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Receptor-dependent formation of endogenous cannabinoids in cortical neurons

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Abstract

We investigated the transduction mechanisms mediating formation of the endogenous cannabinoid (endocannabinoid) lipids, anandamide (arachidonylethanolamide) and 2-arachidonylglycerol, in primary cultures of rat cortical neurons. Unstimulated neurons contained 0.3 ± 0.1 pmol of anandamide and 16.5 ± 3.3 pmol of 2-arachidonylglycerol per mg of protein, as determined by gas chromatography/mass spectrometry. Ca^{2+} entry into the neurons via activated glutamate *N*-methyl-D-aspartate (NMDA) receptors increased 2-arachidonylglycerol levels approximately three times, but had no effect on anandamide levels. By contrast, anandamide formation was stimulated five times by simultaneous activation of NMDA and acetylcholine receptors. Alone, acetylcholine receptor activation had no effect on anandamide or 2-arachidonylglycerol levels. The formation of fatty acid ethanolamides that do not activate cannabinoid receptors, including palmitylethanolamide and oleylethanolamide, was stimulated by coactivation of NMDA and acetylcholine receptors. Pharmacological experiments suggest that the cholinergic contribution to anandamide formation was mediated by $\alpha 7$ nicotinic receptors (antagonized by methyllycaconitine), whereas the contribution to palmitylethanolamide and oleylethanolamide formation was mediated by muscarinic receptors (antagonized by atropine). These findings indicate that cortical neurons produce anandamide and 2-arachidonylglycerol in a receptor-dependent manner, and that brain neurons may generate different endocannabinoid lipids depending on their complement of neurotransmitter receptors.

Keywords: Anandamide; Fatty acid ethanolamide; 2-Arachidonylglycerol; Glutamate; Acetylcholine

1. Introduction

Cannabinoid receptors, the molecular target for the psychoactive constituent of *Cannabis* Δ^9-tetrahydrocannabinol (Piomelli et al., 2000), are activated by a family of lipid molecules that primarily include anandamide (arachidonylethanolamide) (Devane et al., 1992; Di Marzo et al., 1994) and 2-arachidonylglycerol (Mechoulam et al., 1995; Sugiura et al., 1995; Stella et al., 1997). Both anandamide and 2-arachidonylglycerol are produced by brain neurons in an activity-dependent manner (Di Marzo et al., 1994; Stella et al., 1997; Giuffrida et al., 1999), bind to and activate cannabinoid CB_1 receptors (Felder et al., 1993; Vogel et al., 1993; Mechoulam et al., 1995; Sugiura et al., 1996), and undergo rapid biological inactivation (Di Marzo et al., 1994; Beltramo et al., 1997), suggesting that they may act as endogenous modulatory substances (Piomelli et al., 2000).

Unlike classical neurotransmitters, the endogenous cannabinoids (endocannabinoids) may be produced upon demand by enzymatic cleavage of membrane lipid precursors and immediately extruded from neurons without an intermediate step of vesicle storage (Piomelli et al., 2000; Schmid, 2000). This feature—unusual in a brain chemical transmitter, but reminiscent of other lipid-derived mediators—prompted the two questions addressed in the present study. The first is how formation of anandamide and 2-arachidonylglycerol is initiated: is it stimulated by action potentials invading the synaptic nerve endings or by receptor-activated mechanisms? Previous studies have shown that anandamide and 2-arachidonylglycerol can be produced during neural activity in vitro and in vivo, but they have not investigated the cellular mechanisms of this response (Di Marzo et al., 1994; Stella et al., 1997; Giuffrida et al., 1999). The second question is whether, regardless of the mechanism involved, anandamide and 2-arachidonylglycerol can be generated independently of each other. An affirmative answer to this question would

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imply that these compounds may be released under different circumstances and, possibly, serve distinct functional roles in neuronal signaling. To begin to address these questions, we have investigated the mechanisms of anandamide and 2-arachidonylglycerol formation in primary cultures of rat brain cortical neurons. The results of our experiments show that anandamide and 2-arachidonylglycerol are produced in a receptor-dependent manner, and that segregated molecular mechanisms may underlie the formation of each of these compounds.

2. Materials and methods

2.1. Drugs

All drugs were purchased from Research Biochemical (Natick, MA) or Sigma (Saint Louis, MO).

