

**DIFFERENTIAL EFFECTS OF ENDOGENOUS AND SYNTHETIC CANNABINOIDS  
ON  $\alpha_7$ -NICOTINIC ACETYLCHOLINE RECEPTOR-MEDIATED RESPONSES IN  
XENOPUS OOCYTES.**

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**Running title: Effects of cannabinoids on  $\alpha_7$ -nACh receptors**

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**Number of text pages: 28**

**Number of figures: 6**

**Number of references: 44**

**Number of words in the abstract: 212**

**Number of words in the introduction: 325**

**Number of words in the discussion: 1083**

**ABBREVIATIONS: 2-AG, 2-arachidonoylglycerol,  $\Delta^9$ -THC,  $\Delta^9$ -tetrahydrocannabinol; nACh, nicotinic acetylcholine; BAPTA, 1,2-bis (*o*-aminophenoxy) ethane-*N, N, N', N'*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl) piperazineethanesulfonic acid.**

## ABSTRACT

The effects of endogenous and synthetic cannabinoid receptor agonists including 2-arachidonoylglycerol, R-methanandamide, WIN55,212-2, CP55,940 and the psychoactive constituent of marijuana,  $\Delta^9$ -THC, on the function of homomeric  $\alpha_7$  nicotinic ACh receptors expressed in *Xenopus* oocytes was investigated using the two-electrode voltage-clamp technique. The endogenous cannabinoid receptor ligands 2-arachidonoylglycerol and the metabolically stable analogue of anandamide (arachidonylethanolamide), R-methanandamide, reversibly inhibited currents evoked with ACh (100  $\mu$ M) in a concentration-dependent manner ( $IC_{50}$  values of 168 nM and 183 nM, respectively). In contrast, the synthetic cannabinoid receptor agonists CP55,940, the aminoalkylindole, WIN55,212-2, and the phytochemical  $\Delta^9$ -THC did not alter  $\alpha_7$  nicotinic ACh receptor function. The inhibition of  $\alpha_7$ -mediated currents by 2-arachidonoylglycerol was found to be non-competitive and voltage-independent. Additional experiments using endocannabinoid metabolites suggested that arachidonic acid, but not ethanolamine or glycerol, could also inhibit the  $\alpha_7$ -nACh receptor function. Whereas the effects of arachidonic acid were also noncompetitive and voltage-independent, its potency was much lower than 2-AG and anandamide. Results of studies with chimeric  $\alpha_7$ -nACh-5-HT<sub>3</sub> receptors comprised of the amino-terminal domain of the  $\alpha_7$ -nACh receptor and the transmembrane and carboxyl-terminal domains of 5-HT<sub>3</sub> receptors indicated that the site of interaction of the endocannabinoids with the  $\alpha_7$ -nAChR was not located on the N-terminal region of the receptor. These data indicate that cannabinoid receptor ligands that are produced in-situ potently inhibit  $\alpha_7$ -nACh receptor function, whereas the synthetic cannabinoid ligands, and  $\Delta^9$ -THC, are without effect, or are relatively ineffective at inhibiting these receptors.

Endogenous cannabinoids (endocannabinoids) are produced on demand from membrane bound precursors in brain tissue via calcium and/or G-protein dependent processes (for a recent review see Piomelli, 2003). Following release, these molecules bind to cannabinoid CB<sub>1</sub> and/or CB<sub>2</sub> receptors and mimic the effects of synthetic cannabinoids in several in vitro preparations (for a recent review see Freund et al., 2003). However, several reports also indicate that endocannabinoids and the psychoactive active ingredient of marijuana,  $\Delta^9$ -THC, can produce effects that are not mediated by the activation of the cloned CB<sub>1</sub> and/or CB<sub>2</sub> receptors. For example, it has been demonstrated that endocannabinoids such as anandamide and/or 2-AG can inhibit the function of gap junctions (Venance et al., 1995), voltage-dependent-Ca<sup>2+</sup> channels (Oz et al., 2000; Chemin et al., 2001), Na<sup>+</sup> channels (Nicholson et al., 2003) various types of K<sup>+</sup> channels (Poling et al., 1996; Van den Bossche and Vanheel, 2000, Maingret et al., 2001), 5-HT<sub>3</sub> receptor function (Barann et al., 2002; Oz et al., 2002a, Godlewski et al., 2003), and nicotinic ACh receptors (Oz et al., 2003). This suggests that additional molecular targets for certain classes of cannabinoids exist in the CNS, and that these targets may represent important sites for cannabinoids to alter neuronal function.

