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RELEASE OF "NEUROKININ" ON NERVOUS AND ELECTRICAL STIMULATION OF A FROG STOMACH MUSCLE PREPARATION

BY

I. SINGH

From the Department of Physiology, Medical College, Agra, India

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Activation of a frog stomach muscle preparation by electrical stimulation of a vagus nerve or by direct stimulation released two polypeptides. One was destroyed by trypsin or chymotrypsin in about 10 min ; the activity of the other was enhanced by trypsin for about 10 min, but was destroyed by chymotrypsin. Similar stimulation of dog stomach muscle did not release these polypeptides. Correspondingly, the transmission from vagus nerve to stomach muscle in the frog was resistant to atropine, but was blocked by atropine in the dog.

Euler & Lishajko (1961) found substance P in particles sedimented by high speed centrifugation of juice pressed from peripheral nerves. Euler (1963) found that, on some occasions after incubation with low concentrations of trypsin, extracts of sediments of bovine vagus nerves yielded a product which contracted guinea-pig ileum, which suggested that the extract might contain a precursor from which trypsin split off an active substance different from substance P. It was rapidly inactivated by chymotrypsin and thus was presumably a peptide. The active substance was provisionally termed "neurokinin." The formation of the polypeptide was not observed with all extracts. In some of them incubation with trypsin caused only a rapid inactivation of the extract, similar to that seen in a standard solution of substance P ; on other occasions the increase in activity was very great. For extracts of granules from rabbit and guinea-pig ileum, inhibition and potentiation of the actions of substance P by trypsin have been described also by Inouye & Kataoka (1962). Zetler (1963) has shown that substance P from intestine comprises more than one pharmacologically active polypeptide. Frog stomach muscle releases substance P on vagal and direct electrical stimulation (Singh, 1963a). The experiments described here were performed to test whether more than one polypeptide are released when frog stomach muscle is stimulated through the vagus nerve and directly by electric shocks.

METHODS

The stomach of the frog (*Rana tigrina*) was dissected out with a vagus nerve attached. It was then split by cutting along the greater curvature and the mucous membrane gently removed. The muscle layers were then washed for 30 min in Tyrode solution to remove any extraneous substances. Since frog stomach muscle responds well to stimulation in Tyrode solution, the solution can be added without further modification to a test organ-bath containing guinea-pig ileum.

After the preliminary wash the stomach muscle was laid for 30 min in a tilted Petri dish containing 15 ml. of Tyrode solution to study the spontaneous release of substance P polypeptides. Thereafter the vagus nerve was stimulated by rectangular pulses of 0.5 msec duration and 20 V at 12 shocks/sec for the next 30 min to study the release of substance P polypeptides on nervous stimulation. Lastly, immediately after vagal stimulation, the muscle was stimulated directly to study the release of substance P polypeptides on direct stimulation. This was done by suspending the muscle in a glass chamber, 1 cm in diameter and containing 15 ml. of Tyrode solution, between two chlorided silver electrodes 10 cm apart; it was stimulated by sinusoidal alternating current, 15 V, 50 cycles/sec, for 12 sec every min for 30 min. The various solutions were tested for substance P polypeptides on guinea-pig ileum suspended and completely immersed in 3 ml. of Tyrode solution in a 6 ml. organ-bath; thus it was possible to add to the bath a further 3 ml. of Tyrode solution in which frog stomach muscle had been previously stimulated. If less than this volume was added to the solution the responses of the ileum were very feeble. The guinea-pig ileum was immersed in Tyrode solution containing 0.4 mg/l. of atropine sulphate, 0.4 mg/l. of mepyramine and 40 mg/l. of tryptamine (Pernow, 1961). The enzymes used for hydrolysis of polypeptides were amorphous trypsin (Merck), 4 mg/ml., as used by Zetler (1963) for dilute solutions of substance P, and crystalline chymotrypsin (B.D.H.), 0.1 mg/ml. at 37° C, as used by Euler (1963); the trypsin by itself did not produce any contraction. A few experiments were also performed on dog vagus-stomach muscle preparations.

