Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 494 (2004) 55–62

www.elsevier.com/locate/ejphar

Cannabinoid agonists induce contractile responses through $G_{i/o}$-dependent activation of phospholipase C in the bovine ciliary muscle

Marcello D. Lograno*, Maria Rosaria Romano

Department of Pharmacobiology, Section of Pharmacology, University of Bari, Via E. Orabona 4, 70125 Bari, Italy

Received 15 April 2004; accepted 26 April 2004
Available online 28 May 2004

Abstract

This study was undertaken to investigate the effect of some cannabinoid agonists on the bovine ciliary muscle. Both anandamide and CP 55,940 (*cis*-3-(2-hydroxy-4-(1,1-dimethyl heptyl) phenyl)-*trans*-4-(3-hydroxypropyl) cyclohexanol) produced a concentration-dependent contractile response in ciliary muscle. These responses were inhibited by SR 141716A (*N*-[piperidin-1-yl]-5-(4-cholophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide) (0.1 and 1 μM) but not by SR 144528 (*N*-[1*S*)-endo-1,3,3-trimethyl bicyclo[2.2.1] heptan-2-yl] 5-(4-chloro-3-methylphenyl)-1-(4 methoxy benzyl)-pyrazole-3-carboxamide) (1 and 10 μM). A preincubation with $G_{i/o}$ protein inhibitor pertussis toxin (500 ng/ml) for 20 min inhibited the contractile action of anandamide and CP 55,940. In addition, the phospholipase C inhibitor U73122 (1[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl] amino] hexyl]-1*H*-pyrrole-2,5-dione) blocked the anandamide- and CP 55,940-induced contractions, whereas the protein kinase C activator, phorbol 12,13 dibutyrate (PDBu) significantly potentiated the contractions evoked by cannabinoid receptor agonists. We evaluated the binding of [^{3}H]CP 55,940, which specifically labelled a single class of cannabinoid sites with affinity in low subnanomolar range (K_d = 0.6 nM) and the maximal number of binding sites of 1243 fmol/mg protein. Binding of [^{3}H]CP 55,940 was inhibited by ligands having a major selectivity for cannabinoid (CB_1) receptors. These findings provide strong evidence of the involvement of cannabinoid CB_1 receptors promoting contraction in the bovine ciliary muscle. Furthermore, the action of cannabinoid receptor agonists appears to be mediated via phospholipase C. These data also contribute to elucidate the cannabinoid CB_1 receptor pivotal role in the modulation of intraocular pressure and to show that cannabinoid receptor agonists may be regarded as potential antiglaucoma agents.

Keywords: Cannabinoid CB_1 receptor; Anandamide; Receptor binding assay; Glaucoma

1. Introduction

Interest in cannabinoid drugs as antiglaucoma agents began over 30 years ago with an original report which documented intraocular pressure-lowering effects of marijuana in humans (Hepler and Franck, 1971). Since then, many studies have shown cannabinoid intraocular pressure-lowering effects (Colasanti, 1990; Järvinen et al., 2002), which are due to the principal active ingredient in marijuana Delta-9-tetrahydrocannabinol (Δ^9-THC). Investigations on Δ^9-THC led to the discovery of cannabinoid receptors and endocannabinoid compounds, opening new horizons for the use of cannabinoids in medicine, including glaucoma therapy.

At the moment, two cannabinoid or CB receptors have been identified (for review, see Howlett et al., 2002): cannabinoid CB_1 receptors are expressed both inside and outside the brain (Matsuda et al., 1990) and cannabinoid CB_2 receptors are limited to the periphery with functions related to the immune system (Munro et al., 1993). However, another work suggests that cannabinoid CB_2 receptors may be present in the central nervous system, including the retina (Lu et al., 1998). Several effector systems have been identified for cannabinoid receptors. In particular, both receptors are coupled to the $G_{i/o}$ family of G protein and associated with adenylyl cyclase inhibition (Felder et al., 1995). Cannabinoid CB_1 receptors inhibit the presynaptic calcium channels (Twitchell et al., 1997) and stimulate the potassium channels like the A-type and the inwardly rectifying (Mackie et al., 1995) through $G_{i/o}$ protein. In addition, candidate endogenous ligands like anandamide (arachido-

* Corresponding author. Tel./fax: +39-0805442797.
E-mail address: mdlograno@farmbiol.uniba.it (M.D. Lograno).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.04.039

nylethanolamide, AEA) (Devane et al., 1992) and 2-arachidonylglycerol (Stella et al., 1997) have been proposed.

Recent evidence has shown that CB_1 receptor mRNA is highly expressed in the rat's eye (Porcella et al., 1998) as well as in the human's eye (Porcella et al., 2000) and that such expression predominates in the anterior chamber of the eye, particularly in the ciliary body, supporting a substantial role of these receptors in controlling intraocular pressure (Colasanti, 1990). In addition, endogenous cannabinoid receptor ligands, such as anandamide, and other synthetic agonists such as WIN 55,212-2 ((*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo-[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenyl methanone) and CP 55,940 (*cis*-3-[2-hydroxy-4-(1,1-dimethyl heptyl) phenyl]-*trans*-4-(3-hydroxypropyl) cyclohexanol) have been reported to induce a reduction in intraocular pressure (Pate et al., 1996; Mikawa et al., 1997; Song and Slowey, 2000; Porcella et al., 2001); however, the specific mechanisms by which cannabinoids lower intraocular pressure were not completely elicited. The pattern of CB_1 mRNA expression, with the highest concentration occurring in the ciliary body, has supported the possibility that vasodilation-induced decrease of capillary pressure within the ciliary body and reduced intraocular pressure (Randall and Kendall, 1998) can be mediated by cannabinoid CB_1 receptors, although alternative hypotheses should be evaluated.

