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Effects of CP-99, 994, a tachykinin NK_1 receptor antagonist, on abdominal afferent vagal activity in ferrets: evidence for involvement of NK_1 and 5-HT_3 receptors

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Abstract

The effect of CP-99, 994, a tachykinin NK_1 receptor antagonist, on abdominal vagal afferent nerve activity in the ferret was investigated. Substance P (1 μg/kg, i.v.) increased vagal afferent activity by 449.0 ± 51.9% and this was reduced to 145.9 ± 5.7% ($p < 0.01$) by pre-treatment with CP-99, 994 (1 mg/kg, i.v.), and to 149.5 ± 1.5% ($p < 0.001$) by granisetron (1 mg/kg, i.v.), a 5-HT_3 receptor antagonist. In addition, the increase in vagal nerve activity induced by 5-HT (25 μg/kg, i.v., 552.0 ± 57.0% increase from pre-injection level) was significantly reduced (401.3 ± 10.6% increase from pre-injection level, $p < 0.05$) by CP-99, 994 (100 μg/kg, i.v.). These results provide evidence for an involvement of peripheral NK_1 and 5-HT_3 receptors in substance P-induced vagal afferent activation. While the functional consequences (if any) of such peripheral effects were not investigated, they could contribute either directly (e.g. by blockade of receptors on vagal afferents) or indirectly (e.g. modulation of 5-HT release or reduction of local inflammatory response) to the antiemetic effects of CP-99, 994 against cisplatin and other emetic agents acting primarily via the vagus.

Keywords: CP-99, 994; CP-100, 263; Tachykinin NK_1 receptor antagonist; Antiemetic activity; Abdominal afferent nerve activity; Granisetron; Substance P

1. Introduction

Substance P is widely distributed in the central and peripheral divisions of the nervous system and in enteroendocrine cells of the gut (Pernow, 1983) and is the preferred ligand for the tachykinin NK_1 receptor (Petitet et al., 1993). The highly selective and potent non-peptide tachykinin NK_1 receptor antagonist, CP-99, 994 [(+)-(2S,3S)-3-(2-methoxybenzylamino)-2-phenylpiperidine] and other NK_1 receptor antagonists (e.g. L-754, 030, GR 203040) have been shown in a number of pre-clinical studies, in several species including the ferret, to have broad-spectrum antiemetic effects against stimuli with either central (e.g. motion, apomorphine, loperamide) or peripheral (cisplatin, intragastric copper sulphate) sites of action (Watson et al., 1995a,b; Gardner et al., 1995; Rupniak et al., 1997; Tattersall et al., 2000; Zaman et al., 2000). This broad-spectrum of action, binding and c-*fos* studies, central microinjection studies and the lack of effect of non-peptide, poorly brain penetrant tachykinin NK_1 receptor antagonists has led to the conclusion that their site of action is central, most likely in the brainstem (Watson et al., 1995a,b; Tattersall et al., 1996; Rupniak et al., 1997; Rupniak and Kramer, 1999; Zaman et al., 2000). However, Watson et al. (1995b) noted in relation to the antiemetic effects of CP-99, 994 "that the main site of action is in the nucleus tractus solitarius, although this does not exclude involvement of a peripheral site (e.g. vagal afferents responding to mucosally released substance P) for some stimuli (e.g. copper sulphate)."

We recently reported that sendide, a peptide NK_1 receptor antagonist (Sakurada et al., 1993) presumed to be

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poorly brain penetrant, reduced (retching by 44% and vomiting by 62%) but did not block acute cisplatin-induced emesis in the ferret (Minami et al., 1998) when given at a dose of 3 mg/kg, s.c. In contrast to the studies described above, this observation provides a preliminary indication that peripheral NK_1 receptors could be involved in the acute emetic response to cisplatin, a cytotoxic anti-cancer drug. However, a central effect of sendide cannot be excluded as it may access the dorsal brainstem regions involved in emesis such as the nucleus tractus. There is some evidence from rats that medial and ventral zone capillaries of the area postrema may make vascular links with the dorsal aspects of the commissural subnucleus of the nucleus tractus (Roth and Yamamoto, 1968; Gross et al., 1990). In addition, morphological and functional evidence has been presented for unexpectedly high capillary permeability in the nucleus tractus solitarius using [^{14}C]-α-aminoisobutyric acid as a marker (Gross et al., 1990, 1991).

