J. Physiol. (1981), **315**, *pp.* 111–125
With 8 text-figures
Printed in Great Britain

SOME PHYSICAL AND CHEMICAL PROPERTIES OF THE SMOOTH MUSCLE INHIBITORY FACTOR IN EXTRACTS OF THE BOVINE RETRACTOR PENIS MUSCLE

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(*Received 14 July 1980*)

SUMMARY

1. A method of extracting and partially purifying a smooth muscle inhibitory factor from the bovine retractor penis is described. This consists of extraction in methanol followed by adsorption on an anion exchange resin, elution from the resin with 500 mM-sodium chloride solution and, if necessary, removal of adenine nucleotides by adsorption on alumina.

2. The inhibitory factor exists in a stable pharmacologically inactive form and an unstable pharmacologically active form. Conversion to the active form is by a brief exposure to acid at pH 2·0.

3. The inhibitory factor is insoluble in ether or acetone but soluble in methanol. Anhydrous methanol, however, irreversibly destroys pharmacological activity especially if the inhibitory factor is in the active form. This effect of methanol is prevented by the presence of 20–30 % water.

4. The inhibitory factor binds to an anion exchange resin but not to a cation exchange resin. It can be eluted from the resin by 500 mM-sodium chloride solution.

5. The molecular weight of the inhibitory factor, as judged by the ability to pass ultrafiltration membranes, is about 500.

6. Inhibitory activity is unaffected by the proteases trypsin, subtilisin or pepsin or by leucine aminopeptidase, pyroglutamate aminopeptidase or carboxypeptidase. The inhibitory effect of the extract and the inhibitory response to stimulation of the non-adrenergic, non-cholinergic nerves are also unaffected by the protease inhibitor, aprotinin. The active material, therefore, is unlikely to be a peptide.

7. Inhibitory activity is abolished by exposure of the extracts to periodic acid or sodium periodate. Acetic anhydride in pyridine also abolishes activity but the vehicle pyridine is also effective.

8. Sodium borohydride but not borate abolishes inhibitory activity when added to the acid-activated material at pH 2·0 but has no effect or may even potentiate activity if added to the stable inactive form at pH 9·0. When added to the acid-activated but neutralized material at pH 6·8 it usually abolishes inhibitory activity but occasionally has no effect.

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0022-3751/81/5200-1083 $07.50

9. These results suggest the smooth muscle inhibitory factor in these extracts is potent and probably novel. It does not appear to be a peptide or a lipid but may contain a carbohydrate as part of the molecule. Its possible physiological role is discussed.

INTRODUCTION

The retractor penis and anococcygeus muscles in a variety of species have a motor adrenergic innervation and an inhibitory innervation whose transmitter is as yet unknown. Ambache, Killick & Zar (1975) extracted from the bovine retractor penis (BRP) a thermolabile, ether-insoluble substance which powerfully inhibited the BRP and whose action was unaffected by drugs blocking conventional transmitters. This inhibitory material, they believed, could be the inhibitory transmitter. We have confirmed these results and showed that the inhibitory factor exists in two forms, a stable pharmacologically inert form which was converted to an unstable but powerful inhibitory compound by a brief exposure to acid before neutralization and assay (Gillespie & Martin, 1980). We have continued to purify this material and to investigate its physical and chemical characteristics with the ultimate intention of identifying it chemically. This paper describes steps we have found useful in its partial purification, together with some physical and chemical characteristics of the inhibitory material. We have also looked at the effects of a variety of enzymes, particularly proteases and peptidases, on the inhibitory activity. Some of these results have previously been communicated to the Physiological Society (Gillespie & Martin, 1978).

METHODS

Two methods of extraction were used. The first is essentially that of Ambache *et al.* (1975). Fresh BRP muscle from the abattoir was frozen in liquid nitrogen, pulverized in a liquid-nitrogen-cooled stainless steel mortar and the powder extracted in five or ten times its volume of 0·17 M-hydrochloric acid for 30 min. The supernatant was separated by centrifugation, neutralized with 0·5 M-sodium hydroxide and freeze-dried. The second and currently used method was to chop the tissue finely with a microtome knife and extract it overnight in a cold room at 4 °C with five volumes of methanol with constant stirring. The methanol was filtered off and applied to a 5·5 cm × 1 cm column of anion exchange resin (Bio-Rad AG1-X8 in the formate form) which retains the inhibitory factor. The column was washed with 15 ml of distilled water and the inhibitory factor eluted with 500 mM-sodium chloride solution. The first 5 ml fraction was discarded and the next 20 ml containing the inhibitory material was freeze-dried in a flask protected from light by aluminium foil and stored in the deep freeze at −20 °C. This lyophilized powder was reconstituted with distilled water to give a concentration equivalent to 1 g of tissue per millilitre. Occasionally a concentration of 2 g/ml was used. The ionic concentration of these reconstituted extracts varied depending on the weight of tissue extracted and applied to the anion column but was usually isotonic and never more than twice normal tonicity.

