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ACCELERATED COMMUNICATION

Anandamide, an Endogenous Cannabinoid, Inhibits Calcium Currents as a Partial Agonist in N18 Neuroblastoma Cells

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SUMMARY

Anandamide (arachidonyl ethanolamide) has been identified as an endogenous ligand of cannabinoid receptors on the basis of its ability to displace ^{3}H-labeled synthetic cannabinoid in a binding assay. One well characterized cellular action of cannabinoids is inhibition of hormonally stimulated adenylyl cyclase. Another action of synthetic cannabinoids is potent, stereospecific, and reversible inhibition of N-type calcium currents (I_{Ca}) in the NG108–15 neuroblastoma-glioma cell line via a pertussis toxin (PTX)-sensitive pathway, independently of cAMP metabolism. Here we used the N18 neuroblastoma cell line and the whole-cell voltage-clamp technique to show that anandamide also potently inhibits N-type I_{Ca} in a PTX-sensitive fashion. As with the cannabinomimetic aminoalkylindole WIN 55,212–2, inhibition by anandamide was voltage dependent and *N*-ethylmaleimide sensitive. However, anandamide was less efficacious than either WIN 55,212–2 or the nonclassical cannabinoid CP 55,940. Indeed, anandamide appears to act as a partial agonist at the cannabinoid receptor. Application of WIN 55,212–2 always caused further inhibition of I_{Ca} in cells exposed to a maximally effective concentration of anandamide, and application of anandamide always caused a partial recovery of I_{Ca} in cells exposed to a maximally effective concentration of WIN 55,212–2. This partial agonist property of anandamide suggests that, although anandamide inhibits N-type I_{Ca} via a PTX-sensitive G protein, its actions as a neuromodulator in the intact animal may be more complex than would be inferred by extrapolating the results of *in vivo* studies with (−)-Δ^9-tetrahydrocannabinol or synthetic cannabinoids.

The identification of anandamide [5,8,11,14-eicosatetraenamide (*N*-2-hydroxyethyl)] (1) as an endogenous ligand for cannabinoid receptors raises physiological questions. In particular, are the actions ascribed to cannabinoids based on experiments with THC, the nonclassical cannabinoids (exemplified by CP 55,940), or the aminoalkylindoles (such as WIN 55,212–2) valid for the endogenous compound and what is the role of anandamide in normal central nervous system physiology? The one cannabinoid receptor characterized thus far by cloning belongs to the superfamily of G protein-coupled receptors (2). The known intracellular consequences of cannabinoid receptor activation are inhibition of adenylyl cyclase (3) and of voltage-gated calcium channels (4, 5). In several tissues the cannabinoid receptor appears to inhibit hormone-stimulated adenylyl cyclase via the inhibitory G protein G_i. The physiological implications of this inhibition are not known. The most thoroughly studied interaction between cannabinoid receptors and ion channels is the inhibition of voltage-activated I_{Ca} in the NG108–15 cell line. This effect is stereospecific, is mediated via a PTX-sensitive protein (most likely the G proteins G_o or G_i), and is independent of cAMP metabolism, and the majority of the inhibited current appears to be carried by N-type calcium channels (4, 5). Inhibition of N-type I_{Ca} decreases neurotransmitter release in a number of tissues (6, 7). It is likely that cannabinoid-mediated inhibition of calcium channels is responsible for the characteristic inhibition by cannabinoids of electrically stimulated acetylcholine release in ileum (8) and vas deferens (9, 10). A conclusion from these electrophysiological studies is that cannabinoids may exert some of their psychoactive effects by inhibition of neurotransmitter release from cannabinoid receptor-containing neurons in the central nervous system.