Our aim in this study was to demonstrate the existence of cannabinoid CB receptors in the bovine ciliary muscle using a binding assay with the tritiated CP 55,940. Moreover, we investigated whether the activation of cannabinoid CB receptor by endogenous and synthetic agonists could modulate the regulation of the ciliary muscle tone via G-protein coupled signalling pathways.

2. Materials and methods

2.1. Isolation of ciliary muscles

Bovine eyes, obtained from a slaughterhouse, were enucleated within 5 min after death and immediately put in ice-cold oxygenated modified Krebs solution for transport to the laboratory. After removing vitreous and lens, ciliary muscles were quickly isolated under a binocular microscope (Nikon, Japan) and were dissected from the sclear spur, lens and choroid as previously described (Lograno and Reibaldi, 1986; Lograno et al., 1991). The isolated tissues were immediately used for functional experiments or frozen on dry-ice and stored at −80 °C for binding assays.

2.2. Organ bath studies

Ciliary muscle strips of 4–5 mm length were prepared and immediately studied in a miograph system. Samples were mounted in a 10-ml organ bath containing Krebs solution (37 °C) continuously aerated with a mixture of 95% O_2 and 5% CO_2. The Krebs solution was as follows (mM): NaCl, 136.8; KCl, 5.4; $CaCl_2$, 2.7; $MgSO_4 \cdot 7\ H_2O$, 0.8; $NaH_2PO_4 \cdot H_2O$, 1.4; $NaHCO_3$, 12; glucose, 5; Na-ascorbate, 0.2 (pH 7.4). The preparation was linked with a silk thread to an isometric strain gauge under a constant load of 300 mg. Contractile activity was measured using an appropriate transducer (Fort 10, WPI, Sarasota, FL, USA) connected to a PowerLab 4/20 recorder (ADInstruments, Castle Hill, NSW, Australia). Tissues were allowed to equilibrate for at least 90 min during which the Krebs solution was changed several times. Before any drug addition, tissues were challenged with carbachol (10 μM) at least once to assess the functional state of each preparation. Tissues were then washed, and the preload was readjusted just before the onset of the actual study. When more than one concentration of the antagonist was added, a control response to the agonist was recorded, in order to ensure that complete recovery from blockade had taken place. All drugs were added to the Krebs solution to obtain the appropriate concentrations expressed as molar concentration. Contractile responses were expressed as percentages of the maximal effect produced by carbachol in each control condition. EC_{50} of functional data was calculated by fitting four-parameter sigmoidal dose–response curves using Prism GraphPad (version 3.0, San Diego, CA, USA).

2.3. Ciliary muscle membrane preparation

Ciliary muscles were homogenized using Ultra-Turrax at setting 4–5 for 30 s in 20 volumes (based on the wet-weight) of ice-cold Tris–HCl buffer (50 mM, pH 7.4) containing 70 μM bacitracin and 10 mM phenylmethylsulphonyl fluoride (PMSF). The homogenate was filtered on a double-layer cheesecloth and centrifuged at 1000 × *g* for 20 min at 0 °C. The supernatant was further filtered and then centrifuged at 15,000 × *g* for 20 min at 2 °C. The resulting pellet was resuspended in 80 volumes of ice-cold buffer. Protein concentration was determined by the Bradford method using bovine serum albumin as standard (Bradford, 1976).

2.4. [^{3}H]CP 55,940 binding assays

[^{3}H]CP 55,940 saturation binding experiments were performed in triplicate. A range of 0.1–1.5 nM concentrations was used. The unlabelled drug for the determination of non-specific binding was prepared to give a final concentration of 10 μM. A 100 μl aliquot of [^{3}H]CP 55,940 was added to 100 μl of unlabelled drug (non-specific binding) or 100 μl of 50 mM Tris–HCl buffer which includes 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$ (pH = 7.4) (total binding), and the final volume was 1 ml. The incubation was initiated by the addition of 400 μl membrane l membrane suspension (150 μg protein) and allowed to continue at 30 °C for 60 min. In the experiment with anandamide, the incubation mixture also included 30 μM

PMSF. Incubation was ended by vacuum filtration through glass fiber Whatman GF/C filters pre-wet for 30 min in 50 mM Tris–HCl buffer (pH 7.4). The filters were washed three times with 3 ml of 50 mM Tris–HCl buffer (pH 7.4), transferred to vials and 7 ml of Pico-Aqua (Packard) liquid scintillation cocktail was added to each vial. Vials were allowed to set several hours prior to counting on a Beckman LS6000IC liquid scintillation counter. Competition binding experiments were carried out by the incubation of bovine ciliary muscle membranes with 1 nM [^{3}H]CP 55,940 in the presence of different concentrations of unlabelled cannabinoid ligands at 30 °C.

Scatchard analysis was performed by first-order nonlinear regression analysis of the saturation isotherms. Binding parameters, represented by the equilibrium dissociation constant (K_d, in nM) and by the receptor density (B_{max}, in fmol/mg protein) were calculated with GraphPad Prism (version 3.0). Inhibition constants (K_i, in nM) were calculated from displacement curves using the nonlinear least-square curve fitting and the same program as described above.

