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The pharmacology of a new oxytocic principle from ox hypothalamus

R. W. HAWKER, W. G. NORTH AND B. ZERNER

Departments of Physiology and Biochemistry, University of Queensland, St. Lucia, Queensland

1. An oxytocic substance has been isolated from ox hypothalamus by successive gel filtration on Sephadex G-25 and Sephadex G-50, and its pharmacology has been examined on three smooth muscle preparations.

2. The substance has the same order of potency on rat uterus, guinea-pig ileum, and hen rectal caecum.

3. The action of the substance on rat uterus was not abolished by thioglycollate.

4. Atropine (1.0 μg/ml.), phenoxybenzamine (0.1 μg/ml.) and mepyramine (1.0 μg/ml.) did not block the smooth muscle action of the substance.

5. Drug action, relative potency, and log dose-response relationships distinguish the substance from 5-hydroxytryptamine, acetylcholine, oxytocin, vasopressin, angiotensin amide, bradykinin, and purified preparations of substance P.

Meinardi & Craig (1966) have shown that oxytocin, vasopressin, and substance P are not the only peptides with oxytocic action present in the mammalian hypothalamus. In earlier studies, Zetler (1963) and Cleugh, Gaddum, Mitchell, Smith & Whittaker (1964) found that substance P preparations from whole brain contained an unidentified oxytocic peptide (or peptides). Other substances with oxytocic action known to occur in the hypothalamus are acetylcholine (Whittaker, 1963) and 5-hydroxytryptamine (Crawford, 1958).

A preliminary report of a new oxytocic substance present in extracts of bovine hypothalamus has recently appeared from these laboratories (North, Hawker & Zerner, 1968), and in this paper the pharmacology of the material is presented.

Methods

Brains of cows (3–6 yr old) were obtained from the local abattoir. A region of the brain representing the anterior hypothalamus was excised. This was defined as a block (approximately $3 \times 2 \times 3$ cm) with the anterior and posterior boundaries being the optic chiasma and the median eminence, respectively. It was 1 cm wide on either side of the midline, and its depth extended to meet the floor of the lateral ventricles. Tissues were frozen ($-80°$ C) within 20 min of death. Acetone-dried powders of the tissues were prepared after arrival at the laboratory.

Preparation of extracts

The acetone-dried powders were ground in a mortar and added to 10 volumes of dilute acetic acid (pH 2.9) which was preheated to 80° C on a water bath. Heating was continued for 5 min to destroy degradative enzymes (Valtin, Sawyer & Sokol, 1965; Lederis, 1961). The cooled suspension was shaken for 2 hr and centrifuged at 1,200 *g* for 10 min at room temperature. The pellet was washed with half of the original volume of acetic acid and recentrifuged. Supernatants were combined and evaporated under reduced pressure at approximately 20° C.

Gel filtration on Sephadex G-25 at pH 2.9

Sephadex G-25 (Pharmacia) was swollen with several washings of distilled water and fines were removed by successive decantations. The gel was equilibrated with dilute acetic acid, pH 2.9. Columns of the gel were packed without application of external pressure and equilibrated under flow at 4° C for 48 hr. These columns measured approximately 100 × 1.0 cm. Even packing was tested by passing a blue Dextran 2000 (Pharmacia) solution (0.1%) through the columns. The evaporated residues were taken up in a minimum volume of dilute acetic acid (pH 2.9) containing 0.02% blue Dextran 2000, and the solution centrifuged at 1,200 *g* for 10 min at room temperature. The supernatants were applied to the columns and washed into the resin bed with three aliquots (0.2 ml.) of acid. Runs were carried out under the hydrostatic pressure of approximately 20 cm of dilute acetic acid. Fractions (2.0 ml.) were collected and monitored for absorbance at 280 nm. These fractions were combined on the basis of absorbance at 280 nm and evaporated under reduced pressure at approximately 20° C. Each residue was taken up in modified

