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Review

Anandamide as an intracellular messenger regulating ion channel activity

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Abstract

The endocannabinoid anandamide (*N*-arachidonoylethanolamine) was proposed to be an extracellular retrograde messenger, which regulates excitability of neurons by cannabinoid CB_1 receptor-dependent inhibition of neurotransmitter release. Recent findings indicate that the neuromodulatory actions of anandamide might be more complex. Anandamide has been shown to directly modulate various ion channels, such as α7-nicotinic acetylcholine receptors, T-type Ca^{2+} channels, voltage-gated and background K^+-channels and Transient Receptor Potential Vanilloid type 1 (TRPV1) channels. The binding site of anandamide at some of these ion channels appears to be intracellular or at the bilayer interface. This rises the intriguing possibility that anandamide, prior to its release into the synaptic cleft, may regulate ion homeostasis and excitability of neurons as an intracellular modulator of ion channels independent of its action at cannabinoid CB_1 receptors. This possibility might extend the concept of anandamide as an endocannabinoid retrograde messenger and may have profound implications for its role in neurotransmission and neuronal function. Here, we will review the evidence for this hypothesis.

Keywords: Anandamide; Cannabinoid; Endocannabinoid; Vanilloid; Signalling

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Contents

1. Introduction

The endogenous lipid *N*-arachidonoylethanolamine was discovered independently by two groups in the early nineties. Mechoulam and co-workers isolated and characterized the lipid from pig brain in 1992 [1]. They demonstrated that it could bind to and activate the metabotropic cannabinoid CB_1 receptor, thereby the first endogenous ligand for the cannabinoid CB_1 receptor, i.e. 'endocannabinoid', was discovered. They named the lipid anandamide after the Sanskrit *ananda*, which means 'bliss'. Anandamide mimicked to a large extent the actions of Δ^9-tetrahydrocannabinol, the main psychoactive component of marijuana. This discovery, together with the isolation of the second endogenous cannabinoid 2-arachidonoylglycerol, established the foundation of a new signalling system in the brain [2,3]. This system is of importance to the understanding of neuronal communication [4,5].

Endocannabinoids are thought to act as extracellular retrograde messengers, which modulate excitability of neurons [5,6]. They are proposed to be released from the post-synaptic neuron upon depolarisation, diffuse to the presynaptic neuron and activate cannabinoid CB_1-receptors. Presynaptic cannabinoid CB_1 receptors inhibit neurotransmitter release by inducing hyperpolarization and/or closing voltage sensitive Ca^{2+}-channels [7]. The stimulation of cannabinoid CB_1 receptor leads to the inhibition of cAMP formation via G_i-proteins [8]. As a result, ion channels such as A-type and inwardly rectifying K^+ channels are activated, while voltage sensitive N-type and P/Q-type Ca^{2+} channels and D-type K^+ channels are inhibited [4,7–10]. The coupling to A- and D-type K^+ channels is thought to be regulated via cAMP, whereas the voltage sensitive Ca^{2+} and $K_{ir}{}^+$-channels are modulated through the coupling with G_i-proteins [8].

Independently of the work of Mechoulam, *N*-arachidonoylethanolamine was also isolated from rat brain and shown to bind to L-type voltage sensitive Ca^{2+} channels at the 1,4-dihydropyridine site [11]. This finding did not receive much attention and its functional implications are not clear as yet. Nevertheless, it already indicated that *N*-

arachidonoylethanolamine is not selective for the cannabinoid receptors and that its neuromodulatory actions might be more complex than originally thought. In fact, this discovery was the first example in which anandamide was shown to modulate ion channel activity without activation of the cannabinoid CB_1 receptor. To date, the list of ion channels which can be directly regulated by anandamide include various neurotransmitter-gated channels, Ca^{2+} and K^+-channels and TRP-channels. Importantly, the binding site of anandamide at several of these ion channels appears to be intracellular. In this review, we will discuss the hypothesis that anandamide, prior to its release into the synaptic cleft, acts as an intracellular modulator of ion channels independent of its action at cannabinoid CB_1 receptors. This might extend the concept of anandamide as an endocannabinoid retrograde messenger and hence have profound implications for its role in neurotransmission and neuronal function.

