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Vagal Adrenergic Degranulation of Enterochromaffin Cell System in Guinea Pig Duodenum

M. F. TANSY, G. ROTHMAN, J. BARTLETT, P. FARBER, and F. J. HOHENLEITNER

Abstract □ Working under the hypothesis that sympathetic nerve stimulation is involved in the degranulation of enterochromaffin cells in the duodenum of the guinea pig, a series of experiments utilizing both chemical and immunological sympathectomy was performed. Once sympathectomy was established, histological examination of the duodenum revealed enterochromaffin cell granulation was unaffected by peripheral vagal stimulation. This would tend to suggest adrenergic, and not cholinergic, fibers are implicated in the degranulation process in the guinea pig. This does not conflict with previous reports that vagal stimulation induces degranulation, because it has been shown that sympathetic fibers run in the vagal trunk of the guinea pig.

Keyphrases □ Enterochromaffin cell system, guinea pig—vagal adrenergic degranulation □ Sympathectomy, chemical, immunological—guinea pig □ Serotonin release—vagal stimulation effect □ Vagal stimulation effect—enterochromaffin cells

It is well known that the duodenum is the predilection site of the enterochromaffin cell (EC) and serotonin (5-HT) occurrence in the alimentary canal of the guinea pig (1). On the other hand, the exact physiologic release mechanism of liberated 5-HT from the EC in the intestinal tract is still largely unknown. Previously, the authors reported (2) that adequate electrical stimulation of the cervical vagus peripherally produced a reduction in argentaffin-positive EC granularity in guinea pig duodena.

Although no synaptic contacts to EC have been detected in mouse colon glands, in cells containing granules characteristic of EC, continuity has been established in a few cases between their basal processes and mucosal nerve fasciculi (3). By fluorescent microscopy, Gabella and Costa (4) traced adrenergic fibers to epithelial structures in the guinea pig gut. Jacobowitz (5) also claimed to follow some fluorescent fibers from perivascular plexuses to the surface epithelium in the mucosa of the cat and monkey small intestine. Whether the EC system is directly influenced by these nerves or affected only by sympathetic transmitter released from adjacent perivascular nerves remains to be seen.

Data from a previous study (2) seemed to indicate that EC granulation may have occurred *via* a noncholinergic mechanism. This suggestion, plus the fact that sympathetic fibers within the vagus were reported for the cat (6), the dog (7), and others (8), supports the contention that adrenergic fibers present in the guinea pig vagus may have been stimulated. Therefore, the present study is designed to provide further information on the nature of vagal degranulation of the EC system in the guinea pig duodenum.

METHODS

Animals—Albino male guinea pigs of the same strain and about 400 g. were used.[1] After an appropriate acclimatization period and a 24-hr. fast from a commercial pellet food (Purina), each guinea pig

[1] Obtained from Marland Farms, Wayne, N. J.

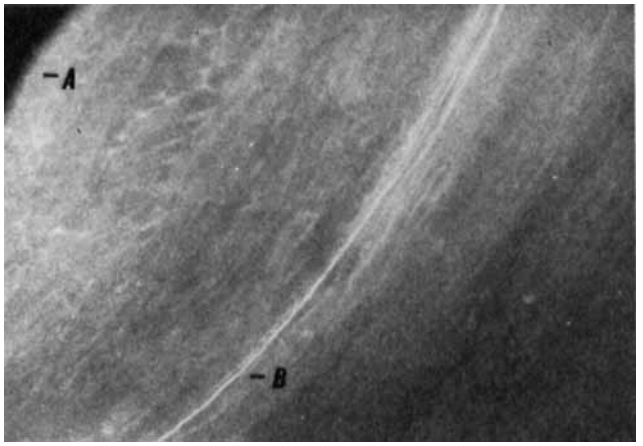

Figure 1—*Longitudinally sectioned vagus nerve of guinea pig. Key: A, edge of cervical trunk (X160); and B, fluorescent slender fiber seen in the nerve trunk 48 hr. before vagectomy.*

was intraperitoneally anesthetized with a preparation[2] containing 100 mg. of urethan and 400 mg. of diallylbarbital/ml. in a dosage of 0.8 ml./kg. of body weight.

