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Expression and biological effects of CB_1 cannabinoid receptor in rat parotid gland

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Abstract

Experiments were designed to determine whether cannabinoids affect salivary gland function. For this purpose, the effect of anandamide on cAMP accumulation, amylase release and Na^+-K^+-ATPase activity was studied in rat parotid glands. Anandamide induced a concentration-dependent increase in cAMP and led to amylase release but inhibited Na^+-K^+-ATPase activity. These effects were blocked by the CB_1 cannabinoid receptor antagonist, AM281. The inhibition of adenylyl cyclase activity by SQ 22536 impaired amylase release and Na^+-K^+-ATPase inhibition. The effect of anandamide on cAMP accumulation significantly correlated with its action either on amylase release or on Na^+-K^+-ATPase activity. Such correlation strongly supports the view that the effect of anandamide on amylase release and Na^+-K^+-ATPase activity is the result of cAMP accumulation. The relative potencies of the CB_1 cannabinoid receptor antagonist, AM281, to block these three functional responses were similar, supporting the view that anandamide actions in parotid glands were achieved through a single receptor subtype, the CB_1. Binding studies using the selective cannabinoid CB_1 receptor antagonist, [^{3}H]SR141716A, indicated the presence of the specific binding site. It may be concluded that in parotid glands the endogenous cannabinoid anandamide, bound to the CB_1 cannabinoid receptor subtype, induces cAMP accumulation which in turn leads to amylase release and Na^+-K^+-ATPase inhibition.

Keywords: Anandamide; Parotid gland; Cyclic AMP; Amylase; Na^+-K^+-ATPase; CB_1 cannabinoid receptor

1. Introduction

As accessory exocrine glands of the digestive tract, the salivary glands supply a variety of proteins, fluids and electrolytes that play key roles in maintaining the environment of the oral cavity, and in facilitating the onset of the digestive process. Under sympathetic and parasympathetic control, the major salivary glands secrete saliva with various components; sympathetic nerve excitation elicits protein-rich saliva, whereas parasympathetic stimulation induces fluid secretion with low protein concentration. These receptor systems seem to act in collaboration with other transmitters as histamine [1], nitric oxide [2] substance P [3] or prostaglandins [4]. On the other hand, salivary gland function can be altered by changes in dietary consistency, radiation therapy, medications, and viral or genetic diseases [5].

Cannabinoids are natural compounds derived from the plant, *Cannabis sativa*. Recently, their presence in the salivary glands of an obligate ectoparasitic arthropod has been demonstrated [6]. Medicinal properties of cannabis were recognized some 5000 years ago and potential therapeutic applications include analgesia, attenuation of nausea and vomiting of cancer chemotherapy, antirheumatic and antipyretic actions decreased bronchial constrictions and decreased intestinal motility [7]. The synthetic cannabinoid drugs nabilone and Δ^9-tetrahydrocannabinol are already used clinically to suppress nausea and vomiting provoked by anticancer drugs or to boost the appetite of AIDS patients [8]. Many of the pharmacological effects reported for cannabinoids are now known to be mediated by CB_1 receptors present in the central nervous system as well as in certain neuronal and non-neuronal peripheral

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tissues or by CB_2 receptors found mainly in cells of the immune system [8]. Recent advances in cannabinoid pharmacology have led to a considerable increase in our understanding of cannabinoid physiology. Both receptor types are members of the super-family of G-protein linked receptors. Among the various physiological actions of cannabinoids, they have been shown to inhibit adenylyl cyclase activity in different systems leading to a reduction in adenosine 3′,5′-cyclic monophosphate (cAMP) levels [9]. However, other studies have shown that cannabinoids increase basal cAMP accumulation in various systems [10].

As stated above, salivary glands are target organs for multiple stimuli including cannabinoids, which were found to modulate mammalian salivary gland function [11,12]. However, up to date there is no evidence of an action of cannabinoids on cAMP levels in salivary glands although in parotid acinar cells cAMP represents a major messenger system that links receptor activation to amylase release [13]. Underlying the ionic mechanism of saliva secretion, the participation of Na^+-K^+-ATPase [14] is essential. Δ-tetrahydrocannabinol was shown to alter various ATPase activities in brain and other tissues [15–17]. On the other hand, recent evidence indicates that the activity of Na^+-K^+-ATPase can be modulated by increasing cAMP levels [18].

In this work we studied the action of the cannabinoids on salivary gland function. For this purpose, we investigated the effect of the endogenous cannabimimetic eicosanoid anandamide on cAMP accumulation, amylase release and Na^+-K^+-ATPase activity in rat parotid gland.