2. Biosynthesis of intracellular anandamide

In order to function as an intracellular messenger, anandamide needs to be produced in a stimulus-dependent fashion in the cell. Indeed, it is widely recognized that anandamide is not stored in vesicles like other neurotransmitters such as dopamine, glutamate and acetylcholine, but is produced "on demand" in a Ca^{2+}-dependent manner [12]. This is the result of a biosynthetic mechanism relying on the existence of a phospholipid precursor for anandamide and of a Ca^{2+}-sensitive phosphodiesterase for the conversion of this precursor into anandamide. Its precursor *N*-arachidonoyl phosphatidylethanolamine (NAPE) is found in the plasma membrane as well as in membranes of the mitochondrion and the endoplasmatic reticulum. The enzyme responsible for its hydrolysis to anandamide has been recently purified, cloned and characterized. It is a membrane-associated hydrolase which belongs to the zinc metallohydrolase family and is found in microsomes [13]. It uses various *N*-acylphosphatidylethanolamines as substrates and can be stimulated by Ca^{2+}. Its subcellular distribution is unknown as yet. The expression of NAPE-PLD is found in brain regions with high cannabinoid CB_1 receptors, but also in regions with low or no cannabinoid CB_1-receptor levels. This suggests that *N*-acylethanolamines, including anandamide, serve also other functions in addition to activation of cannabinoid CB_1-receptors. It was found that strong membrane-depolarizing agents and Ca^{2+}-ionophores could stimulate anandamide biosynthesis in intact neurons. Recently, we have shown that mobilisation of intracellular calcium by the PLC/IP_3 pathway or depletion of intracellular Ca^{2+}-stores with the Ca^{2+}-ATPase inhibitor thapsigargin are also sufficient to produce anandamide and its NAPE biosynthetic precursor intracellularly in HEK-293 cells and sensory neurons [14] (M. van der Stelt and Di Marzo, unpublished observations). These findings are in agreement with electrophysiological studies, which have suggested that endocannabinoid formation can also be elicited by stimulation of metabotropic glutamate receptors and muscarinic receptors [15]. They suggest that triggers for anandamide production are not limited to strong depolarising agents, and that activation of the PLC/IP_3-pathway can also stimulate intracellular anandamide biosynthesis de novo.

3. Controlling the life span of intracellular anandamide

If anandamide is acting as an intracellular messenger, its concentration in the cell should be strictly regulated. Theoretically, intracellular anandamide can be inactivated through two concurrent processes (reviewed by [16]). Firstly, anandamide might be extruded from the cell via a putative transporter selective for endocannabinoids. This might be the same protein that is responsible for the uptake of anandamide from the extracellular space into the cell. Anandamide and other endocannabinoids have indeed been proposed to be transported by a carrier-facilitated diffusion process according to their concentration gradient across the plasma membrane [17,18]. This process might be bi-directional, because it is neither dependent on external Na^+ nor affected by metabolic inhibitors. If so, anandamide release should be blocked by selective inhibitors [19–21]. Indeed, we have demonstrated that the anandamide transport inhibitor, VDM11, significantly increased intracellular anandamide levels in rat dorsal root ganglion neurons stimulated with thapsigargin [14]. Noteworthy, anandamide membrane transport inhibitors also block CB_1-mediated Long Term Depression in the striatum [22], in agreement with the hypothesis of endocannabinoids acting as retrograde messengers, which requires them to be released through a specific transport process.

Secondly, inside the cell anandamide can undergo metabolism via two possible pathways: oxygenation and hydrolysis (for reviews [16,23,24]). Lipoxygenase- and cycloxygenase-catalyzed oxygenation of anandamide has been shown to generate a vast array of compounds, such as hydroperoxy-anandamides and prostamides [24]. At the moment, the target and function, if any, of these putative anandamide metabolites are not characterized; therefore, it is unknown whether oxygenation terminates anandamide's actions or is a source of novel bioactive compounds. For example, it has been suggested that lipoxygenase metabolites of anandamide are natural inhibitors of FAAH and that they can interact with TRPV1 channels [25,26].

The best-studied inactivation pathway of anandamide is the hydrolysis of its amide bond, which yields arachidonic acid and ethanolamine. Fatty acid amide hydrolase, the protein responsible for anandamide hydrolysis in vivo, has been cloned and studied in detail [27,28]. It is associated with the intracellular membrane and has high activity in several brain areas, including brain stem, hippocampus, subtantia nigra and striatum [29]. The distribution of FAAH correlates to a large extent with the expression of cannabinoid CB_1-receptor, but in several brain regions with low or no expression of cannabinoid CB_1 receptor, e.g. the brain stem, FAAH is found in high densities. This suggests that FAAH is not only limiting endocannabinergic signalling, but is also used to terminate signalling of anandamide and its congeners at other molecular targets. In addition, FAAH is localized in the soma and dendrites of pyramidal hippocampal neurons and dopaminergic neurons of the substantia nigra, which appears to be at odds with a presynaptic action of anandamide at cannabinoid CB_1 receptors in the projecting GABAergic neurons. Noteworthy, these principal neurons do express the TRPV1 ion channel, therefore FAAH might inactivate TRPV1-mediated signalling of intracellular anandamide in these neurons. In keeping with this concept, we have evidence that FAAH-inhibitors, as well as anandamide transport inhibitors, can enhance TRPV1-mediated signalling in HEK293 cells [30] and sensory neurons, and that over-expression of FAAH abrogates anandamide- and TRPV1-mediated Ca^{2+}-influx (M. van der Stelt and Di Marzo, unpublished observations).

