Inhibition of Salivary Secretion by Activation of Cannabinoid Receptors

JUAN PABLO PRESTIFILIPPO,*,†,[1] JAVIER FERNÁNDEZ-SOLARI,*,‡ CAROLINA DE LA CAL,‡ MARÍA IRIBARNE,§ ANGELA M. SUBURO,§ VALERIA RETTORI,* SAMUEL M. MCCANN,* AND JUAN CARLOS ELVERDIN‡

Centro de Estudios Farmacológicos y Botánicos (CEFYBO-CONICET), Buenos Aires 1121, Argentina; †Cat de Fisipatología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires 1121, Argentina; ‡Cat de Fisiología, Facultad de Odontología, Universidad de Buenos Aires, Buenos Aires 1121, Argentina; and §Cat de Medicina celular y Molecular, Universidad Austral, Buenos Aires 1121, Argentina

It is known that marijuana use decreases saliva secretion. Therefore, we hypothesized that cannabinoid receptors (CBs) are located in salivary glands to mediate that effect. In these experiments, we used the submandibular gland (SMG) of male rats, which is one of the major salivary glands. Mammalian tissues contain at least two types of CBs, CB_1 and CB_2, mainly located in the nervous system and peripheral tissues, respectively. Both receptors are coupled to Gi protein and respond by inhibiting the activity of adenylyl cyclase. We demonstrated that both CB_1 and CB_2 are present in the SMG, each showing specific localizations. The best-known endocannabinoid is anandamide (AEA), which binds with high affinity to CB_1 and CB_2. We showed that AEA markedly reduced forskolin-induced increase of cAMP content *in vitro*. This effect was blocked by AM251 and AM630 (CB_1 and CB_2 antagonists, respectively), indicating that both receptors are implicated in SMG physiology. In addition, we showed that AEA injected intraglandularly to anesthetized rats inhibited norepinephrine (NE)– and methacholine (MC)–stimulated saliva secretion *in vivo* and that both AM251 or AM630 prevented the inhibitory action of AEA. Also, the intraglandular injection of AM251 increased saliva secretion induced by lower doses of NE or MC. This increase was synergized after coinjection with AM630. Therefore, we concluded that AEA decreases saliva secretion in the SMG acting through CB_1 and CB_2 receptors. Exp Biol Med 231:1421–1429, 2006

Key words: anandamide; submandibular gland; cAMP; AM251; AM630

Introduction

It is well known that Δ^9-tetrahydrocannabinol (Δ^9-THC), the psychoactive principle isolated from *Cannabis sativa*, alters different physiologic parameters in biologic systems. Mammalian tissues contain at least two types of cannabinoid receptors (CBs), CB_1 and CB_2, mainly located in the nervous system (1–3) and in peripheral tissues or immune cells (4), respectively. Both receptors are coupled to Gi protein and respond by inhibiting the activity of adenylyl cyclase (AC) (5). The best-known endocannabinoids are arachidonoyl ethanolamide (anandamide [AEA]) and arachidonoyl glycerol, both derivatives of arachidonic acid that bind with high affinity to CBs (6). Specific and selective antagonists for CBs have been developed, such as AM251 (7), AM281 (8), SR141716A (9, 10), and LY320135 (11) for CB_1 and AM630 (12) and SR144528 (13) for CB_2. For our experiments, we used AM251 because this is a potent selective CB_1 antagonist that has a K_i of 7.49 n*M* for CB_1 and is 306-fold selective over CB_2. We also used AM630, a CB_2 antagonist/inverse agonist (K_i, 31.2 n*M*), which is 165-fold selective over CB_1 and behaves as a weak partial/inverse agonist at CB_1 (14).

The submandibular gland (SMG) is one of the major salivary glands, together with the sublingual and parotid glands. End secretory units, called *acini*, are continuous with a duct system that in rodents has four sequential segments: intercalated ducts (IDs), granular convoluted tubules (GCTs), striated ducts (SDs), and excretory ducts that release the saliva to the oral cavity (15).

Saliva formation is thought to involve a two-stage process (16). First, acinar cells secrete an isotonic "primary fluid" similar in ionic composition to plasma, and second,

This work was supported by grant UBACyT O 025 from the University of Buenos Aires and by grants PICYT 03/14264 and BID 1201 OC-AR from the Agencia Nacional de Promoción Científica Tecnológica y de Innovación.

[1] To whom correspondence should be addressed at Centro de Estudios Farmacológicos y Botánicos (CEFYBO-CONICET) (ex CIBIER), Paraguay 2155, piso 16, Ciudad de Buenos Aires, Buenos Aires 1121, Argentina. E-mail: jprestifilippo@yahoo.com.ar

Received October 17, 2005.
Accepted March 9, 2006.

1535-3702/06/2318-1421$15.00

the ducts then modify this primary fluid by removing sodium and chloride and adding potassium and bicarbonate to produce a final hypotonic solution that enters the mouth (17), without great changes in the volume of secretion.

The secretion of saliva is controlled by the autonomic nervous system. The parasympathetic nervous system exerts its function through the activation of muscarinic receptors *via* impulses in the chorda tympani nerve that releases acetylcholine. Salivation depends on the contraction of myoepithelial cells embracing the acini and IDs (18). The sympathetic nervous system controls salivary secretion by releasing norepinephrine (NE) that acts on α- and β-adrenergic receptors (15). Agonist-stimulated acinar secretion is initiated by the opening of K^+ and Cl^- channels located in the basolateral and apical membranes, respectively. Luminal accumulation of ions generates a transepithelial osmotic gradient that drives the movement of water into the lumen (19). An increase in the intracellular Ca^{2+} is the primary fluid secretion signal in salivary acinar cells.

It has been demonstrated that cannabinoids modulate autonomic neurotransmission. Activation of CB_1 by Δ^9-THC and AEA causes hypotension *via* sympathoinhibitory action in rats (20). These effects are related to presynaptic inhibition of NE exocytosis, as AEA and Δ^9-THC abolished NE release from isolated rat atria and vasa deferentia, whereas SR141716A, a selective CB_1 antagonist, competitively reversed that effect. Moreover, WIN55212–2 and CP55940, synthetic CB agonists, presynaptically inhibited the sympathetic cardioaccelerator response in the rabbit heart by activating CB_1 (21). The same agonist also inhibited the bradycardia evoked by stimulation of the vagus nerve.

In vitro studies on rat SMG slices with ^{3}H-Δ^9-THC showed that this drug was transported into and accumulated by salivary tissue (22). Also, it has been demonstrated that Δ^9-THC decreased electrically stimulated salivary flow in dogs by a mechanism involving a decrease in acetylcholine release, which results in reduction of blood flow to the SMG (23).

