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Capsaicin-like effects of *N*-arachidonoyl-dopamine in the isolated guinea pig bronchi and urinary bladder

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Abstract

A capsaicin-like endogenous ligand of vanilloid (VR1) receptors, *N*-arachidonoyl-dopamine, was recently identified in bovine and rat nervous tissue, and found to be almost as potent as capsaicin, and 5–10-fold more potent than anandamide, on these receptors, both in isolated cells and in vivo. Here we have investigated if *N*-arachidonoyl-dopamine also exerts other capsaicin-like effects at VR1 receptors in some isolated organ preparations. *N*-arachidonoyl-dopamine exerted a potent contractile response of guinea pig isolated bronchi ($EC_{50} = 12.6 \pm 1.7$ μM, $E_{max} = 69.2 \pm 2.4\%$ of carbachol E_{max}), which was blocked by pre-treatment with capsaicin or with the VR1 antagonist capsazepine, as well as by a combination of tachykinin NK1 and NK2 receptor antagonists. In this assay, *N*-arachidonoyl-dopamine was less and more potent and/or efficacious than capsaicin ($EC_{50} = 40.0$ nM; $E_{max} = 93.5\%$) and anandamide ($EC_{50} = 15.2$ μM, $E_{max} = 38.0\%$), respectively. Unlike capsaicin and anandamide, forskolin or ethanol did not enhance *N*-arachidonoyl-dopamine effect in this preparation, whereas epithelial denudation resulted in a 2.5-fold increase in potency without affecting the efficacy. *N*-arachidonoyl-dopamine also contracted the isolated guinea pig urinary bladder, although in this preparation, as well as in the isolated rat urinary bladder, the potency ($EC_{50} = 3.7 \pm 0.3$ and 19.9 ± 0.1 μM) and/or efficacy ($E_{max} = 12.0 \pm 0.1\%$ and $20.7 \pm 0.7\%$ of carbachol E_{max}) of the compound were significantly lower than those of both capsaicin and anandamide. These data suggest that the extent to which exogenous *N*-arachidonoyl-dopamine activates VR1 receptor in isolated organs is largely dependent on pharmacodynamics and bioavailability.

Keywords: Vanilloid VR1 receptor; Cannabinoid CB_1 receptor; Anandamide; Vanilloid; Cannabinoid

1. Introduction

The vanilloid TRPV1 receptor, also known as VR1 receptor (Szallasi and Blumberg, 1999), is the only member of the large family of the "transient receptor potential" (TRP), six *trans*-membrane domain, cation channels discovered to date (Gunthorpe et al., 2002) that responds not only to thermic, acidic or mechanical stimuli, but also to stimulation by natural products, with capsaicin and resiniferatoxin being the best known and most thoroughly studied examples (Sterner and Szallasi, 1999). It is now recognized that the VR1 receptor functions as a molecular integrator of nociceptive stimuli, including heat, protons and plant toxins, as suggested by its relative abundance in peripheral sensory fibres of the C and Aδ type. Studies carried out with transgenic mice lacking functional VR1 receptors implicated this protein in the perception of thermal and inflammatory pain (Caterina et al., 2000; Davis et al., 2000), and the likely role of VR1 receptors in neuropathic pain has also been outlined (Walker et al., 2003). Due to its presence also in sensory neurons innervating smooth muscles in several organs, the VR1 receptor is also thought to be involved in pathological cough (see Chung and Chang, 2002, for review), inflammatory bowel disorders (Yiangou et al., 2001), faecal

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hyperactivity (Chan et al., 2003) and bladder function (Birder et al., 2002). Finally, the recent finding (Mezey et al., 2000; Sanchez et al., 2001) of this channel also in brain nuclei including the hippocampus, the hypothalamus, the nucleus tractus solitarius and the basal ganglia widens considerably its possible biological importance, suggesting that the VR1 receptor is involved in the control of CNS functions, such as neuronal plasticity, body temperature, food intake and movement (Al-Hayani et al., 2001; Doyle et al., 2002; Di Marzo et al., 2001b; Szallasi, 2002).

