Alcohol & Alcoholism Vol. 44, No. 5, pp. 443–448, 2009
Advance Access publication 9 July 2009
doi: 10.1093/alcalc/agp040

PHARMACOLOGY AND CELL METABOLISM

Role of the Endocannabinoid System in Ethanol-Induced Inhibition of Salivary Secretion

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(*Received* 16 *March* 2009; *first review notified* 30 *April* 2009; *in revised form* 27 *May* 2009, 31 *May* 2009 *and* 17 *June* 2009; *accepted* 18 *June* 2009; *advance access publication 9 July 2009*)

Abstract — Aim: The aim of the present study was to determine whether the endocannabinoid system could be involved in the ethanol-induced inhibition of salivation in adult male Wistar rats. **Methods:** Salivary secretion induced by different concentrations of methacholine, a cholinergic agonist, and the endocannabinoid arachidonoyl ethanolamide (anandamide, AEA) production in the submandibular gland (SMG) were determined in rats after ethanol (3 g/kg) administration by gastric gavage. To study the participation of cannabinod receptors in ethanol action, we evaluated methacholine-induced salivary secretion after ethanol administration when CB1 or CB2 receptors were blocked by intra-SMG injections of their selective antagonists AM251 and AM630, respectively. Additionally, we evaluated the *in vitro* effect of ethanol (0.1 M) on SMG production of cAMP, alone or combined with AM251 or AM630. **Results:** Acute ethanol administration increased AEA production in SMG and also inhibited the methacholine-induced saliva secretion that was partially restored by intraglandular injection of AM251 or AM630. In addition, ethanol significantly reduced the forskolin-induced increase in cAMP content in SMG *in vitro* while treatment with AM251 blocked this response. **Conclusion:** We conclude that the inhibitory effect produced by ethanol on submandibular gland salivary secretion is mediated, at least in part, by the endocannabinoid system.

INTRODUCTION

Salivaplays a key role in the local and systemic defense of the oral cavity, the oropharyngeal region, and the upper gastrointestinal tract (Zelles *et al*., 1995). It participates in taste, bolus formation for swallowing (water and mucin) and initiates digestion of starch (amylase) and lipids (lipase) (Nauntofte and Jensen, 1999). Furthermore, saliva components contribute to mucosal coating and provision of antimicrobial action and defense (Pedersen *et al.*, 2002).

The submandibular gland (SMG) is one of the major salivary glands, together with the sublingual and the parotid glands. End secretory units, called acini, are continuous with a ductal system that in rodents has four sequential segments: intercalated ducts, granular convoluted tubules, striated ducts and excretory ducts that release the saliva to the oral cavity. The secretion of saliva is controlled by the autonomic nervous system. The parasympathetic nervous system exerts its function through the activation of muscarinic receptors on salivary glands via impulses in the chorda tympani nerve that releases acethylcholine and salivation depends on the contraction of myoepithelial cells embracing the acini and intercalated ducts (Tandler and Phillips, 1998). The sympathetic nervous system induces salivary secretion by releasing norepinephrine that stimulates α- and β-adrenergic receptors in the acini (Lung, 2003).

Although under normal physiological conditions, the protective potential is sufficiently maintained by saliva, it seems to be disturbed in alcoholics (Abelson *et al.*, 1976; Dutta *et al*., 1992; Proctor and Shori, 1996). Studies have shown positive associations between alcohol consumption and periodontal disease (Sakki *et al*., 1995; Shizukuishi *et al*., 1998; Tezal *et al*., 2001; Pitiphat *et al*., 2003). Moreover, chronic ethanol consumption is considered one of the main causes of oral cancer (Wight and Ogden, 1998).

The ingestion of a high single dose of ethanol causes alteration in saliva flow rate, electrolyte concentration and reduction in SMG protein synthesis in rats and humans (Proctor *et al*., 1993; Shori *et al*., 1994; Enberg *et al.*, 2001). However, the mechanisms by which ethanol exerts its acute effects are not clearly understood.

