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THE ACTION OF SUBSTANCE P ON CONTRACTION, INOSITOL PHOSPHOLIPIDS AND ADENYLATE CYCLASE IN RAT SMALL INTESTINE

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Abstract—The actions of substance P and eledoisin on contraction, [^{3}H]inositol 1-phosphate and cAMP formation in the rat ileum have been compared. Eledoisin was considerably more potent than substance P on both contraction and [^{3}H]inositol 1-phosphate production. Neither peptide altered the cAMP levels in the tissue. These results are discussed in relation to the substance P receptor sub-type present in the rat ileum, and its second messenger.

Two distinct rank orders of potency of substance P and its structural analogues have been observed on contraction of peripheral smooth muscles, and the suggestion has therefore been made [1], and subsequently supported [2], that these results reflect the existence of two receptor sub-types for substance P. On SP–P systems, substance P acts in nanomolar concentrations and is approximately equipotent with eledoisin and substance P methyl ester, while on SP–E systems, substance P acts in micromolar concentrations and is 10–100 times less potent than eledoisin, but approximately 100 times more potent than substance P methyl ester [2].

Substance P, eledoisin and substance P methyl ester have also been shown to have similar potencies on inositol phospholipid hydrolysis in rat parotid gland [3], rat hypothalamus and guinea-pig ileum longitudinal smooth muscle [4], indicating that this event is associated with SP–P receptor activation [4]. This is in accordance with the observation that guinea-pig ileum contraction [1] and rat salivation [3] both appear to be mediated through SP–P receptors.

The present study has been undertaken to investigate the second messenger associated with substance P receptor activation in a SP–E system. If the second messenger system in SP–E systems is not inositol phospholipid hydrolysis, this would provide further support for the contention that SP–P and SP–E systems contain distinct substance P receptor sub-types. The tissue chosen for this study was the rat ileum.

MATERIALS AND METHODS

In vitro *bioassays*. Male Sprague–Dawley rats (180–350 g) were stunned and decapitated. The entire length of the rat ileum was dissected out into freshly gassed (95% O_2:5% CO_2) Krebs solution (composition in mM: NaCl 127, KCl 2.5, $CaCl_2$ 1.8, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 10), and the luminal contents washed away with approximately 40 ml Krebs. Rat ileum longitudinal smooth muscle strips were prepared by placing a 3–4 cm portion of tissue over a glass rod, followed by gently teasing the longitudinal muscle layer away with a piece of moist cotton wool. Tissues were then mounted in 2 ml organ baths, and used as previously described [2]. Atropine (1×10^{-5} M) was present throughout. Full dose response curves to eledoisin and substance P were determined on the same segment of tissue using at least seven different concentrations of each agonist.

Inositol phospholipid breakdown. These experiments were carried out essentially as previously described [4]. Briefly, rat ileum longitudinal smooth muscle strips were cross-chopped at 350 μm on a MacIlwain tissue chopper, and washed with gassed Krebs solution over the next 20 min. They were then pre-labelled with [^{3}H]inositol (8 μCi/ml) in a shaking water bath for 2 hr at 37°. The tissue was then washed several times over the next 60 min to remove [^{3}H]inositol; the final washes were performed with Ca^{2+} free Krebs containing EGTA (0.5 mM) at 37°. The tissue slices were then allowed to settle under gravity, and 25 μl portions were transferred to small vials containing 215 μl of Ca^{2+} free Krebs with EGTA (0.5 mM) and Li^+ (10 mM) present. Li^+ blocks the hydrolysis of inositol 1-phosphate to free inositol [5]. The Ca^{2+} free medium blocks possible indirect effects mediated through the release of other neurotransmitters (see [4]). Agonists (10 μl) were added, and the tubes gassed, capped, and replaced into the shaking water bath for 30 min. The reaction was then stopped by addition of 0.94 ml chloroform/methanol (1:2). Aliquots (750 μl) of the upper phase were taken, and [^{3}H]inositol 1-phosphate was separated from other labelled water soluble metabolites by ion exchange chromatography on Dowex-1 anion exchange columns as previously described [4]. Aliquots (400 μl) of the lower phase were also taken, the chloroform evaporated off, and [^{3}H]inositol phospholipids measured by scintillation counting.

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Experiments were performed in duplicate or triplicate.

Experiments performed on the mouse urinary bladder were carried out in the same manner, with the exception that Ca^{2+} Krebs was used throughout.

Adenylate cyclase assays. These were carried out on cross-chopped slices of rat ileum longitudinal smooth muscle, and also on homogenates of this preparation.