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ABBREVIATIONS: THC, Δ^9-tetrahydrocannabinol; CP 55,940, (*cis*)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-(*trans*)-4-(3-hydroxypropyl)cyclohexanol; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; I_{Ca}, calcium current(s); NEM, *N*-ethylmaleimide; PLA_2, phospholipase A_2; PTX, pertussis toxin; SCG, superior cervical ganglion; WIN 55,212–2, (*R*)-(+)-(2,3-dihydro-5-methyl-3-[(4-morphonolinyl)methyl]pyrol[1,2,3-de]-1,4-benzoxazin-6-yl)(1-napthalenyl)methanone monomethanesulfonate; ω-CgTX, ω-conotoxin GVIA; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

Here we used the technique of whole-cell voltage-clamp and the N18 neuroblastoma cell line to examine the interaction between anandamide and voltage-gated calcium channels. The N18 cells had less phenotypic variability than did the NG108–15 cell line used in earlier studies. In addition to characterizing the signaling pathway and type of calcium channels involved, we have also compared the actions of anandamide with those of WIN 55,212–2. We chose WIN 55,212–2 because most of our previous studies were with this drug and because it has favorable physicochemical properties.

Experimental Procedures

Materials. DMEM was obtained from Biowhittaker and GIBCO, FBS from HyClone, bovine serum albumin (fatty-acid free), dimethylsulfoxide, and NEM from Sigma, PTX from List, ω-CgTX from Peninsula Labs, and tetrodotoxin from Calbiochem. WIN 55,212–2 was a gift from Sterling Research Group. CP 55,940 was a gift from Pfizer Central Research. Anandamide was synthesized as described previously (1). Purity was monitored using thin layer chromatography with an elution system of petroleum ether/ether/methanol (in a ratio of 6:40:4). Anandamide migrated as a single spot with an R_F of 0.5, as expected.[1]

Cell culture and preparation. N18 cells (passages 32–41) were grown on glass coverslip fragments in DMEM plus 5% FBS, using standard cell culture techniques. Six to 14 days before recording, cells were "differentiated" by changing the medium to DMEM plus 0.5% FBS plus 2% dimethylsulfoxide (11). In PTX experiments, differentiated cells were grown for an additional 16–20 hr in medium containing 500 ng/ml PTX. Control cells were treated identically, except that PTX that had been heated to 95° was substituted for native PTX. SCG neurons (12) were provided by M. S. Shapiro (University of Washington, Seattle, WA).

Current recording. Currents were recorded using the whole-cell voltage-clamp technique (13). Pipettes were pulled from hematocrit glass (VWR) and fire polished. The pipette solution contained (in mM) 100 CsCl, 10 EGTA, 5 $MgCl_2$, 40 HEPES, 3 Na_2ATP, and 0.2 GTP, pH 7.30 with CsOH. For recording, a coverslip containing cells was transferred to the recording chamber (200 μl) and constantly perfused at a rate of 1–2 ml/min with an external solution containing (in mM) 160 NaCl, 5 $CaCl_2$, 4 KCl, 1 $MgCl_2$, 10 HEPES, and 8 glucose, pH 7.35 with NaOH. Tetrodotoxin (200 nM) was added to block voltage-gated sodium currents, and bovine serum albumin (3 μM) was present in all recording solutions to decrease adsorption of cannabinoids. I_{Ca} was measured near the end of a 25-msec depolarizing pulse to 0 mV and was defined as that component of the current sensitive to 100 μM $CdCl_2$. Solution reservoirs were selected by means of a series of solenoid valves, and solution changes were accomplished in <1 min. In all experiments the cells were held under voltage clamp at a holding potential of −65 mV. Voltage protocols were generated and data were digitized, recorded, and analyzed using BASIC-FASTLAB (Indec Systems, Capitola, CA). Currents were sampled at 4 kHz and junction potentials are uncorrected. To control for potential response variations with passage number and duration of differentiation, experimental and control measurements were alternated whenever possible. Where appropriate, data are expressed as mean ± standard error.

