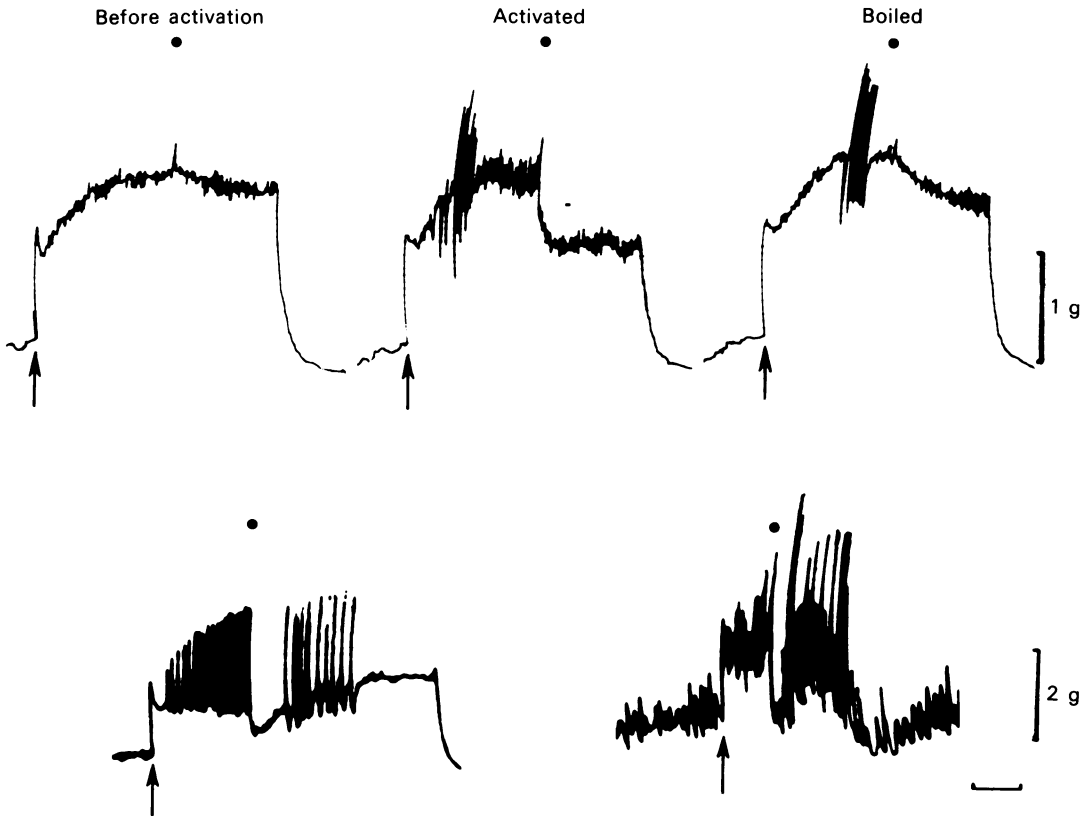

Figure 5   The upper three records show the response of the guinea-pig ileum to 200 µl of inhibitory extract added to the bath. Tone was raised by adding carbachol 3×10^{-8} M to the bath at the arrow. Before acid activation the extract is almost without effect, after acid activation the same volume produces about 40% loss of tone and this inhibitory activity is reduced after 2 min in a bathing water bath. This preparation was the only one of seven to respond to the extract. The two lower records show on the left the response of the duodenum and on the right the colon of the guinea-pig to the addition of 200 µl of inhibitory extract. In both preparations tone and rhythmic activity have been induced by adding 3×10^{-8} M carbachol to the bath. In both the inhibitory extract causes relaxation. Time 2 min.
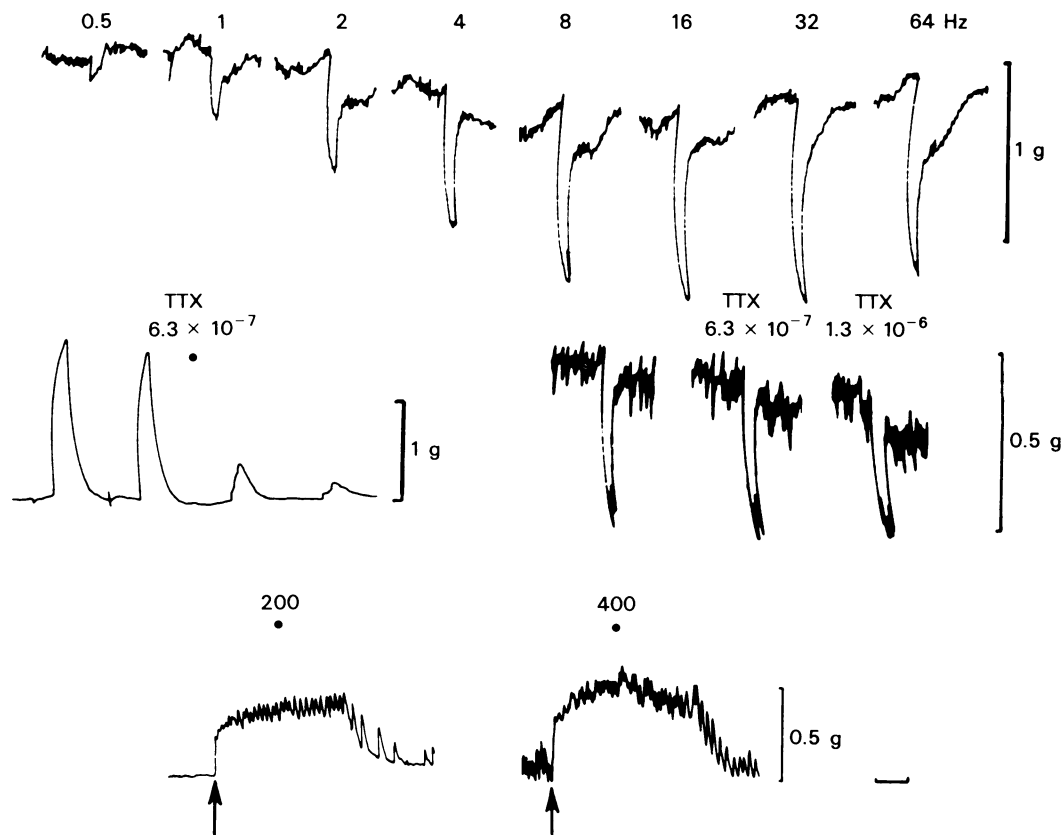
Figure 6 The response of the guinea-pig gall bladder strip to field stimulation and to inhibitory factor. The upper records show the effect of field stimulation for 20 s at the frequencies in Hz shown above each response. Atropine 10^{-6} M was present to block the motor response to cholinergic nerve stimulation and tone had been raised by the addition of histamine 10^{-6} M to the bath. Field stimulation produces rapidly developing relaxation which disappear on cessation of stimulation and which is frequency sensitive with an optimum of between 16 and 32 Hz. The middle records show on the left the motor response to field stimulation of cholinergic nerves in the absence of atropine. These responses are abolished by tetrodotoxin (TTX) 6.3×10^{-7} M. In this same preparation when the TTX was washed out and tone was raised with histamine 10^{-6} M, field stimulation caused relaxation but this was resistant to TTX in double the previous dose. The lower records show that inhibitory extract in doses of 200 and 400 μl have no effect on tone in gall bladder strips in which tone was raised by the addition of histamine 10^{-6} M at the arrows. Time marker 2 min.

The effect of extract on the gall bladder

In the strip preparation of this tissue doses of extract up to 400 μl had no effect either on the atonic strip or on one in which tone was induced by carbachol 10^{-7} M. This was disappointing in view of the report of NANC nerves in this tissue. We tried, therefore, to demonstrate the presence of these nerves by field stimulation of strip preparations. Stimulation in the atonic preparation produced contractions that were abolished by atropine 10^{-6} M and by tetrodotoxin 6.3×10^{-7} M. In the presence of barium chloride 10^{-3} M or histamine 10^{-6} M, the tissue developed maintained tone and field stimulation at frequencies

