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Phoneutria nigriventer spider venom activates 5-HT_4 receptors in rat-isolated vagus nerve

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1 The venom of *Phoneutria nigriventer* spider (PNV) causes intense pain and inflammation following an attack. We have investigated the involvement of capsaicin-sensitive nerve fibres by utilizing an *in vitro* nerve preparation. Extracellular DC potential recordings were made from the rat-isolated vagus nerve, a preparation that is rich in capsaicin-sensitive, that is, nociceptive, C-fibres.

2 PNV (1 – 10 $\mu g\,ml^{-1}$), capsaicin (0.03 – 0.3 μM) or 5-hydroxytriptamine (5-HT) (0.3 – 3 μM) induced dose-dependent depolarizations of vagus nerve fibres. Depolarizing responses to capsaicin were blocked by ruthenium red (RR, 10 μM), but responses to PNV were not. Depolarizing responses to PNV or veratridine (50 μM) were inhibited by tetrodotoxin (TTX, 10 μM), but those to capsaicin were not. This suggests that capsaicin and PNV depolarize the nerve fibres by distinct mechanisms.

3 Depolarization in response to 5-HT (3 μM) was reduced by the 5-HT_3 receptor antagonists Y25130 (0.5 μM) and tropisetron (10 nM) or, to a lesser extent, by the 5-HT_4 receptor antagonist RS39604 (1 or 10 μM). Depolarizing responses to PNV were not affected significantly by Y25130 or tropisetron, but were blocked by RS39604.

4 These data show that 5-HT_4 receptors play a significant role in the activation of nociceptive sensory nerve fibres by PNV and suggest that this is of importance in the development of the pain and inflammation associated with bites from the *P. nigriventer* spider.
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Keywords: *Phoneutria nigriventer*; serotonin; 5-HT_4; vagus nerve; pain; sensory nerve

Abbreviations: PNV, *Phoneutria nigriventer* venom; TTX-R, tetrodotoxin-resistant; TRPV1, capsaicin-gated ion channel; VGSC, voltage-gated sodium channel

Introduction

Species of the genus *Phoneutria* are found throughout South America. Bites by these spiders are the second most common cause of arachnid accidents in Brazil and can be fatal (Lucas, 1988; Bucaretchi *et al*., 2000). Following a bite from *Phoneutria*, patients present mainly with local symptoms that include intense pain, hyperaemia and oedema. In serious cases, peripheral vascular collapse has been reported as well as systemic effects such as tachycardia, hypertension, priapism and pulmonary oedema (Bucaretchi *et al*., 2000). Intradermally injected *Phoneutria nigriventer* venom (PNV) acts as a potent agent producing plasma protein extravasation in rat and rabbit dorsal skin (Antunes *et al*., 1992). In rats, the PNV-induced protein extravasation is blocked by pretreatment of neonatal rats with capsaicin (Costa *et al*., 1997) or by administration of a NK_1 receptor antagonist (Palframan *et al*., 1996). These results suggest an hypothesis that PNV activates nociceptive C-type sensory neurons and causes the release of neuropeptides such as substance P from peripheral sensory nerve endings. This hypothesis is consistent with the symptoms of pain and inflammation that are produced by PNV in humans.

In this study we have used the rat-isolated vagus nerve preparation, which is rich in capsaicin-sensitive C-fibres (Marsh *et al*., 1987), to test directly the hypothesis that PNV activates nociceptive C-fibres and to determine whether the response is because of an action on TRPV1 (VR1, capsaicin) receptors or by some other mechanism.

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Methods

Preparation of rat vagus nerve and electrophysiological recordings

Experiments were performed on vagus nerves taken from male Wistar rats (>250 g). Animals were killed humanely by asphyxiation in a chamber filled with an increasing concentration of carbon dioxide gas. The chest and neck region was carefully opened and a 1.5 – 2 cm length of each vagus nerve was rapidly excised from a point just peripheral to the nodose ganglion down to the mid-thoracic region and placed in a physiological buffer solution (NaCl, 130 mM; $CaCl_2$, 1 mM; $MgCl_2$, 1 mM; KCl, 3 mM; glucose, 11 mM; HEPES, 5 mM, pH adjusted to 7.4 with NaOH) in a Petri dish, where the epineural sheath was removed using fine forceps. Each nerve was then mounted in a custom-made vaseline-gap apparatus that was made according to the design described by Rang & Ritchie (1988) save that the 10 mm long

