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

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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 Gαq-protein-coupled muscarinic M3 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 M3 receptors and cannabinoid CB1 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 M3 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 CB1 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 CB1 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 Gαi/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 CB1 receptors are able to activate Gα protein by stimulating in turn adenylyl cyclase (Glass and Felder, 1997) and to functionally couple to G proteins from the Gα11 family to increase intracellular calcium (Lauckner et al., 2005). Our previous study has shown that activation of the cannabinoid CB1 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 βγ subunits from Gα11 protein (Lograno and Romano, 2004).

Evidences show that the muscarinic M3 and cannabinoid CB1 receptors are co-expressed in different tissues, such as brain, eye...
and cardiovascular system suggesting a functional interaction between these systems (Lau and Vaughan 2008; Marini et al., 2009). In addition, the interactions between cannabinoid CB1 and muscarinic M3 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 M3 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, MgSO4 0.8, NaH2PO4 1.2, NaHCO3 12, CaCl2 2.7, d-glucose 5, Na-ascorbate 0.2) that had been pre-gassed with a mixture of 95% O2 and 5% CO2 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 were 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 CB1 receptor), 4-DAMP (Michel et al., 1989) 100 nM (muscarinic M3 receptor), U73122 (Bleasdale et al., 1990) 0.1 and 1 μM (phospholipase C), Pertussis toxin (Katada and Ui, 1982) 500 ng/ml (Gαi 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 (EC50) was obtained by fitting four-parameter sigmoidal concentration-response curves (GraphPad Prism Software, version 5.0) and was reported as its negative logarithm, pEC50. Emax 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 ± 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 (1H-[11β]-3-methoxyestra-1,3,5(10)-trien-17-yl[amino]hexyl)-1H-pyrole-2,5-dione) and Y27632 (trans-4-[(1R)-1-Aminoethyl]-N-4-pyridinylcyclohexanecarboxamide dihydrochloride) were supplied by Tocris Bioscience (Bristol, UK). Carbachol chloride, 4-DAMP (4-diphenylacetoxy-N-methylpyriderine 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 toxins 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 Emax value of 51.7% ± 1.48 of carbacholmax (anandamide: pEC50 = 6.99 ± 0.03; Fig. 1). A pre-stimulation of M3 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: pEC50 = 7.31 ± 0.14; Emax = 91.2 ± 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).
significance when compared with the control relaxation using Student’s t-test for paired observations.

**Table 1**

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>Contractile effect (%)</th>
<th>CI values</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCh</td>
<td>AEAq</td>
<td></td>
</tr>
<tr>
<td>0.005</td>
<td>0.001</td>
<td>9.3 ± 1.1</td>
</tr>
<tr>
<td>0.005</td>
<td>0.01</td>
<td>29.1 ± 1.5</td>
</tr>
<tr>
<td>0.005</td>
<td>0.1</td>
<td>51.9 ± 2.1</td>
</tr>
<tr>
<td>0.005</td>
<td>1</td>
<td>78.8 ± 1.6</td>
</tr>
<tr>
<td>0.005</td>
<td>10</td>
<td>86.7 ± 1.2</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.E.M. n=8; A CI value significantly less than 1 indicates synergy.

Fig. 1. Effects of pre-incubation for 10 min with carbachol (5 nM) on contraction evoked by anandamide in the bovine ciliary muscle. Data for each concentration are reported as per cent of the maximal effect obtained with CCh 10 µM. Data are given as means with error bars representing S.E.M.; n=10; *P < 0.05, **P < 0.001 significance when compared with the control relaxation using Student’s t-test for paired observations.

3.2. Influence of muscarinic and cannabinoid receptors on the contraction induced by anandamide plus carbachol

The selective muscarinic M3 antagonist 4-DAMP (100 nM) reduced the contractile response anandamide-induced (anandamide plus carbachol: pEC50=7.25 ± 0.09, Emax 90.9 ± 3.24, n=6; in presence of 4-DAMP: pEC50=7.32 ± 0.25, Emax 61.6 ± 7.33, Fig. 2). Similarly, AM251 (100 nM) produced a decrease of the contraction evoked by anandamide plus carbachol (anandamide plus carbachol: pEC50=7.25 ± 0.09, Emax 92.2 ± 3.75, n=6; in presence of AM251: pEC50=6.91 ± 0.03, Emax 36.3 ± 0.65, n=6, Fig. 2). In addition, a pre-treatment with both antagonists to each receptor yielded a complete block of contractile response evoked by anandamide plus carbachol (Fig. 2).

3.3. Effect of signalling blockers

In light of the potentiation of muscarinic M3 and cannabinoid CB1 receptor-mediated signalling in bovine ciliary muscle, it was also sought to clarify whether Pertussis toxin-mediated signalling may actually affect the contractile response induced by anandamide plus carbachol since cannabinoid CB1 receptors have been shown to be coupled to a G protein of the Gi/o subclass. A preincubation for 20 min with Pertussis toxin (500 ng/ml) completely inhibited the contractile action induced by endocannabinoid also in presence of carbachol (5 nM) (Fig. 3).

The contractile response induced by anandamide plus carbachol was also significantly inhibited by pre-treatment with chelethrine (1 and 10 µM) (*P < 0.05, **P < 0.001; Fig. 4). Similar results were obtained with U73122, a well-known inhibitor of PLC. A pre-treatment for 20 min with U73122 (0.1 and 1 µM) significantly inhibited the contractile responses to anandamide plus carbachol in the bovine ciliary muscle (anandamide plus carbachol: pEC50=7.28 ± 0.09, Emax 89.9 ± 4.11, n=5; in presence of U73122 0.1 µM: pEC50=6.78 ± 0.84, Emax 39.6 ± 5.29; in presence of U73122 1 µM: pEC50=6.68 ± 1.32, Emax 16.7 ± 3.91; *P < 0.05, **P < 0.001; Fig. 5). Interestingly, the contractile response evoked by anandamide plus carbachol was also prevented with Y27632 (1 and 10 µM), a Rho-kinase inhibitor, by
suggesting an involvement for Rho-kinase pathway in the contractile process of ciliary muscle (Fig. 6).