It was the recent finding of the VR1 receptor in the brain, where high temperature and low pH are unlikely to occur, that strongly suggested the existence of endogenous ligands for this receptor (Szallasi and Di Marzo, 2000). Several arachidonate derivatives, including 12-lipoxygenase products (Hwang et al., 2000) and the endocannabinoid, anandamide (Zygmunt et al., 1999; Smart et al., 2000), have been proposed as endogenous vanilloid receptor agonists or "endovanilloids" (see Di Marzo et al., 2002, for review). Strong support has been provided for the hypothesis that both types of compounds participate in VR1 receptor gating during pathological conditions (Shin et al., 2002; McVey et al., 2003; Carr et al., 2003; Di Marzo et al., 2002). However, recent studies have identified *N*-arachidonoyl-dopamine (Huang et al., 2002) and *N*-oleoyl-dopamine (Chu et al., 2003) as the two most potent endovanilloids discovered to date. These two compounds exhibit 5–10-fold higher potencies than anandamide at both the human and rat VR1 receptor in isolated cells, and are even more potent than capsaicin at inducing hyperalgesia in vivo (Huang et al., 2002; Chu et al., 2003). Like anandamide, *N*-arachidonoyl-dopamine, and to a much smaller extent *N*-oleoyl-dopamine, can also interact with cannabinoid CB_1 receptors (Bisogno et al., 2000; Chu et al., 2003), thus supporting the hypothesis that these latter receptors and VR1 may work as metabotropic and ionotropic receptors for some fatty acid amides (Di Marzo et al., 2002).

Although *N*-arachidonoyl-dopamine has been tested on both somatosensory (spinal cord and dorsal root ganglia) and central (hippocampal) neurons, very little is known of the capability of this compound to also activate VR1 in sensory neurons innervating, and regulating the tone of, smooth muscles. In particular, whether, and with what efficacy, this compound contracts the bronchi and bladder has not been investigated. This is an important issue, since the gating/desensitisation of VR1 in these two tissues is, in one case (the bronchi), the cause of one of the major undesired side effects of VR1 receptor agonists developed as systemic analgesic drugs, and, in the other case (the bladder), the reason why some VR1 receptor agonists are currently being tested against bladder hypersensitivity and/or hyperactivity in pathological states such as neurogenic detrusor hyperreflexia in patients with spinal cord lesions and multiple sclerosis (Cruz, 2002).

In this study, we have investigated the effect of *N*-arachidonoyl-dopamine, in comparison to capsaicin and anandamide, on guinea pig bronchi and bladder tone, where the effect of VR1 receptor stimulation is due to the release of tachykinins. We report data indicating that potency and efficacy at the VR1 receptor of fatty acid amides such as *N*-arachidonoyl-dopamine and anandamide are strongly dependent on the type of preparation used, and discuss the possible mechanisms underlying this phenomenon.

2. Materials and methods

Male albino Dunkin–Hartley guinea pigs (~ 250 g) and Sprague–Dawley rats (~ 300 g) were used (Morini, Italy). Drugs and reagents were obtained from the indicated companies: anandamide, capsaicin, capsazepine, captopril, carbachol, forskolin, *N*(*G*)-nitro-L-arginine methyl ester (L-NAME) and phosphoramidon (Sigma, Italy); 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-morpholinyl-1*H*-pyrazole-3-carboxamide (AM281, Tocris, Italy); ethanol (Carlo Erba Reagent, Milano). *N*-arachidonoyl-dopamine was synthesized, purified and characterized as previously described (Bisogno et al., 2000). (*S*)-*N*-methyl-*N*-[4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl]benzamide (SR48968) and [(*S*)1-(2-[3-(3,4-dichlorophenyl)-1-(3-*iso*-propoxyphenylacetyl)piperidin-3-yl]etyl)-4-phenyl-1-azoniabicyclo[2.2.2]octane-chloride] (SR140333) were a kind gift from Dr. Xavier Emonds-Alt, Sanofi-Synthlabo', Montpellier, France. The stock concentrations of anandamide (10 mM), capsaicin (10 mM), capsazepine (10 mM) and *N*-arachidonoyl-dopamine (10 mM) were prepared in 100% ethanol. All other drugs were dissolved in distilled water. The appropriate dilutions were then made in Krebs buffer solution. All experiments complied with the national guidelines and were approved by the regional ethics committee.