Because cannabinoid effects were shown in salivary glands and it is known that marijuana use decreases saliva secretion, we had 3 study objectives. We aimed (i) to determine the localization of CBs in the SMG, (ii) to investigate whether both CB_1 and CB_2 are activated by AEA in this gland, and (iii) to determine the effect of AEA on saliva secretion using a novel model of intraglandular injection.

Materials and Methods

Chemicals. AEA, forskolin (FRSK), and NE-HCl were purchased from Sigma Chemical Co. (St. Louis, MO). Chloralose and methacholine (MC) were obtained from FLUKA (Laborchemikalien, Berlin, Germany). AM251 [N-(piperidin-1-yl)-1-(2,4-dichlorophenyl)-5-(4-chlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide] and AM630 6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl-1H-indol-3-yl](4-methoxyphenyl)methanone were obtained from Tocris (Ellisville, MO).

Animals. Adult male Wistar rats (250–300 g) from our own colony were kept in group cages in an animal room having a photoperiod of 12 hrs of light (0700–1900 hrs), room temperature at 22°C–25°C, and free access to rat chow and tap water. The animals were divided into several experimental groups with six to eight animals each and were kept in the laboratory area. The experimental procedures reported herein were approved by the Animal Care Committee of the Center of Experimental Pharmacology and Botanicals of the National Council for Research of Argentina and were carried out in accord with the Declaration of Helsinki.

Immunocytochemical Studies. The rat SMGs were fixed by intracardial perfusion using 4% paraformaldehyde in 0.1 *M* phosphate buffer (pH 7.4). The SMGs were post-fixed for 1 hr and were cryoprotected using graded sucrose solutions (24). Tissue blocks were frozen in N_2-cooled acetone and were sectioned at 16 μm. Primary antibodies against CB_1 (Cayman Chemical, Ann Arbor, MI) and CB_2 (Affinity Bioreagents Antibodies, Golden, CO) were used. After overnight incubation in primary antibody, sections were developed using biotinylated secondary antibodies followed by avidin-biotin-peroxidase complex (Vectastain Elite ABC, Vector Laboratories, Burlingame, CA). A color reaction was obtained using nickel-enhanced diaminobenzidine staining (25). In other sections, bound primary antibodies were labeled with fluorescein-5-isothiocyanate-conjugated goat anti-rabbit IgG and/or goat lissamine rhodamine-conjugated anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA). Glycerol-mounted specimens were observed with a Laser Scanning System Radiance 2000 (BioRad, Hemel Hempstead, UK) using the 488 line of the argon laser and followed by the 543 line of a helium-neon laser, and the emission filters HQ515/30 and HQ590/70, respectively. Optical sections separated by 1 μm were performed in the *z* axis and were processed using Confocal Assistant Software (BioRad).

***In Vitro* Studies.** The animals were killed by cervical dislocation, and the SMGs were removed. The SMGs were cut in half to enhance the penetration of the different substances into the tissue and were preincubated in 500 μl of Krebs-Ringer bicarbonate buffer medium (pH 7.4) containing 0.1% glucose within a Dubnoff metabolic shaker (50 cycles/min, 95% O_2/5% CO_2) for 15 mins before replacement with fresh medium containing the compounds to be tested. The incubation was continued for 30 mins with FRSK (7.6×10^{-5} *M*), an AC activator, or FRSK plus different doses of AEA (1×10^{-10} *M* to 1×10^{-7} *M*). AEA was first diluted in ethyl alcohol (1×10^{-2} *M*), and further dilutions were made in Krebs-Ringer bicarbonate. FRSK was added to the buffer to increase cAMP content because AEA showed no effect on cAMP basal levels.

In other experiments, the SMGs were preincubated for 15 mins in 500 µl of Krebs-Ringer bicarbonate buffer medium alone, with AM630 (6×10^{-5} *M*), a specific antagonist of CB_2, or with AM251 (6×10^{-5} *M*), a specific antagonist of CB_1. AM251 and AM630 were dissolved in dimethyl sulfoxide (2×10^{-3} *M*), and further dilutions were made in saline to reach 6×10^{-5} *M* of AM251 and AM630 before sonication. The incubation was continued for 30 mins with Krebs-Ringer (control), FRSK, FRSK+AEA (1×10^{-9} *M*), FRSK+AEA+AM630 (6×10^{-5} *M*), AM630 alone, FRSK+AEA+AM251 (6×10^{-5} *M*), or AM251 alone.

After that, the SMGs were homogenized in 1 ml of H_2O and centrifuged at 6000 *g* for 10 mins at 4°C. Supernatants were collected, and samples were stored at –20°C before assays. cAMP was measured by radioimmunoassay (RIA).

***In Vivo* Studies.** *Salivary Secretion Studies.* Salivary responses were determined in anesthetized rats (100 mg/kg of chloralose, 0.5 ml of 0.9% NaCl, iv). The ducts of the SMGs were cannulated, and the different sialogogues (NE, NA, and MC) were sequentially injected *via* the right femoral vein, as previously described (26). No resting (unstimulated) flow of saliva was observed. The secretion induced by NE (1, 3, 10, and 30 mg/kg in saline) and MC (1, 3 and 10 mg/kg in saline) during 3 mins after the administration of each dose was colleted on aluminum foil and weighed. Results were expressed as milligrams of saliva per gland.

Effect of AEA and CB Antagonists on Salivary Secretion. To evaluate the participation of the endocannabinoid system on the salivary secretion, intraglandular injections (50 µl) of agonists and/or antagonists were made 15 mins before the measurement of salivary secretion with increasing doses of NE or MC in different animals (4–5 per group). The experimental groups were: 1) saline; 2) AEA (6×10^{-5} *M*); 3) AEA+AM630 (6×10^{-4} *M*), a specific antagonist of CB_2; 4) AM630 alone; 5) AEA+AM251 (6×10^{-4} *M*), a specific antagonist of CB_1; 6) AM251 alone; and 7) AM630+AM251.

Radioimmunoassays. cAMP was measured by RIA by using the highly specific cAMP antibody kindly provided by Dr. A. F. Parlow (National Hormone & Peptide Program, Harbor-UCLA Medical Center, Torrance, CA). The sensitivity of the assay was 0.061 pmol/ml. Intraassay and interassay coefficients of variation were 8.1% and 10.5%.

Statistical Analysis. Data are presented as the mean ± SEM. For *in vitro* studies, comparisons between groups were performed using a one-way analysis of variance, followed by the Student-Newman-Keuls multiple comparisons test for unequal replicates. For *in vivo* studies, when two groups were compared, Student's *t* test was used. All analyses were conducted using the GraphPad Instat software (GraphPad Software, Inc., San Diego, CA). Differences with $P < 0.05$ were considered statistically significant.

