Anandamide: some like it hot

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Anandamide was the first endogenous ligand of cannabinoid receptors to be discovered in 1992. Yet, this compound also efficiently activates receptors specific for capsaicin, known as vanilloid type 1 receptors (VR1). Whether anandamide is a physiological VR1 ligand is controversial. However, very recent reports demonstrate that activation of VR1 by anandamide can be significantly enhanced by various regulatory factors, suggesting that this compound might act as an 'endovanilloid' under certain conditions.

When thinking of anandamide one might wonder: why must things be complicated? Could this compound not just be the long-sought endogenous ligand of cannabinoid receptors? Indeed, when, in 1992, anandamide (Box 1) was first isolated by Devane *et al.* from pig brain[1] and shown to behave as a functional ligand of cannabinoid CB_1 receptors, research on cannabinoids experienced a revival because this finding seemed to mark the end of a long 'battle' and the beginning of a new story. However, it soon became clear that anandamide was not 'la vie en rose' because it behaved as a partial agonist at CB_1 receptors and was almost functionally inactive at cannabinoid CB_2 receptors. The identification of another more abundant and efficacious endocannabinoid, 2-arachidonoyl-glycerol, and the observation that some of the typical cannabimimetic effects of anandamide *in vivo* are not reduced in CB_1 receptor knockout mice (Box 1) suggested that 'better' endocannabinoids could be found. Interestingly, anandamide, which is undoubtedly capable of inducing several pharmacological effects via CB_1 receptors[2], might be an unusual mediator as a result of its ability to interact with more than one molecular transducer.

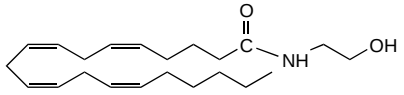
Anandamide and vanilloid receptors

Of the non-CB_1-receptor-mediated effects recently ascribed to anandamide, one is consistently gaining attention from pharmacologists: the capability of acting as a full agonist at vanilloid type 1 receptors (VR1) (Box 2), the sites of action of the pungent component of 'hot' red peppers, capsaicin. It is true that when VR1 is transfected into heterologous expression cell models, anandamide potency at inducing typical VR1-mediated effects (e.g. cation currents, Ca^{2+} influx and cell depolarization) is 5–20-fold lower than its average potency at CB_1

Box 1. Anandamide synthesis and action

Anandamide (Fig. I) is an endogenous eicosanoid with moderate affinity for the two cannabinoid receptor subtypes characterized so far: the CB_1 receptor, with a heterogeneous distribution in the brain, PNS and peripheral organs, and the CB_2 receptor, which is restricted to the immune system[a]. Both cannabinoid receptors are G-protein-coupled receptors. Activation of CB_1 receptors by anandamide leads to partial inhibition of adenylyl cyclase and voltage-activated Ca^{2+} channels[b], among other effects. By contrast, anandamide is functionally ineffective at CB_2 receptors[b]. Recently, three cannabimimetic effects elicited by anandamide *in vivo* (i.e. antinociception on a hot plate, immobility on a ring and suppression of spontaneous activity) were found to persist in mice in which the gene encoding the CB_1 receptor had been genetically disrupted[c]. Although less potent than anandamide, the endocannabinoid 2-arachidonoyl-glycerol[d,e] is fully effective at both CB_1 and CB_2 receptors[b].

Fig. I



Anandamide

Anandamide is formed 'on demand' from the hydrolysis of a phospholipid precursor, catalyzed by a phospholipase D enzyme, and is inactivated via reuptake and enzymatic hydrolysis by cells[f]. Anandamide biosynthesis appears to be induced in neurons (as well as in macrophages and basophils) by agents that cause Ca^{2+} influx into cells[f]. Reuptake occurs via diffusion through the cell membrane, facilitated by a selective, saturable and Na^+-independent carrier that can be stimulated by nitric oxide[g]. Anandamide hydrolysis is catalyzed by fatty acid amide hydrolase, a microsomal enzyme that has been cloned recently, and for which selective inhibitors have been developed[h].

