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Role of cannabinoid CB_1 receptors and $G_{i/o}$ protein activation in the modulation of synaptosomal Na^+,K^+-ATPase activity by WIN55,212-2 and Δ^9-THC

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Abstract

In the present study, we evaluated the effects of the synthetic cannabinoid receptor agonist (*R*)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate (WIN55,212-2) and the active component of *Cannabis* delta-9-tetrahydrocannabinol (Δ^9-THC) on Na^+,K^+-ATPase activity in synaptosomal mice brain preparation. Additionally, the potential exogenous cannabinoids and endogenous opioid peptides interaction as well as the role of $G_{i/o}$ proteins in mediating Na^+,K^+-ATPase activation were also explored. The ouabain-sensitive Na^+,K^+-ATPase activity was measured in whole-brain pure intact synaptosomes (obtained by Percoll gradient method) of female CF-1 mice and was calculated as the difference between the total and the ouabain (1 mM)-insensitive Na^+,K^+-ATPase activities. Incubation *in vitro* of the synaptosomes with WIN55,212-2 (0.1 pM–10 μM) or Δ^9-THC (0.1 pM–0.1 μM), in a concentration-dependent manner, stimulated ouabain-sensitive Na^+,K^+-ATPase activity. WIN55,212-2 was less potent but more efficacious than Δ^9-THC. *N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM-251) (10 nM), a CB_1 cannabinoid receptor selective antagonist, had not effect *per se* but antagonized the enhancement of Na^+,K^+-ATPase activity induced by both, WIN55,212-2 and Δ^9-THC. AM-251 produced a significant reduction in the E_{max} of cannabinoid-induced increase in Na^+,K^+-ATPase activity, but did not significantly modify their EC_{50}. On the other hand, co-incubation with naloxone (1 μM), an opioid receptor antagonist, did not significantly modify the effect of WIN55,212-2 and completely failed to modify the effect of Δ^9-THC on synaptosomal Na^+,K^+-ATPase. Finally, pre-incubation with 0.5 μg of pertussis toxin ($G_{i/o}$ protein blocker) completely abolished the enhancement of ouabain-sensitive Na^+,K^+-ATPase activity induced by WIN55,212-2. A lower dose, 0.25 μg, decreased the E_{max} of WIN55,212-2 by 70% but did not significantly affect its EC_{50}. These results suggest that WIN55212-2 and Δ^9-THC indirectly enhance Na^+,K^+-ATPase activity in the brain by activating cannabinoid CB_1 receptors in a naloxone-insensitive manner. In addition, the effect of WIN55,212-2 on neuronal Na^+,K^+-ATPase is apparently due to activation of $G_{i/o}$ proteins.

Keywords: Cannabinoid; Receptor; Na^+,K^+-ATPase; WIN55,212-2; AM-251; Naloxone

1. Introduction

Na^+,K^+-adenosine triphosphatase (Na^+,K^+-ATPase, EC:3.6.1.3) is present in the plasma membrane of practically every eukaryotic cell, and is known to be concentrated in the synaptic membranes of the central nervous system. The Na^+,K^+-ATPase comprises the enzymatic machinery involved in many aspects of neural activity, *e.g.* restoring the ion gradient disturbed during electrical activity, regulating the resting membrane potential, and providing cation gradients that drive transmitter release and uptake processes (Stahl and Harris, 1986).

Cannabinoids, through their binding to CB_1 receptors (abundant in the brain) and the activation of G-proteins, produce coordinated changes in several cellular effector systems, such as inhibition (Steffens et al., 2004) or stimulation of adenylyl cyclase (Glass and Felder, 1997), inhibition of Ca^{2+} conductance (Shen and Thayer, 1998), activation of K^+ channels (Mackie et al., 1995) and stimulation of mitogen-activated protein kinase (Bouaboula et al., 1995). Recently, some evidence suggests that activation of cannabinoid receptors modulates the activity of Na^+,K^+-ATPase, although the results are inconsistent in this

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respect (Steffens and Feuerstein, 2004; Busch et al., 2004; see Discussion for details). Bearing in mind this fact, the present study was conducted to determine if the synthetic aminoalkylindole cannabinoid receptor agonist, (*R*)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate (WIN55,212-2), and the biologically active component of *Cannabis*, delta-9-tetrahydrocannabinol (Δ^9-THC), modulate synaptosomal Na^+,K^+-ATPase activity and to assess whether CB_1 receptors are involved in these effects.