2.2. Cell cultures

Cortical neurons were prepared as described (Brewer, 1995; Evans et al., 1998), with few modifications. Cerebral cortices were dissected from 18-day-old Wistar rat embryos and the neurons were dissociated mechanically. The neurons (10^6 cells/ml) were then plated on 90-mm Corning® culture dishes (12.5 ml/dish) coated with poly-(L)-ornithine (10 μg/ml; molecular weight: 30,000–70,000) and poly-(DL)-lysine (100 μg/ml; molecular weight: 30,000–70,000). The culture medium was composed of B27-supplemented Neurobasal® containing 500 μM L-glutamine, 24 μM L-glutamic acid, 5 μg/ml streptomycin and 10 U/ml penicillin. The neurons were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. After 3 days, 6 ml of the cell culture media were replaced with 7 ml of B27-supplemented Neurobasal® containing 500 μM L-glutamine, 5 μg/ml streptomycin and 10 U/ml penicillin.

2.3. Cell incubations

In preliminary experiments designed to study the kinetic of endogenous cannabinoid production, we labeled the neurons by overnight incubation with [^{14}C]arachidonic acid and determined the time-course of [^{14}C]2-arachidonylglycerol formation in the presence of glutamate (300 μM) and/or carbachol (1 mM) (Stella et al., 1997). At the end of various incubation periods (0.5 to 15 min), lipids were extracted and analyzed by thin-layer chromatography (Stella et al., 1997). Glutamate significantly increased radioactivity in the 2-arachidonylglycerol fraction at 2.5 min (130 ± 6% of basal, $n = 6$), whereas carbachol had no effect. Coapplication of glutamate and carbachol resulted in a greater [^{14}C]2-arachidonylglycerol accumulation, which reached a maximum of 192 ± 6% of basal at 2.5 min and lasted for at least 15 min (146 ± 16% of basal). Therefore, we selected a 2.5-min incubation time for subsequent gas chromatography/mass spectrometry analyses. After 5–6 days in vitro, the incubation medium was removed and the neurons were washed twice in HEPES-bicarbonate buffer (9 ml, 15 min each) composed of (mM): NaCl, 120; KCl, 5.0; $CaCl_2$, 2.0; $MgSO_4$, 1.0; NaH_2PO_4, 1.0; Glucose, 10; $NaHCO_3$, 5.0; and HEPES, 20 (pH 7.4 at 37 °C). Incubations were started by adding 1 ml of buffer containing drugs at 10 times their final concentration, and by gently shaking the culture dishes for 10 s to allow the drugs to diffuse uniformly. Neurons from two culture dishes were combined for each experimental point (1.17 ± 0.03 mg of protein/dish, $n = 33$).

2.4. Lipid analyses

Incubations were stopped by adding 10 ml of ice-cold methanol. Cells were immediately scraped on ice and the supernatants were transferred into ice-cold chloroform (20 ml) that contained internal standards (0.6 nmol of each [$^2H_4$]acylethanolamide and 0.5 nmol of [$^2H_8$]2-arachidonylglycerol). This rapid procedure significantly reduced the non-enzymatic conversion of 2-arachidonylglycerol into 1(3)-arachidonylglycerol, yielding ≈90% 2-arachidonylglycerol. Organic phases of duplicate samples were pooled, evaporated to dryness under N_2 gas, recovered in chloroform (2 ml), filtered through Ultra-free CL filters (Waters), and evaporated again. Lipids were reconstituted in chloroform (100 μl) for high-performance liquid chromatography (HPLC) fractionation and gas chromatography/mass spectrometry analysis, which were carried out as described (Giuffrida and Piomelli, 1998).

3. Results

3.1. Anandamide formation via coactivation of N-methyl-D-aspartate (NMDA) and acetylcholine receptors

We used isotope dilution gas chromatography/mass spectrometry to investigate anandamide biosynthesis in rat cortical neurons in primary culture (Giuffrida and Piomelli, 1998). Fig. 1A shows the ion current trace obtained after analysis of a lipid fraction purified from these cells. A diagnostic component of mass-to-charge ratio (m/z) 404 (the fragment produced from the molecular ion by loss of one methyl group), eluted from the column at the retention time of authentic standard, confirming that unstimulated neurons contain detectable levels of anandamide (Di Marzo et al., 1994; Cadas et al., 1997). Under basal conditions, we measured on average 0.3 ± 0.1 pmol of anandamide per mg of protein, with values ranging from 0.1 to 1.35 pmol per mg of protein ($n = 17$). This variability appeared to reflect differences amongst culture preparations, rather than within a single preparation, and might result from subtle inequalities in neuronal maturation under the