Results

Immunohistochemical Localization of CBs. In SMG cryosections labeled with the immunoenzymatic procedure, acini and different kinds of ducts were identified by the characteristic structure in cross-sectional and longitudinal sections. As shown in Figure 1, CB_1 immunoreactivity could not be detected in acini, but it was strongly present in the ductal system. CB_1 antiserum homogeneously labeled ID cells, but it showed a distinct basal distribution in GCTs. By contrast, SD cells exhibited moderate immunolabeling of their cytoplasm and a very strong staining of their apical border. A different distribution was found for CB_2 immunoreactivity (Fig. 2). It was present in the periphery of acinar cells but could not be detected in IDs. GCTs exhibited basal CB_2 immunoreactivity in most cells but contained a distinct subpopulation with staining of the whole cytoplasm. Distribution of CB_2 immunoreactivity in SDs was almost identical to that of CB_1 immunoreactivity, with very strong labeling of the apical border and less intense labeling of the cytoplasm. Double immunofluorescent studies combining CB_2 and smooth muscle actin (SMA) labeling demonstrated that peripheral immunostaining of acinar cells reflected the expression of CB_2 in the basal cytoplasm of acinar cells and in surrounding myoepithelial cells.

Inhibitory Effects of AEA *in Vitro* on cAMP Content. *Effect of Different Concentrations of AEA on FRSK-Stimulated cAMP Content.* The cAMP content was increased ($P < 0.01$) by FRSK (7.6×10^{-5} *M*), an activator of AC. This stimulatory effect was significantly blocked ($P < 0.01$, $P < 0.01$, and $P < .001$) by AEA (10^{-7} *M*, 10^{-8} *M*, and 10^{-9} *M*, respectively), with the highest inhibitory effect occurring at 10^{-9} *M* (Fig. 3). However, 10^{-10} *M* AEA had no effect on FRSK-stimulated cAMP content.

Effect of AEA Plus AM630 or AM251 on FRSK-Stimulated cAMP Content. To investigate whether the inhibition of AEA on cAMP content was mediated by CB_1 or CB_2, the specific antagonists of CB_1 and CB_2, AM251 (10^{-5} *M*) and AM630 (10^{-5} *M*), respectively, were added to the media concomitantly with AEA (10^{-9} *M*)+FRSK (7.6×10^{-5} *M*) or alone. The concentration of the antagonists used was 10^{-5} *M* because lower concentrations were not capable of blocking the inhibitory effect of AEA. AM251 partially blocked ($P < 0.01$) the inhibition of AEA on FRSK-stimulated cAMP content (Fig. 4). Similarly, AM630 blocked ($P < 0.05$), but not totally, the inhibition of AEA on FRSK-stimulated cAMP content. Neither AM251 nor AM630 alone had any effect on cAMP content.

Inhibitory Effects of AEA *in Vivo* on Salivary Secretion. *Effect of AEA on MC-Stimulated Salivary Secretion.* The intra-SMG injection of AEA (6×10^{-5} *M*) inhibited the saliva secretion stimulated by three different doses of MC (Fig. 5). At a dose of 3 µg/kg of MC, AEA reduced salivation 2.5-fold ($P < 0.01$). At a dose of 10 µg/kg of MC, AEA reduced salivation 2-fold ($P < 0.01$). The