On the other hand, opioid and cannabinoid agonists share a similar pharmacological profile both in the whole animal and at the cellular level, and *in vivo* interactions between these two groups of drugs have been repeatedly reported (Welch et al., 1995; Manzanares et al., 1999; Pontieri et al., 2001). It has been shown that cannabinoids might increase the synthesis or release of endogenous opioids, or both (Welch, 1997; Pugh et al., 1997). This would be particularly relevant for the cellular and behavioral effects of cannabinoids and could explain the ability of some opioid receptor antagonists to block some effects of the cannabinoid agonists (Gardner and Lowinson, 1991; Reche et al., 1996). Otherwise, several authors, including us, found an activation of brain and spinal cord Na^+,K^+-ATPase by morphine and other opioid agonists (Hajek et al., 1985; Masocha et al., 2002). To rule out a possible synergistic interaction between exogenous cannabinoids and endogenous opioid peptides resulting in a potential modulation on Na^+,K^+-ATPase activity, we tested the effect of naloxone, a competitive antagonist of μ-, δ-, and κ-opioid receptors (Dhawan et al., 1996).

$G_{i/o}$ proteins have been shown to be involved in the regulation of Na^+,K^+-ATPase activity by agonists of different G-protein-coupled receptors such as dopamine D_2 and serotonin $5HT_{1A}$ receptors (Yamaguchi et al., 1996; Peña-Rangel et al., 1999). As stated above, the cannabinoid receptors are G-protein-coupled receptors that seem to couple to inhibitory G_i and or G_o proteins (Prather et al., 2000), but it is not known whether the modulation of cannabinoid receptor agonists on Na^+,K^+-ATPase activity is mediated by $G_{i/o}$ proteins. Pertussis toxin catalyses the ribosylation of α_o, α_i and α_t subunits of G-proteins, irreversibly blocking its activity (Freissmuth et al., 1999). We therefore tested whether pertussis toxin, added directly to assay medium *in vitro*, modifies the effect of WIN55,212-2 on Na^+,K^+-ATPase activity.

2. Materials and methods

2.1. Animals

Female CF-1 Swiss mice (ISP, Chile) weighing 25–30 g were used for all experiments. The animals were housed in a temperature-controlled room at 22±1 °C, with air exchange every 20 min, a 12-h light/dark cycle and free access to food and water.

The animals were handled according to guidelines for the care of laboratory animals in compliance with European Communities Council Directive 86/609. All procedures were approved by the animals care committees.

2.2. Drugs

(*R*)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate (WIN55,212-2), delta-9-tetrahydrocannabinol (Δ^9-THC), pertussis toxin and ouabain were purchased from Sigma-Aldrich S.A. (Chile). *N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM-251) was obtained from Tocris Co. (USA). Naloxone HCl was a generous gift from Cruz Miguel Cendán, (University of Granada, Spain). WIN55,212-2 and AM-251 were dissolved in a mixture of dimethylsulphoxide (DMSO)/water to make up a 1 mM solution from which further dilutions were made with water. The maximum concentration of DMSO, when dissolved in the incubation medium, was no more than 0.05%. Δ^9-THC was dissolved in a mixture of ethanol/water at a concentration of 0.1 mM. Previously, we had not found any effects of these concentrations of DMSO and ethanol on synaptosomal Na^+,K^+-ATPase activity. Naloxone was dissolved in ultrapure water (purer than type I in the National Committee for Clinical Laboratory Standards/College of American Pathologists water quality standards). The cardiac glycoside ouabain was solubilized in the solution medium, directly. Pertussis toxin was prepared in its solvent (sodium phosphate 0.02 M and sodium chloride 0.1 M).

