Archives of Oral Biology 81 (2017) 160–166

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

Immunohistochemical localization of cannabinoid receptor 1 (CB1) in the submandibular gland of mice under normal conditions and when stimulated by isoproterenol or carbachol

Wipawee Thoungseabyoun[a], Apussara Tachow[a], Sawetree Pakkarato[a], Atsara Rawangwong[a], Suthankamon Krongyut[a], Waraporn Sakaew[a], Hisatake Kondo[a,b], Wiphawi Hipkaeo[a,*]

[a] Nanomorphology-based Apply Research Group & Electron Microscopy Unit, Department of Anatomy, Faculty of Medicine, Khon Kaen University, Muang, Khon Kaen, Thailand

[b] Department of Organ Anatomy, Graduate School of Medicine, Tohoku University, Sendai, Japan

ARTICLE INFO

Keywords:
CB1
Isoproterenol
Acinar cell
Granular convoluted duct cell
Mouse submandibular gland

ABSTRACT

Objective: We wished to investigate the subcellular localization of CB1, a receptor for the endocannabinoids in mouse submandibular glands (SMGs) under normal conditions and when stimulated by adrenergic or cholinergic agonists.

Materials and methods: SMGs of both male and female adult mice were utilized for immunoblotting and immuno-light and –electron microscopic analyses. Isoproterenol and carbachol were used as adrenergic and cholinergic stimulants, respectively. SMGs were examined at 15, 30, 60 and 120 min after intraperitoneal injection of these agents.

Results: Selective localization of intense immunoreactivity for CB1 in the granular convoluted ductal cells was confirmed by immunoblotting and the antigen absorption test. In SMGs of control male mice, CB1-immunoreactivity was evident on the basolateral plasma membranes, including the basal infoldings, but was absent on the apical membranes in the ductal cells. Localization and intensity of CB1-immunoreactivity were essentially the same in SMGs of female mice. The immunoreactivity was transiently localized in the apical plasmalemma of some acinar and granular ductal cells of male SMGs shortly after stimulation by isoproterenol, but not by carbachol.

Conclusion: The present finding suggests that CB1 functions primarily in the basolateral membranes of the granular convoluted ductal cells of SMGs under normal conditions, and that the CB1 can function additionally in the apical membrane of acinar and granular ductal cells for modulation of the saliva secretory condition via adrenoceptors.

1. Introduction

Cannabinoids (CBs), natural components derived from the plant *Cannabis sativa*, have been known for many years to induce psychotropic and eventually systemic effects on human bodies. Known as the "body's own" cannabinoid system, the "endo" cannabinoid (eCB) system is involved in a variety of physiological processes such as appetite, pain sensation and immune responses, through two CB receptors (CB1 and CB2) comprising a G protein-coupled receptor superfamily. This occurs in the brain as well as in various peripheral organs and tissues (Kano, Ohno-Shosaku, Hashimotodani, Uchigashima, & Watanabe, 2009). The CB receptors are targeted by ligands such as anandamide and 2-arachidonoylglycerol (2-AG).

The activation of CB receptors inhibits agonist-stimulated salivation and modifies saliva contents, in accord with the fact that marijuana users often experience decreased saliva secretion (Busch, Sterin-Borda, & Borda, 2004; Kopach et al., 2012). In support of the pharmacological findings, there have been several studies demonstrating the localization of immunoreactivities for CB receptors in the salivary glands of several mammals at the light microscopic level (Dall'Aglio et al., 2010; Hipkaeo, Watanabe, & Kondo, 2015; Prestifilippo et al., 2006).