To study the contraction of frog stomach muscle on stimulation of a vagus nerve, frogs weighing about 300 g were pithed and the stomach dissected out with the vagus nerve attached to the lesser curvature. A nerve-muscle preparation was made by cutting out the middle part of the stomach, weighing about 1.5 to 2 g with its nerve attached. This part was then cut longitudinally at the greater curvature, the mucous membrane removed and ligatures tied at the two ends. The preparation was suspended in Ringer solution in a beaker of 250 ml. capacity, and mechanical responses were recorded with an isometric lever. The nerve was laid on a pair of electrodes, and during stimulation the beaker was lowered since the nerve was too short to be pulled out of the solution for stimulation; the preparation was thus suspended in air during stimulation to prevent short-circuiting the current through the solution. When the beaker was again raised, the muscle usually showed a twitch contraction. The nerve was stimulated every 20 to 30 min for 30 sec with the electronic stimulator as described above. The experiments were performed at room temperature (30° C). The Ringer solution contained (in mM): Na 103, K 10, Ca 3, HCO_3 6, and Cl 113.

RESULTS

Release of polypeptides. The description which Euler (1963) has given about the effect of trypsin on extracts of sediments of the bovine vagus nerves is equally applicable to the Tyrode solution in which frog stomach muscle had been stimulated directly or through the vagus nerve; this solution contracted guinea-pig ileum which had been made insensitive to acetylcholine, histamine and 5-hydroxytryptamine. If this solution was treated with trypsin for 10 min, the effects were variable. Sometimes it was inactivated (Fig. 1), while at other times its activity was either unchanged or increased; the remaining activity after incubation with trypsin was abolished by chymotrypsin (Fig. 2). It appears therefore that substance P released on vagal stimulation of frog stomach muscle consists of two polypeptides, one of which is the neurokinin of Euler (1963).

The muscle also released spontaneously some inhibitory substance which interfered with the action of the released polypeptides (Fig. 3). Such an inhibitory substance has been described by Zetler (1963) and by Vogler, Haefely, Hürlimann, Studor, Lergier, Strässle & Berneis (1963) in preparations of substance P.

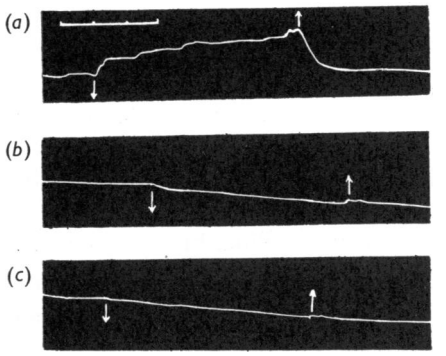

Fig. 1. Responses of guinea-pig ileum preparation in Tyrode solution containing tryptamine, atropine and mepyramine. (*a*) contraction by Tyrode solution in which frog stomach muscle had been stimulated directly and electrically ; (*b*) response after treatment of the same Tyrode solution by trypsin for 10 min ; (*c*) response after treatment of the same Tyrode solution by chymotrypsin for 10 min. Arrows indicate times of addition and removal of each sample of Tyrode solution. Time in minutes.

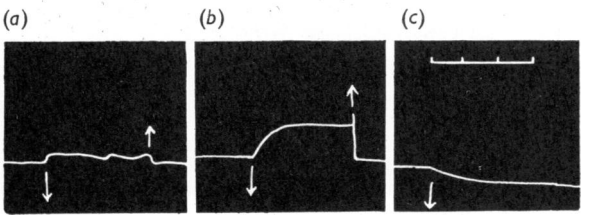

Fig. 2. Responses of guinea-pig ileum preparation in Tyrode solution containing tryptamine, atropine and mepyramine. (*a*) contraction by Tyrode solution in which frog stomach muscle has been stimulated through a vagus nerve ; (*b*) response after treatment of same Tyrode solution by trypsin for 10 min ; (*c*) response after treatment of same Tyrode solution by chymotrypsin for 10 min. Arrows indicate times of addition and removal of each sample of Tyrode solution. Time in minutes.

