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Characterization of the anandamide induced depolarization of guinea-pig isolated vagus nerve

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1 There is considerable interest in elucidating potential endogenously derived agonists of the vanilloid receptor and the role of anandamide in this regard has received considerable attention. In the present study, we have used an electrophysiological technique to investigate the mechanism of activation of vanilloid receptors in an isolated vagal preparation.

2 Both capsaicin and anandamide depolarized de-sheathed whole vagal nerve preparations that was antagonized by the VR1 antagonist, capsazepine ($P<0.05$) whilst this response was unaltered by the cannabinoid (CB1) selective antagonist SR141716A or the CB2 selective antagonist, SR144528, thereby ruling out a role for cannabinoid receptors in this response.

3 The PKC activator, phorbol-12-myristate-13-acetate (PMA) augmented depolarization to both anandamide and capsaicin and this response was significantly inhibited with the PKC inhibitor, bisindolylmaleimide (BIM) ($P<0.05$).

4 The role of lipoxygenase products in the depolarization to anandamide was investigated in the presence of the lipoxygenase inhibitor, 5,8,11-Eicosatriynoic acid (ETI). Depolarization to anandamide and arachidonic acid was significantly inhibited in the presence of ET1 ($P<0.05$). However, in the absence of calcium depolarization to anandamide was not inhibited by ETI.

5 Using confocal microscopy we have demonstrated the presence of vanilloid receptors on both neuropeptide containing nerves and nerves that did not stain for sensory neuropeptides.

6 These results demonstrate that anandamide evokes depolarization of guinea-pig vagus nerve, following activation of vanilloid receptors, a component of which involves the generation of lipoxygenase products. Furthermore, these receptors are distributed in both neuropeptide and non-neuropeptide containing nerves.

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Keywords: Vanilloid receptor; capsaicin; anandamide; protein kinase C; lipoxygenase

Abbreviations: Anandamide, arachidonyl ethanolamide; ANOVA, analysis of variance; BIM, bisindolylmalemide; CB, cannabinoid; CGRP, calcitonin gene-related peptide; DMSO, dimethylsulphoxide; EGTA, ethylene glycol-bis(β-amino ethyl ether)-N,N,N′,N′-tetra acetic acid; ETI, 5,8,11-eicosatriynoic acid; FAAH, fatty acid amide hydrolase; LOX, lipoxygenase; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; VR, vanilloid receptor

Introduction

The existence of a capsaicin receptor was suggested almost 30 years ago (Szolcsányi & Jancsó-Gábor, 1975) and with the discovery of resiniferatoxin as an ultrapotent capsaicin analog and capsazepine as a vanilloid receptor (VR) antagonist, considerable advances have been made concerning the role of VR (Szallasi & Blumberg, 1990; Bevan *et al*., 1992). Recently, VR1 has been cloned (Caterina *et al*., 1997) and targeted disruption of the VR1 gene in mice resulted in an impairment in response to high doses of capsaicin and is implicated in hyperalgesia (Caterina *et al*., 2000; Davis *et al*., 2000).

VR1 is a non-selective cation channel with a high calcium permeability that is activated by a broad spectrum of stimuli, including capsaicin, heat, low pH and the endogenous cannabinoid anandamide (Caterina *et al*., 1997; Tominaga *et al*., 1998; Zygmunt *et al*., 1999). We and others have recently shown that the endogenous cannabinoid (CB) receptor agonist anandamide produces a concentration-dependent contraction of guinea-pig isolated bronchus *via* the activation of the VR1 receptor in this preparation (Craib *et al*., 2001; Tucker *et al*., 2001). However, the mechanism by which anandamide affects airways is far from resolved. The vasodilator response to anandamide in isolated arteries is capsaicin-sensitive and accompanied by release of calcitonin-gene-related peptide (CGRP) (Zygmunt *et al*., 1999). Furthermore, anandamide has been reported to inhibit bronchospasm and cough evoked by capsaicin in the guinea-pig, but to elicit bronchospasm when the constricting tone exerted by the vagus nerve is removed, both effects being mediated through activation of peripheral CB1 cannabinoid receptors (Calignano *et al*., 2000). In contrast, it has recently been suggested that products derived from the

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metabolism of anandamide by lipoxygenase leads to the activation of the vanilloid receptor in airway tissue (Craib *et al*., 2001).

In the present study we have further investigated the role of the vanilloid receptor in the responses to anandamide in isolated vagal airway preparations. We have also investigated the role of cannabinoid receptors, protein kinase C and the lipoxygenase pathway on the response to anandamide in this vagal preparation.

Methods

Tissue preparation

Male albino guinea-pigs (300–400 g) were killed by cervical dislocation after exposure to 100% CO_2 and segments of cervical vagus nerve, approximately 30 to 35 mm long, were excised as rapidly as possible and placed in ice cold HEPES solution. The connective tissue sheath around each isolated vagus nerve was carefully removed, then mounted in a grease-gap recording chamber as described previously (Ireland & Tyers, 1987; Rang & Ritchie, 1988).

The de-sheathed vagus nerve preparation was positioned in a channel (1–2 mm diameter) and perfused with HEPES buffer (10 ml min^{-1}). Each end of the nerve was in contact with a pair of silver–silver chloride (Ag/AgCl) electrodes between which the nerve was surrounded by grease (Dow-Corning high vacuum grease) in order to form a high resistance seal. The temperature of HEPES buffer was maintained at 37°C by passing solutions through a heat exchanger proximal to the nerve. HEPES buffer containing drug solutions were connected *via* a six channel tap to the perfusion stream. The drug solution could be quickly changed by means of a tap, the solution reaching the nerve with a delay of about 15 s. The voltage difference between the two electrodes was recorded using Mac Lab Data Recording System (MacLab BioAmp, MacLab/4e). Bandwidth was set at 0.3–50 Hz and the acquisition rate was 25 ms.