4. Discussion

The present study shows for the first time a functional link between G\textsubscript{i}/G\textsubscript{o}-coupled cannabinoid CB\textsubscript{1} and G\textsubscript{q}-coupled M\textsubscript{3} muscarinic receptors in the bovine ciliary muscle strips and that carbachol synergistically enhances the contractile effects of anandamide since prior activation of muscarinic M\textsubscript{3} receptors by carbachol synergistically enhances the contractile effects of anandamide in absence and in presence of U73122 0.1 and 1 \textmu M. The muscarinic agonist CCh was given 10 min before the endocannabinoid anandamide. Data are given as mean \pm S.E.M; n=8. *P < 0.05; **P < 0.001 significance when compared with the control relaxation using one way ANOVA followed by Bonferroni's post hoc test.

The contractile response induced by anandamide plus carbachol has been significantly inhibited from the selective M\textsubscript{3} muscarinic receptor antagonist 4-DAMP and the selective cannabinoid CB\textsubscript{1} receptor antagonist AM251. In addition, a pre-incubation with both antagonist receptors completely blocked this contractile effect. Taken together, these data strongly indicate that the contraction of bovine ciliary muscle is under control of signals conveyed from M\textsubscript{3} receptors through a G\textsubscript{q}-coupled mechanism to cannabinoid CB\textsubscript{1} receptors and provide evidences for existence of an interesting cross-talk among these receptor pathways involved in the modulation of intraocular pressure.

To examine the mechanisms involved in the transductional signalling pathways has been used specific pharmacological tools. Previously, we have observed that anandamide yielded contractions of ciliary muscle through Pertussis toxin-sensitive G\textsubscript{i/o} \beta\gamma acting via PLC (Lograno and Romano 2004). Given this observation, we have pre-treated the ciliary muscle strips with G\textsubscript{i/o} protein inhibitor Pertussis toxin which caused an inhibition of contraction produced by anandamide plus carbachol confirming that activation of G\textsubscript{i/o} protein was involved. The reinforcement of contractile effects evoked by anandamide plus carbachol was affected from U7312, an inhibitor of PLC, suggesting that messengers derived by phosphoinositide-PLC pathway could be responsible for the contraction and that \beta\gamma dimer of G\textsubscript{i/o} proteins plays a key role in the elevation of intracellular calcium since it has been shown to directly activate PLC\beta isoenzymes. The PLC\beta isoform seems to have distinct binding sites for G\textsubscript{q} and G\textsubscript{b} (Werry et al., 2003) and the simultaneous occupation of these sites can lead to synergistic binding of G\textsubscript{q} and G\textsubscript{b} and the simultaneous occupation of these sites can lead to synergistic activation by giving a possible mechanism for receptor interaction at the level of PLC. In addition, we have investigated the role of protein kinase C as downstream signalling of the phosphoinositide-PLC cascade. In fact, the inhibition of protein kinase C by chelerythrine produced a significant decrease of contraction induced by anandamide plus carbachol confirming that protein kinase C has an important regulator role in the smooth ciliary muscle contractility. It is possible that protein kinase C may be necessary but not sufficient as a messenger of muscle contractility and other event enable the development of contraction.

It has been shown that activation of protein kinase C increases Ca\textsuperscript{2+} sensitivity in a pathway parallel to the Rho-Rho-kinase signalling cascade. In fact, also Rho-kinase is a key regulator of smooth muscle contraction (Uehata et al., 1997; Fukata et al.,...
Our results demonstrate that Y27632, a specific Rho-kinase inhibitor, reduced significantly the contraction induced by anandamide plus carbachol supporting that contraction of ciliary muscle involves not only the Ca^{2+}-dependent activation of myosin light chain kinase (MLCK) but also the Ca^{2+}-independent Rho-Rho-kinase pathway. Therefore, protein kinase C and Rho signalling cascade not only represents two parallel pathways but also they can interact and cooperate. This might depend on the type of smooth muscle, the applied agonist, the concentration of a given agonist and the species.

The use of multiple inhibitors has enabled us to elucidate the mechanisms of this signalling cascade, emphasizing and providing further support on impact mechanism by which a low concentration of carbachol increased the contraction anandamide-induced in the bovine ciliary muscle. More importantly, as depicted in Fig. 7, this higher efficacy might be due to activation of common events; indeed, one possible mechanism may involve the interaction between Gi/o protein and Gq-coupled receptors at the level of PLC which sequentially leads to mobilization of intracellular calcium via inositol 1,4,5-trisphosphate and diacylglycerol and to activation of protein kinase C and Rho/Rho-kinase signalling cascade and both cascades converge at inhibition of myosin light chain phosphatase, MLCP.

We conclude that a strong interaction exists between cannabinoid CB1 and the M3 muscarinic receptors since a co-application of agonists at each of these receptors synergistically enhanced the anandamide-induced contraction. Data shown an intriguing possibility that functional link between these receptors co-expressed on the surface of the muscle cell membrane may produce positive effects on modulation of intraocular pressure and open an attractive therapeutic hypothesis in order to use this compound in association limiting the serious adverse effects of muscarinic agonists.

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**References**