channel containing the vaseline was 1.0 mm rather than 0.3 mm in diameter. One end of the nerve that emerged from the vaseline gap was immersed in a stationary pool of buffer and the other end was continuously superfused at a rate of 0.8 – 1.2 ml min^{-1} at room temperature (20 – 22°C). The extracellular DC potential across the vaseline gap was recorded using Ag/AgCl pellet electrodes that were in contact with the bathing solution around the nerve, one on each side of the gap. The signal was then amplified (WPI, DAM50) and recorded on both a chart recorder and a personal computer via an A/D interface (Axon Instruments) under the control of axotape (v1.1) software. PNV or other test agents were added to the superfusate and applied to one end of the nerve. Usually, depolarizing test agents were applied for 2 – 3 min except for experiments in which cumulative dose – response data were collected (see below). A maximum of three depolarizing stimuli were tested on a single nerve preparation (e.g. 5-hydroxytriptamine (5-HT), 10 mM KCl and PNV) and these were applied at least 25 min apart. DC potential changes evoked by drugs were then measured as the deflection from the baseline that occurred on drug application.

Venom and reagents

PNV was obtained from the Butantan Institute (São Paulo, Brazil). The venom was dialysed using dialysis tubing (MW >12,000, Sigma) to remove low-molecular-weight components including any histamine or 5-HT which may be present in the raw venom (Antunes *et al*., 1992). Following dialysis, samples of venom were analysed using HPLC linked to an electrochemical detector to ensure that 5-HT was absent from test samples. Data were obtained from four different batches of dialysed venom, but at the maximum concentration of PNV tested there was no significant difference in the data obtained with different batches. The drugs and reagents used were obtained as follows: capsaicin, 5-HT, ruthenium red, tropisetron (3-tropanyl-indole-3-carboxylate hydrochloride), veratridine (Sigma); RS39604 hydrochloride, tetrodotoxin and Y25130 hydrochloride (Tocris).

Statistical analysis

Results are presented as means ± s.e.m. unless otherwise indicated. For all statistical comparisons, two-tailed paired and unpaired Student's *t*-tests were used as indicated. $P<0.05$ was considered as significant. The value of n quoted for experiments refers to the number of nerve preparations used.

Results

Depolarizing effect of PNV on the rat vagus nerve

When applied to rat-isolated vagus, PNV (1 – 10 μg ml^{-1}, $n=11$, Figure 1a, b) induced an immediate, dose-dependent depolarization that was qualitatively similar to that produced by capsaicin (0.03 – 0.3 μM, $n=6$, Figure 1a, c). A second application of PNV (10 μg ml^{-1}) repeated 20 – 25 min after the first gave a response of about the same size indicating that