The inhibitory factor was assayed after acid activation by its ability to relax spontaneous tone in the BRP. Extract was activated by acidifying with 0·5 M-hydrochloric acid to pH 2, holding it at this pH for 10 min then returning to pH 6·8 by adding 0·5 M-sodium hydroxide. Assay muscle preparations approximately 2 mm in thickness and 3–4 cm long were suspended in 10 ml baths containing Krebs solution at 37 °C and gassed with 95% oxygen + 5% carbon dioxide. Tension was recorded with Grass FT03 strain gauges and displayed on a Grass four-channel polygraph. The muscle was stretched at intervals but the applied tension was never allowed to exceed 2 g. This process after 30 min to 2 h induces the development of a maintained tone. Extract and drugs were added in volumes between 0·05 and 0·3 ml. Interference by histamine, acetylcholine or noradrenaline in the extracts was prevented by adding appropriate blocking drugs to the Krebs saline solution

(mepyramine 1 μM, atropine 1 μM, phentolamine 5 μM and propranolol 5 μM). The muscles were routinely suspended in ring electrodes so that the response to field stimulation could be compared with the response to inhibitory extract. Field stimulation was by 1·0 ms pulses, at supramaximal voltage and usually 20 Hz frequency. The BRP is readily inhibited by a variety of stimuli including anoxia, hyperosmolarity, acid pH and elevated levels of potassium. To exclude these non-specific inhibitory stimuli we relied on two controls: first, the inhibitory effect should be absent in extracts not activated by acid and, secondly, it should be abolished by exposure of the extract in a sealed test tube to a boiling water bath for 2 min.

In experiments to estimate the molecular weight of the inhibitory factor, extract in Amicon filtration cells was forced through ultrafiltration membranes under a pressure of 50 p.s.i. from a nitrogen cylinder. The cells were half filled to begin with and filtration was continued until half this volume had passed the membrane. Aliquots of the original material, of the ultrafiltrate, and of the residual fluid in the cell were then assayed for inhibitory activity. If the inhibitory factor passed through a particular membrane without restraint then the inhibitory activity of all three should be equal. At the other extreme, if the inhibitory factor was completely prevented from passing, the ultrafiltrate would be free of all activity and the chamber fluid should be twice as active as the starting material.

In experiments on the effects of enzymes on the inhibitory activity 5 ml of extract was incubated at room temperature with the following concentrations of enzyme: trypsin 200–2000 u./ml, subtilisin 20–200 u./ml, pepsin 60 u./ml, pyroglutamate aminopeptidase 5 mu./ml, leucine aminopeptidase 0·5–1·5 u./ml, carboxypeptidase 50 u./ml. The pH of the solution was adjusted to 2·0 with 0·1 M-hydrochloric acid when pepsin was used. All other incubations were at pH 7·0.

Drugs used were aprotinin (Calbiochem), atropine sulphate (BDH), mepyramine maleate (Anthisan, May & Baker), phentolamine (Rogitine, Ciba), propranolol hydrochloride (Sigma), SQ 20881 (Squibb). The enzymes used were carboxypeptidase-A type II (Sigma), leucine aminopeptidase type V (Sigma), pyroglutamate aminopeptidase (Boehringer Mannheim), subtilisin type VI (Sigma), trypsin type I (Sigma).

RESULTS

Physical properties

Three physical characteristics of the inhibitory material – its solubility in solvents less polar than water, its molecular weight and its possible electrical charge – were examined.

Solubility

Three solvents in addition to water were investigated: methanol, ether and acetone. The starting materal was a lyophilized powder originally from an acid extract and equivalent to 10 g of tissue freeze-dried in 50 ml flasks. This powder was extracted with one or other of the organic solvents, the solvent separated from the insoluble residue and evaporated to dryness. This solvent residue and the original solvent-insoluble residue were separately redissolved in distilled water, activated and assayed on the BRP. The results with acetone and ether were clear and are illustrated in Fig. 1. There was no activity in the residue after evaporating off the organic solvent and all of the original activity was recovered from the solvent-insoluble residue. The possibility that during lyophilization a weak acid was converted to an ionizable salt with a reduction in its solubility in organic solvents was excluded by adding ether to an acid extract at pH 2·0, shaking, separating the two phases and then evaporating off each solvent phase. The solid residues were then redissolved in distilled water, activated and assayed on the BRP. All of the inhibitory activity remained in the aqueous phase and none partitioned into the ether.

The results with methanol were more complex. After extraction of a lyophilized powder with anhydrous methanol there was never any inhibitory activity left in the methanol-insoluble residue. This suggested that the inhibitory material was soluble in methanol. However, little or no inhibitory activity was recovered from the methanol. An experiment illustrating this point is shown in the upper records of Fig. 2. This result suggested that methanol was destroying the inhibitory material. The

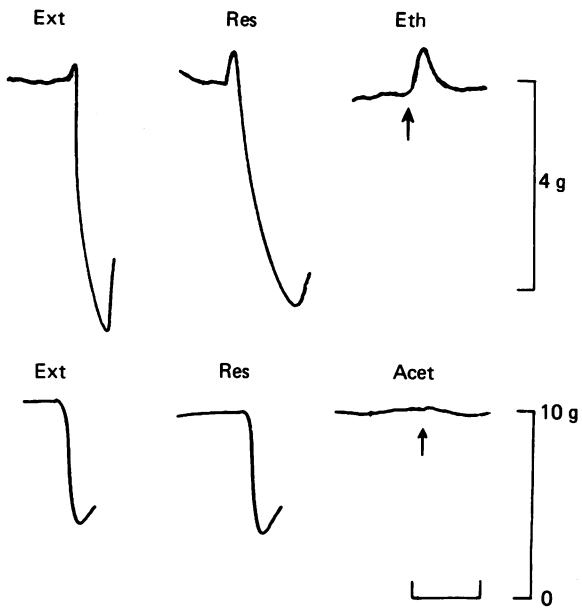

Fig. 1. The upper three records are from one experiment and the lower three from a separate experiment. In each the first record shows the inhibitory activity of the control extract as a fall in spontaneous tone in the BRP muscle. The second record (Res) shows the inhibitory activity remaining in the insoluble material after extraction with either ether (upper row) or acetone (lower row) and the third shows the inhibitory activity of the material extracted by ether (Eth) or acetone (Acet) after their evaporation and reconstitution of the solid residue in saline. No inhibitory activity was extracted by either solvent and all the activity of the original extract was recovered in the ether- or acetone-insoluble fraction. Time marker 2 min.