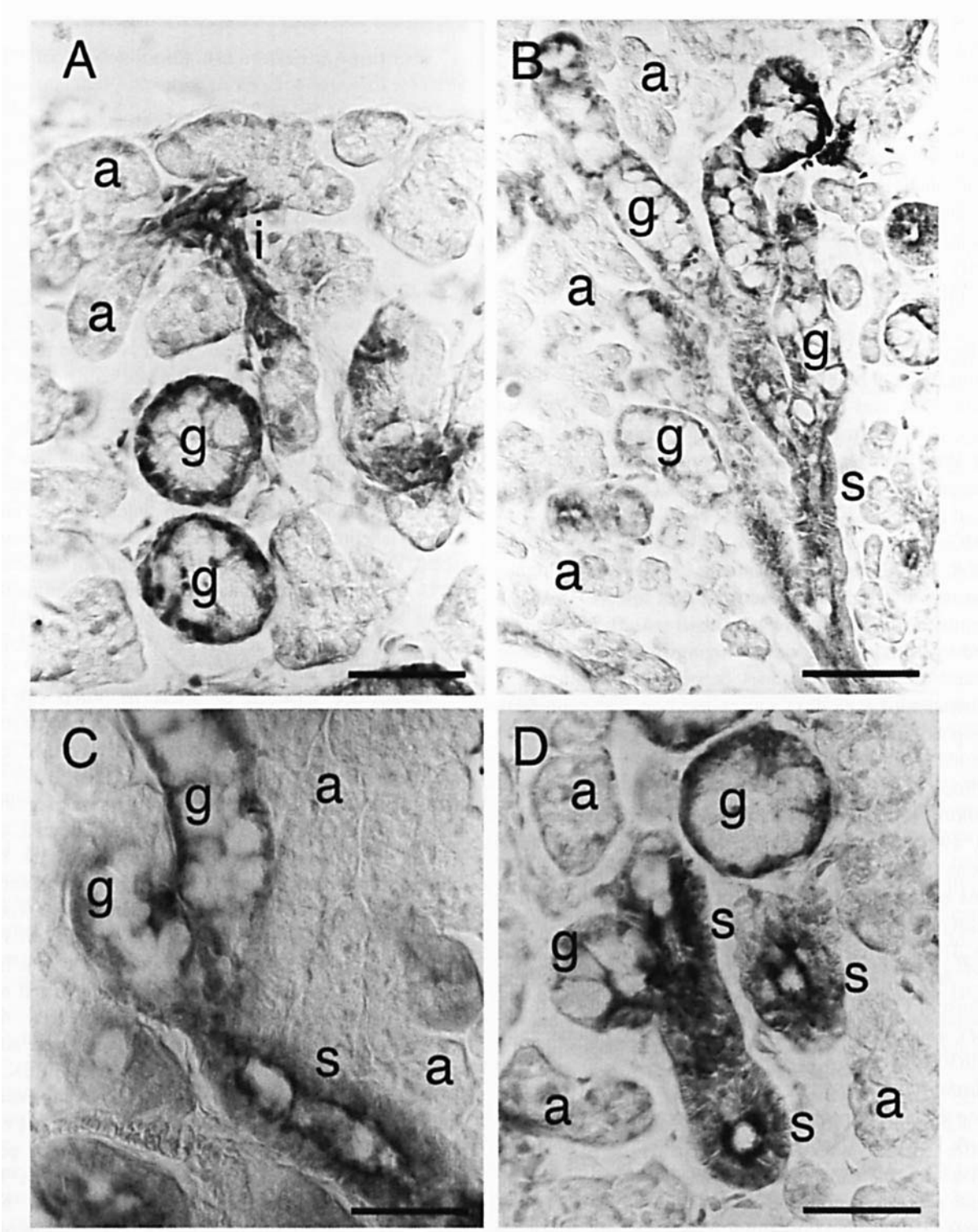

Figure 1. CB_1 immunoreactivity detected in SMG cryosections using an immunoenzymatic procedure with nickel enhancement. Abbreviations for this and the following plate: (a) acinus; (i) intercalated duct; (g) GCT; and (s) SD. In images A through D, CB_1 immunoreactivity appears as a black deposit that is absent in acini but is present along the different segments of the ductal system. (A) Acini show no immunostaining, but moderate labeling is observed in intercalated ducts. Calibration bar, 50 μm. (B) A low-power view showing the continuity of GCTs with more distal SDs. Both ductal segments show strong immunostaining. In GCTs, CB_1 immunoreactivity is mainly localized in basal regions, whereas in SDs it occupies all the cytoplasm but is concentrated in the luminal surface. Calibration bar, 100 μm. (C) A GCT and an SD are shown at higher magnification. Notice the different distribution of CB_1 immunoreactivity in each ductal segment. Calibration bar, 50 μm. (D) Transverse sections of GCTs and SDs confirm the basal distribution of CB_1 immunoreactivity in the former and the strong staining of the apical surface in the latter. Calibration bar, 50 μm.

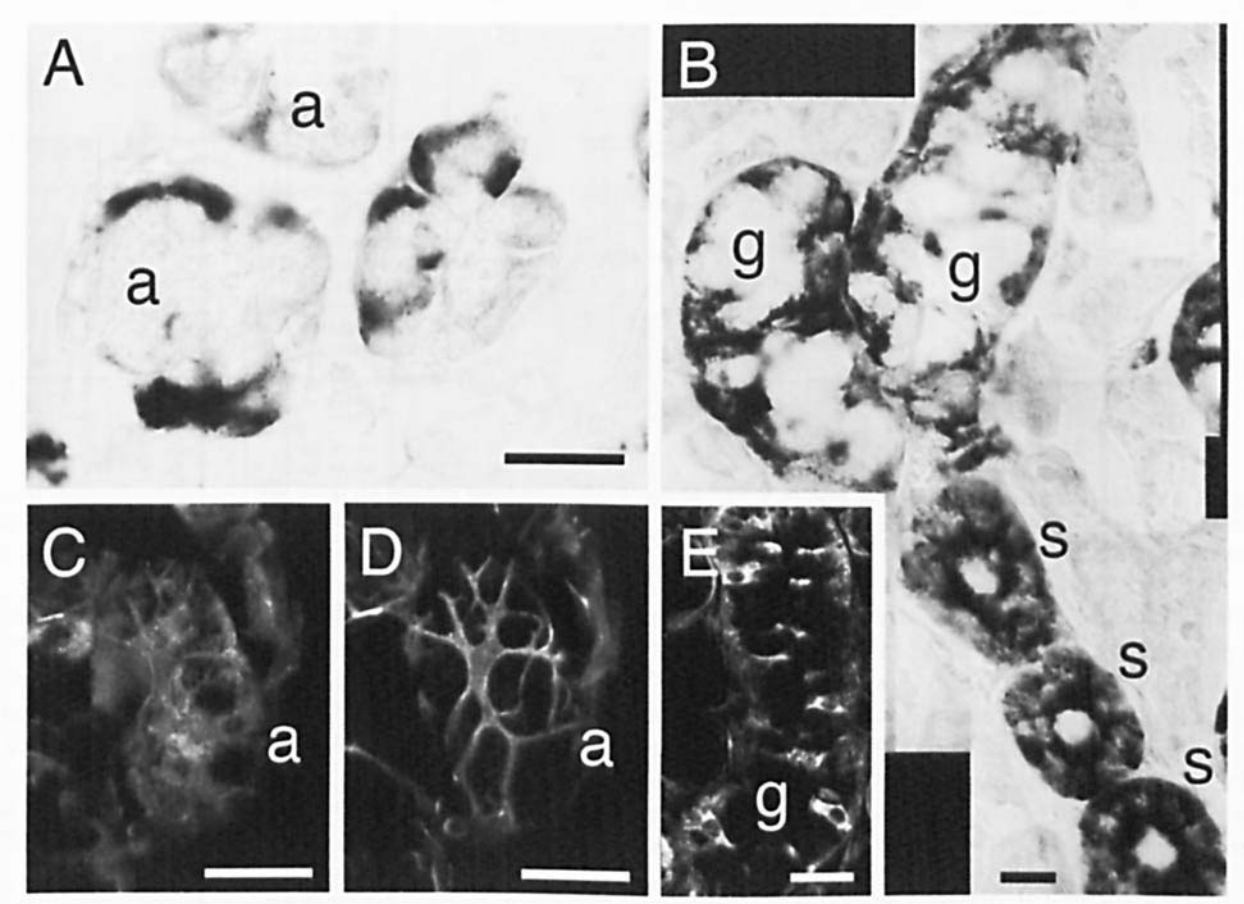

Figure 2. Distribution of CB_2 immunoreactivity in SMG cryosections labeled with immunoenzymatic (A and B) and immunofluorescent (C through E) procedures. Calibration bars for all figures, 20 μm. (A) A high-magnification view of submandibullary acini showing that CB_2 immunoreactivity occupies a peripheral position. (B) A composite image showing the strong immunostaining of GCTs and SDs. Distribution of CB_2 immunoreactivity follows the same pattern of CB_1 immunoreactivity but appears stronger. (C and D). Confocal images of an acinus labeled with CB_2 and SMA antibodies, respectively. SMA immunofluorescence (D) is present in long and slender cell processes wrapped around the acinus and corresponding to myoepithelial cells. The same structure can be seen in (C), demonstrating that myoepithelial cells express CB_2. Notice that CB_2 immunofluorescence is also present in the cytoplasm of acinar cells. (E) Image of GCT illustrating the presence of two different patterns of CB_2 immunofluorescence. Most cells contain basal labeling, but a subpopulation of cells exhibits stronger immunofluorescence that occupies all apicobasal levels of the cytoplasm.

