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Abstract

We have previously demonstrated that intracisternal orexin-A potently stimulated gastric acid secretion through the vagus nerve. Considering its stimulatory action on feeding, we hypothesized that orexin-A is a candidate mediator of cephalic phase gastric secretion. It has also been suggested that the stimulation of acid by central orexin-A may be mediated by orexin 1 receptor (OX_1R) in the brain. In the present study, we tried to clarify whether endogenously released orexin-A in the brain indeed plays a physiological role in gastric secretion. To address the question, the effects of OX_1R antagonist on gastric acid secretion was examined in rats. Intraperitoneal administration of SB334867, a specific OX_1R anatagonist, by itself did not change gastric acid secretion in pylorus-ligated conscious rats. Pretreatment with SB334867 in a dose of 10 mg/kg completely blocked the stimulated acid output by intracisternal orexin-A but not thyrotropin-releasing hormone, suggesting that SB334867 specifically blocked the action of orexin-A in the brain. 2-deoxy-D-glucose (2-DG) -induced stimulation of gastric acid output was significantly blocked by pretreatment with intraperitoneal administration of SB334867. These results suggest that endogenously released orexin-A in the brain plays a vital role in central regulation of gastric secretion. Since 2-DG induces central glucoprivation as a hunger state, the present study furthermore support the speculation that orexin-A may be an important molecule that triggers the cephalic phase gastric aicd secretion.

Key words

orexin, orexin receptor antagonist, SB334867, gastric acid, 2-deoxy-D-glucose, cephalic phase

Introduction

Orexins/hypocretins are novel neuropeptides that are localized in neurons in the lateral hypothalamus [8, 30]. It has been so far demonstrated that orexins may be implicated in a wide variety of physiological functions. These include feeding [10, 30, 35], behavioral activity [16], sleep/awake [4, 20, 22], energy balance [21], neuroendocrinological response [19] and cardiovascular functions [5, 31]. In addition to these functions, we suggested for the first time that orexin-A is involved in central regulation of gastrointestinal functions in that intracisternal but not intraperitoneal injection of orexin-A dose-despondently stimulated gastric acid secretion in conscious rats [34]. 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 [28]. The vagal dependent stimulation of gastric acid secretion of orexin-A furthermore supports 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 [11].

The effect of intracisternal injection of orexin-A or -B on gastric acid secretion was examined and it was clearly demonstrated that the acid stimulation was induced by orexin-A but not orexin-B [34]. It has been so far shown that orexins bind to two specific receptors, named OX_1R and OX_2R. According to in vitro binding and functional assays, OX_1R is selective for orexin-A and OX_2R is non-selective for orexin-A and orexin-B [30]. 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 OX_1R. 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 [30]. 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. Considering the structural property of the peptide, 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. The experiments on the structure-activity relationship suggested that the orexin-A-induced acid stimulation requires OX_1R activation and that disulfide bonds in orexin-A may have a key role in the receptor activation [26]. These pharmacological studies suggest that orexin-A acts on OX_1R in the brain to stimulate gastric acid through the vagus nerve. However, we do not know whether endogenously released orexin-A in the brain indeed plays a physiological role in the regulation of acid production. In the present study, we tried to clarify the above question using a recently-developed OX_1R antagonist, SB334867 [32].

Materials and Methods

Animals

Male Sprague-Dawley rats weighing approximately 120 g were housed under controlled light /dark conditions (lights on: 07:00 - 19:00) with the room temperature regulated to 23-25^{o}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) and thyrotropin releasing hormone (TRH) were purchased from Peptide Institute Inc., Osaka, Japan. An OX_1R antagonist,

1-(2-methylbenzoxazol-6-yl)-3-[1,5]naphthydrin-4-yl urea hydrochloride (SB334867)[32], donated by Glaxo SmithKline Research Center (Verona, Italy), was dissolved in 10 % (w/v) encapsin in sterile water. Orexin-A was dissolved in normal saline just before experiments.
2-deoxy-D-glucose (2-DG) was obtained from Sigma (St Louis, MO, USA).