The experiments on tissue slices were carried out as described by Quik *et al.* [6]. The rat ileum longitudinal smooth muscle was cross-chopped at 350 μm on a MacIlwain tissue chopper, and the slices incubated at 30° for 20–30 min with frequent washings. The slices were then allowed to settle under gravity, and 25 μl portions were transferred into small tubes containing 215 μl Krebs. Agonists (10 μl) were added, and the tubes gassed, capped and placed in a shaking water bath at 30° for 10 min. The tubes were subsequently transferred to a boiling water bath for 3 min, and then centrifuged. Experiments were performed in triplicate.

Experiments carried out on homogenized tissue were performed as described by Clement-Cormier *et al.* [7]. Tissue was homogenized in 15 vol. (w/v) of 2 mM Tris–malcate (pH 7.4) containing 2 mM EGTA. The final incubation mixture contained 50 μl of tissue, 80 mM Tris–maleate (pH 7.4), 0.5 mM ATP, 2.0 mM $MgSO_4$, 10 mM theophylline, 0.6 mM EGTA and various test substances in a volume of 500 μl. All components of the mixture, except ATP, were incubated on ice for 20 min. The reaction was then initiated by addition of ATP, and the tubes transferred to a shaking water bath at 30° for 5 min, before boiling for 3 min followed by centrifugation. Experiments were performed in triplicate.

The concentration of cAMP in the supernatant was estimated using a cAMP radioimmunoassay kit (New England Nuclear).

Analysis of dose response curves. Data from individual dose response curves have been pooled and fitted to the following Hill equation:

$$Y = \frac{\text{max. } D^n}{D^n + EC_{50}{}^n}$$

where Y is the fraction of occupied receptors, max. the maximal response, D the concentration, n the Hill slope and EC_{50} the concentration required to yield 50% of the max.

This equation was fitted using a modified Marguardt approach as implemented in the Harwell library routine VB01A on the Cambridge IBM 3081, with max., n and EC_{50} as unknowns. Each point was weighted according to the reciprocal of its variance. Statistical indications were made using a Student's t-test.

Metabolism studies. The stability of eledoisin and substance P in the lipid studies were compared using the guinea-pig ileum as a bioassay. An initial dose response curve to eledoisin and substance P on the guinea-pig ileum was determined. Samples were then tested on the guinea-pig ileum, and the response observed titrated back to the apparent concentration of peptide in the sample through the use of the appropriate dose response curve. It is important to note that in these studies bioactive metabolites of substance P and eledoisin will also contribute to the activity observed. Experiments were performed in triplicate.

Materials. Theophylline, isoprenaline sulphate, GTP and ATP were purchased from Sigma, Poole. Forskolin was purchased from Calbiochem. The cAMP radioimmunoassay kit was obtained from New England Nuclear. All other materials were obtained from previously described sources [4].

RESULTS

Contractile studies

There was no significant variation in the ability of eledoisin and substance P to elicit contraction along the length of the rat ileum (Fig. 1a). Therefore, the entire length of the rat ileum has been used to prepare longitudinal smooth muscle strips for use in studying the action of substance P on inositol phospholipid and cAMP levels in the tissue (see below).

The dose response curves to substance P and eledoisin for contraction of the rat ileum had similar maximal responses, but the curve for eledoisin was significantly steeper than that for substance P (Table 1; Fig. 1b). Eledoisin was 5.7 times more potent than substance P in eliciting contraction of the rat ileum using their EC_{50} values as reference (Table 1).

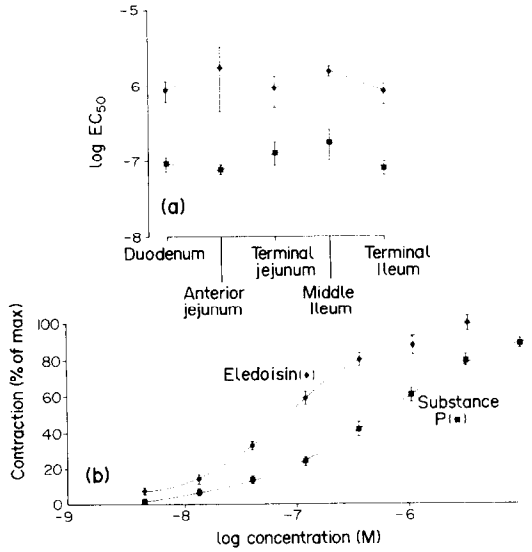

Fig. 1. Absolute potency values of substance P and eledoisin along the length of the rat ileum. (a) Regional sensitivity of rat ileum to substance P and eledoisin. The ileum was divided into regions according to Holzer *et al.* [8]. Full dose response curves were determined to substance P and eledoisin on the same tissue, using at least seven doses of each agonist. Dose response curves were fitted by eye; each point represents the mean ± S.E.M. of three experiments. Atropine (1×10^{-5} M) was present throughout. ■, eledoisin, ◆, substance P. (b) Dose response curves to substance P and eledoisin on rat ileum. The data have been pooled from above; therefore each point represents the mean ± S.E.M. of 15 determinations, i.e. N = 3 for each of the five regions described above. The dose response curves have been fitted to a Hill equation as described in Materials and Methods.

