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BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
(2001) 280(4):976-981.

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Requirement of intact disulfide bonds in orexin-A-induced stimulation of gastric acid secretion that is mediated by OX₁ receptor activation

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Abstract

Orexin-A is a neuropeptide consisting 33 amino acids with two intrachain disulfide bonds, namely Cys6-Cys12 and Cys7-Cys14, and is a potent stimulator of food consumption and gastric acid secretion. In contrast, orexin-B, a peptide containing 28 amino acids without disulfide bond, which has no stimulatory action of gastric acid. The objective of the present study was to characterize the receptor-mediated mechanism of orexin-A-induced stimulation of gastric acid secretion using orexin-A-related peptides with modification of disulfide bonds. Intracisternal injection of orexin-A but not orexin-B or orexin-A (15-33) that does not contain both disulfide bonds stimulated gastric acid secretion in pylorus-ligated conscious rats. The ability of the stimulation of gastric acid output was less in three alanine-substituted orexin-A, [Ala^{6,12}]orexin-A, [Ala^{7,14}]orexin-A and [Ala^{6,7,12,14}]orexin-A, than orexin-A. Orexins-induced calcium increase was measured in CHO-K1 cells expressing OX₁R or OX₂R. Orexin-A induced a transient increase in [Ca²⁺]_i in CHO-K1/OX₁R cells in a dose-dependent manner. EC₅₀ values for OX₁R of orexin-A, orexin-B or orexin-A (15-33) was 0.068, 0.69 or 4.1 nM, respectively, suggesting that peptides containing no disulfide bonds have lower potency for the receptor. Agonistic activity for OX₁R of the three orexin-A analogues with modification of one or both disulfide bonds was significantly reduced as compared with that of orexin-A. EC₅₀ values for OX₂R of

orexin-A and orexin-B was almost equal but potency for the receptor of orexin-A (15-33) and three alanine substituted orexin-A was less than that of orexin-A. A significant inverse relationship between gastric acid output and EC₅₀ values for OX₁R but not OX₂R was observed. These results suggested that the orexin-A-induced acid stimulation requires OX₁R activation and that disulfide bonds in orexin-A may have a key role in the receptor activation.

Introduction

Orexins/hypocretins are novel neuropeptides that are localized in neurons in the lateral hypothalamus (1, 2). It has been so far demonstrated that orexins may be implicated in a wide variety of physiological functions. These include feeding behavior (1, 3, 4), behavioral activity (5), sleep/awake (6, 7, 8), energy balance (9) and neuroendocrinological response (10). In addition to these functions, we have shown that intracisternal but not intraperitoneal injection of orexin-A dose-dependently stimulated gastric acid secretion in conscious rats (11). The acid stimulation by central orexin-A was completely blocked by atropine or surgical vagotomy, suggesting that orexin-A acts in the brain to stimulate gastric acid secretion through the vagal system. Considering the potent orexigenic action of orexin-A, orexin-A may be an important candidate as a mediator of the cephalic phase secretion as proposed by Pavlov (12). The vagal dependent stimulation of gastric acid secretion of orexin-A furthermore support the hypothesis that orexin-A may play a vital role in cephalic phase gastric secretion because it has been recognized the importance of vagus in conveying the neural impulses that mediate cephalic phase gastric secretion.

We have examined the effect of intracisternal injection of orexin-A and -B on gastric acid secretion and demonstrated clearly that the acid stimulation was induced by orexin-A but not orexin-B (13). It has been so

far shown that orexins bind to two specific receptors, named OX₁R and OX₂R. According to in vitro binding and functional assays, OX₁R is selective for orexin-A and OX₂R is non-selective for orexin-A and orexin-B (1). Based upon the finding, the lack of acid stimulatory action of orexin-B may suggest that orexin-A-induced acid stimulation may be mediated by OX₁R. On the basis of amino acid sequence, orexin-A is a 33-amino acid peptide with an N-terminal pyroglutamyl residue and C-terminal amidation. Orexin-B contains 28 amino acid with C-terminal amidation and is 46 % (13/28) identical in sequence to orexin-A (1). In addition to the difference of number of amino acid, the major difference in peptide structure between orexin-A and -B is existence or lack of disulfide bonds, respectively. Little is however known about the structure requirements of orexin-A for biological activity. In the present study, we synthesized orexin-A-related peptides with modification of the disulfide bonds and examined the effect of the peptides on gastric acid secretion and the potency of activation of orexin receptors in order to clarify the receptor-mediated mechanism of the orexin-A-induced acid stimulation.