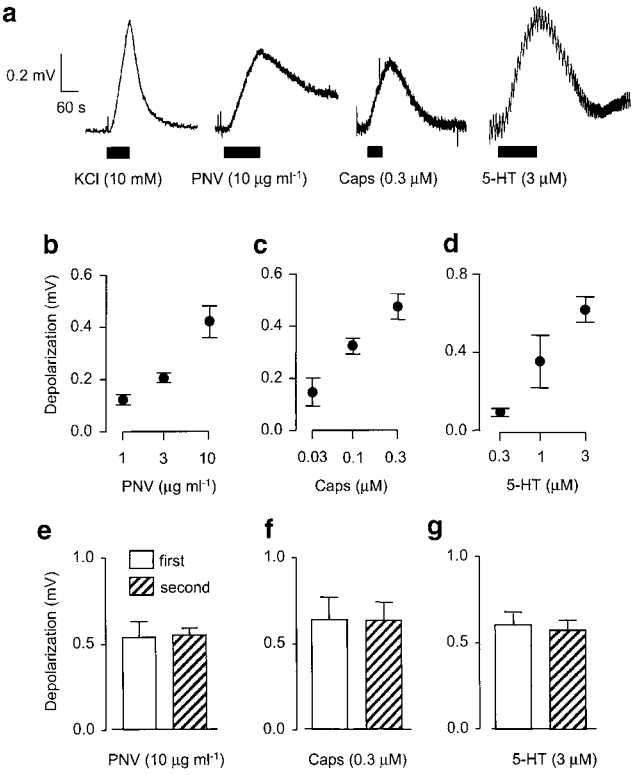

Figure 1 Extracellular DC potential responses of rat vagus nerve to different agents. (a) Representative traces illustrate the depolarization response of vagus nerves in response to superfusion with KCl (10 mM), PNV (10 μg ml^{-1}), capsaicin (Caps, 0.3 μM) or 5-HT (3 μM), respectively. Dose-dependent depolarization data (mean ± s.e.m.) are shown for PNV (b, 1 – 10 μg ml^{-1}; $n=11$), Caps (c, 0.03 – 0.3 μM; $n=6$) and 5-HT (d, 0.3 – 3 μM; $n=6-12$). The DC potentials (mean ± s.e.m.) evoked by a series of two applications (repeated at 20 – 25 min) of PNV (d, $n=9$), Caps (e, $n=5$) or 5-HT (f, $n=9$) to the same vagus nerve are also shown.

responses to PNV do not show significant homologous desensitization (Figure 1d). Similarly, the depolarization observed in response to capsaicin at a dose of 0.3 μM was not significantly different from the second response in the same nerve (Figure 1e). Responses to PNV were not affected significantly by prior exposure of the nerve to 0.3 μM capsaicin nor were capsaicin responses affected by prior application of PNV (data not shown), that is, the responses to the two substances did not cross-desensitize. Also, co-administration of capsaicin with PNV (0.3 μM and 10 μg ml^{-1} respectively, $n=5$) was not significantly larger than the response to PNV alone or to capsaicin alone, that is, the responses were not additive (Figure 2a).

Although capsaicin did not produce any measurable homologous or heterologous desensitization when applied at 0.3 μM, treatment of the nerve with a high concentration of capsaicin (5 μM for 3 min, $n=7$) reduced the response produced by subsequent application of PNV (10 μg ml^{-1}, 3 min, Figure 2b) or capsaicin (0.3 μM, 2 min, Figure 2c) by 53 ± 8.0% and 76 ± 9.5%, respectively. Responses evoked by 10 mM KCl, which were applied as a nonspecific depolarizing stimulus, were unaffected by prior treatment with the high concentration of capsaicin (5 μM for 3 min, data not shown).