4. Novel intracellular role of anandamide: regulation of ion channel activity

4.1. Neurotransmitter-gated channels

Anandamide has been shown to interact with a variety of neurotransmitter gated ion channels, e.g. NMDA-, serotonin (5-HT_3) and 7α-nicotinic acetylcholine receptors [31–34]. These studies were performed in Xenopus oocytes or HEK293 cells, expressing the cloned receptors, but not cannabinoid CB_1-receptors, which suggests that there is a direct interaction of anandamide with these channels. Furthermore, neither the cannabinoid CB_1-receptor antagonist SR141716A, nor pertusis toxin, blocked the effect of anandamide on these channels [31,35,36]. Anandamide augmented the NMDA-induced current [31], whereas it inhibited the serotonin and acetylcholine induced currents [32,33,35,36]. The binding place of anandamide was suggested to be at the transmembrane or intracellular C-terminal site for the 7α-nicotinic acetylcholine receptor [37]. The effect of anandamide was mimicked by arachidonic acid, but anandamide metabolism inhibitors did not prevent the action of anandamide, whereas R-methanandamide, an anandamide more metabolically stable analog, exhibited a higher potency, suggesting that the intact endocannabinoid was altering nicotinic receptor function [32,37]. At the moment it is unknown whether these interactions are also occurring in vivo and little work has been done to assess the physiological importance of these effects.

4.2. Calcium channels

There are three low-voltage-activated, T-type Ca^{2+} channels, which contribute to pacemaker activities in the central nervous system. All three T-type channels are inhibited by anandamide at submicromolar concentrations on a presumed intracellular site [38]. It was shown that T-type Ca^{2+} currents, which could be blocked by anandamide, are involved in neuritogenesis in neuroblastoma-glioma NG108-15 hybrid cells, a cell line that recapitulates early steps of neuronal differentiation [38,39]. Anandamide also inhibits non-competitively L-type Ca^{2+} channels at low micromolar concentrations by binding to the 1,4-dihydropyridine, 1,5-benzothiazepine and phenylalkylamine binding sites [11].

4.3. Potassium channels

Anandamide has been shown to interact with both voltage-gated and background potassium channels:

(1) Voltage-gated K^+ channels shape the action potential by controlling its repolarization phase and determine the membrane potential and duration of the interspike interval. Anandamide and arachidonic acid have been shown to be equipotent at converting non-inactivating delayed rectifiers voltage-gated K^+ channels into rapidly inactivating A-type K^+ channels [40]. Anandamide induced rapid and complete inactivation of these K^+ channels when they are expressed in Xenopus oocytes. It was not tested whether this effect was due to hydrolysis to arachidonic acid. However, there was no lag-time upon application of anandamide, which suggests that anandamide had a di-

rect interaction and did not need to be hydrolysed into arachidonic acid or to alter the phosphorylation state of the channel. Indeed, anandamide has been shown previously to directly inhibit Shaker-related voltage sensitive K^+-channels at low micromolar concentrations [41]. Although the effect was shared by THC and other polyunsaturated N-acylethanolamines, it was insensitive towards blockade by SR141716A. This suggests that the modulation of the K^+-channel was independent of the activation of cannabinoid CB_1-receptors. At the moment it is unknown whether anandamide influences the action potential of neurons through this mechanism in vivo.