Nicotinic acetylcholine (nACh) receptors containing the  $\alpha_7$  subunit belong to the ligand-gated ion channel super family that is found in many brain areas where cannabinoids are known to act (Lindstrom et al, 1996). In addition, the potential involvement of these receptors in pain transmission, neurodegenerative diseases and drug abuse has been extensively reported (Damaj et al., 2000; Orr-Urteger et al., 2000; Picciotto et al., 2001). Biochemical and behavioral studies support the idea that functional interactions between nicotine receptors and cannabinoid receptor ligands exist (Pryor et al., 1978; Valjent et al., 2002). In addition, we have shown that the endogenous cannabinoid, anandamide, inhibits ion currents mediated by the activation of nACh

$\alpha_7$  receptors expressed in *Xenopus* oocytes (Oz et al., 2003). Another receptor that shares a high degree of homology with nACh  $\alpha_7$  receptors is the serotonin 5-HT<sub>3</sub> receptor (for reviews see Jackson and Yakel, 1995; Reeves and Lummis, 2002). As might be expected, earlier studies demonstrated that both endogenous and synthetic cannabinoids could inhibit the function of 5-HT<sub>3</sub> receptors expressed in mammalian cell lines or in *Xenopus* oocytes (Barann et al., 2002; Oz et al., 2002), although the potency of these cannabinoids appears to be lower at 5-HT<sub>3</sub> receptors than at  $\alpha_7$ -nACh receptors.

In addition to the phytochemical  $\Delta^9$ -THC, there are three different classes of cannabinoid receptor ligands that are currently employed in pharmacological research. These include the non-classical cannabinoids, typified by the agonist CP-55,940, the aminoalkylindoles, such as WIN55,212-2, and the arachidonic acid-derived, eicosanoid molecules such as anandamide (arachidonylethanolamide) and 2-arachidonylglycerol (Pertwee, 1997). The present study was performed to compare the effects a range of cannabinoid receptor ligands representing each of these classes. We find that the endocannabinoids potently inhibit  $\alpha_7$ -nACh receptor function, independently of cannabinoid receptor activation, and that the synthetic cannabinoids and  $\Delta^9$ -THC do not appear to significantly disrupt  $\alpha_7$ -nACh signaling.

## MATERIALS AND METHODS

Mature female *Xenopus laevis* frogs were purchased from *Xenopus laevis* I, Ann Arbor, MI and were housed in dechlorinated tap water at 19-21° C with a 12/12 hours light/dark cycling, and fed with beef liver twice a week. Clusters of oocytes were removed surgically under tricaine (Sigma, St.Louis, MO) local anesthesia (0.15 % w/V) and individual oocytes were dissected away manually in a solution containing (in mM): NaCl, 88; KCl, 1; NaHCO<sub>3</sub>, 2.4; MgSO<sub>4</sub>, 0.8; HEPES, 10 (pH 7.5). Later, dissected oocytes were stored 2-7 days in modified Barth's solution (MBS) containing (in mM): NaCl, 88; KCl, 1; NaHCO<sub>3</sub>, 2.4; Ca(NO<sub>3</sub>)<sub>2</sub>, 0.3; CaCl<sub>2</sub>, 0.9; MgSO<sub>4</sub>, 0.8; HEPES, 10 (pH 7.5), supplemented with sodium pyruvate 2 mM, penicillin 10,000 IU/L, streptomycin 10 mg/L, gentamicin 50 mg/L, and theophylline 0.5 mM. Ion currents were recorded as previously described (Oz et al., 2002b). Briefly, oocytes were placed in a 0.2 ml recording chamber and superfused at a constant rate of 3-5 ml/min. The bathing solution consisted of (in mM): NaCl, 95; KCl, 2; CaCl<sub>2</sub>, 2; and HEPES 5 (pH 7.5). The cells were impaled at the animal pole with two standard glass microelectrodes filled with a 3 M KCl (1-10 MΩ). The oocytes were voltage-clamped routinely at a holding potential of -70 mV using GeneClamp-500 amplifier (Axon Instruments Inc., Burlingame, CA), and current responses were directly recorded on a Gould 2400 rectilinear pen recorder (Gould Inc., Cleveland, OH). Current-voltage curves were generated by holding each membrane potential in a series for 30 s, followed by a return to -70 mV for 10 min. Oocyte capacitance was measured by a paired-ramp method described earlier (Schmitt and Koepsell, 2002). Briefly, voltage-ramps were employed to elicit constant capacitive current,  $I_{cap}$ , and the charge associated with this current was calculated by the integration of  $I_{cap}$ . Ramps had slopes of 2 V/s and durations of 20 ms, and started at a holding potential of -90 mV. A series of 10 paired-ramps was delivered at 1 s intervals, and

averaged traces were used for charge calculations. In each oocyte, the averages of 5-6 measurements were used to obtain values for membrane capacitance;  $C_m$ . Currents for  $I_{cap}$  recordings were filtered at 20 kHz and sampled at 50 kHz.

Compounds were applied externally by addition to the superfusate. All chemicals used in preparing the solutions were from Sigma-Aldrich (St. Louis, MO). Anandamide, R-(+)-methanandamide, 2-AG, (-)-nicotine, and  $\alpha$ -bungarotoxin were from Sigma-RBI (St. Louis, MO). Procedures for the injections of pertussis toxin (PTX, 50 nl, 50  $\mu$ g/ml) or BAPTA (50-100 nl, 100 mM) were performed as described previously (Oz et al., 1998). PTX was dissolved in distilled water, BAPTA was prepared in Cs<sub>4</sub>-BAPTA. Injections were performed 1 h prior to recordings using oil-driven ultra microsyringe pump (Micro4, WPI, Inc. Sarasota, FL). Stock solutions of anandamide were prepared in dimethylsulfoxide (DMSO) at a concentration of 100 mM. DMSO, alone, did not affect nicotinic receptors when added at concentrations up to 0.3 % (v/v) in MBS solutions, a concentration twice as high as that resulting from the most concentrated application of the agents used.