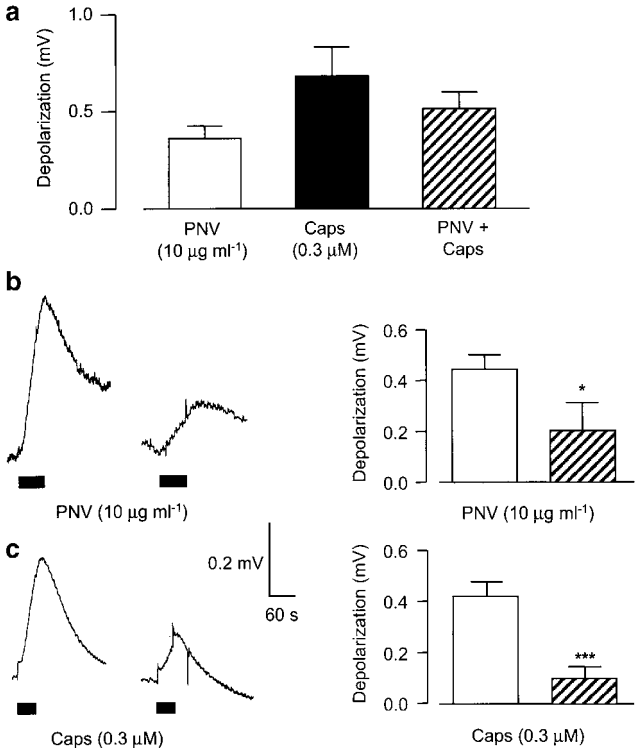

Figure 2 Capsaicin-sensitive sensory nerve mediated PNV-induced depolarization in rat vagus nerve. (a) The responses (mean ± s.e.m., $n = 5$) evoked by PNV (open bar) or Caps alone (closed bar) and together on the same vagus nerves (hatched bar). (b) A representative trace details the effect of a single nerve in response to PNV (10 μg ml^{-1}), before and after pre-treatment with capsaicin (Caps, 5 μM, 3 min). Group data (mean ± s.e.m., $n = 7$) are shown on the right as PNV before (open columns) and after (hatched columns) pretreatment with capsaicin (5 μM). (c) A representative trace from a single nerve in response to Caps (0.3 μM) before and after pretreatment with the high concentration of Caps (5 μM, 3 min). The corresponding graph shows the group data (mean ± s.e.m., $n = 7$) of the response evoked by Caps before (open columns) and after (hatched columns) treatment, respectively. A significant reduction is shown (two-tailed paired Student's *t*-test) in the amplitude of PNV (*$P < 0.05$) or Caps (***$P < 0.001$) after desensitization by Caps (5 μM).

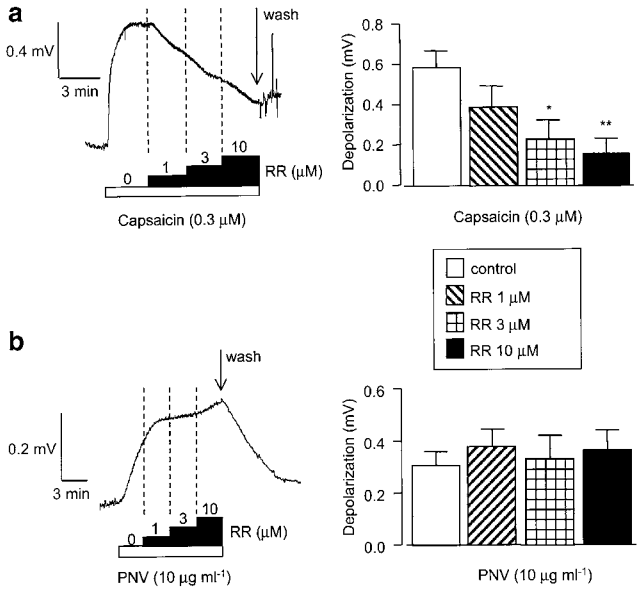

Figure 3 Effect of RR. Cumulative increasing concentrations of RR were applied to Caps or PNV solution, such that the concentration of RR was changed every 3 min. (a) Trace from a single vagus nerve, with a corresponding graph of group data (mV; mean ± s.e.m., $n = 6$) shows the effect of RR (1 – 10 μM) on Caps-induced depolarization. (b) Trace from a single vagus nerve and a corresponding graph of group data (mV; mean ± s.e.m., $n = 5$) to show the effect of RR (1 – 10 μM) on PNV-induced depolarization. A significant reduction is shown (two-tailed paired Student's *t*-test) in the amplitude of Caps plus RR (*$P < 0.05$; **$P < 0.01$) compared to the capsaicin response before RR.

Effect of ruthenium red (RR)

To test whether PNV depolarized the vagus nerve by an action on vanilloid receptors, we used the noncompetitive vanilloid receptor antagonist RR (1 – 10 μM). Capsaicin (0.3 μM, $n = 6$) was applied continuously and after 3 min RR was added cumulatively to the capsaicin solution at concentrations of 1, 3 and 10 μM for 3 min at each concentration. The capsaicin-induced depolarization was decreased dose-dependently by RR (Figure 3a). Using the same protocol in a separate series of experiments, RR had no significant effect on the depolarization produced by PNV (10 μg ml^{-1}, $n = 5$, Figure 3b).

Effect of tetrodotoxin

It has been shown that PNV can influence the kinetics of tetrodotoxin (TTX)-sensitive voltage-gated sodium channels (VGSCs), at least in skeletal muscle (Araujo *et al*., 1993). Accordingly, we tested the effect of TTX on the PNV-induced depolarization using a protocol that was identical to that adopted for the experiments with capsaicin and RR described above. The response to PNV was inhibited dose-dependently by TTX (Figure 4a, $n = 5$). In contrast, TTX (1 – 10 μM) added to capsaicin solution (0.3 μM) had no significant effect on capsaicin-induced depolarization (Figure 4b, $n = 11$). The depolarizing response to veratridine (50 μM, $n = 7$), a chemical activator of VGSCs, was almost abolished by TTX (10 μM, Figure 4c).