Results

Anandamide inhibits I_{Ca}. A step depolarization to 0 mV elicited an inward I_{Ca} of approximately 50–300 pA in most differentiated N18 cells. Bath application of anandamide inhibited this current in a concentration-dependent fashion (Fig. 1, A and B). Inhibition was detectable with 1 nM anandamide, half-maximal with 20 nM anandamide, and maximal with 100

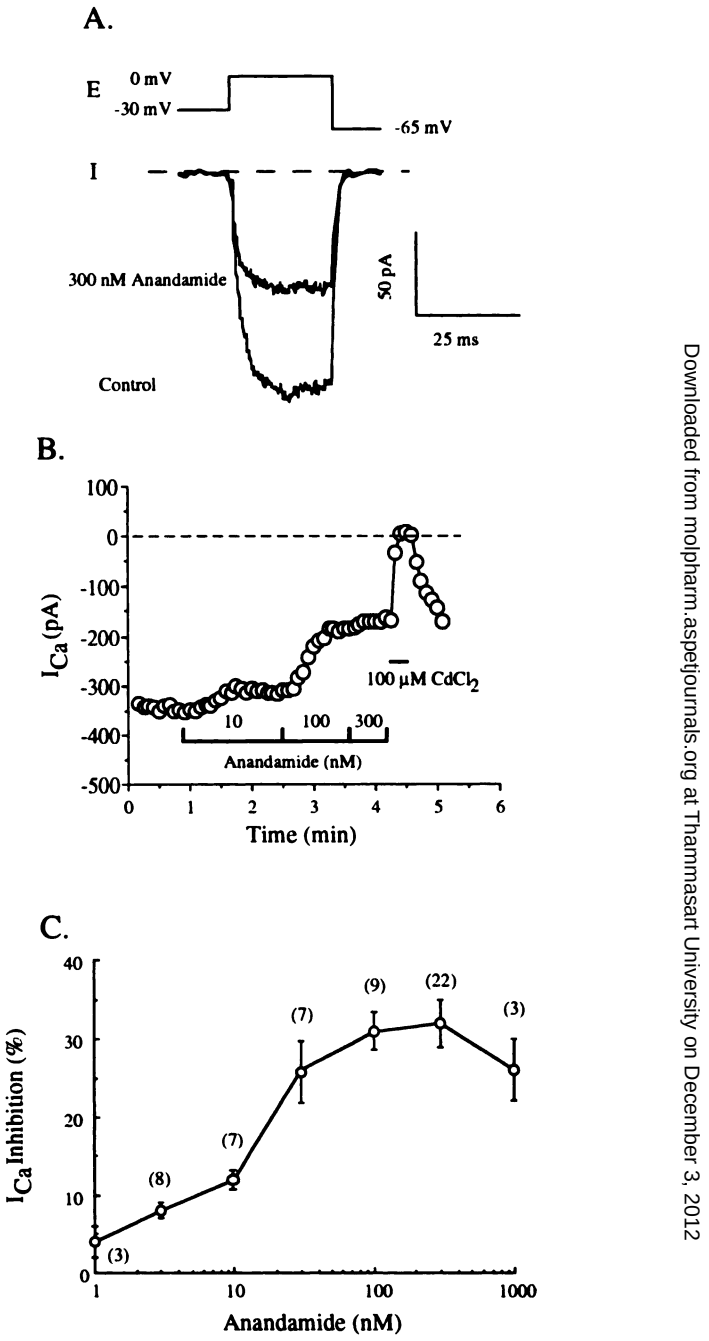

Fig. 1. Inhibition of I_{Ca} by anandamide. A, Bath application of 300 nM anandamide inhibits I_{Ca} evoked during a depolarizing step to 0 mV. *Dashed line*, zero current. The record has been Cd^{2+} subtracted. In this experiment only, a 250-msec prepulse to −30 mV was applied before the test pulse. B, Effect of increasing concentrations of anandamide on I_{Ca}. I_{Ca} was measured near the end of a 25-msec depolarizing step to 0 mV applied every 5 sec from a holding potential of −65 mV. C, Concentration-response curve for I_{Ca} inhibition. *Numbers in parentheses*, number of cells tested at that concentration.