2.5. Statistics

All data in the text are reported as mean ± S.E.M, n refers to the number of preparation. Statistical evaluation was performed using Student's t-test for paired data when applicable or by analysis of variance (ANOVA) (with Bonferroni post hoc test) as appropriate; $P < 0.05$ was considered statistically significant.

2.6. Materials

Drugs and reagents used in the present study were obtained from the following companies: Anandamide, CP 55,940, WIN 55,212-2 and U73122 (1[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl] amino] hexyl]-1*H*-pyrrole-2,5-dione) Tocris Cookson (Bristol BS11 8TA UK); Phorbol 12,13 dibutyrate (PDBu) and Pertussis toxin Sigma Aldrich (St. Louis, MO, USA); SR 141716A (*N*-[piperidin-1-yl]-5-(4-cholophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide) and SR 144528 (*N*-[1*S*)-endo-1,3,3-trimethyl bicyclo[2.2.1] heptan-2-yl] 5-(4-chloro-3-methylphenyl)-1-(4methoxy benzyl)-pyrazole-3-carboxamide) generous gift from Dr. Francis Barth at Sanofi Recherché (Montpellier, France); [^{3}H]CP 55,940 (120 Ci/mmol) Pelkin-Elmer Life Sciences (Milan, Italy). All other reagents were of analytical grade. Stock solution (1 mM) of each drug was prepared in dimethyl sulfoxide and then diluted in the appropriate buffer. The final bath concentration of dimethyl sulfoxide did not exceed 0.1%, which we have found elsewhere to have little or no effect on mechanical activity and binding assays. Both functional and binding experiments were carried out in the dark, using only a sodium lamp to prevent light degradation of anandamide.

3. Results

3.1. Cannabinoid CB agonist-induced contractions

In the bovine ciliary muscle, anandamide, a cannabinoid CB receptor endogenous agonist, produced a concentration-dependent contractile response with EC_{50} value of 8.78 nM. The maximal response was obtained with 10 μM anandamide (47% of carbachol$_{max}$; Fig. 1A; $n = 7$). SR 141716A (0.1 and 1 μM), selective cannabinoid CB_1 receptor antagonist, produced a rightward shift of the anandamide concentration–response curves (Fig. 1A; $n = 7$; $P < 0.001$) and EC_{50} values of 60.2 and 928 nM were respectively calculated. Furthermore, increasing concentrations of SR 141716A decreased the maximal response to anandamide. The effect of SR 144528 (1 and 10 μM), selective cannabinoid CB_2 receptor antagonist, on the contractile response to anandamide was also evaluated. Anandamide on control tissue gave an EC_{50} of 8.90 nM and the maximal response was at 20 μM (49% of carba-

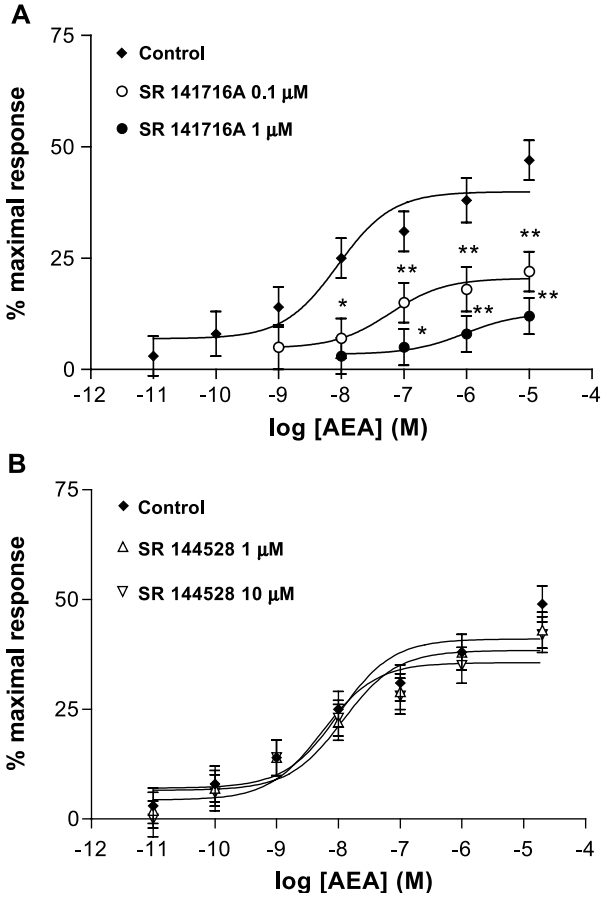

Fig. 1. Effect of cannabinoid receptor antagonist on contractions evoked by anandamide in the bovine ciliary muscle. (A) Concentration–response curves to AEA (control) ($n = 7$) and on tissue previously exposed to SR 141716A (0.1 and 1 μM, $n = 7$). (B) Concentration–response curves to AEA (control) ($n = 8$) and on tissue previously exposed to SR 144528 (1 and 10 μM; $n = 6$). Each point is the mean ± S.E.M. *$P < 0.01$, **$P < 0.001$.

$chol_{max}$). Increasing concentration of the antagonist did not alter the anandamide concentration–response curves (Fig. 1B; $n=6$; $P>0.05$).

CP 55,940, a cannabinoid CB receptor agonist, also significantly contracted the bovine ciliary muscle in a concentration-dependent manner with EC_{50} value of 18.3 nM; the maximum response was estimated with 10 μM CP 55,940 (55% of $carbachol_{max}$). SR 141716A (0.1 and 1 μM) yielded a shift to the right of the CP 55,940 concentration–response curves (Fig. 2A; $n=6$; $P<0.001$) and EC_{50} values of 0.17 and 0.14 μM were respectively calculated. SR 144528 was evaluated and it did not modify the CP 55,940 concentration–response curves (Fig. 2B; $n=6$; $P>0.05$).