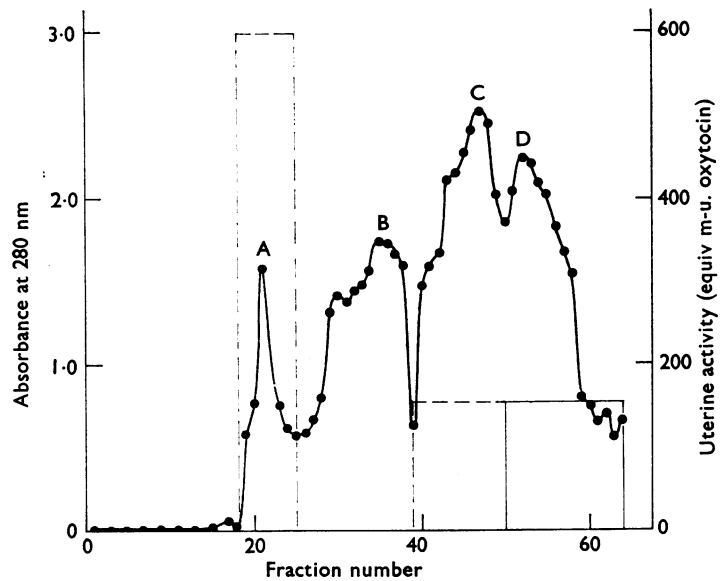

FIG. 1. Gel filtration of ox hypothalamus extract on a column (104 × 1.0 cm) of Sephadex G-25; the eluant was dilute acetic acid (pH 2.9). Histograms of rat uterus activity: ——, oxytocin/hypothalamus; - - - -, thioglycollate-resistant activity/hypothalamus. Blue Dextran 2000 (0.02%) is eluted in peak A. Flow rate, 0.212 ml./cm^2 per min.

Krebs solution (Munsick, 1960), and assayed for oxytocic activity before, and after, incubation with 0.01M sodium thioglycollate for 30 min. This treatment with thioglycollate was shown to inactivate oxytocin and vasopressin.

Gel filtration of Sephadex G-25 at pH 5.3

A similar procedure was used in these experiments. The buffer system was 0.05M ammonium acetate, pH 5.3.

Gel filtration on Sephadex G-50 at pH 2.9

Gel filtration of fraction C obtained from Sephadex G-25 gel filtration at pH 5.3 was carried out on 100 × 1.0 cm columns of Sephadex G-50 in dilute acetic acid at pH 2.9. Fractions (2.0 ml.) were collected and evaporated under reduced pressure at approximately 20° C. Each residue was taken up in magnesium-free Tyrode solution for assay on the isolated guinea-pig ileum and isolated rat uterus. A solution of the Sephadex G-25 fraction C material (Fig. 2) was assayed against synthetic oxytocin on the rat uterus, and was used as the reference standard.

Activity tests

Isotonic contraction of rat uterus. The method was similar to that described by Holton (1948). Rats were injected subcutaneously with 100 μg of stilboestrol in oil, 40 and 16 hr before death. A section of uterine horn, about 1.2 cm long, was suspended in a 2.5 ml. bath containing magnesium-free Krebs solution (Munsick,