2. Materials and methods

2.1. Drugs

Anandamide and 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-morpho-linyl-1H-pyrazole-3-carboxamide (AM281) were from Tocris Cookson Ltd., UK; 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ 22536) and verapamil hydrochloride were from Research Biochemicals Internationals, USA and dibutyryl adenosine 3′,5′-cyclic monophosphate sodium salt (db cAMP) was from Sigma, USA.

2.2. Animals

Male Wistar rats weighing 250–300 g were used throughout. Animals had free access to food and water until the night before experiments when food, but not water, was withdrawn. Animals were handled according to ''The Guide to the Care and Use of Experimental Animals'' (DHEW Publication, NIH 80-23).

2.3. Experimental procedure for determination of cyclic AMP levels

Free connective tissue and fat were gently removed from parotid glands under a magnifying glass, and the anterior lobe was cut into small slices which were incubated in a final volume of 1 ml of Krebs Ringer bicarbonate medium (KRB) with 5% CO_2 in O_2 for 30 min at 37 °C in the presence of 0.1 mmol/l of isobutyl-1-methyl-xanthine (IBMX). The effect o f 1×10^{-10} to 1×10^{-3} M anandamide alone and in the presence of 1×10^{-7}, 3×10^{-7} and 1×10^{-6} M AM281 on cyclic AMP (cAMP) levels was tested. AM281 was added at the beginning of the incubation time while anandamide was allowed to react during the last 15 min. After incubation, tissues were homogenized in 2 ml of absolute ethanol and centrifuged at 6000 × *g* for 15 min at 4 °C. Supernatants were collected and evaporated to dryness, and residues re-suspended in 5 mmol/Tris–HCl (pH 7.4) containing 8 mmol/l theophylline, 5 mmol/l EDTA, and 6 mmol/l 2-mercaptoethanol. cAMP levels were determined using the Biotrak cAMP [^{3}H] assay system (Amersham Life Science-Protocol-cAMP [^{3}H] assay), based on competition between unlabeled cAMP and a fixed quantity of the tritium-labeled compound for binding to a protein with high specificity and affinity for cAMP.

2.4. Experimental procedure for amylase assay

Free connective tissue and fat were gently removed from parotid glands under a magnifying glass, and the anterior lobe was cut into small slices which were placed in tubes containing 500 μl of KRB medium pH 7.4 without glucose and with 5 mM β-hydroxybutyric acid, bubbled with 95% O_2 and 5% CO_2 and incubated at 37 °C for 30 min. When used, inhibitors were included from the beginning of the incubation time and anandamide was added during the last 15 min. Total amylase content or that released into the medium was determined by the method described by Bernfeld [19] using starch suspension as the substrate. Incubation medium and homogenized gland supernatant aliquots were incubated at 20 °C with a 1% starch suspension during 3 min. The reaction was stopped by the addition of dinitro-salicylic acid solution. After 5 min of boiling water bath, absorbance was measured at a wavelength of 540 nm. Amylase activity in the medium was expressed as a percentage of total activity.

2.5. Membrane preparation for ATPase activity determination

Free connective tissue and fat were gently removed from the parotid glands under a magnifying glass, and the anterior lobe was cut into small slices which were placed in tubes containing 500 μl of KRB solution pH 7.4 bubbled with 95% O_2 and 5% CO_2 and incubated at 37 °C for

30 min. When used, inhibitors were included from the beginning of the incubation time and anandamide was added during the last 15 min. The reaction was stopped by removing parotid slices and homogenizing them at 4 °C in tubes containing 750 μl of Tris–HCl 10 mM, EDTA 1 mM, LiBr 0.4 M (hypotonic shock) and supplemented with the protease inhibitor, phenylmethylsulfonyl fluoride 0.1 mM. Homogenates were centrifuged for 10 min at 1000 × *g*, and supernatants collected and spun down for 20 min at 9000 × *g*. Both steps almost completely eliminated nucleus, mitochondrial and lysosomal fractions, and the resultant supernatant was centrifuged for 60 min at 100,000 × *g*. The pellet was then re-suspended in Tris–HCl 10 mM, EDTA 1 mM and the same protease inhibitor and stored at −70 °C until used.