Measurement of the effects of agonists and antagonists

In the present study, concentration-response curves for agonist-induced depolarization were constructed non-cumulatively using serially increasing concentrations of drugs. All nerves were initially exposed to a 40 mM KCl solution to confirm the integrity of the nerve and adequacy of superfusion. All drug containing solutions were applied for 10 s, after which, the tissue was washed with drug free HEPES solution. Non-cumulative concentration-response curves were obtained to KCl (5–40 mM), capsaicin (1–30 μM), anandamide (1–30 μM) and the cannabinoid agonist CP55940 (1–30 μM) with at least 15 min between applications.

In a separate series of experiments, the nerves were perfused with capsaicin (10 μM) or anandamide (10 μM) for 10 min and then perfused with HEPES solution for 5 min. A second response to capsaicin (10 μM) or anandamide (10 μM) was performed in order to test if desensitization had occurred.

In other experiments, the response to capsaicin and anandamide was evaluated in the absence or presence of the vanilloid receptor antagonist, capsazepine (10 μM), the CB1 receptor antagonist, SR141716A (1 μM), and the CB2 receptor antagonist, SR144528 (1 μM). The antagonists were perfused for 10 min prior to agonist application.

In a further set of experiments, isolated vagus nerve preparations were perfused with the protein kinase C (PKC) activator, phorbol-12-myristate-13-acetate (PMA) (10 μM) for 15 s prior to application of agonist and then perfused with HEPES solution for 5 s. After these pretreatments, the depolarization response to capsaicin (10 μM) or anandamide (10 μM) was evaluated. In other experiments tissues were also perfused with the selective PKC inhibitor, bisindolylmaleimide (BIM) (10 μM) in order to inhibit the response to PMA. The inhibitor was perfused for 5 min prior to application of PMA.

In other experiments, the response to capsaicin (10 μM) was evaluated in Ca^{2+} free HEPES solution containing 1 mM EGTA. In these experiments, the duration of exposure to capsaicin was increased to 30 s. The depolarization to repeated consecutive application of capsaicin (10 μM) was evaluated in the absence or presence of Ca^{2+} in solution at 5 min between intervals.

Finally, vagus nerve preparations were perfused with capsaicin (10 μM), anandamide (10 μM) or arachidonic acid (10 μM) in the absence or presence of the lipoxygenase (LOX) inhibitor, 5,8,11 eicosatriynoic acid (ETI) (10 μM). The antagonists were perfused for 10 min prior to agonist application. In other experiments, the response to anandamide (10 μM) or arachidonic acid (10 μM) was evaluated in the absence or presence of ETI (10 μM) in Ca^{2+} free solution.

Immunohistochemistry

The guinea-pig vagus nerve and ganglia were fixed in 4% paraformaldehyde in PBS, (pH 7.2) overnight. Antibodies to VR1 and substance P raised in mouse and rat respectively, were obtained from Chemicon (Temecula, CA, U.S.A.). Secondary antibodies (anti-mouse Oregon Green 488 and anti-rat Alexa 568 raised in goat) were obtained from Molecular Probes (Eugene, OR, U.S.A.). Specimens were cleared in dimethyl sulphoxide 5×10 min, agitated in washing buffer (PBS, pH 7.2) 5×10 min and incubated for 30 min in 1% skim milk in buffer to block nonspecific binding. Specimens were then incubated overnight in a humidified chamber at 4°C with primary antibody, 1 : 200, and then washed 5×30 min. This step was repeated for secondary antibodies. The specimens were mounted in custom-made chambers to reduce compression of the whole mount and were suspended in 90% glycerol containing p-phenylethylenediamine to reduce bleaching of the fluorochromes.

Confocal laser scanning microscopy

The confocal microscope, MRC1024 was run by Laser Sharp-acquisition version 3.2 software (BIORAD, Hemel Hempsted Hertfordshire, U.K.), and an inverted microscope was used to focus the argon 488 nm, GHe/Ne 543 nm and red diode 638 nm lasers. 2D-images were made from confocal z-series by selecting the brightest pixel at each xy location using Confocal Assistant 4.2 software. In double labelling experiments single fields were scanned for each marker, the images were coloured and superimposed using Adobe Photoshop 5.0 software.

Analysis of results

Results from all experiments are expressed as mean ± standard error of mean where n denotes the number of animals. The experiments were repeated in both vagi and the result averaged for each animal. In some cases the depolarization to capsaicin and anandamide is expressed as a percentage of the response elicited by KCl (40 mM). In other experiments, the area under the curve for depolarization to capsaicin was determined by integration (MacLab System, Chart version 3.3) and normalized to the value obtained with KCl (40 mM). Where appropriate Analysis of variance was used and differences between mean values assessed using Dunnett's test. In other cases, Student's paired or non-paired t-test was used. Differences between mean values were considered significant if $P<0.05$.

Drugs

Capsaicin, phorbol-12-myristate-13-acetate (PMA), bisindolylmaleimide (BIM), 5,8,11-eicosatriynoic acid (ETI), arachidonic acid, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′, N′-tetra-acetic acid (EGTA) (Sigma-Aldrich Chemical Co., Dorset, U.K.); anandamide, capsazepine, CP55940 (Tocris Cookson, Bristol, U.K.); SR141716A, SR144528 (a gift from Sanofi Recherche, Montpellier Cedex, France). Composition of HEPES buffer (mM): NaCl 140, KCl 5, $MgCl_2$ 1, Glucose 10, HEPES 10 and $CaCl_2$ 2, pH = 7.4. The calcium free HEPES solution contained no $CaCl_2$ and 1 mM EGTA to chelate calcium. Unless otherwise specified all drugs were prepared in HEPES buffer solution. Anandamide obtained from Tocris Cookson came prepared as a 10 mg ml^{-1} emulsion in soya oil/water (1 : 4) and dilutions made in HEPES buffer solution. Stock concentrations of capsaicin (0.1 M), capsazepine (0.01 M) and PMA (0.01 M) were prepared in ethanol. The resulting bath concentration of ethanol did not exceed 0.1%. Stock concentrations of CP55940, SR141716A, SR144528 and ETI (0.01 M) were prepared in dimethylsulphoxide (DMSO) such that bath concentrations of DMSO did not exceed 0.2%. Arachidonic acid was prepared fresh in 100% ethanol just prior to experimentation.