The predominant mechanism by which cisplatin (and other cytotoxic anti-cancer drugs) induces acute emesis is proposed to be via the release of 5-hydroxytryptamine (5-HT) (and probably other neuroactive agents such as substance P) from gut mucosal enterochromaffin cells, with the 5-HT activating 5-HT_3 receptors located on abdominal (predominantly vagal) visceral afferents (see Andrews and Davis, 1995 and Minami et al., 1995 for review). A peripheral site of action of sendide is supported by the observation that it inhibited both the substance P- and 5-HT-induced increase in abdominal vagal afferent nerve activity in anesthetized ferrets (Minami et al., 1998). Sendide or granisetron (a highly selective and potent 5-HT_3 receptor antagonist) significantly inhibited the increase in 5-HT release from ferret isolated ileum induced by 2-methyl-5-HT, a 5-HT_3 receptor agonist (Minami et al., 1998). Taken together, these results suggest that tachykinin NK_1 receptor antagonists may act to interfere directly and/or indirectly with the peripheral transduction of the emetic signal.

The aim of the present study was to investigate whether a non-peptide NK_1 receptor antagonist (CP-99, 994) could be shown to have similar peripheral effects to those previously demonstrated for the peptide NK_1 receptor antagonist sendide. To elucidate further, the involvement of tachykinin NK_1 and 5-HT_3 receptors, we determined the effects of CP-99, 994 and granisetron on abdominal afferent vagal nerve activity induced by substance P and 5-HT.

2. Materials and methods

2.1. Measurement of abdominal afferent vagal activity

Experiments were performed using ferrets weighing an average of 1.64 (1.5–1.9) kg. Adult male fitch ferrets (*Mustela putorius furo* L.) were supplied by Marshall Research Animals (North Rose, NY, USA). They were fed on cat chow (Purina, Taiyo, Japan) and allowed water ad libitum. This study was conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals by the Animal Research Committee of Health Sciences University of Hokkaido.

Ferrets were deprived of food overnight and then anesthetized with urethane (500 mg/kg, i.p.) and α-chloralose (50 mg/kg, i.p.), immobilized with gallamine triethiodide (10 mg/kg, i.v.) and maintained by artificial respiration. Electrocardiogram and blood pressure were recorded continuously (Nihon-Kohden, AP-641G, Japan). An arterial blood pressure transducer (Nihon-Kohden, TP-400T) was connected to a polyethylene catheter that was placed in the left common carotid artery, as described previously (Minami et al., 1994). Respiration was maintained through a tracheal cannula connected to a respirator (Harvard Apparatus, model 683, USA). Using an expired gas monitor (Nippondenki San-ei, IH26, Japan), ventilation was adjusted to maintain an end tidal O_2 and CO_2 at approximately 15% and 5%, respectively (Yoshioka et al., 1992; Endo et al., 1995; Minami et al., 1997). Rectal temperature was maintained between 37 and 38 °C with a heating pad (Natsume homeothermic blanket control, KN-474, Japan). A cannula was placed in the external jugular vein for administration of drugs. A minimum time of 10 min was allowed between injections of agonists as our earlier study (Minami et al., 1998) showed that this interval is adequate for evoked changes in abdominal vagal afferent activity to return to baseline. Studies of the Bezold–Jarisch reflex (a reflex elicited by activation of cardio-pulmonary vagal afferents) in the ferret by Middlefell and Price (1991) using jugular injection of 5-HT also demonstrated that a 10 min interval between injections was adequate and also used a 10 min pre-treatment time for investigating the effects of a 5-HT_3 receptor antagonist (ondansetron). Doses of 5-HT between 10 and 30 μg/kg, i.v. were used by Middlefell and Price (1991) to evoke "reproducible submaximal Bezold–Jarisch reflex responses." The present study used a 5-HT dose of 25 μg/kg, i.v. The cardiovascular response to 1 μg/kg, i.v. of substance P was a reduction in blood pressure, which returned to baseline levels within 1 min. This hypotensive effect is qualitatively similar to that reported in the dog and cat under anaesthesia (Burcher et al., 1977). An investigation of the cardiovascular effects of 5-HT and substance P was not the main objective of the present study and therefore were not quantified although are commented upon in the discussion.