remaining records in Fig. 2 from another experiment show that this is part of the explanation. In this experiment a lyophilized extract was divided into two portions. In one the inhibitory factor was activated and in the other it was left in its inactive form. Each portion was then separately extracted with anhydrous methanol. The methanol solution was separated and evaporated, and the inhibitory activity of the residue was immediately assayed. As Fig. 2 shows there was an immediate loss of activity which was greater for the active than the inactive form. Fig. 2 also shows that after this treatment with methanol, the stability of the remaining inhibitory material was reduced. Whereas the active material is stable for at least 2 h when kept on ice, this methanol-treated material lost almost all activity after only 30 min on ice. This loss with time was reversible by re-acidification, as the Figure shows, but the initial loss was not reversible and was not, therefore, due to increased instability

and reversion to the inactive form in the period before assay. The destructive effect of methanol was prevented by the presence of 20–30 % water. The possibility that this was due to the simultaneous extraction of some water-soluble substance that is necessary to stabilize the methanol-soluble material was investigated, but no evidence that this was so was obtained. Rather it seemed that anhydrous methanol

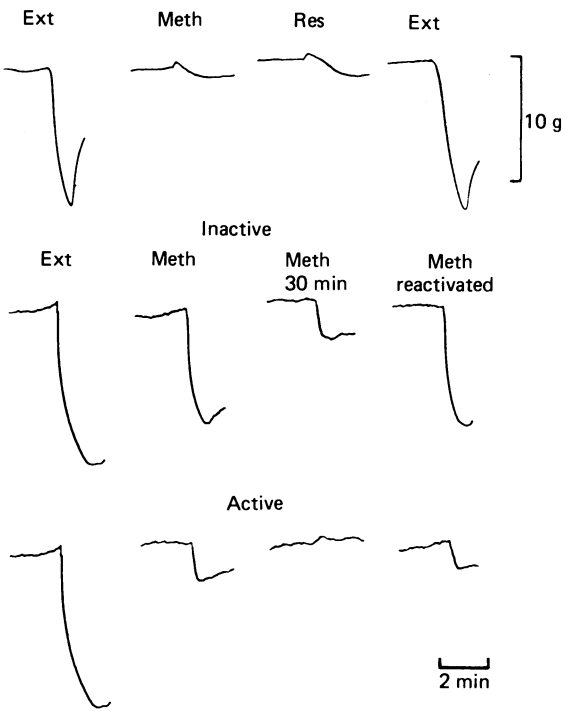

Fig. 2. Assay on spontaneous tone of the BRP of inhibitory extracts from that muscle. The upper row of records shows the effect of extracting a dried extract with anhydrous methanol. No inhibitory activity was recovered from the methanol (Meth) nor could activity be found in the methanol-insoluble residue (Res). The lower two rows are from another experiment in which the inhibitory material was dried either in the stable inactive (Inactive) or the acid-activated form (Active) before extracting with methanol. Some inhibitory activity was recovered from the methanol but compared with the control extract there was some loss with the inactive form and a greater loss with the active form. Furthermore, the methanol-soluble fraction was more unstable so that even on ice much of the activity was lost in 30 min (Meth: 30 min). This loss with time but not the initial loss of activity could be reversed by further acid activation (Meth: reactivated).

had the ability to destroy the inhibitory factor, especially if this were in the active form, and that this effect was prevented by the presence of water. Advantage was taken of this protective effect of water to introduce extraction in anhydrous methanol as the first step in a modified extraction procedure, relying on the tissue water to prevent destruction. This reduced the amount of soluble protein in the extract with no evidence of loss of inhibitory activity.

Ion exchange binding

If the inhibitory factor was a charged molecule it should bind to ion exchange resins. We used two resins, a strong anion resin (Bio-Rad AG1-X8 in the formate form) and the cation resin AG 50-W in both the sodium and hydrogen form. In early experiments the resin was used in short 0·5 × 0·5 cm columns in Pasteur pipettes. Small volumes of about 10 ml of an acid extract reconstituted in distilled water were passed through these columns, and the inhibitory activity in the fluid running through was assayed. The columns, after washing with distilled water, were eluted with increasing concentrations of sodium chloride solution and the activity in these eluates was measured. The results are illustrated in Fig. 3. With the anion column no activity appeared in the extract running through the column, suggesting that the