Treatments

We initially examined the dose-related effects of intraperitoneal injection of SB334867, an OX_1R antagonist on gastric acid secretion. Rats received intraperitoneal injection of several doses of the OX_1R antagonist. Following the intraperitoneal injection and ligation of the pylorus, rats were returned to their cages. Two h after the treatment, gastric contents were collected for determination of gastric acid output.

Next, we examined the effects of intraperitoneal injection of SB334867 on the intracisternal orexin-A-induced stimulation of gastric acid secretion to clarify whether SB334867 blocks the central action of orexin-A . Rats received intraperitoneal administration of several doses of SB334867 (0, 1, 3, or 10 mg/kg) and then intracisternal injection of orexin-A (10 µg/10 µl). Intracisternal injection was performed under brief ether anesthesia with a 10-µl-Hamilton microsyringe after rats were mounted in a stereotaxic apparatus (David Kopf Instruments, Tijunga, CA, USA). Immediately after the intracisternal injection, the pylorus was ligated. Two h later, gastric contents were collected for determination of gastric acid output. To clarify whether SB334867 specifically blocks orexin, the effect of SB334867 on acid secretion stimulated by intracisternal TRH was examined. In the experiment, rats received intraperitoneal injection of SB334867 (10 mg/kg) and then intracisternal TRH (10 µg/10 µl). The doses of orexin-A and TRH were selected according to previous studies [27, 34].

To investigate the role of endogenous orexin-A in the stimulation of gastric acid secretion, we examined the effect of SB334867 on the

stimulated gastric acid secretion by 2-DG-induced hypoglycemia. First, we confirmed the role of the vagus nerve in stimulation of gastric secretion by intravenous 2-DG. The dose and injected route of 2-DG was according to our previous report [25]. In a set of experiment, rats underwent bilateral gastric branch vagotomy or sham operation as described previously [23]. Vagotomized or sham-operated control rats received intravenous administration of 2-DG (100 mg/kg) or saline. Immediately after the injection, the pylorus was ligated. Two h later, gastric contents were collected for determination of gastric acid output. Next, in order to investigate if endogenous orexin-A mediates the 2-DG-induced stimulation of gastric acid, intravenous injection of 2-DG (100 mg/kg) was done in rats that had been pretreated with intraperitoneal administration of SB334867 and the acid output was measured as described below.

Measurements of gastric acid output

Gastric acid secretion was measured using the pylorus-ligation method as described previously [34]. 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.

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.

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

Effects of SB334867 on gastric acid secretion in pylorus-ligated conscious rats.

We first examined the effect of intraperitoneal SB334867 on gastric acid secretion in pylorus-ligated conscious rats. As shown in Fig. 1A, intraperitoneal injection of SB-334867 at the doses of 1-30 mg/kg did not change gastric acid output.

Effects of SB-334867 on the central orexin-A-stimulated gastric acid secretion

Intracisternal injection of orexin-A in a dose of 10 μg/rat potently stimulated gastric acid output, being in good agreement with previous experiments [34]. The stimulated gastric acid output by orexin-A was dose-dependently blocked by pretreatment with intraperitoneal injection of SB334867 (Fig. 1B). Intracisternal injection of TRH in a dose of 10 μg potently stimulated gastric acid even in rats that had been treated with SB334867 (Fig. 1C), suggesting that SB334867 selectively blocks central action of orexin-A.

Effects of SB-334867 on 2-DG-induced gastric acid secretion

Next, we examined the effect of SB334867 on the 2-DG-stimulated gastric acid secretion. As demonstrated in Fig 2A, intravenous administration of 2-DG in a dose of 100 mg/kg significantly increased gastric acid secretion. The acid stimulation by 2-DG was not observed in the vagotomized rats, confirming that 2-DG increases acid secretion via a vagal-dependent pathway. Next, we have examined the effect of SB334867 on the 2-DG-induced stimulation of gastric acid. Pretreatment with intraperitoneal SB334867 in a dose of 10 mg/kg abolished the 2-DG-induced acid stimulation (Fig. 2B), strongly suggesting that endogenously released orexin-A in the brain by 2-DG acted on OX_1R and thereby increased acid production.