2.3. Preparation of brain pure synaptosomes

Mice whole brain crude synaptosomal pellets were isolated as previously described (Gonzalez et al., 2001). Briefly, the mice whole brains were immersed in tubes containing ice-cold isolation medium I [320 mM sucrose; 3 mM ethylendiamine tetraacetic acid tetrasodium salt; 10 mM *N*-2-hydroxyethylpiperazine-*N*′-ethanosulfonic acid (HEPES), pH 7.4] and homogenized with a Polytron (model Ultraturrax T25 basic, Arquimed). The homogenates were centrifuged (model RC2-B, Sorvall Superspeed.) at 1000 ×*g* for 10 min at 4 °C; the resulting pellets were discarded and the supernatants were centrifuged again under the same conditions. The final supernatant was then centrifuged at 17,000 ×*g* for 20 min in order to obtain the crude synaptosomal pellet (P_2 pellet). Then the pellet was dissolved in 375 μl of medium I in order to isolate pure synaptosomes, as described below.

Pure intact synaptosomes were obtained with Percoll density gradients as previously described (Nagy and Delgado-Escueta, 1984). The Percoll (Sigma-Aldrich, Chile) stock solution was made up by adding 0.5 ml of 2.5 M sucrose to 4.5 ml of original Percoll. Solutions of lower Percoll concentration were prepared by appropriate dilution of the stock solution with medium II [250 mM sucrose; 10 mM HEPES; 3 mM EDTA·4 Na, pH 7.4]. Then, in a 16-ml Ultra-Clear centrifuge tube, 3 ml of 10% Percoll solution was layered over 3 ml of 16% Percoll solution, and finally, 3.375 ml of a 7.5% Percoll solution (containing 375 μl of the P_2 pellet solution) was layered over the 10% Percoll solution. All steps were carried out at 4 °C. The tubes were centrifuged at 15,000 ×*g* for 20 min at 4 °C. Synaptosomes (banded at the 10%:16% Percoll interface) were collected from gradients by

wide-tip Pasteur pipette. To clean the synaptosomes from Percoll, the synaptosome/Percoll solution was dissolved (1:1 vol/vol) in a 320-mM sucrose solution and centrifuged at 24,000 ×*g* during 20 min at 4 °C. The supernatant was discarded and the procedure was repeated. The final pellet was dissolved in 1 ml/brain of a 320-mM sucrose solution and the protein concentration was determined by a modified version of Lowry et. al., (1951) method, using bovine serum albumin as the reference standard. After this, synaptosomes were diluted to the required final protein concentration with medium III [320 mM sucrose; 10 mM HEPES, pH 7.4], and were either freshly used for ATPase assays or stored at −20 °C. The stored synaptosomes lost about 4% enzymatic activity after 15 days.

2.4. Na^+,K^+-ATPase assays

To measure the total Na^+,K^+-ATPase activity we added 50 µl of pure intact synaptosome solution (final quantity in the assay medium 0.01 mg protein) to a tube with 350 µl of an incubation medium containing in mM: 100 NaCl, 20 KCl, 2 $MgCl_2$, 5 NaN_3, 0.1 EGTA and 25 HEPES, pH 7.4. To measure the ouabain-insensitive ATPase the medium was the same as above but with 1 mM ouabain. We then added 5 µl of the drug under study (or its vehicle) at the appropriate concentrations and preincubated the mixture for 5 min at 37 °C. For pertussis toxin studies, *in vitro* ADP-ribosylation was previously carried out according to a modification of the method described by Wang et al. (1995) (in ours experiments the tubes were preincubated with pertussis toxin or its solvent [0.01 M Na_2PO_4 buffer, pH 7.0, with 0.05 M NaCl] in a shaking water bath at 37 °C for 30 min). Then the reaction was initiated by adding 50 µl of an ATP disodium salt solution (final ATP concentration in the medium was 2 mM), followed by incubation for 2 min at 37 °C. The reaction was stopped by adding 50 µl of 50% ice-cold tricholoroacetic acid (TCA) and the tubes were put in ice for 10 min. To remove the protein precipitated by TCA, the sample was centrifuged at 1000 ×*g* for 10 min at 4 °C and 400 µl of the supernatant was assayed for the released inorganic phosphate (P_i) by the method of Fiske and Subbarow (1925), modified by Sadrzadeh et al. (1993). Briefly, 400 µl of molybdate solution colour reagent was added to the tubes (final volume of 800 µl), and after incubation for 30 min in the dark at room temperature, the absorbance was read at 810 nm either by a UV–visible spectrophotometer (model Genesys 2, Spectronic Corporation) or a microplate scanning spectrophotometer (Expert 96, Asys/Hitech Instruments, Inc.). Sodium phosphate dibasic solution was used as the reference standard.

