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Differential CB1 and CB2 cannabinoid receptor-inotropic response of rat isolated atria: Endogenous signal transduction pathways

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Abstract

In this study, we have determined the contractile effects of CB1 and CB2 cannabinoid receptor activation on rat isolated atria and the different signaling pathways involved. Anandamide did not has significantly effect on atria contractility, however, the treatment with both CB1 (AM251) or CB2 (AM630) receptor antagonists, the endocannabinoids triggered stimulation or inhibition on contractility respectively. The ACEA stimulation of CB1 receptor exerted decrease on contractility, that significantly correlated with the decrement of cAMP and the stimulation of nitric oxide synthase (NOS) and the accumulation of cyclic GMP (cGMP). On the contrary, JWH 015 stimulation of CB2 receptor triggered positive contractile response that significantly correlated with the increase cAMP production. The inhibiton of adenylate cyclase activity impaired the JWH 015 activation of CB1 receptor induced positive contractile effect, while inhibitors of phospholipase C (PLC), NOS and soluble nitric oxide (NO)-sensitive guanylate cyclase blocked the dose-response curves of ACEA on conntractility. Those inhibitors also attenuated the CB1 receptor-dependent increase in activation of NOS and cGMP accumulation. These results suggest that CB2 receptor agonist mediated positive contractile effect associated with increased production on cAMP while CB1 receptor agonist mediated decrease on contractility associated with decreased cAMP accumulation and increase production of NO and cGMP; that occur secondarily to stimulation of PLC, NOS and soluble guanylate cyclase. Data give pharmacological evidence for the existence of functional CB1 and CB2 cannabinoid receptors in rat isolated atria and may contribute to a better understanding the effects of cannabinoids in the cardiovascular system.

Keywords: Cannabinoid receptors; Atria contractility; NOS; cGMP; Camp

1. Introduction

Cannabinoids include not only plant-derived compounds, but also synthetic agents and endogenous substances termed endocannabinoids which include anandamide [1]. In addition, to having their well-known neurobehavioral effects, anandamide influence a number of other physiological functions, including cardiovascular variables [2,3].

Two types of cannabinoid receptors have been cloned, the CB1 and CB2 [4,5]. Distinction between these receptors is based on differences in their predicted aminoacid sequence, tissue distribution, signaling mechanisms and sensitivity to certain potent agonists and antagonists that show marked selectivity for one or other receptor type [6].

Cannabinoids receptors are widespread including in the cardiovascular system [7]. Both receptor types belong to a group of seven transmembrane-spanning receptors and are coupled to G protein. Studies on signaling events coupled to cannabinoid receptor subtypes have revealed differences in the abilities to modulate many different signal transduction pathways. The signal transduction process to agonist binding to CB1 receptor include the *pertussis toxin* sensitive Gi/o protein [8] that mediate the decrement of cyclic AMP production [9]. However, other studies have shown that cannabinoids increase basal cyclic AMP accumulation in various systems [10,11].

Stimulation of adenylate cyclase has been reported in pertussis toxin treated cells, suggesting that in the absence of functional Gi/o coupling, the CB1 receptor can activated Gs [12]. This might be related to the isoform of adenyllate cyclase expressed in cells. Co-expression of either CB1 or CB2 receptor with adenylate cyclase isoforms 1, 3, 5, 6 or 8 resulted in inhibition of cAMP accumulation, while 2, 4 or 7 enzyme isoforms resulted in stimulation of cAMP production [13]. Neither the cAMP elevating nor the cAMP

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reducing cannabinoid receptors activation have been associated with changes in the direct inotropic effect of the specific receptor activation on isolated myocardium. Thus, the signal transduction mechanisms that mediate the action of CB1 and CB2 receptor agonists on heart contractility have not been clearly defined. It is possible that cannabinoid receptors were coupled to more than one G protein regulating signaling transduction pathways. It is demonstrated that neuronal CB1 but not CB2 receptor activation inhibits D-type and enhances A-type K^+ channel activity [14]; also inhibits P/Q and N-type with different action on L-type Ca^{2+}-channel activity [15,16]. Cannabinoid agonists (CB1) evoked a rapid and transient increase in intracellular free Ca^{2+} by a mechanism whereby a receptor-mediated release of Gi/o βγ subunits might activate PLCβ, leading to inositol-1,4,5-triphosphate (IP3) release [17], that in term triggers cascade reactions involving the stimulation of constitutive nitric oxide synthase (NOS) activity [18].

The cardiovascular action of cannabinoids are complex, and much research has been directed at determining the basis responsible for the selectivity of the action on the nervous system as well as on the heart and blood vessels. Cannabinoid actions on cardiovascular system have been widely interpreted as being mediated by CB1 receptors although, there are growing number of observations particularly in isolated heart and vessel preparations, suggesting the existence of heterogeneity in the distribution of receptors for anandamide in these preparations. Ford et al. [19] suggested that the responses to cannabinoids in Langendorff-perfused rat hearts were mediated by a novel cannabinoid site, but none has been fully characterized. Therefore, there is little pharmacological evidence for the existence of functional CB1 receptors in isolated myocardium and neither can the observations be explained by actions on the CB2 receptor.

The aim of the present study was to demonstrate the existence of functional CB1 and CB2 receptors in isolated rat atria and the signaling events involved in CB1 and CB2 dependent contractile actions by specific agonists. So, we investigated whether: (1) ACEA-stimulation of CB1 receptor exerted decrease contractility correlated with stimulation of NOS-cyclic GMP pathway and decrease production of cAMP accumulation and (2) JWH 015-stimulation of CB2 receptor exerted positive contractile response correlated with increase of cAMP accumulation.

2. Materials and methods

2.1. Animals

Adult male Wistar strain rats (250–300 g) were used. The animals housed in standard environmental conditions were fed with a commercial pelleted diet and water ad libitum. Experimental protocol were done following to the Guide to The Care and Use of Experimental Animals (DHEW Publication, NIH 80-23).