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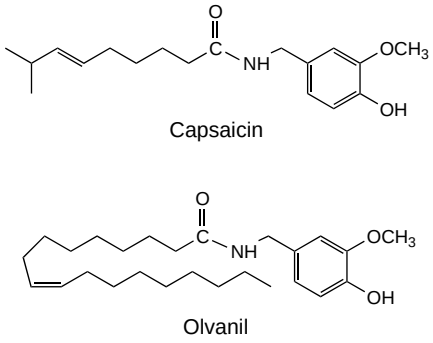
Box 2. Vanilloid receptors

The hypothesis of the existence of receptors for capsaicin (Fig. I) gained support when another natural plant product, resiniferatoxin (RTX), which mimics some of the effects of capsaicin on sensory neurons, was used to demonstrate the existence of specific binding sites[a]. The receptors were called 'vanilloid' receptors because of the presence of the 3-methoxy-4-hydroxy-benzyl moiety in vanillin and in both capsaicin and RTX. However, several ligands for this receptor have been identified that do not possess such a structural feature[b]. Although a possible heterogeneity of vanilloid receptors in rat sensory fibers was suggested[a], the only protein characterized so far that is capable of being activated by capsaicin and RTX is the recently cloned vanilloid type 1 receptor (VR1)[c]. This is a ligand-, heat- and proton-gated nonselective cation channel that is related to the TRP family of ion channels. VR1 was therefore suggested to function as an integrated transducer of nociceptive stimuli[d]. However, VR1 is also expressed in discrete areas of the brain (e.g. hypothalamus and basal ganglia[e,f]), thus possibly explaining some 'central' effects of capsaicin such as hypothermia, regulation of food intake and hypokinesia[a,g].

Fig. I



Vanilloid receptors are easily desensitized by their ligands[h]. Indeed, long-chain capsaicin analogs, of which olvanil (Fig. I) is the most studied example, desensitize the receptor without inducing the typical nociceptive response, and have been proposed as oral analgesics[h]. The chemical resemblance between olvanil and anandamide originally led to the identification of a relationship between the cannabinoid and vanilloid signaling systems[i].

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receptors[3,4]. However, more and more pharmacological actions of anandamide *in vitro* or *ex vivo* that are due to activation of 'native' vanilloid receptors are being observed at more physiologically relevant concentrations. These effects range from local vasodilatation[3] [exerted through the release of calcitonin gene-related peptide (CGRP)] and vas deferens relaxation[5] to sensory neuron excitation[6] and cancer cell apoptosis[7]. Several factors might explain this discrepancy, including the existence of vanilloid receptor subtypes that are different from VR1 (Box 2) or the presence of cofactors in native cells that might be lacking in heterologous expression systems. So, what is it that enhances anandamide activity at vanilloid receptors?

'…apart from being a ligand itself, anandamide might also strengthen the effects of other VR1 activators.'

Recent insights into the regulation of VR1 activity

Despite the initial understandable reluctance to assign physiological relevance to the capsaicin-like effects of anandamide, recent reports suggest that the possibility that this endogenous compound might act as an agonist for VR1 should be reconsidered. For example, ATP can strongly enhance the effect of capsaicin on rat VR1-gated currents by acting as an allosteric factor[8]. Although the effect of this nucleotide on anandamide was not tested, it is likely that ATP also enhances the activity of other VR1 ligands, particularly those compounds that bind to the receptor on the same site as capsaicin. Indeed, anandamide has been shown, using displacement and binding studies in cells that overexpress rat VR1 (Ref. 5), to bind to the same site of VR1 as capsaicin. Substances that increase the intracellular Ca^{2+} concentration might also lead to sensitization of VR1 to anandamide stimulation because they were found to enhance thermal nociception[9], in which VR1 is involved (Box 2), and they stimulate the formation of anandamide (Box 1). An upregulatory effect that might be important was highlighted recently by Premkumar and Ahern[10], who, starting from previous fundamental observations[11], showed that protein kinase C (PKC)-mediated phosphorylation of VR1 leads to sensitization of proton- and ligand-induced simulation of VR1. In this case, a strong (10–15-fold) enhancement of anandamide action on VR1-gated currents was also observed. Indeed, stimulation of PKC alone, in the absence of ligand, elevated temperature or decreased pH, was able to cause VR1 activation. This latter important finding explains, for