Design—One hundred fifteen animals were randomly divided into 18 sham control and eight test groups of approximately 12 animals each subjected to: (*a*) peripheral vagal stimulation (P.V.S.); (*b*) immunosympathectomy (I.M.S.); (*c*) chemical sympathectomy with 6-hydroxydopamine (6-OH-DA); (*d*) P.V.S. after I.M.S.; (*e*) P.V.S. after 6-HO-DA; (*f*) central vagal stimulation (C.V.S.); (*g*) norepinephrine infusion; and (*h*) epinephrine infusion.

Procedures—The surgical, stimulation, histologic, and cell-counting techniques employed in this study were detailed in a previous publication (2). In short, the animals to be stimulated were vagotomized bilaterally, and either the central or peripheral ends were prepared for electrical stimulation. P.V.S. was accomplished with a Grass stimulator using parameters of 10 v. and 25 Hz. with a 1-msec. duration. Central ends of the vagi were alternately stimulated with monophasic squarewave impulses using "pressor" parameters described by Feldman (9). After stimulation the duodenum of each animal was excised, and its most proximal portion was saved for histologic examination. The tissue was immediately fixed in 10% formalin, embedded in paraffin, and sectioned at 6 μ. Sections were stained by the Fontana Masson method for argentaffin cells. Counts were made of the number of argentaffin cells seen in 30 or more high power fields (H.P.F.) at ×430, and the mean number of cells per H.P.F. was taken as a measure of density.

Fluorescent Histochemistry—The histochemical method developed by Falck *et al.* (10), as modified by Csillik and Kalaman (11), was used in these fluorescence studies to determine if the guinea pig vagus contains sympathetic fibers. Under the conditions used, primary catecholamines condense with formaldehyde without diffusion and are readily converted to 6,7-hydroxy-3,4-dihydroisoquinolines, which show an intense green to yellow fluorescence. In each of six guinea pigs under ether anesthesia, both vagus nerves were atraumatically exposed in the neck but only one was tightly ligated with silk thread as far caudal as the tissues would permit. Forty-eight hours after ligation, the neck was surgically reentered, and a 1-cm. segment of nerve most cranial to the ligature was removed for histochemical examination. The isolated nerve specimens were immediately placed on solid CO_2 until completely frozen. The nerve preparations were attached to cryostat holders, and 20–30 16-μ sections were cut at approximately −30°. The sections were transferred to a precooled sealed container and dried over 20 g. anhydrous phosphorus pentoxide for approximately 25 hr. at −30°. After equilibration at room temperature, the tissue was transferred to a 1-l. desiccator containing paraformaldehyde. The desiccator was warmed to 80°, thereby allowing the evolved formaldehyde gas to saturate the tissue sections. The sections were mounted on nonfluorescent glass with nonfluorescent mounting media and cover slip. The monoamine fluorescence, developed by the exposure to formaldehyde gas, was observed under a Zeiss fluorescence microscope and photographed on Panatomic X film with ASA 32. Exposure time depended on fiber intensity and ranged from 4 to 8 min. per exposure. The exciting light was delivered from the Osram HBO 200 high-pressure mercury lamp and was filtered through Schott BG 12 and Zeiss 53 as the primary and secondary filters.

Immunosympathectomy—Salivary gland protein was reported to contain a protein, nerve growth factor (N.G.F.), with a stimulatory effect on the sympathetic nervous system (12). Antiserum to this factor (anti-N.G.F.) destroys most sympathetic nerve cells in newborn animals (13). Guinea pig salivary glands[3] were homogenized in phosphate buffered saline (P.B.S.), pH 7.2, and centrifuged to remove the insoluble residue. Protein was precipitated with 5% trichloroacetic acid and centrifuged, and the precipitate was redissolved in 1 *N* NaOH. This solution was dialyzed for 24 hr. against P.B.S. at 4°. The final protein concentration of this extract was 3.84 mg./ml., using the method of Lowry (14).

Antiserum to guinea pig salivary protein was prepared in rabbits; 1 ml. of extract was mixed with an equal volume of Freund adjuvant and injected subcutaneously in two sites on the shaved flank of the rabbit. Two additional intravenous injections of 1.0 ml. of extract were given at weekly intervals, starting one after the initial injections. The rabbit was bled from the ear vein 2 weeks after the last injection. Several precipitin lines were noted when the resulting antiserum was tested against the salivary gland extract using the Ochterlony method (15). Newborn guinea pigs were injected intraperitoneally with 0.01 ml. anti-N.G.F. serum/g. body weight within 36 hr. of birth and again 24 hr. later or, in some instances, during the first 5 days of postnatal life. Control guinea pigs received normal rabbit serum (N.R.S.).