Animals were sacrificed by cervical dislocation and the airways and urinary bladder of the guinea pig, and urinary bladder of the rat were removed. In the guinea pig, rings from main bronchi (approximately 2 mm in width) were suspended with a resting tension of 1.5 g. In both the guinea pig and rat urinary bladders, vertical halves were suspended with a resting tension of 1 g. The tissues were bathed and aerated (95% O_2 and 5% CO_2) with Krebs solution (described above) that was maintained at 37 °C, and contained phosphoramidon (1 μM) and captopril (1 μM) to minimize peptide degradation. Tissues were allowed to equilibrate for 60 min prior to the beginning and between each set of experiments (washed every 5 min). In all experiments, the tissues were first contracted with carbachol (1 μM). Cumulative concentration–response curves were performed with *N*-arachidonoyl-dopamine (0.1–100 μM), anandamide (0.1–100 μM) and capsaicin (1 nM–10 μM) either following pretreatment with capsaicin (10 μM, desensitisation), in the presence of the VR1 receptor antagonist, capsazepine (10 μM), the tachykinin NK1 and NK2 receptor antagonist, SR48968 and SR140333, respectively (both at 1 μM), the

nitric oxide synthase inhibitor, L-NAME (100 μM), the cannabinoid CB_1 receptor antagonist, AM281 (10 μM), forskolin (10 nM), an activator of adenylyl cyclase, ethanol (0.3%) or their respective vehicles. In another set of experiments, the epithelial was mechanically removed.

The arithmetic mean ± S.E.M. was calculated throughout. Contractile responses are expressed as a percentage (%) of the response to carbachol (1 μM), E_{max}. Statistical analysis was performed by means of the Student's *t*-test or analysis of variance (ANOVA) and the Dunnett's test when required. If $P < 0.05$, the results were considered significant.

3. Results

3.1. Effect of *N*-arachidonoyl-dopamine on guinea pig bronchi

N-arachidonoyl-dopamine potently contracted the guinea pig bronchi (Fig. 1). This compound was significantly more potent than anandamide (Fig. 1A). Its effect was blocked by pre-treatment with 10 μM of capsaicin (Fig. 1B) or with the VR1 receptor antagonist capsazepine (10 μM, Fig. 1C), and by a combination of the tachykinin NK1 and NK2 inhib-