Materials and Methods

Animals

Male Sprague-Dawley rats weighing approximately 200 g were housed under controlled light /dark conditions (lights on: 07:00 - 19:00)

with the room temperature regulated to 23-25°C. Rats were allowed free access to standard rat chow (Solid rat chow, Oriental Yeast Co., Tokyo, Japan) and tap water. All experiments were performed in conscious animals deprived of food for 24 h but with free access to water up to the initiation of the experiments.

Chemicals

Synthetic orexin-A (human/bovine/rat/mouse), the 33-amino acid peptide, human orexin-B, the 28-amino acid peptide and a truncated peptide, orexin-A (15-33), were purchased from Peptide Institute Inc., Osaka, Japan. Three alanine substituted orexin-A, [Ala^{6,12}] orexin-A, [Ala^{7,14}] orexin-A and [Ala^{6,7,12,14}] orexin-A, were synthesized in our laboratory using Pioneer peptide synthesizer (Applied Biosystems, Foster City, CA, USA). Amino acid sequence and disulfide bonds of each peptide were shown in Figure 1. These peptides were dissolved in normal saline just before experiments.

Measurements of gastric acid output

Gastric acid secretion was measured using the pylorus-ligation method as described previously (11, 13). Rats received intracisternal injection of orexin-A, orexin-B, orexin-A (15-33) or orexin-A analogues in a dose of 10 µg/10 µl. The doses used in this study were basically selected

according to our previous study (11). Control animals were injected with normal saline (10 μ l) intracisternally. Intracisternal injection was performed under brief ether anesthesia with a 10- μ l-Hamilton microsyringe after rats were mounted in a stereotaxic apparatus (David Kopf Instruments, Tijuana, CA, USA). Following the intracisternal injection and ligation of the pylorus, rats were returned to their cages. Two h after the treatment, the animals were reanesthetized with ether and sacrificed. The stomachs were removed, and the gastric contents were collected and centrifuged. The volume of gastric secretion was measured and the amount of gastric acid determined by titration with 0.01 N NaOH to a pH of 7.0.

Cell culture

CHO-K1 cells (ATCC, Rockville, MD, USA) were stably transfected with human OX₁ R and OX₂ R expression vectors as described previously (1). CHO/OX₁R and CHO/OX₂R cells were grown in D-MEM/F-12 (1:1) medium (Life Technologies, Rockville, MD, USA) supplemented with 10 % fetal bovine serum (Sigma, Tokyo, Japan), 50 units/ml penicillin, 50 μ g/ml streptomycin and 1 mg/ml G418 (Geneticin; Life Technologies) in a 95 % air and 5 % CO₂ humidified atmosphere at 37°C.

Measurement of intracellular calcium mobilization

The agonistic potency of orexin-A-related peptides to orexin receptors was examined by calcium mobilization in the CHO/OX₁R and CHO/OX₂R cells as reported by Sakurai et al. (1). A fluorometric imaging plate reader, FLIPR™ (Molecular Devices, Sunnyvale, CA, USA) was employed to measure the mobilization of intracellular Ca²⁺ in response to the orexin related peptides. CHO/OX₁R and CHO/OX₂R cells were seeded into 96-well black-wall clear-bottom plate (Packard, Meriden, CT, USA) at 5 x 10⁴ cells /well 20 h before assay. The cells were incubated with a Ca²⁺-sensitive fluorescent dye, fluo-3 acetoxymethyl ester (4 μM final concentration; Molecular Probes, Eugene, OR, USA) in an assay buffer (Hank's Balanced Salts Solution (HBSS) containing 20 mM HEPES, 0.5 % BSA and 2.5 mM probenecid at pH 7.4, supplemented with 0.04 % pluronic acid at 37°C for 1 h. Then, cells were washed 4 times with the assay buffer, before setting on a FLIPR™. Orexin-B-induced change in fluorescence over baseline at a dose of 2 μM was determined as a maximum agonist response. The concentration-response curves were fitted and EC₅₀ values are calculated as an index of potency using GraphPad Prism™ (Graphpad Software, San Diego, CA, USA).