Results

Control experiments

In control experiments, drug vehicles including ethanol ($n=4$), DMSO ($n=5$) and soya oil ($n=4$) failed to induce depolarization of isolated vagus nerve (data not shown). In contrast, KCl induced a concentration-dependent depolarization of guinea-pig isolated vagus nerve (Figure 1a). The amplitude of the response to 40 mM KCl was 762 ± 5 μV ($n=268$ preparations). Capsaicin and anandamide also depolarized guinea-pig vagal nerve preparations (Figure 1) with an amplitude of 147 ± 4 μV (10 μM; $n=180$) and 107 ± 5 μV (10 μM; $n=89$). Of the total number of guinea-pig vagal nerves tested, capsaicin (10 μM) elicited depolarization in 87% of these nerves (180/207), whilst the frequency for anandamide (10 μM) was 54% (89/164). Of the total number of capsaicin-sensitive vagal preparations, anandamide induced depolarization in 61% (52/85) of these nerves. Depolarization induced by capsaicin was significantly greater than the response elicited by anandamide ($P=0.017$, ANOVA, Figure 1b).

Role of vanilloid receptor in depolarization response to agonist

The vanilloid receptor antagonist, capsazepine (10 μM) significantly attenuated the depolarization to capsaicin (10 μM) (control, 19.5 ± 1.3% vs capsazepine, 10.3 ± 1.6%, $n=5$ animals, $P<0.01$; Figure 2a). Similarly, the response to anandamide (10 μM) was also antagonized by capsazepine (control, 14.2 ± 1.3% vs capsazepine, 7.9 ± 0.6%, $n=7$, $P<0.01$; Figure 2b). Vehicle (0.1% ethanol) failed to significantly alter depolarization to capsaicin (control, 19.2 ± 0.9% vs vehicle, 18.4 ± 1.2%, $n=5$; $P>0.05$) or anandamide (control, 14 ± 1.2% vs vehicle, 13.3 ± 1.1%, $n=4$; $P>0.05$).

Acute desensitization effect between capsaicin and anandamide

Acute desensitization with capsaicin (10 μM, 5 min) significantly inhibited depolarization to anandamide (control, 14.7 ± 3.2% vs capsaicin treated, 6.5 ± 1.8%, $n=4$, $P=0.011$) and capsaicin (control, 21.4 ± 3.4% vs capsaicin treated, 7.2 ± 2.0%, $n=4$, $P=0.04$; Figure 3a). In contrast, pretreatment with anandamide (10 μM), while inducing desensitization to itself (control, 14.9 ± 1.2% vs anandamide treated, 7.2 ± 0.9%, $n=4$, $P=0.02$), failed to significantly alter depolarization to capsaicin (control, 19.9 ± 1.5% vs anandamide treated, 17.5 ± 1.0%, $n=4$, $P>0.05$; Figure 3b).

Involvement of cannabinoid receptors

The cannabinoid agonist, CP55940 (10 μM) failed to induce the depolarization of guinea-pig vagus nerve (2.3 ± 0.5%, $n=6$). The CB1 selective antagonist, SR141716A (1 μM) failed to significantly alter depolarization to capsaicin (10 μM) (control, 17.9 ± 1.9% vs SR141716A, 17.2 ± 2.1%, $n=4$, $P>0.05$) and anandamide (10 μM) (control, 12.5 ± 2.5% vs SR141716A, 12.0 ± 1.8%, $n=4$, $P>0.05$; Figure 4a). Similarly, the CB2 selective antagonist, SR144528 (1 μM) failed to significantly alter depolarization to capsaicin (control, 18.1 ± 1.2% vs SR144528, 16.5 ± 1.0%, $n=4$, $P>0.05$) and anandamide (control, 12.5 ± 1.9% vs SR144528, 11.8 ± 1.6%, $n=4$, $P>0.05$; Figure 4b).

PKC experiments

The PKC activator, PMA (10 μM) failed to depolarize isolated vagus nerve preparations ($n=5$) but augmented depolarization to capsaicin ($P=0.003$, ANOVA). This was reflected by an increase in the amplitude of the response to capsaicin (10 μM) (vehicle, 18.2 ± 0.7% vs PMA, 28.6 ± 3.4%, $n=6$; $P<0.05$, Figure 5a). Furthermore, this increase in depolarization to capsaicin in the presence of PMA (10 μM) (vehicle, 18.9 ± 1.4% vs PMA, 28.2 ± 2.7%, $n=5$ each, $P<0.01$) was significantly inhibited by the selective PKC inhibitor, BIM (10 μM) (PMA ± BIM, 17.2 ± 1.8%, $n=5$; $P>0.05$ compared to control; Figure 5b).

Similarly, PMA (10 μM) significantly augmented depolarization to anandamide (10 μM) (vehicle, 14.8 ± 0.6% vs PMA,

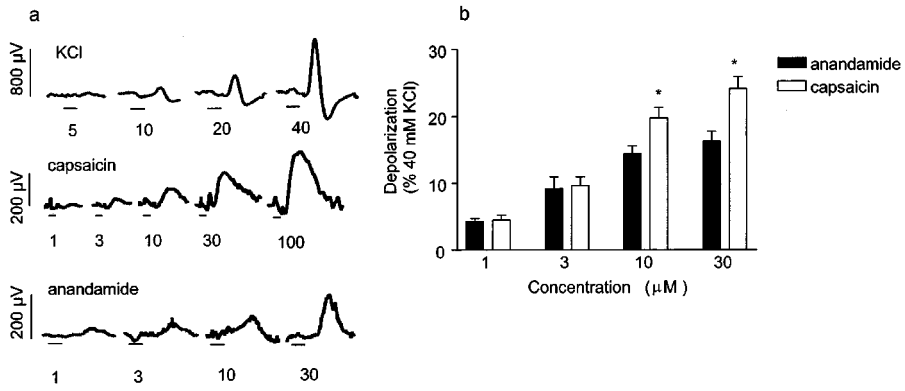

Figure 1 (a) Recording of the depolarization of vagus nerve to KCl (mM), capsaicin (μM) and anandamide (μM). Horizontal line represents period of drug exposure (10 s). (b) Bar graph representing non-cumulative normalized concentration dependent depolarization to capsaicin ($n=4$) and anandamide ($n=4$). Each column represents the mean and vertical line the standard error of the mean. $^*P<0.05$ compared to anandamide.

