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H_1-histamine receptors may mediate the contractile response of guinea-pig ileum to 'histamine-free' splenic extracts

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1 A water-soluble splenic factor, which produces a contractile response of the guinea-pig ileum, that is resistant to cholinoceptor and adrenoceptor antagonists is described.

2 The ileal contractions elicited by the splenic extract showed some significant differences from those elicited by 5-hydroxytryptamine. The responses to splenic extract were not affected by the D-tryptamine-receptor antagonist, methysergide.

3 The effect of the splenic extract on the guinea-pig ileum was similar to that of histamine. The H_1-histamine antagonists, (+)-chloropheniramine and diphenhydramine, caused a parallel shift to the right of the splenic extract dose-response curve without suppression of the maximum response. A pA_2 value of 8.97 ± 0.03 for (+)-chloropheniramine and 7.55 ± 0.1 for diphenhydramine was calculated.

4 Significant histamine levels, as determined by fluorometric methods, could not be detected in the splenic extract. Likewise, the splenic factor did not release histamine from the intestinal preparation.

5 These results support the view that: (i) the splenic factor acts through H_1-histamine receptors; (ii) it is not histamine; (iii) it does not have any histamine releasing effect on the ileal smooth muscle.

Introduction

Since Gandarias (1959) obtained water-soluble splenic material with striking activity on smooth muscle, there has been a continued interest in this smooth muscle-stimulating agent derived from the splenic tissue of rats and rabbits. Further experiments have demonstrated a similar activity on smooth muscle with extracts of bovine spleen (Ainz, 1974; Gandarias, Ainz, Fernandez, Goiriena, Lacort & Rabanal, 1978; Gil-Rodrigo, 1981). This activity has been shown to persist after treatment with α- and β-adrenoceptor blocking agents or muscarinic cholinoceptor antagonists, such as atropine (Gonzalez, 1970; Gandarias, 1971; Ainz, 1974; Gandarias *et al.*, 1978; Gil-Rodrigo, 1981).

The aim of this paper has been to search for mechanisms other than adrenergic or cholinergic, to explain the effects of these splenic extracts. Our attention has been focused mainly on possible histaminergic or tryptaminergic mechanisms. The presence of D-tryptamine and H_1-histamine receptors in the guinea-pig ileum is well-documented (Gaddum & Picarelli, 1957; Ash & Schild, 1966). Specific agonists for both types of receptors have been shown to induce contraction in this preparation (Gaddum & Picarelli, 1957; Ash & Schild, 1966; Costa & Furness, 1979a, b). Since the results obtained in the guinea-pig ileum with splenic extracts in the presence of specific antagonists for H_1-histamine receptors showed consistent inhibition, further research was carried out which revealed that this splenic factor was not histamine and was devoid of any histamine releasing effect on the ileum.

Methods

Preparation of tissue extracts

Extracts were obtained from fresh bovine spleen. The raw material was bruised and salted out by addition of 5 ml of 0.3 N NaOH and 5 ml 5% $ZnSO_4$ solution (Ashwell, 1957) per g of wet tissue. The material was then homogenized in a MSE ultrasonic homogenizer (6 kHz × 3 min) and centrifuged at

5200 g for 5 min. The precipitate was discarded and the supernatant collected and stored at 2–4°C. Under these conditions the extract remained active for at least one week. When lyophilized, the activity remained unchanged for several months.

Pharmacological assays

Guinea-pigs of either sex weighing about 400 g were stunned by a blow on the back of the head and exsanguinated. The terminal ileum was quickly dissected out and transferred to a bath containing atropinized Tyrode solution at 37°C. The Tyrode solution had the following composition (g/l): NaCl 8, KCl 0.2, $CaCl_2.H_2O$ 0.18; $MgSO_4.7H_2O$ 0.26, $NaH_2PO_4.2H_2O$ 0.013, $NaHCO_3$ 1.0, glucose 1.0 and atropine sulphate 0.002. After removal of mesenteric attachments, pieces of ileum about 25 mm in length were mounted along their longitudinal axis in a muscle chamber (20 ml volume) containing Tyrode solution at 37°C continuously bubbled with 95% O_2 and 5% CO_2. Isotonic contractions of the ileum were recorded after allowing a period of 30 min for equilibration. A linear isotonic transducer at a tension of 0.5 g (Ainz, 1974) connected to a Hewlett-Packard 7754A multi-pen recorder was used.

Experiments ($n = 30$) carried out in order to determine the influence of the chemicals used in the treatment of the splenic material during the extraction procedure showed that there were no significant differences in the basal motility of the ileum before and after addition of the pooled extracting reagents.