Statistical analysis

The results are expressed as mean ± SEM. Statistical analysis was performed by analysis of variance and subsequent Fisher's LSD test. P <

0.05 was considered statistically significant. Correlation analysis was performed by Spearman correlation.

Ethical considerations

Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the Public Health Service. The approval of the Research and Development and Animal Care committees at the Asahikawa Medical College was obtained for all studies.

Results

We examined the effects of intracisternal injection of synthetic orexin-A, orexin-B or orexin-A (15-33) on gastric acid secretion in pylorus-ligated conscious rats. Intracisternal injection of orexin-A in a dose of 10 μ g significantly stimulated gastric acid secretion when compared with saline (Figure 2). In contrast, neither orexin-B nor orexin-A (15-33) in the same dose stimulated acid production (Figure 2). The effect of Ala-substituted orexin-A on gastric acid secretion was also assessed. As shown in Figure 2, the mean value of gastric acid output was increased by all the three tested orexin-A analogues, [Ala^{6,12}]orexin-A, [Ala^{7,14}] and [Ala^{6,7,12,14}], but the stimulation of acid secretion by [Ala^{7,14}]orexin-A or [Ala^{6,7,12,14}] orexin-A did not reach to the statistical significant when compared with saline.

Orexin-A and its related peptides induced a transient increase in $[Ca^{2+}]_i$ in CHO/OX₁R or OX₂R cells in a dose-dependent manner (Fig. 3). Table 1 illustrates the EC₅₀ values for OX₁R and OX₂R of orexin-A and related analogues. Agonistic potency of orexin-B to OX₁R was significantly lower than that of orexin-A, confirming the previous data by Sakurai et al. (1). Orexin-A (15-33), which does not contain both disulfide bonds, to OX₁R was much less potent than that of orexin-A. It was also shown that the tested three alanine-substituted orexin-A peptides lacking either or both disulfide bonds had reduced potency to OX₁R when compared with the full molecule of orexin-A. On the other hand, orexin-A induced a transient increase in $[Ca^{2+}]_i$ dose-dependently in CHO/OX₂R cells. An EC₅₀ value of orexin-B in OX₂R expressing cells was 0.057 nM which is equal to that of orexin-A. Orexin-A (15-33) in CHO/OX₂R cells had the lowest potency to OX₂R among the tested analogues. It was also demonstrated that the potency of the three alanine-substituted orexin-A in CHO/OX₂R was lower when compared with intact orexin-A.

Figure 4 shows the relationship between EC₅₀ values of orexin-A-related peptides in CHO/OX₁R or CHO/OX₂R to increase intracellular $[Ca^{2+}]_i$ and the ability of gastric acid output detected by *in vivo* pylorus ligation method. There was a significant negative correlation between EC₅₀ values for OX₁R and gastric acid output ($p = 0.019$). In contrast, a

significant relationship between EC₅₀ values for OX₂R and gastric acid output was not observed ($p = 0.658$).

Discussion

The present study first clearly demonstrated that the stimulation of gastric acid secretion was observed by orexin-A but not orexin-A (15-33) that does not contain two disulfide bonds. These results may suggest that N-terminal region of orexin-A containing disulfide bonds are necessary for activating a neuronal system to stimulate gastric acid secretion. From the data of intracellular calcium mobilization, orexin-A (15-33) showed weak potency for OX₁R as compared to that of orexin-A. This *in vitro* data furthermore support our above speculation concluded from *in vivo* experiments.

To furthermore clarify our hypothesis that disulfide bonds plays a key role in activating orexin receptors to stimulate gastric acid secretion, we synthesized three ala-substituted analogues of orexin-A lacking either or both disulfide bonds and examined their activity to stimulate gastric acid secretion. When compared with intact orexin-A, the acid stimulatory effect was weaker in rats treated with three synthesized ala-substituted orexin-A, [Ala^{6, 12}] orexin-A, [Ala^{7, 14}] orexin-A and [Ala^{6, 7, 12, 14}] orexin-A. These results suggest that loss of disulfide bonds resulted in reducing the acid stimulatory action of orexin-A. It was also demonstrated that *in vitro*

potency of the alanine-substituted orexin-A was sufficiently reduced in OX₁R and OX₂R expressing cells. This result indicates that disulfide bonds in orexin-A may have a key role in activating orexin receptors.