3.2. Activation of cannabinoid CB_1 receptor

Cannabinoid CB_1 receptors have been shown to couple to G_i as well as G_s protein (Glass and Felder, 1997). A preincubation with $G_{i/o}$ protein inhibitor pertussis toxin (500 ng/ml) for 20 min completely abolished anandamide-induced contraction of ciliary muscle strips (Fig. 3A; $n=6$). Pertussis toxin also completely prevented the contractile responses to CP 55,940 in the concentration utilized (data not shown). In addition, the initial maximal contraction yielded by anandamide (10 μM, $n=5$) or CP 55,940 (10 μM, $n=4$) could be repeated after about 1 h waiting period. These results suggest that the anandamide- and CP 55,940-induced contractions were initiated by the activation of $G_{i/o}$ protein-coupled signalling pathway.

We have explored the role of phospholipase C in anandamide-induced contractions assessing the effect of the phospholipase C inhibitor U73122. The action of anandamide (1 nM to 10 μM) was inhibited in the presence of increasing concentrations of U73122 (1, 5 and 10 nM) (Fig. 4; $n=6$). The higher concentration utilized (10 nM) completely blocked the contractile responses of anandamide (Fig. 3B). The same results were obtained using CP 55,940 (from 1 nM to 10 μM) and U73122 (1, 5 and 10 nM) (data not shown).

In another series of the experiment, the sensitivity of the anandamide- and CP 55,940-induced contraction to protein kinase C modulation was examined in the bovine ciliary muscle, using the protein kinase C activator phorbol 12,13 dibutyrate (PDBu). A preincubation with PDBu (10 or 100 nM) for 20 min before anandamide and CP 55,940 significantly increased the contractile responses (80% of AEA_{max}, $P<0.0001$ and 74% of CP $55{,}940_{max}$, $P<0.001$, $n=7$) (Fig. 3C).

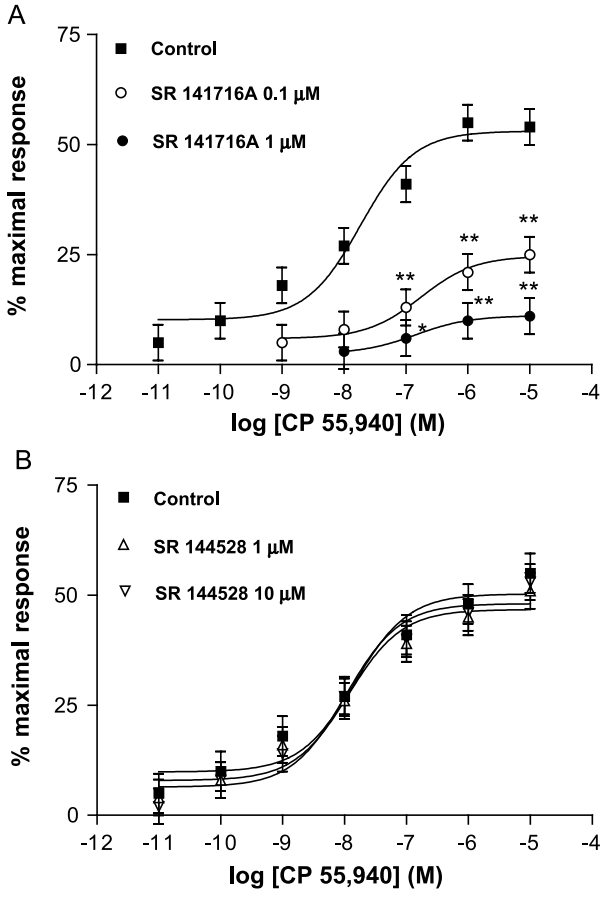

Fig. 2. Effect of cannabinoid receptor antagonist on contractions evoked by CP 55,940 in the bovine ciliary muscle. (A) Concentration–response curves to CP 55,940 (control) ($n=6$) and on tissue previously exposed to SR 141716A (0.1 and 1 μM, $n=6$). (B) Concentration–response curves to CP 55,940 on control strips ($n=6$) and on tissue previously exposed to SR 144528 (1 and 10 μM, $n=7$). Each point is the mean ± S.E.M. *$P<0.01$, **$P<0.001$.

3.3. Biochemical characterization of cannabinoid receptor in the bovine ciliary muscle

Time course studies of [^{3}H]CP 55,940 binding in ciliary muscle membranes were initially performed. Non-specific binding for [^{3}H]CP 55,940 represented less than 10% of the total binding. Specific binding of [^{3}H]CP 55,940 linearly increased with membrane protein concentration over a range of 80–230 μg/ml. Scatchard transformation of the saturation data produced a linear plot indicating a single population of saturable binding sites. An average K_d value of 0.61 ± 0.072 nM was obtained, whereas the B_{max} was 1243 ± 87.0 fmol/mg protein (Fig. 5).

