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Effects of endogenous cannabinoid anandamide on cardiac Na^+/Ca^{2+} exchanger

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ABSTRACT

Endocannabinoid anandamide (N-arachidonoyl ethanolamide; AEA) has been shown to cause negative inotropic and antiarrhythmic effects in ventricular myocytes. In this study, using whole-cell patch clamp technique, we have investigated the effects of AEA on cardiac Na^+/Ca^{2+} exchanger (NCX1)-mediated currents. AEA suppressed NCX1 with an IC_{50} value of 4.7 μM. Both inward and outward components of exchanger currents were suppressed by AEA equally. AEA inhibition was mimicked by the metabolically stable analogue, methanandamide (metAEA, 10 μM) while it was not influenced by inhibition of fatty acid amide hydrolase with 1 μM URB597 incubation. The effect of AEA, was not altered in the presence of cannabinoid receptor 1 and 2 antagonists AM251 (1 μM) and AM630 (1 μM), respectively. In addition, inhibition by AEA remained unchanged after pertussis toxin (PTX, 2 μg/ml) treatment or following the inclusion of GDP-β-S (1 mM) in pipette solution. Currents mediated by NCX1 expressed in HEK-293 cells were also inhibited by 10 μM AEA a partially reversible manner. Confocal microscopy images indicated that the intensity of YFP-NCX1 expression on cell surface was not altered by AEA. Collectively, the results indicate that AEA directly inhibits the function of NCX1 in rat ventricular myocytes and in HEK-293 cells expressing NCX1.

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1. Introduction

Endocannabinoids are a class of polyunsaturated fatty acid-based compounds that mimic most of the effects of Δ^9-tetrahydrocannabinol (THC), the active ingredient of the marijuana plant *Cannabis sativa*. N-arachidonoyl ethanolamide (AEA) or anandamide and 2-arachidonylglycerol (2-AG) are the most widely studied endogenous cannabinoids [1,2]. In recent years, extensive research focusing on the biological actions of endocannabinoid compounds indicated that they have important regulatory roles in several physiological and pathological conditions [1,3,4]. It has been shown that the endocannabinoid system consists of at least the endocannabinoid receptors (CB_1 and CB_2 cannabinoid receptors), the enzymes regulating the synthesis (such as phospholipase-D, and monoacylglycerol lipase), and the degradation (such as fatty-acid amide hydrolase and lipases) processes, and the proteins involved in their transport across the biological membranes [1,4]. CB_1 receptors are located in the brain and several peripheral tissues including the heart and the vasculature [4,5]. The CB_2 receptors, on the other hand, are expressed primarily in the immune system but recently their presence in the brain, myocardium, and smooth muscle cells has also been demonstrated [4].

Recent studies suggest that endocannabinoids have important modulatory roles on the function of the cardiovascular system under various pathological conditions, such as hypertension, myocardial infarction and heart failure [6,7]. AEA, the most studied endocannabinoid, has a complex set of actions on cardiac functions. Experiments with AEA performed in isolated Langendorff rat hearts and in isolated, electrically stimulated human atrial appendages [5,8] have revealed a negative inotropic effect which

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may underlie its ability to decrease cardiac output as observed in studies performed in vivo [9]. Moreover, AEA and other cannabinoids have been reported to have antiarrhythmic effects in in vivo animal models [10,11]. However, electrophysiological mechanisms underlying these cardiac actions of AEA remain largely unknown. We hypothesized that some of the actions of AEA on cardiomyocytes can be mediated by the modulation of NCX1 which plays a major role in Ca^{2+} homeostasis and generation of arrhythmias [12,13]. Thus, in the present study, using whole-cell patch clamp, we investigated the actions of AEA on NCX1-mediated currents (I_{NCX1}) in acutely dissociated rat ventricular myocytes and in HEK-293 cells expressing NCX1.

2. Materials and methods

2.1. Ventricular myocyte isolation

The work was performed with approval of the Animal Research Ethics Committee of the College of Medicine and Health Sciences (Al Ain, UAE). Animals were bred at our own Animal Facility from the original stock. The animals were housed in polypropylene cages in climate and access controlled rooms (22–24 °C; 50% humidity). The day/night cycle was 12 h/12 h. Food and water were provided ad libitum.

Ventricular myocytes were isolated from adult male Wistar rats (250–270 g) according to previously described techniques [14]. Briefly, the animals were euthanized using a guillotine and hearts were removed rapidly and mounted for retrograde perfusion according to the Langendorff method. Hearts were perfused at a constant flow rate (8 ml/min/g weight of tissue) at 36–37 °C with a solution containing (mM): 130 NaCl, 5.4 KCl, 1.4 $MgCl_2$, 0.75 $CaCl_2$, 0.4 NaH_2PO_4, 5 HEPES, 10 glucose, 20 taurine, and 10 creatine set to pH 7.3 with NaOH. Once the heart had stabilized, perfusion was continued for 4 min with Ca^{2+}-free isolation solution containing 0.1 mM EGTA, and then for 6 min with cell isolation solution containing 0.05 mM Ca^{2+}, 0.75 mg/ml collagenase (type 1; Worthington Biochemical Corp, USA) and 0.075 mg/ml protease (type X1 V; Sigma, Germany). Ventricles were excised from the heart, minced and gently shaken in collagenase-containing isolation solution supplemented with 1% BSA. Cells were filtered from this solution at 4 min intervals and re-suspended in isolation solution containing 0.75 mM Ca^{2+}.