[1] R. Mechoulam, personal communication.

nM anandamide, where 30 ± 4% (n = 9) of I_{Ca} at 0 mV was inhibited (Fig. 1C). There was no obvious change in the time course of the current. N18 cells contain both low and high voltage-activated I_{Ca}. Only the high voltage-activated current was inhibited by 300 nM anandamide (n = 4) (data not shown). I_{Ca} inhibition by WIN 55,212-2 is readily reversible in both NG108-15 cells (4) and N18 cells. In contrast, the inhibition by anandamide was reversible in only about 20% of cells examined.

Anandamide inhibits N-type I_{Ca} via a PTX-sensitive G protein. At least two components of high voltage-activated I_{Ca} (L and N) are readily distinguished in N18 cells. They can be separated by their differential sensitivities to dihydropyridines and ω-CgTX. In differentiated N18 cells, 26 ± 4% (n = 15) of I_{Ca} at 0 mV was sensitive to 2 μM nifedipine, whereas 62 ± 7% (n = 9) was sensitive to 1 μM ω-CgTX (data not shown). Prior exposure to 2 μM nifedipine did not affect the subsequent response to 300 nM anandamide (Fig. 2, A and C). On the other hand, pretreatment with 1 μM ω-CgTX abolished anandamide inhibition of I_{Ca} (Fig. 2, B and C). Some control experiments were done with SCG neurons, which are not known to express cannabinoid receptors and which have no response to WIN 55,212-2 (4). Because bath application of 300 nM anandamide did not inhibit the N-type I_{Ca} in these cells (2 ± 2%; n = 4), it is unlikely that anandamide has a direct action on N-type calcium channels.

Inhibition of I_{Ca} by cannabinoids in NG108-15 cells occurs via a PTX-sensitive pathway (4, 5). Similarly, in N18 cells anandamide inhibition of I_{Ca} was removed by overnight incubation with PTX (500 ng/ml), whereas incubation with heat-inactivated PTX was ineffective (Fig. 2D). PTX-sensitive G proteins are typically sensitive to alkylation by NEM (14). Indeed, a 30-sec exposure to 100 μM NEM in the bath decreased inhibition of I_{Ca} by 300 nM anandamide to 6 ± 1% (n = 4) (data not shown).

Inhibition of I_{Ca} is voltage dependent. One common form of modulation of N-type calcium channels is a voltage-dependent inhibition that can be transiently overcome by application of large positive prepulses (15, 16), i.e., "prepulse facilitation." In mammalian cells, receptors coupled to PTX-sensitive G proteins typically produce such voltage-dependent inhibition (17). In some cases, I_{Ca} is subject to prepulse facilitation even in the absence of agonists, an effect thought to be due to a tonic level of agonist-independent G protein activation (18). Indeed, this is the case with N18 cells (Fig. 3A, *control*). Consistent with the interpretation that prepulse facilitation without agonist involves G proteins, treatment of N18 cells with 100 μM NEM increased I_{Ca} and markedly reduced (>80%) prepulse facilitation in the absence of agonist (n = 5) (data not shown). N18 cells also showed prepulse facilitation of I_{Ca} during exposure to cannabinoids. Using the double-pulse paradigm of Fig. 3A, we found that inhibition of I_{Ca} by 100 nM WIN 55,212-2 was decreased after a strong depolarizing pulse to 100 mV for 50 msec, compared with I_{Ca} preceding the facilitating pulse. On average, 62 ± 10% (n = 4) of the anandamide response and 70 ± 4% (n = 6) of the WIN 55,212-2 response was voltage dependent (Fig. 3B).