The above *in vivo* and *in vitro* evidence led us a speculation that a lack of disulfide bonds in orexin-A lowers the activity to orexin receptors, thereby reducing the acid stimulatory action of orexin-A. We therefore next examined the relationship between the potency to stimulate orexin receptors and the ability of acid secretion. The significant inverse relationship between gastric acid output and *in vitro* functional potency for OX₁R but not for OX₂R suggested that acid stimulatory activity of orexin-A depends on its potency for OX₁R. This evidence may indicate that orexin-A-induced stimulation of gastric acid secretion is indeed mediated by OX₁R. So far, it has been postulated that orexin-A may play roles in a variety of body function such as feeding, energy consumption, neuroendocrinological response and gastric secretion (1, 3, 5, 9, 10, 11). However, these evidence came from experimental data obtained by injection of synthetic orexins into the cerebrospinal fluid, indicating that we could not exclude the possibility that these orexin-A-induced changes exert through known orexin receptors-independent pathways. It might be novel for the present study to suggest that the stimulation of gastric acid secretion may be mediated indeed by its specific receptor, OX₁R.

Trivedi et al. have reported the distribution of orexin receptor mRNA in the rat brain (14). According to their data, OX₁R and OX₂R exhibited markedly differential distribution. For instance, high levels of OX₁R mRNA is detected in locus coeruleus whereas OX₂R is not expressed in the brain site. Contrary, neurons in the paraventricular hypothalamic nucleus express OX₂R but not OX₁R. In addition to the locus coeruleus, the lateroanterior hypothalamic nucleus, ventromedial hypothalamic nucleus, amygdalohippocampal area, and dorsal and median raphe highly express OX₁R. Immunohistochemical studies have shown that orexin-immunoreactive fibers and terminals are richly distributed in these brain sites (15, 16, 17). It is therefore speculated that the site of action of orexin-A to stimulate gastric acid secretion should be in the brain nuclei that express OX₁R because the orexin-A-induced stimulation of gastric acid should be mediated through the action of OX₁R as shown in this study.

In summary, the present study suggest that orexin-A-induced stimulation of gastric acid secretion is exerted through the action of OX₁R and that the two disulfide bonds in orexin-A have a key role in activating OX₁R to stimulate the neuronal system for gastric acid production.

Acknowledgments

This work was supported in part by grants-in-aid from the Ministry of Education, Science, Sports and Culture of Japan.

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Figure legends

Figure 1

Amino acid sequence of orexin-A and related peptides used in this study.

Orexin-A is a 33-amino acid peptide with an N-terminal pyroglutamyl residue and C-terminal amidation, and contains two disulfide bonds (Cys6-Cys12 and Cys7-Cys14). Orexin-B is a 28-amino acid peptide with C-terminal amidation and contains no disulfide bond. A synthetic orexin-A fragment, Orexin-A (15-33), does not contain disulfide bond. Three alanine substituted orexin-A, [Ala 6, 12]orexin-A, [Ala 7, 14]orexin-A and [Ala 6, 7, 12, 14]orexin-A, lacks one or both disulfide bonds.

Figure 2

Effect of intracisternal injection of orexin-A and its related peptides on gastric acid output in conscious rats. Under brief ether anesthesia, rats received intracisternal injection of either saline (10 μ l), orexin-A, orexin-B, orexin-A (15-33), [Ala6,12]orexin-A, [Ala7,14]orexin-A or [Ala6,7,12,14]orexin-A in a dose of 10 μ g/10 μ l, and the pylorus was ligated. Amino acid sequence of the tested peptides is shown in Figure 1. Two h after intracisternal injection, the animals were sacrificed and the stomach was removed. Gastric acid output was determined. Each column represents the mean \pm SEM of 5 animals. * $p < 0.01$, when compared with saline.

Figure 3

Dose-response curves of $[Ca^{2+}]_i$ response evoked by orexin-A and its related peptides in cells expressing human OX₁R (upper panel) and OX₂R (lower panel). Amino acid sequence of the tested peptides is shown in Figure 1. Values are the means of a typical experiment (n=3) in quadruplicate.

Figure 4

Correlation between gastric acid output and in vitro functional potency for OX₁R (A) or OX₂R (B) by orexin-A and its related peptides. Amino acid sequence of the tested peptides is shown in Figure 1. A statistical significant relation between the both parameters was observed (A) but not in (B).