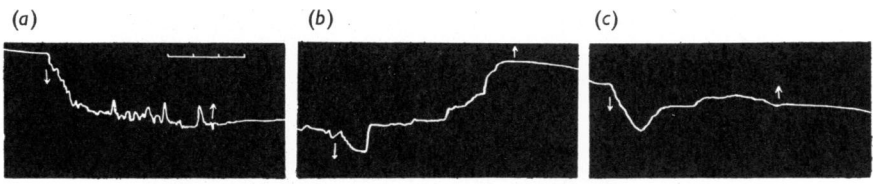

Fig. 3. Responses of guinea-pig ileum preparation in Tyrode solution containing tryptamine, atropine and mepyramine. (*a*) inhibition and spontaneous contractions induced by Tyrode solution in which frog stomach muscle had been stimulated directly and electrically ; (*b*) response to the same Tyrode solution but after treatment by trypsin for 10 min ; (*c*) response to the same Tyrode solution but after treatment by chymotrypsin for 10 min. Arrows indicate times of addition and removal of each sample of Tyrode solution. Time in minutes.

In contrast to frog stomach muscle no release of any polypeptide could be detected from dog stomach muscle on direct or vagal stimulation.

Physiological evidence for the release of polypeptides. This evidence can only be indirect as there are no known antagonists to substance P polypeptides. It was assumed that the contraction produced would be by substance P polypeptides, if it was not abolished by the combined action of atropine (10^{-7} g/ml.), mepyramine (10^{-7} g/ml.) and 2-bromolysergic acid diethylamide (10^{-7} g/ml.), which drugs abolish respectively the responses to acetylcholine, histamine and 5-hydroxytryptamine.

The contraction of frog stomach muscle produced by stimulation of a vagus nerve is completely resistant to atropine (Singh, 1963b). In winter it may be abolished by bromolysergic acid diethylamide (10^{-7} g/ml.) but in summer it was completely resistant to the combined action of the three drugs (Fig. 4).

In contrast to the contraction of frog stomach muscle, that of dog stomach muscle produced by stimulation of a vagus nerve was abolished by atropine (10^{-8} to 10^{-7} g/ml., Fig. 5), so that transmission is cholinergic, but morphine (10^{-6} g/ml.) greatly increased the response if it was initially small, the action of the drug persisting

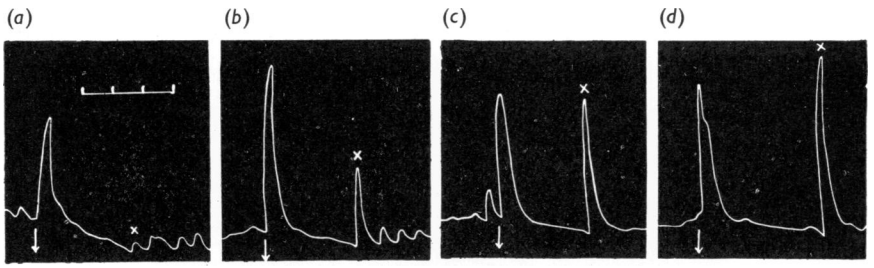

Fig. 4. Contractions of a frog stomach muscle preparation produced by stimulation of a vagus nerve (at each of the arrows) in summer (room temperature 30° C). (*a*) a control response; (*b*) response after atropine (10^{-7} g/ml.) had been applied for 20 min; (*c*) response after atropine and mepyramine (10^{-7} g/ml.) had been applied for a further 20 min; (*d*) response after atropine, mepyramine and 2-bromolysergic acid diethylamide (10^{-7} g/ml.) had been applied for a further period of 3 hr. The muscle was suspended in air during vagal stimulation; the contractions marked × were produced when it was reimmersed in Ringer solution. Time in minutes.

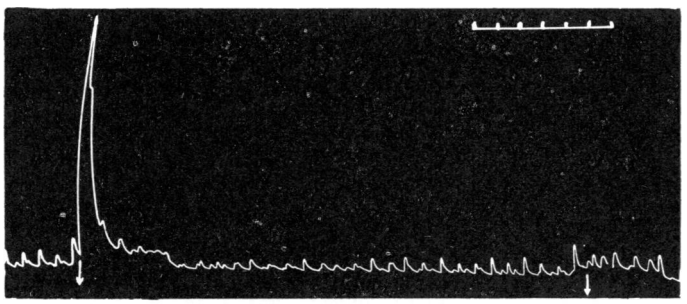

Fig. 5. Contractions of a dog stomach muscle preparation produced by stimulation of a vagus nerve (VS at arrows), and their abolition by atropine (Atr, 10^{-7} g/ml. at black dot). Time in minutes.