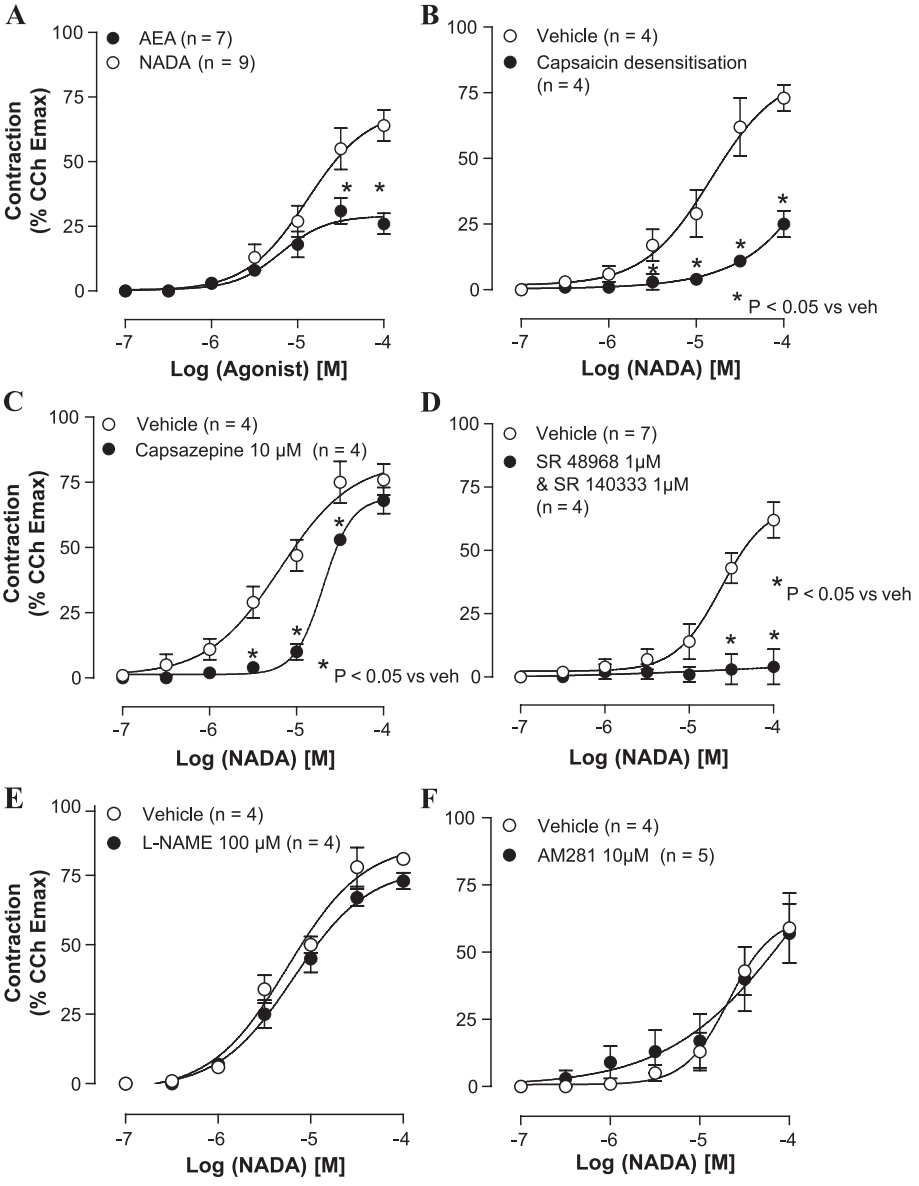

Fig. 1. Effect of *N*-arachidonoyl-dopamine (NADA) on the isolated guinea pig bronchi. The contraction was measured as percent of the maximal contraction elicited by 1 μM of carbachol (CCh). Data are means ± S.E.M. of $n \geq 4$ experiments. (A) Effect of NADA compared to that of anandamide (AEA). (B) Effect of NADA with or without pre-treatment of the bronchi with capsaicin (VR1 desensitisation). (C) Effect of NADA with or without pre-treatment of the bronchi with capsazepine (VR1 antagonism). (D) Effect of NADA with or without pre-treatment of the bronchi with a combination of the NK1 and NK2 receptor inhibitors, SR48968 and SR140333. (E) Effect of NADA with or without pre-treatment of the bronchi with the nitric oxide synthase inhibitor L-NAME. (F) Effect of NADA with or without pre-treatment of the bronchi with the cannabinoid CB_1 receptor antagonist AM281.

itors, SR48968 and SR140333 (1 μM, Fig. 1D). The nitric oxide synthase inhibitor L-NAME (100 μM, Fig. 1E) and the CB_1 receptor antagonist AM281 (10 μM, Fig. 1F) did not modify the response to *N*-arachidonoyl-dopamine.

3.2. Regulation of the effect of N-arachidonoyl-dopamine on guinea pig bronchi

The adenylyl cyclase inhibitor forskolin (10 nM) and ethanol (0.3%) did not significantly modify *N*-arachidonoyl-dopamine effect on guinea pig bronchi (Fig. 2A,B). However, epithelial denudation of the bronchi enhanced the effect of *N*-arachidonoyl-dopamine while leaving unaltered the effect of anandamide (Fig. 2C,D).

3.3. Effect of N-arachidonoyl-dopamine on guinea pig urinary bladder

N-arachidonoyl-dopamine weakly contracted the guinea pig urinary bladder (Fig. 3A). Its effect was blocked by pre-treatment with 10 μM of capsaicin (Fig. 3A) or with the VR1 receptor antagonist capsazepine (10 μM, Fig. 3C), and by a combination of the tachykinin NK1 and NK2 inhibitors, SR48968 and SR140333 (1 μM each, Fig. 3B). *N*-arachidonoyl-dopamine was significantly less potent than both capsaicin and anandamide (Fig. 3D).

3.4. Effect of N-arachidonoyl-dopamine on rat urinary bladder

N-arachidonoyl-dopamine weakly contracted the rat urinary bladder (Fig. 4A). Its effect was blocked by pre-treatment with 10 μM capsaicin (Fig. 4A) or with the VR1 receptor antagonist capsazepine (10 μM, Fig. 4C), and by a combination of the tachykinin NK1 and NK2 inhibitors, SR48968 and SR140333 (1 μM each, Fig. 4B). *N*-arachidonoyl-dopamine was significantly less potent than both capsaicin and anandamide (Fig. 4D).