The dorsal abdominal vagus nerve was exposed and sectioned just below the diaphragm. The ventral vagus remained intact. Afferent abdominal vagus nerve activity was recorded 5 mm from the peripheral cut end of the nerve with bipolar platinum–iridium wire electrodes as described by Yoshioka et al. (1992). This portion of the nerve was immersed in a pool of warm paraffin oil. The nerve activity was amplified, passed through a filter (time

Table 1
Effects of intravenous administration of CP-99, 994 and granisetron on the substance P-induced increase in abdominal afferent vagal nerve activity in anesthetized ferrets

Drugs (μg/kg, i.v.)	*n*	Change from pre-injection level (%)
SP(1)	9	449.0 ± 51.9
SP(5)	7	754.7 ± 94.5
SP(10)	9	1030.1 ± 160.0
SP (1) + CP-99, 994 (10)	4	453.9 ± 66.9
SP (1) + CP-99, 994 (100)	6	266.7 ± 57.9 *
SP (1) + CP-99, 994 (1000)	4	145.9 ± 25.6 * *
SP (1) + CP-100, 263 (100)	6	473.7 ± 48.0
SP (1) + granisetron (1000)	4	149.5 ± 6.7 * * *

Mean ± S.E.
Statistically significant differences were assessed by one way ANOVA, followed by Dunnett's multiple range test (for multi-groups) or Student's *t*-test (for two groups).
* $p < 0.05$.
* * $p < 0.01$.
* * * $p < 0.001$ vs. SP (1).

constant of 6.7 ms, and a high cut-off filter of 1000 Hz) and displayed on a thermal array recorder (Nihon-Kohden, RTA-1300A). The discharge rates (Hz) were monitored using a real-time data analyzer (ATAC-450, Nihon Kohden) and the effects of agonists calculated as the percentage increase relative to spontaneous baseline activity. A dose of 25 μg/kg, i.v. of 5-HT was used as the test stimulus, as in a previous study of the dose-dependent increase in abdominal vagal afferent activity in the ferret (Minami et al., 1998, data not presented) identified 25 μg/kg, i.v. as a submaximal dose, as is the case for its cardiovascular effects (see above). Indirect support for this as a submaximal dose comes from a study of gastric vagal afferents in the ferret in which the dose sensitivity to injection of 5-HT into the gastric arterial supply was investigated (Blackshaw and Grundy, 1993). There was no indication that the afferent response was reaching a plateau at a dose of 20 μg given by this direct route. A previous study had shown that a dose of substance P of 1 μg/kg, i.v. elicited a submaximal vagal afferent response (Minami et al., 1998), this was confirmed in the present study (see Section 3.1) and therefore this dose was selected to investigate the effects of the NK_1 and $5\text{-}HT_3$ receptor antagonists.

The receptor antagonists CP-99, 994 or granisetron were injected 10 min before intravenous administration of 5-HT or substance P. The doses of antagonists used have previously been demonstrated to have antiemetic effects against cisplatin in the ferret when given systemically (e.g. Reynolds et al., 1991; Watson et al., 1995b). Nerve activities were expressed as the percentage change in baseline value evoked by agonist (i.e. substance P, 5-HT) and compared with the responses to the same agonists over the same time period following administration of either CP-99, 994, CP-100, 263 or granisetron.

2.2. Drugs

CP-99, 994, (+)-(2S,3S)-3-(2-methoxybenzylamino)-2-phenylpiperidine (Pfizer, Groton, CT, USA) and its enantiomer CP-100, 263, (−)-(2R,3R)-3-(2-methoxybenzylamino)-2-phenylpiperidine, which has a lower affinity for the NK_1 receptor (Watson et al., 1995b), were dissolved in normal saline. Granisetron hydrochloride (SmithKline Beecham, Tokyo, Japan), 5-HT creatinine sulphate (Sigma, St. Louis, MO, USA) and substance P (Research Biochemicals, Natick, MA, USA) were dissolved in normal saline. An equal volume of physiological saline was administered to control animals. Doses are expressed in terms of the salt.

2.3. Statistical analysis

All values are given as mean ± S.E. Statistically significant differences were assessed by one way ANOVA followed by Dunnett's multiple range test (for multi-groups) or Student's *t*-test (for two groups) (Wallenstein et al., 1980). A $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effects of CP-99, 994 on abdominal afferent vagus nerve activity

Intravenous bolus injection of substance P (1–10 μg/kg, i.v.) produced a dose-dependent increase in abdominal afferent nerve activity (Table 1). 5-HT (25 μg/kg, i.v.) also produced an increase in abdominal afferent nerve activity. The increased vagal activity induced by repeated administration of substance P or 5-HT was reproducible (data not shown). Basal vagal afferent activity was not affected by CP-99, 994 or granisetron alone at the doses used.