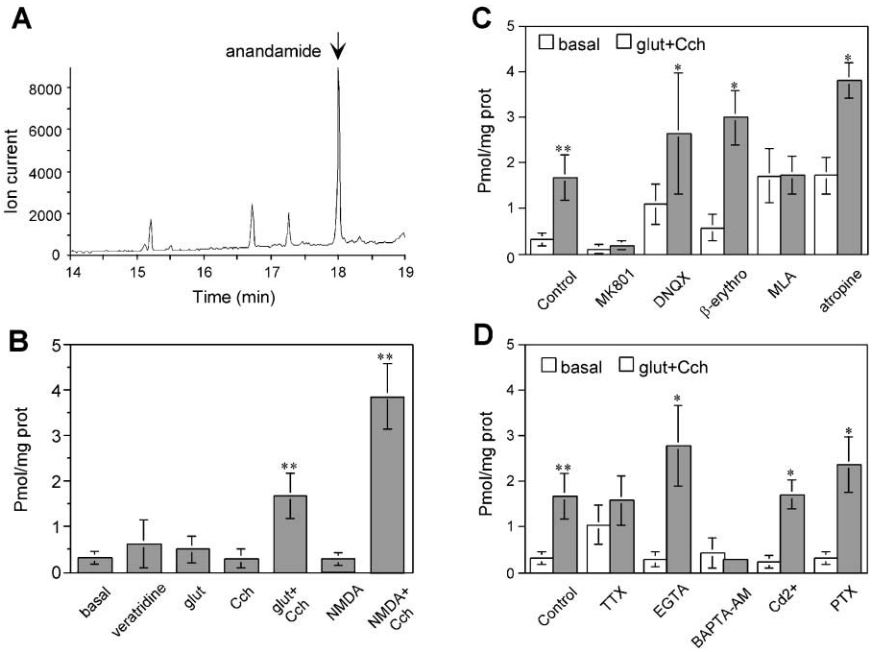

Fig. 1. Coactivation of glutamate NMDA and acetylcholine α7 nicotinic receptors elicits anandamide formation in rat cortical neurons. (A) Identification of anandamide by selected ion monitoring gas chromatography/mass spectrometry. Anandamide was purified from neurons by HPLC and analyzed by gas chromatography/mass spectrometry as the trimethylsilylether (TMS) derivative. The fragment monitored was produced from the anandamide-TMS molecular ion by the loss of methyl group ($[M-15]^+$, m/z 404). The arrow indicates the retention time of standard anandamide. (B) Effects of glutamate and carbachol on anandamide levels. Neurons were incubated for 2.5 min in the presence of glutamate (glut, 300 μM), carbachol (Cch, 1 mM), veratridine (10 μM), and/or NMDA (300 μM; in the presence of 100 μM D-serine (Johnson and Ascher, 1987), and in the absence of extracellular magnesium (Nowak et al., 1984)). $^{*}P < 0.05$ and $^{**}P < 0.01$ significantly different from basal (ANOVA followed by Dunnett's test). (C) Effects of various receptor antagonists on anandamide production. Neurons were pre-incubated for 30 min in buffer (control), or in buffer containing one of the following drugs: MK801 (1 μM), DNQX (10 μM), dihydro-β-erythroidine (β-erythro, 10 μM), methyllycaconitine (MLA, 30 nM) or atropine (1 μM); and then incubated for 2.5 min with buffer (basal) or glutamate (300 μM) plus carbachol (1 mM) (glut + Cch). $^{*}P < 0.05$ and $^{**}P < 0.01$ significantly different from basal (unpaired Student's *t*-test). (D) Effects of tetrodotoxin (TTX), pertussis toxin (PTX) and treatments that affect intracellular Ca^{2+} on anandamide production. Neurons were pre-incubated for 30 min in the presence of buffer (control), tetrodotoxin (TTX, 1 μM), EGTA (1 mM; in the absence of Ca^{2+}), BAPTA-AM (10 μM), cadmium chloride (Cd^{2+}, 100 μM), or treated for 18 h with pertussis toxin (PTX, 1 μg/ml). Neurons were incubated for 2.5 min in buffer (basal) or in buffer containing glutamate (300 μM) plus carbachol (1 mM) (glut + Cch). $^{*}P < 0.05$ and $^{**}P < 0.01$ significantly different from basal (unpaired Student's *t*-test). Results represent the mean ± S.E.Mm. of 3–17 experiments.

serum-free culture conditions of these experiments. To limit the impact of variability on data interpretation, all treatment groups were compared to appropriate controls within the same cell culture preparation.