Anandamide acts as a partial agonist at the cannabinoid receptor. Because inhibition of I_{Ca} by WIN 55,212-2 occurs via the cannabinoid receptor, we wanted to determine whether maximal concentrations of WIN 55,212-2 and anandamide would fully occlude each other's actions. This was determined not to be the case. Anandamide (300 nM) inhibited I_{Ca} by 33 ± 5% (n = 7), whereas WIN 55,212-2 (100 nM) inhibited I_{Ca} by 54 ± 5% (n = 8) (Fig. 4A). Thus, WIN 55,212-2 is more efficacious in inhibiting I_{Ca} than is anandamide. We

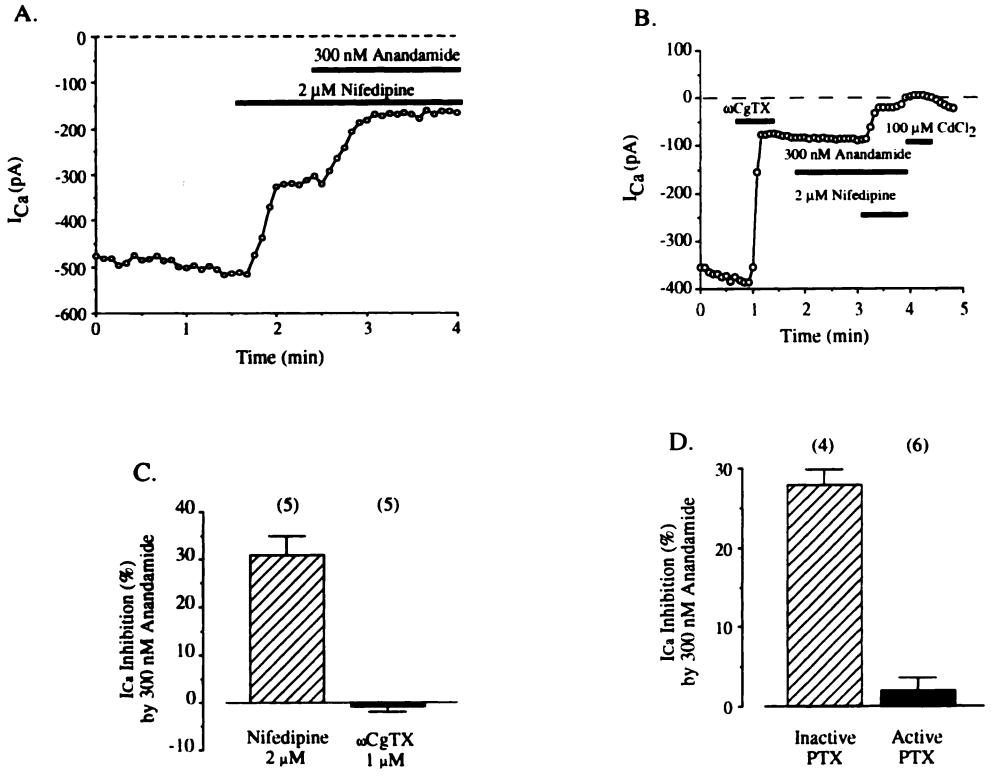

Fig. 2. Characterization of anandamide inhibition of I_{Ca}. A, Anandamide inhibits I_{Ca} in the presence of an L-type calcium channel blocker. Bath application of 2 μM nifedipine reduces I_{Ca} evoked by a depolarizing pulse to 0 mV, and addition of 300 nM anandamide further inhibits the current. B, N-type calcium channel blockers prevent inhibition of I_{Ca} by anandamide. Bath application of 1 μM ω-CgTX inhibits a large fraction of I_{Ca} and 300 nM anandamide does not inhibit the residual current. Most of the remaining current is nifedipine sensitive. C, Summary of nifedipine and ω-CgTX sensitivity of the anandamide inhibition of I_{Ca}. D, PTX pretreatment (500 ng/ml) eliminates anandamide inhibition of I_{Ca}.