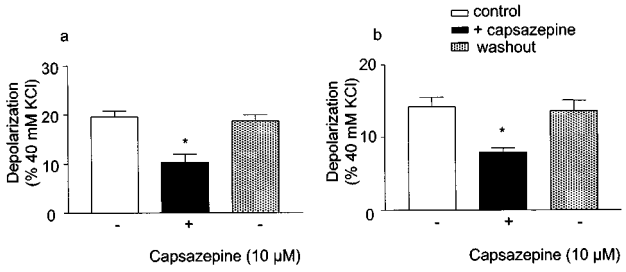

Figure 2 Bar graph representing the normalized depolarization for (a) capsaicin (10 μM, $n=5$ each) and (b) anandamide (10 μM, $n=7$ each) in the absence or presence of the vanilloid receptor antagonist, capsazepine (10 μM). Data expressed as a percentage of the response to 40 mM KCl in each preparation. Each column represents the mean and vertical lines represent standard error of the mean. $^*P<0.05$ compared to control.

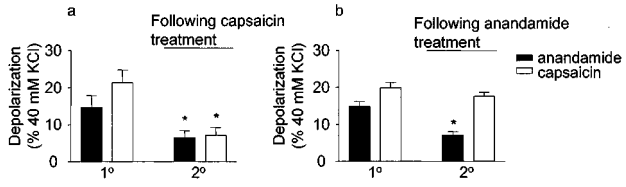

Figure 3 Bar graph representing the normalized depolarization for anandamide and capsaicin prior to (1°) and following treatment (2°) with (a) capsaicin (10 μM, $n=4$ each) and (b) anandamide (10 μM, $n=4$ each). Data expressed as a percentage of the response to 40 mM KCl in each preparation. Each column represents the mean and vertical lines represent standard error of the mean. $^*P<0.05$ compared to the 1° response.

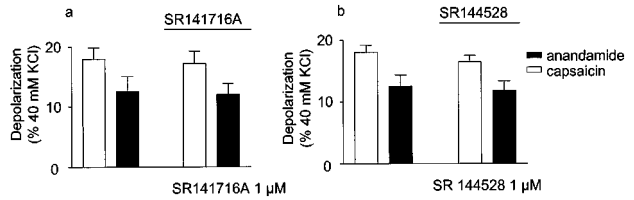

Figure 4 Bar graph representing the normalized depolarization for capsaicin (10 μM, $n=4$) and anandamide (10 μM, $n=4$) in the absence or presence of (a) the CB1 receptor antagonist, SR141716A (1 μM) and (b) the CB2 receptor antagonist, SR144528 (1 μM). Data expressed as a percentage of the response to 40 mM KCl in each preparation. Each column represents the mean and vertical lines represent standard error of the mean.

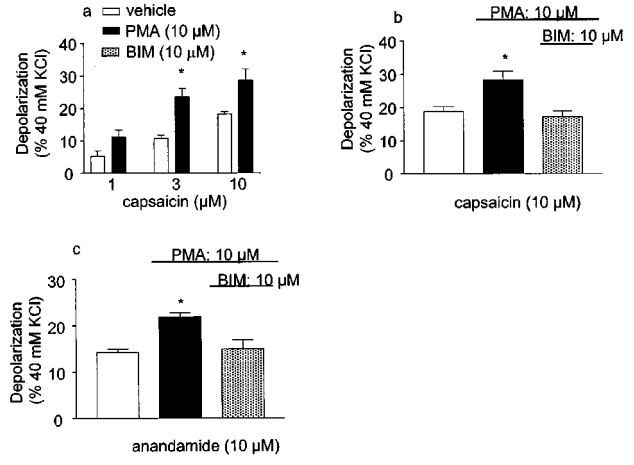

Figure 5 Bar graph (a) showing mean non-cumulative normalized concentration depolarization for capsaicin in the presence of vehicle ($n=6$) or PKC activator, PMA (10 μM, $n=6$). Bar graph representing the normalized depolarization for (b) capsaicin (10 μM, $n=5$) and (c) anandamide (10 μM, $n=5$) in the absence (vehicle, $n=5$ each), or presence of the PKC activator, PMA (10 μM, $n=5$ each) or PMA (10 mM) and PKC inhibitor, BIM (10 μM, $n=5$ each). Each column represents the mean and vertical lines represent standard error of the mean. $P<0.05$.

22.5±1.0%, $n=5$ each, $P<0.01$). The selective PKC inhibitor, BIM (10 μM) suppressed the augmented response to anandamide in the presence of PMA (PMA±BIM 15.5±2.0%, $n=5$; $P>0.05$ compared to control; Figure 5c).

The role of calcium in the response of isolated vagal nerve preparation to capsaicin

In the absence of calcium, depolarization to capsaicin was significantly greater than in its presence ($P=0.002$, ANOVA) and the effect of raising duration of exposure, significantly increased the magnitude of depolarization to capsaicin

($P=0.009$, ANOVA). The amplitude of depolarization to capsaicin was significantly increased in calcium free HEPES buffer (30 s application; 2 mM Ca^{2+}, $22.9\pm1.5\%$ vs 0 mM Ca^{2+}, $31.7\pm2.9\%$, $n=4$ each, $P<0.05$; Figure 6a). Similarly the integral data for capsaicin was significantly greater in the absence of calcium ($P=0.0002$, ANOVA; Figure 6b) that was also dependent upon the duration of exposure ($P=0.0001$, ANOVA). The magnitude of the integral data was significantly increased in calcium free HEPES buffer (30 s application; 2 mM Ca^{2+}, $74.1\pm8.8\%$ vs 0 mM Ca^{2+}, $127.1\pm15.7\%$, $n=4$ each, $P<0.05$; Figure 6b).