Ouabain-sensitive Na^+,K^+-ATPase activity was calculated as the difference between the values of activity in the absence and in the presence of ouabain (*i.e.*, total Na^+,K^+-ATPase - ouabain-insensitive Na^+,K^+-ATPase). The data were plotted as the percentage increase in basal Na^+,K^+-ATPase activity (*i.e.* ATPase activity without drug), which was calculated as follows: % Increase = [(SA with drug − SA without drug)/SA without drug] × 100, where SA is the ouabain-sensitive Na^+,K^+-ATPase specific activity for synaptosomes incubated with each drug concentration or without the drug (with solvent).

2.5. Data analysis

Experiments were performed in duplicate and repeated at least three times. The values of EC_{50} (concentration of drug that produced half of the maximum enhancement of Na^+,K^+-ATPase activity) and E_{max} (maximum increase in Na^+,K^+-ATPase activity) were calculated from the concentration–response curves using a non-linear regression analysis with the GraphPad Inplot computer program (GraphPad Software Inc., USA). Values quoted are given as means ± S.E.M. for the number of independent experiments indicated. A two-tailed Student's *t*-test (unpaired) was used to evaluate differences between two experimental groups (level of significance, $p < 0.05$). One-way analysis of variance (ANOVA), followed by Newman–Keuls multiple comparisons test were used for statistical significance of differences between multiple groups.

3. Results

3.1. Effects of WIN55,212-2 and Δ^9-THC on synaptosomal ouabain-sensitive Na^+,K^+-ATPase specific activity

The incubation of whole brain synaptosomes with different concentrations of WIN55,212-2 (0.1 pM to 10 µM) stimulated ouabain-sensitive Na^+,K^+-ATPase activity in a concentration-dependent way (Fig. 1). Using non-linear regression analysis, the data was fitted to a sigmoid curve, which allows to calculate E_{max} as 70.88 ± 2.94% of stimulation and EC_{50} as 19.1 pM (95% confidence interval [CI] = 11.6 to 31.4 pM). Δ^9-THC (0.1 pM to 0.1 µM) also stimulated ouabain-sensitive Na^+,K^+-ATPase activity of the synaptosomes (Fig. 1), but it was less efficacious (E_{max} = 22.58 ± 2.86% of stimulation) and more potent (EC_{50} = 1.12 pM; CI = 0.23 to 5.43 pM) than WIN55,212-2. Their corresponding solvents did not significantly modify synaptosomal Na^+,K^+-ATPase activity (Fig. 1).

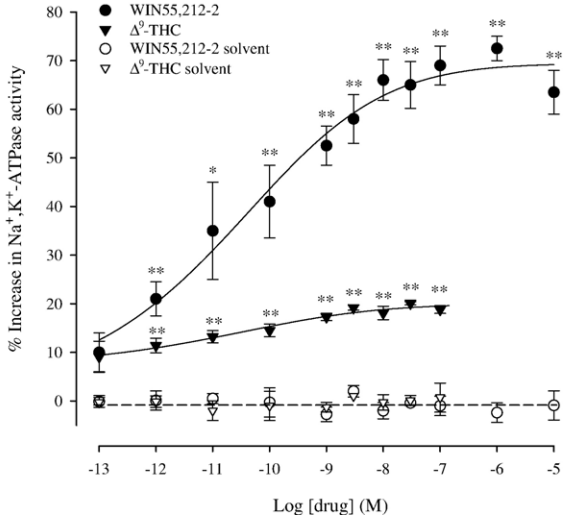

Fig. 1. Stimulatory effect *in vitro* of WIN55,212-2 and Δ^9-THC on mice whole brain synaptosomal ouabain-sensitive Na^+,K^+-ATPase specific activity. Each point represents the mean ± S.E.M. of the values from three to five independent experiments. Statistically significant differences in comparison to the effect of respective drug solvents: $^*P < 0.05$, $^{**}P < 0.01$ (two-tailed Student's *t*-test).