In our previous immuno-light microscopic study (Hipkaeo et al., 2015), intense immunoreactivity for CB1 was shown to be confined to the basolateral cell domains in the ductal cells, especially in the granular convoluted portion, but it was not discerned in the acinar

* Corresponding author at: Electron Microscopy Unit, Department of Anatomy, Faculty of Medicine, Khon Kaen University, Muang, Khon Kaen, 40002, Thailand.
E-mail address: wiphawi@kku.ac.th (W. Hipkaeo).

http://dx.doi.org/10.1016/j.archoralbio.2017.05.003
Received 21 December 2016; Received in revised form 5 May 2017; Accepted 10 May 2017

cells, of the submandibular gland (SMG) of mice at stages of postnatal 4–6 weeks. A similar finding had been reported in rats by Prestifilippo et al. (2006). However, it remains to be confirmed by immunoblotting analysis and the antigen-absorption test among others whether the previously reported immunoreaction was due to the authentic CB1. Since the previous findings were all at the light microscopic level, the exact localization of CB1 at the ultrastructural level also remains to be elucidated. In addition, it has been reported that the pharmacological activity of CB1 is regulated by testosterone in the parotid gland of male rats (Busch, Sterin-Borda, & Borda, 2006). In this regard, it will be interesting to determine the expression and localization of CB1 in female. Furthermore, in order to further confirm that CB1 is really involved in the control of saliva secretion, it is worth examining whether or not any changes in its localization may occur after enhancement of the gland secretion by external chemical stimuli. The present study was undertaken to clarify these issues described above.

2. Materials and methods

2.1. Animals and tissue preparation

Male and female ICR mice were purchased from the National Laboratory Animal Center (NLAC), Bangkok, Thailand. Twenty seven male mice at the stage of postnatal 6 weeks were divided into two groups; one for analysis under normal conditions (3 mice, acting as controls), another (24 mice) for analysis under extrinsic stimulation. The stimulation was performed by intraperitoneal injection in physiological saline of isoproterenol as a β-adrenergic receptor agonist (15 mg/kg body weight, Sigma-Aldrich; Gillingham, UK), or carbachol as a cholinergic muscarinic receptor agonist (1 mg/kg body weight, Sigma-Aldrich; Gillingham, UK). At each of various time intervals after injection (15 min, 30 min, 60 min and 120 min), 3 mice injected with isoproterenol and 3 injected with carbachol were sacrificed. All male mice, experimental and control, were perfused with 10 ml physiological saline, under thiopental sodium anesthesia (50 mg/kg body weight). The right SMG of each mouse was extirpated and put into liquid nitrogen and kept at −80C for immunoblotting analysis. Thereafter, each animal was perfused with 10 ml 4% paraformaldehyde/PBS, and the left SMG was removed, then postfixed with the same fixative overnight. The specimens were then dipped into 30% sucrose/PBS for cryoprotection until use for immunohistochemical analysis.

In addition, three female mice at the stage of postnatal 6 weeks were processed in the same way as the male mice described above. All mice were provided with free access to foods and water until the morning of the day of injection.

2.2. Immunohistochemistry

Cryosections of the specimens were made on a cryostat at 20 μm thickness and incubated with 0.3% H_2O_2/methanol for 10 min to inhibit intrinsic peroxidase activity. Ten percent normal goat serum/PBS was then added for 30 min to prevent non-specific antibody binding. Sections were incubated at room temperature overnight with the rabbit polyclonal anti-CB1 antibody (a kind gift of Prof M. Watanabe, Hokkaido University, JAPAN) at 1 μg/ml as used in our previous study (Hipkaeo et al., 2015). The specificity of the antibody was confirmed previously using CB1 gene knockout mice (Fukudome et al., 2004; Uchigashima et al., 2007). The sections were incubated for 1 h at room temperature with biotinylated goat anti-rabbit IgG (Abcam; Cambridge, MA USA). For visualization of the antigen-antibody reaction sites with diaminobenzidine (DAB) reaction as the marker, the sections were then treated using a VECTASTAIN Elite ABC kit (Vector Laboratories, Burlingame, CA USA).