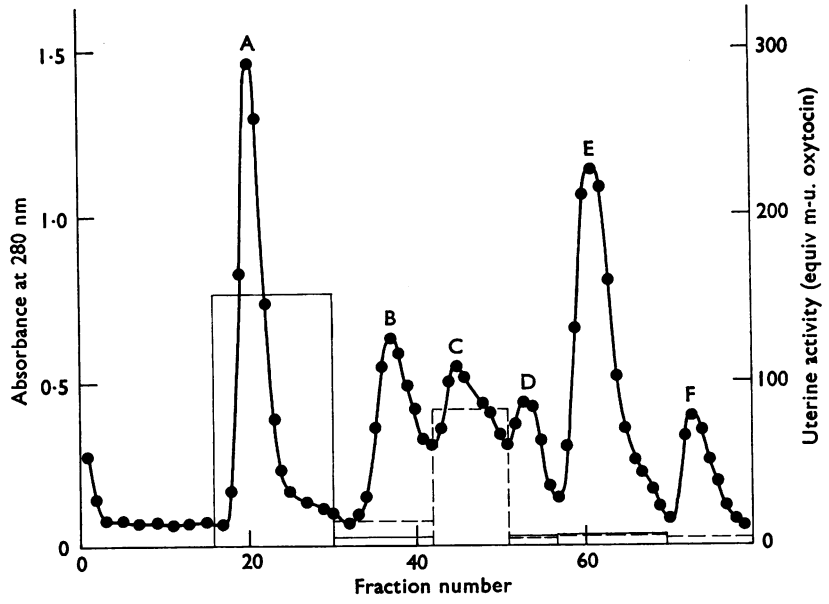

FIG. 2. Gel filtration of ox hypothalamus extract on a column (101 × 1.0 cm) of Sephadex G-25; the eluant was dilute ammonium acetate (pH 5.3). Histograms of rat uterus activity: ——, oxytocin/hypothalamus; - - - - -, thioglycollate-resistant activity/hypothalamus. Blue Dextran 2000 (0.02%) is eluted in peak A. Flow rate, 0.170 ml./cm² per min.

1960). The bath was aerated with 95% oxygen and 5% carbon dioxide, and the temperature was maintained at 28° C. Test doses were added at 3 min intervals and washed out by overflow. Logarithmic dose-response relationships were established for each substance on a number of tissues using three doses over eighteen responses. Linear relationships ($P<0.05$) were compared on each tissue for deviations from parallelism ($P<0.05$).

Atropine (1 μg/ml.) was administered by addition to the Ringer reservoir. The effects of phenoxybenzamine (0.1 μg/ml.) and mepyramine (1.0 μg/ml.) were measured after contact with the organ for 45 to 60 min. A frontal lever was used to give a 12-fold magnification, and responses were recorded on a smoked drum.

The method used in the studies with the potassium-depolarized uterus was the same as that described above, with the exception that sodium chloride was replaced by potassium chloride in the bathing fluid.

Guinea-pig ileum. The terminal ileum of guinea-pigs weighing 200–300 g was suspended in a 2.5 ml. bath containing magnesium-free Tyrode solution maintained at a temperature of 28° C. Test doses were added at 2 min intervals and washed out by overflow. Logarithmic dose-response relationships were established for each substance on a number of tissues in the same manner as that described for the rat uterus. The methodology used for studying the effects of drugs, the aeration of the bath and the recording apparatus were the same as for the rat uterus. On this preparation the effects of neostigmine (1 μg/ml.) on responses to various stimulants were studied.

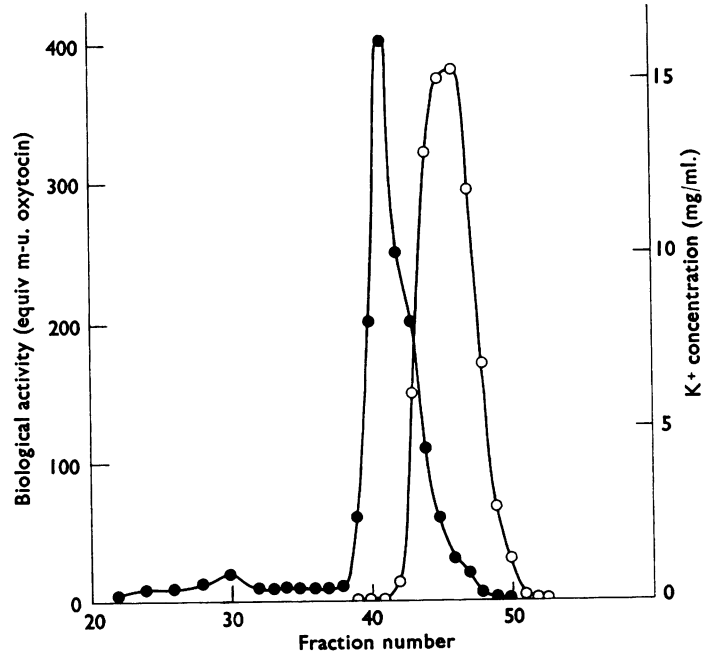

FIG. 3. Gel filtration of peak C material on a column (114 × 1.0 cm) of Sephadex G-50; the eluant was dilute acetic acid (pH 2.9). ●——●, Guinea-pig ileum activity; ○——○, potassium ion concentration. Flow rate, 0.427 ml./cm² per min.