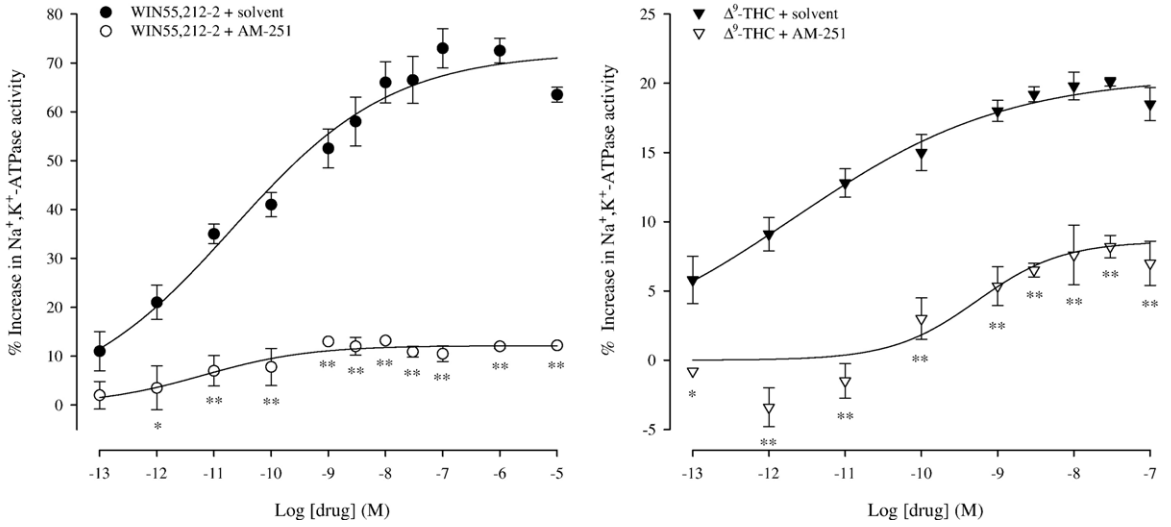

Fig. 2. Antagonism by *in vitro* co-incubation with AM-251 (10 nM) of the stimulatory effects of different concentrations of WIN55,212-2 (left side) and Δ^9-THC (right side) on mice whole brain synaptosomal Na^+,K^+-ATPase specific activity. Each point represents the mean±S.E.M. of the values from four to five independent experiments. Statistically significant differences in comparison to the effect of each cannabinoid compound+AM-251 solvent for the same dose: $^*P<0.05$, $^{**}P<0.01$ (two-tailed Student's *t*-test).

3.2. Effect of AM-251 on the cannabinoid-induced increase in synaptosomal ouabain-sensitive Na^+,K^+-ATPase specific activity

Fig. 2 (left side) shows the effect of co-incubation of synaptosomes with WIN55,212-2 and AM-251 (10 nM) or its solvent. Synaptosomes incubated with WIN55,212-2+solvent showed a concentration-dependent enhancement of ouabain-sensitive Na^+,K^+-ATPase activity, with an E_{max} of 72.20±3.47% and an EC_{50} of 21.11 pM (CI=11.90 to 37.30 pM). Co-incubation with AM-251 (10 nM) produced an antagonism of the ability of WIN55,212-2 to increase synaptosomal Na^+,K^+-ATPase activity. The maximum efficacy of WIN55,212-2 in the presence of AM-251 was diminished by approximately six times (E_{max}=12.14±0.64%), whereas the EC_{50} was not significantly changed (6.37 pM; CI=3.29 to 12.3 pM).

Fig. 2 (right side) shows the effect of co-incubation of synaptosomes with Δ^9-THC and AM-251 (10 nM) or its solvent. The presence of AM-251 significantly reduced E_{max} of the Δ^9-THC-induced increase in Na^+,K^+-ATPase activity (E_{max} of 20.52±0.80% and 8.70±1.96% for solvent and AM-251 co-incubation, respectively). The EC_{50} of Δ^9-THC in the presence of AM-251 increased by approximately two orders of magnitude (2.12 pM [CI=1.40 to 3.20 pM] and 191 pM [66.10 to 550 pM] for solvent and AM-251 co-incubation, respectively). AM-251 alone (0.1 pM to 10 μM) had no effect on ouabain-sensitive Na^+,K^+-ATPase activity (data not shown).