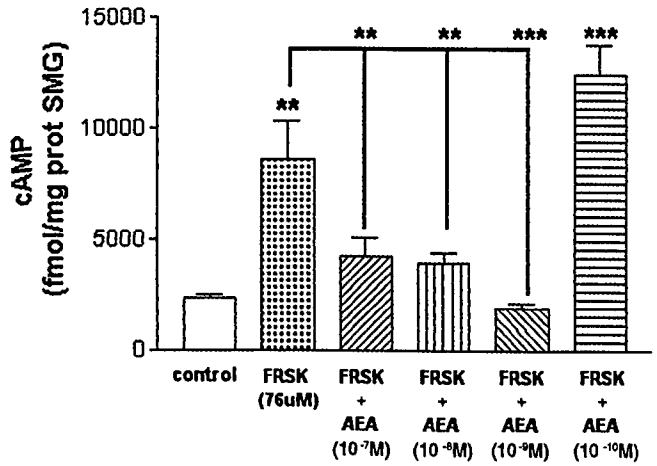

Figure 3. Effect of FRSK (7.6×10^{-5} *M*) and different doses of AEA (10^{-7} *M* to 10^{-10} *M*) on cAMP content from SMG incubated *in vitro*. $^{**}P < 0.01$; $^{**}P < 0.001$ versus control; $++P < 0.01$; and $+++P < 0.001$ versus FRSK.

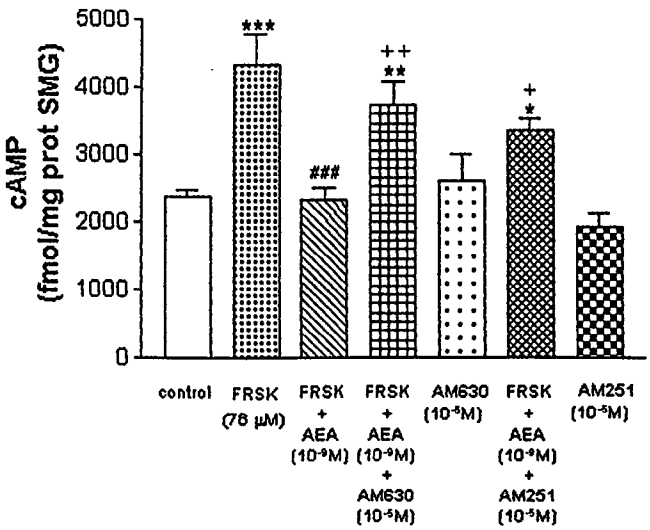

Figure 4. Effect of AM251 (10^{-5} *M*) and AM630 (10^{-5} *M*) on AEA-inhibited cAMP content. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus control; $+P < 0.05$; $++P < 0.01$ versus FRSK+AEA; and $^{###}P < 0.001$ versus FRSK.

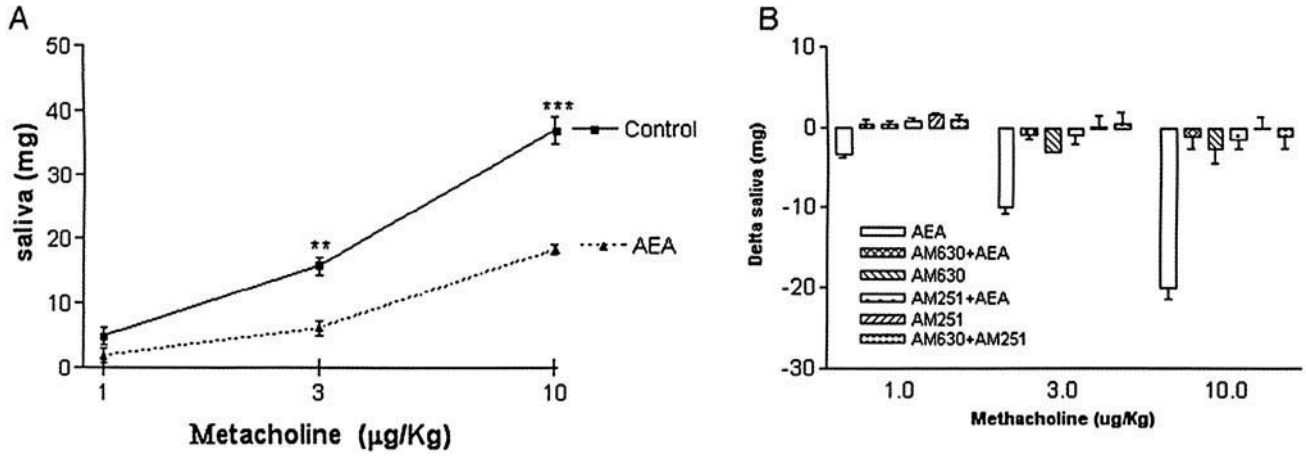

Figure 5. Effect of intraglandular injection of AEA (6×10^{-5} *M*) on (A) MC-stimulated salivary secretion and (B) Δ saliva was calculated by subtracting the baseline level in stimulated salivary secretion for control rats. Values are mean ± SE. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus respective control.

inhibitory effect of AEA on MC-induced saliva secretion was prevented by injecting AM251 (6×10^{-4} *M*) or AM630 (6×10^{-4} *M*) at three MC doses. The injection of AM251 alone had a stimulatory effect ($P < 0.05$) on the saliva secretion induced by the lowest MC dose (1 µg/kg), whereas AM630 alone did not modify MC-induced salivation. However, simultaneous injection of both antagonists increased the salivation ($P < 0.01$) induced by the lowest MC dose over the volume obtained with AM251 injected alone. The injection of saline had no significant effect on any dose (data not shown). These results are also given in more detail in Table 1.

Effect of AEA on NE-Stimulated Salivary Secretion. At the dose of 1 µg/kg of NE, AEA reduced salivation 9-fold ($P < 0.01$) in comparison to the control curve (Fig. 6). At a dose of 3 µg/kg of NE, AEA reduced salivation 4-fold ($P < 0.001$). At a dose of 10 µg/kg of NE, AEA reduced salivation 2.5-fold ($P < 0.01$). At a dose of 30 µg/kg of NE, AEA reduced salivation 2-fold ($P < 0.001$). The inhibitory effect of AEA on saliva secretion was prevented by injecting AM251 (6×10^{-4} *M*) or AM630 (6×10^{-4} *M*) at all the doses observed. The injection of AM251 alone had a stimulatory effect ($P < 0.05$) on the saliva secretion induced by the lowest dose of NE utilized (1 µg/kg), which was synergized ($P < 0.01$) after the coinjection with AM630. The injection of AM630 alone as well as saline (data not shown) had no significant effect at any dose. These results are also given in more detail in Table 2.

Discussion

The present work shows for the first time, to our knowledge, the presence of CBs in the SMG. Both CB_1 and CB_2 were immunohistochemically detected in acinar and ductal components of the gland, and evidence of their role in saliva secretion was obtained.