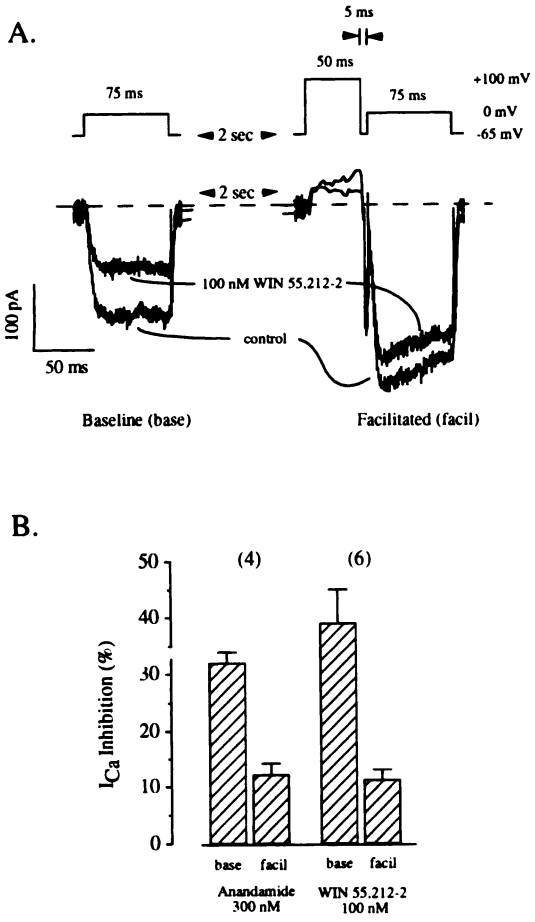

Fig. 3. Voltage sensitivity of the cannabinoid inhibition of I_{Ca}. A, A 100-mV depolarizing prepulse increases I_{Ca} during the subsequent test pulse (*control*) and removes a substantial fraction of the WIN 55,212-2-induced inhibition of I_{Ca} (100 nM WIN 55,212-2). *Dashed line*, zero current. The record has been Cd^{2+} subtracted. B, Summary of the voltage dependence of the inhibition of I_{Ca} by anandamide and WIN 55,212-2. *base*, current before a 100-mV, 50-msec facilitating pulse; *facil*, current after pulse (see text for details).

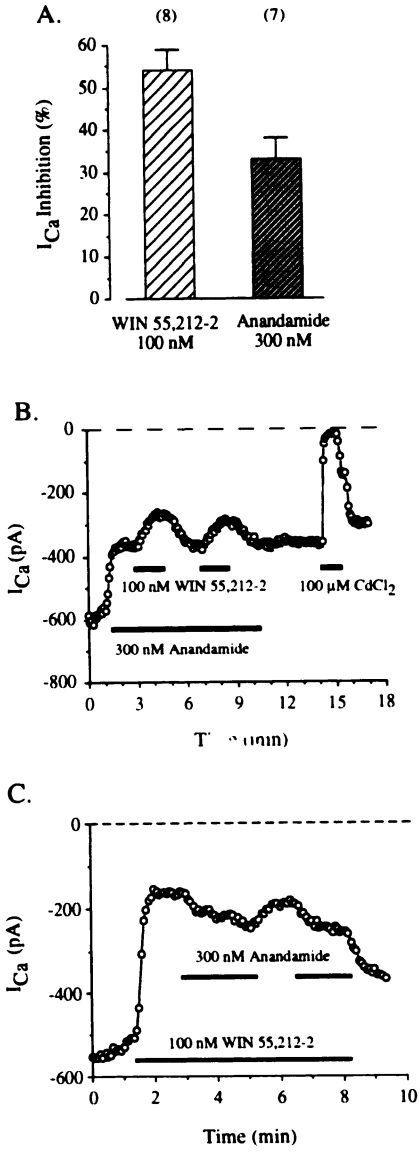

Fig. 4. Anandamide acts as a partial agonist at the cannabinoid receptor. A, Anandamide inhibits a smaller fraction of I_{Ca}, compared with WIN 55,212-2. B, WIN 55,212-2 is more efficacious than anandamide. After inhibition by a maximal concentration of anandamide, WIN 55,212-2 further reduces I_{Ca}. The effect is reversible and repeatable. C, Anandamide acts as a partial agonist. Inhibition by WIN 55,212-2 is decreased by simultaneous application of 300 nM anandamide.