Table 1

Pharmacological characterization of synthetic orexin-A related peptides on human orexin receptors stably expressed CHO cells

	[Ca ²⁺] _i response (EC ₅₀ : nM)	
	OX ₁ R	OX ₂ R
orexin-A	0.068 ± 0.0029	0.057 ± 0.0036
orexin-B	0.69 ± 0.082	0.057 ± 0.0081
orexin-A (15-33)	4.1 ± 0.81	1.3 ± 0.24
[Ala ^{6, 12}] orexin-A	0.17 ± 0.0003	0.13 ± 0.0043
[Ala ^{7, 14}] orexin-A	0.66 ± 0.070	0.35 ± 0.020
[Ala ^{6, 7, 12, 14}] orexin-A	0.42 ± 0.036	0.27 ± 0.038

The concentration of orexin-A and related peptides required to induce half-maximum response (EC₅₀) represents the mean ± SE of three individual determinants performed in quadruplicate.

Figure 1

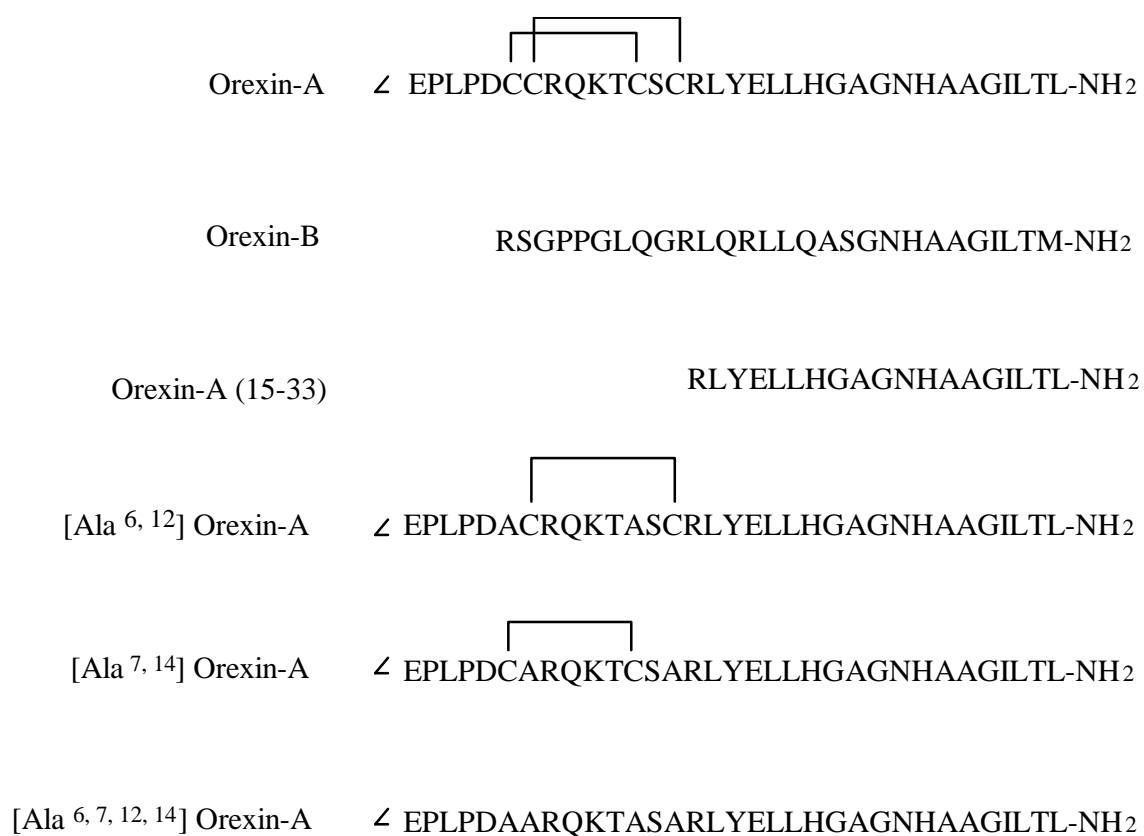


Figure 2

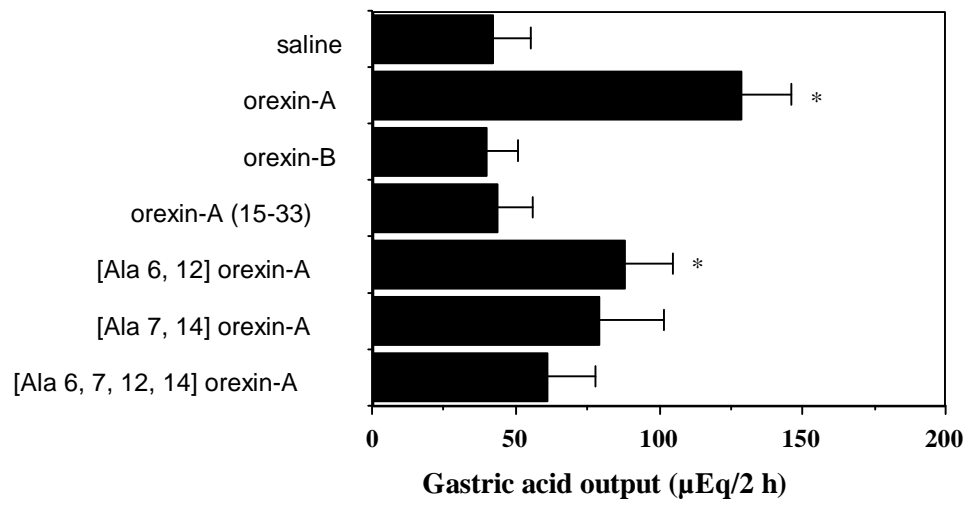


Figure 3

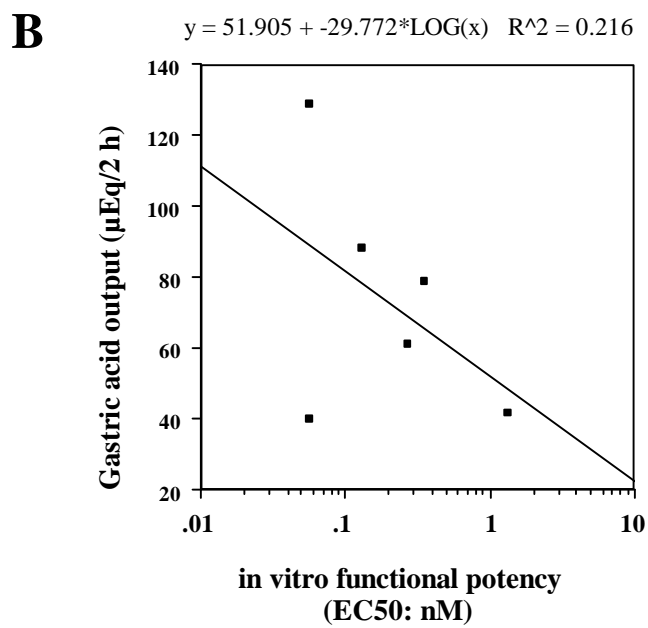
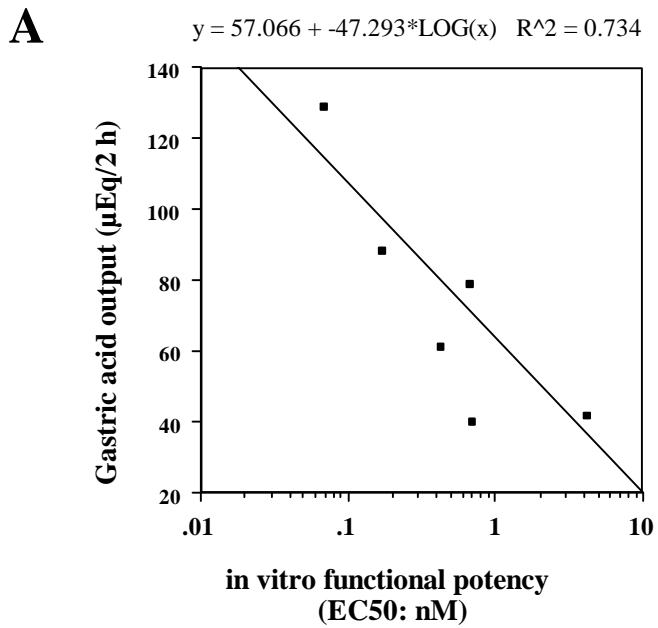


Figure 4