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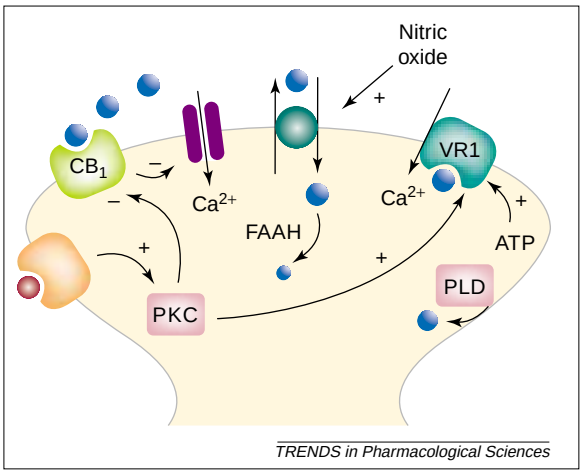

Fig. 1. Hypothetical function of anandamide as either an endocannabinoid or an 'endovanilloid'. Extracellular anandamide (blue circles) activates cannabinoid CB_1 receptors first and is then transported into cells by a selective carrier. Intracellular anandamide, either transported from outside or produced from intracellular membrane precursors by the action of phospholipase D (PLD), might be present in amounts sufficient to stimulate phosphorylated vanilloid type 1 receptors (VR1). Furthermore, anandamide produced inside cells might activate VR1 first and then, after release from cells, CB_1 receptors. The anandamide membrane transporter, which is activated by nitric oxide, might function as a 'gate' between the effect of anandamide on either CB_1 receptors or VR1 (Ref. 17). Hydrolysis by fatty acid amide hydrolase (FAAH) limits anandamide actions, particularly those exerted at intracellular targets. Protein kinase C (PKC), activated by bradykinin or other agonists (red circle), sensitizes VR1 to the effect of anandamide and protons[10], while inactivating CB_1 receptors. ATP might enhance the effect of VR1 ligands, such as anandamide, that are capable of binding to the capsaicin site[5]. Anandamide, via PKC, might also sensitize VR1 to the effect of protons[10]. CB_1 receptors and VR1 often have opposing effects on intracellular signals. For example, activation of CB_1 receptors leads to inhibition of voltage-activated Ca^{2+} channels, whereas stimulation of VR1 leads to Ca^{2+} influx into the neuron. However, whether the VR1-mediated effect of anandamide opposes the CB_1-receptor-mediated effect depends on whether anandamide rapidly desensitizes VR1 (Refs 4,6), as do some other VR1 agonists.

example, why the algesic peptide bradykinin, which stimulates the ε isoform of the kinase via bradykinin B_2 receptors, induced VR1-mediated currents in the presence of PKC-ε and in a manner sensitive to a PKC inhibitor[10]. Premkumar and Ahern proposed that production of relatively weak VR1 ligands, such as anandamide and lipoxygenase products[12], and concomitant VR1 phosphorylation might result in a synergic response, thus providing a possible resolution of the controversy regarding the effect of anandamide on VR1. Furthermore, the authors showed that anandamide, via activation of PKC, enhanced the response of VR1 to protons and anandamide itself. This latter finding suggests that, apart from being a ligand itself, anandamide might also strengthen the effects of other VR1 activators.

The findings of Premkumar and Ahern are all the more exciting if one considers the possibility that other Ser/Thr protein kinases might also enhance ligand (and anandamide)-activated stimulation of VR1. In fact, Premkumar and Ahern showed that the introduction of ATPγS into the assay solution, with subsequent irreversible protein phosphorylation, leads to the activation of and/or sensitization of VR1 (Ref. 10). Indeed, stimulation of the cAMP-dependent kinase also lowers by approximately fivefold the concentration of anandamide necessary for the activation of VR1 and native vanilloid receptors[13]. However, it remains to be established whether PKC-ε and other protein kinases catalyze the phosphorylation of VR1 directly and, if so, on which residue(s).