Table 1. EC_{50} values and Hill slopes for substance P and eledoisin on various responses in the rat ileum

Tissue	Response	Peptide	N	EC_{50} value (M)	Hill slope	Potency relative to substance P
Rat ileum	Contraction	Substance P	15	$5.4 \pm 0.7 \times 10^{-7}$	0.75 ± 0.03	1
		Eledoisin	15	$9.5 \pm 1.0 \times 10^{-8}$	0.92 ± 0.05*	5.7
Rat ileum longitudinal muscle	Inositol phospholipid hydrolysis	Substance P	8	$1.5 \pm 0.7 \times 10^{-7}$	0.76 ± 0.15	1
		Eledoisin	7	$4.8 \pm 2 \times 10^{-9}$	1.36 ± 0.42†	31

Dose response curves have been generated using at least seven doses of each agonist, and data have been pooled from all experiments and fitted to a Hill equation as described in Materials and Methods. Results are represented as means ± S.E.M. Relative potencies have been determined using the EC_{50} values as reference.

* $P < 0.05$.

† Variances significantly different ($P < 0.05$).

Inositol phospholipid hydrolysis

On pre-labelled rat ileum longitudinal smooth muscle slices in Ca^{2+}-free Krebs, a maximally effective dose of substance P (30 μM) produced a 86.9 ± 13.6% (N = 17) increase in the level of [^{3}H] inositol 1-phosphate above basal during a 30-min incubation period. No significant change in [^{3}H] inositol phospholipids was observed (1.8 ± 4.8% increase above basal; N = 17), and so unequal division of tissue could be corrected relative to the lipid levels.

The dose response curves for substance P and eledoisin on [^{3}H]inositol 1-phosphate accumulation in rat ileum longitudinal muscle slices appeared to possess the same maximal responses although the curve for eledoisin was steeper than that for substance P (Table 1; Fig. 2). Eledoisin was estimated to be 31 times more potent than substance P using their EC_{50} values as reference. These EC_{50} values for substance P and eledoisin on inositol phospholipid hydrolysis were approximately one order of magnitude lower than the corresponding values on contraction (Table 1).

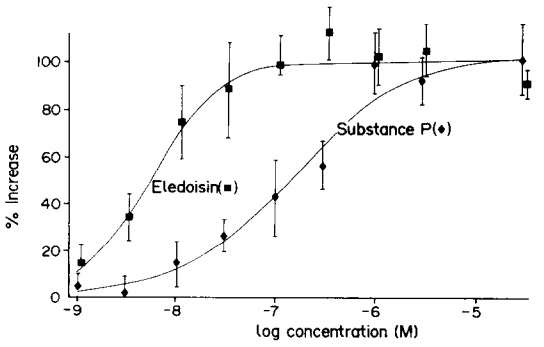

Fig. 2. Dose response curves to substance P and eledoisin on [^{3}H[inositol 1-phosphate accumulation in rat ileum longitudinal muscle slices. Experiments were carried out on pre-labelled tissue in Ca^{2+} free Krebs containing 0.5 mM EGTA. Results are expressed relative to the response elicited by 30 μM substance P (= 100%) and represent the mean ± S.E.M. of seven to eight experiments. Lines have been fitted to a Hill equation as described in Materials and Methods.

The higher potency of eledoisin relative to substance P on inositol phospholipid hydrolysis compared with contraction may be due, at least in part, to a greater stability of eledoisin in the lipid studies. At the end of the 30-min experimental period, aliquots of the incubation fluid from slices exposed to 3 μM eledoisin or 3 μM substance P had 80 ± 2.5% and 23 ± 4.6% respectively (N = 5 in both cases) of their original bioactivity as assayed on the guinea-pig ileum.

Substance P (30 μM) also evoked a significant increase ($P < 0.05$) in the levels of [^{3}H]inositol 1-phosphate in tissue slices prepared from the mouse urinary bladder (114 ± 35% increase above basal; N = 4).

cAMP levels

Substance P and eledoisin had no significant effect on cAMP levels in either homogenates or slice preparations prepared from the rat ileum longitudinal smooth muscle (Table 2). Further, substance P and eledoisin did not interfere with adenylate cyclase activation induced by isoprenaline or forskolin in tissue slices prepared from the rat ileum (Table 2).