As shown in Table 1, the increase in abdominal afferent nerve activity of the vagus induced by substance P (1

Table 2
Effects of CP-99, 994 on the 5-HT-induced increase in abdominal afferent vagal nerve activity in anesthetized ferrets

Drugs (μg/kg, i.v.)	*n*	Increase from pre-injection level (%)
5 HT (25)	7	552.0 ± 57.0
5 HT (25) + CP-99, 994 (10)	7	512.8 ± 34.8
5 HT (25) + CP-99, 994 (100)	6	401.3 ± 58.5 *
5 HT (25) + CP-100, 263 (100)	7	589.5 ± 53.5

Mean ± S.E.
Statistically significant differences were assessed by one way ANOVA, followed by Dunnett's multiple range test (for multi-groups) or Student's *t*-test (for two groups).
* $p < 0.05$ vs. 5-HT (25 μg/kg).

μg/kg, i.v.) was significantly reduced by CP-99, 994 (100 and 1000 μg/kg, i.v.) but not by CP-100, 263 (100 μg/kg, i.v.), the enantiomer of CP-99, 994. Intravenous administration of granisetron (1000 μg/kg) also significantly inhibited the increase induced by substance P (1 μg/kg, i.v.) (Table 1). Furthermore, the increase in abdominal afferent nerve activity of the vagus induced by 5-HT (25 μg/kg, i.v.) was significantly inhibited by CP-99, 994 (100 μg/kg, i.v.) but not by CP-100, 263 (100 μg/kg, i.v.) (Table 2).

4. Discussion

The present study has shown that the highly selective and potent non-peptide tachykinin NK_1 receptor antagonist CP-99, 994 had a similar effect to the peptide tachykinin NK_1 receptor antagonist sendide (Minami et al., 1998) in inhibiting in a dose-related manner both the substance P- and 5-HT-induced increase in abdominal vagal afferent nerve activity in anesthetized ferrets. This study also confirmed that granisetron, a highly selective 5-HT_3 receptor antagonist, significantly inhibited substance P-induced increase in the abdominal afferent vagal nerve activity. The effects of CP-99, 994 on vagal afferent activity will be discussed before considering the possibility of a peripheral contribution to the site of antiemetic action of NK_1 receptor antagonists.

4.1. CP-99, 994 and vagal afferent activity

The reduction in substance P-induced increase in vagal afferent discharge by CP-99, 994 indicates an involvement of tachykinin NK_1 receptors. The response to substance P was not abolished even at a CP-99, 994 dose of 1 mg/kg, i.v., implicating other tachykinin receptors in the response and such receptors have been identified in the gastrointestinal tract (Grady et al., 1996). The results of the present study do not allow us to distinguish direct effects of substance P on abdominal vagal afferent NK_1 receptors to be separated from indirect effects on enteric neurons, smooth muscle or enterochromaffin cells, which could also result in an increase in afferent activation (e.g. via muscle contraction or 5-HT release). Although the enantiomer (CP-100, 263) was without effect at the same dose as CP-99, 994 (i.e. 100 μg/kg) it would have been desirable to study higher doses to draw firmer conclusions regarding the involvement of NK_1 receptors. The reason for this is that although CP-100, 263 is >1000 times less potent than CP-99, 994 at displacing [^{125}I]-Bolton–Hunter conjugate of substance P from ferret brain membranes and human fibroblast (IM-9) cells (Watson et al., 1995b), in vivo studies of emesis (e.g. ipecac) have shown that it may have limited antiemetic effects at doses 10-fold greater than CP-99, 994 (Watson et al., 1995b). The pharmacological mechanism involved is not known but may be due to an effect on ion channels (e.g. Ca^{2+}, McLean et al., 1993) and such effects could perhaps account for some of the non-specific effects of CP-99, 994, which have been reported at higher doses (Smith et al., 1994).

The increase in abdominal vagal afferent activity induced by substance P was reduced by granisetron to a similar extent to that produced by CP-99, 994. This implies that substance P is activating a pathway which contains a 5-HT_3 receptor presumably activated by released 5-HT. While we are not aware of studies demonstrating the presence of NK_1 receptors on enterochromaffin cells, they have been located on myenteric neurons (Southwell et al., 1998). In the longitudinal muscle myenteric plexus preparation of guinea-pig ileum, substance P has been shown to stimulate the release of [^{3}H]-choline. Acetylcholine acting via a muscarinic receptor has been shown to stimulate the release of 5-HT from enterochromaffin cells (Schwörer et al., 1991). Thus, exogenous substance P could evoke a release of 5-HT from enterochromaffin cells via activation of an enteric neuron and the 5-HT released could in turn increase vagal afferent activity via 5-HT_3 receptor activation. Such a mechanism could account for the reduction in substance P-induced afferent activity by a selective 5-HT_3 receptor antagonist.