Effect of 5-HT and 5-HT receptor antagonists

5-HT (0.3 – 3 μM, at least 3 min) depolarized the vagus nerve (Figure 1a). Responses to 5-HT were dose-dependent (Figure 1d) and were reproducible, that is, they did not desensitize, when 5-HT was applied at 25 min intervals (Figure 1f). Superfusion (10 min) of the nerves with either Y-25130 (0.5 μM) or tropisetron (0.1 or 10 nM), which are 5-HT$_3$ receptor antagonists (Richardson *et al*., 1985; Sato *et al*., 1992), strongly inhibited the depolarization evoked by 5-HT (3 μM, Figure 5a, b). The small, residual 5-HT-induced depolarization that remained in the presence of 10 nM tropisetron was further reduced by RS39604 (10 μM, Figure 5b), an antagonist of 5-HT$_4$ receptors (Hegde *et al*., 1995) although the reduction was not significant statistically. Similar data were obtained using granisetron (10 nM) which reduced responses to 5-HT (3 μM) from 0.43 + 0.08 to 0.08 + 0.05 mV ($n = 7$) or granisetron (10 nM) in combination with RS39604 (10 μM) which abolished the response. Superfusion (10 min) of the nerves with RS39604 (1 or 10 μM) caused

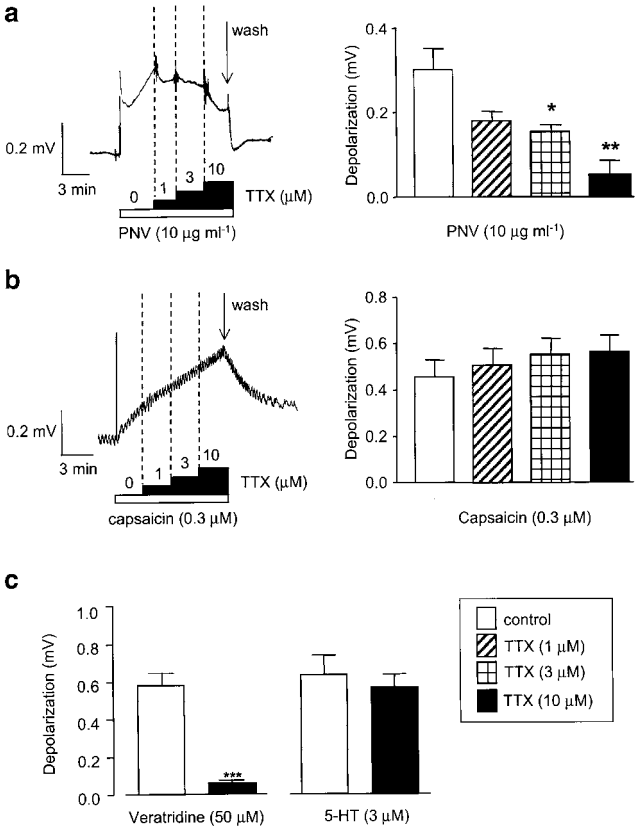

Figure 4 Effect of TTX. Cumulative increasing concentrations of TTX were applied to Caps or PNV solution, such that the concentration of TTX was changed every 3 min. (a) Shows a trace from a single nerve and a corresponding graph of group data (mean ± s.e.m., $n = 5$) to show the effect of TTX (1 – 10 μM) on PNV-induced depolarization. (b) Shows a trace from a single nerve and a corresponding graph of group data (mean ± s.e.m., $n = 11$) to show the effect of TTX (1 – 10 μM) on Caps-induced depolarization. A significant reduction is shown (two-tailed paired Student's t-test) in the amplitude of PNV plus TTX (*$P<0.05$; **$P<0.01$) compared to PNV response before TTX. (c) Shows the response (mean ± s.e.m.) evoked by veratridine ($n = 7$) and 5-HT ($n = 4$) before (open bars) and after addition of TTX (10 μM, closed bars). A significant reduction is shown (two-tailed unpaired Student's t-test) in the amplitude of veratridine plus TTX (**$P<0.001$) compared to response without TTX.