Discussion

Orexin-A binds to two specific G protein-coupled receptors, OX_1R and OX_2R [30]. With regard to the receptor activation by orexin-A to stimulate acid secretion, we have already performed a couple of experiments on a structure-activity analysis. From the experiments, the orexin-A-induced acid stimulation requires OX_1R activation and disulfide bonds in orexin-A may have a key role in the receptor activation [26]. SB334867 is a non-peptide selective OX_1R anatagonist [29, 32, 33]. Haynes et al. have demonstrated that intraperitoneal administration of SB334867 reduced both centrally injected orexin-A-induced food intake and feeding stimulated by an overnight fast in rats [14]. These results suggest that the OX_1R antagonist, SB334867, made it possible to examine a physiological role of orexin- OX_1R pathways in the biological actions induced by exogenous orexin. We have already demonstrated that exogenously administered orexin-A acts in the brain to stimulate acid secretion [34]. However, little is known whether endogenous orexin-A indeed acts in the central nervous system to increase acid secretion. To clarify the above speculation, we have done experiments using a recently developed selective OX_1R antagonist, SB334867. The major finding of this study is that endogenously released orexin-A induced by 2-DG has a stimulatory effect on gastric acid secretion. These results strongly support that orexin-A plays a physiological role in the central regulation of gastric acid secretion.

In the present study, we administered the OX_1R antagonist peripherally to block the binding of endogenous orexin to OX_1R in the brain. According to the previous reports, OX_1R in the brain could be anatagonized by intraperitoneal injection of SB334867. For instance, intraperitoneal administration of SB334867 blocks the feeding by intracerebroventricular injection of orexin-A in rats [14]. These results must show peripherally administered SB334867 could enter across the blood brain barrier to exert its antagonistic action. Although OX_1R mRNA is highly expressed in the brain, Johren et al. have demonstrated

using RT-PCR that small amounts of OX_1R mRNA are found in other peripheral tissues such as kidney, adrenal, testis, thyroid, ovary and jejunum [17]. One may speculate that effects of OX_1R antagonist administered peripherally as in the present study may be mediated by the blockade of OX_1R in the peripheral sites. However, the possibility that peripheral sites of action of SB334867 resulted in blocking the 2-DG-induced stimulation of gastric acid is unlikely as following. Our previous data clearly demonstrated that centrally but not peripherally administered orexin-A stimulates gastric acid secretion [34], suggesting the site of action of orexin-A must be located in the brain. The present evidence that the stimulation of gastric acid by centrally injected orexin-A was completely blocked by intraperitoneal injection of SB334867, strongly indicating that SB3348867 acts in OX_1R in the central nervous system. Furthermore, if the blockade of OX_1R in the peripheral sites by SB334867 is essential to block the stimulation of acid secretion by 2-DG, centrally administered TRH-induced acid secretion should be inhibited. As shown in the study, SB334867 failed to block the TRH-induced acid stimulation. These results further suggested that the blockade of SB334867 is specific to the action of orexin. With regard to the point that intrapertoneally administered SB334867 could enter into the brain, earlier investigators showed that brain levels of SB334867 after intraperitoneal injection of 10 mg/kg SB334867 is 13.7 μM at 0.5 h and 3.8 μM at 2 h [29], confirming that peripherally administered SB334867 indeed acted in the central nervous system. From these evidence, we would suggest that SB334867 acts in the brain to block the action of endogenous orexin-A.