2.2. Recordings of NCX1 currents in cardiomyocytes

Currents through NCX1 were recorded from rat isolated ventricular myocytes using the patch clamp technique in whole cell configuration. Currents were filtered at 5 kHz and digitized using a Digidata 1322 interface. Briefly, I_{NCX1} was recorded using a descending voltage ramp from +100 mV to −100 mV from a holding potential of −40 mV for 2 s duration. As described previously [15], I_{NCX1} was measured as current sensitive to nickel (Ni^{2+}), therefore, Ni^{2+}-insensitive components were subtracted from total currents to isolate I_{NCX1}. External solution contained (in mM): 150 NaCl, 5 CsCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 glucose (pH = 7.4). Nifedipine (10 μM), oubain (100 μM), and niflumic acid (30 μM) were used to block Ca^{2+}, Na^+- K^+-ATPase, and Cl^- currents, respectively. 10 mM nickel chloride solution was used to block I_{NCX1}. K^+ currents were minimized by Cs^+ substitution for K^+ in both pipette and external solutions. The pipette solution contained (in mM): 120 CsCl, 20 $NaCl_2$, 10 tetraethylammonium chloride (TEACl), 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 1 MgATP and 10 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) (pH = 7.2) with CsOH. The combination of 10 mM BAPTA and 1 mM Ca^{2+} in the pipette solution gave a free $[Ca^{2+}]_i$ of 20 nM (calculated with the "Max-chelator program"; WEBMAX v 2.10, Stanford, CA, USA, which was supplied by Dr. D. Bers). For experiments including pertussis toxin (PTX) pretreatment, cells were incubated with PTX (2 μg/ml) for 3 h at 37 °C (control cells to this group were incubated in the same conditions with distilled water only). Changes of external solutions and application of drugs were performed using a multi-line perfusion system with a common outflow connected to the recording chamber. The pipettes were fabricated from filamented BF 150-86-10 borosilicate glass (OD = 1.5 mm, ID = 0.86 mm) (Sutter Instruments Co., CA, USA) on a horizontal puller (Sutter Instruments Co., CA, USA). Electrode resistance ranged from 2.0 to 4.0 MΩ. Experiments were performed at room temperature (22–24 °C). Electrophysiological data were analyzed using pClamp 10.2 (Molecular Devices, Union City, CA, USA) and Origin 7.0 (OriginLab Corp., Northampton, MA, USA) software. The amplitudes of the currents were normalized to the cell membrane capacitance to provide current densities (pA/pF).

2.3. Western blot analysis

Ventricles were obtained from normal Wistar rats. Tissue samples were flash-frozen in liquid nitrogen and stored at −80 °C. After thawing, tissue extracts were prepared by homogenization on ice with RIPA buffer (Pierce Biotechnology, IL, USA) supplemented with protease inhibitors (Roche, GmbH, IN, USA). Later, the extracts were clarified to remove the cellular debris by centrifugation at 13,000 r.p.m. for 15 min at 4 °C. Protein content in the extracts was determined using the Lowry assay (BioRad). A measure of 50 μg protein was resolved in 12% SDS-PAGE and was transferred onto nitrocellulose membranes (GE Healthcare, UK). Blocking was performed for 2 h at room temperature with 5% non-fat skimmed milk powder prepared in phosphate buffer solution (PBS) containing 0.1% Tween 20 (Sigma, CA, USA). After washing with phosphate-buffered saline 0.1% Tween 20 (PBST), the membranes were probed with either rabbit polyclonal CB1 (Cayman Chemicals, 1:1000 dilution) or with an antibody raised against the last 15 residues of rat CB1 or CB2 antibody (Cayman Chemicals, 1:1000 dilution) overnight at 4 °C. After washing with PBST, membranes were incubated for 1 h at room temperature in HRP (horseradish peroxidase)-coupled secondary antibody (goat anti-rabbit) (GE Biosciences, UK). Subsequently, the membranes were washed with PBST and were developed using chemiluminescence detection kit (Super Signal-West Pico Substrate, Pierce). To confirm uniform loading, the membranes were stripped and re-probed with β-actin (Chemicon, CA, USA).

2.4. Cell culture

Human Embryonic Kidney 293 (HEK-293) cells were maintained in DMEM/Ham's F-12 medium (50:50; Mediatech, Inc., Herndon, VA, USA) supplemented with 10% FBS. They were grown in a humidified atmosphere at 37 °C and 5% CO_2. Twenty four hours after plating, cells were transiently transfected with cDNA for NCX1 using Lipofectamine LTX reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Experiments were performed 48 h after transfection when cells were 70–80% confluent.

2.5. Image analysis

Fluorescence signal was determined from the average pixel intensity within the cell using NIH-image-J. Cell surface pixel intensity was determined from the region of interest drawn inside and outside the cell surface. Pixel intensities were determined from the initial time point of YFP fluorescence (excitation, 488 nm; emission, 525–575 nm) and were compared after 10 min exposure to

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