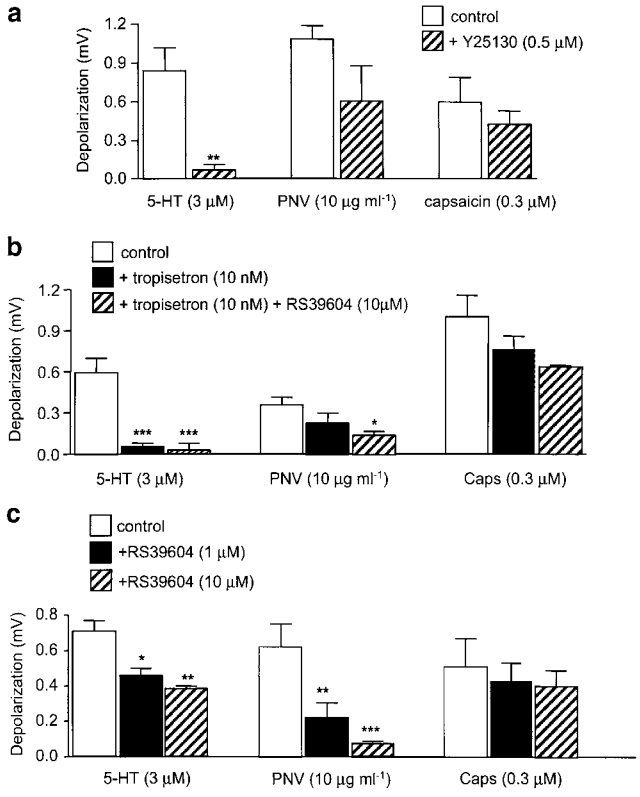

Figure 5 Effects of 5-HT$_3$ (Y25130 and tropisetron) and 5-HT$_4$ (RS39604) receptor antagonists. (a) Shows the depolarizing response (mean ± s.e.m.) evoked by 5-HT ($n = 5$), PNV ($n = 4$) and Caps ($n = 4$) before (open bars) and after treatment of the nerves with Y25139 (hatched bars). (b) shows the depolarizing response (mean ± s.e.m.) evoked by 5-HT ($n = 6 – 8$), PNV ($n = 5$) and Caps ($n = 3 – 4$) in the absence (open bars) and presence of tropisetron alone (closed bars, 10 nM) and tropisetron plus RS39604 (hatched bars, 10 μM). (c) shows the depolarizing response (mean ± s.e.m.) evoked by 5-HT ($n = 13$), PNV ($n = 5 – 14$) and Caps ($n = 6$) before (open bars) and after treatment of the nerves with RS39604 at 1 μM (closed bars) and 10 μM (hatched bars). Data are compared by two-tailed paired Student's t-test in panel (a) and two-tailed, unpaired Student's t-test in panels (b) and (c) (*$P<0.05$, **$P<0.01$ and ***$P<0.001$) compared to control (open bars).

a small but significant reduction in the response to 5-HT (3 μM, Figure 5c) which recovered after washing the preparation for 60 min. These data are in agreement with previous reports that suggest that the effects of 5-HT on the vagus nerve are mediated predominantly by 5-HT$_3$ receptors with a relatively small contribution from 5-HT$_4$ receptors (Rhodes *et al.*, 1992). Responses to PNV (10 μg ml^{-1}) were not reduced significantly by either Y-25130, tropisetron or granisetron, but were significantly reduced by RS39604 at 1 μM and strongly inhibited at 10 μM (Figure 5c). Responses to either capsaicin (0.3 μM, Figure 5a–c) or to KCl (10 mM, $n = 8$, not shown) were not significantly reduced by Y-25130, tropisetron or RS39604.