2.2. Atrial preparation for contractility

Male Wistar rats were killed by decapitation. The left atria were carefully dissected from the ventricles, attached to a glass holder and immersed in a tissue bath containing Krebs–Ringer bicarbonate (KRB) solution gassed with 5% CO_2 in oxygen and maintained at pH 7.4 and 30 °C. KRB solution was composed as described previously [20]. A preload tension of 750 mg was applied to the atria and tissues were allowed to equilibrate for 1 h. The initial control values for contractility of the isolated atria were recorded by use of a force transducer coupled to an ink writing oscillograph. The preparations were paced with a bipolar electrode and an SK4 Grass Stimulator. The stimuli had a duration of 2 ms and the voltage was 10% above threshold. Contractility (dF/dt) were assessed by recording the maximum rate of isometric force development during electrical stimulation at a fixed frequency of 150 beats min^{-1}. Control values (=100%) refer to the dF/dt before the addition of drugs. The absolute value for dF/dt at the end of the equilibration period (60 min) was 7.8 ± 0.5 g s^{-1}. Cumulative dose-response curves were obtained according to the method of Van Rossum [21]. A maximal effect was achieved within 5 min after each dose. Dose-response curves of CB1 and CB2 cannabinoid receptor agonists were done on untreated atria and those from chemically sympathectomized rats injected intravenously 24 h prior to sacrifice with 6-hydroxydopamine (6-OHDA) (16.5 mg kg^{-1}). In order to assess an adequate denervation, the in vitro influence of tyramine (1×10^{-6} M) and norepinephrine (NE) (1×10^{-8} M) were assayed. As expected 6-OHDA-treated atria showed supersensitive to NE and refractory to tyramine [22].

2.3. Determination of nitric oxide synthase activity

Nitric oxide synthase (NOS) activity was measured in atria by production of [U^{-14}C]-citrulline from [U^{-14}C]-arginine according to the procedure described by Bredt and Snyder [23] for brain slices and by Sterin-Borda et al. [20] for rat atria. Briefly, after 20 min preincubation in KRB solution, atria were transferred to 500 μl of prewarmed KRB equilibrated with 5% CO_2 in O_2 in the presence of [U^{-14}C]-arginine (0.5 μCi). Appropriate concentrations of drugs were added and the atria were incubated for 20 min under 5% CO_2 in O_2 at 37 °C. Measurement of basal NOS activity in whole atria by the above mentioned procedure was inhibited 95% in the presence of 0.5 mM N^G-monomethyl-L-arginine (L-NMMA). The results (pmol g^{-1} tissue wet weight) obtained for whole atria were expressed as the difference between values in the absence (252 ± 12; $n = 9$) and in the presence (12 ± 2, $n = 9$) of L-NMMA.

2.4. Cyclic AMP and GMP assay

Tissues were incubated in 1 ml KRB for 30 min and ACEA or JWH 015 were added in the last 5 min. When blockers were used, they were added 25 min before the addition of cannabinoid agonists. After incubation, atria were homogenized in 2 ml of absolute ethanol and centrifuged at 6.000 × *g* for 15 min at 4 °C. Pellets were then rehomogenized in ethanol–water (2:1) and supernatants collected and evaporated to dryness as indicated above. Cyclic AMP or cGMP in the residue was dissolved in 400 μl of 0.05 M sodium acetate buffer pH 6.2. Aliquots of 100 μl were taken for the nucleotides determination using RIA procedure with a cAMP ^{3}H or cGMP ^{125}I RIA KIT from Dupont/New England Nuclear.

2.5. Drugs

Anandamide, ACEA, JWH 015, AM251, AM630, ODQ and SQ 22536 from Tocris Cookson Inc.; L-arginine and N^{G}-monomethyl-L-arginine (L-NMMA) from Sigma Chemical Company and U-73122 from ICN Pharmaceuticals Inc. Stock solutions were freshly prepared in the corresponding buffers. The vehicles used were: for ACEA and JWH 015 ethanol and for AM251 and AM630 dimethyl-sulfoxide (DMSO) in distilled water. The drugs were diluted in the bath to achieve the final concentration stated in the text. The final concentration of DMSO and ethanol were more than 1:1000 and they lacked pharmacological action.

2.6. Statistical 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 test was applied. Differences between means were considered significant if $P < 0.05$.

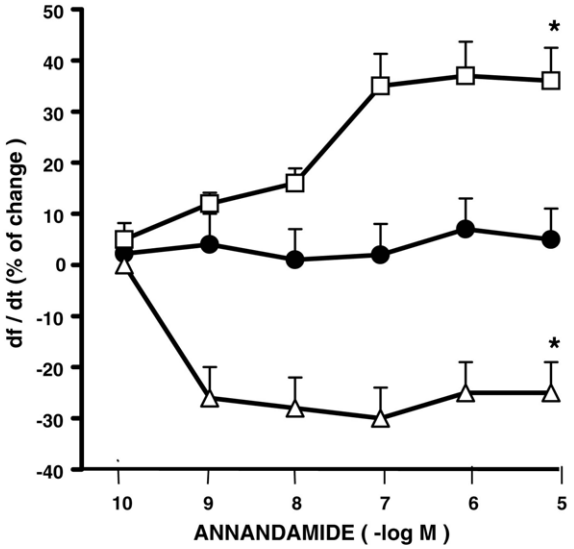

Fig. 1. Effect of increasing concentrations of anandamide on the contractility (d*F*/d*t*) of rat isolated atria. The action of CB_1 (AM251) or CB_2 (AM630) receptor antagonists is also shown. Tissue were incubated for 30 min in absence (●) or presence of 1×10^{-7} M AM251 (□) or 1×10^{-7} M AM630 (△) and then, dose-response curves of anandamide were performed. Values are expressed as percentage of changes calculated by comparison with the absolute values prior to the addition of anandamide. None of the receptor blockers had any significant effect on basal d*F*/d*t* g.s. (7.8 ± .5) after 30 min pretreatment at the concentration used (d*F*/d*t* g.s: AM251 7.6 ± 0.4; AM630 8.1 ± 0.8). For all curves, values are means ± S.E.M. of eight experiments. $^{*}p < 0.0001$ vs. anandamide alone (Student's *t*-test).