Cells of origin innervating the stomach through the vagus nerve are located in the dorsal motor nucleus of the vagus nerve (DMN) in the medulla oblongata [24]. Since intracisternally injected orexin-A-induced acid production is mediated by the vagus nerve [34], the DMN neurons projecting their axon terminals to the stomach should be activated by the injection of orexin-A into the cisterna magna. Although orexin-immunoreactive neurons are located only in the hypothalamus [30],

orexin-immunoreactive fibers are widely distributed in the central nervous syetem including the DMN [7, 13, 35]. The fact that orexin-immunoreactive fibers are identified in the DMN may support the idea that the site of action of intracisternally injected orexin-A to induce acid production is on the DMN neurons. Results from recent immunohistochemical studies indicate that the majority of DMN neurons in the rat express orexin receptors, with the OX_1R found in greater abundance than OX_2R [18]. In addition, Krowicki et al., showed by combining immunostaining for OX_1R with retrograde labeling of neurons following injections of fluorescently tagged cholera toxin into the gastric wall that OX_1R is expressed in a majority of preganglionic vagal motor neurons that innervate the stomach [18]. These neuroanatomical studies indicate that orexin-A is capable to binding to OX_1R on DMN neurons innervating the stomach. Moreover, recordings obtained in rat medullary slices revealed that orexin directly depolarize a fraction of DMN neurons, including some that were identified as preganglionic parasympathetic neurons based on their retrograde labeling following intraperitoneal administration of Fluorogold [15]. Grabauskas and Moises have recently demonstrated using whole-cell recordings obtained from DMN neurons in rat brainstem slices that orexins act preferentially within the DMN to directly excite vagal motor neurons that project to gastric fundus and corpus [12]. These electrophysiological studies together with the neuroanatomical evidence described above and the present pharmacological results suggest that endogenous orexin-A from descending hypothalamic projections into the DMN activates OX_1R on the DMN, followed by stimulating the vagal flow that should cause the increase in acid output from the stomach

2-DG has been used as a tool for central activation of the vagal pathway. It has been reported that 2-DG administered peripherally acts in the brain especially in the hypothalamus to increase vagal tone, thereby stimulating gastric acid secretion [6, 9]. However little is known about the precise molecular mechanism in the brain by which the 2-DG stimulated acid secretion. In the present study, we confirmed that

intravenous 2-DG significantly stimulated gastric acid output through the vagus nerve. The stimulatory action by orexin-A was not observed in rats that had been received vagotomy and had been pretreated with atropine [34], strongly suggesting that the orexin-A-induced acid production is a vagus-dependent mechanism. Because both 2-DG and orexin-A stimulate acid secretion through the vagus nerve, we made a hypothesis that orexin-A in the brain might be involved in the 2-DG induced stimulation of acid secretion. The present evidence that SB334867 blocked the 2-DG-evoked stimulation of acid indicated that orexin-A may mediate the 2-DG-induced acid secretion through the vagus nerve.

A couple of reports showed hypoglycemia activates orexin-A neurons. Cai et al., have demonstrated that insulin-induced hypoglycemia stimulated c-fos expression in orexin-A neurons in the LHA in rats [3]. 2-DG is known as a compound that inhibits glucose utilization (glucoprivation) and causes intracellular glucopenia [2]. Briski and Sylvester have examined the effect of 2-DG on c-fos expression in neurons containing orexin-A in the hypothalamus and demonstrated that a large majority of orexin-A neurons in the lateral hypothalamic area were immunostained for c-fos, while orexin-A neurons expressed negligible c-fos immunoreactivity following vehicle administration [1], suggesting that central glucopenia induced by 2-DG activates orexin-A neurons in the LHA. These immunohistochemical studies strongly support our speculation that orexin-A may mediate the 2-DG-induced acid secretion. From an another point of view, orexin-A plays a vital role in the hypoglycemia-induced acid production.

The cephalic phase of gastrointestinal secretion, which occurs in response to the sight, smell, taste, and anticipation of feeding, produces a coordinated secretory response that primes the gut to assist digestion of the impending meal. The most important component of the cephalic phase responses is gastric acid secretion as first characterized by Pavlov [28]. The present study suggested that endogenously released orexin-A possibly induced by central glucoprivention plays a key role in the gastric acid

stimulation, supporting the hypothesis that orexin-A in the brain triggers the cephalic phase stimulation.