2.6. Determination of Na^+-K^+-ATPase activity

Membrane aliquots (approximately 10–20 μg of protein) were transferred to the Na^+-K^+-ATPase assay medium (final volume 172 μl) containing (in mM): NaCl, 100; KCl, 20; $MgCl_2$, 3, Tris–HCl, 160 (pH 7.4); and Na_2ATP, 4; and incubated for 30 min at 37 °C in the absence or presence of 2×10^{-3} M ouabain. When ouabain was present, NaCl and KCl were omitted from the incubation medium and replaced by Tris–HCl. The reaction was stopped by the addition of 40 μl of cold trichloroacetic acid 30%. Samples were centrifuged at 3000 × *g* for 10 min and the inorganic phosphate released (total ATPase activity) was measured. Na^+-K^+-ATPase activity was calculated as the difference between the means of total ATPase activity and ouabain-sensitive ATPase activity and expressed as μmol Pi/ mg protein hour [20].

2.7. Radioligand binding assays

Parotid glands were freed of connective tissue, fat and lymph nodes and then homogenized in Ultraturrax in six volumes of 5 mM Tris–HCl buffer pH 7.4, 1 mM $MgCl_2$, 0.25 M sucrose supplemented with 1 mM sodium ethylenediaminotetra-acetate (EDTA) and 1 mM DL-Dithiothreitol (DTT). The slurry was centrifuged at 4 °C for 10 min at 3000 × *g* twice, the supernatant was collected and spun down 10 min at 12,000 × *g* and the resulting supernatant was centrifuged for 2 h at 30,000 × *g*. The pellet was then resuspended in 10 mM phosphate buffer (pH 7.4) supplemented with 1 mM EDTA, 0.1 mM phenylmethyl-sulfonyl fluoride, 1 mM DTT, 2 μg/ml leupeptin and 2 μM pepstatin A and stored at −70 °C until used. Saturation assay was determined by incubation of 100–200 μg of protein with 0.080 to 3.22 nM of the CB_1 receptor antagonist [^{3}H]SR141716A (Amersham Biosciences UK Limited) in a total volume of 150 μl of 50 mM Tris–HCl, 10 mM $MgCl_2$ (pH 7.4), for 1 h at 30 °C with continuous shaking. Binding was stopped by adding 2 ml of ice-cold buffer followed by rapid filtration (Whatman GF/c). Filters were rinsed with 6 ml of ice-cold buffer, transferred into vials containing 10 ml of scintillation cocktail and counted in a liquid scintillation spectrometer. Non-specific binding was determined in the presence of 1×10^{-6} M AM281.

2.8. Data analysis

Student's *t*-test for unpaired values was used to determine the levels of significance. When multiple comparisons were necessary after analysis of variance, the Student–Newman–Keuls multiple comparison test was applied. Differences between means were considered significant if $P < 0.05$.

3. Results

As shown in Fig. 1A, anandamide induced a concentration-dependent increase in cAMP production in rat parotid glands. The CB_1 cannabinoid receptor antagonist, AM281 1×10^{-6} M inhibited the effect of anandamide 1×10^{-7} M (Fig. 1B). It should be pointed out that anandamide 1×10^{-7} M was chosen to study the effect of inhibitory drugs because it was the concentration that induced 60–80% of maximal effect in cAMP accumulation, amylase release and Na^+-K^+-ATPase inhibition.

Fig. 2A shows the ability of anandamide to induce amylase release in a concentration-dependent manner in rat parotid glands, and Fig. 2B shows the inhibition of this effect by the CB_1 cannabinoid receptor antagonist AM281 1×10^{-6} M, by the adenylyl cyclase inhibitor, SQ 225365 $\times 10^{-6}$ M and by the calcium influx blocker, verapamil 1×10^{-5} M. On the other hand, incubation of parotid glands in the presence of the cAMP analog, dibutyryl cAMP 1×10^{-4} M resulted in amylase release (Fig. 2B).

As seen in Fig. 3A, Na^+-K^+-ATPase activity decreased in the presence of increasing anandamide concentrations. The inhibitory effect of anandamide on enzyme activity was prevented by the CB_1 cannabinoid receptor antagonist AM281 1×10^{-6} M and by the adenylyl cyclase inhibitor, SQ 225365 $\times 10^{-6}$ M. Dibutyryl cAMP (1×10^{-4} M) mimicked the inhibitory action of anandamide (Fig. 3B). Verapamil (1×10^{-5} M) failed to modify the effect of the cannabinoid (1×10^{-7} M) on enzyme activity (Fig. 3B).

At the concentration used, none of the inhibitory agents had any effect upon basal values of cAMP accumulation, amylase release and Na^+-K^+-ATPase activity (Table 1). The vehicles used were distilled water and dimethyl sulfoxide (DMSO, final concentration 1:1000) and they lacked pharmacological effects.