The main psychoactive ingredient of *Cannabis sativa*, delta-9-tetrahydrocannabinol (THC), affects different physiological functions. Twenty years ago, two subtypes of G-protein-coupled cannabinoid (CB) receptors were identified: the CB1 central receptor subtype, which is mainly expressed in the brain (Devane *et al.*, 1988; Herkenham *et al.*, 1990), and the CB2 peripheral receptor subtype, which appears to be particularly abundant in the immune system (Munro *et al.*, 1993). A few years later, arachidonylethanolamide (anandamide, AEA) and arachidonoyl glycerol, the best-known endocannabinoids, were discovered and purified. Both endocannabinoids derive from arachidonic acid and bind with high affinity to CB receptors (Mechoulam *et al.*, 1998). Selective antagonists have been developed for CB1 receptor, such as AM251 (Gatley *et al.*, 1996) and SR141716A (Rinaldi-Carmona *et al.*, 1995), and for CB2 receptor, such as AM630 (Pertwee *et al.*, 1995) and SR144528 (Griffin *et al.*, 1999).

Furthermore, the CB1 and CB2 receptors were described in the gastrointestinal tract. Neuronal activation of CB1 receptors reduces motility, diarrhea, pain and emesis, and induces eating, while activation of CB2 receptors, acting mostly via immune cells, reduces inflammation (Sanger, 2007).

Recently, we demonstrated that CB1 and CB2 receptors are present in the SMG exhibiting specific localizations (Prestifilippo *et al.,* 2006). Both CB1 and CB2 receptors are functionally linked to the inhibition of adenylyl cyclase (Howlett and Fleming, 1984) and AEA binds with high affinity to both CB receptors decreasing cAMP production in the SMG

(Mechoulam *et al.*, 1998, Prestifilippo *et al.*, 2006). In addition, AEA injected intraglandularly inhibited norepinephrine and methacholine-stimulated saliva secretion in rats (Prestifilippo *et al.*, 2006). Also, it has been reported that chronic ethanol exposure increases the levels of endocannabinoids in different tissues (Basavarajappa, 2007). Therefore, since acute ethanol ingestion as well as AEA injected in the SMG reduces salivary secretion, the aim of this study was to determine whether the endocannabinoid system could be involved in the inhibition of salivary secretion produced by ethanol.

MATERIAL AND METHODS

Animals

Adult male Wistar rats (250–300 g) were kept in an animal room having a photoperiod of 12 h of light, at 22–25°C and free access to rat chow and tap water. The experimental procedures reported here were approved by the Animal Care Committee of the Center for Pharmacological and Botanicals Studies of the National Council of Scientific and Technical Research of Argentina and were carried out in accordance with the National Institute of Health (NIH) guidelines.

AEA production

The production of AEA in the SMG was assayed as described by Paria *et al*. (1996) with minor modifications. The SMGs (7–8 per group) were removed after 60 min of ethanol (3 g/kg of body weight in a final volume of 5 ml per rat) or vehicle (5 ml of water) administered by gastric gavage. Each SMG was homogenized in 500 μl of buffer [20 mM Tris–HCl/1 mM EDTA (pH 7.6)] and centrifuged at 2000$\times g$ for 15 min. Supernatant protein (150–100 μg) was incubated in a total volume of 200 μl of 50 mM Tris-HCl (pH 9.0) with 40 μM (0.1 μCi) of [1-^{14}C]arachidonic acid (40–60 μCi/mmol, Perkin–Elmer, Waltham, MA, USA) and 20 mM ethanolamine (Sigma Chemicals, St. Louis, MO, USA) during 5 min at 37°C. The reaction was terminated by the addition of 400 μl of chloroform–methanol (1:1) mixture. Two additional washes of the aqueous phase with 400 μl of chloroform were performed. Organic phases were evaporated to dryness under nitrogen gas and dissolved in 40 μl of chloroform–methanol (1:1) mixture. Samples and standards were applied on Silica Gel 60 plates (Merck, Darmstadt, Germany). The synthesized [^{14}C]AEA was resolved by using the organic layer of an ethyl acetate–hexane–acetic acid–water (100:50:20:100) mixture. The plate was exposed to an x-ray film at −70°C. After autoradiography, distribution of radioactivity on the plate was counted in a scintillation counter by scraping off the corresponding spots of the plate. The retardation factor values of AEA and arachidonic acid were 0.33 and 0.78, respectively.

