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Signaling cross-talk between cannabinoid and muscarinic systems actives Rho-kinase and increases the contractile responses of the bovine ciliary muscle

Maria Rosaria Romano*, Marcello Diego Lograno

Department of Pharmacy - Science of Drug, Section of Pharmacology and Toxicology, University of Bari "Aldo Moro", Bari, Italy

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ABSTRACT

The aim of the present study was to evaluate the role of a possible interaction between cannabinoid and muscarinic systems, both widely expressed in the ocular structure and involved in the control of bovine ciliary muscle contractility and intraocular pressure modulation. The ciliary muscle strips isolated by bovine eyes were exposed cumulatively to anandamide in the presence and in the absence of carbachol (5 nM), in a miograph system for isometric recording. The experiments were also conducted in the presence of AM251 (100 nM), 4-DAMP (100 nM), *Pertussis* toxin (500 ng/ml), U73122 (0.1 and 1 μM), chelerythrine (1 and 10 μM) and Y27632 (1 and 10 μM). Contractile responses were expressed as the percentage of 10 μM carbachol-induced contraction. The anandamide-induced contraction on bovine ciliary muscle strips was enhanced by the previous stimulation of G_q-protein-coupled muscarinic M_3 receptors with carbachol. The contractile response to anandamide plus carbachol was affected by different inhibitors such as *Pertussis* toxin, phospholipase C, protein kinase C and Rho-kinase. The key results of the present study show that sequential activation of muscarinic M_3 receptors and cannabinoid CB_1 receptors produce synergistic contractile effects of the bovine ciliary muscle by involving the activation of Rho-kinase and protein kinase C.

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1. Introduction

The ciliary muscle has smooth muscle-like properties and in the mammalian eyes the cellular contraction and the relaxation properties of ciliary muscle influence aqueous humour outflow and consequently the intraocular pressure (Woodword and Gil, 2004; Gabelt and Kaufman, 2005). The homoeostatic regulation of intraocular pressure has an important role for the optic nerve health, the retinal ganglion cell survival and a normal vision; in addition, an elevated intraocular pressure is considered the main risk factor of glaucoma disease (Kass et al., 2002). The ciliary muscle is essentially innervated by parasympathetic nerve fibres (Glasser and Kaufman, 2003) and its contraction is initiated and supported by muscarinic receptor stimulation, mostly of the M_3 subtype (Glasser and Kaufman, 2003), which cause an increase in aqueous humor drainage (Woodword and Gil, 2004; Gabelt and Kaufman, 2005).

Emerging studies demonstrated that also cannabinoids modulate the production and the drainage of humor aqueous (Porcella et al., 2000; Lograno and Romano, 2004; Tomida et al., 2004; Nucci et al., 2008). In fact, the cannabinoid CB_1 receptors are widely expressed in anterior segment of the eye, in particular the trabecular meshwork, the Schlemm's canal and ciliary muscle supporting the crucial physiological role for cannabinoids in the ocular hydrodynamic (Straiker et al., 1999; Lograno and Romano, 2004; Tomida et al., 2004; Romano and Lograno, 2007). Cannabinoid CB_1 receptors are members of the superfamily of the G-protein-coupled receptors with seven-*trans*-membrane-domain (Howlett, 2005; Pacher et al., 2006). It couples *via* pertussis toxin-sensitive $G_{i/o}$ protein to inhibit adenylyl cyclase and L-, N-, and P/Q-type calcium channel (Twitchell et al., 1997; Gebremedhin et al., 1999). However, it has been reported that cannabinoid CB_1 receptors are able to activate G_s protein by stimulating in turn adenylyl cyclase (Glass and Felder, 1997) and to functionally couple to G proteins from the $G_{q/11}$ family to increase intracellular calcium (Lauckner et al., 2005). Our previous study has shown that activation of the cannabinoid CB_1 receptor by anandamide and CP55,940 caused contraction of ciliary muscle by activation of phospholipase (PL) C in a Pertussis toxin-sensitive manner involving the $\beta\gamma$ subunits from $G_{i/o}$ protein (Lograno and Romano, 2004).

Evidences show that the muscarinic M_3 and cannabinoid CB_1 receptors are co-expressed in different tissues, such as brain, eye

* Correspondence to: Department of Pharmacy, Section of Pharmacology, University of Bari "Aldo Moro", Via Orabona 4, 70125 Bari, Italy.
Tel./fax: +39 080 5442797.
E-mail address: mariarosariaromano@hotmail.com (M.R. Romano).

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and cardiovascular system suggesting a functional interaction between these systems (Lau and Vaughan 2008; Marini et al., 2009). In addition, the interactions between cannabinoid CB_1 and muscarinic M_3 receptors might result intriguing in the ocular tissues where these systems are widely expressed (Choppin and Eglen, 2001; Lograno and Romano, 2004). The present study examines the effect of anandamide in presence of non-selective muscarinic M_3 receptor agonist carbachol by investigating the cross-talk between these two receptor subtypes and the mechanism(s) of this putative interaction.