Fig. 4 and Table 1 demonstrate a significant correlation between cAMP accumulation and both amylase

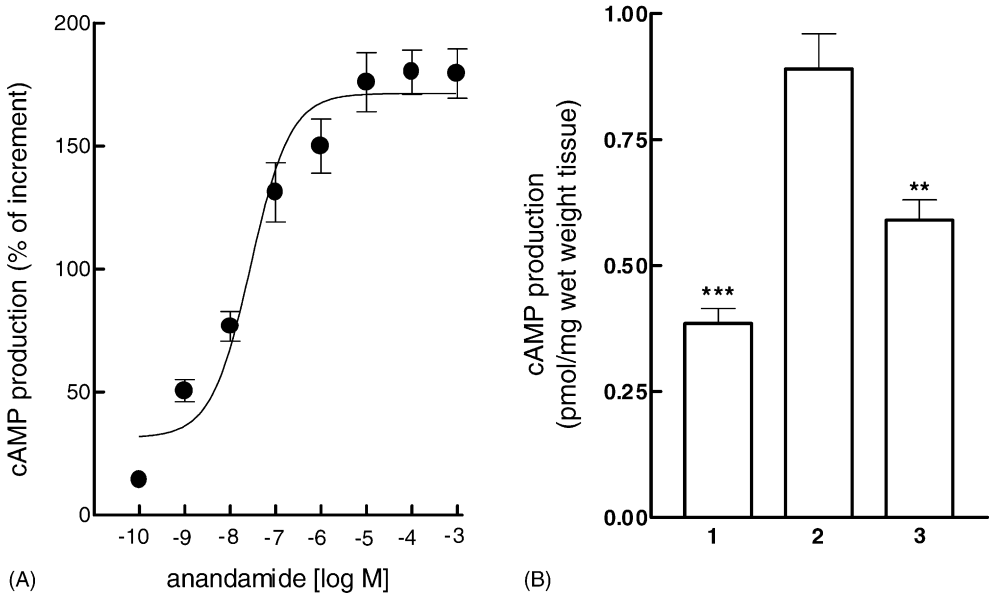

Fig. 1. Panel A: Concentration-dependent increase in cAMP production by anandamide in rat parotid glands represented as percentage of increment from basal conditions. Each point represents the mean ± S.E.M. of four experiments. Panel B: Effect of AM281 1×10^{-6} M on anandamide (1×10^{-7} M)-induced cAMP production in rat parotid glands. Basal cAMP production is also shown. Values are the means ± S.E.M. of four experiments of each group. 1: Basal; 2: anandamide; 3: AM281. $^{**}P < 0.01$; $^{***}P < 0.001$ compared with the effect of anandamide.