DISCUSSION

The rat duodenum has been previously described as a SP–E system based on the rank order of potency values of tachykinins and substance P alkyl esters in eliciting contraction [2]. The present study has shown that the entire length of the rat small intestine can also be considered a SP–E tissue because eledoisin was considerably more potent than substance P in contracting all regions studied. Further, the EC_{50} values for substance P on these preparations lie in the range previously described for SP–E systems, but are considerably greater than those described for SP–P systems [2].

Using this same criterion, it would appear that inositol phospholipid hydrolysis in the rat small intes-

Table 2. Substance P effects on adenylate cyclase activity in rat ileum longitudinal smooth muscle

Tissue condition	Agonist(s)	Concentration (μM)	N	cAMP level (%)
Slices	—	—	—	100
Slices	Substance P	20	4	104 ± 7
Slices	Eledoisin	20	2	115 ± 4
Slices	Isoprenaline	100	3	199 ± 24*
Slices	Forskolin	10	1	432
Slices	Substance P + Isoprenaline	as above	3	200 ± 22*
Slices	Eledoisin + Isoprenaline	as above	2	186 ± 2*
Slices	Substance P + Forskolin	as above	1	455
Homogenates	—	—	—	100
Homogenates	Substance P	100	2	91 ± 2
Homogenates	Eledoisin	100	1	118
Homogenates	GTP	10	1	92
Homogenates	Substance P + GTP	as above	1	110
Homogenates	Eledoisin + GTP	as above	1	114

Experiments were carried out on either rat ileum longitudinal smooth muscle slices or homogenates of this tissue as described in the Methods. Results are expressed relative to basal (100% = no effect), and are expressed as the mean ± S.E.M. Individual experiments were performed in triplicate.
* $P < 0.05$.

tine can also be considered as a SP–E system because eledoisin was 31 times more potent than substance P in stimulating [^{3}H]inositol 1-phosphate production. However, if inositol phospholipid hydrolysis is the second messenger system for substance P induced contractions in this tissue, then the dose response curve to substance P on this response should either be superimposable on the curve for contraction or lie to the right of it (thereby suggesting the existence of spare receptors). This is clearly not the case as the dose response curves to both substance P and eledoisin on [^{3}H]inositol 1-phosphate accumulation lie approximately one order of magnitude to the *left* of their corresponding curves for contraction.

The explanation for this discrepancy is not obvious. It may be that the different experimental conditions used in the two assays have affected the potencies of the two peptides. For example, access to the receptor site in the tissue slices used in the lipid studies may be less restricted than in the intact muscle strips used for contraction; or alternatively, perhaps the much longer incubation period used in the lipid studies enables a greater concentration of peptide to be reached in the vicinity of the receptor site. However, it is important to stress that these comments are also likely to be applicable to a similar study previously carried out on the guinea-pig ileum, and in that case such discrepancies were not apparent [4]. Therefore, other explanations for the present results should be considered. For example, the formation of [^{3}H]inositol 1-phosphate in rat ileum may not be mediated through SP–E receptors located on smooth muscle cells but may be linked to another receptor sub-type found on other tissue types in this preparation, such as nerve cells.

Clearly much more work is needed to evaluate whether inositol phospholipid hydrolysis is the second messenger system for substance P induced contractions of the rat ileum. However, it should be stressed that this possibility is supported by the observation that the potency of eledoisin relative to substance P on contraction is similar to that on [^{3}H] inositol 1-phosphate production. Further, the present study has also shown that substance P is able to evoke [^{3}H]inositol 1-phosphate production in mouse urinary bladder, a tissue which has also been characterized as an SP–E system based on the potencies of tachykinins and substance P alkyl esters in eliciting contraction [2]. It would therefore be of interest to extend this study to ascertain whether eledoisin can also stimulate inositol phospholipid hydrolysis in this tissue.

If inositol phospholipid hydrolysis is found not to be the second messenger for substance P induced contractions in rat ileum, the question would then arise as to what is? The answer would not be expected to be cAMP production as this is normally associated with muscle relaxation, and this has been shown to be the case in the present study. Disappointingly though, the present study has shown that adenylate cyclase inhibition does not appear to be the second messenger, because substance P and eledoisin did not interfere with the level of activity of adenylate cyclase in the presence of isoprenaline, GTP and forskolin (for examples of agonist mediated inhibition of adenylate cyclase in the presence of either GTP or forskolin see [9] and [10] respectively).

In conclusion, the present study has shown that substance P is able to evoke [^{3}H]inositol 1-phosphate production in two previously characterized SP–E systems, the rat ileum and mouse bladder [2]. The possibility therefore arises that inositol phospholipid

hydrolysis may be the second messenger system for substance P receptors in SP–E systems, although more work is needed to verify this tentative suggestion. This study has not provided any further support for the concept that SP–E and SP–P systems may contain different substance P receptor sub-types.

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