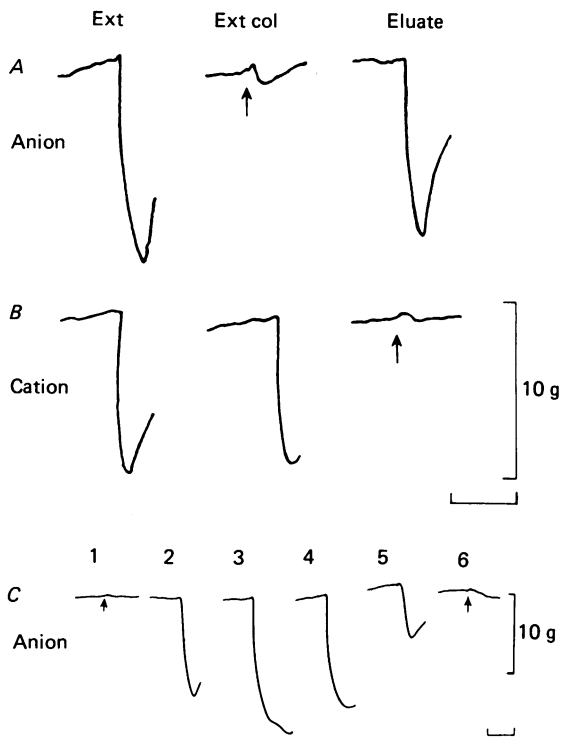

Fig. 3. Assay on the BRP of inhibitory extract before and after passing through (*A*) an anion exchange resin and (*B*) a cation resin, and (*C*) its distribution in successive samples from an anion column eluted with 500 mM-sodium chloride solution. In *A* and *B* the first record is the activity of the extract applied to the column (Ext), the next activity is the extract leaving the column (Ext col) and the last the activity in a 100 mM-sodium chloride solution used to elute the column (Eluate). There is no inhibitory activity in the extract leaving the anion column, suggesting it has been retained, and this is supported by the activity in the 100 mM-sodium chloride eluate. In contrast the extract leaving the cation column is active and no activity appears in the eluate. The records in *C* are from another experiment in which a larger anion column and a more concentrated sodium chloride solution (500 mM) were used. The records show the inhibitory activity in successive 5 ml samples; there is none in the first, which includes the void volume, but all of the activity is then recovered in the next four samples.

inhibitory factor had been retained. This was confirmed by the appearance of inhibitory activity almost equal to the original in the eluate with 100 mM-sodium chloride. The opposite results were obtained with the cation resin: all of the inhibitory activity was present in the extract as it left the column, and no activity could be eluted with sodium chloride solution. These experiments were repeated with methanol extracts and the inhibitory material was found to bind to the anion resin as readily from methanol as from distilled water. Methanol extraction followed by binding to an anionic resin was, therefore, adopted as the first two steps in purification. The methanol extracts were applied in volumes up to 250 ml, equivalent to 50 g of original tissue. This meant scaling up the columns to 5·5 × 1 cm. With these larger columns, 100 mM-sodium chloride solutions were found inadequate to elute the inhibitory factor in a compact volume. Increasing the concentration to 500 mM corrected this and the inhibitory activity appeared in four successive 5 ml samples as the final records in Fig. 3 show.

Molecular weight

Our first attempt to estimate the molecular weight of the inhibitory factor was with gel chromatography on Sephadex beads. These experiments were unsuccessful since no inhibitory activity was recovered from the columns. We turned, therefore, to the more direct technique of ultrafiltration through Amicon filters with various molecular size retention values. The first experiments filtering acid extracts through membranes of large pore size resulted in muco-protein and inhibitory factor being trapped on the membrane. The resolution of this problem led to our realization that acid activation was necessary before inhibitory activity appeared. These experiments have been described earlier (Gillespie & Martin, 1980). With this new information, we re-examined the ability of the inhibitory factor to pass membranes with a range of retention values, but now re-activated each sample before assay. Both acid extracts which contain soluble proteins, and methanol extracts which do not, were used. The results are shown in Fig. 4. With an acid extract as starting material, inhibitory activity appeared in the ultrafiltrate from the 300000 membrane but this was less than the activity of the starting material. The chamber fluid showed a corresponding increase in activity, suggesting that the inhibitory factor was not all freely filterable. With the 10000 similar results were obtained. Inhibitory activity appeared in the ultrafiltrate but it was less than in the starting material and activity in the chamber fluid rose. With the 500 membrane, no activity appeared in the ultrafiltrate and there was a corresponding doubling of activity in the chamber fluid. Similar experiments with protein-free methanol extracts reconstituted in distilled water gave slightly different results. With the 300000 and 10000 membranes inhibitory activity readily passed through the membrane, with no loss of activity in comparison with the starting material and no increase in activity in the chamber fluid. With the 500 membrane variable results were obtained; sometimes activity appeared in the ultrafiltrate and on other occasions no activity appeared. Fig. 4 illustrates the commonest outcome: some activity in the ultrafiltrate but less than the starting material, with little or no evidence of concentration in the chamber fluid. These experiments were duplicated with the inhibitory factor in the active form to see whether acid activation involved a significant change in molecular weight. There was no evidence that this was so

though these experiments were less satisfactory because there was a loss of activity in the chamber fluid during filtration. This seemed to be due to time and possibly the constant stirring since, if the outflow tube was clipped to prevent filtration and the gas pressure applied with constant stirring for the period normally necessary for filtration, a similar loss of activity was found in the chamber fluid.