For construction of dose-response curves, the agonists were added cumulatively (except in the case of 5-hydroxytryptamine), to the organ bath using the geometric sequence of concentration 1; 2; 4; 8; etc. The dose producing a 50% maximal effect (ED_{50}) was estimated graphically from dose-response curves.

Dose-ratios (DR) were estimated as the ratio of the ED_{50} in the presence of antagonist compared with the control ED_{50}. The pA_2 values were calculated using the equation: $pA_2 = pAx + \log (DR - 1)$ (Van Rossum, 1963).

The antagonists were added at least 5 min before the agonists were tested.

Drugs used were: histamine dihydrochloride (Sigma) (H), 5-hydroxytryptamine creatinine sulphate (Sigma) (5-HT), (+)-chloropheniramine maleate (Schering), diphenhydramine hydrochloride (Substancia) and methysergide bimaleate (MSD) (Sandoz). All doses given in the text are expressed as final molar (M) bath concentrations except for doses of splenic extract (SE), which are given as mg of fresh tissue per ml of saline in the bath.

Biochemical assays

The histamine concentration in the splenic extract and in nutrient solution bathing the ileal smooth muscle were measured by the fluorometric method described by Udenfriend (1962).

To measure the fluorometric recovery, histamine, at concentration of 0.16 μg/ml (similar to one of the standard samples which was taken as 100%), was added to several samples of SE and the nutrient solution. In all cases the recovery was close to 60%. The values given in the text have been adjusted accordingly.

Statistical evaluation

The results are expressed as means ± s.e.mean for the number of experiments indicated (n). Significant differences between means were calculated by Student's t test.

Results

Agonist assays

The addition of increasing doses of SE (1, 2, 4, 8 etc. mg/ml) caused graded contractions of the guinea-pig ileum in a dose-related manner (Figures 1 and 2). The effect of SE was compared to that of 5-HT and histamine in concentrations of 0.5, 1, 2, 4 etc. $\times 10^{-5}$ M and 10^{-7} M respectively (Figure 2). The ileal responses to SE showed some significant differences from those obtained with 5-HT. (i) The effect of 5-HT was partially blocked by atropine, whereas the smooth muscle response to SE was not. (ii) The guinea-pig ileum developed tachyphylaxis to 5-HT but not to SE. On the other hand, the effect of SE was

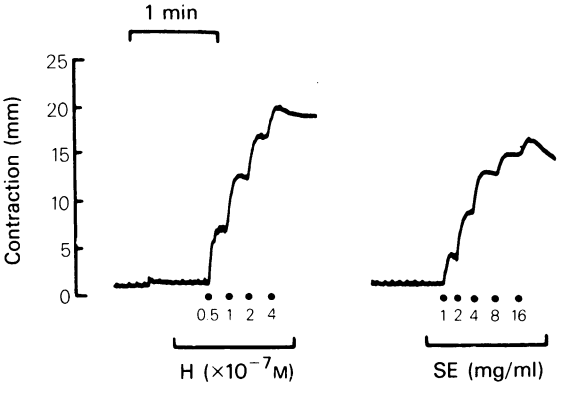

Figure 1 Responses of the guinea-pig ileum to graded doses of splenic extract (SE) and histamine (H).

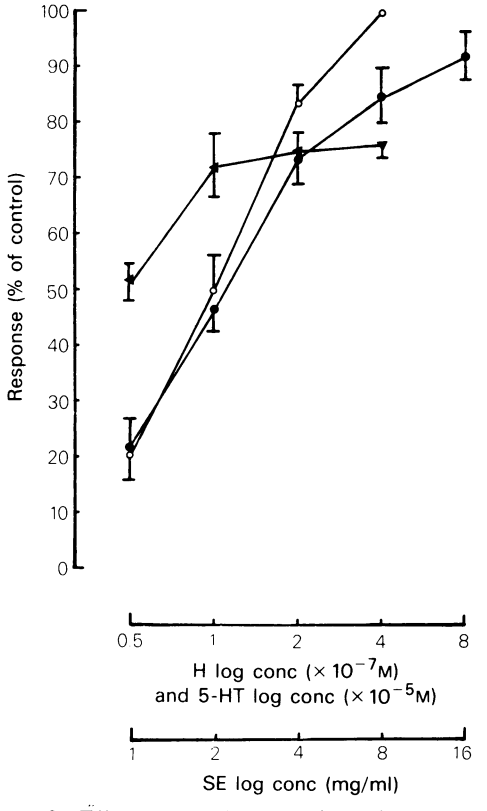

Figure 2 Effects of splenic extract (SE, ●), histamine (H, ○) and 5-hydroxytryptamine (5-HT, ▲) on the guinea-pig ileum. Each point represents the mean of at least three values. Vertical lines indicate s.e.means.

similar to that of histamine. A comparison of the contractions to SE and histamine is shown in Figure 1.

