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MINI REVIEW
Anandamide and vanilloid TRPV1 receptors

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A large body of evidence now exists to substantiate that the endocannabinoid, anandamide, activates TRPV1 receptors. It is a low intrinsic efficacy TRPV1 agonist that behaves as a partial agonist in tissues with a low receptor reserve, while in tissues with high receptor reserve and in circumstances associated with certain disease states, it behaves as a full agonist. The efficacy of anandamide as a TRPV1 agonist is influenced by a succession of factors including receptor reserve, phosphorylation, metabolism and uptake, CB_1 receptor activation, voltage, temperature, pH and bovine serum albumin. There are indications that the endocannabinoid system may play a role in the modulation of TRPV1 receptor activation. The activation of TRPV1 receptors by anandamide has potential implications in the treatment of inflammatory, respiratory and cardiovascular disorders. The relative importance of anandamide as a physiological and/or pathophysiological TRPV1 receptor agonist in comparison to other potential candidates has yet to be revealed.
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Introduction

The vanilloid VR1 or TRPV1 receptor is part of a family of transient receptor potential (TRP) channels (see Benham *et al.*, 2002), whose expression is largely associated with small diameter primary afferent fibres. This receptor is a nonselective cation channel that integrates multiple noxious stimuli and is associated with the pathophysiology of various major diseases (Szallasi, 2002). It is activated by the naturally occurring vanilloids, capsaicin and resiniferatoxin (RTX), noxious heat and acid (see Szallasi, 2002). Anandamide (*N*-arachidonoyl-ethanolamine) is an 'endocannabinoid', as defined by its ability to bind to and activate cannabinoid CB_1 and CB_2 receptors (see Pertwee & Ross, 2002); however, the pharmacology of this compound is complex (see Di Marzo, 2002; Di Marzo *et al.*, 2002b). The search for endogenous TRPV1 receptor activators or 'endovanilloids' is ongoing and recent advances suggest that anandamide may be one such compound (Di Marzo *et al.*, 2001). Since the first revelations of the structural similarity of anandamide to capsaicin and of its ability to activate TRPV1 receptors (Melck *et al.*, 1999; Zygmunt *et al.*, 1999; Smart *et al.*, 2000), this topic has been the focus of exciting and sometimes controversial debate.

Various reviews cover the interaction of anandamide with TRPV1 receptors in pain pathways (see Di Marzo *et al.*, 2002a; Iversen & Chapman, 2002; Rice *et al.*, 2002; Walker & Huang, 2002), the cardiovascular system (see Högestätt & Zygmunt, 2002; Ralevic *et al.*, 2002; Randall *et al.*, 2002) and immune

cells (see Parolaro *et al.*, 2002). This article will focus on the most recent findings and, in particular, those aspects relating to the pharmacology of anandamide.

The basics: affinity, potency and efficacy

There is ample evidence that the interaction of anandamide with TRPV1 receptors is specific: TRPV1 actions are blocked by receptor-specific antagonists and not by antagonists of CB_1 and CB_2 receptors; desensitisation of TRPV1 *via* capsaicin pretreatment abrogates the effects of anandamide; neonatal capsaicin treatment prevents anandamide activation of TRPV1; anandamide-mediated TRPV1 effects are absent from untransfected cells that do not express TRPV1 receptors; anandamide displaces radiolabelled RTX from specific binding sites (Zygmunt *et al.*, 1999; Smart *et al.*, 2000; Ross *et al.*, 2001b).

It seems appropriate to begin this article by reviewing the literature relating to the pharmacology of anandamide and TRPV1. Traditionally, the foundations of the pharmacological analysis of any compound are the three basic parameters of affinity, potency and efficacy. Numerous studies confirm that anandamide activates TRPV1 receptors; those that specifically measure the affinity, efficacy or potency at native and recombinant TRPV1 receptors are shown in Tables 1 and 2.

Affinity

Radioligand displacement assays in recombinant cell lines using the high-affinity TRPV1 agonist [^{3}H]RTX demonstrate

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Table 1 Studies measuring the affinity, potency and efficacy of anandamide at recombinant TRPV1 receptors

Cell line	Affinity (K_i)	Measured response	pH	Potency (pEC_{50})	Efficacy (E_{max}) (%)[a]	Reference
HEK 293(rat)		Inward current	7.4	5.31 ± 0.06	$\sim 30-50$	Zygmunt et al. (1999)
		[Ca^{2+}]$_i$ (FLIPR)	7.4	5.73 ± 0.04	100	Ralevic et al. (2001)
			6.4	5.76 ± 0.04	—	Ralevic et al. (2001)
		[Ca^{2+}]$_i$ (FLIPR)	—	5.85 ± 0.01	100	Jerman et al. (2002)
		[Ca^{2+}]$_i$ (Fura-2)	—	5.85 ± 0.12 (22°C)	63	Sprague et al. (2001)
				5.15 ± 0.04 (37°C)	72	Sprague et al. (2001)
				<4 (50°C)	120	Sprague et al. (2001)
HEK 293 (human)		[Ca^{2+}]$_i$ (FLIPR)	7.4	5.94 ± 0.06	100	Smart et al. (2000)
			6.4	5.76 ± 0.04	100	Smart et al. (2000)
			7.4	5.60 ± 0.10	100	Ralevic et al. (2001)
			7.4	6.66 (with LEA)[b]	—	Smart et al. (2000)
			7.4	5.74 (no LEA)	—	Smart et al. (2002)
	—	[Ca^{2+}]$_i$ (Fluro-3 imaging)	7.4	6.20	100	De Petrocellis et al. (2001a)
	5.40 (with PEA)		7.4	6.66 (with PEA)[c]	—	De Petrocellis et al. (2001b)
	4.72 (no PEA)			6.36 (no PEA)	—	De Petrocellis et al. (2001b)
CHO (rat)	5.78 ± 0.06 (with PMSF)	[Ca^{2+}]$_i$ ([^{45}Ca^{2+}] uptake)	7.4	5.80 ± 0.04 (with PMSF)[d]	100	Ross et al. (2001b)
	<5 (no PMSF)		7.4	<5 (no PMSF)	—	Ross et al. (2001b)
CHO (guinea-pig)		[Ca^{2+}]$_i$	7.5	5.01	~ 22	Savidge et al. (2002)
NIH 3T3 (rat)		[Ca^{2+}]$_i$ ([^{45}Ca^{2+}] uptake)	5.5	4.85	—	Olah et al. (2001)
			6.0	4.60	—	Olah et al. (2001)
			6.5	4.20	—	Olah et al. (2001)

[a]Relative to that of capsaicin (100%).
[b]Lauroylethanolamide (LEA) is an N-acylethanolamide.
[c]Palmitoylethanolamide (PEA) is an N-acylethanolamide.
[d]Phenylmethylsulphonyl fluoride (PMSF) is an inhibitor of FAAH.