**Synthesis of cRNA and chimeric construct:** The cDNA clones of the chick nACh $\alpha_7$  subunit and 5-HT<sub>3A</sub> subunit were provided by Dr. Lindstrom (University of Pennsylvania, PA) and Dr. David Julies (University of California, San Francisco, CA), respectively. Capped cRNA transcripts were synthesized *in vitro* using a mMMESSAGE mMACHINE kit from Ambion (Austin, TX) and analyzed on 1.2 % formaldehyde agarose gel to check the size and the quality of the transcripts. The chimeric  $\alpha_7$ -nACh - 5-HT<sub>3A</sub> receptor was constructed as described previously (Eisele et al., 1993, Yu et al., 1996).

**Data analysis:** Average values were calculated as mean  $\pm$  standard error (SE). Statistical

significance was analyzed using Student's *t* test or ANOVA as indicated. Concentration-response curves were obtained by fitting the data to the logistic equation,

$$y = E_{\max}/(1+[x/EC_{50}]^{-n}),$$

where *x* and *y* are concentration and response, respectively,  $E_{\max}$  is the maximal response,  $EC_{50}$  is the half-maximal concentration, and *n* is the slope factor (apparent Hill coefficient).



## RESULTS

*Xenopus* oocytes that were either not injected with  $\alpha_7$ -nAChR mRNA (n=6) or injected with distilled water (n=4) did not demonstrate ion currents when 1-3 mM ACh in the presence of 1  $\mu$ M atropine was applied. In oocytes injected with  $\alpha_7$ -nAChR mRNA, a 4-5 s application of ACh activated a fast inward current that rapidly desensitized. These ACh-induced inward currents were elicited at 10 min intervals to avoid receptor desensitization, and were irreversibly abolished by 10 nM  $\alpha$ -bungarotoxin (n=3, data not shown), indicating that these responses were mediated by neuronal  $\alpha_7$  nACh receptor-ion channels.

In earlier studies, endogenous cannabinoids such as anandamide and 2-AG were shown to release intracellular  $\text{Ca}^{2+}$  in endothelial and NG108-15 cells (Sugiura et al., 1996; Mombouli et al., 1999; for a review Howlett and Mukhopadhyay, 2000). In the oocyte expression system this increased level of intracellular  $\text{Ca}^{2+}$  would be detected by the activation of  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels and concomitant alterations in the membrane input resistance. For this reason, we examined the effects of 2-AG and R-methanandamide, a metabolically stable chiral analogue of anandamide that is resistant to hydrolytic inactivation by fatty acid amide hydrolase (Abadji et al., 1994), on membrane resistance ( $R_m$ ), membrane capacitance ( $C_m$ ) and resting membrane potential ( $V_m$ ) in uninjected oocytes. The results presented in Table 1 demonstrate that neither 2-AG nor R-methanandamide had a significant effect on the passive membrane properties of the oocytes.

In our earlier studies we found that anandamide potently inhibited the function of  $\alpha_7$ -nAChRs expressed in *Xenopus* oocytes ( $\text{IC}_{50} = 229$  nM; Oz et al., 2003). In the present study, the application of 10 nM to 3  $\mu$ M of 2-AG also concentration-dependently inhibited ACh (100  $\mu$ M)-induced ion currents in  $\alpha_7$ -nACh receptor expressing oocytes (Fig 1A). The ACh-induced

currents were maximally inhibited by 2-AG within 4-5 minutes of its initial bath application, and it was applied for 30-40 min to ensure equilibrium concentrations (Fig. 1). In contrast, a high concentration of WIN55,212-2 (10  $\mu$ M), a synthetic full agonist at CB<sub>1</sub> receptors, did not affect the maximal amplitudes of the ACh-induced ion currents at application times up to 30 min (Fig. 1B). Results of experiments demonstrating the time course of the effects of 2-AG and WIN55,212-2 on the mean amplitudes of the ACh-induced currents from 4-6 oocytes are presented in Figure 1C. In addition, in the absence of agonist neither 2-AG nor WIN55,212-2 (both at 30  $\mu$ M) had effects on holding currents in oocytes voltage clamped at  $-70$  mV ( $n = 3-5$ ), suggesting that passive membrane properties of the oocytes were unaffected.

In the next series of experiments we examined the concentration-response relationships of the effects 2-AG, R-methanandamide, CP 55,940, WIN55,212,2 and  $\Delta^9$ -THC on the function of nicotinic receptors (Fig 2). Two arachidonoylglycerol and R-methanandamide inhibited the nicotinic receptor-mediated response with IC<sub>50</sub> values of 168 nM and 183 nM, respectively. These values were slightly lower than the IC<sub>50</sub> values of 229 nM previously described for anandamide (Oz et al., 2003). In contrast to the relatively potent inhibition by 2-AG and R-methanandamide, neither  $\Delta^9$ -THC nor WIN55,212-2 altered the maximal amplitudes of the ACh-induced inward currents. Furthermore, although the synthetic agonist CP 55,940 had no effect at concentrations up to 1  $\mu$ M, at much higher concentrations it inhibited the nicotinic receptor response, demonstrating an IC<sub>50</sub> of 3.4  $\mu$ M (slope = 2.6).