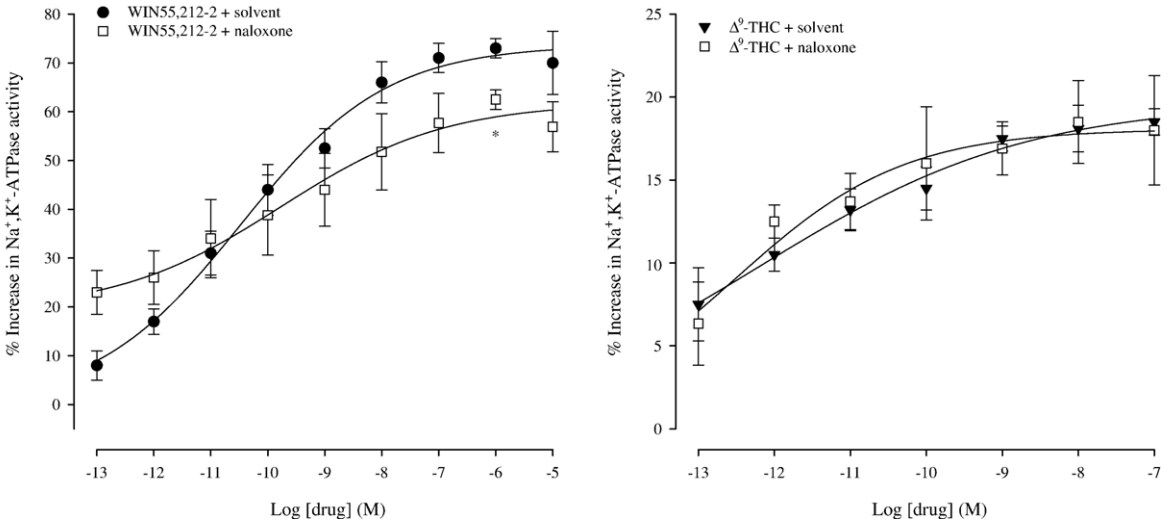

Fig. 3. Antagonism by *in vitro* co-incubation with naloxone (1 μM) of the stimulatory effects of different concentrations of WIN55,212-2 (left side) and Δ^9-THC (right side) on synaptosomal Na^+,K^+-ATPase specific activity. Each point represents the mean±S.E.M. of the values from four to five independent experiments. Statistically significant differences in comparison to the effect of WIN55,212-2+naloxone-solvent for the same dose: $^*P<0.05$ (two-tailed Student's *t*-test). No statistically significant differences were observed in comparison with Δ^9-THC+naloxone-solvent.

3.3. Effect of naloxone on the cannabinoid-induced increase in synaptosomal ouabain-sensitive Na^+,K^+-ATPase specific activity

The co-incubation of brain synaptosomes with naloxone at a concentration of 1 μM, did not significantly modify neither the efficacy nor potency of WIN55,212-2 in stimulating ouabain-sensitive Na^+,K^+-ATPase activity (E_{max}=73.67±1.83% *versus* 67.08±5.84%; EC_{50}=33.40 pM [CI=25 to 44.70 pM] *versus* 9.80 pM [2.82 to 34.10 pM] for solvent- and naloxone-co-incubated synaptosomes, respectively). Nevertheless, a different slope of the Na^+,K^+-ATPase increase under WIN55,212-2 and opioid antagonist combination appeared (Fig. 3, left side). On the other hand, naloxone completely failed in antagonizing the effect of Δ^9-THC (E_{max}=19.69±0.82% *versus* 18.07±0.76%; EC_{50}=0.67 pM [CI=0.44 to 1.01 pM] *versus* 0.30 pM [0.19 to 0.48 pM] for solvent- and naloxone-co-incubated synaptosomes, respectively) on Na^+,K^+-ATPase activity (Fig. 3, right side). Naloxone alone (1 μM) had no effect on ouabain-sensitive Na^+,K^+-ATPase activity (data not shown).

3.4. Effect of pertussis toxin on the cannabinoid-induced increase in synaptosomal ouabain-sensitive Na^+,K^+-ATPase specific activity

Preincubation *in vitro* for 30 min of brain synaptosomes with pertussis toxin at a concentration of 0.05 μg/μl, added in a volume of 5 μl to the reaction medium (*i.e.* 0.25 μg), decreased the maximum WIN55,212-2-induced increase in Na^+,K^+-ATPase by approximately 70% (E_{max}=74.89±2.22% and 20.77±2.62%, for the solvent- and pertussis toxin-pre-treated synaptosomes, respectively) (Fig. 4), but did not significantly modify its EC_{50} (23.5 pM [CI=16.60 to 33.20 pM] and 109 pM [CI=27.10 to 438 pM] for solvent- and pertussis toxin-pre-treated synaptosomes, respectively).