4. Discussion

In this study, we have examined the effect of *N*-arachidonoyl-dopamine, in comparison to capsaicin and ananda-

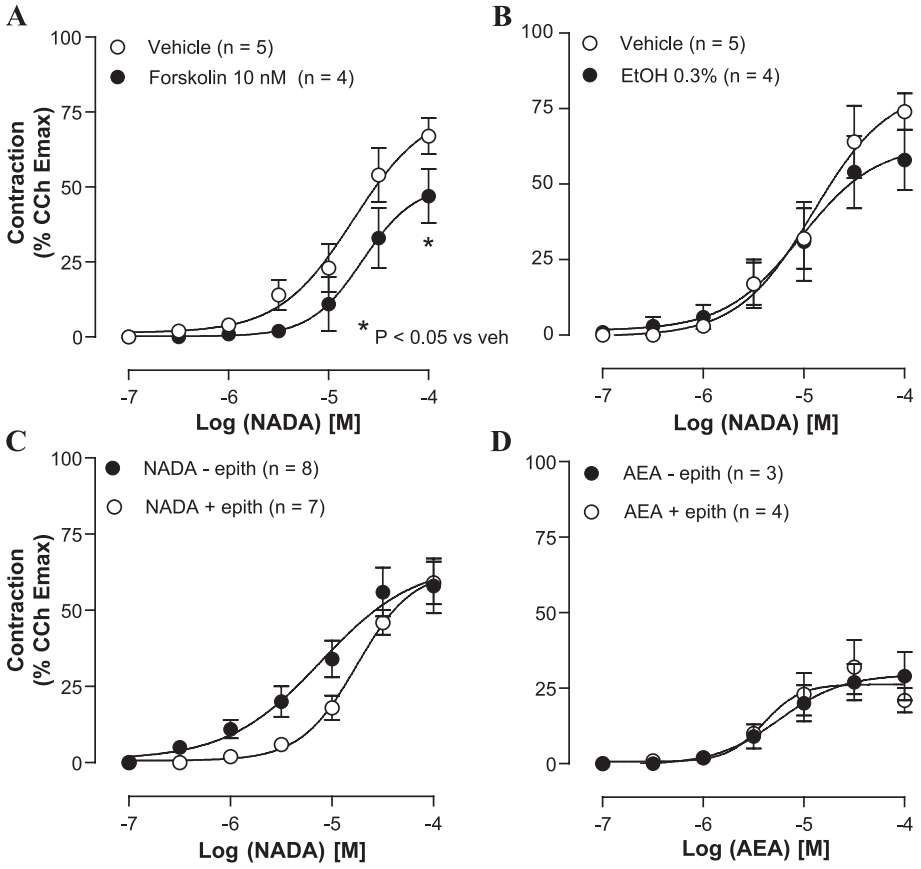

Fig. 2. Regulation of the effect of *N*-arachidonoyl-dopamine (NADA) on the isolated guinea pig bronchi. The contraction was measured as percent of the maximal contraction elicited by 1 μM of carbachol (CCh). Data are means ± S.E.M. of $n \geq 3$ experiments. (A) Effect of NADA with or without pre-treatment of the bronchi with forskolin (sensitisation via protein phosphorylation). (B) Effect of NADA with or without pre-treatment of the bronchi with ethanol (EtOH) (sensitisation via unknown mechanism). (C) Effect of NADA with or without epithelial denudation of the bronchi. (D) Effect of anandamide (AEA) with or without epithelial denudation of the bronchi.