3. Results

To determine the cannabinoids receptor subtypes involved in the biological effect of anandamide in rat atria,

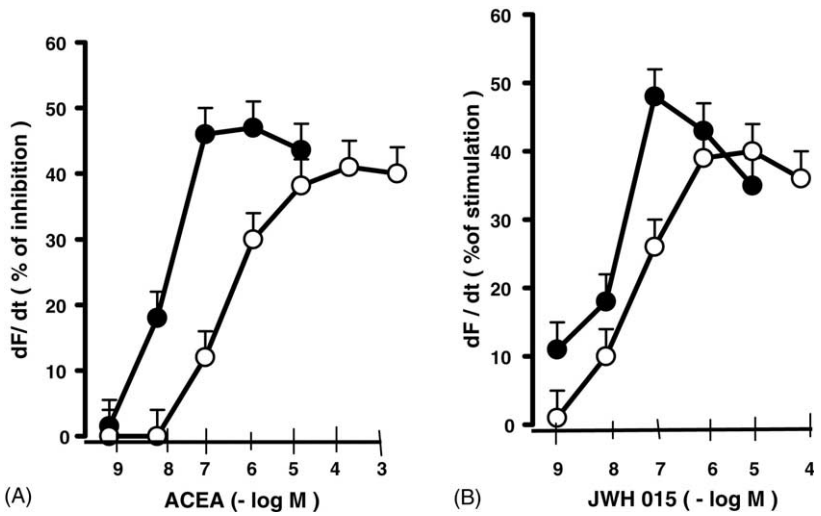

Fig. 2. Effects of increasing concentrations of A: CB1 agonist (ACEA) alone (●) or in presence of CB_1 antagonist (AM251, 1×10^{-7} M) (○); B: CB2 agonist (JWH 015) alone (●) or in presence of CB2 antagonist (AM630, 1×10^{-7} M) (○). Values are expressed as percentage of decrease (ACEA) or increase (JWH 015) calculated by comparison with the absolute values prior to the addition of CB_1 or CB_2 receptor agonists. For all curves values are means ± S.E.M. of six experiments. For more details see Fig. 1.

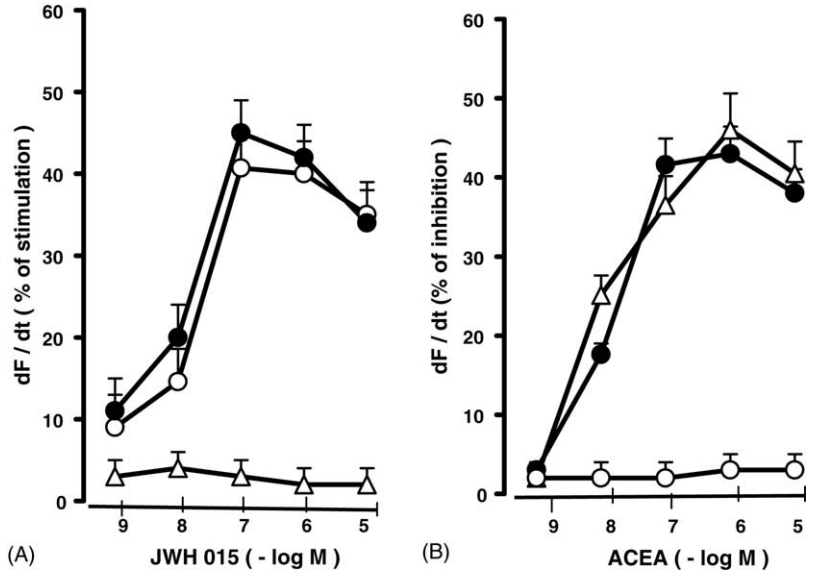

Fig. 3. Effect of 5×10^{-5} M SQ 22536 and ODQ on the dose-response curves of (A) JWH 015 or (B) ACEA upon atria d*F*/d*t*. Tissues were incubated for 30 min in absence (●) or in presence of SQ 22536 (△) or ODQ (○) and then, dose-response curve of JWH 015 or ACEA were obtained. Values represent of means ± S.E.M. of seven experiments in each group. No contractile effects were observed with enzymatic inhibitors at the concentration used (d*F*/d*t* g.s: basal 7.7 ± 0.4; SQ 22536 7.4 ± 0.5; ODQ 8.0 ± 0.7; $n = 7$). For more details see Fig. 1.

the concentration-response curves of anandamide in presence of both CB_1 or CB_2 receptor antagonists were performed. Fig. 1 shows that anandamide did not have a significant effect on atria contractility. However, a 30 min pretreatment of atria with CB_1 or CB_2 receptor antagonists triggered anandamide concentration-dependent changes on contractility. Under these conditions, anandamide induced a biphasic action: positive effect in presence of AM251 (CB_1 antagonist) or negative inotropism in presence of AM630 (CB_2 antagonist). Results suggest that decrease on contractility is triggered by CB_1 receptor activation, while CB_2 mediates the positive contractile effect.