The results of the competition curves are shown in Fig. 6 and Table 1. Binding was inhibited in a concentration-dependent manner by various ligands with a rank order of potency of CP 55,940>SR 141716A>anandamide>WIN 55,212-2≫SR 144528. CP 55,940 had the greatest apparent affinity when tested in competition studies with K_i value of 0.95 nM. SR 141716A effectively appears to compete for [^{3}H]CP 55,940 binding with K_i value of 3.55 nM. The apparent affinity of anandamide ($K_i=9.52$) was 10-fold higher than CP 55,940; moreover, when anandamide was assayed without the inclusion of PMSF to inhibit amidase activity (Compton and Martin, 1997), the K_i

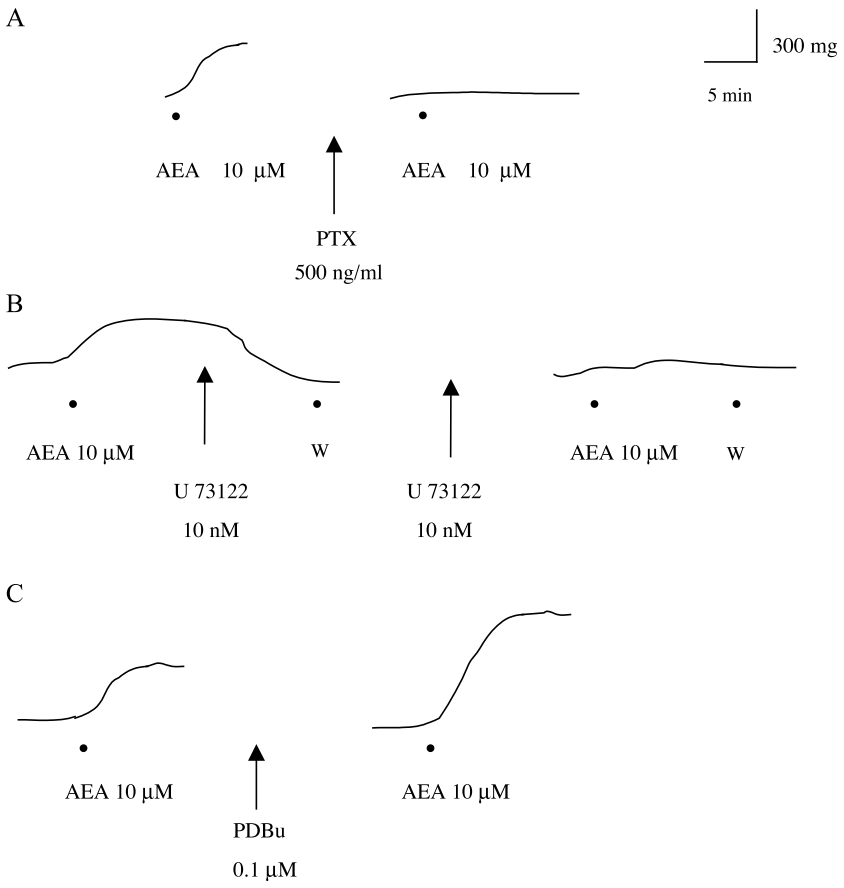

Fig. 3. Typical tracings showing the AEA response on bovine ciliary muscle; the effect of AEA is mediated via a $G_{i/o}$ protein signalling pathway. (A) Trace shows one experiment in which the AEA (10 μM) response was blocked by pretreatment (20 min) with pertussis toxin (500 ng/ml); (B) one experiment showing that U73122 (10 nM) was efficacious to inhibit the response to AEA. U73122 (10 nM) was applied on AEA-induced tone and with preincubation for 15 min before AEA; (C) AEA (10 μM) response was potentiated by PBDu (0.1 μM) preincubated for 20 min, as shown in this representative trace.

increased to 487 ± 79 nM, which indicates the susceptibility of this compound to enzymatic hydrolysis in membrane preparations. WIN 55,212-2 had a lower ability to compete for the receptor labelled with [^{3}H]CP 55,940 than other compound. Furthermore, one can note that SR 144528, a selective antagonist of cannabinoid CB_2 receptor, did not

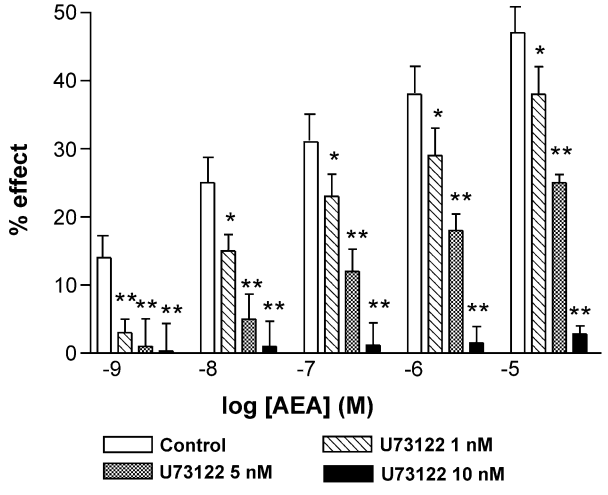

Fig. 4. Effect of U73122 pretreatment on anandamide-induced contractions in the bovine ciliary muscle. Increasing concentrations of AEA (1 nM to 10 μM) in absence and in presence of 1, 5 and 10 nM U73122. Values shown are mean ± S.E.M. of five experiments *P<0.01, **P<0.001.