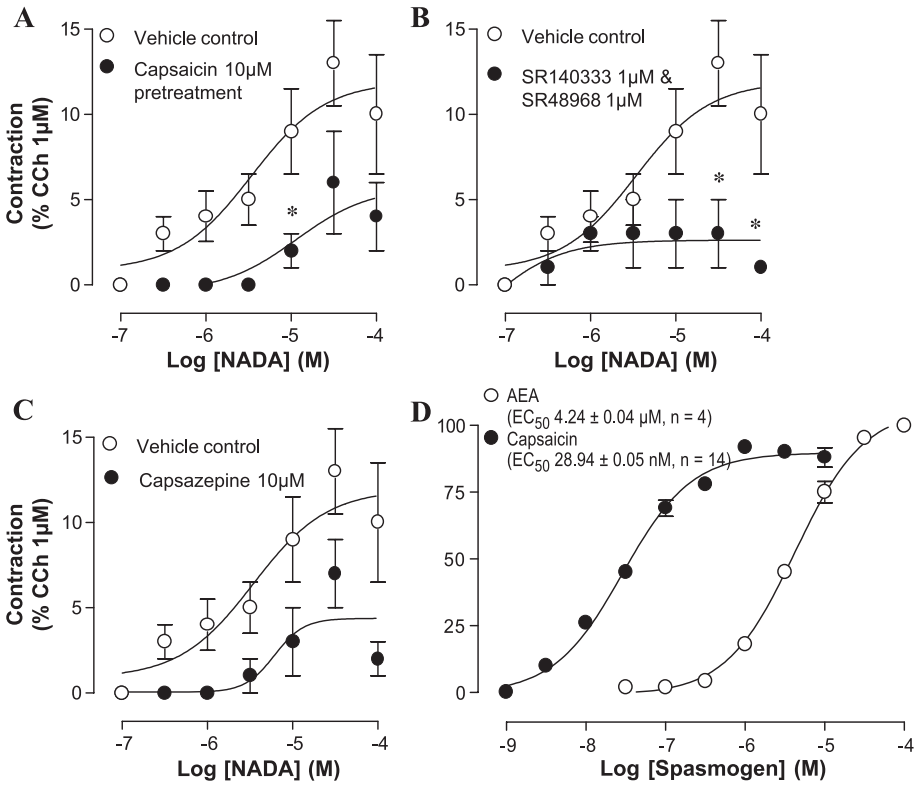

Fig. 3. Effect of *N*-arachidonoyl-dopamine (NADA), capsaicin and anandamide (AEA) on the isolated guinea pig urinary bladder. The contraction was measured as percent of the maximal contraction elicited by 1 μM of carbachol (CCh). Data are means ± S.E.M. of $n \geq 4$ experiments. (A) Effect of NADA with or without pre-treatment of the bladder with capsaicin (VR1 desensitisation). (B) Effect of NADA with or without pre-treatment of the bladder with a combination of the NK1 and NK2 receptor inhibitors, SR48968 and SR140333. (C) Effect of NADA with or without pre-treatment of the bladder with capsazepine (VR1 antagonism). (D) Effect of capsaicin and anandamide. (A) Vehicle $n=6$, capsaicin $n=4$; (B) vehicle $n=5$, SR $n=4$; (C) vehicle $n=6$, capsazepine $n=5$.

mide, on two typical VR1-mediated responses, the contraction of isolated guinea pig bronchi and urinary bladder. The action of *N*-arachidonoyl-dopamine on VR1, to date, has been analysed in vitro only in heterologous expression systems, where high levels of human or rat VR1 are present, or in somatosensory rat neurons, where the direct effect of VR1 gating on intracellular Ca^{2+} concentration has been measured (Huang et al., 2002). In these tests, *N*-arachidonoyl-dopamine was found to be almost as potent and efficacious as capsaicin, and at least 5-fold more potent than anandamide. In the past, the potency and/or efficacy in vitro of anandamide, but not capsaicin, on VR1 was shown to be dependent, to a large extent, on pharmacodynamic factors (see Di Marzo et al., 2001a, for review). In particular, it was found that the anandamide membrane transporter plays a major role in allowing extracellular anandamide to access the cytosolic binding site of VR1 (De Petrocellis et al., 2001a; Andersson et al., 2002). Therefore, the cellular complexity of the tissue used to test VR1-mediated effects, and, for example, the cell types and number of cell layers that separate the exogenous compound from the sensory fibres containing the receptor, might determine the efficacy and apparent potency of vanilloid long chain fatty acid amides. These compounds, unlike capsaicin, depend on the presence of a membrane transporter to enter the cell. Indeed, Andersson et al. (2002) provided evidence that the possible lack, or relative low abundance, of an active transporter might explain why anandamide and olvanil are much less potent at activating VR1 receptors in guinea pig bronchi than in the rat or mouse mesenteric artery. Furthermore, it was found that the cloned guinea pig VR1 receptor is less sensitive to anandamide than its rat or human counterpart (Savidge et al., 2002). For all these reasons, it was deemed important to test the effects of *N*-arachidonoyl-dopamine also on isolated guinea pig bronchi and urinary bladder. Furthermore, in these assays, the potency of *N*-arachidonoyl-dopamine depends not only on its capability of stimulating VR1 gating, but also on VR1 functional coupling to the release of substance P and calcitonin gene-related peptide (CGRP) from the sensory neurons that innervate the smooth muscle, and on the effects of these neuropeptides on smooth muscle cell NK1 and NK2 receptors.