To assess cannabinoid receptor subtypes involved in the atria contractile variation, the concentration-response curves of both CB_1 (ACEA) and CB_2 (JWH015) receptor specific agonists were performed. Fig. 2 shows that ACEA diminished and JWH 015 stimulated the atria d*F*/d*t*. Moreover, the selective CB_1 antagonist AM251 or the selective CB_2 antagonist AM630 shifted to the right the ACEA negative or the JWH 015 positive dose-response curves upon contractility, respectively. The relative potencies of the AM251 or AM630 in inhibiting the CB1 or CB2 cannabinoid receptor agonist effects, were determined by increasing concentration of the antagonists (1×10^{-7} M,

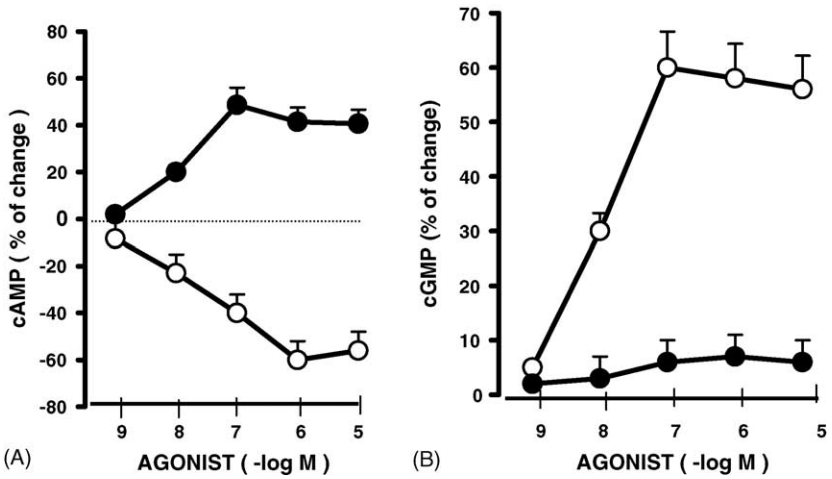

Fig. 4. Effect of increasing concentration of CB1 agonist (ACEA) (○) or CB2 agonist (JWH 015) (●) on cAMP (A) and cGMP (B) production of rat isolated atria. Cyclic nucleotides were measured incubating atria for 20 min and then, for an additional 10 min with different concentrations of CB1 or CB2 receptor agonists. Values are expressed as percentage of changes calculated by comparison with the absolute values prior the addition of agonists. Basal values of cAMP: 1.5 ± 0.1/mg/tissue wet weight and cGMP: 47 ± 3.1 pmol/g/tissue wet weight. For all curves, values are mean ± S.E.M. of six experiments performed in duplicate.

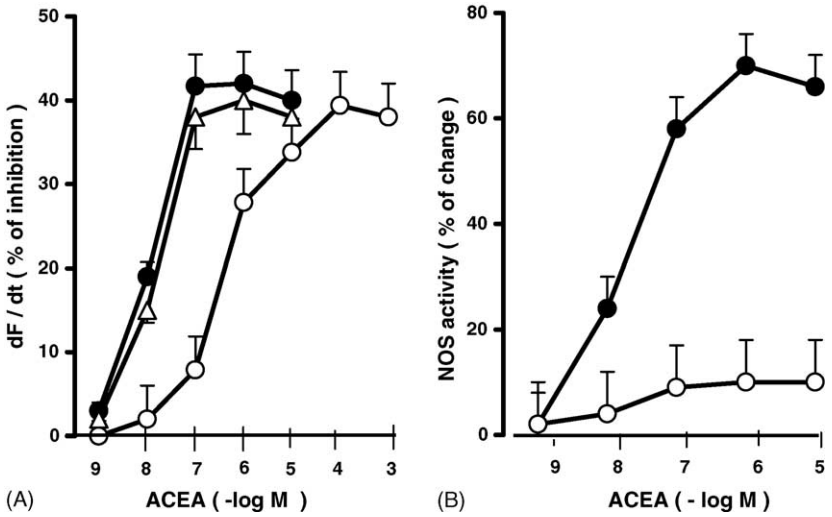

Fig. 5. A: Decreasing in contractility (dF/dt) of rat isolated atria by increasing concentration of ACEA alone (●) or in presence of 5×10^{-6} M L-NMMA (○). The reversal of the L-NMMA action by L-arginine (5×10^{-5} M) is also shown (△). B: Stimulation of NOS activity by increasing concentration of ACEA alone (●) or in the presence of 5×10^{-7} M AM251 (○). Tissue were incubated for 30 min in presence or absence of enzymatic inhibitor or AM251 and then, for an additional 10 min with different ACEA concentrations. No contractile effects 'per se' were observed with either L-NMMA (dF/dt g.s: 7.9 ± 0.6) or L-arginine (dF/dt g.s: 7.5 ± 0.4) at the concentrations used. Values are mean ± S.E.M. of five experiments in each group. NOS activity was performed by duplicates. For more details see Fig. 1.

3×10^{-7} M and 1×10^{-6} M) (pA2: CB1, 7.29 ± 0.16 and CB2, 6.98 ± 0.19). The pA2 values show that AM251 and AM630 relative potencies were equal for inhibiting ACEA and JWH 015 – induced inhibition or stimulation of atria contractility, respectively. Atropine (1×10^{-7} M) or propranolol (1×10^{-7} M) were without effects on ACEA or JWH 015 contractile effects, respectively (data not shown). Moreover, the effect of CB1 or CB2 agonists were not modified when experiments were carried out on atria from rats that had been chemically sympathectomized with 6-OHDA (EC_{50} normal: JWH 015 $7.1 \times 10^{-8} \pm 0.16$ M; ACEA $7.8 \times 10^{-8} \pm 0.31$ M and EC_{50} 6-OHDA: JWH 015 $6.9 \times 10^{-8} \pm 0.21$ M; ACEA $7.3 \times 10^{-8} \pm 0.40$ M; $n = 6$).

To characterize the involvement of cyclic nucleotides in the contractile effects of CB1 and CB2 receptors stimulation, we determined whether the cAMP and cGMP inhibitor modified the contractile effects of the corresponding cannabinoid agonists.