To determine whether activation of neurotransmitter receptors increased anandamide levels, we incubated the neurons for 2.5 min with glutamate (300 μM) or carbachol, an acetylcholine receptor agonist (1 mM). Although these agents had no effect separately, they stimulated anandamide formation more than 5 times when applied together (Fig. 1B). Three pharmacological experiments suggest that anandamide biosynthesis resulted from the concomitant activation of glutamate NMDA and cholinergic α7 nicotinic receptors. First, the combination NMDA (300 μM)/carbachol was more effective at stimulating anandamide production than glutamate/carbachol (Fig. 1B). Second, the NMDA receptor antagonist dizocilpine (MK801, 1 μM), but not the amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate (AMPA/kainate) receptor antagonist 6,7-dinitroquinoxaline-2,3 dione (DNQX, 10 μM), prevented anandamide formation (Fig. 1C). Finally, the α7 nicotinic receptor antagonist methyllycaconitine (30 nM) (Alkondon et al., 1992) blocked the response, whereas dihydro-β-erythroidine (a nicotinic receptor antagonist that does not affect α7 nicotinic receptors, 1 μM) or atropine (a muscarinic receptor antagonist, 1 μM) had no effect (Fig. 1C). Pretreatment of cells with pertussis toxin (1μg/ml, overnight) was also ineffective, which is consistent with a lack of $G_{i/o}$ protein involvement (Fig. 1D).

Veratridine (10 μM), a Na^+ channel activator, did not increase anandamide levels in neurons (Fig. 1B) although it elevated intracellular Ca^{2+} in a cadmium-sensitive manner, as assessed by Ca^{2+} imaging (N. Stella and D. Piomelli, unpublished results). However, receptor-dependent anandamide production was significantly reduced by the Na^+ channel blocker tetrodotoxin (1 μM) (Fig. 1D). Together, these results suggest that activation of voltage-gated Na^+ channels is necessary, but not sufficient to stimulate anandamide biosynthesis. Buffering intracellular Ca^{2+} with 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (acetoxymethyl) ester (BAPTA-AM, 10 μM) prevented anandamide formation, whereas removal of ex-

ternal Ca^{2+} with EGTA (1 mM) or addition of cadmium chloride (100 μM) to block voltage-activated Ca^{2+} channels had no effect (Fig. 1D). Thus, NMDA and α7 nicotinic receptors may act synergistically to evoke anandamide biosynthesis via a tetrodotoxin-sensitive mechanism that requires mobilization of Ca^{2+} from intracellular stores.

3.2. Production of other fatty acid ethanolamides

In cortical neurons, activity-dependent hydrolysis of saturated and monounsaturated species of *N*-acyl phosphatidylethanolamine generates several fatty acid ethanolamides in addition to anandamide (Di Marzo et al., 1994; Cadas et al., 1997; Hansen et al., 1997; Schmid, 2000). These compounds, of which palmitylethanolamide and oleylethanolamide are two prominent examples, do not activate cannabinoid receptors and have no established signaling function (Piomelli et al., 1998). Incubating the neurons with glutamate plus carbachol, not glutamate or carbachol alone, stimulated the formation of both palmitylethanolamide and oleylethanolamide (Figs. 2A,B and 3A,B). This response was mediated in all likelihood by co-activation of NMDA receptors and muscarinic receptors because, (i) NMDA was as effective as glutamate in eliciting palmitylethanolamide and oleylethanolamide production (Figs. 2B and 3B); (ii) MK801 and atropine blocked the response (Figs. 2C and 3C), whereas (iii) methyllylcaconitine and dihydro-β-erythroidine had no effect (Figs. 2C and 3C). Pertussis toxin had no significant effect on palmitylethanolamide and oleylethanolamide formation (Figs. 2D and 3D). Additional experiments will be necessary to determine the G protein coupling of the putative muscarinic receptor involved in this response. Receptor-dependent biosynthesis of palmitylethanolamide and oleylethanolamide was not affected by tetrodotoxin or by removal of external Ca^{2+} with EGTA, but was blocked by chelating intracellular Ca^{2+} with BAPTA-AM (Figs. 2D and 3D). The results indicate that coactivation of NMDA and muscarinic receptors stimulates palmitylethanolamide and oleylethanolamide formation by mobilizing Ca^{2+} ions from intracellular stores.