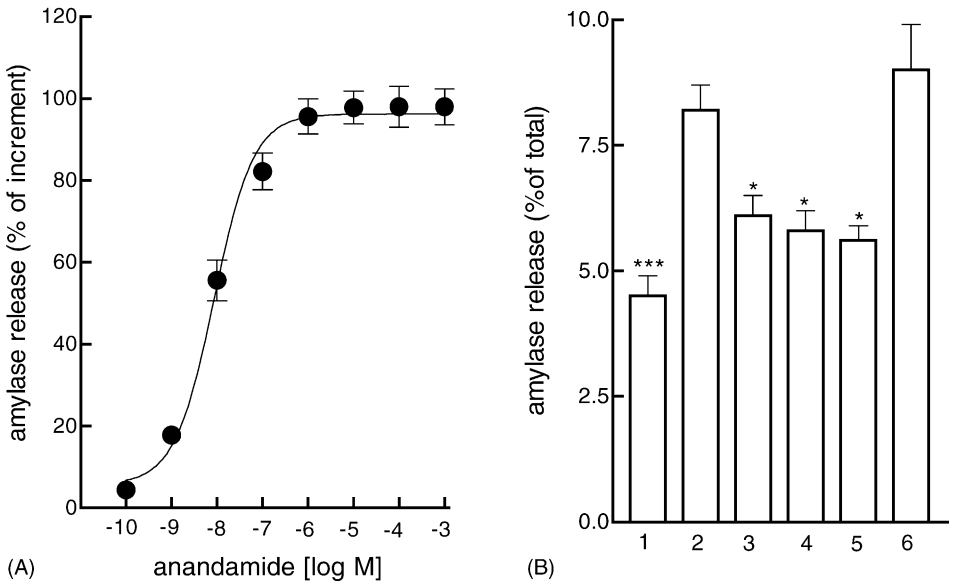

Fig. 2. Panel A: Concentration-dependent increase in amylase release by anandamide in rat parotid glands represented as percentage of increment from basal conditions. Each point represents the mean ± S.E.M. of four experiments. Panel B: Effect of AM281 1×10^{-6} M, SQ-225365 $\times 10^{-6}$ M and verapamil 1×10^{-5} M on anandamide (1×10^{-7} M)-induced amylase release in rat parotid glands represented as the percentage release from total amylase content in the gland. Basal amylase release and the effect of dibutyryl cAMP (1×10^{-4} M) are also shown. Values are the means ± S.E.M. of four experiments of each group. 1: Basal; 2: anandamide; 3: AM281; 4: SQ 22536; 5: verapamil; 6: db-cAMP. $^{*}P < 0.05$; $^{***}P < 0.001$ compared with the effect of anandamide.

Table 1
Influence of inhibitory agents upon basal values of parotid glands

Inhibitors	cAMP (pmol/mg wet wt)	Amylase (% of total)	Na^+-K^+-ATP-ase (μmol Pi/mg protein h)	*n*
None	0.41 ± 0.04	4.5 ± 0.36	2.5 ± 0.20	6
AM281(1×10^{-6} M)	0.39 ± 0.04	4.7 ± 0.50	2.8 ± 0.25	3
SQ 22536(5×10^{-6} M)	0.43 ± 0.03	4.3 ± 0.41	2.6 ± 0.26	3
Verapamil (1×10^{-5} M)	0.38 ± 0.03	4.4 ± 0.37	2.3 ± 0.27	3

Values are the means ± S.E.M. of *n* experiments.

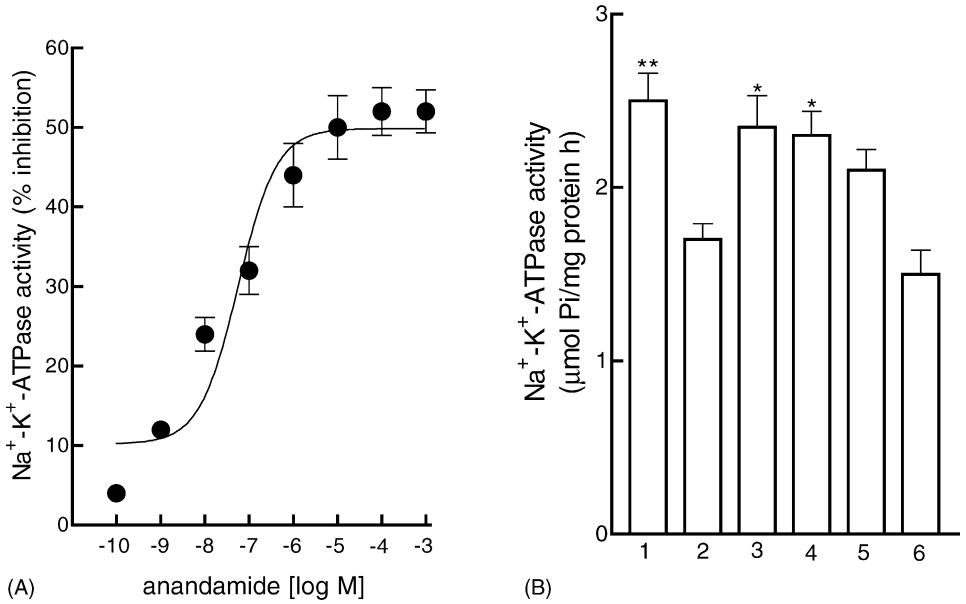

Fig. 3. Panel A: Concentration-dependent inhibition in Na^+-K^+-ATPase activity by anandamide in rat parotid glands represented as percentage of inhibition from basal conditions. Each point represents the mean ± S.E.M. of four experiments. Panel B: Effect of AM281 1×10^{-6} M, SQ-225365 $\times 10^{-6}$ M and verapamil 1×10^{-5} M on anandamide (1×10^{-7} M)-induced inhibition in Na^+-K^+-ATPase activity in rat parotid glands. Basal enzyme activity and the effect of dibutyryl cAMP (1×10^{-4} M) are also shown. Values are the means ± S.E.M. of four experiments of each group. 1: Basal; 2: anandamide; 3: AM281; 4: SQ 22536; 5: verapamil; 6: db-cAMP. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with the effect of anandamide.

release and Na^+-K^+-ATPase inhibition. This result indicates that anandamide-induced cAMP accumulation resulted in amylase release and Na^+-K^+-ATPase inhibition.