The dose-response curves to histamine and SE (Figure 2) show that near maximal responses were reached with histamine at a concentration of 4×10^{-7} M and SE at a concentration of 16 mg/ml. The maximum response to histamine (4×10^{-7} M) was taken as control, 100%. The effects of different doses of SE and 5-HT were expressed as percentages in relation to this control.

The data in Figure 2 were used to determine ED_{50} values and to evaluate the intrinsic activity of SE with reference to histamine. Concentrations of SE and histamine required to produce half-maximal (ED_{50}) responses of ileum were: 2.17 ± 0.2 mg/ml and $0.97 \pm 0.08 \times 10^{-7}$ M, respectively, and therefore 2.17 mg of fresh tissue contained a concentration of active factor with approximately the same activity as histamine 1×10^{-7} M. The intrinsic activity of SE estimated as the ratio between the maximum effect of SE and that of the histamine (Van Rossum, 1963) was: 0.92.

Antagonist assays

Effects of methysergide 5-HT at a concentration of 1×10^{-5} M produced responses of the ileum similar to those obtained with the intermediate dose of 4 mg/ml of SE (Figures 2 and 3). Doses of MSD (2.5×10^{-6} M) which completely abolished responses to 5-HT (10^{-5} M) did not inhibit the response to SE (4 mg/ml) (Figure 3). The amplitudes of contraction to SE (4 mg/ml) expressed as a % of control (see above) were: 74 ± 4.04, (5) in the absence of MSD, and 72.9 ± 3.31, (5) in the presence of MSD. Contractions elicited by SE (4 mg/ml) were not significantly affected by MSD.

Effects of (+)-chloropheniramine and diphenhydramine The H_1 antagonists, (+)-chloropheniramine and diphenhydramine, in concentrations of 0.5, 1 and 2×10^{-9} M for (+)-chloropheniramine and 0.5, 1, 2 and 4×10^{-8} M for diphenhydramine, caused a paral-

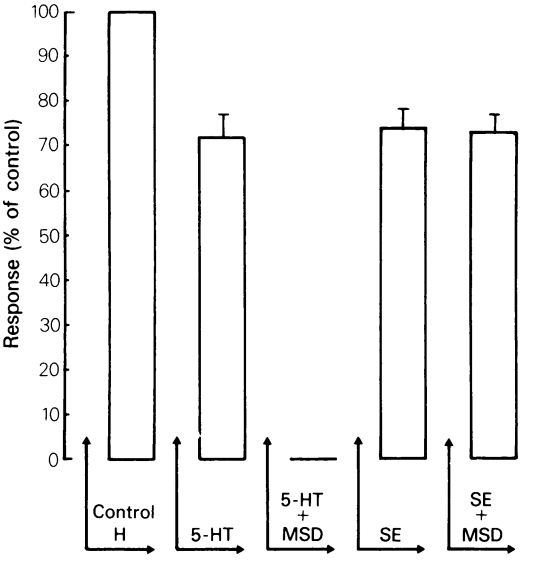

Figure 3 Effects of methysergide (MSD, 2.5×10^{-6} M) on responses of the guinea-pig ileum to 5-hydroxytryptamine (5-HT, 10^{-5} M) and splenic extract (SE, 4 mg/ml). The responses to 5-HT and SE are expressed as percentages relative to the maximum response obtained in each experiment for histamine (H, 4×10^{-7} M), which was taken as (control) 100%. The heights of the columns indicate the means obtained from at least five experiments with the s.e. given by the vertical lines.