In vivo studies

Salivary secretion was assessed in anesthetized rats (chloralose 100 mg/kg, 0.5 ml NaCl 0.9% i.v.) after 60 min of ethanol (3 g/kg of body weight in a final volume of 5 ml per rat) or vehicle (5 ml of water) administered by gastric gavage or after 15 min of AEA (1 μg/50 μl) injected into the SMG. The SMG ducts were cannulated with a fine glass cannula, and salivary secretion was induced by different concentrations of methacholine (1, 3 and 10 μg/kg in saline) (FLUKA, Berlin, Germany) during 3 min after the administration of each dose sequentially injected via the right femoral vein. The saliva was collected on aluminum foil and weighed, as previously described (Bianciotti *et al.*, 1994). Resting flow of saliva (unstimulated) was not observed in rats. Results were expressed as milligrams of saliva/3 min and six rats per group were used.

To evaluate the participation of the endocannabinoid system in salivary responses to ethanol, AM251 (15 μg/50 μl), a selective antagonist for CB1 receptors (Gatley *et al*., 1996), and AM630 (15 μg/50 μl), a selective antagonist for CB2 receptors (Pertwee *et al.*, 1995), were injected into the SMG 45 min after ethanol administration. Fifteen minutes after the injection of the antagonists, dose response curves to methacholine were performed to evaluate salivary secretion. The doses of AEA and CB receptor antagonists employed were obtained from our previous reports (Prestifilippo *et al.*, 2006).

In vitro studies

The animals were killed by decapitation, and SMGs were removed. SMGs (5–6 per group) were cut into halves to enhance the penetration of the different substances into the tissue and preincubated in 500 μl of Krebs–Ringer bicarbonate buffer medium (pH 7.4) containing 0.1% glucose within a Dubnoff metabolic shaker (50 cycles per minute, 95% O_2/5% CO_2) for 15 min before replacement with a fresh medium containing the compounds to be tested. The incubation was continued for another 30 min with forskolin (76 μM) (Sigma Chemicals, St. Louis, MO, USA), an adenylyl cyclase stimulator, forskolin plus AEA (1 nM) (Sigma Chemicals, St. Louis, MO, USA) or forskolin plus ethanol (0.1 M). In other experiments, SMGs were pre-incubated for 15 min with Krebs–Ringer alone and with AM630 (10 μM) (Tocris Ellisville, MO, USA) or AM251 (10 μM) (Tocris Ellisville, MO, USA). AM251 and AM630 were dissolved in dimethyl sulphoxide, and further dilutions were made in saline. Then, incubation was continued for another 30 min with Krebs–Ringer (control), forskolin, forskolin plus ethanol (0.1 M), forskolin plus ethanol plus AM630, AM630 alone, forskolin plus ethanol plus AM251 or AM251 alone. After the incubations, the SMGs were homogenized in 1 ml of H_2O and centrifuged at 6000$\times$ g for 10 min at 4°C. Supernatants were collected and samples were stored at −20°C until cAMP measurements.

cAMP was measured by radioimmunoassay by using the highly specific cAMP antibody kindly provided by Dr A. F. Parlow (National Hormone & Peptide Program, CA, USA). The assay sensitivity was 0.061 pmol/ml. Intra- and inter-assay coefficients of variation were 8.1% and 10.5%, respectively.