Chemical Sympathectomy—It was reported that a single injection of 6-hydroxydopamine (6-OH-DA) markedly reduces the norepinephrine content in various sympathetically innervated organs of different species for several days or weeks (16–18). For destruction of adrenergic nerve endings in this study, guinea pigs were given 20 mg./kg. of 6-OH-DA intraperitoneally for 3 consecutive days; on the 4th day they were given 30 mg./kg. i.v. and were sacrificed 4 hr. after the last injection. In some animals, P.V.S. was performed at that time. The purpose of this part of the study was to investigate the action of 6-OH-DA on the adrenergic nerve endings in the guinea pig duodenum, employing, in principle, the histochemical method developed by Falck *et al.* (10).

Drugs—Each of 18 guinea pigs received an intravenous infusion of 1/100 norepinephrine[4] over 15 min. at a rate of 0.1 ml./min. Seven others received 1/100 epinephrine[5] comparably infused. The 6-hydroxydopamine (2,4,5-trihydroxyphenethylamine) was obtained as the hydrobromide salt.[6]

RESULTS

Whole-mount preparations of the guinea pig vagus under fluorescence microscopy revealed the presence of adrenergic fibers (Fig. 1). An abundance of fluorescent material was also found in Auerbach's and Meissner's plexus. However, no fluorescent fibers were found to innervate the EC scattered throughout the duodenal mucosa. Most of the fluorescent nerves in the mucosal connective tissue seemed to be associated with the vasculature. Conversely, in animals injected with anti-N.G.F. or 6-hydroxydopamine, there was little nerve fluorescence in the duodenal preparations.

In certain of the experiments, comparable EC granulation was found in immunosympathectomized and chemical sympathectomized animals as compared to normal guinea pigs. In subsequent experiments, adequate P.V.S. at the cervical level was unable to reduce 5-HT stores in both chemical (Fig. 2) and immunosympathectomized guinea pigs, but the reduction occurred as reported (2) in normal animals (Fig. 3).

Data presented here show that with C.V.S., EC granulation also disappeared from the duodena. But the magnitude of the fall produced by C.V.S. was not as great as that produced by P.V.S. or norepinephrine infusion. Following epinephrine infusion, similar 5-HT stores were found in an equivalent number of fields as compared to the controls. A summary analysis of this data is exhibited in Fig. 4.

[2] Dial Urethane, Ciba Pharmaceutical Co.

[3] Pel-Freez Biologicals Inc., Rogers, Ark.
[4] Levophed, Winthrop.
[5] Adrenalin, Parke Davis.
[6] Regis Chemical Co., Chicago, Ill.

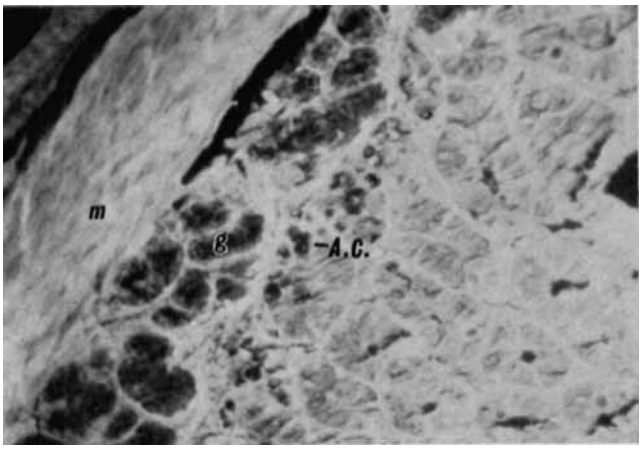

Figure 2—*Photomicrograph of a section of duodenum from a peripheral vagally stimulated animal after pretreatment with 6-OH-DA. Note the numbers of argentaffin cells (A.C.) apparent adjacent to Brunner's glands (g); m indicates the tunica muscularis (fluorescent microscopy, X160).*