As can be seen in Fig. 3 adenylate cyclase inhibitor (SQ 22536, 5×10^{-5} M) blunted the JWH 015 positive contractile effect while the selective inhibition of nitric oxide (NO)-sensitive guanylate cyclase by ODQ (5×10^{-5} M), prevented the decrease on contractility triggered by ACEA. No effects were observed with SQ 22536 on ACEA or ODQ on JWH 015 contractile effects. Moreover, the effects of CB_1 or CB_2 receptor agonists on contractility were studied in parallel with their ability to modify cyclic nucleotides production. Fig. 4 shows that CB1 receptor agonist decreased cAMP (A) but increased cGMP (B) production in a concentration-dependent manner. On the contrary, the CB_2 receptor agonist increase cAMP (A) without effect on cGMP (B) accumulation.

To determine if an endogenous NO signaling system participated in the contractile action of CB_1 receptor activation, rat isolated atria were incubated with the NOS inhibitor L-NMMA (5×10^{-6} M). As can be seen in Fig. 5 this NOS inhibitor shifted to the right the dose-dependent decrement on contractility induced by ACEA. The action of the inhibitor was more effective at low concentrations of the agonist. The inhibitory action of L-NMMA on ACEA contractile effect was reversed by L-arginine (5×10^{-5} M). The CB_1 receptor agonist also

Table 1
Maximal effect of ACEA upon NOS activity and cGMP production by rat isolated atria

Drugs	NOS activity (pmol/tissue wet weight)	cGMP (pmol/g/tissue wet weight)
None	148 ± 11	47 ± 3
ACEA	262 ± 18*	72 ± 5*
ACEA + AM251	162 ± 12**	52 ± 4**
AM251	140 ± 10	45 ± 3
ACEA + L-NMMA	95 ± 6**	49 ± 4**
L-NMMA	98 ± 5	46 ± 4
ACEA + ODQ	253 ± 20*	42 ± 4**
ODQ	150 ± 13	40 ± 3
ACEA + U-73122	136 ± 10**	41 ± 3**
U-73122	146 ± 12	44 ± 4

Influence of different blockers. Activity of NOS and cGMP accumulation were measured incubating atria with or without inhibitors for 30 min and then, for an additional 10 min with 5×10^{-7} M ACEA. Values are means ± S.E.M. of five experiments performed in duplicate in each group. The final concentration of inhibitors were: AM251 5×10^{-7} M; L-NMMA 5×10^{-5} M; ODQ 5×10^{-5} M and U-73122 5×10^{-6} M.

* Statistically different from basal value (none) with $p < 0.0005$.

** Statistically different from ACEA alone with $p < 0.0005$ for NOS or cGMP respectively (Student's t-test).

increased in a concentration-dependent manner the NOS activity and this effect was blocked by AM251 (5×10^{-7} M).

Table 1 shows that the specific CB1 receptor antagonist inhibited the stimulatory action of ACEA on both NOS activity and cGMP accumulation. In addition, the inhibitor of PLC U-73122 (5×10^{-6} M) attenuated the CB1 receptor-dependent activation of cGMP levels and NOS activity, indicating the participation of PLC in these actions.

None of the inhibitors at the concentration used had any effect per se on basal values of NOS activity and cGMP (Table 1). It is important to note that on contractile experiments L-NMMA was used at 5×10^{-6} M, concentration that inhibited by 66% basal NOS activity but did not modify basal dF/dt values.

Fig. 6 demonstrates a significant correlation ($\alpha = 0.05$) between ACEA inhibited dF/dt, increased NOS-cGMP and inhibited cAMP (Pearson r: NOS, −0.9781; cGMP, −0.9981; cAMP, −0.9784). This result indicated that CB1 receptor activation-induced decrease in contractility as a result of NOS-cGMP pathway activation and cAMP inhibition.

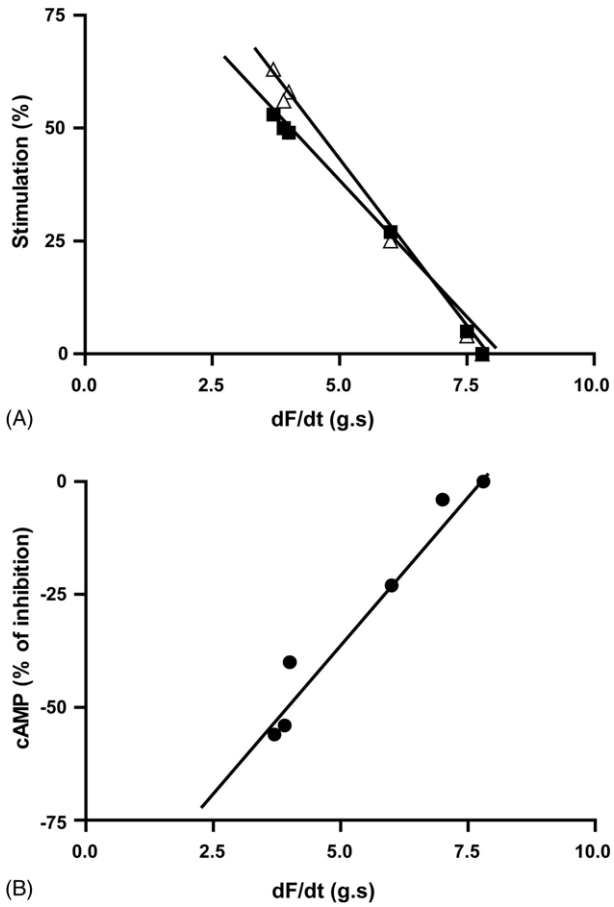

Fig. 6. Correlation in the modulatory effect of ACEA 1×10^{-10} to 1×10^{-6} M on contractility (dF/dt), NOS activity, cGMP and cAMP accumulations. (A) dF/dt was plotted as a function of either of NOS (■) ($p < 0.0001$) or cGMP (△) ($p < 0.0001$); (B) dF/dt was plotted as a function of cAMP (●) ($p < 0.0007$). Values are mean of six experiments of each group.