CB_1 was mainly localized in the ductal system, from IDs to SDs. CB_2 immunoreactivity was also detected in the acini, cellular structures that are responsible for the production and

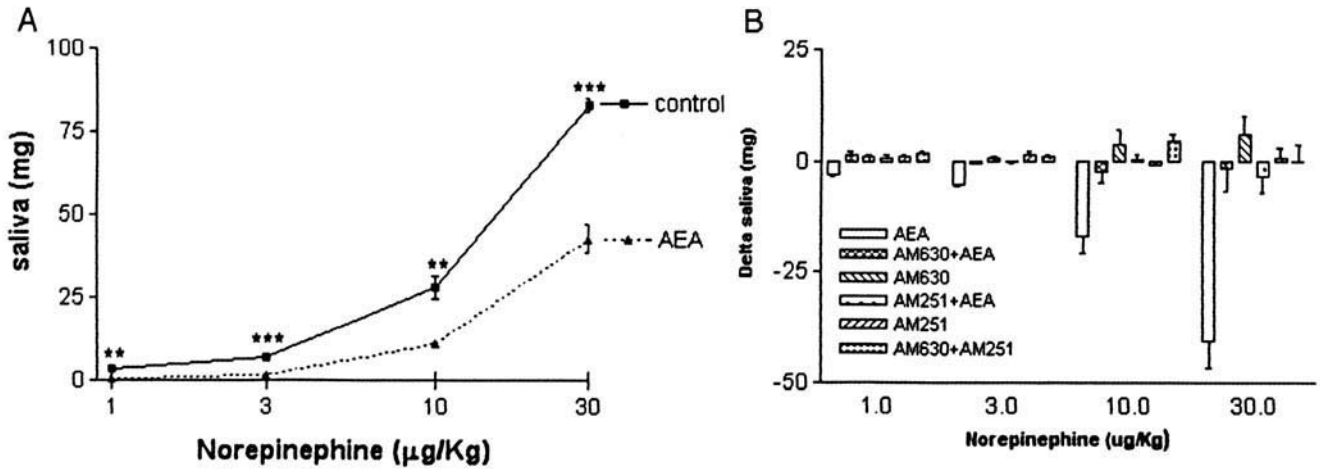

Figure 6. Effect of intraglandular injection of AEA (6×10^{-5} *M*) on (A) NE-stimulated salivary secretion and (B) Δ saliva. Values are mean ± SE. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus respective control.

Table 1. Effect of Intraglandular Injection of AEA and/or CB Antagonist on MC-Stimulated Salivary Secretion. The results are expressed in milligrams of saliva. Values are mean ± SE. *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$ versus respective control

	AEA		AM630+AEA		AM630		AM251+AEA		AM251		AM630+AM251	
MC (μg/kg)	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
1	5.0 ± 1.4	2.0 ± 1.1	4.7 ± 1.3	5.0 ± 1.1	4.8 ± 1.3	5.7 ± 1.7	4.7 ± 1.3	6.7 ± 0.8	4.8 ± 0.9	7.8 ± 0.7*	5.2 ± 0.3	8.6 ± 0.4**
3	15.8 ± 1.4	6.3 ± 1.1**	14.6 ± 1.3	13.7 ± 1.0	15.0 ± 1.4	11.4 ± 2.0	14.8 ± 1.3	14.2 ± 1.3	15.2 ± 1.4	15.5 ± 1.3	16.5 ± 1.4	18.2 ± 0.3
10	37.0 ± 2.2	18.3 ± 0.8***	34.3 ± 2.0	34.9 ± 2.0	35.2 ± 2.1	31.9 ± 2.9	34.7 ± 2.0	33.3 ± 1.5	35.6 ± 2.1	36.1 ± 1.7	39.3 ± 1.4	37.6 ± 1.6

Table 2. Effect of Intraglandular Injection of AEA and/or CB Antagonist on NE-Stimulated Salivary Secretion. The results are expressed in milligrams of saliva. Values are mean ± SE. *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$ versus respective control

	AEA		AM630+AEA		AM630		AM251+AEA		AM251		AM630+AM251	
NE (μg/kg)	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
1	3.5 ± 0.7	0.4 ± 0.2**	3.8 ± 0.7	7.1 ± 0.4	3.9 ± 0.7	6.5 ± 1.2	3.4 ± 0.7	5.7 ± 2.3	3.6 ± 0.7	5.9 ± 0.5*	3.3 ± 0.3	7.0 ± 0.6**
3	7.0 ± 0.6	1.7 ± 0.3***	7.5 ± 0.7	7.2 ± 0.3	7.8 ± 0.7	8.7 ± 0.9	6.8 ± 0.6	6.5 ± 0.3	7.2 ± 0.6	8.5 ± 0.3	6.7 ± 0.3	8.8 ± 0.7
10	28.0 ± 3.6	11.0 ± 0.9**	30.0 ± 3.9	27.0 ± 2.8	31.1 ± 4.0	37.1 ± 5.4	27.2 ± 3.5	29.0 ± 4.7	28.8 ± 3.7	26.6 ± 0.4	26.6 ± 2.6	33.7 ± 3.2
30	83.3 ± 2.0	42.8 ± 4.3***	89.1 ± 2.2	84.8 ± 9.3	92.4 ± 2.2	103.3 ± 7.6	81.0 ± 2.0	75.3 ± 6.9	85.8 ± 2.1	86.6 ± 3.7	79.1 ± 2.5	79.1 ± 2.9

release of the primary saliva, and in myoepithelial cells, cells that are located close to the acini and whose contraction facilitates the release of the acinar secretion. The acini have rounded nuclei, typical of serous acini and basal cytoplasmic basophilia. Two different morphologic classes of acini as described in humans are not observed in the rat. CB_2 immunoreactivity was not detected in IDs, but it was present in more distal ducts in the same localizations as CB_1. Therefore, CBs could be associated with the two fundamental mechanisms of salivary secretion: acinar release of protein and fluid and ductal modification of primary secretion.

Immunohistochemical studies have shown the presence of CB_1 immunoreactivity in the epithelia of the prostate, where it also negatively regulates AC activity (27). CB_1 immunoreactivity has also been demonstrated in follicular and parafollicular cells of the thyroid, where they can decrease the release of triiodothyronine and thyroxine (28). Although in the different organs along the gastrointestinal tract, CB_1 has been mainly found on neurons and nerve fibers (29, 30), no evidence of the colocalization of CBs with neuronal markers was found in the SMG.

There are two major signal transduction pathways implicated in salivary gland cells. One involves the generation of cAMP, and the other involves the breakdown of plasma membrane polyphosphoinositides (31). It is known that cAMP mediates the mobilization of intracellular Ca^{+2} by β-adrenergic stimulation in acinar cells as well as GCTs (32). Moreover, an increase in the intracellular level of cAMP stimulates some salivary functions, such as salivary flow rate and secretion of proteins (33).