In immuno-DAB electron microscopy, some of the sections were postfixed with 0.5% OsO_4 in 0.1 M phosphate buffer and embedded in Epon after *en-bloc* staining with 0.1% uranyl acetate. Ultrathin sections were observed under a JEM1010 transmission electron microscope (Jeol; Tokyo, Japan).

In the antigen-absorption test acting as a control for the immunohistochemistry, the antibody was pre-absorbed with CB1 antigen (100 μg/ml, a kind gift of Prof M. Watanabe) and sections were incubated with the absorption solution for 1 h at room temperature and subsequently treated in the same procedure as the regular immunoreaction described above.

2.3. Immunoblotting analysis

Frozen mouse SMGs were homogenized in a lysis buffer composed of 20 mM Tris-HCl (pH 8.5), 20 mM KCl, 10 mM EDTA (pH 8.0), 250 mM sucrose and 1 x complete protease inhibitor cocktail (Pierce, Thermo Scientific; Waltham, MA, USA). After centrifugation at 3000 rpm, the protein concentration in the supernatants was examined using a NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific; Wilmington, DE, USA). The total proteins of 40 μg from each lysate were individually boiled for 10 min in 2 x SDS sample buffer and subjected to SDS/10% PAGE electrophoresis. They were then electrophoretically transferred to a PVDF membrane (GE Healthcare; Buckinghamshire, UK). Non-specific binding sites were blocked using 5% skim milk (wt/vol)/TBS/0.3%tween-20, and the membrane was incubated overnight at 4 ˚C with the same antibody at 0.1 μg/ml in 5% skim milk (wt/vol)/TBS/0.1% tween-20 and then treated with HRP-goat anti-rabbit IgG (Invitrogen; Camarillo, CA) for 1 h, at room temperature. Immunoreactive proteins were visualized using the ECL prime immunoblotting substrate (GE Healthcare; Buckinghamshire, UK). Anti-goat beta actin (Santa Cruz Biotechnology, Texas, USA) was used as a control. The intensities of all immunoblotting bands were quantified using NIH's Image J, and the relative intensities of CB1 and β-actin were determined. Statistical differences were analyzed between two mean values with the *Student's t test*, and among multiple mean values with post hoc tests in the ANOVA. Differences with a *P value* less than 0.05 were considered significant.

All procedures were conducted in accordance with Guidelines for the Care and Use of Laboratory Animals at Khon Kaen University. This study was approved by the Animal Ethics Committee of the Khon Kaen University, based on the Ethics of Animal Experimentation of the National Research Council of Thailand (Reference No.AEKKU 4/2560).

3. Results

Immuno-light microscopy for CB1-immunoreactivity in SMG of normal male mice was in accord with our previous study (Hipkaeo et al., 2015). Intense CB1- immunoreactivity was primarily detected in the ductal cells throughout the gland, but no significant immunoreaction was seen in the acinar cells (Fig. 1a). In the granular convoluted ductal cells, intense immunoreactivity was distinct in the basolateral plasmalemmas, but not in the apical ones (Fig. 1b). On the other hand, the immunoreactivity was evident throughout the cytoplasm without cell polarity in the excretory ducts, whereas the intercalated ductal cells showed only weak immunoreactivity throughout the cytoplasm.

Likewise, in SMGs of female mice, expression of CB1 was primarily in the ductal cells, but not in the acini. The striated duct of female rodents is characterized by much less differentiation of the granular ductal cells as compared to the male counterpart, and the ductal cells contain less numerous granules in mixture with typical striated ductal cells scanty of granules (Amano, Mizobe, Bando, & Sakiyama, 2012). In accord with such intrinsic structural characteristics, some ductal cells in female showed the distinct immunoreactivity in the basolateral plasmalemmas similar to the male counterpart, while others sometimes showed the immunoreactivity throughout the entire cytoplasm without any intracellular polarity, as was the case in male (Fig. 1c, d). In spite of such somehow heterogenous appearance of the immunoreactivity, no clear difference between female and male was discerned in the