3.3. 2-Arachidonylglycerol formation via NMDA receptor-mediated CA^{2+} entry

To quantify 2-arachidonylglycerol in cortical neurons, we developed an isotope dilution gas chromatography/mass spectrometry assay similar to that used for anandamide. The electron-impact mass spectra of synthetic unlabeled and [$^2H_8$]-labeled 2-arachidonylglycerol (analyzed as *bis* trimethylsilylether derivatives) included the molecular ions ($[M]^+$, m/z 530), and ions produced by loss of one methyl group ($[M\text{-}15]^+$, m/z 515) or one derivatizing trimethylsilenol group ($[M\text{-}90]^+$, m/z 440). We monitored the $[M\text{-}90]^+$ fragments, because of their reasonable abundance and high diagnostic value: rectilinear mass spectrometry responses ($r^2 > 0.99$) were obtained with these fragments when amounts of synthetic

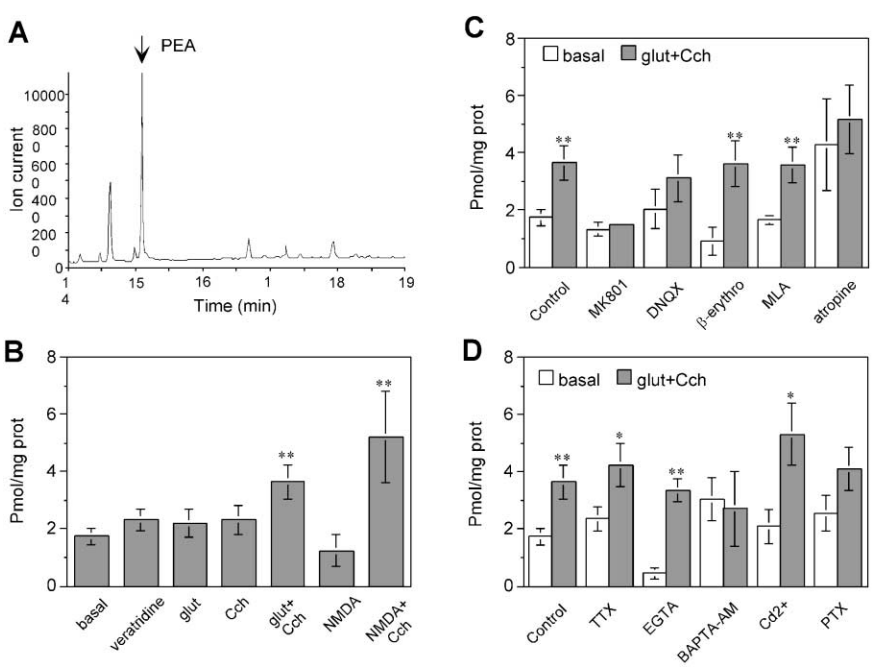

Fig. 2. Formation of palmitylethanolamide in cortical neurons. Experiments were carried out as described in Fig. 1 legend. (A) Identification of palmitylethanolamide by selected ion monitoring gas chromatography/mass spectrometry (m/z 356, $[M\text{-}15]^+$). The arrow indicates the retention time of standard palmitylethanolamide (PEA). (B) Effects of glutamate and carbachol on palmitylethanolamide levels. (C) Effects of various receptor antagonists on palmitylethanolamide production. (D) Effects of tetrodotoxin (TTX), pertussis toxin (PTX) and treatments that affect intracellular Ca^{2+} on palmitylethanolamide.