Table 2 Studies measuring the affinity, potency and efficacy of anandamide at native TRPVI receptors

Tissue	Measured response	Potency (pEC_{50})	Efficacy (E_{max}) (%)[a]	Reference
Guinea-pig basilar artery	Relaxation	6.02 ± 0.11	100 ± 1	Zygmunt et al. (1999)
Rat hepatic artery	Relaxation	6.45 ± 0.11	92 ± 3	Zygmunt et al. (1999)
Rat mesenteric artery	Relaxation	6.1 ± 0.1	98 ± 1	Andersson et al. (2002)
Rat mesenteric bed	Relaxation	6.32 ± 0.04	$\sim 100\%$	Ralevic et al. (2001)
Guinea-pig bronchi	Contraction	5.23 ± 0.1 (with PMSF)[b]	46.5 ± 6.0	Tucker et al. (2001)
		5.26 ± 0.05 (no PMSF)	41.6 ± 5.8	Tucker et al. (2001)
		5.18 ± 0.11 (with PMSF)	49.5 ± 3.9	Craib et al. (2001)
		4.15 ± 0.64 (no PMSF)	—	Craib et al. (2001)
		$<5.4\pm0.1$	$>28\pm6$	Andersson et al. (2002)
Guinea-pig ileum	[^{3}H]Ach release	6.3	—	Mang et al. (2001)
	Increased muscle tone	6.3	—	
Mouse trigeminal neurones	Outward current	5.3 ± 0.1	38 ± 2	Roberts et al. (2001)
Rat DRG	Inward current	—	$10-50$	Smart et al. (2001)
	Single channel currents (inside-out patches)	4.93	—	Hwang et al. (2001)
	[Ca^{2+}]$_i$ ([^{5}Ca^{2+}] uptake)	5.15 (pH 5.5)	—	Olah et al. (2001)
		5 (pH 6)	$\sim 23\%$	Olah et al. (2001)
		Inactive (pH 6.6)	—	Olah et al. (2001)
	[Ca^{2+}]$_i$ (FLIPR)	—	$\sim 63\%$	Jerman et al. (2002)

[a]Relative to that of capsaicin (100%).
[b]Phenylmethylsulphonyl fluoride (PMSF) is an inhibitor of FAAH.

that anandamide has a low affinity for the receptor. While the K_i value of $\sim 2\,\mu M$ for anandamide is high, it is similar to that of the established receptor agonist capsaicin (De Petrocellis *et al.*, 2001b; Ross *et al.*, 2001b). This assay is, of course, reliant on RTX binding to the same site on TRPV1 as other agonists. It has been observed that RTX has a significantly lower K_i/EC_{50} ratio than capsaicin as measured in radioligand binding and functional Ca^{2+} uptake assays (see Di Marzo *et al.*, 2002a) in both native and recombinant TRPV1 expression systems. Such findings can be explained on the basis that RTX has a lower intrinsic efficacy than capsaicin. Indeed, there is direct evidence that RTX is a lower efficacy agonist at TRPV1 than capsaicin (Wardle *et al.*, 1997; Ross *et al.*, 2001b; Andersson *et al.*, 2002). However, it is possible that RTX and capsaicin bind to different domains on the receptor. There is now evidence that RTX behaves differently from capsaicin: the activation of TRPV1 by RTX leads to a Ca^{2+}-independent Ca^{2+} mobilisation that is not observed with capsaicin (Marshall *et al.*, 2003). Such data question the usefulness of [³H]RTX as a radioligand for the direct measurement of the TRPV1 receptor 'affinity' of anandamide and capsaicin: the inhibition of binding of [³H]RTX does not necessarily imply a direct competition and may be explained by allosteric binding sites on the TRPV1 receptor.

There is good evidence that the structural determinants for capsaicin binding and sensitivity are also essential for the interaction of anandamide with the receptor (Jordt & Julius, 2002). Consequently, in mutant receptors rendered insensitive to capsaicin, anandamide is also inactive, even at low pH. While the wild-type VRL-1 receptor does not normally bind either capsaicin or anandamide; VRL-1 receptor mutants, in which capsaicin displaces the specific binding of [³H]RTX, also have an affinity for anandamide (Jordt & Julius, 2002).

Potency

While anandamide has a similar affinity for TRPV1 to that of capsaicin, it has a significantly lower potency. In high expression recombinant cell lines using various methods, the potency (EC_{50}) of anandamide has been measured in the range of $0.7 - 5\,\mu M$ (Table 1). In native systems, the potency of anandamide ranges from 0.3 to $0.8\,\mu M$ in blood vessels (relaxation) compared with $6 - 10\,\mu M$ in bronchus (contraction) and DRG neurones ($[Ca^{2+}]_i$ and inward current) (Table 2).

Efficacy: full or partial agonist?