Taking into account the importance of AC activity for salivary function, and because the mechanism of action of cannabinoids acting on its receptors is by inhibition of AC with consequent decrease in cAMP (5), we measured the effect of AEA on FRSK-induced cAMP levels. This endocannabinoid markedly reduced FRSK-induced increase of cAMP, and this effect was blocked by the CB_1 and CB_2 antagonists, indicating that both receptors are implicated in SMG physiology. The inhibitory effect of AEA on cAMP accumulation was not blocked completely by incubating the selective antagonists separately, constituting additional evidence for the presence and function of both CB_1 and CB_2 in the SMG.

The cAMP response element (CREB) located at the 5′-promoter region of cAMP-regulated genes is phosphorylated by cAMP protein kinase inducing their transcription (34). In the SMG, CREB elements are concentrated in GCTs (35), where we detected the highest immunoreactivity for CB_1 and CB_2. This localization suggests that CBs could modulate hormonal effects on these specialized ducts.

Salivation is controlled by the autonomic nervous system not only by direct activation of salivary gland receptors but also by activation of brain mechanisms (36–39). Moreover, it has been reported that atropine, a muscarinic antagonist, injected intracerebroventricularly reduced the salivation induced by pilocarpine, a muscarinic agonist, injected ip, suggesting that pilocarpine enters the brain and acts on central muscarinic receptors, activating autonomic efferents to induce salivation (37). In addition, it was shown that the injection intracerebroventricularly of isoproterenol, a β-adrenoreceptor agonist, produced an increased salivary flow, demonstrating the participation of a central adrenergic system in the regulation of the salivary flow. Therefore, it is possible that the sialogogues injected through the femoral vein acted not only on their receptors in the SMG but also on their receptors at the brain level, stimulating efferent responses in the periphery that could be decreased by endocannabinoids. Therefore, the AEA injected intraglandularly, acting mainly through CB_1, could inhibit the release of neurotransmitters from presynaptic terminals on the SMG, as it has been demonstrated that the endocannabinoid system decreases autonomic neurotransmission. Moreover, it was shown that THC decreased salivary flow from the SMG during electrical stimulation by a mechanism involving a decrease in the release of acetylcholine (23). Also, evidence exists that AEA and THC abolished NE release in other systems by presynaptic inhibition (20). Although CB immunoreactivities have been detected in the enteric innervation (40), we were not able to detect them in SMG nerves. Our results showed that AEA injected intraglandularly inhibited NE- and MC-stimulated saliva secretion *in vivo* and that both AM251 and AM630 prevented that inhibition. These results provided evidence that both CB_1 and CB_2 are implicated in the modulation of saliva secretion. The CB_2 mainly located peripherally to the acini could regulate the release of saliva from these cells to the salivary ducts. The function of CB_1 in the SMG is more difficult to explain, as these receptors were not immunodetected in the vicinity of the acini. However, the immunodetection of CB_1 at the ductal system suggests a paracrine effect from the duct cells to the acinar cells to reduce the volume of saliva.

In addition, CB_1 and CB_2 antagonists increased the induced salivation at lower doses of sialogogues. These findings indicate that there is an endogenous endocannabinoid tone that could regulate salivary secretion.

In summary, the inhibitory effects of AEA on saliva secretion could be exerted at different levels 1) as a relaxant of myoepithelial cells or 2) within the cytoplasm of the secretory cells, blocking the intracellular signaling pathways activated by NE or MC. 3) Finally, because intravenous sialogogues could also induce some central activation of salivation, AEA may also control the release of cholinergic, adrenergic, or peptidergic neurotransmitters from the presynaptic terminals to the salivary glands.

We thank Ricardo Horacio Orzuza for technical assistance.

1. Devane WA, Dysarz FA III, Johnson MR, Melvin LS, Howlett AC. Determination and characterization of a cannabinoid receptor in rat brain. Mol Pharmacol 34:605–613, 1988.
2. Herkenham M, Lynn AB, Little MD, Johnson MR, Melvin LS, de Costa BR, Rice KC. Cannabinoid receptor localization in brain. Proc Natl Acad Sci U S A 87:1932–1936, 1990.