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Figure legends

Figure 1

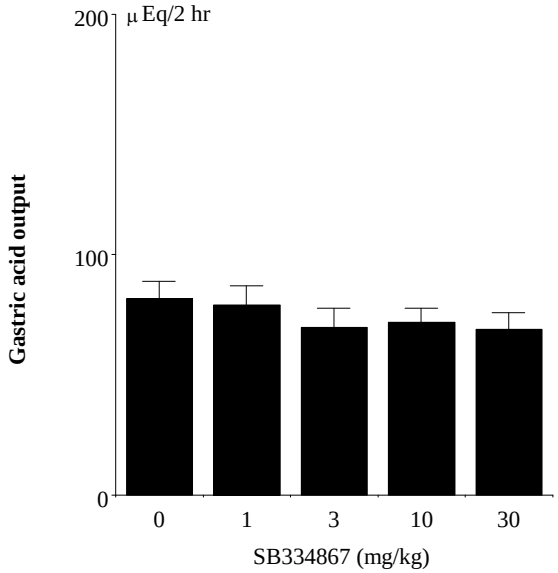
Effects of intraperitoneal injection of an orexin 1 receptor antagonist, SB334867, on gastric acid secretion in pylorus-ligated conscious rats. Dose-response effects of SB334867 on basal (Fig 1A) or intracisternal orexin-A(10 μg/10 ul/rat)-stimulated acid secretion (Fig. 1B). Fig. 1C shows the effect of SB334867 (10 mg/kg) on the intracisternal TRH-stimulated acid secretion. Gastric juice was collected 2 hr after the pylorus-ligation. Each bar represents the mean ± SEM of 5 animals. In Figure 1B, * $p < 0.01$ when compared with control without orexin-A (ic). ** $p < 0.01$, when compared with SB334867 (0 mg/kg) with orexin-A (ic). In Figure 1C, *, ** $p < 0.01$ when compared with saline control, respectively.

Figure 2

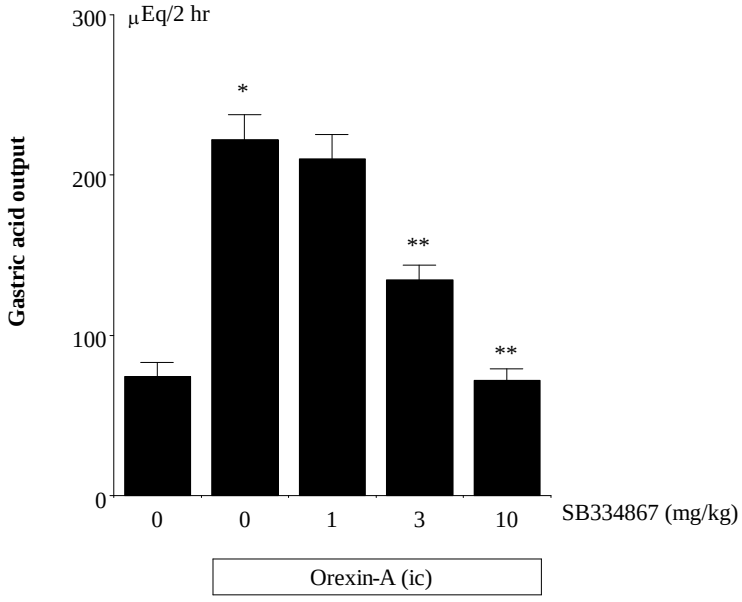
Effects of intraperitoneal injection of an orexin 1 receptor antagonist, SB334867, on 2-DG-stimulated gastric acid secretion. Fig. 2A shows the effect of surgical vagotomy on the 2-DG-induced acid stimulation. Effect of SB334867 on the 2-DG-evoked acid production was examined (Fig.2B). Gastric juice was collected 2 hr after the pylorus-ligation. Each bar represents the mean ± SEM of 5 animals. * $p < 0.01$, when compared with saline.