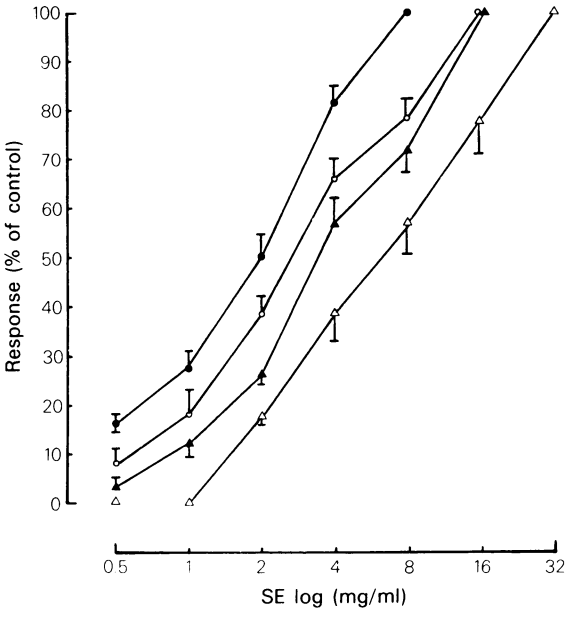

Figure 4 Effects of (+)-chloropheniramine on the dose-response curve to splenic extract (SE): (●) control; (+)-chloropheniramine (○) 0.5×10^{-9}M; (▲) 1×10^{-9}M; (△) 2×10^{-9}M. Each point represents the mean of at least four values; vertical bars shows s.e.mean.

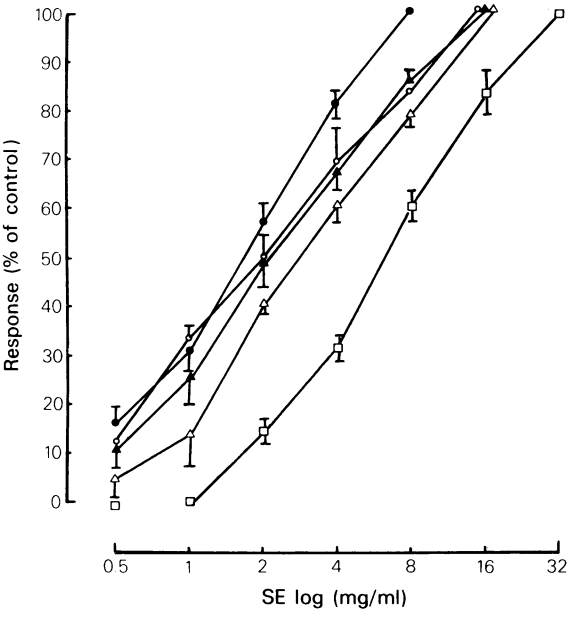

Figure 5 Effects of diphenhydramine on the dose-response curve to splenic extract (SE): (●) control; diphenhydramine (○) 0.5×10^{-8}M; (▲) 1×10^{-8}M; (△) 2×10^{-8}M; (□) 4×10^{-8}M. Each point represents the mean of at least three values; vertical bars indicate s.e.mean.

lel shift to the right in the dose-response curve to SE with no suppression of the maximum response (Figures 4 and 5). The apparent pA_2 values for the antagonism of (+)-chloropheniramine and diphenhydramine to SE were: 8.97 ± 0.03 and 7.55 ± 0.1. These pA_2 values were not significantly different, under our experimental conditions, from those obtained with histamine as agonist: 8.98 ± 0.1 for (+)-chloropheniramine and 7.61 ± 0.05 for diphenhydramine. The relationships between log (dose-ratio -1) of SE and $-\log H_1$ antagonist concentration had slopes of 1.034 ± 0.095 for (+)-chloropheniramine and 1.22 ± 0.12 for diphenhydramine which were not significantly different from 1 ($P < 0.05$), being compatible with an antagonism of a competitive nature. These results are consistent with the involvement of H_1-receptors in the guinea-pig ileum responses to SE.

Histamine concentration determination

Splenic extracts When the SE was assayed fluorometrically to detect the histamine content, a mean value (μg/ml) of 0.0117 ± 0.0007, $n = 41$ (from 6 different spleens) was obtained. According to the weight/volume proportion given in the extraction procedure (see above): 1 g wet tissue per 10 ml, the histamine level detected fluorometrically represents the amount in 100 mg of fresh tissue. The dose-response curves to SE, in the agonist assays, were carried out using a dose-range from 1 to 16 mg/ml, which is between 100 to 6 times smaller than the dose of SE assayed fluorometrically. Therefore, the histamine content, estimated in the dose of SE which produced the half-maximal response (ED_{50}) was approx. 75 times smaller than that of histamine required to obtain the same half-maximal response. Thus it seems unlikely that the active factor present in SE, is histamine.

Nutrient solution The histamine content of several nutrient solution samples was analysed in 5 assays with pieces of ileum from different animals, to study a possible histamine releasing mechanism. The concentration of histamine detected was very low, in the order of 0.0065 ± 0.0006 μg/ml ($n = 29$) before the addition of SE (4 mg/ml) and 0.0063 ± 0.0006 μg/ml ($n = 28$) after it. These concentrations are close to the lower limit of linearity of the fluorometric method used and therefore the histamine levels measured should not be taken as absolute values. The histamine values detected before and after SE administration were not significantly different.