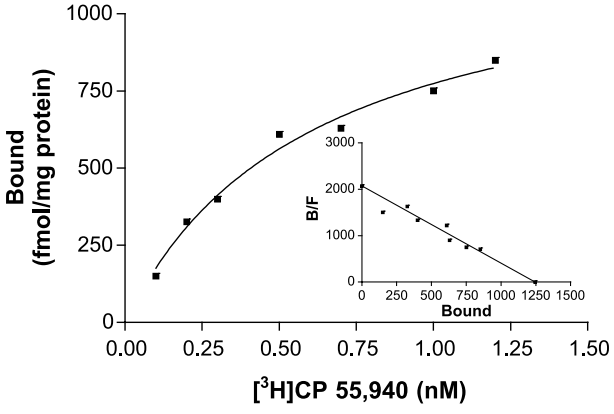

Fig. 5. Representative saturation analysis and Schatchard plot (inset) of [^{3}H]CP 55,940 to binding to bovine ciliary muscle membranes. K_d = 0.6 ± 0.072 nM, B_{max} = 1243 ± 87.0 fmol/mg of protein. Data were analysed with GraphPad Prism program and a one site analysis gave the best fit. CP 55,940 10 μM was used to define non-specific binding. Assays were performed as described in Materials and methods. Values represent a mean ± S.E.M. of six experiments, each performed in triplicate.

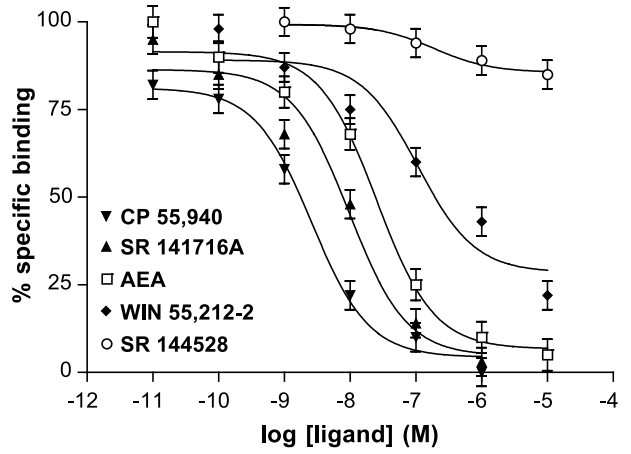

Fig. 6. Displacement of [^{3}H]CP 55,940 binding to membranes of bovine ciliary muscle by various cannabinoid receptor ligands. Assays were performed as described in Section 2. Data points represent percent of specific [^{3}H]CP 55,940 bound in absence of compound tested; and represent mean ± S.E.M. of four or five separate experiments, each performed in triplicate.

produce any displacement of [^{3}H]CP 55,940 binding sites in the membrane preparations examined.

4. Discussion

Several preclinical and clinical studies suggest that the cannabinoids may be regarded as a new class of antiglaucoma agents (Hepler and Franck, 1971; Colasanti, 1990; Porcella et al., 2001). The high level of cannabinoid CB_1 receptors in appropriate intraocular structures reinforces the hypothesis of a physiological role for cannabinoids in the control intraocular pressure. In fact, the CB_1 receptor expression in the ciliary pigment epithelium suggests that cannabinoids may have an effect on aqueous humor production, whereas their presence in the trabecular meshwork and Schlemm's canal suggests that they can influence conventional outflow.

This study clearly demonstrates the presence of functional cannabinoid CB_1 receptors in the bovine ciliary muscle. Other studies by reverse transcriptase polymerase chain reaction and immunohistochemical techniques (Porcella et al., 1998; Straiker et al., 1999) have established the existence of cannabinoid CB_1 receptors in the rat and human ciliary muscle. We have found that both the endogenous ligand anandamide and the highly potent synthetic cannabinoid CP 55,940 induced contractions in the bovine ciliary muscle strips. The contractile effects of anandamide and CP 55,940 were inhibited by SR 141716A, the selective cannabinoid CB_1 receptor antagonist (Rinaldi-Carmona et al., 1995), in a concentration-dependent manner. Furthermore, the inability of SR 144528, a selective cannabinoid CB_2 receptor antagonist, to alter the anandamide- and CP 55,940-induced concentration–contraction curves suggests that CB_2 receptor are not involved in this effect.

A binding study conducted with [^{3}H]CP 55,940 allowed us to selectively identify a population of cannabinoid receptors in the membrane preparations of the bovine ciliary muscle. Saturation data show that [^{3}H]CP 55,940 binding was saturable and of high affinity and occupying a single class of binding sites with K_d values in the low subnanomolar range (K_d value of 0.6 nM). The ability of a number of type-selective cannabinoid ligands to displace [^{3}H]CP 55,940 binding from bovine ciliary muscle membranes was assessed. Unlabelled CP 55,940 displaced [^{3}H]CP 55,940 binding with 10 times greater potency than anandamide and SR 141716A. Furthermore, WIN 55,212-2 showed a lower potency to displace [^{3}H]CP 55,940 binding as indicated by the affinity values (K_i value of 60.2 nM). In contrast to the potent displacement of [^{3}H]CP 55,940 binding to CB_1 observed for various CB_1 selective agonist and antagonists, SR 144528 was inactive. The relative potencies of cannabinoid ligands in displacing [^{3}H]CP 55,940 binding (CP 55,940>anandamide>SR 141716A>WIN 55,212-2) is consistent with what has been found in functional assay. Taken together, these data strongly support the hypothesis that the contractions evoked by anandamide and CP 55,940 in the bovine ciliary muscle involved the cannabinoid CB_1 receptor.