Since activation of  $\alpha_7$ -nACh receptors permit sufficient Ca<sup>2+</sup> entry to activate endogenous Ca<sup>2+</sup>-dependent Cl<sup>-</sup> channels in *Xenopus* oocytes (Séguéla et al., 1993; Sands et al., 1993), it was important to determine in the present study whether the effect of 2-AG was due to the inhibition

of these currents or secondarily, on other currents induced by  $\text{Ca}^{2+}$  entry. Thus, extracellular  $\text{Ca}^{2+}$  was replaced with  $\text{Ba}^{2+}$ , since  $\text{Ba}^{2+}$  can pass through nACh  $\alpha_7$  receptors (Sands et al., 1993) but causes little, if any, activation of  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channels. In addition, because a small  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  current remains, even in  $\text{Ba}^{2+}$ , we injected oocytes with the  $\text{Ca}^{2+}$  chelator, BAPTA (Sands et al., 1993). Under these conditions, 2-AG (300 nM) produced the same level of inhibition ( $43 \pm 4$  % of controls) of ACh-induced currents when compared to control oocytes (Fig. 3A).

Examination of the voltage-dependence of the 2-AG and R-methanandamide inhibition indicated that the degree of inhibition of the ACh (100  $\mu\text{M}$ )-induced currents by 2-AG (200 nM) or R-methanandamide (200 nM) did not vary with membrane potential (Fig. 3B and 3C). In addition, there was no change on the reversal potential of the ACh-activated ion currents ( $4 \pm 2$  mV in controls versus  $6 \pm 3$  mV in 2-AG and  $5 \pm 4$  in R-methanandamide), indicating that neither the ionic selectivity of the channel nor the driving force on  $\text{Na}^+$  and  $\text{Ca}^{2+}$  were affected by these molecules.

Another way 2-AG and R-methanandamide may alter  $\alpha_7$ -nAChR-channel activity might be through the competitive inhibition of ACh binding to the receptor. To test this possibility, the effects of 2-AG and R-methanandamide on  $\alpha_7$ -nAChR function were examined at different concentrations of ACh. Neither 2-AG nor R-methanandamide altered the potency of ACh (Fig 3D). Thus, in controls and in the presence of 2-AG or R-methanandamide, the  $\text{EC}_{50}$  values for ACh were  $142 \pm 8$   $\mu\text{M}$ ,  $137 \pm 7$   $\mu\text{M}$  and  $149 \pm 8$  (n=4), and slope values were  $1.4 \pm 0.1$ ,  $1.6 \pm 0.2$  and  $1.3 \pm 0.2$ , respectively. However, both of these molecules inhibited the maximal response to ACh by approximately 45 % (n=4-7), suggesting that the inhibition of  $\alpha_7$ -nAChR currents was

noncompetitive. The effect of 2-AG (300 nM) on currents induced by a more potent agonist at  $\alpha_7$ -nACh receptors, nicotine (10  $\mu$ M and 100  $\mu$ M), was also examined. The degree of inhibition of nicotine-induced currents was identical to ACh and did not vary with nicotine concentration (10  $\mu$ M=  $44 \pm 5$  % (n=4) and 100  $\mu$ M=  $41 \pm 6$  % (n=5) of controls, respectively).

The endocannabinoids tested in these experiments contain several major chemical moieties: arachidonic acid and ethanolamine are contained in anandamide, whereas glycerol and arachidonic acid are found in 2-AG. In order to determine whether these chemical moieties could by themselves alter  $\alpha_7$ -nAChR function, we examined the effects of arachidonic acid, ethanolamine and glycerol on the function of  $\alpha_7$ -nACh receptor. Arachidonic acid (1  $\mu$ M) reduced the maximal amplitudes of  $\alpha_7$ -nACh receptor-mediated ion currents to  $54 \pm 7$  of controls (n=5). Conversely, 35 min incubation in ethanolamine or glycerol did not alter the maximal amplitudes of ACh-induced currents (Fig 4A). We have shown that anandamide (Oz et al., 2003), R-methanandamide and 2-AG (present study Fig. 3C) inhibit the function of  $\alpha_7$ -nACh receptors in voltage-independent and noncompetitive manners. If the endocannabinoid metabolite arachidonic acid mediated the effects of these molecules, it would be expected that the arachidonic acid inhibition of  $\alpha_7$ -nACh receptor response would also be voltage-independent and noncompetitive. For this reason, we examined the voltage-dependency of the inhibition of  $\alpha_7$ -nACh receptor, by arachidonic acid. Similar to endocannabinoids, the inhibition of the ACh induced currents by arachidonic acid (1  $\mu$ M) did not vary with membrane potential (Figs. 5A and 5B), and the reversal potential of the ACh-activated ion currents was not changed ( $3 \pm 2$  mV in controls versus  $5 \pm 3$  mV in arachidonic acid). In addition, we examined the inhibitory effect of arachidonic acid at increasing concentrations of ACh to determine whether its inhibitory effects

on the  $\alpha_7$ -nACh receptor-mediated currents were competitive (Fig. 5C). Similar to the endocannabinoid precursors, arachidonic acid did not cause any shift on the concentration-response curve, but inhibited the maximal oocyte response to ACh to about 45 to 55 % of controls (n=4-7). In controls and in the presence of arachidonic acid, the  $EC_{50}$  values for ACh were  $138 \pm 6 \mu\text{M}$  and  $142 \pm 7 \mu\text{M}$  (n=5-6), and slope values were  $1.4 \pm 0.3$  and  $1.5 \pm 0.3$ , respectively, suggesting that similar to anandamide, R-methanandamide and 2-AG, arachidonic acid inhibited the function of the nicotinic receptors in a noncompetitive manner.