Hen rectal caecum. The preparation of the isotonic hen rectal caecum was carried out as described by Cleugh, Gaddum, Holton & Leach (1961). Tissues were stored at 4° C for 18 hr before use. A length of tissue (about 1.5 cm) was suspended in a 2.5 ml. bath maintained at 28° C. Test doses were added at 4 min intervals and washed out by overflow. Logarithmic dose-response relationships were established for oxytocic substances and histamine, and compared in the manner described in the rat uterus studies. The action of atropine (1 μg/ml.) on the responses of the caecum to the substances was recorded.

Drugs

The following preparations were used: a synthetic oxytocin (Syntocinon, Sandoz); a synthetic bradykinin (in ampoules of 1 mg/ml., BRS 640, Sandoz); arginine-vasopressin (Pitressin, Parke-Davis); a synthetic angiotensin amide (l-L-asparaginyl-5-valyl angiotensin octapeptide, supplied as the solid in ampoules of 1 mg, Ciba). Acetylcholine chloride, atropine sulphate and histamine acid phosphate were supplied by British Drug Houses. 5-Hydroxytryptamine creatine sulphate and mepyramine maleate were supplied by May & Baker. Phenoxybenzamine hydrochloride was obtained from Smith, Kline & French Laboratories, and neostigmine methylsulphate from Mann Research Laboratories. All drug concentrations are expressed in terms of the salt.

Results

Sephadex G-25 gel filtration

The results of a typical separation of activities on Sephadex G-25 at pH 2.9 are shown in Fig. 1. Oxytocic activity, abolished by sample incubation with thioglycollate, was found in peak D. This activity is believed to be due to oxytocin, because synthetic oxytocin (Sandoz) was shown to have the same elution volume with respect to the external void volume on Sephadex G-25 in these conditions. The amount of activity recovered from this peak was approximately 100 m-u./hypothalamus.

Oxytocic activity, not reduced by sample incubation with thioglycollate, was found in peaks A and C. Peak A activity, which comprised almost half of the total activity of the extract, could be due to substance P (Meinardi & Craig, 1966). The activity in peak C was of special interest, because this has apparently not been described previously (North, Hawker & Zerner, 1968).

Gel filtration on Sephadex G-25 at pH 5.3 gave the pattern shown in Fig. 2. The activity due to oxytocin (abolished by sample incubation with thioglycollate) was eluted in peak A (Fig. 2). This is presumably due to the association of oxytocin with a protein at this pH (Sawyer, 1961; Ginsburg & Ireland, 1963). Only a trace of oxytocic activity not reduced by sample incubation with thioglycollate was found in peak A.

Peak C contained amounts of thioglycollate-resistant activity similar to those found in separations at pH 2.9. There is, however, a much better separation of the peak C active fraction at pH 5.3, because oxytocin is associated with a protein at this pH. Considerable concentrations of potassium and sodium ions are found in peak C.

Sephadex G-50 gel filtration

Figure 3 shows the results of a typical filtration of peak C activity (isolated on Sephadex G-25 at pH 5.3) on Sephadex G-50 at pH 2.9. The biological activity was eluted before potassium ion, but a considerable overlapping of the two occurred. The pharmacology of salt-free cuts of the active fraction C was investigated on three biological preparations. The salt-free active fraction will be referred to as fraction C′.

Activity tests

Isotonic contraction of rat uterus. Fraction C′ has a potent oxytocic action on the rat uterus. If left in the organ bath, it induces a series of contractions as shown in Fig. 5. A similar pattern of response was shown by oxytocin, bradykinin and angiotensin amide (Fig. 4). The time from administration of the substance to maximum contraction of the uterus was considerably different for a number of oxytocic

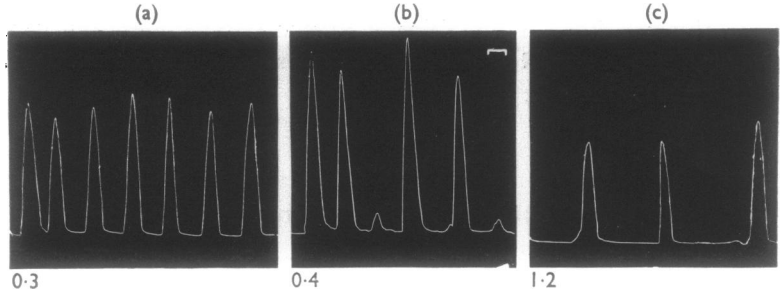

FIG. 4. Isolated rat uterus preparation suspended in 2.5 ml. of magnesium-free Krebs solution. Responses to (a) bradykinin (0.3 ng); (b) angiotensin amide (0.4 ng); (c) oxytocin (1.2 ng). Each substance induced a series of contractions when left in contact with the tissue. Drum speed, 0.12 mm/sec.