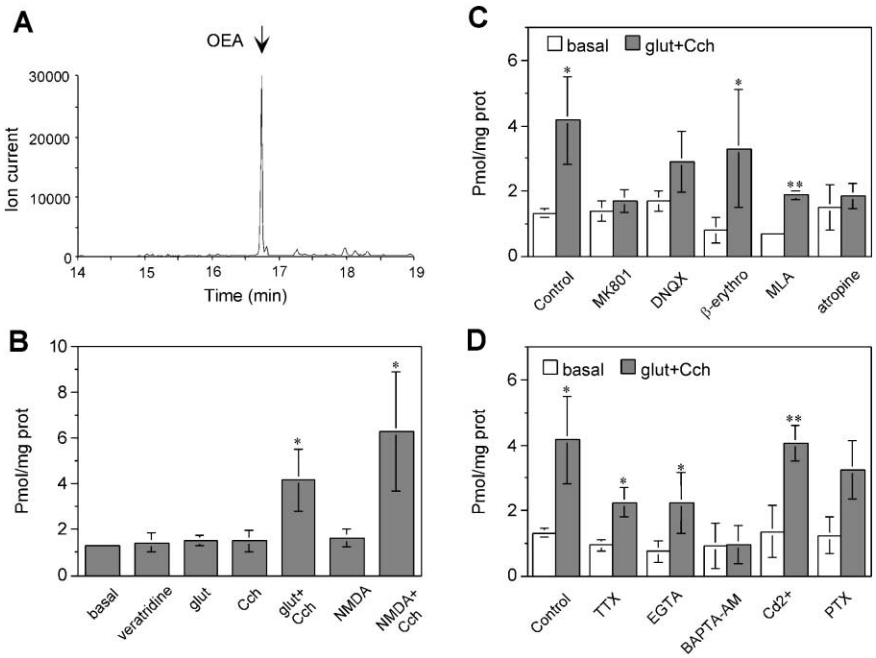

Fig. 3. Formation of oleylethanolamide in cortical neurons. Experiments were carried out as described in Fig. 1 legend. (A) Identification of oleylethanolamide by selected-ion monitoring gas chromatography/mass spectrometry (m/z 382, $[M\text{-}15]^+$). The arrow indicates the retention time of standard oleylethanolamide (OEA). (B) Effects of glutamate and carbachol on oleylethanolamide levels. (C) Effects of various receptor antagonists on oleylethanolamide production. (D) Effects of tetrodotoxin (TTX), pertussis toxin (PTX) and treatments that affect intracellular Ca^{2+} on oleylethanolamide production.

2-arachidonylglycerol ranging from 0 to 1 nmol were injected into gas chromatography/mass spectrometry together with a fixed amount of $[^2H_8]$2-arachidonylglycerol (1 nmol).

Cortical neurons contained, on average, 16.5 ± 3.3 pmol of 2-arachidonylglycerol per mg of protein ($n = 17$). In these analyses, only a minor peak was detectable at the retention time of 1(3)-arachidonylglycerol (Fig. 4A), a

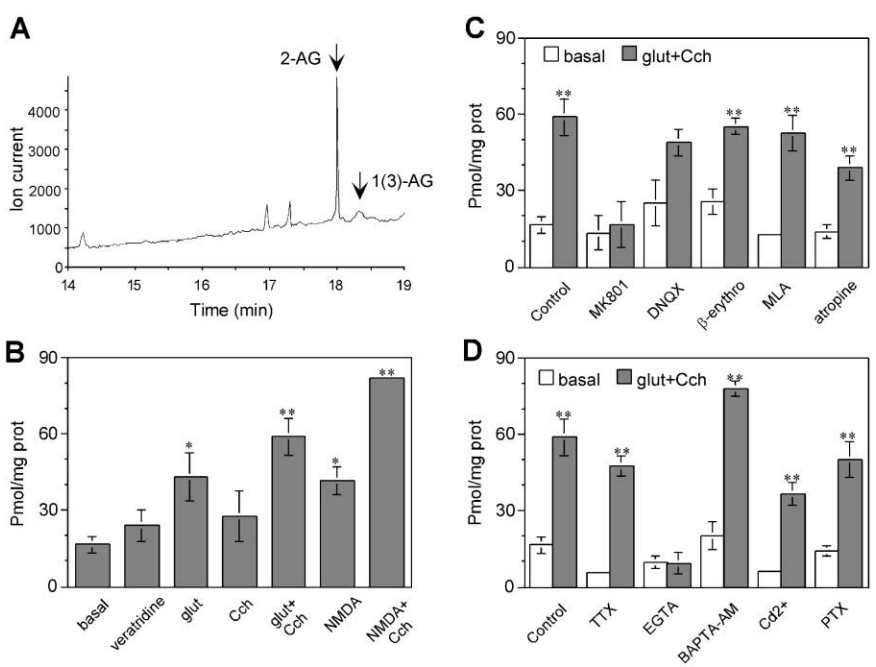

Fig. 4. NMDA receptor activation stimulates 2-arachidonylglycerol formation. Experiments were carried out as described in Fig. 1 legend. (A) Identification of 2-arachidonylglycerol by selected ion monitoring gas chromatography/mass spectrometry (m/z 432, $[M\text{-}90]^+$). The arrows indicate the retention times of standard 2-arachidonylglycerol (2-AG) and 1(3)-arachidonilglycerol (1(3)-AG). (B) Effects of glutamate and carbachol on 2-arachidonylglycerol levels. (C) Effects of various receptor antagonists on 2-arachidonylglycerol production. (D) Effects of tetrodotoxin (TTX), pertussis toxin (PTX) and treatments that affect intracellular Ca^{2+} on 2-arachidonylglycerol production.

compound that originates from the non-enzymatic isomerization of 2-arachidonylglycerol (Serdarevich, 1967; Mechoulam et al., 1995; Bisogno et al., 1997; Stella et al., 1997). 2-Arachidonylglycerol isomerization to 1(3)-arachidonylglycerol is typically fairly high (Stella et al., 1997), and its limited occurrence in the present experiments was probably due to the rapid extraction procedure utilized (see Materials and methods).