The preceding data suggested that the metabolite, arachidonic acid, might be responsible for the presumed endocannabinoid action on  $\alpha_7$ -nACh receptor-mediated currents in the oocytes. However, a more critical test of this hypothesis would be achieved by comparing the potency of arachidonic acid with the endocannabinoid molecules in this system. Figure 4B demonstrates the effects of increasing concentrations of arachidonic acid, ethanolamine and glycerol on the amplitudes of ACh (100  $\mu\text{M}$ )-activated currents. Although, ethanolamine and glycerol were ineffective up to concentrations of 100  $\mu\text{M}$ , arachidonic acid inhibited ACh-induced currents with an  $EC_{50}$  value of 1.2  $\mu\text{M}$ , indicating that the potency of arachidonic acid was lower than that of anandamide or 2-AG. Therefore, it is unlikely that the arachidonic acid metabolite was responsible for the inhibition of  $\alpha_7$ -nACh receptors observed during anandamide application.

In earlier studies we found that anandamide inhibited  $\alpha_7$ -nACh receptor-mediated responses with a potency that was at least one order of magnitude higher than at 5-HT<sub>3</sub> receptors expressed in *Xenopus* oocytes (Oz et al., 2002; 2003). Thus, in these studies, the  $IC_{50}$  values for anandamide were 229.0 nM and 3.7  $\mu\text{M}$  at  $\alpha_7$ -nACh and 5-HT<sub>3</sub> receptors, respectively. The development of chimeric  $\alpha_7$ -nACh-5HT<sub>3</sub> receptors (Eisele et al., 1993, Zhang et al., 1997), and

the differences in anandamide potency at these distinct receptor-operated ion channels provided an opportunity to evaluate the location of the anandamide interaction with the  $\alpha_7$ -nACh receptor. Therefore, we utilized a functional chimeric receptor-ion channel constructed with the N-terminal domain of the  $\alpha_7$ -nACh receptor and the C-terminal and transmembrane domains of the 5-HT<sub>3</sub> receptor (Eisele et al., 1993, Zhang et al., 1997). The properties of these chimeric receptors are similar to the native  $\alpha_7$ -nACh receptor with regard to the potency and efficacy of ACh. However, they are different from the native  $\alpha_7$ -nACh receptors in that they display slower activation and inactivation kinetics (Eisele et al., 1993). In agreement with our earlier results (Oz et al., 2003), application of 300 nM anandamide inhibited the ACh-induced currents mediated by  $\alpha_7$ -nACh receptors in a noncompetitive manner (Fig 6A). Thus, in the presence of anandamide, maximal ACh-induced currents were inhibited to 40-45 % of control, and the EC<sub>50</sub> values of ACh were not significantly altered ( $122 \pm 6 \mu\text{M}$  and  $117 \pm 5 \mu\text{M}$ ; n=4-6). In contrast, this same concentration of anandamide had no significant effect on 5-HT<sub>3</sub> receptor-mediated currents in oocytes injected with cRNA coding for this receptor (Fig. 6B). Similarly, when the ability of 300 nM anandamide to inhibit ACh-induced currents mediated by the  $\alpha_7$ -nACh-5-HT<sub>3</sub> chimeric receptors was examined, it was found that the endocannabinoid did not significantly inhibit these currents (Fig. 6C). Thus in the presence and absence of anandamide the EC<sub>50</sub> values for ACh were  $132 \pm 5 \mu\text{M}$  and  $127 \pm 6 \mu\text{M}$  (n=5-6), respectively. These data suggest that the N-terminal domain of the  $\alpha_7$ -nAChRs was not the critical target for the anandamide inhibition of  $\alpha_7$ -nAChRs, and further that this site must be located either at one of the transmembrane domains or near the carboxyl terminal of this receptor.

## DISCUSSION

The present results indicate that endogenous and synthetic cannabinoids have differential effects on the function of neuronal  $\alpha_7$ -nACh receptors expressed in *Xenopus* oocytes. Whereas the endogenous cannabinoid receptor ligands such as 2-AG and R-methanandamide inhibited the function of  $\alpha_7$ -nACh receptors at relatively low concentrations, the synthetic cannabinoid ligands, including WIN55,212-2 and CP 55,940, and the plant-derived  $\Delta^9$ -THC, were virtually ineffective in modulating  $\alpha_7$ -nAChR-mediated ion currents. These results therefore suggest that whereas  $\alpha_7$ -nACh receptors may represent molecular targets for endogenous cannabinoids in the mammalian brain, they are not likely to be affected by the psychoactive constituent in marijuana.