2. Materials and methods

2.1. Ciliary muscle preparation

Bovine eyes were obtained from a local slaughterhouse, enucleated within 5 min after death and immediately put in ice-cold modified Krebs solution (composition in mM: NaCl 136.8, KCl 5.4, $MgSO_4$ 0.8, NaH_2PO_4 1.2, $NaHCO_3$ 12, $CaCl_2$ 2.7, D-glucose 5, Na-ascorbate 0.2) that had been pre-gassed with a mixture of 95% O_2 and 5% CO_2 and kept at 4 °C during transportation. They were brought to the laboratory within 30 min. The techniques for isolation and preparation of ciliary smooth muscle have been performed according to the method previously described (Lograno and Romano, 2004). Briefly, after removal of the vitreous body and crystalline lens, ciliary muscle was quickly isolated under a binocular microscope (Nikon, Japan) and was dissected from the sclera. Ciliary muscle strips of 4–5 mm length were prepared and immediately placed in 10 ml tissue bath filled with pre-aerated Krebs solution at 37 °C. The upper end of the preparation was linked with a silk thread to an isometric transducer (Fort 10, WPI, Sarasota, FL, USA). The experimental protocol was performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and in compliance with the Italian law on Animal Care no. 116/1992 and the Directive 2010/63/EU.

2.2. Myograph experiments

Ciliary muscle strips were stretched to an optimal passive tension of 3 mN and allowed to stabilize for at least 90 min during which the Krebs solution was changed every 15 min before the beginning of each experiment. Before any drug addition, all strips were constricted submaximally with carbachol (10 μM) to check the functionality of the tissue. After the carbachol challenge, tissues were washed, and the preload was readjusted just before the onset of the actual study.

To examine the synergic properties between anandamide and carbachol, the contractile activity of anandamide was investigated after a preincubation for 10 min with carbachol (5 nM), then cumulative concentration-response curves to anandamide was constructed.

To evaluate the mechanisms involved in the contractile effects, the strips were treated for 20 min with the following antagonists and enzyme inhibitors: AM251 (Lan et al., 1999) 100 nM (cannabinoid CB_1 receptor), 4-DAMP (Michel et al., 1989) 100 nM (muscarinic M_3 receptor), U73122 (Bleasdale et al., 1990) 0.1 and 1 μM (phospholipase C), *Pertussis* toxin (Katada and Ui, 1982) 500 ng/ml ($G_{i/o}$ protein), chelerythrine (Herbert et al. 1990) 1 and 10 μM (protein kinase C) and Y27632 (Uehata et al., 1997) 1 and 10 μM (Rho-kinase).

2.3. Isobologram analysis

Synergy between anandamide and carbachol was studied and the combined effects of the drugs were calculated by using the combination index (CI) isobologram method (Chou and Talalay, 1984). Assessment of synergy was performed using CalcuSyn software (Biosoft, Cambridge, UK). Combination index (CI) values <1, $=1$ and >1 indicate synergy, additivity and antagonism, respectively.

2.4. Data and statistical analysis

Contractile effects induced by cannabinoids were expressed as a percentage of the maximal response evoked by carbachol (10 μM). The concentration of contraction, giving a half-maximal response (EC_{50}) was obtained by fitting four-parameter sigmoidal concentration-response curves (GraphPad Prism Software, version 5.0) and was reported as its negative logarithm, pEC_{50}. E_{max} refers to the maximal response achieved. Assessment of synergy was performed using CalcuSyn software (Biosoft, Cambridge, UK). Combination index values <1, $=1$ and >1 indicate synergy, additivity and antagonism, respectively. All data were expressed as mean values $\pm$ S.E.M. The letter n refers to the experimental animals. Statistical analysis was performed by analysis of variance (ANOVA) followed by Bonferroni *post hoc* test (GraphPad Prism Software, version 5.0). Student's *t*-test for paired data was used when appropriate. P values of less than 0.05 were considered to indicate a statistical significant.

2.5. Drugs used

Anandamide (in Tocrisolve 100) water-soluble emulsion, AM251 (N-(piperidin-1.yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide), chelerythrine chloride, U73122 (1[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino] hexyl]-1H-pyrrole-2,5-dione) and Y27632 (*trans*-4-[(1*R*)-1-Amino-ethyl]-*N*-4-pyridinylcyclohexanecarboxamide dihydrochloride) were supplied by Tocris Bioscience (Bristol, UK). Carbachol chloride, 4-DAMP (4-diphenylacetoxy-*N*-methylpiperidine methiodide) and *Pertussis* toxin were obtained from Sigma Aldrich (St Louis, MO, USA). All drugs were dissolved in dimethyl sulphoxide or ethanol or distilled water as appropriate. The working solutions were freshly prepared on the day of the experiments by diluting the stock solutions with Krebs solution. The final bath concentration of dimethyl sulfoxide or ethanol was 0.1% which we have found elsewhere to have no effect on the tonus or mechanical function of preparation.

3. Results

3.1. The effect of carbachol on cumulative anandamide response curves

Anandamide (0.1 nM–10 μM) produced a concentration-dependent contraction of bovine ciliary muscle with E_{max} value of 51.7% $\pm$ 1.48 of $carbachol_{max}$ (anandamide: $pEC_{50}=6.99 \pm 0.03$; Fig. 1). A pre-stimulation of M_3 muscarinic receptors with carbachol (5 nM) for 10 min developed a small but not significant contraction ($<10\%$) in bovine ciliary muscle strips, but contraction significantly increased by adding subsequent cumulative concentrations of anandamide (in presence of carbachol: $pEC_{50}=7.31 \pm 0.14$; E_{max} 91.2 $\pm$ 3.39; $^{*}P<0.05$, $^{**}P<0.001$; Fig. 1). Sample calculation for the combination index (CI) values of carbachol at 5 nM plus anandamide from 0.1 to 10 μM, demonstrated synergism (CI $<$ 1) at all effect levels (Table 1).

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