indicates that the cleavage of the Gly[4]-Tyr[5] bond may decrease the antinociceptive activity of dermorphin.

We have recently demonstrated that resistance to the cleavage of Tyr[1]-D-Ala[2] was strengthened by amastatin and that captopril inhibited the cleavage of Gly[4]-Tyr[5] and Pro[6]-Ser[7] bonds in the dermorphin molecule by endopeptidase. Furthermore, both peptidase inhibitors prevented three sites of peptide bonds upon incubation with rat brain extracts (unpublished data).

In the present experiment, dermorphin with captopril or amastatin, produced an antinociceptive effect which lasted longer than that produced by dermorphin alone when compared with Ringer control, but there was no significant increase in potency. The finding that dermorphin-induced antinociceptive activity was dramatically enhanced when the heptapeptide was concurrently administered with captopril and amastatin means, at least, that either cleavage of the Tyr[1]-D-Ala[2] or Gly[4]-Tyr[5] bonds may decrease dermorphin-induced antinociceptive activity.

Our present findings suggest that one of the catabolic pathways modulating dermorphin-induced antinociceptive activity by means of endopeptidase, is the cleavage of Tyr[1]-D-Ala[2] bond. The other produces the *N*-terminal tetrapeptide which is less potent than its parent heptapeptide.

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The effect of ascorbate on the acetylcholine release from guinea-pig ileal myenteric plexus

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The effect of ascorbate on the release of acetylcholine from the longitudinal muscle-myenteric plexus preparation of the guinea-pig isolated ileum has been investigated using a bioassay and an isotopic technique. Ascorbate at 5 mM increased the spontaneous output of acetylcholine and enhanced DMPP-induced output of acetylcholine, while iso-ascorbate, 5 mM, did not. Ascorbate did not influence either the spontaneous or the DMPP-induced release of acetylcholine from synaptosomes of the ileal myenteric plexus. These results suggest that ascorbate promotes acetylcholine release from intramural cholinergic nerves.

We have previously shown that ascorbate elicits a contractile response in the guinea-pig isolated ileum and have suggested that this response is due to a release of acetylcholine(ACh) from the myenteric plexus (Terada et al 1980). We have also demonstrated that ascorbate augments the contraction induced by 1,1-dimethyl-4-phenylpiperazinium(DMPP), a nicotinic agonist, in the ileal longitudinal muscle of guinea-pig, and proposed that it might promote the ACh-releasing action of DMPP at the ganglion cells in the myenteric plexus (Hayashi et al 1983). Kuo et al (1979) and Kuo & Yoshida (1980) have reported that ascorbate causes a release of ACh from isolated synaptic vesicles of brain in rat, guinea-pig and rabbit. Furthermore, Pinchasi et al (1979) demonstrated that ascorbate induces Ca^{2+}-dependent release of ACh from synaptic vesicles isolated from *Torpedo* spp. In the present study, we report on the effect of ascorbate on ACh release from the longitudinal muscle strip of guinea-pig isolated ileum and its influence on ACh release from the

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synaptosomal preparations derived from the ileal myenteric plexus.

Methods

ACh bioassay. Male guinea-pigs, ca 300 g, were killed by a blow to the head and 20 cm of terminal ileum was excised after discarding the 5 cm nearest to the ileocaecal junction. The longitudinal muscle strip with the myenteric plexus was prepared from the ileal segments according to Paton & Zar (1968). ACh output from the strips was determined using the method of Knoll & Vizi (1971) with slight modification. The preparations, of ca 30–40 mg wet weight, were set up in an organ bath containing 3 ml Tyrode solution at 37 °C bubbled with a mixture of 95% oxygen and 5% carbon dioxide. Physostigmine salicylate (3 μM) was added to the Tyrode to prevent enzymatic hydrolysis of ACh. The preparations were allowed to equilibrate for 60 min. Samples were then collected at 15 min intervals. Ascorbate or iso-ascorbate and DMPP were added for the last 5 min and 3 min, respectively, of a 15 min period of incubation.