Statistics

Data are presented as the means ± SEM. Comparisons between groups were performed using a one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls multiple comparison test for unequal replicates or Student's *t*-test, when appropriate. All analyses were conducted with the GraphPad Prism Version 4.00 software (San Diego, CA, USA). Differences with P values < 0.05 were considered statistically significant.

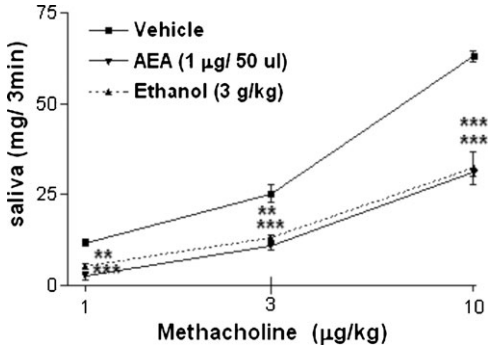

Fig. 1. Effect of ethanol (3 g/kg) and AEA (1 μg/50 μl) on methacholine-stimulated salivary secretion from the SMG. Data are reported as means ± SEM (6 rats per group). $^{**}P < 0.01$ and $^{***}P < 0.001$ versus vehicle (ANOVA, followed by the Student–Newman–Keuls multiple comparisons post-test).

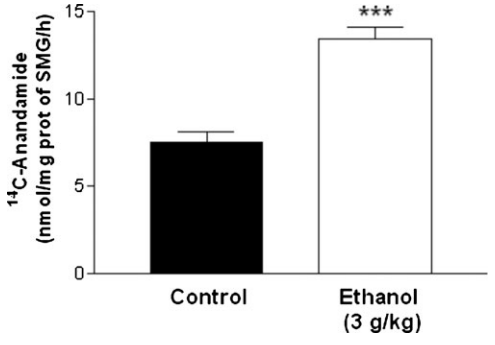

Fig. 2. Production of AEA in the SMG measured 1 h after ethanol (3 g/kg) administration. Values are means ± SEM (7–8 rats per group). $^{***}P < 0.001$ versus control (Student's *t*-test).

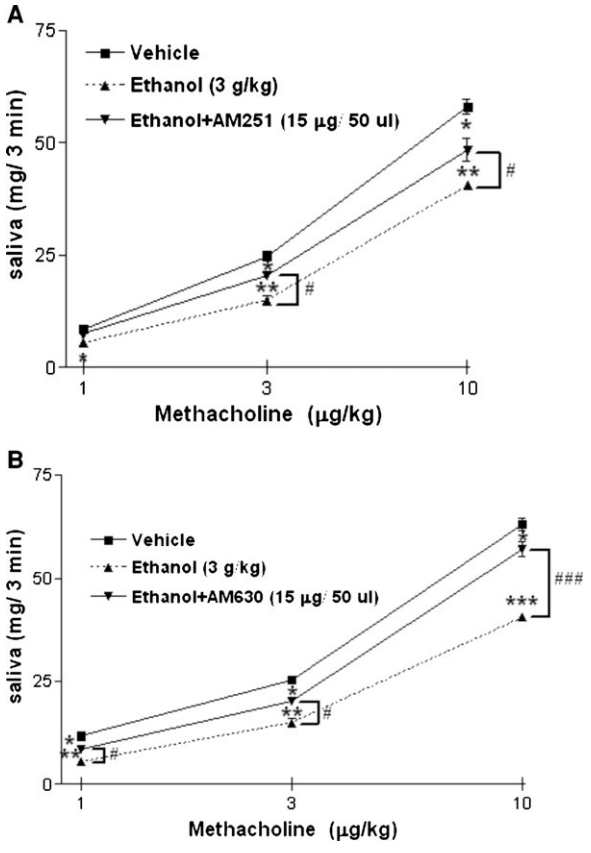

Fig. 3. Effect of (A) AM251 (15 μg/50 μl) or (B) AM630 (15 μg/50 μl) on methacholine-stimulated salivary secretion injected into the SMG combined with ethanol (3 g/kg). Values are means ± SEM (6 per group). $^{*}P < 0.05$; $^{**}P < 0.01$ and $^{***}P < 0.001$ versus vehicle. $^{\#}P < 0.05$, ethanol + AM251 versus ethanol; $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$, ethanol + AM630 versus ethanol. ANOVA followed by the Student–Newman–Keuls multiple comparisons post-test.