CP-99, 994 but not the enantiomer CP-100, 263 (see above) significantly reduced the vagal afferent response to 5-HT by about 27%. This result suggests that 5-HT may act partially via the release of substance P, which in turn acts on a tachykinin NK_1 receptor leading directly or indirectly to afferent activation. Although substance P and 5-HT evoked similar increases in vagal afferent discharge (substance P: $449.0 \pm 51.9\%$; 5-HT: $552.0 \pm 57.0\%$) CP-99, 994 had a more marked effect on the response to substance P than 5-HT. This may indicate that 5-HT releases a relatively small amount of endogenous substance P to act on NK_1 receptors in comparison to the effect of 1 μg/kg of exogenous substance P. While we cannot identify either the site at which 5-HT is acting or the source of the substance P, the enteric nervous system, the enterochromaffin cells or axon collaterals of visceral afferents are worthy of investigation as all contain substance P and have responses mediated by 5-HT_3 receptors (Sundler et al., 1977; Holzer, 1988; Sanger, 1992; Costa et al., 1996).

It is worth noting that the reflex bradycardia and respiratory changes induced by jugular injection of 2-methyl-5-HT acting on cardio-pulmonary vagal afferents in the ferret was not affected by intravenous administration of CP-99, 994 at the same dose used in the present study (Watson et al., 1995b) but was abolished by 5-HT_3 receptor antagonists (Andrews et al., 1992). In the present study using 5-HT, the decrease in blood pressure appeared unaffected by CP-99, 994 but was clearly abolished by granisetron (unpublished observation). These observations argue against the effects of 5-HT on abdominal vagal

afferent discharge being secondary to systemic cardiovascular changes. Similarly, while CP-99, 994 markedly reduced the vagal afferent response to substance P (see Table 1), it was without apparent effect on the hypotensive response (unpublished observation). Granisetron was also without effect on the cardiovascular response to substance P (unpublished observation).

4.2. Central vs. peripheral site(s) of action of CP-99, 994

The broad-spectrum antiemetic effects of tachykinin NK_1 receptor antagonists against both central and peripherally acting emetic agents has been ascribed to an action at an as yet unidentified site in the brainstem. This conclusion is based upon the broad-spectrum of action, binding studies, the requirement for central penetration, central microinjection and neurophysiological studies (Gardner et al., 1994; Hargreaves et al., 1994; Watson et al., 1995a,b; Tattersall et al., 1996, 2000; Fukuda et al., 1999).

While the weight of evidence supports a central site of action for non-peptide tachykinin NK_1 receptor antagonists including CP-99, 994, the results from the present and the previous study (Minami et al., 1998) shows that some consideration should be given to a contribution from a peripheral site of action for tachykinin NK_1 receptor antagonists against cisplatin and perhaps other emetics acting via abdominal vagal afferents. A peripheral site of action would not be readily apparent from in vivo studies of emesis with brain penetrant agents such as CP-99, 994, and central injection studies with such agents do not exclude an additional peripheral site of action as this would be obscured by the potent central blockade of all emetic inputs.

The results from this study, although only providing circumstantial evidence for a peripheral contribution to the antiemetic action for CP-99, 994, do provide direct evidence that systemically administered CP-99, 994 can have a peripheral effect in modulating abdominal vagal afferent activation. As substance P and 5-HT are well-known to be involved in epithelial inflammatory responses, and gut inflammation has been implicated in the delayed emetic response to cytotoxic drugs (Andrews and Davis, 1995), an action of CP-99, 994 to modulate responses mediated by 5-HT and substance P could indirectly contribute to its efficacy against the delayed phase of cisplatin-induced emesis in the ferret (Watson et al., 1995a; Rudd et al., 1996). Tattersall et al. (1996) reported that the tachykinin NK_1 receptor antagonists L-741, 671 and L-743, 310 inhibited plasma protein extravasation (a component of an inflammatory response) induced by resiniferatoxin from the guinea-pig esophagus. In the clinic, the non-peptide tachykinin NK_1 receptor antagonist L-754, 030 has been shown to be efficacious in the delayed phase of cisplatin-induced emesis when used in combination with dexamethasone and granisetron (Navari et al., 1999). Further studies are required to investigate whether a peripheral action of a NK_1 receptor antagonist could contribute to such clinical effects.

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