Cannabinoid CB_1 receptors are known to exert their biological functions by interacting with G_i/G_o proteins to inhibit adenylate cyclase (Howlett et al., 1986; Vogel et al., 1993). However, it is not clear whether the anandamide- and CP 55,940-induced contractions of ciliary muscle were the result of the decreased cAMP levels due to adenylate cyclase inhibition, or the increased cytosolic calcium concentrations. In our study, the contraction of ciliary smooth muscle induced by anandamide or CP 55,940 was completely blocked by pretreatment with pertussis toxin, indicating that coupling of cannabinoid receptor to G_i/G_o proteins in ciliary smooth muscle cells is necessary to initiate the motor response. In addition, we have found that phospholipase C inhibitor U73122 blocked the contractions of cannabinoid receptor agonists in the bovine ciliary muscle. Activation of phospholipase C leads to a mobilization of intracellular Ca^{2+} and an activation of protein kinase

Table 1
Potencies of various cannabinoid ligands in inhibiting [^{3}H]CP 55,940 binding to bovine ciliary muscle membranes

Ligand	K_i (nM)*	Hill coefficient	n
Anandamide	9.52 ± 0.15	0.75 ± 0.17	5
CP 55,940	0.95 ± 0.01	0.77 ± 0.14	4
SR 141716A	3.55 ± 0.07	0.86 ± 0.02	4
WIN 55,212-2	60.2 ± 1.31	0.56 ± 0.10	4
SR 144528	>1000[a]		4

Bovine ciliary muscle membranes were incubated for 60 min at 30 °C with 1 nM [^{3}H]CP 55,940 in the presence of increasing concentrations of cannabinoid ligands. *K_i values were calculated by fitting displacement binding curves using the GraphPad Prism program. Values represent the mean ± S.E.M. of four and five independent experiments, each performed in triplicate.

[a] Indicates less than 50% displacement when tested at 1 mM.

C as a result of inositol-1,4,5-triphosphate and diacylglycerol formation, respectively. Thus, our results with U73122 indicate that cannabinoids modulate the contractions of the bovine ciliary muscle by enhancing Ca^{2+} intracellular levels and this effect involves a phospholipase C-sensitive mechanism. To confirm this hypothesis, the role of protein kinase C in contractile effect induced by cannabinoid agonists was also evaluated utilizing PDBu, an activator of protein kinase C, which produced a significant increase of contractile effect to anandamide and CP 55,940 in the bovine ciliary muscle. Previous studies have shown an action of cannabinoids on Ca^{2+} release from intracellular Ca^{2+} stores. For example, Delta-9-tetrahydrocannabinol elicited Ca^{2+} release from thapsigargin-sensitive Ca^{2+} stores in DDT_1MF-2 smooth muscle cells (Filipeanu et al., 1997), a cell type that does not express VSCCs. In those studies, the cannabinoid effect on Ca^{2+} release was sensitive to blockade by SR 141716A, indicating the involvement of the cannabinoid CB_1 receptor.

Our data show that phospholipase C have a key role in contraction process and that βγ subunit of G_i/G_o proteins could be the possible candidate to activate phospholipase C. It is interesting to bear in mind that involvement of phospholipase C is a pathway typically associated with G_q (Exton, 1996). Indeed, studies performed in expression systems have shown that βγ subunit of G_i/G_o proteins can activate phospholipase C (Exton, 1996), providing a pathway by which G_i/G_o-coupled receptors such as the cannabinoid receptors could enhance intracellular Ca^{2+} levels and produce the contractile effects in the bovine ciliary muscle. Other G_i/G_o-coupled receptors such as M_2 muscarinic, somatostatin and opioid receptors, have been shown to activate phospholipase C.

In conclusion, the present study shows that cannabinoid CB_1 receptors are present in bovine ciliary muscle and that their activation by cannabinoid ligands modulates the muscular tone. Ciliary muscle alters the accommodation of the lens allowing us to focus on objects at various distances. Moreover, the contraction of ciliary muscles causes a significant change in the shape of trabecular meshwork to which they are attached. This facilitates the escape of aqueous humor by conventional outflow via Schlemm's canal, reducing intraocular pressure. These results further contribute to elucidate the functional role of cannabinoid receptors in the ocular pathophysiology.

Acknowledgements

The authors thank Dr. Vito Masciopinto, vet of the slaughterhouse of the AUSL BA/5 (Bari, Italy), Dr. Mariangela Dimatteo for the help in the preparation of this manuscript and Sanofi Recherché (Montpellier, France) for having provided SR 141716A and SR 144528. Supported by "Ministero dell'Università e della Ricerca Scientifica e Tecnologica" (Cofinanziamento 2001).

References

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein dye binding. Anal. Biochem. 72, 248–254.

Colasanti, B.K.J., 1990. A comparison of the ocular and central effects of delta 9-tetrahydrocannabinol and cannabigerol. Ocul. Pharmacol. 6, 259–269.

Compton, D.R., Martin, B.R., 1997. The effect of the enzyme inhibitor phenylmethylsulfonyl fluoride on the pharmacological effect of anandamide in the mouse model of cannabimimetic activity. J. Pharmacol. Exp. Ther. 283, 1138–1143.

Devane, W.A., Hanus, L., Breuer, A., Pertwee, R.G., Stevenson, L.A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., Mechoulam, R., 1992. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 258, 1946–1949.

Exton, J.H., 1996. Regulation of phosphoinositide phospholipases by hormones, neurotransmitters, and other agonists linked to G proteins. Annu. Rev. Pharmacol. Toxicol. 36, 481–509.

Felder, C.C., Joyce, K.E., Briley, E.M., Mansouri, J., Makie, K., Blond, O., Lai, Y., Ma, A., Mitchell, R.L., 1995. Comparison of the pharmacology and signal transduction of the human cannabinoid CB_1 and CB_2 receptors. Mol. Pharmacol. 48, 443–450.