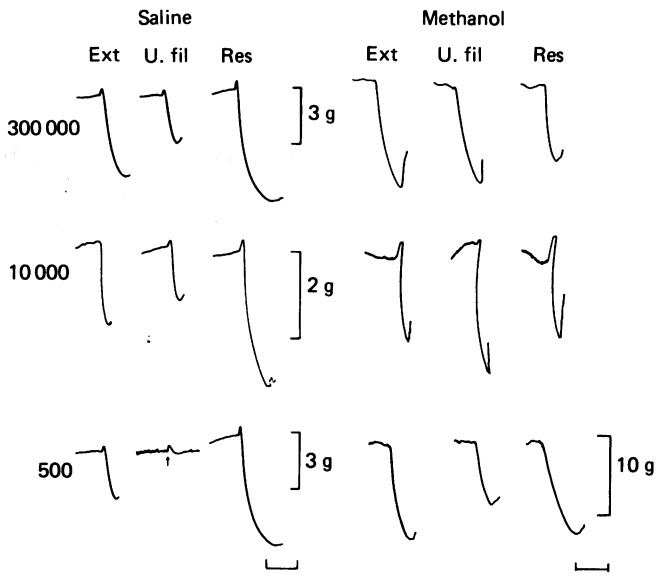

Fig. 4. The ability of the inhibitory factor to pass ultrafiltration membranes with the retention values shown on the left of each row. Two types of extract were used, acid extracts which after neutralization gave a solution in approximately normal saline, and methanol extracts. All samples were activated or re-activated before assay. In each group of three records the first shows the activity of the extract before filtration (Ext), the second the activity in the ultrafiltrate (U.Fil) and the third the activity in the fluid remaining in the filtration chamber (Res). In the saline extracts the ultrafiltrate from the 300000 membrane was used as the starting material for the 500 membrane. With saline extracts the activity in the ultrafiltrate is less than that of the starting material and the chamber fluid is more concentrated except for the 500 membrane where no activity appeared in ultrafiltrate. In contrast the inhibitory material in protein-free methanol extracts passed all three membranes with some evidence of restraint with the 500. Time marker 2 min.

We have interpreted the results with the two high-molecular-weight retention filters and acid extracts as indicating a degree of binding to soluble protein, so that reduced activity in the ultrafiltrate was a measure of the free concentration of inhibitory factor in the chamber fluid. With similar filters and methanol extracts without protein the activity of the ultrafiltrate and chamber fluid was equal. The inability of the inhibitory factor in saline extracts to pass the 500 molecular weight retention membrane and its variable ability to pass from a methanol extract we have interpreted as indicating a molecular weight of about 500.

Chemical properties

We have previously shown that the inhibitory factor in these extracts, unlike ATP, is not retained on an alumina column at pH 9·0. It is, therefore, presumably not ATP (Bowman, Gillespie & Martin, 1979). Another possibility was that it was a polypeptide,

and so we looked at the ability of a group of non-specific proteases – trypsin, subtilisin and pepsin – together with the peptidases, pyroglutamate aminopeptidase, leucine aminopeptidase and carboxypeptidase, to destroy the inhibitory activity. Reconstituted extracts at a concentration equivalent to 1 g/ml and approximately isotonic were incubated at room temperature for 1 h with the concentrations of enzymes given in the Methods. The results are shown in Fig. 5. None of the proteases or aminopeptidases had any effect on the inhibitory activity. Carboxypeptidase did

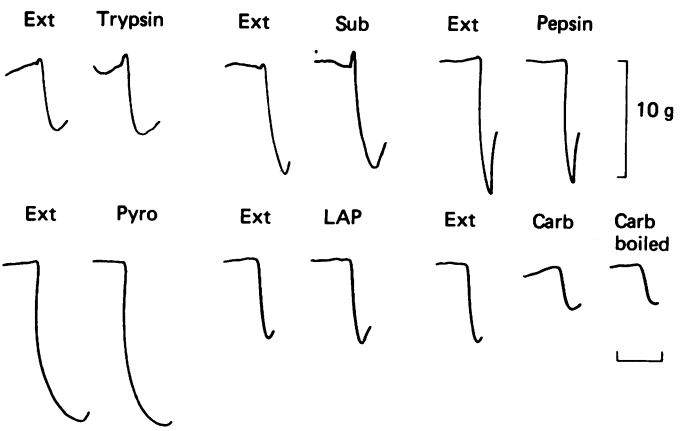

Fig. 5. The effect of some proteases and peptidases on the inhibitory activity of extracts from the BRP. Each pair of records is from a different experiment and in each the first shows the inhibitory activity on the BRP of the extract and the second the activity after incubation at room temperature for 1 h with trypsin (200 u./ml), subtilisin (Sub: 200 u./ml), pepsin (60 u./ml), pyroglutamate aminopeptidase (Pyro: 5 mu./ml), leucine aminopeptidase (Lap: 1·5 u./ml) and carboxypeptidase (Carb: 50 u./ml). Only with carboxypeptidase was there a reduction in effect and this was as great with boiled enzyme. Time marker 2 min.

reduce activity but incubation with boiled enzymes was equally effective, suggesting the the inactivation was not due to enzyme action. In the course of other experiments we have incubated inhibitory material with galactosidase and collagenase and these enzymes also have failed to destroy the inhibitory activity.

The converse approach, an attempt to potentiate the effect of inhibitory extract and of inhibitory nerve stimulation by inhibiting general tissue proteases with aprotinin or the specific bradykinin/angiotensin converting enzyme with SQ 20881, was also tried. Fig. 6 illustrates the results with aprotinin. In concentrations from 100 to 10000 kallikrein inactivation units per millilitre of bath fluid it had no effect on the inhibitory nerve response in either the BRP or the rat anococcygeus muscle, and no effect on the inhibitory activity of extract on the BRP; SQ 20881 was similarly ineffective.