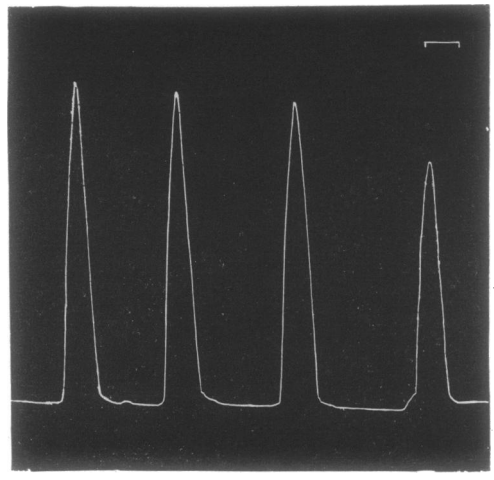

FIG. 5. Isolated rat uterus preparation suspended in 2.5 ml. of magnesium-free Krebs solution. Responses to fraction C′ left in contact with the tissue. The tissue used was the same as that used for Fig. 4. Drum speed, 0.12 mm/sec.

materials tested. The action of acetylcholine was most rapid (25–50 sec). The times for the other agents were as follows: 40–60 sec (bradykinin); 50–70 sec (angiotensin amide and vasopressin); 60–100 sec (oxytocin); 70–120 sec (fraction C′).

The general range of sensitivity of the uterus to the above substances was: 0.04–0.20 ng/ml. (angiotensin amide and bradykinin); 0.01–0.40 μg/ml. (acetylcholine and 5-hydroxytryptamine); 0.04–0.40 ng/ml. (oxytocin); and 0.10–1.0 μg/ml. (vasopressin). Some uterus preparations, however, gave no response to vasopressin less than 1.0 μg/ml.

The relationship between the height of uterine contraction and the logarithm of the dose for each substance over the range tested, in nearly all cases, showed no departure from linearity ($P<0.05$). The slopes of this relationship distinguished fraction C′ from 5-hydroxytryptamine, angiotensin amide and acetylcholine, but not from oxytocin ($P<0.05$). Logarithmic dose-response curves for bradykinin and for fraction C′ showed a significant deviation from parallelism in four of six experiments. Contractions induced by 5-hydroxytryptamine had a regression with dose significantly different from those of all other oxytocic principles in all uterine preparations tested.

Atropine abolished the action of acetylcholine and diminished contractions induced by 5-hydroxytryptamine by about 50%. It had variable, but approximately equal, effects on the responses of the uterus to bradykinin and to fraction C′; for some uteri, a 10–20% decrease in sensitivity to these substances occurred, while for others, a 3-fold increase in sensitivity was observed.

Incubation of the uterus for 45 to 60 min with phenoxybenzamine (0.1 μg/ml.) blocked the action of 5-hydroxytryptamine. Standard responses to bradykinin and to fraction C′ were reduced by approximately 20%, and the slopes of their dose-response relationships increased. Mepyramine (1.0 μg/ml.) increased contractions induced by bradykinin and fraction C′ by about 30%.

The potassium-depolarized uterus gave a graded response with dose to fraction C′, oxytocin and bradykinin as shown in Fig. 6. Histamine did not contract the isotonic rat uterus preparation.