While it is clear that anandamide can activate TRPV1 receptors, significant differences emerge when comparisons are made of the efficacy of anandamide in native and recombinant receptor systems. There are also significant differences between tissues. Using the measurement of $[Ca^{2+}]_i$ in high expression recombinant cell lines expressing either rat or human TRPV1, anandamide appears to be a full agonist as defined by an E_{max} value that is not significantly different from that of capsaicin (De Petrocellis *et al.*, 2001a; Smart *et al.*, 2000; Ralevic *et al.*, 2001; Ross *et al.*, 2001b; Sprague *et al.*, 2001; Jerman *et al.*, 2002). Some authors find the E_{max} value of anandamide to be less than 100% in recombinant expression systems. Measurements of inward current using whole-cell patch-clamp electrophysiology in HEK 293 cells Zygmunt *et al.*

(1999) estimate the efficacy of anandamide to be $30 - 50\%$ that of capsaicin. In the same cells, Sprague *et al.* (2001) find that, at 22 and 37°C, the E_{max} value for anandamide is 63 and 72% of the response to capsaicin. In CHO cells expressing guinea-pig TRPV1, anandamide has a significantly lower efficacy (22%) than that of capsaicin (Savidge *et al.*, 2002). Similar differences are found in tissues expressing native TRPV1 receptors and the efficacy of anandamide appears to be tissue dependent (Table 2). Thus, it is a full agonist in blood vessels and mesenteric arterial bed; it is a partial agonist in the bronchus, dorsal root ganglion (DRG) and trigeminal neurones (Table 2). In the substantia gelatinosa neurones of the dorsal horn, anandamide activates TRPV1 receptors to increase the frequency of mEPSPs and stimulate neuropeptide release, but only at high concentrations of 10 and $50\,\mu M$ (see Morisset *et al.*, 2001).

The differences in the efficacy of anandamide between tissues and as measured using different pharmacological end points can be accounted for by a low intrinsic efficacy. Agonist potency is a function of both affinity and efficacy. The ratio of K_i (inhibition constant) to EC_{50} value is indicative of the relative intrinsic efficacy of a compound. In CHO cells expressing rTRPV1, Ross *et al.* (2001b) found that, although anandamide and AM404 have E_{max} values of 100%, they display a low K_i/EC_{50} ratio as determined from radioligand binding and [$^{45}Ca^{2+}$] uptake data. This is indicative of a low intrinsic efficacy. As a consequence of the low intrinsic efficacy of anandamide at TRPV1, the E_{max} value will vary with the pharmacological end point that is measured. Thus, while the elevation of $[Ca^{2+}]_i$ has a high degree of signal amplification, measurement of the TRPV1 current has no downstream amplification. Accordingly, analyses of TRPV1 currents in sensory neurones reveal that anandamide is a partial agonist (Zygmunt *et al.*, 1999; Roberts *et al.*, 2002). When the response in the elevation in $[Ca^{2+}]_i$ is measured, anandamide is found to be either a partial or a full agonist depending on the tissue used (see Tables 1 and 2).

The low intrinsic efficacy of anandamide at the TRPV1 receptor has important physiological implications. A low intrinsic efficacy agonist will attenuate the effects of a full agonist. Indeed, in trigeminal neurones, coapplication of anandamide with capsaicin significantly reduces the currents produced by capsaicin (Roberts *et al.*, 2002). If endogenous activators of TRPV1 exist that are full agonists, then compounds such as anandamide may have an inhibitory role and serve as anti-inflammatory and analgesic compounds. Indeed, a high efficacy TRPV1 agonist, *N*-arachidonoyl-dopamine (NADA), has recently been isolated from the central nervous system (Huang *et al.*, 2002).

Factors influencing the efficacy and potency of anandamide at TRPV1

The intrinsic efficacy of anandamide at TRPV1 is relatively low in comparison to that observed for this compound at the CB_1 receptor (see Pertwee & Ross, 2002) and as a result, the physiological relevance of anandamide as an endogenous TRPV1 receptor agonist is controversial. Recently, however, a succession of factors have been shown to influence the efficacy and potency of anandamide at TRPV1 significantly.

Receptor reserve

The receptor reserve in a given tissue or cell line will influence the efficacy of a low intrinsic efficacy agonist, whereby such a compound may behave as a full agonist or a partial agonist/antagonist in situations of high or low receptor reserve, respectively. Thus, although anandamide and AM404 may behave as full agonists in certain systems, one would predict that they may behave as partial agonists in tissues with a low receptor reserve. Andersson et al. (2002) performed computer simulations of the impact of the receptor reserve on the concentration – response curves for various TRPV1 agonists, their hypothesis being that the bronchus has a significantly lower receptor reserve than mesenteric vessels. The simulations accurately modelled the experimental data from these tissues. Thus, while capsaicin is equally potent in both tissues, RTX, olvanil and anandamide are less potent in the bronchi.

The low intrinsic efficacy of anandamide indicates that its efficacy is significantly affected by changes in the receptor reserve in a given tissue. Circumstances that increase TRPV1 expression levels will conspire to increase the efficacy of anandamide such that it behaves as a full agonist. In vitro, the nerve growth factor (NGF) and glial-derived neurotrophic factor regulate DRG neurone responses to capsaicin (Winter et al., 1988) and the treatment of DRG neurones with NGF increases the levels of TRPV1 mRNA (Winston et al., 2001). This has two important implications for the activation of TRPV1 by anandamide. Firstly, the levels of NGF used in cultures of DRG neurones may vary between labs and this may affect the efficacy and potency of anandamide measured by different research groups. Secondly, alterations in the levels of these growth factors may be associated with the development of chronic pain (for review see Di Marzo et al., 2002a) and any associated changes in the TRPV1 receptor expression will alter the efficacy of anandamide.

The TRPV1 receptor is associated with the pathophysiology of various major diseases (Szallasi, 2002). Alterations in the expression and sensitivity of TRPV1 receptors is concurrent with the development of chronic pain (see Di Marzo et al., 2002a) and inflammation (Amaya et al., 2003). The expression of TRPV1 is dramatically increased in colonic nerve fibres of patients with active inflammatory bowel disease (Yiangou et al., 2001). Patients with asthma show a significantly enhanced sensitivity to capsaicin as compared to normal subjects (see Spina & Page, 2002), and TRPV1 may be a key mediator in the pathophysiology of asthma (see Hwang & Oh, 2002). Hudson et al. (2001) demonstrate that in an animal model of neuropathic pain, VR1 immunoreactivity increases significantly in both C and A fibres in undamaged DRG neurones.