Three simple chemical reagents did abolish the inhibitory activity of our extracts and these are illustrated in Figs. 7 and 8. Sodium periodate or periodic acid, 0·5 ml of a 0·054 or 0·54 M solution added to a reconstituted methanol extract equivalent to 10 g of tissue in either the active or inactive form completely abolished activity (Fig. 7, upper row). Acetic anhydride, 0·5 ml of a 1:2 dilution in pyridine added to a reconstituted methanol extract equivalent to 10 g of tissue, also completely abolished the inhibitory effect (Fig. 7, lower row). Unfortunately, pyridine alone in

these concentrations was equally effective so that no conclusions can be drawn about the effect of acetic anhydride. Finally, sodium borohydride in suitable conditions irreversibly abolished the inhibitory activity of extracts. Originally the borohydride was added to acid-activated extracts. In ten such experiments 5 mg borohydride added to 2·5 ml of extract completely abolished the inhibitory activity but in a further four experiments it was ineffective. This was not due to insufficient borohydride,

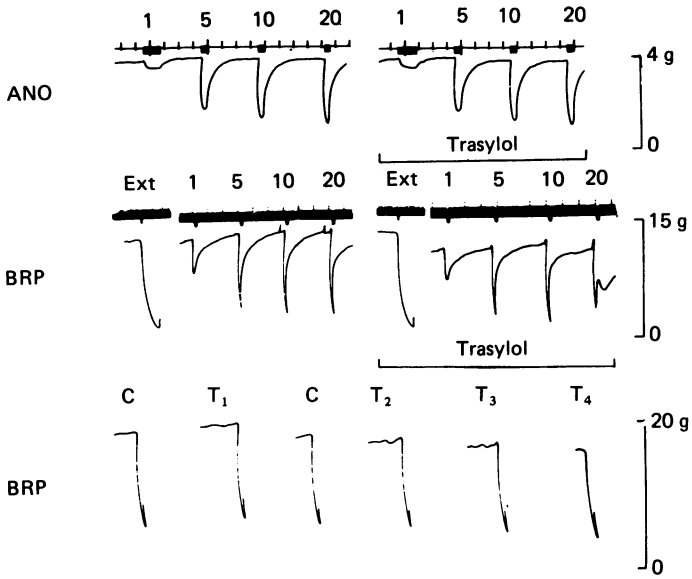

Fig. 6. The upper row shows the response of the rat anococcygeus (ANO) to field stimulation at the frequencies in hertz shown above each record. Tone has been raised and the adrenergic nerves blocked by the previous addition of guanethidine at 5×10^{-5} M. The next row shows the effect on spontaneous tone in the BRP of either field stimulation at the frequencies shown or of 0·1 ml of inhibitory extract. The final row shows the response of the BRP to inhibitory extract. In the upper two rows the first panel shows control responses and the second responses in the presence of trasylol at 2520 kallikrein units per millilitre. Trasylol had no effect on the inhibitory response to either nerve stimulation or the inhibitory extract. The bottom row shows the effect of increasing concentrations of trasylol on the response to inhibitory extract. C represents the response to 0·1 ml of extract alone, T1 the response to 0·1 ml of extract in the presence of 630 u., T2 in the presence of 3150 u., T3 in the presence of 5040 u. and T4 in the presence of 10080 u. trasylol. The response is unchanged. Time 1 min.

since in extracts whose activity was destroyed, 1 mg borohydride added to 2·5 ml was also completely effective and 0·1 mg produced considerable reduction in inhibitory activity. On the other hand, in experiments in which inhibitory activity was not abolished, doubling the borohydride to 10 mg per 2·5 ml of extract was still ineffective. Such results suggested that 2 mg of borohydride per millilitre of extract represented a reasonable safety margin. The explanation of these variable results apparently lies in the effect of acid activation and is illustrated in Fig. 8. When borohydride was added to the extract before activation (the pH in these circumstances is about 9) it was ineffective; subsequent acid activation produced almost full

inhibitory activity. When the borohydride was added after activation and still at pH 2 then inhibitory activity was destroyed. If the activated extract was first neutralized and then the borohydride added, the results, like our original results, were variable; activity was sometimes lost and sometimes, as in Fig. 8, unaltered. Not only was borohydride ineffective in destroying inhibitory activity when added to the stable,

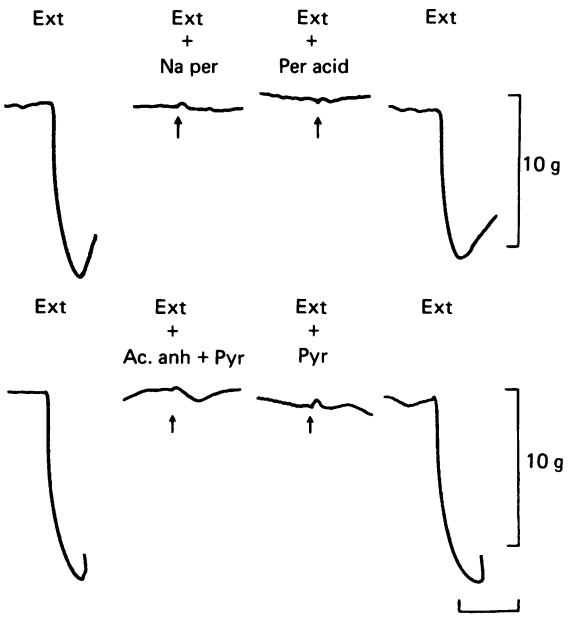

Fig. 7. The effect of 0·54 M-sodium periodate (Na per), periodic acid (Per acid), acetic anhydride in pyridine (Ac. anh + Pyr) and pyridine alone (Pyr) on the inhibitory effect on the BRP of extracts from that muscle. The first and last responses in each row are controls to 0·1 ml of extract alone. In the upper row both periodate and periodic acid abolished the inhibitory activity of extract. The lower records show that while acetic anhydride in pyridine abolished activity, pyridine alone in a similar concentration was as effective. Time marker 2 min.