(2) Background K^+ channels are not voltage-gated and play an essential role in the setting of the neuronal resting membrane potential and input resistance. Anandamide is a direct and selective blocker of the background K^+ channel TASK-1 [42]. TASK-1 channels set the resting membrane potential of both cerebellar granule neurons and somatic motoneurons and are sensitive to low pH and anesthetics. Noteworthy, TASK-1 is abundantly expressed in the brain stem, where very low cannabinoid CB_1-receptors are found. Anandamide and methanandamide could block TASK-1 mediated currents independent of G-proteins at submicromolar concentrations in CHO-cells overexpressing this K^+-channel [42]. In cerebellar granule neurons the anandamide-mediated inhibition of TASK-1 standing-outward K^+-current induced depolarisation. Interestingly, TASK-1-like background K^+ currents in motoneurons and cerebellar granule neurons are inhibited by the activation of several Gq-coupled receptors, including muscarinic receptors. In light of the finding that muscarinic receptor stimulation leads to anandamide production (see above), it might be speculated that anandamide is acting as an intracellular messenger for TASK-1, which results in excitation of neurons.

4.4. TRPV1 channels

Cation channels of the transient receptor potential (TRP) family are critically involved in the Ca^{2+} homeostasis of cells. In contrast to many neurotransmitter-gated channels, TRP-channels are often gated by cytoplasmic ligands and integrate multiple chemical and physical stimuli. TRP-channels are candidates to mediate store-operated Ca^{2+} entry, which is a process in which depletion of intracellular Ca^{2+} stores leads to influx of extracellular Ca^{2+} into the cell [43–46]. In 1999, it was reported that anandamide could activate the Vanilloid type 1 TRP channel (TRPV1) [47,48]. TRPV1 is highly expressed in small diameter primary afferent fibers [49]. It is a molecular integrator of noxious stimuli, such as heat and low pH, and can also be activated by the pungent ingredient of hot chilli peppers, capsaicin [50]. In primary sensory neurons, TRPV1 is essential for the development of inflammatory hyperalgesia [51,52]. Furthermore, TRPV1 has also been found in various brain areas, including dopaminergic neurons of the substantia nigra, hippocampal pyramidal neurons, hypothalamic neurons, the locus coeruleus in the brainstem and in various layers of the cortex, where it might be involved in modulation of synaptic plasticity [53,54]. The physiological role of TRPV1 in the central nervous system is unknown, but exogenous anandamide induces, in hippocampal slices, a TRPV1-mediated enhancement of paired-pulse depression, which is a form of short-term synaptic plasticity [55]. Tonically active TRPV1 receptors have been found in the substantia nigra compacta [56], and the paraventricular nucleus of the hypothalamus [57].

Anandamide has a low micromolar affinity for TRPV1 in recombinant cell lines, which is similar to that of capsaicin [58,59]. However, its potency in various assays is usually five to ten fold lower than that of capsaicin. For example, in high recombinant expression systems the EC_{50} value for anandamide-induced Ca^{2+}-influx ranges from 0.4 to 5 μM and anandamide appears to act as a full agonist, while in native systems such as Ca^{2+}-influx and inward current in DRG neurons anandamide is a partial agonist with a potency varying from 6 to 10 μM [60–62]. Due to its low potency and partial agonism in some assays, anandamide's ability to be a physiological relevant activator of TRPV1 was originally controversial [63]. However, it is now well established that the potency and efficacy of (exogenous) anandamide at TRPV1 are influenced by a multitude of different factors, ranging from assay conditions and species differences to TRPV1 modification and the ability of anandamide to reach the intracellular binding site on TRPV1. Thus: (i) due to its low intrinsic efficacy, anandamide is a partial agonist in tissues with a low ion channel reserve, but it appears to be a full agonist in tissues with a high ion channel reserve such as the mesenteric artery [64,65]. In several pathological conditions, TRPV1 activity/expression is up-regulated, and this results in a significantly higher efficacy of anandamide [66]; (ii) it has been shown that ethanol, which is frequently used as a vehicle to dissolve anandamide, can potentiate TRPV1 to anandamide via an unknown mechanism [67], whereas bovine serum albumin and plastic may prevent anandamide from reaching the intracellular binding site [59]; (iii) apart from high temperature (>43 °C); and (iv) low (<7.2) pH, which may activate and/or sensitize TRPV1, multiple signalling pathways have also been shown to interact with TRPV1 to modify its gating properties and response to anandamide [49]. The channel might be sensitised by: (v) removal of its inhibition by phosphatidylinositolbisphosphate via PLC-mediated hydrolysis [68], (vi) protein kinase C-catalysed phosphorylation following PLC-mediated diacylglycerol release [69,70], (vii) protein kinase A-mediated phosphorylation [71], (viii) phosphorylation induced by calmodulin-dependent protein kinase II [72]; (ix) voltage-dependent priming [73]. By converse, TRPV1 can be: (x) rapidly desensitised subsequent to activating stimuli by a calmodulin-dependent step [74]; (xi) the level of expression and the activity of the putative anandamide transporter, which may vary in different types of cells, is of crucial importance for exogenous anandamide to reach its cytosolic binding's site at TRPV1 and vice versa to clear anandamide from the intracellular compartment [30,65]. Indeed, it has been demonstrated that when anandamide (100 nM) is injected into the cell, it is very efficacious at inducing TRPV1-mediated plasma membrane currents in DRG neurons [75]; (xii) the rapid metabolism of anandamide inside cell may also limit its activation of TRPV1 [30,76] or potentiate its effect in case of lipoxygenase-mediated conversion [25,77] (see above). (xiii) *N*-Acyl-ethanolamine anandamide congeners, which are co-biosynthesized with anandamide from the phospholipase D-dependent hydrolysis of the corresponding *N*-acyl-phosphatidylethanolamines [13], also significantly enhance ananadamide effects at TRPV1 [78]. One of these compounds, *N*-oleoylethanolamine, was even found to be able to activate TRPV1 per se under certain conditions [79]. (xiv) Anandamide functional activity at TRPV1 can be significantly masked by its concomitant activity on cannabinoid CB_1 receptors, particularly in those brain regions and neurons where the two receptors are co-expressed and are coupled to opposing biological effects [80]. In this case, a significant enhancement of the potency of anandamide at TRPV1 can be observed in the presence of CB_1 antagonists [80].