The ACh in the sample (0·1 ml) was assayed on other ileal strips suspended in 3 ml of oxygenated Tyrode, the bath temperature being kept at 20 °C to reduce release of endogenous ACh. To eliminate any influence of DMPP carried over in the test sample, hexamethonium (100 μM) was added to the solution. The contractile responses of the ileal strips were recorded isotonically. Active substance in the sample was considered to be ACh since contractions were abolished by atropine (0·2 mM). Estimation of ACh content of samples was based on control concentration-response curves for ACh, the output of which was expressed as pmol g^{-1} tissue wet weight min^{-1}.

Release of [^{3}H]ACh from ileal longitudinal muscle. An ileal longitudinal muscle strip was equilibrated in Tyrode for 30 min and then incubated for 60 min at 37 °C in Tyrode containing [^{3}H]choline (1 μM, 1 μCi). The strip was then transferred to a superfusion bath (2 ml) and perfused with prewarmed (37 °C) oxygenated Tyrode containing 10 μM physostigmine, at 1 ml min^{-1}, by means of a Watson-Marlow MHRE-22 pump with delta attachment (Watson-Marlow Ltd). After 30 min perfusion, when the output of tritium became relatively constant, perfusates were collected (one 1 ml sample min^{-1}) for the determination of [^{3}H]ACh.

Determination of [^{3}H]ACh. [^{3}H]ACh was separated from labelled choline by the method of Marchi et al (1981, 1983). A 100 μl sample of either supernatant was incubated with 10 mM Tris-HCl buffer (pH 8·5), 5 mM $MgCl_2$, 1 mM ATP and 2·0 m unit ml^{-1} choline kinase in a final volume of 200 μl at 37 °C for 45 min. This was extracted with 200 μl of 10 mg ml^{-1} tetraphenylboron in butyronitrate and the [^{3}H]ACh in the organic phase was counted by an Aloka liquid scintillator and expressed as d min^{-1} mg^{-1} tissue.

Preparation of synaptosomes from ileal myenteric plexus. This was according to Shinozuka et al (1985). The longitudinal muscle strips were minced and homogenized in 5 volumes of 0·32 M sucrose containing 3 mM sodium phosphate buffer (pH 7·2) using a Teflon-glass homogenizer (type 3, Omega Electric). The homogenate was centrifuged at 1000*g* for 10 min, and the supernatant further centrifuged at 17 000*g* for 20 min. The pellet (P2-fraction) was gently suspended in 3 ml of a buffer solution having the following composition (mM); NaCl 132, KCl 5, $CaCl_2$ 1·2, $MgCl_2$ 1·3, NaH_2PO_4 1·2, glucose 10, Tris base 20, and used for experiments of ACh release. The normal buffer solution was buffered to pH 7·4 (at 25 °C) by titration with maleic acid and saturated with 95% oxygen and 5% carbon dioxide.

[^{3}H]ACh release from synaptosomes. The P2-preparation (synaptosomal fraction) was incubated with 1 μM [^{3}H]choline(1 μCi ml^{-1}) for 30 min at 37 °C under 95% oxygen and 5% carbon dioxide, after preincubation for 30 min. The [^{3}H]ACh-loaded synaptosomes were cooled to 0–4 °C, then centrifuged at 5000*g* for 10 min. The pellet was then resuspended, washed twice in the fresh buffer containing 10 μM physostigmine and then resuspended in 1·5 ml of the physostigmine containing the buffer solution saturated with 95% oxygen and 5% carbon dioxide. Aliquots (100 μl) of the suspension were added to chilled polyethylene tubes containing 400 μl buffer solution, with or without appropriate drugs, with gentle mixing. Then [^{3}H]ACh release from the preparation was initiated by transferring the tubes to a bath at 37 °C, for 10 min incubation. The release was terminated by chilling the tubes in an ice bath. After centrifugation of the tubes at 5000 *g* for 10 min, supernatants were collected for determination of [^{3}H]ACh. Synaptosomal protein concentration was estimated according to Lowry et al (1951).

Drugs. The drugs used were; acetylcholine chloride (Sigma), adenosine 5′-triphosphate (Sigma), atropine sulphate (Merck), butyronitrate (Wako), choline kinase (Sigma), 1,1-dimethyl-4-phenyl-piperazinium iodide: DMPP (Aldrich), ethylene glycol-bis [β-aminoethyl ether] *N,N′*-tetraacetic acid: EGTA (Wako), hexamethonium (Sigma), physostigmine salicylate (Tokyo-Kasei), tetrodotoxin (Sankyo) sodium L-ascorbate and sodium iso-ascorbate (Wako). [Methyl-^{3}H] choline chloride and protosol were obtained from the New England Nuclear, Boston, USA.