In another series of experiments, the depolarization to capsaicin (10 μM) was repeated in calcium free buffer and compared to that obtained in normal HEPES buffer. Tachyphylaxis to capsaicin was more resistant in calcium free solution (Figure 6c).

Lipoxygenase experiments

The lipoxygenase inhibitor, ETI (10 μM) failed to reduce the depolarization to capsaicin (control, $21.4\pm1.7\%$ vs ETI, $19.0\pm1.8\%$, $n=5$, Figure 7a). In contrast, ETI (10 μM) significantly reduced the depolarization to anandamide ($P=0.012$, ANOVA; control, $14.9\pm2.3\%$ vs ETI, $7.2\pm1.1\%$, $n=5$, $P<0.01$, Figure 7b). In further experiments, arachidonic acid (10 μM) depolarized guinea-pig vagal preparations in 48% of nerves tested (10/21). The number of capsaicin-sensitive vagal nerves responding to arachidonic acid was 90% (9/10). ETI significantly attenuated this response ($P=0.029$, ANOVA; control, $17.0\pm3.0\%$ vs ETI, $4.7\pm1.6\%$, $n=4$, $P<0.05$; Figure 7c,d).

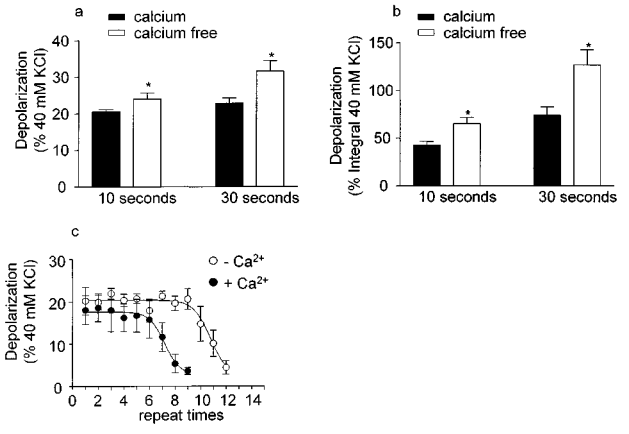

Figure 6 Bar graph representing (a) the normalized depolarization for capsaicin in the presence and absence of calcium in HEPES buffer. Capsaicin (10 μM) was applied for 10 s ($n=7$ each) and 30 s ($n=4$ each). Data expressed either as a percentage of the response to 40 mM KCl in each preparation. Alternatively, (b) integration of each response as a percentage of the integral data to 40 mM KCl is presented. Each column represents the mean and vertical lines represent standard error of the mean. *$P<0.05$ compared to Ca^{2+} presence. (c) Tachyphylaxis to capsaicin (10 μM) in presence (closed circles, $n=4$) and absence (open circles, $n=4$) of calcium in HEPES buffers Capsaicin (10 μM) was applied for 10 s separated by 5 min intervals. Data expressed as a percentage of the response to 40 mM KCl in each preparation. Horizontal axis represents the number of successive applications of capsaicin. Each circle represents the mean and vertical lines represent standard error of the mean.

In calcium free HEPES buffer the depolarization to anandamide was not significantly different to the response obtained in calcium-containing HEPES buffer ($P>0.05$). Moreover, the depolarization to anandamide in calcium free conditions was unaffected by the presence of ETI ($P>0.05$, ANOVA, Figure 8a). In contrast, ETI significantly inhibited depolarization to arachidonic acid ($P=0.001$, ANOVA) under calcium free conditions (control, $17.1\pm0.9\%$ vs ETI, $5.9\pm1.1\%$, $n=4$, $P<0.01$; Figure 8b).

Confocal microscopy

The localization of vanilloid receptors was demonstrated in cell bodies contained within the jugular ganglion (Figure 9a). These receptors could be colocalized on cell bodies containing substance P (Figure 9a). Furthermore, a subpopulation of non-neuropeptide containing cell bodies also expressed vanilloid receptors (Figure 9b).

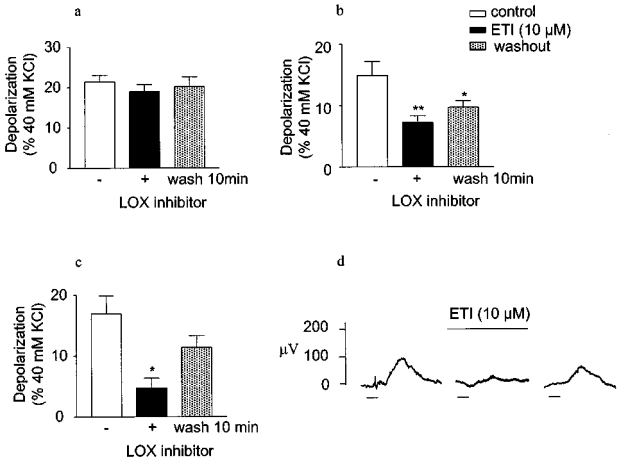

Figure 7 Bar graph representing the normalized depolarization for (a) capsaicin (10 μM, $n=5$), (b) anandamide (10 μM, $n=5$) and (c) arachidonic acid (10 μM, $n=4$) in the absence or presence of the lipoxygenase inhibitor, ETI (10 μM). Data expressed as a percentage of the response to 40 mM KCl in each preparation. Each column represents the mean and vertical lines represent standard error of the mean. *$P<0.05$ compared to control. (d) Recording of the depolarization of vagus nerve to arachidonic acid. Horizontal line represents duration (10 s) of drug administration.