non-activated material, but in several experiments it definitely potentiated this activity. One such experiment is shown in the lower records of Fig. 8. This effect was particularly noticeable with extracts of low potency. The ability of sodium borohydride to abolish the inhibitory effect on the BRP is selective in that it acts only on extracts at acid pH, and it is ineffective in preventing the inhibitory activity of other drugs such as papaverine. We excluded the possibility that it was the borate ion formed from the borohydrate which reacted with the inhibitory factor in the extracts. Sodium tetraborate added to extracts in concentrations equivalent to that of sodium borohydride had no effect on the inhibitory activity of the extracts.

DISCUSSION

Tissue extraction with methanol followed by anion exchange represents a considerable improvement in purification compared with acid extraction. Methanol greatly reduces soluble protein, and anion exchange largely removes lipid and tissue amines,

particularly noradrenaline. Further purification is possible. The lyophilized extract contains adenine nucleotides, and methods of removing these without removing the inhibitory factor have previously been described (Bowman *et al.* 1979). Removal is necessary if the activity of the inhibitory factor on tissues sensitive to ATP is under investigation; if all assays are on the BRP or anococcygeus as in the present experiments then this is unnecessary as the tissues are relatively insensitive to ATP.

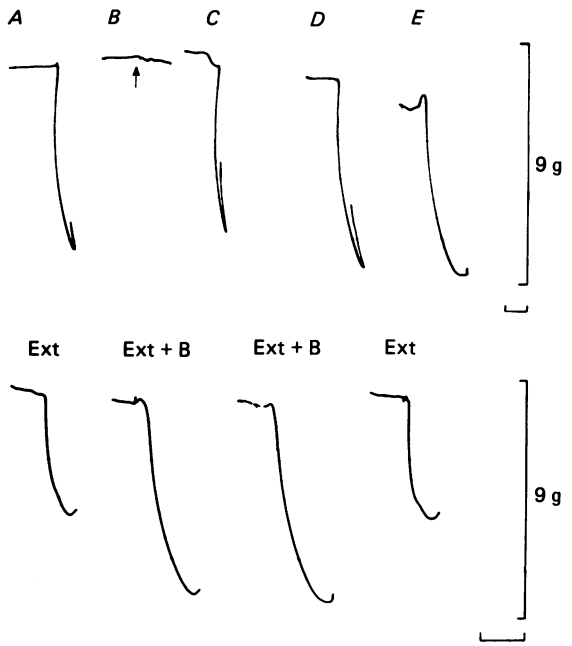

Fig. 8. The effect of sodium borohydride (5 mg in 2·5 ml) on the inhibitory activity of extract. The records in the upper row are from the same experiment: *A* is the control response to 0·1 ml of extract, *B* the response to 0·1 ml of extract to which borohydride was added at pH 2, and *C* the response to 0·1 ml of extract to which borohydride was added at pH 9 before activation. Borohydride abolished activity when added at pH 2·0 but not at pH 9. Records *D* and *E* later in the experiment when tone had begun to fall show again the response to 0·1 ml control extract (*D*) and extract to which borohydride was added after activation and neutralization to pH 6·8. Inhibitory activity was not reduced. The lower row of records from another experiment show another effect of borohydride: potentiation of the inhibitory response. The first and last responses are controls to 0·1 ml extract (Ext), the middle two are separate 2·5 ml aliquots to each of which 5 mg borohydride was added at pH 9·0 before activation (Ext + B). After activation 0·1 ml produced a bigger response.

After purification, doses of extract equivalent to 2 mg wet weight of tissue will produce relaxation of a sensitive preparation of BRP. Given that 70% or so of the tissue wet weight is water, that structural proteins are left behind and that soluble proteins are removed on the anion column, the total quantity of organic material remaining is not likely to exceed 100–200 μg/g wet weight. The amount of this material accounted for by the inhibitory factor is unknown but it is probably a small fraction. Consequently, it seems likely that the material is potent. From the results described here, and previously, we believe it is also likely to be novel. Its insensitivity