Anandamide uptake and metabolism

Anandamide membrane transporter   The agonist binding site on TRPV1 is thought to be intracellular (Jung et al., 1999) and consequently the potency of exogenously added anandamide is affected by its ability to enter the cell. The anandamide membrane transporter (AMT) is responsible for the uptake of extracellular anandamide and compounds that alter the function of the AMT modulate the potency of anandamide in HEK cells expressing TRPV1 (De Petrocellis et al., 2001a; and see Di Marzo et al., 2002a). The AMT is activated by nitric oxide (Maccarrone et al., 2000a) and the TRPV1

receptor-mediated component of anandamide-induced vasorelaxation in the mesenteric arterial bed is attenuated by the nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) (Harris et al., 2002). A recent study by Andersson et al. (2002) provides evidence that primary afferent fibres express the AMT and that variability in the expression levels of the transporter affects the potency of anandamide. Thus the AMT inhibitor, VDM13, causes a 2.3-fold rightward shift in the log concentration – response curve for vasodilatation of mesenteric arteries by anandamide, but does not affect the contractile response to anandamide in the bronchus. However, even in the presence of the uptake inhibitor, anandamide is still a full agonist at TRPV1 in mesenteric arteries as compared to a partial agonist in the bronchi. As discussed earlier, the authors demonstrate that the remaining tissue differences are due to a lower receptor reserve in the bronchus. It should be noted that the existence of the AMT is controversial (see Glaser et al., 2003). A number of AMT inhibitors are also inhibitors of fatty acid amide hydrolase (FAAH) and as such, may increase the intracellular anandamide levels by inhibiting hydrolysis.

Fatty acid amide hydrolase (FAAH)   Inside the cell, anandamide is rapidly metabolised by FAAH to yield arachidonic acid and ethanolamide. There is evidence that FAAH inhibitors enhance, attenuate or have no effect on TRPV1 activation by anandamide. Thus, as has been demonstrated for CB_1 receptors (Cravatt et al., 2001), the affinity and potency of anandamide at TRPV1 is enhanced by the inhibition of FAAH in certain tissues including CHO cells (Ross et al., 2001b), HEK 293 (De Petrocellis et al., 2001a), rat ileum (McVey et al., 2002) and neuroblastoma cells (Maccarrone et al., 2000c). On the other hand, in the mesenteric bed, FAAH inhibitors attenuate the TRPV1 receptor-mediated relaxation by anandamide (Orliac et al., 2003). In the bronchus, however, the potency of anandamide as a TRPV1 agonist is unaffected by FAAH inhibitors (Tucker et al., 2001; Andersson et al., 2002).

The intraluminal administration of anandamide causes inflammation of the rat ileum resulting in enteritis. The effect involves stimulation of substance P release from sensory fibres and is antagonised by capsazepine (McVey et al., 2002). It is more potent in the presence of FAAH inhibitors. Inflammation of the ileum by Clostridium diffificle toxin A is known to involve the TRPV1 receptor activation and McVey et al. (2002) have also demonstrated that toxin A stimulates the release of anandamide. Moreover, FAAH inhibitors significantly increase the inflammatory effects of this toxin, indicating that endocannabinoids may mediate its inflammatory actions. In endotoxaemic rats in which LPS has been used to mimic Gram-negative sepsis, anandamide causes a reduction in the contractile responses of mesenteric beds to noradrenaline (Orliac et al., 2003). In this case, the effect of anandamide is inhibited by capsazepine and an FAAH inhibitor and thus appears to involve a metabolite of anandamide that is as a TRPV1 receptor agonist.

Lipoxygenase

The lipoxygenase metabolites of arachidonic acid, particularly 12-(S)-hydroperoxyeicosatetraenoyl acid (12-(S)-HPETE), 5-(S)HETE and leukotriene B_4 (LTB$_4$), are agonists of the

TRPV1 receptor (Hwang *et al.*, 2001; Piomelli, 2001). Recent studies of the action of the potent inflammatory mediator bradykinin, provide more compelling evidence for the role of lipoxygenase metabolites in the activation of TRPV1. Thus, bradykinin activation of TRPV1 receptors in both cultured DRG neurones and the skin is significantly attenuated by lipoxygenase inhibitors (Shin *et al.*, 2002). Of the neurones that respond electrophysiologically to bradykinin, 75% coexpress mRNAs encoding the TRPV1, B2 receptor and 12-lipoxygenase. Furthermore, extracellular recording from C fibre receptive fields in guinea-pig isolated airways reveals that lipoxygenase inhibitors dramatically inhibit bradykinin-induced action potentials that are TRPV1 receptor mediated (Carr *et al.*, 2002): the nonselective lipoxygenase inhibitor, ETYA, and the 12-lipoxygenase inhibitor, baicalein, reduce the effect of bradykinin by 76 and 60%, respectively. It is known that 15-HPETE is synthesised by bronchial epithelial cells and induces persistent airway hyper-responsiveness that is sensitive to pretreatment with capsaicin (see Spina & Page, 2002). In addition, protease-activated receptor 2 causes TRPV1 receptor-mediated coronary vasodilation that involves a lipoxygenase-derived product (McLean *et al.*, 2002).