Figure 1

A


200
μEq/2 hr
100
0
Gastric acid output
0
1
3
10
30
SB334867 (mg/kg)

B


300
μEq/2 hr
200
100
0
Gastric acid output
*
**
**
0
0
1
3
10
SB334867 (mg/kg)
Orexin-A (ic)

C

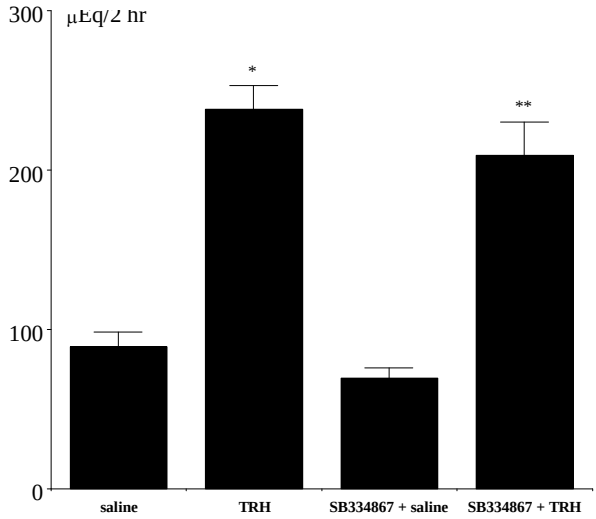
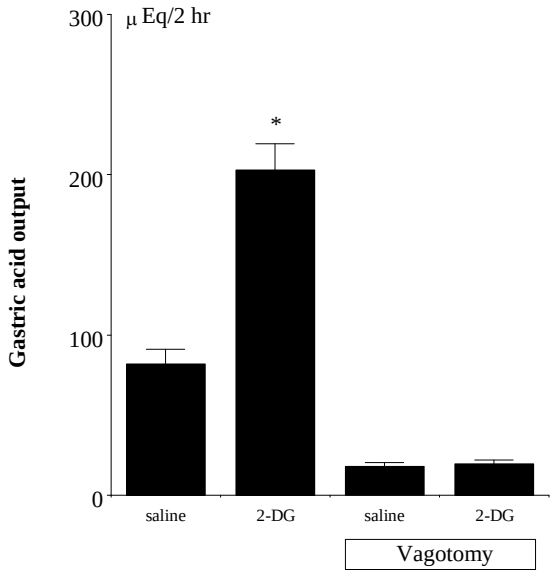
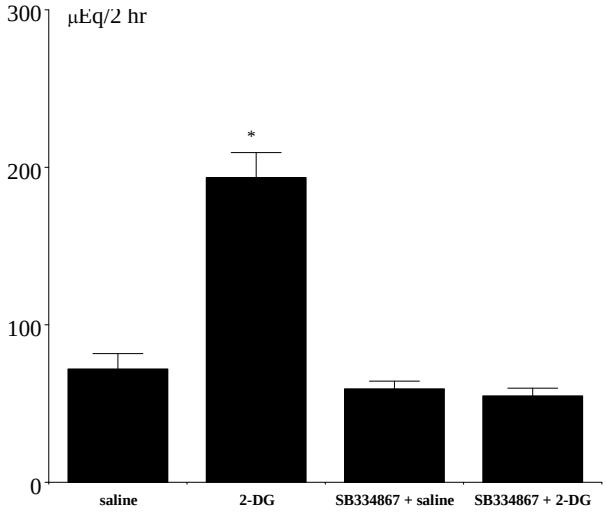

300
µEq/2 hr
200
100
0
*
**
saline
TRH
SB334867 + saline
SB334867 + TRH

Figure 2

A


300
μ Eq/2 hr
*
200
100
0
Gastric acid output
saline
2-DG
saline
2-DG
Vagotomy

B


300
μEq/2 hr
*
200
100
0
saline
2-DG
SB334867 + saline
SB334867 + 2-DG