We found that *N*-arachidonoyl-dopamine was significantly more potent and, particularly, more efficacious than anandamide in contracting the isolated guinea pig bronchi, an effect that was blocked not only by pre-treatment with capsaicin or with the VR1 receptor antagonist capsazepine, but also by a combination of the tachykinin NK1 and NK2

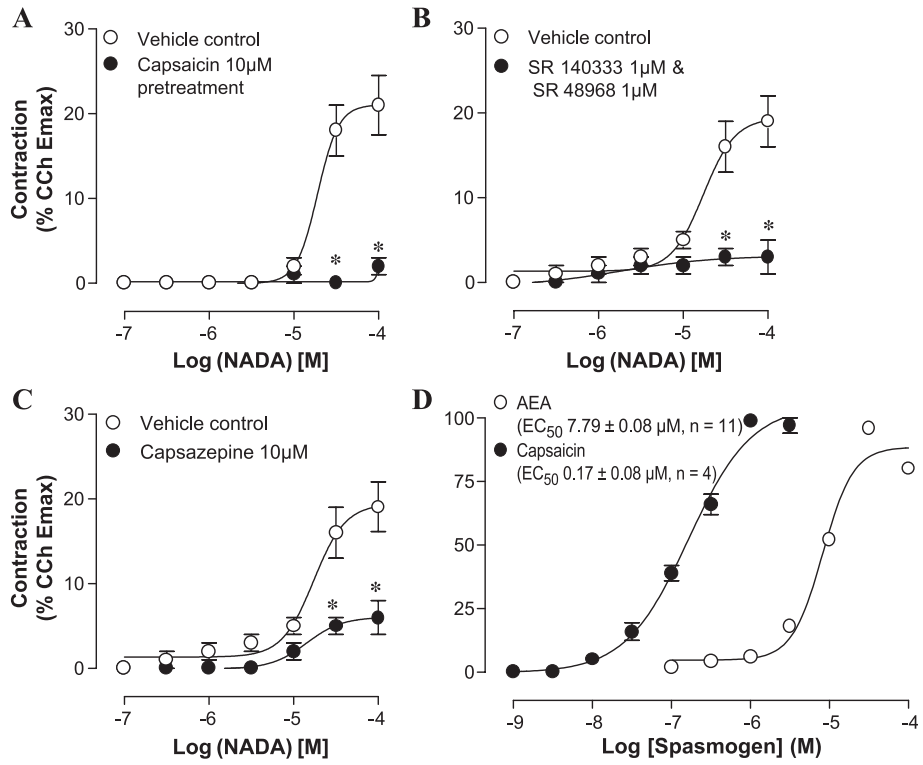

Fig. 4. Effect of *N*-arachidonoyl-dopamine (NADA), capsaicin and anandamide on the isolated rat urinary bladder. The contraction was measured as percent of the maximal contraction elicited by 1 μM of carbachol (CCh). Data are means ± S.E.M. of $n \geq 4$. (A) Effect of NADA with or without pre-treatment of the bladder with capsaicin (VR1 desensitisation). (B) Effect of NADA with or without pre-treatment of the bladder with a combination of the NK1 and NK2 receptor inhibitors, SR48968 and SR140333. (C) Effect of NADA with or without pre-treatment of the bladder with capsazepine (VR1 antagonism). (D) Effect of capsaicin and anandamide. (A) Vehicle $n = 6$, capsaicin $n = 5$; (B) vehicle $n = 6$, SR $n = 4$; (C) vehicle $n = 6$, capsazepine $n = 5$.

inhibitors, SR48968 and SR140333. We also tested the nitric oxide synthase inhibitor L-NAME, since it has been reported that some VR1-mediated effects of anandamide require NO to be fully evident (De Petrocellis et al., 2001a). However, the inhibitor did not affect the contraction exerted by *N*-arachidonoyl-dopamine. These findings indicate that *N*-arachidonoyl-dopamine does activate VR1 receptor in peri-bronchial sensory fibres of the guinea pig. However, in this assay, *N*-arachidonoyl-dopamine was significantly less potent and, to some extent, less efficacious than capsaicin ($EC_{50} = 40.0$ nM; $E_{max} = 93.5\%$, De Petrocellis et al., 2001b). We hypothesised that this could be due to the fact that *N*-arachidonoyl-dopamine also activates cannabinoid CB_1 receptors (Bisogno et al., 2000), and that this action could result in a relaxing effect (Calignano et al., 2000), thereby masking the VR1-mediated contraction. However, against this hypothesis, we found that the CB_1 receptor antagonist AM281 did not enhance the effect of *N*-arachidonoyl-dopamine on guinea pig bronchi, thus indicating that under our conditions, *N*-arachidonoyl-dopamine predominantly activates VR1 and exerts no relaxing effect via CB_1 receptors.