In addition to hydrolysis by FAAH, anandamide can also be metabolised by a range of oxygenase enzymes that are already known to convert arachidonic acid into potent biologically active compounds (see Kozak & Marnett, 2002; Ross *et al.*, 2002). These include cyclooxygenase-2, lipoxygenase and *P*450 enzymes. *In vitro*, 12- and 15-lipoxygenase convert anandamide into 12- and 15-HPETE ethanolamide (HPETEE), respectively, the reaction rates being similar to those for arachidonic acid (see Kozak & Marnett, 2002). Thus, the pharmacology of anandamide may be directed by tissue-specific differences in the balance of metabolic enzymes. For example in platelets, which lack significant expression of COX-2 or FAAH, the primary metabolism of anandamide is by lipoxygenase (Edgemond *et al.*, 1998). As an alternative substrate for lipoxygenase enzymes, anandamide may attenuate the production of metabolites of arachidonic acid that are TRPV1 receptor agonists. Alternatively, anandamide may activate TRPV1 *via* lipoxygenase metabolites of arachidonic acid formed subsequent to its metabolism by FAAH. A further possibility is that anandamide lipoxygenase metabolites may themselves activate TRPV1 receptors (Figure 1). In the bronchus, the TRPV1 receptor-mediated contractile action of anandamide is little affected by FAAH inhibitors, but is significantly attenuated by lipoxygenase inhibitors (Craib *et al.*, 2001). These data suggest that, in this tissue, anandamide may be metabolised to hydroperoxyeicosatetraenoyl ethanolamides (HPETEEs) and LTB$_4$ ethanolamides that, like the hydroperoxy derivatives of arachidonic acid (HPETEs) and LTB$_4$, may be vanilloid receptor agonists. In hTRPV1-HEK cells, the anandamide lipoxygenase metabolites, 11(*S*)-HPETEE and 5(*S*)-HPETEE, do not induce an increase in $[Ca^{2+}]_i$ and 15(*S*)-HPETEE has only a modest effect at concentrations above 10 μM (De Petrocellis *et al.*, 2001a). However, it is feasible that these compounds and related molecules are more potent as TRPV1 agonists if produced intracellularly *via* the metabolism of arachidonic acid and/or anandamide close to the intracellular TRPV1 agonist binding site.

In the bronchus, lipoxygenase inhibitors modestly attenuate the contractile action of capsaicin (Craib *et al.*, 2001), raising the possibility that the increase in intracellular calcium elicited by TRPV1 receptor activation leads to the release of arachidonic acid and/or anandamide, whose hydroxylation by lipoxygenase may lead to the formation of compounds that are themselves vanilloid agonists. Indeed, mass spectrometric analysis shows that capsaicin and depolarisation (KCl) induce a significant release of anandamide in DRG cultures (Ahluwalia *et al.*, 2003). It is perhaps notable that the capsaicin-evoked release of anandamide is significantly attenuated when the FAAH inhibitor, MAFP, is excluded from the buffer, demonstrating that anandamide is rapidly metabolised in DRG neurones. Metabolic products of anandamide have also been implicated in anandamide-induced depolarisation of the guinea-pig isolated vagus nerve that is TRPV1 receptor mediated (Kagaya *et al.*, 2002). In this preparation, depolarisation by anandamide, but not capsaicin, is inhibited by lipoxygenase inhibitors, but only in the presence of calcium. It is not clear, however, whether these active metabolites are produced *via* direct lipoxygenase metabolism of anandamide or *via* the metabolism of arachidonic acid, because these experiments did not include FAAH inhibitors.

Potent FAAH inhibitors have recently been synthesised, which enhance the levels of anandamide significantly, and these compounds may be of considerable therapeutic benefit (Boger *et al.*, 2000). In the event of inhibition of FAAH metabolism of anandamide, increased levels of endogenous anandamide may lead to the production of significant levels of the HPETE ethanolamides. It is also notable that specific inhibitors of 5-lipoxygenase markedly enhance the level of hydrolysis of anandamide by FAAH in human mast cells (Maccarrone *et al.*, 2000b). Thus, Maccarrone *et al.* hypothesise that 5-lipoxygenase metabolites of anandamide may be acting as endogenous inhibitors of FAAH. To complement the hypothesis, they have demonstrated that various HPETE and HETE ethanolamides are potent endogenous inhibitors of FAAH (Maccarrone *et al.*, 2000d; van der Stelt *et al.*, 2002). Consequently, lipoxygenase metabolism of anandamide may enhance TRPV1 receptor activation by increasing the levels of available anandamide.

Entourage effects

Anandamide is one of a group of *N*-acylethanolamides that include palmitoylethanolamide (PEA). PEA is cosynthesised with anandamide and acts as an 'entourage' compound to inhibit the degradation of anandamide and thereby increase the available levels. PEA enhances both the affinity and the potency of anandamide at TRPV1 receptors (De Petrocellis *et al.*, 2001b), an effect shared by other *N*-acylethanolamides (e.g. lauroylethanolamide) (Smart *et al.*, 2002) (see Table 1). The enhancement of anandamide-induced TRPV1 receptor activation by these compounds does not correlate with their ability to inhibit anandamide metabolism by FAAH (Smart *et al.*, 2002). This suggests that these compounds enhance the action of anandamide by a mechanism other than the inhibition of enzymatic degradation. Therefore, in circumstances that increase the synthesis of *N*-acylethanolamides, the actions of anandamide at TRPV1 may be enhanced.

Phosphorylation

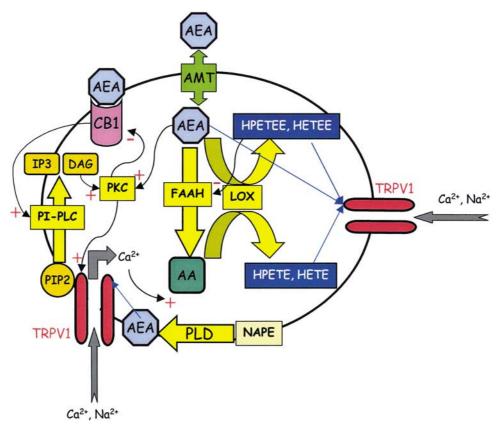Phosphorylation of TRPV1 by protein kinase C (PKC) and protein kinase A (PKA) sensitises TRPV1 receptors to