Three main features distinguished the mechanisms governing the receptor-dependent formation of 2-arachidonylglycerol from that of anandamide. First, activation of NMDA receptors was sufficient to cause a marked accumulation of 2-arachidonylglycerol in neurons (Fig. 4B). The application of glutamate or NMDA increased 2-arachidonylglycerol production about three times (Fig. 4B), and MK801 completely prevented this effect (Fig. 4C). DNQX partially impaired 2-arachidonylglycerol production, indicating that AMPA/kainate receptors may also be involved (Fig. 4C). Second, carbachol had little effect on 2-arachidonylglycerol biosynthesis when applied alone or in combination with glutamate, albeit it enhanced the effect of NMDA (Fig. 4B). The pharmacological basis of this enhancement was not investigated. Third, incubation with a medium containing the Ca^{2+} chelator EGTA abolished 2-arachidonylglycerol formation, whereas depleting internal Ca^{2+} with BAPTA-AM had no effect (Fig. 4D). The results suggest that flux of external Ca^{2+} allowed by the activation of ionotropic NMDA receptor channels is sufficient to elicit 2-arachidonylglycerol formation in cortical neurons.

4. Discussion

Despite their similar chemical structures, anandamide and 2-arachidonylglycerol are produced through distinct biochemical pathways. Formation of anandamide may result from hydrolysis of the phospholipid precursor *N*-arachidonyl phosphatidylethanolamine, catalyzed by a phosphodiesterase such as phospholipase D (Di Marzo et al., 1994; Cadas et al., 1997; Schmid, 2000). 2-Arachidonylglycerol, on the other hand, may be produced by cleavage of 1,2-diacylglycerol generated by phospholipase C acting on phosphatidylinositol bisphosphate (Gammon et al., 1989; Stella et al., 1997), although the participation of alternative pathways cannot be excluded (Piomelli et al., 2000). The existence of different enzymatic routes for the formation of anandamide and 2-arachidonylglycerol suggests that under certain circumstances, these two endocannabinoid substances might operate independently of each other. This possibility is supported by two findings. In hippocampal slices, stimulation of glutamate-releasing fibers in the Schaffer collaterals increases the levels of 2-arachidonylglycerol, but not those of anandamide (Stella et al., 1997). Conversely, in vivo microdialysis experiments show that activation of striatal D_2-family dopamine receptors enhances release of anandamide, but not of 2-arachidonylglycerol (Giuffrida et al., 1999). It is unclear, however, whether this discrepancy reflects different experimental conditions, regional segregation of the phospholipase C and phospholipase D pathways, or receptor-activated mechanisms linked to the generation of specific endocannabinoid lipids. To examine these different possibilities, in the present study we have investigated the receptor mechanisms underlying the formation of anandamide and 2-arachidonylglycerol in primary cultures of rat cortical neurons.

Our findings indicate that the excitatory neurotransmitter glutamate triggers 2-arachidonylglycerol biosynthesis by allowing external Ca^{2+} to enter cortical neurons through activated NMDA receptor channels. In these cells, 2-arachidonylglycerol formation is likely to be mediated by the phospholipase C/diacylglycerol lipase pathway, because selective drug inhibitors of these enzyme activities prevent the accumulation of 2-arachidonylglycerol elicited by a Ca^{2+} ionophore (Stella et al., 1997). This conclusion is also in agreement with previous work showing that NMDA receptor activation stimulates phospholipase C and diacylglycerol lipase activities in cultured neurons (Nicoletti et al., 1986; Farooqui et al., 1993). Noteworthy, we found that activation of acetylcholine receptors with carbachol had little or no effect, per se, on 2-arachidonylglycerol levels. This result, at variance with the ability of cholinergic agonists to stimulate phospholipase C activity in many tissues and to cause 2-arachidonylglycerol accumulation in vascular endothelium (Mechoulam et al., 1998), underscores the differences in 2-arachidonylglycerol biosynthesis between neurons and other non-neuronal cell types.