Subsequently, we investigated the relative potencies of the CB_1 cannabinoid receptor antagonist AM281 in inhibiting anandamide effects, by determining its pA_2. Fig. 5 shows that AM281 induced a parallel right shift of the concentration–response curves to anandamide. The drug blocked anandamide effects competitively, as demonstrated by the slopes of the Schild plots, which were close to unity (Table 2). Listed in Table 2 and derived from Fig. 5, pA_2 values show that AM281 relative potencies were equal for inhibiting anandamide-induced cAMP accumulation, amylase release and Na^+-K^+-ATPase inhibition (Table 3).

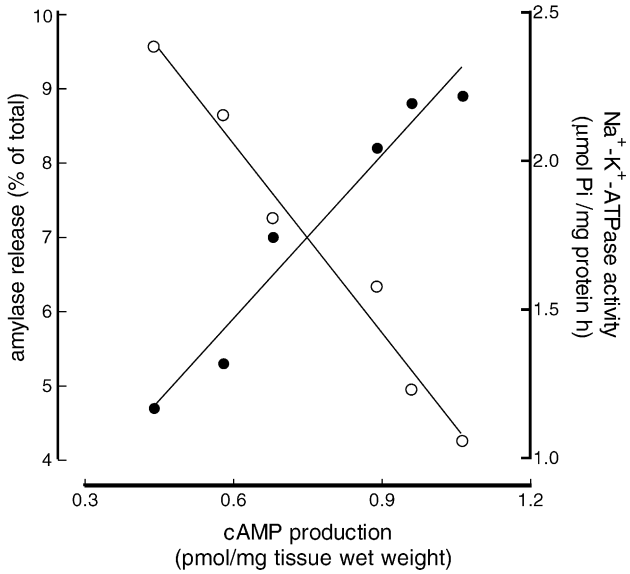

Fig. 4. Correlation in the modulatory effect of anandamide 1×10^{10} M to 1×10^{-5} M on cAMP production, amylase release and Na^+-K^+-ATPase activity, in rat parotid glands. Cyclic AMP production was plotted as a function either of amylase release (●) or Na^+-K^+-ATPase activity (○). Values are the means of four experiments of each group.

Table 2
Correlation parameters between cAMP production and either amylase release or Na^+-K^+-ATPase activity

Parameters	Amylase release	ATPase inhibition
Number of XY pairs	6	6
Pearson *r*	0.9776	−0.9880
95% confidence interval	0.8037 to 0.9977	−0.9987 to −0.8902
P value (two tailed)	0.0007	0.0002
R squared	0.9558	0.9761

Correlation was performed by using the GraphPad Prism program. These data derive from Fig. 4.

Finally, we ascertained the presence of the CB_1 cannabinoid receptor subtype in rat parotid glands by determining the ability of [^{3}H]SR141716A to bind to parotid membranes. Results obtained from saturation binding studies indicated the presence of a single class of specific binding site (Fig. 6). These sites were found to be present at a density (B_{max}) of 0.93 ± 0.18 pmol/mg of protein with K_d value of 0.41 ± 0.03 nM.