Filipeanu, C.M., de Zeeuw, D., Nelemans, S.A., 1997. Delta9-tetrahydrocannabinol activates $[Ca^{2+}]_i$ increases partly sensitive to capacitative store refilling. Eur. J. Pharmacol. 336, R1–R3.

Glass, M., Felder, C.C., 1997. Concurrent stimulation of cannabinoid CB_1 and dopamine D_2 receptors augments cAMP accumulation in striatal neurons: evidence for a Gs linkage to the CB_1 receptor. J. Neurosci. 17, 5327–5333.

Hepler, R.S., Franck, I.R., 1971. Marihuana smoking and intraocular pressure. JAMA 217, 173–180.

Howlett, A.C., Qualy, J.M., Khachatrian, L.L., 1986. Involvement of Gi in the inhibition of adenylate cyclase by cannabinomimetic drugs. Mol. Pharmacol. 29, 307–313.

Howlett, A.C., Barth, F., Bonner, T.I., Cabral, G., Casellas, P., Devane, W.A., Felder, C.C., Herkenham, M., Martin, B.R., Mechoulam, R., Pertwee, R.G., 2002. International union of pharmacology: XXVII. Classification of cannabinoid receptors. Pharmacol. Rev. 54, 161–202.

Järvinen, T., Pate, D.W., Laine, K., 2002. Cannabinoids in the treatment of glaucoma. Pharmacol. Ther. 95, 203–220.

Lograno, M.D., Reibaldi, A., 1986. Receptor–responses in fresh human ciliary muscle. Br. J. Pharmacol. 87, 379–385.

Lograno, M.D., Daniele, E., Trabucchi, M., Govoni, S., 1991. Evidence for protein kinase C modulation of the ciliary muscle response to carbachol and desensitization. Eur. J. Pharmacol. 204, 49–53.

Lu, Q.J., Straiker, A.J., Lu, Q.X., Maguire, G., 1998. Expression of CB_2 cannabinoid receptor mRNA and protein in adult rat retina. [ASCB Abstract]. Mol. Biol. Cell 9, 354a.

Mackie, K., Lai, Y., Westenbroek, R., Mitchell, R., 1995. Cannabinoids activate an inwardly rectifying potassium conductance and inhibit Q-type calcium currents in AtT20 cells transfected with rat brain cannabinoid receptor. J. Neurosci. 15, 6552–6561.

Matsuda, L.A., Lolait, S.J., Brownstein, M.J., Young, A.C., Bonner, T.I., 1990. Structure of cannabinoid receptor and functional expression of the cloned cDNA. Nature 346, 561–564.

Mikawa, Y., Matsuda, S., Kanagawa, T., Tajika, T., Ueda, N., Minura, Y., 1997. Ocular activity of topically administered anandamide in the rabbit. Jpn. J. Ophthalmol. 41, 217–220.

Munro, S., Thomas, K.L., Abu-Shaar, M., 1993. Molecular characterization of a peripheral receptor for cannabinoids. Nature 365, 61–65.

Pate, D.W., Järvinen, K., Urtti, A., Jarbo, D., Fich, M., Mahadevan, V., Järvinen, T., 1996. Effects of topical anandamides on intraocular pressure in normotensive rabbits. Life Sci. 21, 1849–1860.

Porcella, A., Casellas, P., Gessa, G.L., Pani, L., 1998. Cannabinoid receptor CB_1 mRNA is highly expressed in the rat ciliary body: implications

for the antiglaucoma properties of marihuana. Mol. Brain Res. 58, 240–245.

Porcella, A., Maxia, C., Gessa, G.L., Pani, L., 2000. The human eye expresses high levels of CB_1 cannabinoid receptor mRNA and protein. Eur. J. Neurosci. 12, 1123–1127.

Porcella, A., Maxia, C., Gessa, G.L., Pani, L., 2001. The synthetic cannabinoid WIN55212-2 decreases the intraocular pressure in human glaucoma resistant to conventional therapies. Eur. J. Neurosci. 13, 409–412.

Randall, M.D., Kendall, D.A., 1998. Endocannabinoids: a new class of vasoactive substances. Trends Pharmacol. Sci. 19, 55–58.

Rinaldi-Carmona, M., Barth, F., Heaulme, M., Alonso, R., Shire, D., Congy, C., Soubrie, P., Breliere, J.C., Le Fur, G., 1995. Biochemical and pharmacological characterization of SR 141716A, the first potent and selective brain cannabinoid receptor antagonist. Life Sci. 56, 1941–1947.

Song, Z.H., Slowey, C.A., 2000. Involvement of cannabinoid receptors in the intraocular pressure-lowering effects of WIN55212-2. J. Pharmacol. Exp. Ther. 292, 136–139.

Stella, N., Schweitzer, P., Piomelli, D., 1997. A second endogenous cannabinoid that modulates long-term potentiation. Nature 388, 773–778.

Straiker, A.J., Maguire, G., Mackie, K., Lindsey, J., 1999. Localization of cannabinoid CB_1 receptors in the human anterior eye and retina. Invest. Ophthalmol. Vis. Sci. 40, 2442–2448.

Twitchell, W., Brown, S., Makie, K., 1997. Cannabinoids inhibit N- and P/Q-type calcium channels in cultured rat hippocampal neurons. J. Neurophysiol. 78, 43–50.

Vogel, Z., Barg, J., Levy, R., Saya, D., Heldman, E., Mechoulam, R., 1993. Anandamide, a brain endogenous compound, interacts specifically with cannabinoid receptors and inhibits adenylate cyclase. J. Neurochem. 61, 352–355.