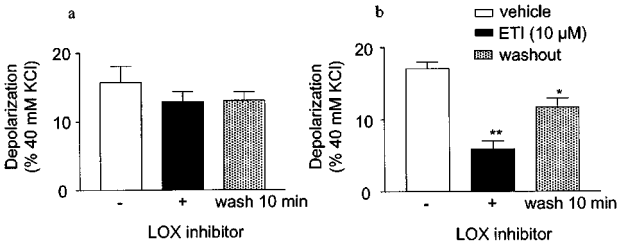

Figure 8 Bar graph representing the normalized depolarization for (a) anandamide (10 μM, $n=5$) and (b) arachidonic acid (10 μM, $n=4$) in the absence or presence of the lipoxygenase inhibitor, ETI (10 μM) in the absence of calcium. Data expressed as a percentage of the response to 40 mM KCl in each preparation. Each column represents the mean and vertical lines represent standard error of the mean. *$P<0.05$ compared to control.

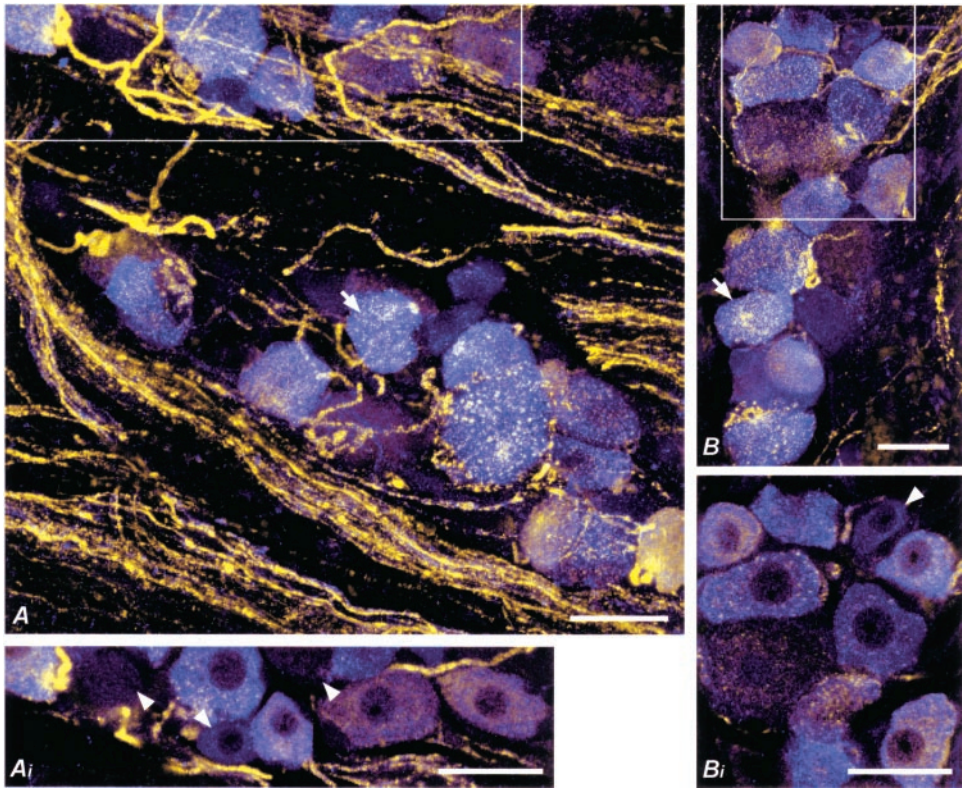

Figure 9 Confocal images of the jugular ganglion concurrently stained to demonstrate vanilloid receptor (VR1-IR) and substance P immunoreactive (SP-IR) neurons shown in blue and yellow respectively. Bar = 50 μm. (A) and (B): VR1-IR soma are seen in clusters within the jugular ganglion. Many of these cells co-localize with SP-IR (arrows) and give rise to SP-IR fibres that can be seen joining thick nerve bundles that pass through the ganglia. Scan depth = 25 μm. (Ai) and (Bi): Insets from (A) and (B) showing single optical section through the ganglion to reveal cells that are VR1-IR but not SP-IR (arrow heads).

Discussion

In the present study, we have provided further evidence that the putative endogenous cannabinoid agonist anandamide can activate vanilloid receptors. Our results show that anandamide is able to induce depolarization of guinea-pig isolated vagus nerve preparations, which although less than that induced by capsaicin, was inhibited by the VR1 antagonist capsazepine, but not by the CB1 receptor antagonist SR141716A or the CB2 receptor antagonist SR144528 and may in part be due to the generation of lipoxygenase products. Consistent with the observation that the depolarization of isolated vagus nerve was CB1/CB2 receptor independent, the cannabinoid receptor agonist, CP55940 failed to depolarize the vagus nerve. Our work also confirms that the anandamide-induced response was attenuated by capsaicin pretreatment, while anandamide failed to cross-desensitize the response to capsaicin. Moreover the ability of the PKC activator PMA to augment responses to both capsaicin and anandamide also points to a common mechanism of action.

The grease gap recording technique has previously been used to measure depolarization of vagus nerve to various substances (Ireland & Tyers, 1987; Rang & Ritchie, 1988; Butler *et al*., 1990). Using a similar technique, we have demonstrated reproducible depolarization responses to KCl, capsaicin, anandamide and arachidonic acid. This technique offered us the advantage of studying the activation of VR1 receptors directly, rather than indirectly *via* the release of neuropeptides and subsequent contraction of bronchial smooth muscle (Tucker *et al*., 2001), although this technique does not allow one to distinguish between the type of afferent nerves being activated. Recently, it has been shown that anandamide activates cardiac vagal nerve endings (Malinowska *et al*., 2001) and pulmonary vagal C-fibre afferents (Lin & Lee, 2002) in anaesthetized rats *via* a vanilloid receptor dependent mechanism. In contrast, anandamide did not activate rapidly adapting pulmonary receptors (RARs), suggesting that anandamide selectively excites C-fibres (Lin & Lee, 2002). However in the guinea-pig, capsaicin can induce depolarization of both vagal C and Aδ fibres whose cell bodies reside in the jugular ganglion (Holzer, 1991; Szallasi, 1995a; Riccio *et al*., 1996; Kajekar *et al*., 1999). Therefore, in the present study, our confocal microscopy observations have provided clear evidence that VR1 receptors are present on both neuropeptide containing nerves as well as nerves that do not stain for substance P. The nature of the neurotransmitter in the VR1 positive nerves that do not stain substance P remains to be established, but it is likely that both would be stimulated by anandamide. In 1997, the vanilloid receptor was identified by using an expression cloning strategy based on calcium influx to isolate a functional cDNA encoding a capsaicin receptor from sensory neurons (Caterina *et al*., 1997). VRs are expressed along the entire length of vanilloid-sensitive sensory neurons (Szallasi, 1995a) and nerve ligation studies suggest that VRs are transported from the cell bodies