The role of the anandamide membrane transporter

Advances have also been made in the identification of the VR1 domains that interact directly with protons and ligands[14–16]. These new data strongly suggest, for example, that the capsaicin binding site is on the cytosolic side of the receptor, which opens intriguing possibilities for the capsaicin-like actions of anandamide. In fact, the release of anandamide is not vesicle-mediated but, instead, occurs via facilitated diffusion through the cell membrane only after its synthesis[2]. An as yet uncharacterized membrane transporter, selective for anandamide, has been identified (Box 1). This carrier protein works in both directions and, hence, also mediates the rapid uptake of extracellular anandamide for its subsequent inactivation by intracellular enzymes. Thus, in principle, anandamide synthesized *de novo* might act at intracellular targets, such as VR1, before being released from cells. Conversely, if extracellular anandamide is to activate VR1, it might do so more rapidly and efficaciously if it is transferred into cells by the membrane transporter. In either case, anandamide action on VR1 is likely to be limited by intracellular metabolism to a greater extent than its action at CB_1 receptors, this phenomenon probably being responsible in part for the weak activity of exogenous anandamide as a VR1 agonist (Fig. 1). These considerations are supported by recent experiments showing that selective inhibitors of the anandamide transporter block with the same potency both the uptake of anandamide by VR1-expressing cells and its action at VR1 in the same cells[17]. Furthermore, inhibition of the enzymatic hydrolysis of anandamide has also been shown to enhance the potency of this compound as a VR1 agonist by at least fivefold[5,17]. More importantly from a physiological point of view, it was found that, in agreement with the previous observation that nitric oxide (NO) enhances the activity of the anandamide transporter (Box 1), an NO donor could augment anandamide uptake by VR1-expressing cells and enhance the activity of anandamide at VR1 to the same extent[17]. This latter finding raises the intriguing possibility that substances that stimulate NO release, by enhancing anandamide cellular uptake, might divert anandamide action from cannabinoid receptors to VR1.

If these data are confirmed by experiments carried out in native cells, one could propose a functional coupling between the anandamide membrane transporter and vanilloid receptors (Fig. 1). The overlap between the ligand recognition properties of these two proteins, which might explain

why some synthetic substrates for the transporter are 10–100-fold more potent than capsaicin as VR1 agonists, also supports this concept[18,19]. Furthermore, a double function for anandamide as either an endocannabinoid or an 'endovanilloid', depending on the activity of the transporter, the site of production of the mediator, and the phosphorylation state of VR1 and CB_1 receptors (which, unlike VR1, are downregulated by PKC-mediated phosphorylation[20]), represents an intriguing possibility (Fig. 1). This might occur not only in neurons of the dorsal root ganglia, where VR1 and CB_1 receptors are colocalized[21], but also in some CNS regions (Box 2).

A lasting controversy?

The discoveries discussed above are not likely to quench the heated debate (elegantly summarized by Szolcsanyi[22]) on whether or not anandamide is a physiological VR1 ligand. It now appears likely that upregulation of VR1, stimulation of the anandamide transporter and inhibition of anandamide metabolism can lower the concentration threshold for VR1 activation by anandamide. However, it remains to be explained why opposite peripheral actions, for example on pain perception, are observed following administration of either anandamide or capsaicin. One possible explanation might be that anandamide, in addition to its sensitizing effect[10], also desensitizes VR1, as some preliminary results seem to indicate (Refs 4,6, and L. De Petrocellis and V. Di Marzo, unpublished). Indeed, several synthetic and natural vanilloids immediately desensitize native vanilloid receptors to the point that no pungency can be perceived with these compounds, whose use as analgesics or anti-inflammatory agents has been proposed (Box 2). It should be noted that VR1 is not restricted to peripheral sensory neurons and is also found in brain nuclei through which capsaicin produces effects that are, in some cases, similar to those induced by anandamide (Box 2). Clearly, several questions remain to be answered before a definitive conclusion can be drawn as to whether anandamide, particularly in the CNS (Ref. 19), is, together with other eicosanoids[12], an 'endovanilloid'. Thus, future studies might reveal not only an 'inhibitory' role for this endogenous lipid in neuronal transmission through CB_1 receptors, as the Sanskrit word 'ananda' for 'internal bliss' would suggest, but also an 'excitatory' function through the activation of VR1, or the more recently described direct inhibition of background K^+ (TASK-1) channels[23].

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