examined this phenomenon further by applying combinations of WIN 55,212-2 and anandamide. Fig. 4B shows that, during maximal inhibition of I_{Ca} by anandamide, applications of 100 nM WIN 55,212-2 inhibited I_{Ca} further. Conversely, during maximal inhibition of I_{Ca} by WIN 55,212-2, application of 300 nM anandamide resulted in partial recovery of I_{Ca} (Fig. 4C). In seven cells, application of 300 nM anandamide resulted in recovery of 37 ± 6% of the 100 nM WIN 55,212-2-inhibited I_{Ca}. With this finding in mind, we re-examined the interaction between WIN 55,212-2 and CP 55,940. Previously, we found that maximally effective concentrations of these two compounds occluded one another in NG108–15 cells (4). A similar occlusion occurred in N18 cells. Application of WIN 55,212-2 after a maximally effective inhibiting concentration of CP 55,940 decreased I_{Ca} by only an additional 3 ± 2% (n = 4), whereas the opposite order of application decreased I_{Ca} by an additional 2 ± 1% (n = 3). We conclude that anandamide is unique among the cannabinomimetic compounds we have examined electrophysiologically thus far, with the property of being a partial agonist at the cannabinoid receptor.

Discussion

The actions of anandamide on I_{Ca} have several similarities to those of other cannabinoid receptor ligands. Anandamide is potent, with half-maximal inhibition of I_{Ca} occurring at 20 nM, close to the K_i (39 nM) determined for anandamide in binding experiments using rat forebrain synaptosomal membranes (1). The inhibition uses a PTX-sensitive G protein and is voltage dependent and NEM sensitive. In neuroblastoma cells, all of the current inhibited by anandamide appears to be carried though N-type calcium channels. About 45% of the N-type I_{Ca} is inhibited.

Inhibition by anandamide was irreversible in the majority of cells studied. Similar irreversibility was found in previous studies that used CP 55,940, THC, and other potent cannabinoid agonists to investigate the inhibition of I_{Ca} as well as the inhibition of electrically stimulated ileum and vas deferens contraction (4, 5, 10). The lack of reversibility may be a consequence of bath application of highly lipophilic compounds

that dissociate slowly from the cannabinoid receptor. Its relevance in normal physiology is uncertain. In the brain small quantities of anandamide are presumably released from localized sources; thus, its actions may be quickly limited by metabolism or dilution. However, final resolution of this issue will require further experimentation.

There is a major difference between the inhibition of I_{Ca} by anandamide and that by synthetic cannabinoids in N18 cells. In these cells anandamide appears to act as a partial agonist at the cannabinoid receptor. Thus, anandamide has a lower intrinsic efficacy than WIN 55,212–2 or CP 55,940. One way of conceptualizing a partial agonist is to propose that the cannabinoid receptor exists in (at least) two conformations in equilibrium with each other. One conformation (CR_A) catalyzes GDP-GTP exchange on the α subunit of the G protein that inhibits I_{Ca}, whereas the other (CR_I) does not. Full agonists, such as WIN 55,212–2 and CP 55,940, have a much higher affinity for CR_A than for CR_I; thus, at full receptor occupancy GDP-GTP exchange is maximally stimulated and I_{Ca} is maximally inhibited. On the other hand, partial agonists, such as anandamide, have more similar affinities for CR_A and CR_I; thus, at full receptor occupancy a smaller fraction of the receptors catalyze GDP-GTP exchange and thus I_{Ca} may be less than maximally inhibited. However, in cells expressing a high density of cannabinoid receptors, anandamide might activate enough G proteins to inhibit I_{Ca} maximally.