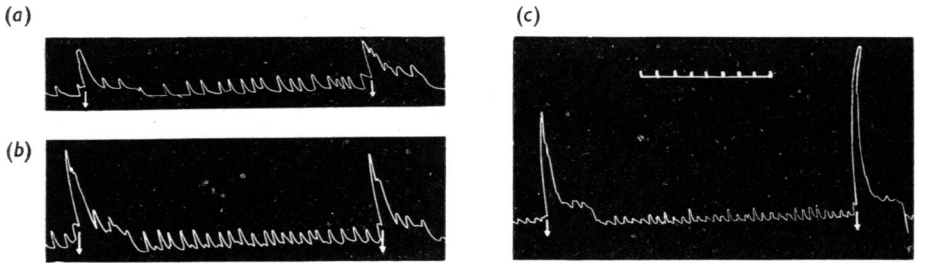

Fig. 6. Contractions of a dog stomach muscle preparation produced by stimulation of a vagus nerve (at each of the arrows). (*a*) two control responses ; (*b*) two responses after morphine (10^{-6} g/ml.) applied for 20 min ; (*c*) two responses after morphine had been withdrawn, when the potentiating effect of morphine still persisted. Time in minutes.

after it was withdrawn (Fig. 6); these enhanced responses in the presence of morphine were abolished by atropine. Morphine abolishes similar response in frog stomach muscle (Singh, 1963b).

DISCUSSION

The transmission from vagus nerve to stomach muscle in the frog is noncholinergic, but in the dog it is cholinergic. Traces of the noncholinergic mechanism still remain in mammalian tissues, as nerve-smooth muscle transmission resistant to atropine has been reported (Ambache, 1955) and may be responsible for the atropine-resistant vasodilation in the submandibular salivary glands of the cat on stimulation of the chorda tympani nerve.

One can only speculate on the role of "neurokinin." Such polypeptides would be useful in situations where proteolytic enzymes are present as their effect would be enhanced. The release of such polypeptides would be likely where they stimulate secretion of juices containing proteolytic enzymes. Hormones of the alimentary canal, secretin and gastrin, are polypeptides. It is, therefore, possible that parasympathetic nerves to the stomach, intestines and pancreas release such polypeptides. The abnormal secretion of these polypeptides might underlie the pathology of gastric ulceration.

REFERENCES

AMBACHE, N. (1955). The use and limitations of atropine for pharmacological studies on autonomic effectors. *Pharmacol. Rev.*, **7**, 567–494.

EULER, U. S. VON (1963). Substance P in subcellular particles in peripheral nerves. *Ann. N.Y. Acad. Sci.*, **104**, 449–463.

EULER, U. S. VON & LISHAJKO, F. (1961). Presence of substance P in subcellular particles of peripheral nerves. Proc. Sci. Soc. Bosnia and Herzagovina, Dept. of Med. Sci., vol. 1, pp. 109–112, *Symposium on Substance P*, Sarajevo, June 9–11, 1961.

INOUYE, A. & KATAOKA, K. (1962). Subcellular distribution of Substance P in the nervous tissues. *Nature (Lond.)*, **193**, 585.

PERNOW, B. (1961). Estimation of Substance P. *Meth. Med. Res.*, **9**, 220–223.

SINGH, I. (1963a). Release of Substance P on nervous and electrical stimulation of frog's stomach muscle. *Arch. int. Pharmacodyn.*, **143**, 138–143.

SINGH, I. (1963b). Tryptaminergic fibres in frog's vagus nerve. *Arch. int. Physiol.*, **71**, 371–381.

VOGLER, K., HAEFELY, W., HÜRLIMANN, A., STUDOR, R. O., LERGIER, W., STRÄSSLE, R. & BERNEIS, K. H. (1963). A new purification procedure and biological properties of Substance P. *Ann. N.Y. Acad. Sci.*, **104**, 378–390.

ZETLER, G. (1963). New pharmacologically active polypeptides present in impure preparations of Substance P. *Ann. N.Y. Acad. Sci.*, **104**, 416–436.