3. Pertwee RG, Ed. Cannabinoid Receptors. London: Academic Press, pp1–34, 1995.
4. Munro S, Thomas KL, Abu-Shaar M. Molecular characterization of a peripheral receptor for cannabinoids. Nature 365:61–65, 1993.
5. Howlett AC, Fleming RM. Cannabinoid inhibition of adenylate cyclase: pharmacology of the response in neuroblastoma cell membranes. Mol Pharmacol 26:532–538, 1984.
6. Mechoulam R, Fride E, Di Marzo V. Endocannabinoids. Eur J Pharmacol 359:1–18, 1998.
7. Gatley SJ, Gifford AN, Volkow ND, Lan R, Makriyannis A. ^{123}I-labeled AM251: a radioiodinated ligand which binds *in vivo* to mouse brain cannabinoid CB_1 receptors. Eur J Pharmacol 307:331–338, 1996.
8. Lan R, Gatley J, Lu Q, Fan P, Fernando SR, Volkow ND, Pertwee R, Makriyannis A. Design and synthesis of the CB1 selective cannabinoid antagonist AM281: a potential human SPECT ligand. AAPS PharmSci 1:E4, 1999.
9. Rinaldi-Carmona M, Barth F, Heaulme M, Alonso R, Shire D, Congy C, Soubrie P, Breliere JC, Le Fur G. Biochemical and pharmacological characterisation of SR141716A, the first potent and selective brain cannabinoid receptor antagonist. Life Sci 56:1941–1947, 1995.
10. Rinaldi-Carmona M, Barth F, Heaulme M, Shire D, Calandra B, Congy C, Martinez S, Maruani J, Neliat G, Caput D, Ferrara P, Soubrie P, Lefue G. SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. FEBS Lett 350:240–244, 1994.
11. Felder CC, Joyce KE, Briley EM, Glass M, Mackie KP, Fahey KJ, Cullinan GJ, Hunden DC, Johnson DW, Chaney MO, Koppel GA, Brownstein M. LY320135, a novel cannabinoid CB1 receptor antagonist, unmasks coupling of the CB1 receptor to stimulation of cAMP accumulation. J Pharmacol Exp Ther 284:291–297, 1998.
12. Pertwee R, Griffin G, Fernando S, Li X, Hill A, Makriyannis A. AM630, a competitive cannabinoid receptor antagonist. Life Sci 56: 1949–1955, 1995.
13. Griffin G, Wray EJ, Tao Q, McAllister SD, Rorrer WK, Aung MM, Martin BR, Abood ME. Evaluation of the cannabinoid CB_2 receptor–selective antagonist, SR144528: further evidence for cannabinoid CB_2 receptor absence in the rat central nervous system. Eur J Pharmacol 377:117–125, 1999.
14. Hosohata Y, Quock RM, Hosohata K, Makriyannis A, Consroe P, Roeske WR, Yamamura HI. AM630 antagonism of cannabinoid-stimulated [^{35}S]GTPγS binding in the mouse brain. Eur J Pharmacol 321:R1–R3, 1997.
15. Tandler B, Phillips CJ. Microstructure of mammalian salivary glands and its relationship to diet. In: Garret JR, Ekstrom J, Anderson LC, Eds. Glandular Mechanisms of Salivary Secretion: Oral Frontiers on Biology. Basel: Karger, Vol 10:pp21–35, 1998.
16. Thaysen JH, Thorn NA, Schwartz IL. Excretion of sodium, potassium, chloride and carbon dioxide in human parotid saliva. Am J Physiol 178: 155–159, 1954.
17. Cook DI, Young JA. Fluid and electrolyte secretion by salivary glands. In: Handbook of Physiology: The Gastrointestinal System: Salivary, Gastric, Pancreatic, and Hepatobilary Secretion. Bethesda: American Physiological Society, Vol 3:pp1–23, 1989.
18. Lung MA. Autonomic nervous control of myoepithelial cells and secretion in submandibular gland of anaesthetized dogs. J Physiol 546(Pt 3):837–850, 2003.
19. Man YG, Ball WD, Marchetti L, Hand AR. Contributions of intercalated duct cells to the normal parenchyma of submandibular glands of adult rats. Anat Rec 263:202–214, 2001.
20. Ishac EJ, Jiang L, Lake KD, Varga K, Abood ME, Kunos G. Inhibition of exocytotic noradrenaline release by presynaptic cannabinoid CB1 receptors on peripheral sympathetic nerves. Br J Pharmacol 118:2023–2028, 1996.
21. Szabo B, Nordheim U, Niederhoffer N. Effects of cannabinoids on sympathetic and parasympathetic neuroeffector transmission in the rabbit heart. J Pharmacol Exp Ther 297:819–826, 2001.
22. McConnell WR, Borzelleca JF. A study of the mechanism of transport of Δ^9-tetrahydrocannabinol in the rat submaxillary gland *in vitro*. Arch Int Phamacodyn Ther 235:180–186, 1978.
23. McConnell WR, Dewey WL, Harris LS, Borzelleca JF. A study of the effect of Δ^9-tetrahydrocannabinol (Δ^9-THC) on mammalian salivary flow. J Pharmacol Exp Ther 206:567–573, 1978.
24. Barthel LK, Raymond PA. Improved method for obtaining 3-microns cryosections for immunocytochemistry. J Histochem Cytochem 38: 1383–1388, 1990.
25. Suburo AM, Wheatley SC, Horn DA, Gibson SJ, Jahn R, Fischer-Colbrie R, Wood JN, Latchman DS, Polak JM. Intracellular redistribution of neuropeptides and secretory proteins during differentiation of neuronal cell lines. Neuroscience 46:881–889, 1992.
26. Bianciotti LG, Elverdin JC, Vatta MS, Colatrella C, Fernandez BE. Atrial natriuretic factor enhances induced salivary secretion in the rat. Regul Pept 49:195–202, 1994.
27. Ruiz-Llorente L, Sanchez MG, Carmena MJ, Prieto JC, Sanchez-Chapado M, Izquierdo A, Diaz-Laviada I. Expression of functionally active cannabinoid receptor CB_1 in the human prostate gland. Prostate 54:95–102, 2003.
28. Porcella A, Marchese G, Casu MA, Rocchitta A, Lai ML, Gessa GL, Pani L. Evidence for functional CB1 cannabinoid receptor expressed in the rat thyroid [published correction appears in Eur J Endocrinol 147: 434, 2002]. Eur J Endocrinol 147:255–261, 2002.
29. Casu MA, Porcella A, Ruiu S, Saba P, Marchese G, Carai MA, Reali R, Gessa GL, Pani L. Differential distribution of functional cannabinoid CB_1 receptors in the mouse gastroenteric tract. Eur J Pharmacol 459: 97–105, 2003.
30. MacNaughton WK, Van Sickle MD, Keenan CM, Cushing K, Mackie K, Sharkey KA. Distribution and function of the cannabinoid-1 receptor in the modulation of ion transport in the guinea pig ileum: relationship to capsaicin-sensitive nerves. Am J Physiol Gastrointest Liver Physiol 286:G863–G871, 2004.
31. Baum BJ. Neurotransmitter control of secretion. J Dent Res 66:628–632, 1987.
32. Valdez IH, Turner RJ. Effects of secretagogues on cytosolic Ca^{2+} levels in rat submandibular granular ducts and acini. Am J Physiol 261:G359–G363, 1991.
33. Abdollahi M, Simaiee B. Stimulation by theophylline and sidelnafil of rat submandibular secretion of protein, epidermal growth factor and flow rate. Pharmacol Res 48:445–449, 2003.
34. Johannessen M, Delghandi MP, Moens U. What turns CREB on? Cell Signal 16:1211–1227, 2004.
35. Kim J, Amano O, Wakayama T, Takahagi H, Iseki S. The role of cyclic AMP response element–binding protein in testosterone-induced differentiation of granular convoluted tubule cells in the rat submandibular gland. Arch Oral Biol 46:495–507, 2001.
36. Moreira TS, Thomaz AC, De Luca LA Jr, Renzi A, Menani JV. Moxonidine reduces pilocarpine-induced salivation in rats. Auton Neurosci 91:32–36, 2001.
37. Renzi A, Colombari E, Mattos Filho TR, Silveira JE, Saad WA, Camargo LA, De Luca LA Jr, Derobio JG, Menani JV. Involvement of the central nervous system in the salivary secretion by pilocarpine in rats. J Dental Res 72:1481–1484, 1993.
38. Renzi A, De Luca LA Jr, Menani JV. Lesions of the lateral hypothalamus impair pilocarpine-induced salivation in rats. Brain Res Bull 58:455–459, 2002.
39. Moreita TS, Takakura AC, De Luca LA Jr, Renzi A, Menani JV. Inhibition of pilocarpine-induced salivation in rats by central noradrenaline. Arch Oral Biol 47:429–434, 2002.
40. Wright K, Rooney N, Feeney M, Tate J, Robertson D, Welham M, Ward S. Differential expression of cannabinoid receptors in the human colon: cannabinoids promote epithelial wound healing. Gastroenterology 129:437–453, 2005.