In order to improve the potency and efficacy of *N*-arachidonoyl-dopamine, as previously observed with anandamide (and to a smaller extent with capsaicin) in this assay (De Petrocellis et al., 2001b), we pre-treated the bronchi with forskolin. This compound, by activating adenylate cyclase and, subsequently, protein kinase A, was shown to sensitise, by direct phosphorylation, VR1 receptors to the action of endogenous agonists, protons and heat (Bhave et al., 2002; Rathee et al., 2002). However, in the case of *N*-arachidonoyl-dopamine, we observed no potentiation, and indeed we found a significant, albeit small, reduction of the effect at the highest dose. Ethanol, which also sensitises VR1 receptors to the action of agonists, protons and heat by an as-yet unknown mechanism, did not potentiate the effect of *N*-arachidonoyl-dopamine on guinea pig bronchi. These findings indicate that *N*-arachidonoyl-dopamine potency and efficacy in this tissue might have been limited not so much by reduced VR1 phosphorylation/sensitisation, but rather by bioavailability factors (such as, e.g., penetration into the fibres). In agreement with this hypothesis, epithelial denudation of the bronchi resulted in enhanced potency, but not efficacy, of *N*-arachidonoyl-dopamine, not anandamide. This finding was not surprising in view of the fact that anandamide is also significantly less potent in the guinea pig bronchi, possibly due to the lack of an active anandamide transporter in this preparation (Andersson et al., 2002). Accordingly, also *N*-arachidonoyl-dopamine, like anandamide, becomes significantly more efficacious, and at least as potent as capsaicin, when its relaxant action, again via a substance P- and CGRP-mediated mechanism, is measured in the isolated rat mesenteric artery (personal communication by E. Hogestatt). In-

deed, in this preparation, penetration of *N*-arachidonoyl-dopamine and anandamide through endothelial cells, where a very active anandamide membrane transporter has been described (Hillard and Jarrahian, 2000; Maccarrone et al., 2000), is likely to occur more efficiently and to play a major role in the efficacy of the two compounds. Furthermore, if *N*-arachidonoyl-dopamine, like anandamide (Savidge et al., 2002), was intrinsically less potent on guinea pig VR1 than on rat VR1, this could also provide an additional explanation for the smaller effects of *N*-arachidonoyl-dopamine and anandamide in guinea pig bronchi.

In order to assess whether bioavailability or species-dependent differences play a role in determining *N*-arachidonoyl-dopamine efficacy and/or potency at VR1 in isolated organs, we tested the effect of *N*-arachidonoyl-dopamine on guinea pig and rat urinary bladder, in comparison again with anandamide and capsaicin. We found that *N*-arachidonoyl-dopamine contracted the guinea pig bladder in a way sensitive to capsazepine, capsaicin pre-treatment, and a combination of SR48968 and SR140333. However, in this assay, *N*-arachidonoyl-dopamine was a much weaker agonist than in the bronchi, to the point of being significantly less potent and efficacious than anandamide. It is unlikely that this strong decrease in potency was due to a higher degradation rate of *N*-arachidonoyl-dopamine, since this compound is considerably more resistant than anandamide to enzymatic hydrolysis (Bisogno et al., 2000; Huang et al., 2002). Therefore, also in consideration of the strong component of epithelial cells present in the urinary bladder, limited access of *N*-arachidonoyl-dopamine to sensory fibres might have played a major role in limiting its effects in this preparation. When tested in the urinary bladder from another species, the rat, *N*-arachidonoyl-dopamine exerted a contraction that was again sensitive to capsazepine, capsaicin pre-treatment, and SR48968 and SR140333. However, the potency in this preparation did not increase (and, instead, it decreased), although the efficacy was significantly increased. This suggests that species differences might cause pharmacodynamic changes rather than a decrease in potency of the compound.

In conclusion, data presented here show for the first time that *N*-arachidonoyl-dopamine activates VR1, and subsequently tachykinin release, also in sensory neurons that innervate the smooth muscle in isolated bronchi and urinary bladder preparations. They suggest that the pharmacological actions of *N*-arachidonoyl-dopamine on VR1 receptors, like those of anandamide and olvanil (Andersson et al., 2002), are largely dependent on bioavailability and pharmacodynamic factors, and that caution is needed when interpreting experiments carried out with the exogenously administered compound.

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