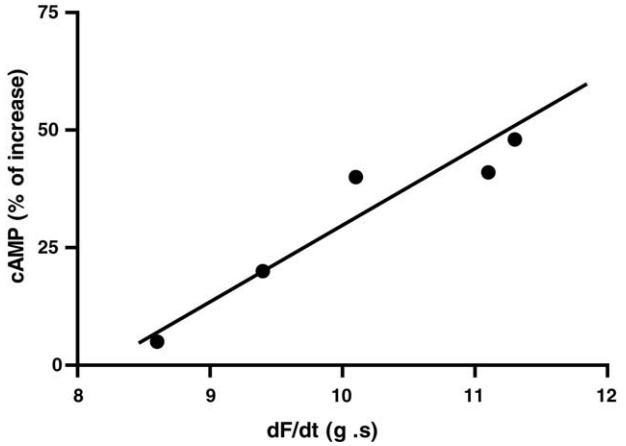

Fig. 7. Correlation in stimulatory effect of JWH 015 1×10^{-9} to 1×10^{-5} M on dF/dt and cAMP accumulation ($p < 0.012$). Values are mean of six experiments of each group.

Finally, we ascertained a significant correlation ($\alpha = 0.05$) between JWH 015 increased dF/dt and cAMP accumulation (Pearson r: 0.9518). Fig. 7 demonstrated that CB2 receptor activation-induced increase in cAMP resulted in increase on contractility.

4. Discussion

The current study gives pharmacological evidence for the existence of functional CB1 and CB2 receptors in rat isolated atria and characterizes the differential signaling events involved in the CB1 and CB2 specific receptor activation.

Agonist stimulation of CB1 and CB2 cannabinoid receptors triggered differential regulation on atria contractility. The CB1 receptor agonist-mediated decrease on contractility while CB2 receptor agonist-mediated positive contractile effect. Both these effects were receptor-mediated actions demonstrated by virtue of blockade by the selective cannabinoid CB1 (AM251) or CB2 (AM630) receptor antagonists. Interestingly, the endogenous cannabinoid anandamide had not consistent actions on atria contractility, but triggered significant positive or negative contractile effect in the presence of either CB1 or CB2 receptor antagonist respectively. These results suggest the co-expression of CB1 and CB2 receptors on rat atria. The existence of CB1 receptor in the rat heart was demonstrated [24,25] and had shown that anandamide exerts a CB1 sensitive cardiodepressive effect in vitro [3,19,24] and in vivo [25,26].

The CB1 and CB2 anandamide effects were studied on rat heart [19]. Specifically the latter paper demonstrated clear CB1 receptor-mediated decrease on contractility but the CB2 antagonist or the selective CB2 agonist JW 015 had not significantly effect. Since, the effect of CB1 selective agonist ACEA did not summate with those of JWH 015, it was concluded that the cannabinoid response

in rat heart were not mediated by anandamide acting at both CB1 and CB2 receptors [19]. Our results give considerable evidence that the actions of cannabinoids on rat isolated atria show a pharmacological profile which is consistent with two opposite contractile effects on the recognized CB1 and CB2 receptors.

A factor that plays a pivotal role in developing either CB1-negative or CB2-positive contractile response is the finding showing evidence of differential regulation of cannabinoid receptor coupling pathway that could either inhibit or stimulate adenylate cyclase. Thus, here we provide experimental evidence that cannabinoid CB1decrease on contractility correlates with the decrease in cAMP production while cannabinoids CB2 positive contractile effects correlates with increase cAMP production. The participation of cAMP in the CB2-positive inotropism was evident by the reversion with SQ 22536, an inhibitor of adenylate cyclase activity. Rhee et al. [13] demonstrated that the inhibition or stimulation of cAMP accumulation in cell that co-express either CB1 or CB2 receptors might be related to adenylate cyclase isoforms expressed in cells.

The present data also indicated that both nitric oxide (NO) and cGMP are involved in CB1-receptor mediated cardiodepressive effect, pointing to the role of PLC in the rapid activation of NOS and in the accumulation of cGMP. This conclusion are supported by the fact that the concentration response curves of ACEA acting on CB1 receptor were shifted to the right when NOS activity were inhibited. The mechanism seem to involve an activation of PLC whose intermediates would turn on the NOS activity, which inducing release of NO that in term activates a NO-sensitive-guanylate cyclase with increase production of cGMP, that accounts for the decrease on contractility by ACEA. Bonz et al. [24] demonstrated in human atrial preparation that the NOS inhibiton by NAME did not prevent the anadamide CB1-sensitive (0.1–1 μM) cardiodepressive effect in vitro. The nature of the inhibition showed in this paper with L-NMMA suggests that NO-mediated effect would be more relevant at lower than at higher concentration of ACEA. Thus, it seem likely that NO-mediated pathway predominates at low concentration of CB1 agonist would more than one signaling cascade accounts for the maximal cardiodepressive effect of CB1 agonist. The role of NO on the cardiodepressive effect of ACEA was further confirmed by the ability of the CB1 agonist to increase NOS activity and cGMP accumulation. The ACEA-induced decrease on contractility and increase NOS activity and cGMP production, were blunted by stereospecific NOS inhibitor, by the selective inhibition of NO-sensitive-guanylate cyclase and the inhibition of PLC. Moreover, there is a correlation between myocardial CB1 receptor stimulation promoted increase in NOS activation cGMP accumulation and decrease in contractility.

The fact that the specific CB1 antagonist was able to inhibit both ACEA-promonted increases in cGMP production and NOS activity demonstrates that they were CB1 receptor-mediate action. An interaction between CB1 cannabinoid receptors and PLC activation was shown in neurons in which cannabinoid agonist augmented the release of calcium associated with IP_3 receptor sensitive pool [27].