Figure 1 Metabolic pathways of anandamide and arachidonic acid related to the TRPV1 receptor. Anandamide (AEA) is rapidly hydrolysed by FAAH to yield arachidonic acid and ethanolamide. Arachidonic acid (AA) is oxygenated by lipoxygenase enzymes: the products include 12-(S)- and 15-(S)-HPETE, 5-(S)HETE and LTB$_4$ that are TRPV1 agonists (Hwang et al., 2001). Anandamide is also a substrate for lipoxygenase (see Kozak & Marnett, 2002), yielding equivalent HPETE ethanolamides (HPETEE) and HETE ethanolamides (HETEE) that may also be TRPV1 agonists (Craib et al., 2001). The lipoxygenase products of anandamide are potent inhibitors of FAAH (Maccarone et al., 2000d). PKC can activate the TRPV1 receptor directly (Premkumar & Ahern, 2000; Olah et al., 2002), and it will also sensitise the receptor to other agonists (Vellani et al., 2001). Anandamide can activate PKC directly (De Petrocellis et al., 1995; Premkumar & Ahern, 2000). CB$_1$ receptor activation is coupled to the stimulation of PLC, hydrolysis of PIP2 and release of the TRPV1 receptor from the tonic inhibitory effect of this compound (see Hermann et al., 2003). Anandamide formation occurs through phosphodiesterase-mediated cleavage of a phospholipid precursor, N-arachidonoyl-phosphatidyletha-nolamine (NAPE), and its production in and release from neurones is calcium dependent (Di Marzo et al., 1994). In cultured neurones, anandamide is synthesised in response to TRPV1 receptor activation (Ahluwalia et al., 2003).

vanilloids, anandamide, heat and protons (see Di Marzo et al., 2002a). Premkumar & Ahern (2000) report that the repeated application of anandamide evokes progressively larger TRPV1 currents in oocytes and that anandamide treatment enhances proton-evoked currents. The potentiation by anandamide is significantly attenuated by compounds that inhibit PKC, and the authors suggest that anandamide stimulates PKC to enhance agonist-evoked responses (Figure 1). These experiments were performed in an oocyte expression system injected with TRPV1, thus anandamide actions are presumably cannabinoid receptor independent. Indeed, there is evidence that by binding directly to the diacylglycerol site, anandamide has a dual modulatory effect on PKC in the rat brain *in vitro*: while anandamide increases phosphatidylserine-induced PKC activation, it also inhibits dioleylglycerol-induced potentiation of Ca^{2+}-induced PKC activation (De Petrocellis et al., 1995).

PKC is a series of isozymes that are specific for different substrates and there is controversy as to which isozyme is responsible for PKC-mediated enhancement of TRPV1 responses (see Cesare et al., 1999; Numazaki et al., 2002; Olah

et al., 2002). In cultured DRG neurons, the PKC activator, phorbol 12,13 dibutyrate (PBDu), induces TRPV1 receptor activation; in cultures treated chronically with PBDu, there is a significant downregulation of the PKCα isoform (and only a partial downregulation of PKC β, δ, ε isoforms) with concomitant loss of TRPV1 receptor activation (Olah et al., 2002). While anandamide activates TRPV1 receptors in cultures in which PKCα is downregulated, it is four-fold less potent and this may implicate PKCα in modulating the potency of anandamide (Olah et al., 2002).

Phosphorylation of TRPV1 is likely to be associated with inflammation (Cesare et al., 1999). A comparative study of PKCγ immunoreactivity in the dorsal horn before and after the induction of inflammation in the rat hind paw reveals that there are persistent alterations that parallel the time course of allodynia (Martin et al., 1999). Kamei et al. (2001) have produced evidence that allodynia and hyperalgesia in diabetic mice may be due to the sensitisation of VR1 receptors in primary sensory neurons and there is evidence of an upregulation of certain isoforms of PKC in diabetes (Borghini et al., 1994; Koya & King, 1998). Furthermore, PKC

inhibitors have been shown to have beneficial effects on diabetic neuropathy (Cameron et al., 1999).

As discussed above, PKC and PKA facilitate the activation of TRPV1 by anandamide. In contrast, PKC-mediated phosphorylation of the CB_1 receptor prevents cannabinoid-mediated activation of K_{ir} currents and the inhibition of P/Q-type Ca^{2+} channels (Garcia et al., 1998). The CB_1 receptor inhibits adenylyl cyclase via the α-subunit of Gi resulting in the inhibition of PKA activation. Thus, one may anticipate that PKC and PKA are pivotal in regulating the balance of CB_1 and TRPV1 activation by anandamide (see Di Marzo et al., 2002a; Hermann et al., 2003) (Figure 1).

Phosphatidyl-inositol-bis phosphate

The TRPV1 receptor is under the inhibitory control of phosphatidyl-inositol-bis phosphate (PIP2); both antibody sequestration of plasma membrane PIP2 and phospholipase C (PLC) hydrolysis of PIP2 potentiate the TPRV1 channel activation (Chuang et al., 2001). The potent inflammatory mediators, bradykinin and NGF, activate PLC signalling pathways in DRG neurones leading to the hydrolysis of PIP2 to inositol-tris-phosphate and diacylglycerols (DAGs) (Chuang et al., 2001). The authors suggest that, in addition to sensitisation via a PKC-dependent mechanism, TRPV1 is also sensitised by a PKC-independent mechanism consequent to the hydrolysis of PIP2.

In HEK-293 cells overexpressing both the CB_1 and TRPV1 receptors, pretreatment with a cannabinoid receptor agonist significantly enhances capsaicin-evoked increases in $[Ca^{2+}]_i$ (Hermann et al., 2003). This effect is inhibited by SR141716A and is absent from cells that express TRPV1 only, indicating that CB_1 receptor activation leads to an enhanced activation of TRPV1. Furthermore, anandamide produces a significantly greater increase in intracellular calcium in CB_1-TRPV1-HEK as compared to TRPV1-HEK cells. The CB_1 receptor-mediated enhancement of TRPV1 activation is attenuated by the inhibitors of phosphoinositide-3-kinase (PI-3-K) and PLC. PI-3-K is responsible for the formation of PIP2, while PI-PLC catalyses PIP2 hydrolysis and the result of inhibition of these enzymes will be the attenuation of the turnover and hydrolysis of PIP2. CB_1 receptor activation is coupled to the stimulation of PLC and PI-3-K (Ho et al., 1999; Netzeband et al., 1999; Gomez Del Pulgar et al., 2002) and hence may release TRPV1 from tonic inhibition by PIP2. PKC is also activated as a consequence of the hydrolysis of PIP2 to DAG. Hermann et al. (2003) suggest that these mechanisms may underlie the CB_1 receptor-mediated enhancement of TRPV1 receptor activation (Figure 1).