Endogenous cannabinoids, at the concentration range used in this study, have been shown to activate native cannabinoid receptors (for reviews; DiMarzo et al., 2002; Sugiura et al., 2002). However, binding studies conducted in *Xenopus* oocytes indicate that the cloned cannabinoid receptors, CB<sub>1</sub> and CB<sub>2</sub>, are not expressed endogenously in these cells (Henry and Chavkin, 1995). In addition, our previous work has demonstrated that the CB<sub>1</sub> receptor antagonist SR-141716A and the CB<sub>2</sub> receptor antagonist SR144528 did not affect the anandamide-induced inhibition of  $\alpha_7$ -nACh receptors, nor did pertussis toxin alter the inhibitory effects of anandamide on the  $\alpha_7$ -nACh receptors (Oz et al., 2003). Thus, it is unlikely that the observed effects of the endocannabinoids on  $\alpha_7$ -nAChR function in the present study were due to the activation of cannabinoid receptors or other G<sub>i</sub>/G<sub>o</sub> G-protein-coupled receptors.

During our experiments, the application of endocannabinoids even at the highest concentrations did not cause a significant change in baseline holding currents, suggesting that the intracellular concentration of Ca<sup>2+</sup> was not affected. Since Ca<sup>2+</sup>-activated Cl<sup>-</sup> channels are highly

sensitive to intracellular levels of  $\text{Ca}^{2+}$  (for a review Dascal, 1988), its release would be reflected by changes in holding currents under voltage-clamp conditions. In addition, other passive membrane properties, such as membrane capacitance, were not significantly altered (Table 1), suggesting that endocannabinoids, at the concentrations used in this study, did not alternatively disrupt the integrity of the lipid membrane.

The present data strongly suggest that the effects of the endocannabinoids were due to a non-competitive interaction with the  $\alpha_7$ -nAChR. This is supported by data demonstrating that increases in the concentration of ACh could not overcome the endocannabinoid inhibition of the ACh-induced ion currents, and by the observation that the  $\text{EC}_{50}$  for ACh was unchanged by these molecules. Although the noncompetitive nature of the inhibition by R-methanandamide (Fig. 3C) or anandamide (Oz et al., 2003) suggested that the ACh binding site itself was not targeted by the endocannabinoids, we used the chimeric  $\alpha_7$ -nACh-5-HT<sub>3</sub> receptor to further examine the possible site of this interaction in more detail. The functional chimeric  $\alpha_7$ -nACh-5-HT<sub>3</sub> receptor consisted of an N-terminal domain from the  $\alpha_7$ -nACh receptor and the transmembrane and C-terminal domains from the 5-HT<sub>3</sub> receptor (Eisele et al., 1993, Zhang et al., 1997). Previous studies have demonstrated that anandamide inhibits the function of  $\alpha_7$ -nACh receptors with a potency that was an order of magnitude higher than at 5-HT<sub>3</sub> receptors in *Xenopus* oocytes (Oz et al., 2002a; 2003). In the present study, the observed resistance of the chimeric  $\alpha_7$ -nACh-5-HT<sub>3</sub> receptor to inhibition by a concentration of anandamide (300 nM) that potently inhibited the  $\alpha_7$ -nACh receptor-mediated response indicated that the site of interaction with the  $\alpha_7$ -nACh receptor must be at the transmembrane domain, and/or the C-terminal receptor domains, but not at the ligand binding N-terminal domain. Thus, these data seem to confirm our previous observation



that anandamide does not interfere with  $\alpha_7$ -nACh receptor-mediated function by inhibiting the binding of Ach to this receptor, but rather, by disrupting some process in the plasma membrane, or within the cell at the C-terminal tail.

Earlier studies on 5-HT<sub>3</sub> receptors indicated that both endogenous and synthetic cannabinoids are effective inhibitors of this ligand-gated ion channel (Barann et al., 2002; Oz et al., 2002a; Godlewski et al., 2003). This is in contrast with the results of the present study where only the endogenous ligands were capable of inhibiting the function of  $\alpha_7$ -nAChR, at reasonable concentrations. This suggests that, despite the high degree of homology of  $\alpha_7$ -nACh receptors and 5-HT<sub>3</sub> receptors (Reeves and Lummis, 2002) that a distinct site for endocannabinoid versus synthetic cannabinoid interaction with these proteins must exist.

The endocannabinoid, 2-AG, is hydrolyzed in situ to form arachidonic acid and glycerol, whereas anandamide is metabolized to arachidonic acid and ethanolamine by fatty acid amide hydrolase (for reviews see Piomelli et al., 1998, Piomelli, 2003). Also, earlier studies on nACh receptors at the neuromuscular junction demonstrated that many fatty acids, including arachidonic acid and prostaglandin E<sub>2</sub> could modulate the function of neuronal nicotinic ACh receptors via a direct action (Vijayaraghavan et al., 1995, Nishizaki et al., 1998; Tan et al., 1998; Du and Role, 2001; for review see Arias, 1998). Because of these known effects, we wanted to determine whether the metabolites of the endocannabinoids could also alter the  $\alpha_7$ -nAChR function. Under the present experimental conditions, we found that only arachidonic acid had significant effects on  $\alpha_7$ -nACh receptor-mediated function in the oocytes. Thus both the intact endocannabinoids and arachidonic acid shared similar time courses for the inhibition, as well as similar noncompetitive and voltage-independent modulation. However, the potencies of 2-AG or R-methanandamide were higher than

that of arachidonic acid on  $\alpha_7$ -nAChRs, suggesting that it was the intact endocannabinoid and not the metabolite, arachidonic acid that altered  $\alpha_7$ -nACh receptor function. In addition, since glycerol and ethanolamine had virtually no effect at these receptors, it can also be concluded that the endocannabinoid, and not these other metabolites, mediated the inhibitory effect of anandamide and 2-AG on  $\alpha_7$ -nACh receptor function.