It is known that myocardial contractility is regulated by changes in the free intracellular calcium concentrations which are determined by concerted interaction of calcium influx through voltage dependent calcium channel, release of calcium from intracellular pools and calcium extrusion system [28]. Cannabinoid CB1 receptor activation decreases the L-type calcium channel [16] and simultaneously transient increases intracellular free calcium concentration [17]. According to our results the increment of intracellular calcium concentration by CB1 receptor activation could be related to the activation of PLC followed by the generation of inositolphosphates, which releases calcium from intracellular stores. This in term, activates constitutive atrial NOS with cGMP accumulation. Both messengers mediate the ACEA-induced decrease on contractility. Moreover, anandamide-activated CB1 receptors stimulated NO production in vein segments [18,29], in arterial endothelial cells [30,31] and in monocytes [32]. The NO generation was preceded by a rapid increase in intracellular calcium concentration [30,31], consistent with the stimulation of a calcium-regulated constitutive NOS. It has been reported that myocytes produced endogenous NO and the negative inotropic effect of NO in rat isolated atria appears to be mediated in part by activation of soluble guanylate cyclase with accumulation of cGMP [20,33]. Our results pointing to a role for NOS-cGMP in the cardiodepressive action induced by ACEA are in agreement with previous studies reporting that the generation of NO by sodium nitroprusside or a cyclic GMP analogue have the ability to decrease heart contractility [20,34].

Anandamide has been demonstrated that decreased release of noradrenaline by atrium subjected to electrical field stimulation [35] or appear to act increasing vagal activity [36]. However, in this work, neither atropine nor propranolol modified the CB1 or CB2-induced contractile actions, indicating a direct action on atria cannabinoid receptors. Moreover, the fact that in 6-OH dopaminized rat atria persisted the contractile effects of both CB1 and CB2 agonists, assess that these effects are independent of endogenous adrenergic mechanism. It is note that our preparation were paced with punctate electrodes and the voltage was 10% above threshold to avoid endogenous catecholamines release. However, cannabinoids activating CB1 receptors has several muscarinic interactions. Anandamide causes hypotension and bradycardia in rat and dog [37], which are thought to be vagally mediated since they are blocked by atropine or vagal section [36,38,39]. Cardiac muscarinic M_2 receptor and adenosine A_1 receptor activation mediated inotropic negative response via NOS-cGMP pathway in rat isolated atria [20,21].]

So far, adenosine [40], NO [41], cGMP [42] and also endocannabinoids [1] have been identified to have important protective role in cardiovascular system, particularly in preconditioning, shock and myocardial ischaemia.

Here, we give a new insight into the pathway by which cannabinoid receptor activation induced rapid stimulation of NO with subsequent accumulation of cGMP that may be the final mediators of protection in myocardial pathophysiological conditions. Therefore, understanding their cardiovascular effects and the receptors mediating them, will open up new clinical applications of cannabinoids in the treatment of cardiovascular disorders.

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References

[1] Robin Hiley C, Ford WR. Cannabinoid pharmacology in the cardiovascular system: potential protective mechanisms through lipid signaling. Biol Rev 2004;79:187–205.
[2] Ralevic V, Kendall DA, Randall MD, Smart D. Canabinoid modulation of sensory neurotransmission via cannabinoid and vanilloid receptors: roles in regulation of cardiovascular function. Life Sci 2002;71:2577–94.
[3] Randall MD, Kendall DA. Involvement of a cannabinoid in endothelium-derived hyperpolarizing factor-mediated coronary vasorelaxation. Eur J Pharmacol 1997;335:205–9.
[4] Matsuda LA, Lolait SJ, Brownstein MJ, Young AG, Bonner TI. Structure of cannabinoid receptor and functional expression of the cloned cDNA. Nature 1990;346:561–4.
[5] Munro S, Thomas KL, Abu-Shaar M. Molecular characterization of a peripheral receptor for cannabinoids. Nature 1993;365:61–5.
[6] Howlett AC, Barth TI, Bonner G, Cabral P, Casellas WA, Devane CC, Felder M, Herkenhian K, Mackie K, Martin BR, Mechoulam R, Pertwee RG. International Union of Pharmacology, XXVII. Classification of cannabinoid receptors, Pharmacol Rev 202;54:161–202.
[7] Pertwee RG. Pharmacology of cannabinoid CB1 and CB2 receptors. Pharmacol Ther 1997;74:129–80.
[8] Howlett AC, Qualy JM, Khachatrian LL. Involvement of Gi in the inhibition of adenylate cyclase by cannabimimetic drugs. Mol Pharmacol 1986;29:307–13.
[9] Childers SR, Deadwyler SA. Role of cyclic AMP to the actions of cannabinoid receptors. Biochem Pharmacol 1996;52:819–27.
[10] Pertwee RG. Pharmacology of cannabinoid CB1 and CB2 receptors. Pharmacol Ther 1997;74:129–80.
[11] Busch L, Sterin-Borda L, Borda E. Expresión and biological effects of CB1 cannabinoid receptor in rat parotid gland. Biochem Pharmacol 2004;68:1767–74.
[12] Glass M, Felder CC. Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors augments cAMP accumulation in striatal neurons: evidence for a Gs linkage to the CB1 receptor. J Neurosci 1997;17:5327–33.
[13] Rhee MH, Bayewitch M, Avidor-Reiss T, Levy R, Vogel Z. Cannabinoid receptor activation diferentially regulates the various adenylate cyclase isozymes. J Neurochem 1998;71:1525–34.
[14] Mu J, Zhuang SY, Kirby MT, Hampson RE, Deadwyler SA. Cannabinoid receptors differentially modulate potassium A and D currents in hippocampal neurons in culture. J Pharmacol Exp Ther 1999;291:893–902.
[15] Ho BY, Stadnicka A, Prather PL, Buckley AR, Current LL, Bossjar ZJ, et al. Cannabinoid CB1 receptor-mediated inhibition of prolactin release and signaling mechanisms in GH4CI cells. Endocrinology 2000;141:1675–85.
[16] Gebremedhin D, Lange AR, Camprell WB, Hillard CJ, Harder DR. Cannabinoid CB1 receptor of cat cerebral arterial muscle functions to inhibit L-type calcium channel current. Am J Physiol 1999;276:H2085–93.
[17] Sugiura T, Kodaka T, Kondo S, Nakane S, Kondo H, Waku K, et al. Is the cannabinoid CB1 receptor a 2-arachidonylglycerol receptor? Structural requirements for triggering a calcium transient in NG108-15 cells. J Biochem 1997;122:890–5.
[18] Maccarrone M, Barri M, Lorenzon T, Bisogno T, Di Marzo V, Finazzi-Agro A. Anandamide uptake by human endothelial cells an its regulation by nitric oxide. J Biol Chem 2000;275:13484–92.
[19] Ford WR, Honan SA, Whiote R, Hiley CR. Evidence of a novel site mediating anandamide-induced negative inotropic and coronary vasodilator responses in rat isolated hearts. Br J Pharmacol 2002;135:1191–8.
[20] Sterin-Borda L, Vila Echagüe A, Perez Leiros C, Genaro A, Borda E. Endogenous nitric oxide signaling system and the cardiac muscarinic acetylcholine receptor-inotropic response. Br J Pharmacol 1995;115:1525–31.
[21] Van Rossum JM. Cumulative dose-response curves. Arh Int Pharmacodyn Ther 1963;143:299–330.
[22] Sterin-Borda L, Gomez R, Borda E. Role of nitric oxide/cyclic GMP in myocardial adenosine A1 receptor-inotropic response. Br J Pharmacol 2000;135:444–50.
[23] Bredt DS, Snyder SH. Nitric oxide mediates glutamate-linked enhancement of cyclic GMP levels in the cerebellum. Proc Natl Acad Sci USA 1989;86:9030–3.
[24] Bonz A, Laser M, Kullmer S, Kniesch S, Balbin-Ebell J, Popp V, et al. Cannabinoids acting on CB1 receptors decrease contractile performance in human atrial muscle. J Cardiovasc Pharmacol 2003;41:657–64.
[25] Batkai S, Pacher P, Jarai Z, Wagner JA, Kunos G. Cannabinoid antagonist SR-141716 inhibits endotoxic hypotension by a cardiac mechanism not involving CB1 or CB2 receptors. Am J Physiol Heart Circ Physiol 2004;287:H595–600.
[26] Pacher P, Batkai S, Kunos G. Haemodynamic profile and responsiveness to anandamide of TRPV1 receptor knock-out mice. J Physiol 2004;558:647–57.
[27] Netzeband JG, Conroy SM, Parsons KL, Gruol DL. Cannabinoid enhances NMDA-elicited calcium signals in cerebellar granule neurons in culture. J Neurosci 1999;19:8765–77.
[28] Xu A, Narayanan N. Reversible inhibition of the calcium-pumping ATPase in native cardiac sarcoplasmic reticulum by a calmodulin-binding peptide. Evidence for calmodulin-dependent regulation of the Vmax of calcium transport. J Biol Chem 2000;275:4407–16.
[29] Stefano GB, Salzet M, Magazine HI, Bilfinger TV. Antagonism of LPS and IFN-gamma induction of iNOS in human saphenous vein endothelium by morphine and anandamide nitric oxide inhibition of adenylate cyclase. J Cardiovasc Pharmacol 1998;31:813–20.
[30] Fimiani C, Mattocks D, Cavani F, Salzet M, Deutsch DG, Pryor S, et al. Morphine and anandamide stimulate intracellular calcium transients in human arterial endothelial cells: coupling to nitric oxide release. Cell Signal 1999;11:189–93.