Since the depolarizing response to PNV was blocked by either the 5-HT$_4$ antagonist RS39604 or by TTX (see above), we checked whether there were any nonspecific or unexpected interactions between RS39604 and sodium channels and also whether TTX had any effect on 5-HT receptors. Pretreatment with TTX (10 μM; 15 min) had no significant effect on 5-HT-induced depolarization, but abolished the response induced by veratridine (Figure 4c). Neither RS39604 ($n = 4$) nor tropisetron ($n = 4$) had significant effects on veratridine-induced depolarization (veratridine response was 0.53 ± 0.15 mV before and 0.67 ± 0.16 mV after 10 nM tropisetron and 0.48 ± 0.12 mV before and 0.63 ± 0.27 mV after 10 μM RS39604).

Discussion

We have used the rat-isolated vagus nerve preparation to test the hypothesis that the proinflammatory effects of PNV include a neurogenic component via an action on capsaicin-sensitive sensory nerve fibres. The vagus nerve contains a high proportion of capsaicin-sensitive C fibres (Marsh *et al.*, 1987) and is therefore a convenient preparation in which to test this hypothesis as well as being a potential target for the effects of

the venom *in vivo*. Like capsaicin, PNV depolarized the nerve. Using a low concentration of capsaicin, we could find no evidence for cross-desensitization between capsaicin and PNV responses, but nerves that were conditioned by exposure to a high concentration of capsaicin were clearly less sensitive to either PNV or capsaicin even though nonspecific depolarizing responses produced by increasing extracellular K^+ were unchanged. The conditioning effect produced by the high concentration of capsaicin probably was because of a functional desensitization of sensory Aδ- and C-fibres in the preparation (see review by Holzer, 1991). Responses to capsaicin and PNV were not additive, that is, the responses occluded each other. Taken together, these data suggest either that PNV and capsaicin act by the same mechanism or, if they act by different mechanisms, then they act on the same population of nerve fibres. If PNV and capsaicin act by the same mechanism, it would be expected that responses to PNV, like responses to capsaicin, would be blocked by RR. We show clear evidence that this is not the case. Thus, even though they act on the same population of fibres, it is unlikely that PNV and capsaicin act by the same mechanism. These data are consistent with data obtained *in vivo* where RR did not affect PNV-induced oedema formation in rats (Costa *et al*., 2000).

PNV-induced depolarization was inhibited by TTX, while the response to capsaicin was not changed significantly. This result points to the same conclusion as the experiments with RR discussed above, that is, that PNV and capsaicin depolarize the nerve fibres by different mechanisms. Either PNV activates VGSCs directly or it provokes a response that somehow depends on VGSCs. Previously published work suggests that PNV contains distinct classes of toxins that act on ionic channels in both muscle and nerve (see review Brazil & Fontana, 1993) and PNV has been shown to slow the inactivation and deactivation processes of VGSCs in skeletal muscle (Araujo *et al*., 1993). Activation of VGSCs has been suggested as the mechanism underlying the progressive morphological alterations that occur in muscle and nerve fibres of mice that have been exposed to PNV (Mattiello-Sverzuta & Cruz-Hofling, 2000). A direct effect of PNV on VGSCs in the vagus nerve, whereby PNV affects the kinetics of VGSCs, that is, a veratridine-like effect, is therefore an attractive possible mechanism that could contribute to PNV-induced depolarization.