RESULTS

Acute effect of ethanol and AEA on salivary secretion

Resultsindicate that ethanol administered by gastric gavage (3 g/kg) significantly inhibited SMG salivary secretion stimulated by all doses of methacholine tested [ANOVA: 1 μg/kg, $F(2,15)$: 26.23, $P < 0.01$; 3 μg/kg, $F(2,15)$: 22.57, $P < 0.01$; 10 μg/kg, $F(2,15)$: 34.17, $P < 0.001$] (Fig. 1). The intra-SMG injection of AEA (1 μg/50 μl) also inhibited the saliva secretion stimulated by the same doses of methacholine ($P < 0.001$) (Fig. 1).

Acute effect of ethanol on AEA production in the SMG

In order to investigate the participation of the endocannabinoid system in salivary responses to ethanol, we first evaluated the effect of ethanol (3 g/kg) on AEA production in the SMG. Ethanol increased AEA production (Student's *t*-test, $P < 0.001$) measured 1 h after administration (Fig. 2).

Involvement of the endocannabinoid system in ethanol inhibition of salivation

We evaluated the effect on salivation of the selective antagonists for CB1 and CB2 receptors, AM251 and AM630, respectively. The inhibitory effect of ethanol (3 g/kg) on methacholine-induced salivary secretion [ANOVA: 1 μg/kg, $F(2,15)$: 8.616, $P < 0.05$; 3 μg/kg, $F(2,15)$: 32.17, $P < 0.01$; 10 μg/kg, $F(2,15)$: 24.52, $P < 0.01$] was completely prevented by the intra-glandular injection of AM251 (15 μg/50 μl) at 1 μg/kg of methacholine dose, and was partially reversed when higher doses of methacholine were injected ($^{\#}P < 0.05$ versus ethanol) (Fig. 3A).

Injection of AM630 (15 μg/50 μl) partially prevented (1 μg/kg and 3 μg/kg, $^{\#}P < 0.05$ versus ethanol; 10 μg/kg, $^{\#\#\#}P < 0.001$ versus ethanol) the decrease in salivation exerted by ethanol at the three methacholine doses used [ANOVA: 1 μg/kg, $F(2,15)$: 34.17, $P < 0.01$; 3 μg/kg, $F(2,15)$: 22.02, $P < 0.01$; 10 μg/kg, $F(2,15)$: 31.96; $P < 0.001$] (Fig. 3B). The injection of AM251 and AM630 alone did not modify methacholine-induced salivation (data not shown).

Effect of ethanol on cAMP production in the SMG: participation of the endocannabinoid system

Since activation of both CB1 and CB2 receptors inhibits adenilate cyclase activity, we investigated whether ethanol could modulate forskolin-increased cAMP production through the activation of CB receptors *in vitro*. Results showed that ethanol (0.1 M) as well as AEA (1 nM) significantly blocked [ANOVA: $F(3,33)$: 7.310, $P < 0.05$] the forskolin (76 μM)-induced increase in cAMP content ($P < 0.001$). (Fig. 4A). In order to