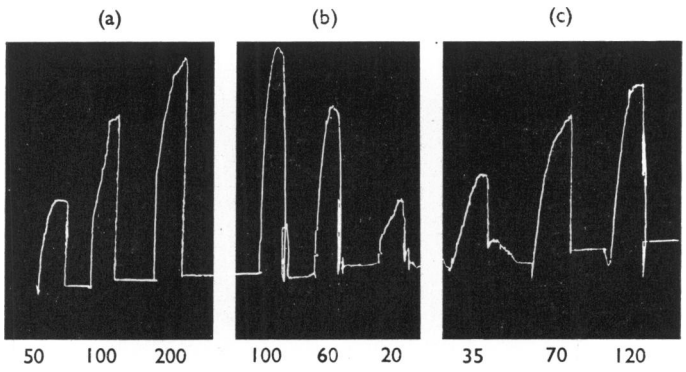

FIG. 6. Isolated potassium-depolarized rat uterus preparation suspended in 2.5 ml. of magnesium-free Krebs solution in which potassium chloride replaced sodium chloride. Responses to (a) bradykinin; (b) oxytocin; (c) fraction C′. Doses of bradykinin and oxytocin expressed as ng; fraction C′ in equivalent m-u. of oxytocin referred to the rat uterus. Drum speed, 0.12 mm/sec.

Isotonic contraction of guinea-pig ileum. Fraction C′ has almost the same potency on the ileum as on the uterus. It caused rapid contractions (6–8 sec), similar to those induced by acetylcholine and 5-hydroxytryptamine (7–9 sec). On the other hand, the actions of bradykinin and angiotensin amide on the ileum were slow (30–60 sec and 20–60 sec, respectively). Contractions induced by vasopressin were of an intermediate character (9–18 sec). Responses of the ileum to fraction C′, acetylcholine and bradykinin are shown in Fig. 7.

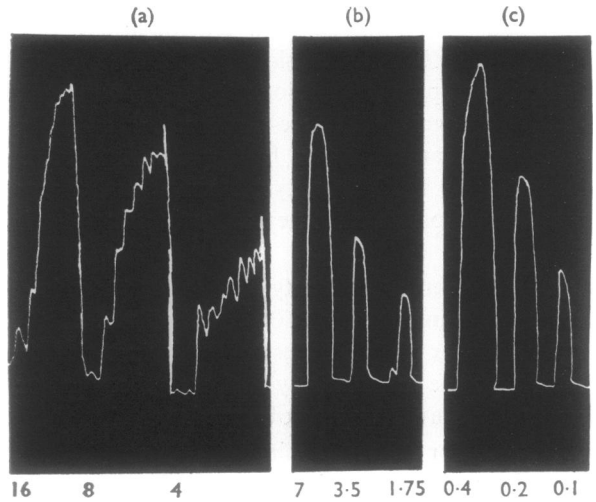

FIG. 7. Isolated guinea-pig ileum preparation in 2.5 ml. of magnesium-free Tyrode solution. Responses to (a) bradykinin; (b) fraction C′; (c) acetylcholine. Doses of bradykinin and acetylcholine expressed in ng; fraction C′ in equivalent m-u. of oxytocin referred to the rat uterus. Drum speed, 0.12 mm/sec.

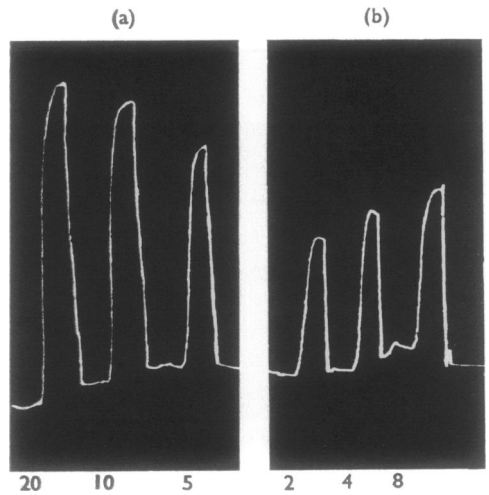

FIG. 8. Isolated hen rectal caecum preparation in magnesium-free Cleugh-Tyrode solution. Responses to (a) acetylcholine; (b) fraction C′. Doses of acetylcholine expressed in ng; fraction C′ in equivalent m-u. of oxytocin referred to the rat uterus. Drum speed, 0.12 mm/sec.

The general range of sensitivity of the ileum to these substances was: 0.8–8.0 ng/ml. (bradykinin); 0.04–4.0 ng/ml. (angiotensin amide); 0.01–0.4 ng/ml. (acetylcholine); 0.1–1.0 μg/ml. (5-hydroxytryptamine); 2.0–9.0 ng/ml. (vasopressin); and 0.02–1.0 μg/ml. (histamine). As much oxytocin as 1.0 μg/ml. failed to give a significant response on the ileum.