Despite this wealth of evidence that exogenous anandamide can activate TRPV1, it has never been shown that endogenous anandamide can also signal through TRPV1. In view of the possible role of TRP channels in store-operated Ca^{2+} entry, we hypothesize that anandamide may function as a store-operated messenger signalling to TRPV1 to gate extracellular Ca^{2+}. This hypothesis is based on our findings that intracellular Ca^{2+} mobilisation by thapsigargin, or by receptors coupled to the PLC/IP_3 pathway leads to: (a) the formation of intracellular anandamide in HEK293 cells and primary sensory neurons, and (b) anandamide-induced TRPV1-dependent influx of extracellular Ca^{2+} in these cells (M. van der Stelt and Di Marzo, unpublished observations). Thus, anandamide can act as an intracellular messenger capable of modulating TRPV1 channel activity.

5. Anandamide cross-talks with well-established intracellular signals

If anandamide behaves as an intracellular signal, it should cross-talk also with major intracellular signalling pathways other than IP_3 and Ca^{2+}, e.g. the cAMP-dependent and diacylglycerol-dependent signalling cascades, which are initiated with the activation of protein kinase A (PKA) and C (PKC), respectively. Indeed, this compound was described to exert a dual action (both inhibitory and excitatory, depending on the co-presence of phosphatidylserine or diacylglycerols) on rat brain PKC preparations [80], and is known to inhibit forskolin-stimulated adenylyl cyclase via CB_1 receptors [8]. Furthermore, anandamide stimulates the PLD enzyme involved in generating phosphatidic acid working as an intracellular signal [81]. In turn, anandamide levels can be significantly enhanced in both HEK-293 cells and sensory neurons following stimulation with forskolin or with phorbol miristoyl acetate (PMA), a typical PKC activator, this latter effect being significantly counteracted by a selective PKC inhibitor (V. Vellani and V. Di Marzo, unpublished observations). Whether these effects are due to stimulation of anandamide biosynthesis or inhibition of its inactivation has not been investigated. However, it is worthwhile noting that the enzyme responsible for NAPE biosynthesis was found to be activated by PKA [82]. At any rate, these data strengthen the hypothesis that anandamide, by being capable of cross-talking with several second messenger pathways, is itself an intracellular signal.

6. Conclusions and perspectives

From the data discussed in this article, there seem to be several pieces of evidence clearly pointing to an intracellular, non-cannabinoid receptor-mediated, function for anandamide, in addition to its commonly accepted role as an endocannabinoid. Both the biosynthetic and catabolic enzymes for this compound are entirely intracellular, and the pathway for its formation “on demand”, by being strictly dependent on the presence of membrane phospholipid precursors, closely resembles those of other intracellular signals. This is in agreement with the finding, reviewed here, of non-cannabinoid receptor targets for anandamide, many of which appear to interact with this compound at the level of their intracellular domains. The hypothesis that anandamide indeed functions as an intracellular messenger will have to be challenged by future experiments, which will have to aim particularly at finding

physiological and pathological conditions where this phenomenon occurs. At any rate, the intracellular and extracellular actions of this intriguing mediator should not be seen as mutually exclusive, but rather as sequential to each other, and represent yet another example of the functional plasticity of lipid mediators.

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