to the periphery in a form capable of ligand binding (Szallasi *et al*., 1995b) and it is also clear that these receptor are functionally expressed along vagal nerve fibres as demonstrated in this study.

Both cloned (VR1) and native VRs are non-selective cation channels that are gated by heat, low pH and vanilloid agonists (Tominaga *et al*., 1998). However, in recent years considerable attention has been focused on identifying endogenous ligands for the vanilloid receptor. It has recently been demonstrated that certain lipoxygenase products of arachidonic acid metabolism, in particular 12- and 15-(S)-hydroperoxyeicosatetraenoic acids, 5- and 15-(S)-hydroxyeicosatetraenoic acids and leukotriene B4 can activate the VR1 receptor (Hwang *et al*., 2000). Furthermore, anandamide displays a high structural similarity to the vanilloids, especially olvanil, a nonpungent, capsaicin analogue (Di Marzo *et al*., 1998). It is perhaps not surprising therefore, that the putative endogenous cannabinoid receptor agonist anandamide is also capable of activating VR1 receptors in transfected cells (Zygmunt *et al*., 1999; Smart *et al*., 2000; Ross *et al*., 2001), DRG neurones (Hwang *et al*., 2000; Smart *et al*., 2000; Tognetto *et al*., 2001) and in airway tissue (Craib *et al*., 2001; De Petrocellis *et al*., 2001b; Tucker *et al*., 2001). Recently, these findings have been extended with the suggestion that anandamide itself may be metabolized by a lipoxygenase-dependent pathway, and whose products activate the vanilloid receptor (Craib *et al*., 2001). A number of biochemical studies have shown that hydroxyl derivatives of anandamide are formed by 12-,15- but not 5-lipoxygenase (Ueda *et al*., 1995; Hampson *et al*., 1995; Edgemond *et al*., 1998). The pharmacology of these derivatives has been investigated and like anandamide, they bind to CB1-receptors (Edgemond *et al*., 1998). Interestingly, the 15-hydroxy derivative of anandamide is considerably less potent than anandamide in stimulating the vanilloid receptor. Therefore, the role of these metabolites as endogenous activators of the vanilloid receptor is unclear (De Petrocellis *et al*., 2001a), although it remains to be established whether the 12-hydroxy derivative activates the vanilloid receptor.

Anandamide is metabolised by fatty acid amide hydrolase (FAAH) to arachidonic acid (Cravatt *et al*., 1996) and lipoxygenase products generated from this pathway could have the potential to activate the vanilloid receptor (Hwang *et al*., 2000; Olah *et al*., 2001). Thus, we have demonstrated that anandamide induces depolarization of vagal nerves, which in part, is mediated by lipoxygenase metabolism. However, the contribution of lipid mediators derived from the lipoxygenase metabolism of anandamide (Craib *et al*., 2001) or of arachidonic acid, following the metabolism of anandamide by FAAH, in this response remains to be established.

In order to further explore the mechanism by which anandamide stimulates the vanilloid receptor, experiments were performed in the absence of calcium. Under such conditions, anandamide would still be subject to metabolism by FAAH (Hillard *et al*., 1995; Karava *et al*., 2001). Whilst depolarization to anandamide was unaffected by calcium removal, lipoxygenase inhibition failed to inhibit this response suggesting that anandamide directly activated native vanilloid receptor under these conditions. A further possibility is that calcium entry *via* the vanilloid receptor might also facilitate the mobilization of arachidonic acid with subsequent metabolism by lipoxygenase, resulting also in the activation of the vanilloid receptor. This possibility was supported by the fact that, although anandamide was previously shown to cause arachidonate release *via* CB1- and CB2-receptors (Shivachar *et al*., 1996; Hunter & Burstein, 1997; Pestonjamasp & Burstein, 1998), we found that depolarization of vagal nerves by anandamide was inhibited by the VR1 antagonist capsazepine, but not by CB1- or CB2-selective antagonists. Furthermore, the possibility that VR1-mediated calcium entry leads to arachidonate mobilization is supported also by previous findings in several cell lines (Maccarrone *et al*., 2000). Finally, in support of this hypothesis, we found that when the calcium requirement of arachidonate release was bypassed by treating our preparations directly with arachidonic acid, the subsequent depolarization of vagal nerves was sensitive to the lipoxygenase inhibitor ETI. However, we also observed that depolarization of vagal nerves by capsaicin which is a more potent agonist at native VR1 than anandamide, was unaffected by ETI, although in a previous study it was observed that capsaicin-induced contraction of guinea-pig bronchial preparations tended to be suppressed by lipoxygenase inhibition (Craib *et al*., 2001), although this was not significant. Clearly, further work is required to determine the importance of calcium entry following vanilloid receptor activation, on the generation of endogenous lipid activators of this ion channel.

There is currently some dispute as to the physiological significance of the role of anandamide as a CB and VR selective agonist, as the high concentration of anandamide reported to be required in order to activate the VR1 receptor is greater than that required to activate CB receptors (Szolcsányi, 2000). Whether local concentrations generated within the microenvironment of the nerve are sufficient to activate VR receptor is unknown at present, although it remains plausible that sensitization of afferent nerves may augment the response to endogenously liberated anandamide (Di Marzo *et al*., 2001; Olah *et al*., 2001; Vellani *et al*., 2001).