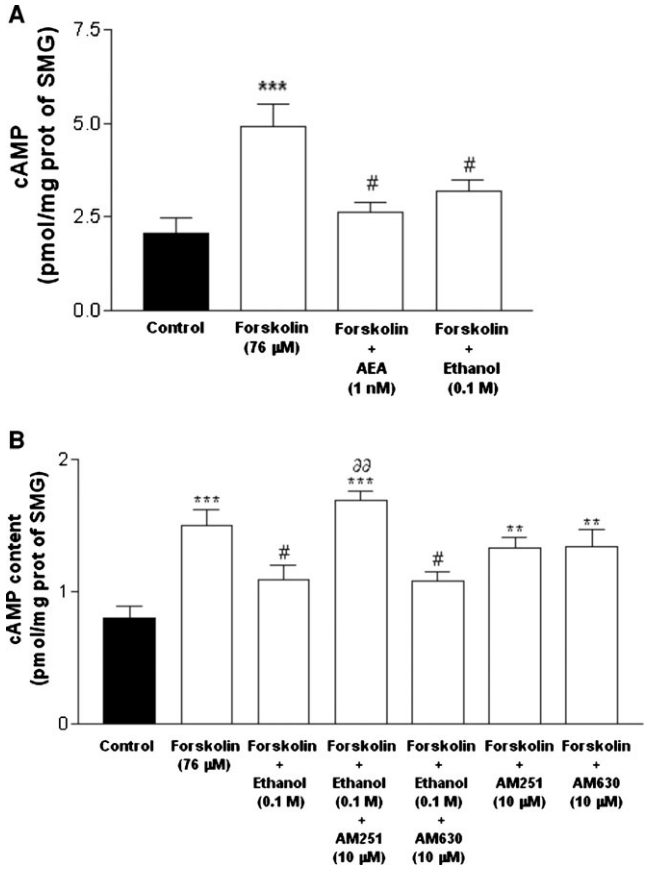

Fig. 4. (A) Effect of AEA (1 nM) and ethanol (0.1 M) on cAMP production stimulated by forskolin (76 μM). (B) Effect of AM251 (10 μM), AM630 (10 μM), ethanol + AM251 or ethanol + AM630 on cAMP production stimulated by forskolin (76 μM). Values are means ± SEM (5–6 per group). $^{**}P < 0.01$ and $^{***}P < 0.001$ versus control. $^{\#}P < 0.05$ versus forskolin, $^{\partial\partial}P < 0.01$ versus forskolin + ethanol. ANOVA followed by the Student–Newman–Keuls multiple comparisons post-test.

confirm the participation of the endocannabinoid system in the ethanol actions in the SMG, we studied whether the decrease in forskolin-stimulated cAMP production exerted by ethanol could be mediated by CB receptors. For this purpose, AM251 (10 μM) and AM630 (10 μM) were used. Figure 4B shows that treatment with AM251 completely blocked the ethanol-induced inhibition of forskolin-stimulated cAMP content [ANOVA: $F(6,27)$: 8.392, $P < 0.01$]. In contrast, AM630 was unable to reverse the ethanol effect on cAMP production.

DISCUSSION

It is well recognized that ethanol consumption decreases salivary secretion (Enberg *et al.*, 2001). However, the mechanism by which ethanol exerts this noxious effect is not clearly understood. The present work shows for the first time the participation of the endocannabinoid system in the inhibition of salivary secretion provoked by acute ethanol administration.

Our results demonstrate that ethanol administration by gastric gavage increased AEA production in the SMG and also inhibited the methacholine-induced salivary secretion. Although ethanol has been shown to modulate a variety of functions (Mukherjee *et al.*, 2008; Harper, 2009), it does not seem to have a specific receptor site for its action. It is known that acute ethanol ingestion alters salivary flow rate and saliva composition (Shori *et al.*, 1994; Enberg *et al.*, 2001). It was reported that alcohol modifies membrane lipid composition; however, these alterations do not explain the changes observed in saliva composition and secretion. In addition, it was previously described that acute alcohol intake not only did affect the autonomic innervations but also produced different responses in rat parotid, sublingual and submandibular gland function (Proctor *et al.*, 1993).

We hypothesized that the increased AEA production observed in the SMG after 1 h of ethanol administration may be responsible for the reduction of salivation. We have previously reported that CB1 or CB2 receptors were expressed in the main cellular structures involved in saliva production and secretion that are acini and ducts. Most CB1 receptors were present in ducts, while CB2 receptors were located in ducts and acini (Prestifilippo *et al.*, 2006). To further investigate the involvement of AEA in the ethanol-induced inhibition of salivation, we employ CB receptor antagonists. Results demonstrated that ethanol effect on salivary secretion was partially prevented by blocking CB1 and CB2 receptors.