Hermann et al. (2003) also found that if cAMP-mediated signalling is activated, CB_1 agonists mediate the inhibition and not the enhancement of TRPV1 receptor activation: when PKA is activated via forskolin stimulation of adenylyl cyclase, CB_1 receptor agonists inhibit TRPV1 receptor activation by capsaicin. In cells in which there is coexpression of these receptors, the findings of Hermann et al. (2003) raise two possibilities for CB_1 receptor influence on the activation of TRPV1. Firstly, CB_1 receptor activation may lead to an enhanced TRPV1 receptor activation. One may predict such an outcome in cells in which the CB_1 receptor couples to pathways that facilitate that gating of TRPV1. Alternatively, CB_1 receptor activation may lead to an inhibition of TRPV1 receptor activation. Such an outcome may be observed in cells

in which CB_1 receptors are not coupled to PI-PLC or PI-3-K or in which the TRPV1 receptor is phosphorylated by cyclic AMP-dependent PKA activation.

CB_1 receptor activation

Within the DRG, there is a heterogeneous population of cells containing small, intermediate and large diameter neurones that give rise to C, Aδ or Aβ fibre axons. Both in culture and in situ, DRG neurones express both CB_1 and TRPV1 receptors. The localisation of CB_1 and TRPV1 receptor is controversial (see Rice et al., 2002); some studies suggest a high degree of colocalisation (Ahluwalia et al., 2000; 2002) and others suggest little colocalisation (Farquhar-Smith et al., 2000; Khasabova et al., 2002). Anandamide has a dual effect on neuropeptide release from cultured DRG neurones: at low concentrations, anandamide induces a CB_1 receptor-mediated inhibition of electrically stimulated neuropeptide release, presumably by the inhibition of voltage-gated Ca^{2+} channels (Ross et al., 2001a), while at higher concentrations anandamide evokes a TRPV1 receptor-mediated release (Tognetto et al., 2001). Anandamide also attenuates capsaicin-evoked neuropeptide release from DRG neurones and isolated paw skin (Richardson et al., 1998; Nagy et al., 2002). Furthermore, in the presence of a CB_1 receptor antagonist, anandamide becomes equipotent with capsaicin as a TRPV1 receptor agonist (Nagy et al., 2002). Similarly, in the guinea-pig ileum, anandamide increases acetylcholine release in a capsazepine-sensitive manner and this effect is enhanced in the presence of the CB_1 receptor antagonist, SR141716A (Mang et al., 2001). Finally, in human neuroblastoma cells, cannabinoid receptor antagonists potentiate the TRPV1 receptor-mediated cell death induced by anandamide (Maccarrone et al., 2000c). The implication of such data is that the activation of the CB_1 receptor by anandamide may attenuate its TRVP1 receptor-mediated action. In the spinal cord, superfusion of the CB_1 receptor antagonist significantly enhances the release of neuropeptide evoked by capsaicin (Lever & Malcangio, 2002). The activation of the TRPV1 receptor may evoke the release of endocannabinoids that subsequently activate CB_1 receptors to attenuate the TRPV1 receptor-mediated release of neuropeptide. This hypothesis is supported by the finding that capsaicin induces a significant release of anandamide in DRG cultures (Ahluwalia et al., 2003). Cannabinoid receptors are constitutively active and there are numerous examples of the CB_1 receptor antagonist, SR141716A, producing inverse cannabimimetic effects (see Pertwee, 2003; Hurst et al., 2002). In DRG neurons, a cannabinoid agonist inhibits and antagonist enhances voltage-gated Ca^{2+} currents (Ross et al., 2001a). It is possible that in DRG neurones and the spinal cord, constitutively active CB_1 receptors may exert a tonic inhibitory effect on the TRPV1 receptor by attenuating cAMP-dependent PKA activation. In this scenario, inverse agonists may enhance TRPV1 receptor activation.

Ellington et al. (2002) find that the CB_1 receptor agonist, CP55940, inhibits capsaicin-evoked calcitonin gene-related peptide (CGRP) release from the paw skin of both the control and diabetic animals. While anandamide inhibits CGRP release from the paw skin of control animals, it produces a small stimulation of CGRP release in diabetic animals. This demonstrates that there is an alteration in the pharmacology of anandamide in the disease state that may be associated with

changes in TRPV1 receptor expression and/or sensitivity in sensory fibres of diabetic animals.

Anandamide also has bidirectional effects on cough in conscious guinea-pigs (Jia *et al.*, 2002). When given by aerosol, anandamide induces cough, an effect that is prevented by pretreatment with capsazepine and not CB_1 or CB_2 receptor antagonists. When airways are pretreated with the ligand for a longer time in experimental conditions, anandamide inhibits cough through CB_1 receptors.

Voltage

Voltage-dependent priming of TRPV1 receptors is yet another mechanism by which the activation by anandamide may be enhanced. Ahern & Premkumar (2002) demonstrate that depolarisation acts synergistically with agonists to enhance the TRPV1 current in both oocytes expressing TRPV1 and cultured DRG neurones. Hence, voltage modulates the TRPV1 currents activated by anandamide, protons, heat and PKC (PBDu treatment). Intriguingly, voltage evokes a significantly larger current rise with anandamide and PKC as compared to that observed with capsaicin. Furthermore, the extent of the activation of TRPV1 receptors by voltage is inversely related to the agonist concentration. Consequently, this mechanism is significantly more pronounced for low efficacy agonists such as anandamide.