Discussion

Although splenic extracts have sometimes been used in therapeutics, their pharmacological activity, particularly with respect to its contractile action on smooth muscle, was relatively unknown. In 1948 Salva made reference to the therapeutic use of water-soluble splenic extracts in some forms of intestinal atony. Salva (1948) and later Krontil & Klabusay (1961) described a vasodilator and hypotensive effect of the splenic extracts in several species. This effect was blocked by atropine and other similar agents. However, the splenic extract obtained by Gandarias (1959) preserves its effectiveness in the presence of atropine (Gonzalez, 1970; Gandarias, 1971; Ainz, 1974; Gandarias *et al.*, 1978; Gil-Rodrigo, 1981). In addition, its activity on smooth muscle was not blocked by α- and β-adrenoceptor antagonists (Gonzalez, 1970; Gandarias, 1971; Ainz, 1974).

In this paper we have attempted to study the possible involvement of tryptaminergic and histaminergic mechanisms in the contractile response of the ileal smooth muscle brought about by the water-soluble splenic extracts. From our results it can be concluded that the H_1-histamine receptors mediate this contractile response. This statement can be interpreted in several ways, three of which are considered below: (i) the extract contains a sufficient level of histamine which mediates the smooth muscle contraction; (ii) the addition of the splenic extract to the nutrient bath solution induces a release of histamine from the piece of ileum, which acts on the H_1-receptors and triggers the contractile response; (iii) the extract contains a substance different from histamine with a molecular configuration able to interact with the H_1-receptors inducing contraction of the ileal smooth muscle. The first two hypotheses seem unlikely. As can be seen from the results, the extract contains an insignificant amount of histamine and it appears improbable that the contractile response could be elicited by a release of endogenous histamine from the ileal preparation through stimulation by the splenic extract. Therefore, the third possibility seems to be the most likely.

Brown, Drum & Hollenberg (1977) have described the presence of a water-soluble product in the rabbit renal cortex active on certain smooth muscle preparations. This factor seems to be a thermostable low molecular weight (700 daltons) product. These authors have discarded the possibilities that it could be a prostaglandin, renin, angiotensin, catecholamine, 5-HT, histamine, bradykinin, a nucleotide, a small organic product of local metabolism or an ion. By analogy with Brown's description (1977) the active factor in our splenic extract is water-soluble, thermostable and its molecular weight, calculated by elution on Sephadex G-15, is less than 1000 daltons (Ainz, 1974). It also seems probable that the active factor in the splenic extracts is not a prostaglandin, given the extraction conditions and that the treatment of the extract with liposolvents does not modify its activity (Gandarias, 1971). Neither is it a catecholamine, because its activity is not affected by α- and β-adrenoceptor antagonists (Gonzalez, 1970; Gandarias, 1971; Ainz, 1974). It cannot be acetylcholine, because it is unaffected by atropine (Ainz, 1974; Gandarias *et al.*, 1978; Gil-Rodrigo, 1981), nor 5-HT, because it is resistant to methysergide (in the present work), nor histamine because the histamine level detected is too low to explain its activity (in the present work). It does not appear to be a polypeptide, given its thermostability (Gandarias *et al.*, 1978) and its negligible content of proteins (less than 1%; unpublished observation), or an ion because the extract desalted by filtration on Sephadex gels still remains active (Ainz, 1974); it is not a nucleotide like cyclic AMP or cyclic GMP because the extract does not contain significant amounts of cyclic GMP (Gil-Rodrigo, 1981) and the level of cyclic AMP detected (Gil-Rodrigo, 1981) is able to produce only a very weak and occasional relaxant effect on the guinea-pig ileum (unpublished observation).

Despite the obvious similarities between the extract described by Brown *et al.* (1977) and our splenic extract, some differences should be noted: (i) the extraction procedure is different; (ii) extracts of spleen, lung and heart obtained by Brown *et al.* (1977) have low activity and can be blocked by methysergide, in the case of spleen, and by atropine, in the case of heart and lung. However, using the extraction procedure of Gandarias (1959), extracts with similar characteristics to those of spleen can be obtained even if the original organ is the lung or the pancreas; (iii) the α-adrenoceptor antagonist, phenoxybenzamine blocks the active factor of Brown *et al.* (1977) but not the splenic factor of Gandarias (1971). On the other hand, diphenhydramine, an H_1-receptor antagonist, inhibits the action of our product but not that of Brown *et al.* (1977).

All these considerations suggest the interesting possibility that a still unidentified amine is involved. Further experiments are needed to identify this factor with marked activity on smooth muscle.

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