The synthesis of endocannabinoids can be triggered in response to membrane depolarization and the subsequent influx of  $\text{Ca}^{2+}$  (Piomelli et al., 1998; Freund et al., 2003). Alternatively, postsynaptic activation of neurotransmitter receptors such group I metabotropic glutamate receptors (Varma et al., 2001; Maejima et al., 2001),  $M_1$  and  $M_3$  muscarinic receptors (Ohno-Shosaku et al., 2003),  $D_2$  dopamine receptors (Giuffrida et al., 1999), or the co-activation of NMDA and  $\alpha_7$ -nACh receptors (Stella and Piomelli, 2001; Freund et al., 2003) can induce endocannabinoid synthesis in neuronal preparations. Recent studies also indicate that endocannabinoids are synthesized postsynaptically, and that they can modulate synaptic transmission in a retrograde fashion, by occupying  $\text{CB}_1$  receptors (for a review see; Wilson and Nicoll, 2002). The evidence to date is also quite strong that in the central nervous system,  $\alpha_7$ -nACh receptors are located presynaptically and play an important modulatory role in synaptic transmission (McGehee et al, 1995; Girod et al., 2000; Dani, 2001). Because of the potency of the endocannabinoid modulation and the presence of  $\alpha_7$ -nAChRs in these critical locations, it is possible that the activity of the  $\alpha_7$ -nACh receptors may be modulated by endocannabinoids released from postsynaptic sites in situ.

In conclusion, our results indicate that endocannabinoids, but not synthetic or plant-derived cannabinoid ligands, inhibit the function of  $\alpha_7$  nicotinic receptors expressed in *Xenopus* oocytes. In addition, the results of studies with chimeric  $\alpha_7$ -nACh-5-HT<sub>3</sub> receptor suggest that the site of this

interaction is at the transmembrane or C-terminal domain of the  $\alpha_7$ -nACh receptor. Collectively, these data suggest that  $\alpha_7$ -nACh receptors may represent targets for the endocannabinoids in the intact nervous system.

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## FOOTNOTES

**Acknowledgement:** The authors wish to thank Dr. Lindstrom for kindly providing the cDNA clone of  $\alpha_7$  subunit of nACh receptors and Dr. David Julius for providing 5-HT<sub>3</sub> receptor cDNA.

## FIGURE LEGENDS

**Figure 1.** The effects of 2-AG and WIN 55,222-2 on  $\alpha_7$ -nACh receptor-mediated ion currents. **(A)** Currents activated by ACh (100  $\mu$ M) in control extracellular media (*left*), during bath application of 300 nM 2-AG (*middle*), and during a 10 min recovery (*right*). **(B)** Records of currents activated by ACh (10  $\mu$ M) in control (*left*), during application of 10  $\mu$ M WIN 55,212-2 (*middle*), and after a 10 min Win 55,212-2 washout (*right*). **(C)** Time-courses of the effects of 2-AG and WIN 55,212-2 on the peak Ach-induced currents. Each data point represents the normalized means and s.e.m. of 4 to 6 experiments. The duration of the 2-AG or WIN 55,212-2 application is indicated by the horizontal bar.

**Figure 2.** Concentration-dependence of the effect of cannabinoid receptor ligands on  $\alpha_7$ -nACh receptor-mediated ion currents. Data points represent the mean  $\pm$  s.e.m. of 4-7 oocytes. The curve is the best fit of the data to the logistic equation described in the methods. Currents were activated by applying ACh (100  $\mu$ M). The  $IC_{50}$  and slope factors for 2-AG, R-methanandamide and CP 55,940 were 118 nM; 0.92 and 163 nM; 1.03 and 2.7  $\mu$ M and 3.2, respectively.

**Figure 3.** Inhibition of ACh-induced currents by 2-AG is not due to an effect on endogenous  $Ca^{2+}$ -activated  $Cl^-$  channels, is independent of membrane-potential, and is due to a noncompetitive interaction with the  $\alpha_7$ -nACh receptor. **(A)** Comparison of the effect of 2-AG (200 nM) on the amplitudes of ACh (100  $\mu$ M)-induced currents in the absence or presence of factors contributing the activation of  $Ca^{2+}$ -dependent  $Cl^-$  channels. Oocytes were either injected with 50 nl distilled water and recorded in a 2 mM  $Ca^{2+}$ -containing MBS solution (black bar), or injected with 50 nl of BAPTA (100 mM, gray bar) and recorded in a 2 mM  $Ba^{2+}$ -containing MBS solution. Each data point represents the normalized mean  $\pm$  s.e.m. of 5 to 6 experiments. **(B)** Current-voltage relationships of

ACh-activated currents in the absence and presence of 2-AG or R-methanandamide. Normalized currents activated by 100  $\mu$ M ACh. Each data point represents the normalized means and s.e.m. of four to six experiments. **(C)** Quantitative evaluation of the effects of 2-AG and R-methanandamide are presented as percent inhibition at different voltages indicated in the figure. **(D)** Concentration-response curves for peak ACh-induced currents in the presence and absence of 200 nM 2-AG or R-methanandamide. Oocytes were voltage-clamped at -70 mV and currents were activated by applying ACh (0.01-3 mM). Paired concentration-response curves were constructed and responses normalized to the maximum response under control conditions.  $EC_{50}$  and slope values were determined by fitting the curves from 4 to 6 oocytes to the standard logistic equation as described in the methods section.