Alternatively, the high concentrations required to activate native vanilloid receptor might be a consequence of the metabolism of anandamide by lipoxygenase (Craib *et al*., 2001), by FAAH to arachidonic acid and subsequent metabolism by lipoxygenase and/or the generation of lipoxygenase products *via* a calcium dependent process. Further studies measuring the release of lipoxygenase products generated by these different pathways in sensory nerves will be required for definitive proof of this mechanism.

We have previously reported that the contractile response to anandamide in guinea-pig isolated bronchus was inhibited by the NK2-selective antagonist, SR48968 but not by the NK1-selective antagonist, SR140333 (Tucker *et al*., 2001). These results provided indirect evidence that anandamide induced contraction of airway smooth muscle was a consequence of activating VR1 receptors on peripheral sensory nerves with subsequent release of endogenous neuropeptides. Here we provide further direct evidence that anandamide can activate VR1 receptors by showing that depolarization to anandamide was inhibited by the VR1 receptor antagonist capsazepine. Similarly, the vanilloid receptor antagonist capsazepine inhibited anandamide-induced vasodilation, release of CGRP and activation of the cloned receptor (Zygmunt *et al*., 1999). Whilst capsazepine is routinely used as a vanilloid receptor antagonist there is evidence that this agent may also induce a time dependent

block of voltage-gated calcium channels (Docherty *et al*., 1997) and nicotinic acetylcholine (nACh) receptors (Liu & Simon, 1997). Potassium (Kuenzi & Dale, 1996) and hyperpolarization-activated cyclic nucleotide gated (HCN) currents (Gill *et al*., 2002) are also inhibited by capsazepine. It would seem unlikely that nACh receptors or HCN channels are present in the axons in the vagus nerve and contribute to the capsaicin-induced depolarization. On the other hand, if the capsaicin-induced depolarization is dependent on subsequent activation of voltage-gated sodium channels then this could confound interpretation of the data. However, capsaicin-mediated depolarization in mammalian vagus is still present in sodium free solution (Marsh *et al*., 1987). Furthermore, we have demonstrated that depolarization to capsaicin is not blocked by the removal of extracellular calcium. Therefore, voltage-gated sodium and calcium channels do not contribute greatly to the responses observed to capsaicin.

It is known that capsaicin causes a rapid desensitization of vanilloid receptors (Szallasi & Blumberg, 1999). In the present study, pretreatment with capsaicin to cause desensitization of VR1 receptors also abolished the anandamide-induced depolarization response as well as the capsaicin-induced depolarization response in the isolated vagal nerve preparation. However, anandamide failed to cross-desensitize the depolarization response to capsaicin, confirming our earlier results with guinea-pig isolated bronchus (Tucker *et al*., 2001). It is of interest that 64 and 72% of capsaicin sensitive afferent fibres was activated by anandamide in normal and arthritic rat joints respectively (Gauldie *et al*., 2001), which is similar to our findings that only 61% of capsaicin sensitive nerves responded to anandamide. This suggests that anandamide is less efficacious than capsaicin in stimulating the vanilloid receptor as the inward current of anandamide was significantly smaller ($<50\%$) than that of capsaicin (Zygmunt *et al*., 1999; Hwang *et al*., 2000; Smart *et al*., 2000). Similarly, at normal pH anandamide behaves as a partial agonist (Olah *et al*., 2001) and therefore would be relatively poor at inducing desensitization to an agonist with greater efficacy like capsaicin. In relation to desensitization of the capsaicin response to repeated stimulation with this agonist, it was reported that acute desensitization was facilitated by calcium entry (Docherty *et al*., 1996; Koplas *et al*., 1997), a finding which we have confirmed in guinea-pig isolated vagus nerve.

There is a possibility that gating and phosphorylation of VR1 is regulated by Ca^{2+}, neurotrophin receptor tyrosine kinase, protein kinase A and protein kinase C (Kress & Guenther, 1999; Winston *et al*., 2001; Lopshire & Nicol, 1998; Vellani *et al*., 2001). Nociceptors are either coupled directly to ionotropic P2X receptors or to the G protein-mediated metabotropic P2Y receptors and most P2Y receptors are coupled to Ca^{2+}-sensitive (PKC-β, γ) or insensitive (PKC-ε, δ) forms of PKC (Premkumar, 2001). Bradykinin sensitizes the heat-gated current by activating PKC-ε (Cesare *et al*., 1999), and PKC-ε gene has a role in regulating thermal and acid-induced hyperalgesia (Khasar *et al*., 1999). On the other hand, PKC-γ also appears to play a role in regulating neuropathic pain (Malmberg *et al*., 1997), and extracellular ATP potentiates VR1 responses through metabotropic ATP (P2Y1) receptors in a PKC-dependent manner (Tominaga *et al*., 2001). These results suggest that PKC may link a range of stimuli to the activation of VR (Di Marzo *et al*., 2001). Similarly, activation of the vanilloid receptor by anandamide was PKC-dependent in rat VR1 expressing *Xenopus laevis* oocyte and neonatal rat dorsal root ganglia cell (Premkumar & Ahern, 2000). Furthermore, phosphorylation of VR1 by PKC increases the probability of channel gating by agonists including anandamide in neonatal rat dorsal root ganglia cells and in HEK293 cells expressing human vanilloid receptors (Vellani *et al*., 2001). In the present study, we have shown that PKC plays a role in augmenting the vanilloid response to anandamide using an electrophysiological technique in guinea-pig vagus nerve, consistent with recent findings in guinea-pig isolated bronchus (De Petrocellis *et al*., 2001b).

In conclusion, we have demonstrated that the endogenous cannabinoid receptor agonist, anandamide evokes depolarization following activation of vanilloid receptors in guinea-pig vagus nerve that in part may also be due to the liberation of lipoxygenase products. The finding of vanilloid receptors on neuropeptide and non-neuropeptide nerves suggests that endogenous activation of this receptor generated in the lung may activate these different nerve types.

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