to cholinoceptor, adrenoceptor and histamine receptor antagonists excludes the obvious transmitters. The two most attractive alternatives are ATP or a polypeptide, the former on the basis of the considerable evidence assembled by Burnstock for ATP as a transmitter of non-adrenergic, non-cholinergic nerves in several tissues (Burnstock, 1972, 1979) and the latter on the increasing evidence for polypeptide-containing nerves in viscera such as the gut (Larsson, Fahrenkrug, Schaffalitzky de Muckadell, Sundler, Håkanson & Rehfeld, 1976). Reinforcing the latter is the recent report on the ultrastructure of the anococcygeus (Gibbins & Haller, 1979) together with earlier reports on the BRP (Eränkö, Klinge & Sjöstrand, 1976) which describe nerve profiles containing opaque vesicles consistent with known peptidergic nerves (Bargmann, Lindner & Andres, 1967; Baumgarten Holstein & Owman, 1970). Further, though rather indirect, support for a polypeptide comes from the ability of bradykinin to produce muscle relaxation in the rat anococcygeus, one of the few agents to do so (Gillespie & McKnight, 1978). Nevertheless, the inhibitory factor does not appear to be either ATP or a peptide. ATP can be removed almost completely from the extracts without reducing the inhibitory activity on the BRP (Bowman *et al.* 1979) and the present results show that incubation with either proteases or peptidases has no effect on inhibitory activity, and aprotinin, a non-specific protease inhibitor, or SQ 20881, an antagonist of the angiotensin converting enzyme, applied to the assay tissue did not potentiate the inhibitory response to nerve stimulation or to tissue extract. Such negative findings are, of course, less convincing and we have no direct evidence that these added enzymes were active. What can be said is that they were present in adequate amounts and at a temperature, pH and ion concentration at which activity would be expected and that one, collagenase, which was incubated with tissue as well as reconstituted extract, clearly caused rapid disruption of the muscle tissue. Other characteristics of this inhibitory factor are also inconsistent with either ATP or a polypeptide. Its most unusual feature is the requirement for acid activation. This is not necessary for the activity of ATP. It could be a requirement of a protein if changing pH produced some changes in the tertiary structure. It could also be consistent with a peptide if changing pH activated some enzyme which acted on a precursor, rather like kallikrein or angiotensin converting enzymes acting on their precursors. Neither possibility seems tenable. Methanol extracts contain little protein, there is less after passing through the anion exchange column, and even less after filtration through a 10000 molecular weight retaining membrane, yet after such a sequence there is no diminution in inhibitory activity. In any case the number of peptide bonds in even a small protein would make it susceptible to one or other of the proteases we used. A small polypeptide could be extracted and pass these filters and might, if in a suitable conformation and with only a few peptide bonds, be resistant to enzyme attack. There is, however, the difficulty of explaining acid activation. This is rapid. We have standardized on 10 min for convenience but the process is already near complete in the shortest period studied, that is 2 min; it takes place on ice and the pharmacologically active form produced easily reverts to the stable inactive form (Gillespie & Martin, 1980). None of these properties is compatible with enzyme conversion of a precursor molecule. In any case, the enzyme would have to be in the extract and would, like other proteins, be lost during purification and destroyed by proteases. Another attractive possibility would be an acid lipid, by analogy with the prostaglandins and SRSA. Against this is the insolubility of the

inhibitory factor in lipid solvents even at acid pH, the easily reversible acid-activation process, and the pharmacological properties which are almost the opposite of those of SRSA (Morris, Piper, Taylor & Tippins, 1979). Since the inhibitory factor falls neither in the broad group of proteins and polypeptides, nor apparently in that of lipids, the possibility that it is carbohydrate in nature should be considered. Supporting such a possibility is the reaction with periodic acid, a reaction fairly specific for carbon–carbon bonds with glycols, and the reaction with borohydride, a fairly specific reducing agent for aldehydes and ketones. If a sugar is part of the molecule then activation by acid may represent either hemiacetal formation, lactone formation or the formation of the enol form. All of these transformations are catalysed by acid and all represent reversible reactions. Hemiacetal formation has the added attraction that the hemiacetal might react with methanol to form a full acetal and so lose activity. It was the assumption that the inactive stable compound extracted from the tissues contained a sugar in the open chain form which, in an acid environment, formed a hemiacetal, that suggested testing borohydride. On this hypothesis, borohydride should react with the inactive material, since the aldehyde would be freely available, but it should not react with the active form since internal cyclization of the aldehyde with a hydroxyl would prevent the reaction. The results were exactly the opposite. It was the active form which reacted with the borohydride while the inactive form was either unaffected or sometimes potentiated. These results suggest that acid exposes the active aldehyde or ketone, and that it is the stable material which is in the hemiacetal or lactone ring form; the conversion of this to the open chain may then be catalysed by acid. This interpretation relies very much on the reaction with periodic acid indicating the presence of a carbohydrate and on that with borohydride indicating an aldehyde or ketone group. We have tried to control the possibility that either reagent acted directly on the muscle to make it unresponsive to inhibitory agents in general, by showing that the effects of other inhibitory drugs such as papaverine and IBMX were not reduced. We have excluded the possibility that it is the borate ion formed after the oxidation of borohydride that reacts with the inhibitory factor, by showing that the borate ion alone is ineffective.

Finally, there is the important question of the physiological function of this substance and, in particular, its relationship to non-adrenergic, non-cholinergic inhibitory nerves. In favour of a neurotransmitter role are its presence in tissue containing such nerves, its potency and evanescent action and its ability to mimic closely the inhibitory effect of nerve stimulation. Particularly important is its ability to inhibit the rat anococcygeus, since in previous investigations we have tested a wide range of compounds of natural origin and found only one, bradykinin, able to inhibit this muscle. Against a role as neurotransmitter is the ability to extract this or a similar compound from tissue lacking such an innervation (Gillespie & Martin, 1980). It may be, as with histamine and 5-hydroxytryptamine, that this material has functions in addition to neurotransmission which will account for this wide distribution. Whether this particular function as neurotransmitter is established it remains true that historically few potent substances isolated from mammalian tissue have failed eventually to find a physiological role.

In summary, we have described some of the physical and chemical properties of what appears to be a novel and potent smooth muscle inhibitory material extracted

from the BRP muscle. This does not seen to be either a peptide or a lipid but may contain a carbohydrate, a component which is involved in the process of acid activation which contributes biological activity to the compound.

Our thanks are due to the M.R.C. for Research Studentships for two of us (J.C.H. and W.M.), to the Medical Research Funds of Glasgow University for help with apparatus and to Professor C. J. W. Brooks for helpful discussions and suggestions.

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