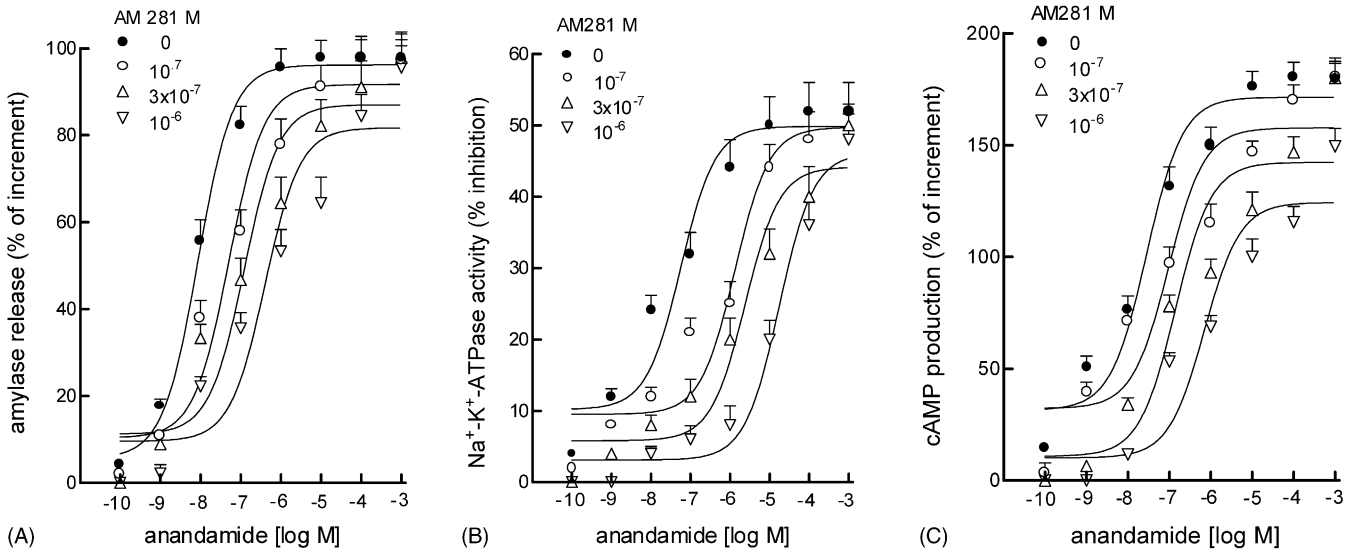

Fig. 5. Effect of increasing concentration of AM281 on anandamide-induced amylase release (A), Na^+-K^+-ATPase inhibition (B) and cAMP production (C). Each point represents the mean ± S.E.M. of four experiments of each group.

Table 3
Potencies of the cannabinoid CB_1 receptor antagonist, AM281, in inhibiting the effect of anandamide on cAMP production, amylase release and Na^+-K^+-ATPase activity

Parameters	CAMP	Amylase	Na^+-K^+-ATPase
pA_2	7.40 ± 0.26	7.74 ± 0.14	8.14 ± 0.14
K_a (M)	$5.25 \times 10^{-8} \pm 2.30 \times 10^{-8}$	$2.00 \times 10^{-8} \pm 5.80 \times 10^{-9}$	$8.00 \times 10^{-9} \pm 2.30 \times 10^{-9}$
Slope	−1.001 ± 0.32	−0.9630 ± 0.06	−1.140 ± 0.34
r^2	0.9077	0.9961	0.9165

Data shown derive from Schild plots constructed with data obtained from Fig. 5 and analyzed by linear regression using a GraphPad Prism program. Results were obtained from four independent experiments.

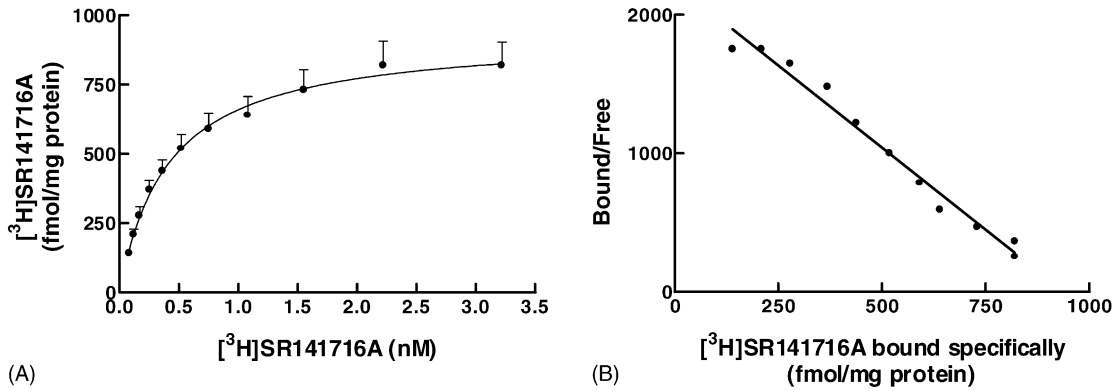

Fig. 6. Saturation curves (A) and Scatchard plots (B) on rat parotid gland membranes incubated with different concentrations of [^{3}H]SR161417A. Results shown are the means ± S.E.M. of a single experiment performed in duplicate, as representative of three separate experiments.

4. Discussion

In the present study we discerned a direct effect of the endocannabinoid, anandamide, on rat parotid gland function. Our findings demonstrated that the cannabinoid CB_1 receptor subtype is expressed in rat parotid glands and, when activated, induces cAMP accumulation, amylase release and Na^+-K^+-ATPase inhibition.