The functional role of 2-arachidonylglycerol formation in NMDA receptor signaling is unclear at present. However, experiments with superfused hippocampal slices suggest that 2-arachidonylglycerol may act as a localized feedback signal within the hippocampus (Stella et al., 1997). Electrical stimulation of the Schaffer's collaterals, a glutamatergic fiber tract that projects from CA3 to CA1 neurons, produced a marked increase in 2-arachidonylglycerol accumulation in the slices, which was prevented by the Na^+ channel blocker tetrodotoxin or by removal of external Ca^{2+} (Stella et al., 1997). In the same preparation, exogenous 2-arachidonylglycerol inhibited the induction of long-term potentiation at CA3–CA1 synapses by activating local cannabinoid CB_1 receptors, whereas it had no effect on basal synaptic transmission (Stella et al., 1997). The possible role of 2-arachidonylglycerol as a negative feedback regulator of *N*-methyl-D-aspartate-mediated responses, suggested by these results, is reinforced by the ability of cannabinoid drugs to reduce glutamate transmission (Shen et al., 1996; Shen and Thayer, 1999), inhibit long-term potentiation (Collins et al., 1994; Terranova et al., 1995; Misner and Sullivan, 1999), and alleviate glutamate-induced neurotoxicity (Nagayama et al., 1999).

In the present experiments, the simultaneous application of glutamate and carbachol, but not of either agent alone, caused a marked stimulation of anandamide biosynthesis in cortical neurons. Pharmacological experiments suggest that this synergistic effect may result from the coactivation of NMDA and α7 nicotinic receptors, and may depend both on membrane depolarization and on mobilization of Ca^{2+} ions from intracellular stores. Though necessary for the response, membrane depolarization was insufficient per se to initiate anandamide biosynthesis. This observation appears to contradict previous results, which showed that membrane-depolarizing agents increase accumulation of radioactive fatty acid ethanolamides in neuronal cultures maintained in a serum-containing medium and labeled with [^{3}H]ethanolamine (Di Marzo et al., 1994; Cadas et al., 1996). Differences in cell culture models may provide, however, a plausible explanation for this discrepancy: neurons cultured in the presence of serum actively release neurotransmitters when they are challenged with membrane-depolarizing agents, whereas neuronal cultures maintained in a defined medium, such as those used in the present experiments, do not (Pfrieger and Barres, 1997; Evans et al., 1998). Thus, fatty acid ethanolamide biosynthesis in the former cultures might result, not from a direct effect of membrane depolarization, but from the action of neurotransmitters released in the extracellular medium. A similar indirect mechanism may be responsible for the release of anandamide induced in vivo by a depolarizing concentration of KCl, administered in dorsal striatum by reverse dialysis (Giuffrida et al., 1999).

Receptor-dependent anandamide formation may be distinguished pharmacologically from that of other fatty acid ethanolamides that do not activate cannabinoid receptors. Indeed, though biosynthesis of all fatty acid ethanolamides is contingent on NMDA receptor occupation, anandamide formation requires the coactivation of NMDA and α7 nicotinic receptors, while oleylethanolamide and palmitylethanolamide formation requires the coactivation of NMDA and muscarinic receptors. Thus, glutamate and acetylcholine may elicit the biosynthesis of different fatty acid ethanolamides, depending on the complement of acetylcholine receptors expressed in their target neurons. The finding that palmitylethanolamide and oleylethanolamide may be produced by dissimilar molecular mechanisms and may exert biological effects that are not mediated by cannabinoid receptors (Calignano et al., 1998; Jaggar et al., 1998) raises the possibility that they might serve signaling functions independent from those of anandamide. Testing this possibility will require a thorough investigation of the pharmacological effects of palmitylethanolamide and oleylethanolamide in neurons, as well as the identification of the putative cellular targets that may mediate these effects.

In conclusion, biosynthesis of anandamide and 2-arachidonylglycerol in cortical neurons may be triggered by activation of membrane receptors for the neurotransmitters, glutamate and acetylcholine. This suggests that a key function of the endocannabinoid system in the brain may be to modulate the effects of primary neurotransmitters by a localized feedback action. In agreement with this idea, recent electrophysiological experiments have suggested that endocannabinoid compounds may serve as retrograde messengers in both hippocampus and cerebellum (Kreitzer and Regerh, 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). Finally, the finding that anandamide and 2-arachidonylglycerol may be produced through divergent molecular mechanisms indicates that these two endocannabinoid lipids may exert their modulatory effects in separate and possibly complementary ways.

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