**Figure 4.** The effects of endocannabinoid metabolites on  $\alpha_7$ -nACh receptor-mediated ion currents. **(A)** The effects of arachidonic acid (AA, 1  $\mu$ M), ethanolamine (EA, 10  $\mu$ M), and glycerol (GL, 10  $\mu$ M) on the maximal amplitudes of  $\alpha_7$ -nACh receptor-mediated ion currents activated by 100  $\mu$ M ACh. Each data point represents the normalized means  $\pm$  s.e.m. of 4 to 6 experiments. The horizontal bar in the figure indicates duration of application. **(B)** Concentration-dependence of the effect of endocannabinoid metabolites on  $\alpha_7$ -nACh receptor-mediated ion currents. Data points are the mean  $\pm$  s.e.m. of 4-5 oocytes. The curve is the best fit of the data to the logistic equation described in the methods. The  $IC_{50}$  and slope factor for arachidonic acid were 1.2  $\mu$ M and 0.9, respectively. The same data sets for 2-AG and R-methanandamide, shown in Fig 2, is presented for comparison. The  $IC_{50}$  and slope factors for 2-AG and R-methanandamide were 118 nM, and 0.9, and 163 nM, and 1, respectively.

**Figure 5:** The inhibition of  $\alpha_7$ -nACh receptor-mediated ion currents by arachidonic acid is

independent of membrane-potential, and is non-competitive. **(A)** Current-voltage relationships of  $\alpha_7$ -nACh receptor-mediated ion currents before (control; ●) and after 30-min treatment with arachidonic acid (1  $\mu$ M; ○). Normalized currents were activated by 100  $\mu$ M ACh. Each data point presents the normalized means  $\pm$  s.e.m. of 4 to 5 experiments. **(B)** The quantitative evaluation of the effects of arachidonic acid is presented as the percent inhibition at different voltages indicated in the figure. **(C)** Concentration-response curves for peak ACh-induced currents in the presence (●) and absence of 1  $\mu$ M arachidonic acid (○). Oocytes were voltage-clamped at -70 mV and currents were activated by applying ACh (0.01-3 mM). Paired concentration-response curves were constructed and responses normalized to the maximum response under control conditions.  $EC_{50}$  and slope values were determined by fitting the curves from 4 to 5 oocytes to the standard logistic equation as described in the methods section.

**Figure 6.** Effect of anandamide on responses mediated by  $\alpha_7$ -nACh receptors, 5-hydroxytryptamine type 3 receptors (5-HT<sub>3</sub>), and chimeric  $\alpha_7$ -nACh-5-HT<sub>3</sub> receptors. Agonist concentration-response curves for  $\alpha_7$ -nACh receptors **(A)**, 5-HT<sub>3</sub> receptors **(B)**, the chimeric receptors **(C)**, and the effect of anandamide (300 nM) on the responses mediated by each of these receptors is shown. Each data point represents the average of 4 to 6 cells (mean  $\pm$  s.e.m.). The error bars that are not visible are smaller than the size of the symbols. Paired concentration-response curves were constructed and responses normalized to the maximum response under control conditions. Current traces illustrating the effect of 300 nM anandamide (ANA) on currents mediated by  $\alpha_7$ -nACh receptors, 5-HT<sub>3</sub> receptors, and chimeric receptors are presented as insets. The bar above each record indicates the period of agonist application. Vertical calibration bars for the  $\alpha_7$ -nACh receptors, the 5-HT<sub>3</sub> receptors, and the chimeric receptors represent 200 nA, 100 nA, and 200 nA, respectively.

Horizontal calibration bars indicate 2 s, 10 s, and 3 s, respectively. The  $\alpha_7$ -nACh receptor and chimeric receptor-mediated currents were activated by 100  $\mu$ M ACh; the 5-HT<sub>3</sub> receptor-mediated currents were activated by 0.1  $\mu$ M 5-HT.

**TABLE 1.**

The effects of 2-AG and R-methanandamide on the changes in passive membrane properties of the *Xenopus* oocytes.

	$R_m$ (M $\Omega$ )	$C_m$ (nF)	$V_m$ (mV)
Control (n=12)	$0.9 \pm 0.2$	$186 \pm 18$	$-34.6 \pm 3.1$
30 min 2-AG (n=8)	$1.2 \pm 0.3$	$189 \pm 16$	$-35.2 \pm 2.9$
30 min R-methanandamide (n=6)	$1.1 \pm 0.3$	$191 \pm 19$	$-33.1 \pm 2.8$