Temperature, pH, BSA, ethanol

Heat is known to enhance the TRPV1 receptor-mediated responses. Accordingly, the pEC_{50} values for the elevation of $[Ca^{2+}]_i$ in HEK293 cells by anandamide are significantly lower at $37^{\circ}C$ compared to $22^{\circ}C$ (Sprague *et al.*, 2001). Hence, temperature may contribute to the low efficacy of anandamide in experiments that are conducted at room temperature, including patch-clamp electrophysiology. Olah *et al.* (2001) found that in both cultured DRG neurones and an NIH3T3 cell line stably expressing TRPV1 receptors, low acidic pH (6.0 and 5.5) markedly enhances the stimulation of $[^{45}Ca^{2+}]$ uptake by anandamide. However, in TRPV1-transfected HEK 293 cells, lowering the pH of the buffer from 7.4 to 6.4 does not affect the potency of anandamide (Ralevic *et al.*, 2001; Smart *et al.*, 2001). De Petrocellis *et al.* (2001c) demonstrate that the potency of anandamide at TRPV1 is markedly reduced in the presence of bovine serum albumin (BSA): $1 \mu M$ elevates $[Ca^{2+}]_i$ by ~ 65, ~ 10 and $\sim 2\%$ in the presence of 0, 0.1 and 0.2% BSA, respectively. The authors suggest that BSA prevents the uptake of anandamide, interfering with the carrier-mediated internalisation of this compound, and the subsequent activation of intracellular TRPV1. Ethanol (0.1–3%) enhances TRPV1 receptor activation by capsaicin, heat, protons and anandamide as measured using either FLIPR or electrophysiology (Trevisani *et al.*, 2002). It is conceivable that TRPV1 receptor activation by anandamide may be greater if ethanol is used as the vehicle as opposed to DMSO.

Central nervous system

TRPV1 receptors have been thought to be primarily associated with the peripheral nervous system and in particular, primary afferent fibres, yet PCR studies show that TRPV1 mRNA is present in brain tissue and is especially high in the CA1 and CA3 regions of the hippocampus (Mezey *et al.*, 2000). When paired-pulse stimulation is applied to hippocampal slices, there is a depression of the second population spike evoked at interpulse intervals of 5–200 ms. The inhibitory feedback is due to the release of GABA following the first stimulation. Al-Hayani *et al.* (2001) demonstrate that the TRPV1 receptor agonists RTX, capsaicin and anandamide increase paired-pulse depression in the CA1 region of the rat hippocampal slice. The effect is blocked by the TRPV1 receptor antagonist, capsazepine. In contrast, the CB_1 receptor agonist, WIN55212, and the endocannabinoid 2-arachidonoyl glycerol, reduce paired-pulse depression, an effect that is blocked by the CB_1 receptor antagonist AM251 and is consistent with the CB_1 receptor-mediated inhibition of GABA release (see Davies *et al.*, 2002). In view of the low intrinsic efficacy of anandamide at TRPV1 as compared to CB_1, it is unexpected that anandamide should preferentially activate the TRPV1 receptor in this preparation. An interesting possibility is that anandamide activation of the CB_1 receptor leads to an enhancement of TRPV1 activation by liberating TRPV1 from tonic inhibition by PIP2 (see Hermann *et al.*, 2003). The experiments were performed in the absence of an FAAH inhibitor and another explanation may be the rapid conversion of anandamide to an active metabolite. Other possibilities include those discussed earlier, in particular receptor reserve and the phosphorylation states of TRPV1 and CB_1 in the CA1 region of the hippocampus. A further possibility is that, despite displaying the characteristics of TRPV1, this may be a novel site at which anandamide has a higher potency. Additional effects of vanilloids have been noted in the hippocampus. Hájos & Freund (2002a) found that the CB_1/CB_2 receptor agonists (WIN55212 and CP55940) and capsaicin modulate excitatory transmission, an effect that is antagonised by capsazepine. The results suggest the existence of a novel site on the hippocampal excitatory axons at which both cannabinoids and vanilloids act to suppress glutamatergic transmission (see Davies *et al.*, 2002; Hájos & Freund, 2002b).

Vanilloid effects have also been reported in the brain stem: microinjection of capsaicin into the sensory nerve terminals of the commissural nucleus of the solitary tract (cNTS), significantly reduces the respiration rate (Geraghty & Mazzone, 2002). The finding that RTX is significantly more potent than capsaicin and that the effects are blocked by capsazepine suggest that this is TRPV1 receptor-mediated. While anandamide and olvanil do not inhibit respiration in this preparation, these compounds significantly attenuate the effect of RTX in a concentration-dependent manner. TRPV1 receptor activation in the cNTS is desensitised by very low concentrations of RTX and the authors suggest that anandamide may be desensitising the receptor. Alternatively, anandamide may be behaving as a partial agonist to antagonise RTX in this preparation.

Marinelli *et al.* (2002) demonstrate that capsaicin causes an increase in miniature excitatory postsynaptic currents in the rat locus coeruleus, an effect that is mediated by presynaptic TRPV1 receptors that potentiate glutamate and adrenaline/noradrenaline release. Similarly, there is evidence that the substantia nigra pars compacta (SNc) contains presynaptic TRPV1 receptors on glutamatergic synapses to dopaminergic neurones. Spontaneous EPSCs (sEPSCs) are increased and inhibited by a TRPV1 agonist and antagonist respectively (Marinelli *et al.*, 2003). The tissue contains high levels of endogenous anandamide and the authors suggest that the stimulation of TPRV1 in the SNc by

endocannabinoids may effect tonic facilitation of glutamate release. In the presence of the CB_1 receptor antagonist AM281, anandamide increases the frequency of sEPSCs, albeit at the high concentration of $30\,\mu M$. At the lower concentration of $10\,\mu M$, anandamide is only active when PKA is stimulated by the adenylyl cyclase activator, forskolin.

Conclusions

A large body of evidence now exists to substantiate that anandamide activates TRPV1 receptors. It is a low intrinsic efficacy TRPV1 agonist behaving as a partial agonist in tissues with a low receptor reserve, while in tissues with high receptor reserve and in circumstances associated with certain disease states, it behaves as a full agonist. Furthermore, there are indications that the endocannabinoid system may play a role in the modulation of TRPV1 receptor activation. The activation of TRPV1 receptors by anandamide has potential implications in the treatment of inflammatory, respiratory and cardiovascular disorders. The relative importance of anandamide as a physiological and/or pathophysiological TRPV1 receptor agonist in comparison to other potential candidates, such as 15-HPETE and NADA, has yet to be revealed.

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