The relationship between the height of contraction of the ileum and the logarithm of the dose, for each substance over the range tested, showed no significant departure from linearity in almost all cases ($P<0.05$). The slopes of the regression lines of fraction C′, acetylcholine, 5-hydroxytryptamine, bradykinin and angiotensin amide were not significantly different ($P<0.05$). Contractions induced by histamine had a logarithmic dose regression significantly different from those of all other substances tested, including that of fraction C′.

Atropine blocked the action of acetylcholine in concentrations of less than 1.0 μg/ml., and reduced the ileal contractions induced by both fraction C′ and bradykinin by about 10%. The responses to 5-hydroxytryptamine were reduced by about 50% and those to angiotensin amide were reduced by about 80%. In the presence of neostigmine, the small reduction of the ileal responses to fraction C′ and bradykinin, caused by atropine, was reversed. On the other hand, the contractions induced by angiotensin amide were increased to twice the height of the responses to the same doses given by the ileum before incubation with atropine.

Mepyramine increased the height of ileal responses to fraction C′ by approximately 20%. It reduced the height of responses to acetylcholine, angiotensin amide and bradykinin by 30%, and responses to histamine by 70%.

Isotonic contractions of hen rectal caecum. This preparation is known to be relatively insensitive to kinins. Bradykinin caused graded contractions in the range 0.02–0.08 μg/ml., and angiotensin amide in the range 0.2–0.8 μg/ml.

The potency of fraction C′ on the caecum is almost the same as its potency on the rat uterus and guinea-pig ileum. The general range of sensitivity of the caecum was: 2.0–8.0 ng/ml. (acetylcholine and histamine) and 0.2–0.8 μg/ml. (5-hydroxytryptamine). Responses of the caecum to acetylcholine and fraction C′ are shown in Fig. 8.

The relationship between the height of contraction of the caecum and the logarithm of the dose, for each substance over the range tested, did not show a significant departure from linearity in the majority of cases ($P<0.05$). The slopes of the regression lines for fraction C′, acetylcholine, 5-hydroxytryptamine, histamine, bradykinin and angiotensin amide were not significantly different ($P<0.05$).

Atropine blocked the action of acetylcholine (at concentrations less than 1 μg/ml.) on the caecum, and reduced the responses of the organ to 5-hydroxytryptamine by about 50%. Standard responses to angiotensin amide were either reduced to one-tenth of their original height or were abolished by atropine, while responses induced by fraction C′ were reduced by about 30%. Atropine almost abolished responses of the caecum to histamine.

Discussion

The oxytocic activity of mammalian hypothalamic extracts has been attributed in the past to oxytocin, vasopressin, substance P, acetylcholine and 5-hydroxytryptamine (Pernow, 1953; Lederis, 1961). More recent studies, however, indicate that most of the oxytocic activity of crude substance P preparations obtained from this

source is due to another substance, possibly a peptide (Hawker, Blackshaw, Roberts & Goodricke, 1962 ; Cleugh, Gaddum, Mitchell, Smith & Whittaker, 1964). Indeed, Meinardi & Craig (1966) demonstrated that substance P-oxytocic action of a single Sephadex G-25 fraction was due to at least two peptides which could be separated by counter current distribution. The active fraction obtained in our studies (fraction C′) does not correspond to the fraction studied by Meinardi & Craig (1966).

Fraction C′ has the same order of potency on the rat uterus, guinea-pig ileum and hen rectal caecum. If the responses induced in these three organ preparations are due to a single substance, then it is neither acetylcholine nor histamine, because acetylcholine has a low potency on the rat uterus compared to its potencies on the other two preparations, and histamine does not contract the uterus.

Bradykinin and angiotensin amide are equipotent on the rat uterus and guinea-pig ileum, but the caecum has a relatively high threshold for both substances. Vasopressin is considerably more potent on the guinea-pig ileum than on the magnesium-free rat uterus, and oxytocin has little action on the gut preparations. Moreover, both the latter peptides have their action abolished by thioglycollate incubation. 5-Hydroxytryptamine, however, has similar potencies when tested on these three preparations and, hence, is not distinguished in these tests from fraction C′ activity.