The presence of cannabinoid CB_1 receptor subtype in rat parotid glands was demonstrated by the ability of the selective cannabinoid CB_1 receptor antagonist, [^{3}H]SR141716A, to bind to parotid membranes. B_{max}

and K_d values were in the same order as those obtained from brain membrane preparations [21]. A single class of specific high-affinity cannabinoid binding sites was detected in pregnant mouse uterus [22] and in guinea pig small intestine [23].

Our results showed that anandamide-induced cAMP accumulation in rat parotid glands. This effect was a receptor-mediated action demonstrated by virtue of blockade by the selective cannabinoid CB_1 receptor antagonist AM281. cAMP has played a pivotal role in developing our understanding of cannabinoid receptors, since the discovery that cannabinoids inhibited adenylyl cyclase became the basis of the concept that cannabinoid actions are mediated through G-protein-coupled receptors [24]. However, findings showing evidence of differential regulation of the cannabinoid receptor coupling pathway that could either stimulate or inhibit adenylyl cyclase were demonstrated in CHO cells expressing the CB_1 receptor [25]. Thus, here we provide unequivocal experimental evidence that the cannabinoid CB_1 receptor can indeed mediate cAMP accumulation with concomitant effects on a peripheral tissue as the parotid gland.

We observed that anandamide induced amylase release from rat parotid glands. The inhibitory effect of AM281, SQ 22536 and verapamil demonstrated a receptor-cAMP-Ca^{2+}-mediated pathway for amylase release. It is well known that in parotid glands, secretagogues promote amylase secretion by cAMP and Ca^{2+}-mediated processes [13]. Certain investigations favor the notion that Ca^{2+} and cAMP function as distinct cellular second messengers in regulating salivary amylase secretion [13]. However, more recent experimental findings demonstrated that dual activation of cAMP and Ca^{2+} phosphoinositide messenger systems potentiates amylase release by enhancing Ca^{2+} availability [26] and, as a matter of fact, it has been suggested that in parotid cells cAMP induced direct Ca^{2+} mobilization [27]. Anandamide was shown to induce CB_1 receptor-mediated mobilization of cytosolic Ca^{2+} [28], while cannabinoids evoke capacitative Ca^{2+} entry [29]. Therefore, our findings are consistent with the interpretation that in parotid glands, anandamide induces cAMP accumulation and Ca^{2+} influx, both messengers involved in amylase secretion.

Our results also disclosed that anandamide, acting through the CB_1 receptor, inhibits Na^+-K^+-ATPase activity in rat parotid glands. The participation of cAMP in this effect was evident by reversion with SQ 22536. Cyclic AMP involvement in acute sodium pump regulation has been documented in 20 different mammalian tissues and in lower vertebrates [30]. The modulation of Na^+-K^+-ATPase activity by the cyclic nucleotide was shown to be tissue-specific and the result can be either pump stimulation or inhibition. Thus, in parotid glands as observed in pancreatic acinar cells [31], cAMP induced a decrease in Na^+-K^+-ATPase activity.

The results presented in this study indicate that anandamide induced cAMP accumulation, amylase release and Na^+-K^+-ATPase inhibition in rat parotid glands. The effect of anandamide on amylase release and Na^+-K^+-ATPase inhibition was shown to be cAMP-dependent. Thus, the effect of anandamide on cAMP accumulation significantly correlated with its action either on amylase release or on Na^+-K^+-ATPase activity. The ability of cAMP to induce amylase release and Na^+-K^+-ATPase inhibition, in rat parotid glands, was evidenced by the result obtained with exogenous dibutyryl-cAMP.

The three cannabinoid receptor-mediated functional responses were achieved through a single receptor subtype, the CB_1, since the CB_1 cannabinoid receptor antagonist, AM281, inhibited anandamide action on cAMP, amylase and Na^+-K^+-ATPase. The relative potencies of the antagonist to block these three functional responses were similar, supporting the above statement. In agreement with such evidence, the CB_1 cannabinoid receptor subtype was described in non-neuronal peripheral tissues [32,33] and unlike the CB_2, proved capable of stimulating cAMP accumulation [25].

It may be concluded that in parotid glands the endogenous cannabinoid anandamide, by coupling to CB_1 cannabinoid receptor subtype, induces cAMP accumulation, which in turn leads to amylase release and Na^+-K^+-ATPase inhibition. In addition, Katayama et al. [34] described the presence of anandamide amidohydrolase in rat parotid gland. Taken together, these results emphasize the role of cannabinoids in the control of salivary gland function and provide new insight into parotid gland physiology. Furthermore, these findings lead to speculation that endocannabinoids may be involved in the pathophysiological mechanisms of salivary gland diseases.

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