[31] Monbouli JV, Schaeffer G, Holzmann S, Kostner GM, Graier WF. Anandamide induced mobilization of cytosolic calcium in endothelial cells. Br J Pharmacol 1999;120:1593–600.
[32] Stefano GB, Liu Y, Goligorsky MS. Cannabinoid receptors are coupled to nitric oxide release in invertebrate immunocytes, microglia and human monocytes. J Biol Chem 1996;271:19238–42.
[33] Balligand JL, Kelly RA, Marsden PA, Smith TW, Michel T. Control of cardiac muscle cell function by an endogenous nitric oxide signaling system. Proc Natl Acad Sci USA 1993;90:347–51.
[34] Shah AM, Lewis MJ, Henderson AH. Effect of 8-bromo cyclic GMP on contractions and on inotropic response of ferret cardiac muscle. Mol Cell Cardiol 1991;23:55–64.
[35] Ishac EJ, Jiang L, Lake KD, Abood ME, Kunos G. Inhibiton of exocytotic noradrenaline release of presynaptic cannabinoid CB1 receptor on peripheral sympathetic nerves. Br J Pharmacol 1996;118:2023–8.
[36] Varga K, Lake KD, Huangfu D, Guyenet PG, Kunos G. Mechanism of hypotensive action of anandamide in anesthetized trats. Hypertension 1995;28:682–6.
[37] Dewey WL. Cannabinoid pharmacology. Pharmacol Rev 1986;38: 151–78.
[38] Varga K, Lake KD, Martin BR, Kunos G. Novel antagonists implicated the CB1 Cnnabinoid receptor in the hypotensive action of anandamide. Eur J Pharmacol 1995;278:279–83.
[39] Lake KD, Compton DR, Varga K, Martín BR, Kunos G. Cannabinoid-induced hypotension and bradycardia in rats is mediated by CB1-like cannabinoid receptors. J Pharm Exp Ther 1997;281:1030–7.
[40] Liu GS, Thornton J, Von Winkle DM, Stanley AW, Olsson RA, Downey JM. Protection against infarction afforded by preconditioning is mediated by A1 adenosine receptors in rabbit heart. Circulation 1991;84:350–6.
[41] Vegh A, Szekeres L, Parratt J. Preconditioning of the ischaemic myocardium involvement of the L-arginine nitric oxide pathway. Br J Pharmacol 1992;107:648–52.
[42] Szilvassy Z, Ferdinandy P, Bor P, Jakab I, Lonovics J, Koltai M. Ventricular overdrive pacing-induced anti - ischaemic effect: a conscious rabbit model of preconditioning. Am J Physiol 1994;266: H2033–41.