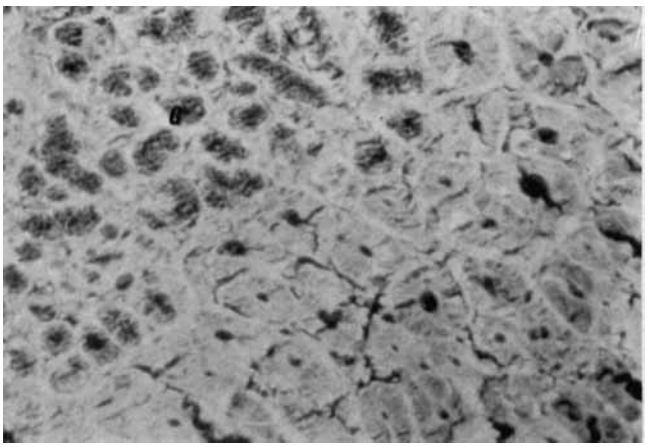

Figure 3—*Photomicrograph of a representative section from a peripheral vagally stimulated animal. Note the complete absence of argentaffin cell granulation in the area adjacent to Brunner's glands (g)(fluorescent microscopy, X160).*

DISCUSSION

The present experiments confirm 5-HT release by vagal stimulation, at least as measured by argentaffin cell granulation, in the guinea pig duodenum. An interesting supposition made in the initial study was that vagal degranulation occurs *via* a noncholinergic mechanism. In support of this, the data presented here show EC granulation practically disappearing with P.V.S., but pretreatment of the guinea pig with 6-hydroxydopamine or anti-N.G.F. completely abolishes the response. These findings are perhaps less surprising in view of the fact that numerous adrenergic fibers were detected in the guinea pig vagus.

Although the role of histologically demonstrated sympathetic axons in the vagus is unknown, it seems quite plausible that these fibers could have been excited by the stimulus parameters used in these experiments and, in turn, could have been responsible for degranulating EC. Unfortunately, because the present experiments were designed primarily to ascertain the presence of adrenergic fibers in the guinea pig vagus, no attempt was made to determine their origin or ultimate course. Nevertheless, while no experimental data support the presumption, it is to be expected that some of the adrenergic fibers in the cervical vagus proper terminate in the duodenum.

In certain experiments with animals injected with anti-N.G.F., there was little nerve fluorescence in the duodenal preparations and occasionally a few scattered fibers remained. Curiously, all of the guinea pigs did not react alike in this respect to the I.M.S. On the other hand, pretreatment of all guinea pigs with 6-OH-DA led to ultramorphological alterations of adrenergic nerve terminals similar to those previously described in detail for cats (16). The superiority over I.M.S. is evident. In this connection, an informative series of experiments was recently conducted by McGregor and Phelan (19) who tried to discover if the ability to evoke vasoconstrictor responses returns to the damaged but regenerating nerve endings before tissue noradrenaline stores are fully replenished. Essentially, they found vasoconstrictor responses of mesenteric arteries to sympathetic nerve stimulation were virtually abolished at 2, 3, and 4 days after administration of 6-OH-DA; by 6 days the response had partially returned and by 8 days was almost normal. From the standpoint of this study's data and these morphological and physiological observations, the possibility of a direct effect of the vagal adrenergic transmitter on the EC system in the intestinal mucosa of the guinea pig is a very real one. Experimental evidence has shown that a decrease in the granularity of these cells can be obtained with C.V.S.

Recent work by Ben-Ishay *et al.* (20) identified the pressor agent released from the sympathetic nerve terminals in response to C.V.S. as norepinephrine. Therefore, it should only follow that norepinephrine infusion can also degranulate EC in the guinea pig small intestine. While norepinephrine is taken up better than epinephrine in the rat adrenergic nerves, the reverse holds true for frog adrenergic nerves which contain adrenaline as the transmitter (21). This, plus the fact that Singh (22) earlier suggested that vagal stimulation results in release of 5-HT from the frog's stomach, prompted a study on the effects of epinephrine infusion on EC granularity. Following epinephrine infusion, however, the concentration of granulated argentaffin cells in the guinea pig duodena numerically remained the same.

Analyses of the data also reveal that 5-HT granulation persists in the apparent absence of sympathetics. From this observation, one might presume that adrenergic innervation has no influence on 5-HT metabolism in the guinea pig. Yet, 5-HT levels were reported to be increased in certain intestinal tissues following I.M.S. in mice (23). According to Thompson (24), this increase was probably not due to a greater population of argentaffin cells but to augmented amine storage. From the denervated preparations, it becomes evident that the elevated 5-HT levels observed in the gastrointestinal mucosa of the immunosympathectomized mouse were undoubtedly the result of an increased concentration of 5-HT per argentaffin cell rather than due to a larger population of cells.