The number (n) of experiments represents the number of preparations isolated from different animals. Some data were statistically analysed by Student's *t*-test.

Results

Table 1 shows the results which were obtained with ACh-bioassay. In the presence of ascorbate 5 mM, the

Table 1. Effects of ascorbate and iso-ascorbate on the acetylcholine output from the myenteric plexus-longitudinal muscle preparation from guinea-pig ileum.

	Acetylcholine output (pmol g^{-1} min^{-1})			
	Spontaneous (A)	DMPP-induced (B)	B − A	n
Control	236·4 ± 11·6	1520·3 ± 67·4†††	1283·9	19
Ascorbate (0·5 mM)	229·5 ± 11·6	1542·6 ± 108·2		6
Ascorbate (5 mM)	403·5 ± 30·8***	2476 ± 260·5*†††	2073·3	6
Iso-ascorbate (5 mM)	203·2 ± 10·2	1535·8 ± 106·6		7

Significantly different from control (***$P < 0.001$, * $P < 0.05$).
Significantly different from spontaneous output (†††$P < 0.001$).
Values are given as mean ± s.e.m.
n = number of experiments.

Table 2. Effect of ascorbate on the [^{3}H]acetylcholine output from [^{3}H]choline preloaded into the myenteric plexus-longitudinal muscle preparation from guinea-pig.

	[^{3}H]Acetylcholine output (×1000 d min^{-1} (mg tissue)$^{-1}$ min^{-1})			
	Spontaneous (A)	DMPP-induced (B)	B − A	n
Control	42·9 ± 1·1	74·8 ± 6·3††	31·1	9
Ascorbate (5 mM)	59·4 ± 7·4**	97·9 ± 10·1**††	38·5	9

Significantly different from control (**$P < 0.01$).
Significantly different from spontaneous output (††$P < 0.01$).
Values are given as mean ± s.e.m.
n = number of experiments.

spontaneous ACh output significantly increased, the extent of increase being 71%. DMPP at 10 μM also induced an increase in ACh output over control values of 1285 pmol g^{-1} min^{-1}; this was augmented by ascorbate at 5 mM to 2075 pmol g^{-1} min^{-1} the extent of augmentation being 61·5%. At 0·5 mM, ascorbate had no effect on either the spontaneous or DMPP-induced ACh output. Iso-ascorbate (5 mM), an epimer of ascorbate, did not affect ACh output. In the presence of hexamethonium (0·5 mM) or tetrodotoxin (0·2 μM), the ACh release induced by ascorbate, DMPP and DMPP plus ascorbate was abolished. The ACh output was also completely suppressed at 10 °C.

In the ileal longitudinal muscle strips preloaded with [^{3}H]choline, ascorbate at 5 mM significantly increased the spontaneous [^{3}H]ACh output by 38·5%. DMPP (10 μM)-induced output of [^{3}H]ACh was augmented by ascorbate (5 mM), to the extent of rate 24% (Table 2). These effects of ascorbate and DMPP on the [^{3}H]ACh output were abolished when calcium-free Tyrode with 1·2 mM EGTA was used (data not shown). Table 3 shows the influence of ascorbate on the output of [^{3}H]ACh from synaptosomal preparations of the ileal myenteric plexus. DMPP at 10 μM increased the [^{3}H]ACh output significantly, and the effect was suppressed completely by the calcium free buffer solution with 1·2 mM EGTA. Ascorbate had no effect on either the spontaneous or the DMPP-induced [^{3}H]ACh output in this preparation.

Discussion

Ascorbate elicits contractile responses in the guinea-pig ileum (Dawson et al 1967; Terada et al 1980). We have examined its effects on the contractile responses induced by ACh, histamine, DMPP and high concentrations of potassium in the ileal longitudinal muscle strip of guinea-pig, and found that it potentiated only the DMPP-induced contractile response in a concentration-dependent manner (Hayashi et al 1983).