The regression lines for 5-hydroxytryptamine, acetylcholine, and bradykinin on the rat uterus, and for histamine on the guinea-pig ileum, are sufficiently different from those for fraction C′ to attribute to them, at most, a partial role in the activities. The action of atropine on the oxytocic responses to fraction C′ is little different from its action on responses to bradykinin. As the concentration of atropine used was sufficient to inhibit responses of the uterus to acetylcholine, and to reduce responses to 5-hydroxytryptamine by half, it would seem that very little of the oxytocic activity of the extract is due to these substances. This conclusion is supported by the results obtained on incubation of the uterus with phenoxybenzamine. Concentrations which abolished contractions to 5-hydroxytryptamine reduced responses to fraction C′ and to bradykinin only by small (and similar) amounts.

The potentiation of uterine responses to fraction C′ by mepyramine might be explained as an action on histamine present in the extract. A similar potentiation was observed for responses to bradykinin, however, so no histamine need be present to account for this effect. Because fraction C′ gave graded contractions of the potassium-depolarized uterus, it would seem to have a mode of action similar to that of oxytocin and bradykinin.

Angiotensin amide has an action on the guinea-pig ileum which is chiefly indirect (the release of acetylcholine from Auerbach's plexus), and bradykinin has a direct action on the muscle fibre (Khairallah & Page, 1961). The results of atropine administration on responses of the ileum to these substances are in agreement with the above statement, although the small reduction of bradykinin responses by atropine suggests that a minor amount of the action of this peptide could result from the release of endogenous acetylcholine. The ileal responses to bradykinin, and to fraction C′, are reduced to the same extent. The effects of atropine demonstrate that acetylcholine, 5-hydroxytryptamine and histamine make little, if any, contribution to the ileum-stimulating activity of the extract.

Khairallah & Page (1961) have reported that incubation of the tissue with neostigmine returns to normal the ileal responses induced by angiotensin amide in the

presence of atropine. In our study, neostigmine incubation gave a large potentiation of responses to the peptide, although this occurred chiefly at higher doses. The ileal responses to bradykinin, and to fraction C′, returned to normal in the presence of neostigmine.

Atropine and neostigmine have little effect on contractions of the ileum induced by fraction C′, so this clearly indicates that a significant amount of angiotensin is not present in the extract.

The presence of atropine has little effect on contractions of the hen rectal caecum induced by fraction C′. The large reduction of the responses of the caecum to acetylcholine, 5-hydroxytryptamine, angiotensin amide, and histamine, induced by atropine suggests, therefore, that these substances play little part in this activity of the extract. The effects of atropine on the caecal responses to angiotensin amide are of interest. Atropine almost abolished these responses, indicating that angiotensin amide acts chiefly via the release of endogenous acetylcholine. Acetylcholine, however, had the same order of potency on the ileum and caecum, while angiotensin was approximately 1,000 times more potent on the ileum than on the caecum. This implies that most of the angiotensin is destroyed, or its action is blocked, before it reaches the nerve plexus in the caecum, by a substance (or substances) which is not present in the ileum. These observations could also suggest that atropine is not a specific antagonist of acetylcholine receptors in the hen rectal caecum.

Fraction C′ possesses similar pharmacological properties to crude preparations of substance P (Bisset & Lewis, 1962). Purified substance P, however, is almost 100 times more active on the isotonic guinea-pig ileum than on the rat uterus (Zuber & Jaques, 1962). Moreover, the elution of substance P from Sephadex G-25 in similar conditions is considerably different (Meinardi & Craig, 1966). The elution of fraction C′ from Sephadex G-25 suggests that it is a small molecule, but its elution from Sephadex G-50 demonstrates that adsorption by the gel is affecting its behaviour on Sephadex.

Although the pharmacological tests carried out fail to demonstrate that bradykinin does not contribute to the rat uterus and the guinea-pig ileum activities of fraction C′, the behaviour of bradykinin on Sephadex has been shown in our laboratories to be considerably different.

The activities of fraction C′ on the three organ preparations are believed to be due to a single substance. This seems to be a peptide of low molecular weight. The physiological role of this substance has not as yet been evaluated because sufficient material has not been accumulated. The isolation of the substance in quantity sufficient for chemical characterization is being actively pursued in this laboratory.

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