The CB1 and CB2 receptors belong to the family of the seven transmembrane-spanning receptors, and are coupled to $G_{i/o}$ proteins (Howlett and Fleming, 1984). Since the mechanism of action of endocannabinoids acting on its receptors is by inhibiting adenylyl cyclase with the consequent decrease in cAMP and taking into account the importance of adenylyl cyclase activity for salivary function, we measured the effect of ethanol on forskolin-induced cAMP levels in SMG slices. Ethanol markedly reduced forskolin-induced increase in cAMP level and this effect was blocked by the CB1 but not by CB2 receptor antagonist, indicating that ethanol actions were mediated preferentially by CB1 receptors *in vitro*. Coincidently, only the CB1 receptor antagonist completely reversed the inhibitory effect of ethanol on methacholine-induced salivary secretion at the lowest dose of the sialogogue (1 μg/kg).

On the other hand, an important center for eliciting salivary secretion exists in the hypothalamus and involves cholinergic pathways. Numerous projections exist from the lateral hypothalamus to salivary nuclei located in the brain stem (Matsuo and Kusano, 1984; Hainsworth and Epstein, 1996). Sialogogues injected through the femoral vein act not only on their receptors in the SMG but also on their receptors in the brain, stimulating efferent responses to the periphery. It was shown that THC decreased salivary flow from the SMG during electrical stimulation of the chorda tympani by a mechanism involving a decrease in the release of acetylcholine (Mc Connell *et al.*, 1978). Acute and chronic exposure to ethanol has been proposed to induce neuroadaptations in the endocannabinoid system. The exposure of cultured neuronal cells or cerebellar granular neurons to chronic ethanol resulted in an accumulation of AEA (Basavarajappa and Hungund, 1999) and 2-arachidonoylglycerol (Basavarajappa *et al.*, 2000). In this line, we have previously demonstrated that ethanol administration by gastric gavage (3 g/kg) increased AEA in the medial basal hypothalamus (Rettori *et al.*, 2007). It was also reported that ethanol increased plasma AEA, which peaked 90 and 240 min after its administration (Giuffrida *et al.*, 1999). Furthermore, the

presence of CB1 receptors were shown in the lateral hypothalamus, which is an important area that controls salivary secretion (Matsuo and Kusano, 1984; Hainsworth and Epstein, 1996) and recently, we have reported that the intracerebroventricular administration of AEA significantly decreased salivation (Fernandez-Solari *et al*., 2009). Therefore, ethanol administration by gastric gavage increases AEA production in the SMG and also in the hypothalamus suggesting that the produced AEA could activate not only the CB receptors in the SMG but also hypothalamic CB receptors and thus affecting both peripheral and central control of salivation.

It is known that ethanol can also alter the release of different neurotransmitters in the nervous system. It enhances the release of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) in the hypothalamus (Lomniczi *et al*., 2000) that could attenuate autonomic neurotransmission to the SMG with a consequent inhibition of salivary secretion (Fernandez-Solari *et al.*, 2009). Thus, the ethanol action on the hypothalamic salivary nuclei could explain the partial blocking of the ethanol inhibitory effect on salivary secretion produced by the CB1 and CB2 receptor antagonists injected in the SMG. Additionally, we cannot discard a direct non-specific effect of ethanol, CB receptor independent, on submandibular gland function.

Therefore, we conclude that the inhibitory effect produced by ethanol on submandibular gland salivary secretion is mediated, at least in part, by the endocannabinoid system.

Acknowledgements — This work was supported by grants from the University of Buenos Aires (UBACyT O 007), ANPCyT (03-14264) and Fundación Alberto J, Roemmers. The authors are greatly indebted to Ricardo Horacio Orzuza for technical assistance.

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