In the present study, ascorbate increased both the spontaneous and DMPP-induced ACh output from guinea-pig longitudinal muscle strips. This output may be considered to be derived from cholinergic nerve terminals since it is abolished by treatments such as

Table 3. Effect of ascorbate on the [^{3}H]acetylcholine output from [^{3}H]choline preloaded into synaptosomes from ileal myenteric plexus of guinea-pig.

	[^{3}H]Acetylcholine output (×1000 d min^{-1} (mg protein)$^{-1}$ min^{-1})			
	Spontaneous (A)	DMPP-induced (B)	B − A	n
Control	21·9 ± 0·4	27·4 ± 0·5***	5·5	9
Ca^{2+} free	22·6 ± 1·9	21·9 ± 1·8		4
Ascorbate (5 mM)	21·7 ± 0·9	26·9 ± 1·7*	5·2	4

Significantly different from spontaneous output (***$P < 0.001$, *$P < 0.05$).
Values are given as mean ± s.e.m.
n = number of experiments.

cooling, tetrodotoxin and hexamethonium. Ascorbate also increased both the spontaneous and DMPP-induced output of [^{3}H]ACh, and this was suppressed in the absence of calcium. The increase of spontaneous ACh release induced by ascorbate was 71% as measured by bioassay, and 38·5% in the tritium experiments, and that induced by DMPP was increased by ascorbate 61·5 and 24%, as measured by the respective assays. With both assays, ascorbate augmented the total endogenous ACh release more than the labelled ACh release. Ascorbate may therefore preferentially promote ACh release from the pool of newly synthesized ACh.

It has been generally accepted that DMPP depolarizes ganglion cells in the intestine through a nicotinic receptor, which in turn leads to firing of post ganglionic fibres resulting in ACh release. Possibly, ascorbate potentiates the action of DMPP in the cholinergic ganglion cells of ileal myenteric plexus. The effect of ascorbate on spontaneous ACh release might be explained in terms of (i) ascorbate causing excitation of cholinergic neuron by itself and (ii) potentiating the nicotinic action of endogenous ACh released spontaneously from cholinergic interneurons. There is good evidence for intrinsic cholinergic input to enteric neurons; when reflexes are elicited in the guinea-pig duodenum, fast excitatory postsynaptic potentials (EPSPs) are observed in neurons of the myenteric plexus. These are blocked by nicotonic antagonists (Hirst et al 1974; Hirst & Mckirdy 1974). The peristaltic reflex elicited in isolated segments of small intestine by distension is antagonized by nicotinic blocking drugs (Kosterlitz & Lees 1964; Costa & Furness 1976). We found ascorbate action to be prevented by hexamethonium so explanation (ii) seems the most probable.

Ascorbate effects the function of the nervous tissue through its peroxidizing activity of membrane lipids (Boxall & Phizackerley 1973; Sharma & Murti 1976 Matsuda et al 1979; Leslie et al 1980). But a relation between ascorbate and lipid peroxidation is not supported by our results since iso-ascorbate, which also promotes lipid peroxidation, did not affect ACh output.

Fosbraey & Johnson (1980) reported evidence in support of the existence of prejunctional nicotinic receptors on the cholinergic nerve endings of guinea-pig ileum which increased the release of ACh. Furthermore, Briggs & Cooper (1981) demonstrated that the nicotinic agonist, DMPP, stimulated calcium-dependent release of [^{3}H]ACh from synaptosomal preparations derived from the myenteric plexus of guinea-pig ileum. We examined the effect of ascorbate on the [^{3}H]ACh release from ileal synaptosomes (P2-fraction) and found that DMPP at 10 μM caused an increase in [^{3}H]ACh output from the preparation and that the release was calcium-dependent, and temperature-dependent (data not shown). It is therefore suggested that [^{3}H]ACh released from ileal synaptosomes originated in the cholinergic nerve terminals. Ascorbate had no effect on the DMPP-induced release of ACh, nor was spontaneous [^{3}H]ACh output increased by ascorbate. Thus, inactivity of ascorbate on synaptosomes led us to the view that the site of action of ascorbate is not on cholinergic nerve varicosities. This agrees with our previous report that ascorbate may act on the nerve cell bodies rather than terminals and fibres. Probably, ascorbate facilitates the excitability of cholinergic nerves in the ileal myenteric plexus via some specific mechanisms other than lipid peroxidation or reduction.

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