If different G proteins couple the cannabinoid receptor to adenylyl cyclase and to calcium channels, and the receptor can exist in conformations that may activate one and not the other G protein, then it is possible that a ligand such as anandamide might be a partial agonist for I_{Ca} inhibition and a full agonist for adenylyl cyclase inhibition. Thus, it will be interesting to determine whether anandamide is a partial agonist for inhibition of adenylyl cyclase in N18 cells. Some of the compounds in the aminoalkylindole series are weak antagonists, binding to the cannabinoid receptor but antagonizing the actions of WIN 55,212–2 in mouse vas deferens and adenylyl cyclase assays (9, 19). Perhaps comparison of these structures with the structure of anandamide will give additional insight into the development of effective cannabinoid receptor antagonists.

It is unusual for an endogenous agonist to be a partial agonist. The effects of anandamide may be milder and more subtle than those caused by THC and the synthetic cannabinoids. Thus, caution should be used when extrapolating from experiments performed with ligands that are full agonists at the cannabinoid receptor to the normal physiological functions of anandamide. It is possible that anandamide actually limits the action of other (yet to be identified) endogenous cannabinoids. An alternative interpretation of the lower intrinsic efficacy of anandamide is that it may be a more stable metabolite of another endogenous cannabinoid that is a full agonist at the cannabinoid receptor.

We previously found that the majority of I_{Ca} inhibited by cannabinoids in NG108–15 and N18 cells was N-type, as defined by ω-CgTX sensitivity (4).[2] As we speculated above, inhibition of N-type calcium channels may explain the inhibition of electrically stimulated vas deferens contraction by anandamide, as well as some of the behavioral effects of this compound (20). In addition to inhibition of N-type calcium channels, Caulfield and Brown (5) found inhibition by THC and CP 55,940 of L- and T-type calcium channels in NG108–15 cells, a finding we have been unable to reproduce. Possible explanations for this discrepancy may lie in different culture conditions (17, 21) or the higher (micromolar) concentrations of cannabinoids those authors used. The action of cannabinoids on P-type calcium channels is unknown. Judging from the functional similarities of N- and P-type channels (22), it would not be surprising if cannabinoids could also inhibit P-type channels.

Another similarity between the actions of anandamide and those of the potent synthetic cannabinoids is the NEM sensitivity of the response. Although NEM is expected to alkylate many proteins in the cells from which we record, it has proven to be a useful and rapid tool for dissecting G protein-mediated responses because it alkylates, and inactivates, G proteins of the G_o and G_i classes (14, 23). Thus, NEM sensitivity provides presumptive evidence for G_i or G_o involvement in anandamide-mediated inhibition of I_{Ca}.

Several intracellular signaling pathways couple to N-type calcium channels in SCG cells (24). In N18 cells, the inhibition of I_{Ca} by both anandamide and WIN 55,212–2 is voltage dependent and PTX sensitive. Thus, we would assign the cannabinoid receptor to the widely studied intracellular signaling pathway designated *fad* in SCG neurons (24). However, compared with other ligands (i.e., norepinephrine) operating through this pathway in NG108–15 cells, the slow onset of I_{Ca} inhibition by cannabinoids (4) and the limited efficacy of even the potent synthetic cannabinoids in inhibiting I_{Ca} are distinctive.

It is possible that anandamide is a neuromodulator at cannabinoid receptor-containing synapses, and it may serve to dampen neurotransmitter release during periods of intense stimulation. Although many details remain to be elucidated, the first step in the primary pathway for the synthesis of anandamide is probably the activation of PLA_2 and the release of arachidonic acid. PLA_2 can be activated either by increases in intracellular calcium (25) as might occur during a period of intense postsynaptic activity, directly by a G protein-coupled receptor (26), or by tyrosine kinase activation (27). Anandamide might function either as a presynaptic "automodulator" (if presynaptic PLA_2 is stimulated) or as a retrograde modulator (if synthesized postsynaptically). In both cases, anandamide and related compounds would inhibit voltage-dependent calcium channels, thus serving to decrease neurotransmitter release and quieting synaptic activity.

Acknowledgments

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