of 0.5 Hz and above, then produced inhibitory responses with a maximum at 16 Hz (Figure 6). These responses, however, were not abolished by tetrodotoxin 1.26×10^{-6} M or by high concentrations of lignocaine (10^{-4} M) and their magnitude continued to increase with increasing pulse duration from 0.5 up to 50 ms. These inhibitory responses could not be obtained in every preparation and their presence or absence was unrelated to the agonist used to induce tone. Because of the inconsistency between these results and those reported by Davison *et al.* (1978) in the whole gall bladder preparation, we repeated the experiments with that preparation and raising tone with caerulin 0.3 μg/ml as Davison, *et al.* had done.
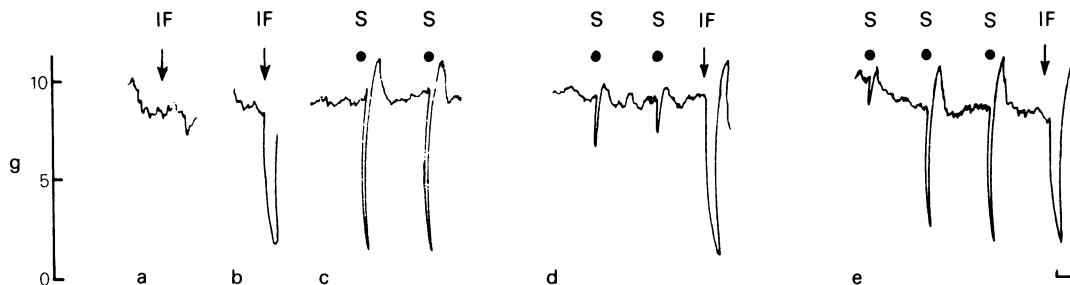
Figure 7 The effect of apamin 5×10^{-8} M on the response of the guinea-pig taenia coli to field stimulation and to the inhibitory factor. Cholinergic and adrenergic nerve activity was blocked by the addition of atropine 2×10^{-7} M and guanethidine 2×10^{-6} M respectively. Tone has been raised by the addition of an extra 7 mM KCl. In panel (a) 200 μl of inhibitory extract was added before activation, this had no effect; in (b) the same dose of activated extract produced relaxation. Panel (c) shows the inhibitory response to field stimulation at 2 Hz for 10 s. Between panel (c) and (d), apamin 5×10^{-8} M was added to the bath and panel (d) shows two responses to field stimulation in which the response is almost completely blocked, whereas the response to inhibitory extract is unaltered. Between (d) and (e), the apamin was washed from the bath and the response to field stimulation is almost completely restored. Time marker 1 min.

Inhibitory responses were small and infrequently observed and, like the strip preparation, when present were not abolished by tetrodotoxin in concentrations up to 1.26×10^{-6} M.

Apamin on inhibitory responses in the taenia coli

The effects of apamin on the response of the guinea-pig taenia coli to inhibitory factor are illustrated in Figure 7. As others have found, the inhibitory response to transmural stimulation of the NANC nerves or to ATP are blocked by low doses of apamin of 5×10^{-8} M which had no effect on the response to the inhibitory factor.

Discussion

There are clearly problems in attributing the effects of a semi-purified material of biological origin to a single component in that mixture. The present extract contains sodium, chloride and formate ions, the latter from the ion exchange column used in purification. This limits the range of bath concentrations which can be studied since hypertonicity can itself cause inhibition. Though a nuisance, this problem when present is easily detected since the inhibitory effect is heat stable and can be reduced or eliminated by dilution with distilled water. Other known constituents which cause difficulties in interpretation are adenine nucleotides to which the taenia in particular is very sensitive. We believe these nucleotides are largely removed by alkaline alumina adsorption and, in any case, unlike the extract, the inhibitory effect of ATP is little affected by 2 min exposure to a boiling water bath. For these reasons we believe the inhibitory effects we describe here are due to the same novel

inhibitory factor we have previously reported as causing relaxation in the BRP, the rat anococcygeus and in various vascular smooth muscles. This is based on its thermolability and, in particular, its requirement for acid activation before pharmacological activity appears. Acid activation was a requirement in all experiments but in some only part of the inhibitory activity was abolished by boiling. It is not possible to say whether this residual activity was due to some other substance in the extract or whether the compound produced from the inhibitory factor by boiling has itself some inhibitory activity. These inhibitory effects were obtained in many preparations tested over a period of two years. The requirement for acid activation and the thermolability were also confirmed many times. Nevertheless, inhibitory effectiveness of extract could not be demonstrated on every preparation. On occasions an extract shown simultaneously to be highly effective in relaxing the BRP was without effect on, for example, the taenia coli. These variations in response were not related to the sex, age or maturity of the guinea-pigs and, indeed, we have no ready explanation for those failures to produce relaxation. However, we have noted great variability in the inhibitory effectiveness of both ATP and isoprenaline in the guinea-pig taenia coli. In some preparations concentrations of ATP in the 10^{-7} M range are effective yet in others no inhibition was obtained till concentrations of 10^{-5} M were reached. It may be there is a corresponding variability in the response to the inhibitory factor.

The inhibitory factor in our extracts mimics well the inhibitory effect of stimulation of the NANC nerves in the gut. Its effectiveness in different regions is consistent with the known distribution of these nerves. On these grounds it would be a possible candidate as inhibitory transmitter. The observation

that apamin blocks the response to NANC nerve stimulation and to ATP without altering the response to the inhibitory extract, however, makes it unlikely that the latter is the transmitter of these nerves in the gut.

We have been unable to confirm a NANC innervation in the guinea-pig gall bladder. Inhibitory responses to field stimulation in the presence of guanethidine and atropine were certainly obtained and looked very like nerve responses. However, the resistance of these responses to tetrodotoxin and high concentrations of lignocaine and their increase in amplitude with very long stimulating pulses casts doubts upon their neuronal origin.

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