In sum, it is the opinion of the authors that sufficient attention has not been paid to the physiologic release of 5-HT from EC. The complexity of the results reported preclude an explanation on the basis of any very simple theory. The mechanism whereby norepinephrine and adrenergic but not cholinergic nerve stimulation degranulate the EC system in the guinea pig duodenum is beyond the scope of this paper. However, the correlation between morphological and biochemical data, together with the failure of atropine to

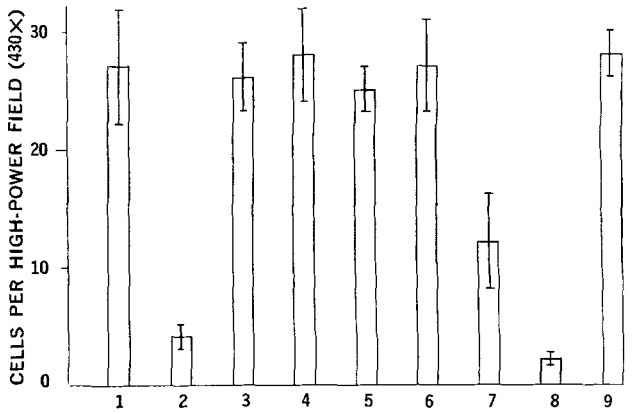

Figure 4—*Bar 1, controls; Bar 2, peripheral cervical vagal stimulation; Bar 3, immunosympathectomized animals; Bar 4, peripheral cervical vagal stimulation after immunosympathectomy; Bar 5, chemical sympathectomy with 6-hydroxydopamine; Bar 6, peripheral cervical vagal stimulation after chemical sympathectomy; Bar 7, central cervical vagal stimulation; Bar 8, norepinephrine infusion; Bar 9, epinephrine infusion. Each bar represents the mean value ± standard deviation.*

block vagal degranulation, provides a basis for a hypothesis on adrenergic release of 5-HT from the bowel.

CONCLUSION

The experiments were designed to provide additional information on the effects of vagal stimulation on the granulation of the EC system as a link to the release of 5-HT in the bowel. As evidenced by the data, it was confirmed that peripheral electrical stimulation of the cervical vagus produced a reduction of argentaffin-positive EC granulation. This effect was not blocked by atropinization of the animal. C.V.S. and infusion of norepinephrine but not epinephrine produced a similar degranulation. This effect was blocked by both chemical and immunological sympathectomy. Finally, catecholamine-fluorescent fibers were found to be present in the trunk of the cervical vagus in the guinea pig. In short, the mutual agreement in the morphological, histochemical, and physiological observations tested provides a basis for the hypothesis of the noradrenergic release of 5-HT from EC of the guinea pig small bowel.

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Topical Mosquito Repellents III: Carboxamide Acetals and Ketals and Related Carbonyl Addition Derivatives

P. TSAKOTELLIS*, H. L. JOHNSON†, W. A. SKINNER†, D. SKIDMORE‡, and H. I. MAIBACH‡

Abstract □ Slow release of topically applied mosquito repellents was attempted *via* formation of low volatility carbonyl addition derivatives of repellent alcohols and carbonyl compounds. Sufficiently rapid hydrolytic release of volatile, repellent moieties on the skin surface did not occur. Vapor repellency was due to the intact derivatives and was found to be dependent upon volatility characteristics. It was concluded that optimum volatility for prolonged topical repellency varies with each homologous series. Compounds incorporating both an acetal and an amine function, although not comparable to diethyltoluamide in topical repellency, appear to be worthy of additional study.

Keyphrases □ Mosquito repellants, topical—synthesis □ Carboxamide acetals, ketals—synthesis □ Repellency, mosquito—volatility relationship

In previous studies of mosquito repellency among structural analogs of diethyltoluamide (DEET), the dominant significance of volatility was demonstrated as a factor in both potency and duration of topical repellent action (1, 2). A limitation is imposed on the concept that duration is maximized by decreased volatility owing to a concomitant decline in potency (1). In the present study an alternative means of prolongation of repellent action through slow release was examined. In addition, the authors examined the effect on repellency of functional group alteration of various repellent carbonyl compounds and extended studies on the relationship between volatility and repellency to classes of compounds other than amides.

Attempts at slow release were based on the assumption that hydrolysis (chemical or enzymatic) of acetal-type derivatives on the skin surface could result in a prolonged release of the parent alcohol and carbonyl moieties as repellent agents. Choice of repellent moieties was based partly on preliminary estimations of "in-