5-HT depolarizes the vagus nerve by an action on 5-HT$_3$ and 5-HT$_4$ receptors (Rhodes *et al*., 1992; Coleman & Rhodes, 1995; Nemoto *et al*., 2001). The involvement of 5-HT$_3$ and 5-HT$_4$ receptor subtypes in the mediation of PNV-induced depolarization was investigated here because previous data obtained *in vivo* with the relatively nonselective antagonist methysergide suggested a possible involvement of 5-HT receptors in PNV-induced oedema (Costa *et al*., 2000; 2001). In the present experiments, we used Y25130, tropisetron and granisetron which are selective antagonists at 5-HT$_3$ receptors (Miyata *et al*., 1991, Sato *et al*., 1992) and RS39604 which is a selective antagonist at 5-HT$_4$ receptors (Hegde *et al*., 1995). Our data show that 5-HT$_3$ antagonists inhibited 5-HT-induced depolarization, but had no significant effect on responses to PNV, capsaicin or high extracellular K^+ confirming previous data which has shown that 5-HT-induced depolarization is mediated predominantly by 5-HT$_3$ receptors (Richardson *et al*., 1985; Rhodes *et al*., 1992) and showing also that PNV-induced depolarization does not depend on 5-HT$_3$ receptors. The 5-HT$_4$ receptor antagonist RS39604 inhibited the response evoked by 5-HT and abolished the residual response to 5-HT that was evoked in nerves pretreated with tropisetron or granisetron. This result is in agreement with previous studies which show that activation of 5-HT$_4$ receptors contributes to 5-HT-induced depolarization of the vagus nerve (Rhodes *et al*., 1992). RS39604 blocked the PNV-induced depolarization of the rat vagus nerve, but did not affect responses to capsaicin or high extracellular K^+ which shows that the blocking effect of RS39604 is selective for PNV and therefore suggests that PNV acts at 5-HT$_4$ receptors. Since PNV might directly activate VGSCs (see above), we were concerned that RS39604 might have a nonselective action at VGSCs but RS39604 had no significant effect on veratridine-induced depolarization. Likewise, tropisetron had no significant effect on veratridine-induced depolarization. Taken together, the data suggest strongly that PNV acts at 5-HT$_4$ receptors. Unlike 5-HT$_3$ receptors, which are ion channels, 5-HT$_4$ receptors are metabotropic receptors. It is a reasonable speculation that the 5-HT$_4$ receptors are coupled indirectly to VGSCs via depolarization or directly via second messenger systems since VGSCs clearly contribute to 5-HT$_4$-receptor-mediated depolarization. Thus, TTX has relatively little effect on responses to 5-HT which acts predominantly through 5-HT$_3$ receptors but blocks PNV which acts selectively at 5-HT$_4$ receptors. Interestingly, previous work has suggested that 5-HT$_4$ receptors are coupled to VGSCs in somatosensory capsaicin-sensitive sensory neurones (Cardenas *et al*., 1997; 2001) although the VGSCs involved were somatic TTX-resistant (TTX-R) VGSCs, presumably NaV1.8, which is not the case here. Vagal fibres do not express functional TTX-resistant VGSCs in any significant amounts (Farrag *et al*., 2002) even though the somata of these neurones express TTX-R VGSCs (Schild & Kunze, 1997), so it is possible that the PNV response is because of a 5-HT$_4$-receptor-mediated effect on TTX-sensitive VGSCs in sensory neurones that express somatic TTX-R VGSCs. Alternatively, the PNV-induced depolarization may combine effects of PNV on 5-HT$_4$ receptors which normally mediate a relatively modest depolarization with a veratridine-like effect on TTX-S VGSCs which amplifies the response.

In the light of recent evidence that suggests a role for 5-HT$_4$ receptors in peripheral nociception and visceral nociception in particular (Espejo & Gil, 1998) and the demonstration of neurogenic inflammatory responses provoked by PNV (Costa *et al*., 1997; 2000; 2001), the present results contribute to a growing argument that implicates 5-HT$_4$ receptors in mechanisms of peripheral sensory nerve activation. The PNV used in this study was dialysed to remove small molecules (molecular weight less than 12,000), but the venom is nevertheless a complex substance and it will be important to determine what are the active fractions of the venom and to isolate the chemicals responsible for this important biological activity. Further experiments *in vivo* and on isolated nodose or jugular ganglion cellular somata will be required to investigate this interesting possibility further.

Activation of vagal afferent fibres may contribute to the pulmonary oedema that is a serious and sometimes fatal complication of PNV envenomation (Bucaretchi *et al*., 2000). Further, our data are consistent with results from studies that show that PNV acts as a potent stimulant of sensory nerves *in vivo* where it causes neurogenic inflammation that is blocked

by NK_1 receptor antagonists or by capsaicin desensitization (Palframan *et al*., 1996, Costa *et al*., 1997; 2000; 2001). In this study, we have demonstrated clearly that PNV depolarizes the isolated vagus nerve and shown that this is because of activation of 5-HT$_4$ receptors in a mechanism involving TTX-sensitive VGSCs in the nerve fibres. These insights suggest that a 5-HT$_4$ antagonist or an NK_1 antagonist might be a useful addition to antivenom and analgesic therapy in cases of PNV accidents.

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