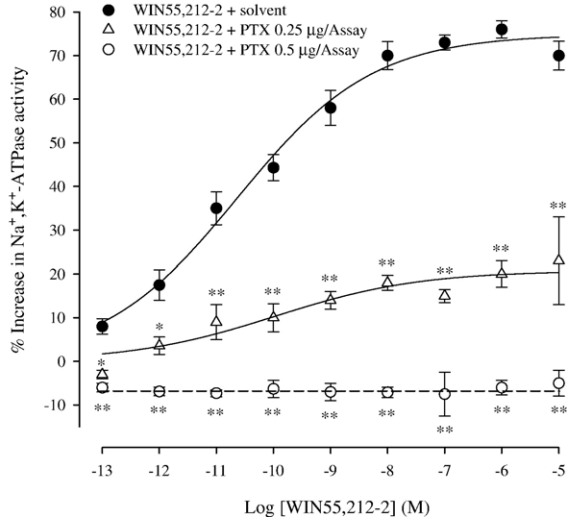

Fig. 4. Antagonism by *in vitro* pre-incubation with pertussis toxin (0.25 and 0.5 μg/assay, 30 min before) of the stimulatory effect of different concentrations of WIN55,212-2 on synaptosomal Na^+,K^+-ATPase specific activity. Each point represents the mean±S.E.M. of the values from three independent experiments. Statistically significant differences in comparison to the effect on synaptosomes pre-treated with pertussis toxin-solvent: *P<0.05, **P<0.01, (one-way ANOVA, followed by Newman–Keuls test).

Preincubation with a higher concentration of pertussis toxin (0.1 μg/μl, added in a volume of 5 μl; *i.e.*, 0.5 μg) completely abolished the enhancement of synaptosomal ouabain-sensitive Na^+,K^+-ATPase activity induced by WIN55,212-2 (Fig. 4).

4. Discussion

The present study found that WIN55,212-2 and Δ^9-THC enhance synaptosomal Na^+,K^+-ATPase activity in an AM-251-sensitive and naloxone-insensitive manner. We also found that the effect of WIN55,212-2 on Na^+,K^+-ATPase was blocked by pertussis toxin, suggesting the involvement of $G_{i/o}$ proteins.

Incubation of synaptosomes with WIN55,212-2 and Δ^9-THC increases Na^+,K^+-ATPase activity in a concentration-dependent way. The only one previous study of the interaction between the synthetic cannabinoid receptor agonist WIN55,212-2 and Na^+,K^+-ATPase was reported by Steffens and Feuerstein (2004). These authors did not observe any significant modulation of WIN55,212-2 on isolated (non-membrane-bound) purified Na^+,K^+-ATPase. This fact, apparently in contradiction with our results, argue in favour of the idea that WIN55,212-2 has to interact with some components of the cell membrane to modulate the Na^+,K^+-ATPase activity (*i.e.* a cannabinoid receptor, in agreement with our results). In other recent study it has been indicated that anandamide, an endocannabinoid, evoked a concentration-dependent increase in cAMP in rat parotid glands, which in turn leads to Na^+,K^+-ATPase inhibition (Busch et al., 2004). Furthermore, the CB_1 antagonist AM-281 inhibited this effect. These differences, particularly between anandamide and WIN55,212-2, were not totally unexpected as previous studies suggested dissimilarities between the mechanisms involved in the cellular effects of anandamide and WIN55,212-2 (for review see, Demuth and Molleman, 2006). Theoretically, the activation of different mechanisms by two agonists of a receptor may be due to their action on different subtypes of the receptor, or to their action on the same receptor coupled to different transducer proteins (Milligan, 1993). Both possibilities may be considered in explaining these discrepancies.

The effect of Δ^9-THC on Na^+,K^+-ATPase had not previously been explored, and we found that it shows less efficacy but greater potency than WIN55,212-2 in stimulating ouabain-sensitive Na^+,K^+-ATPase activity. The lower efficacy but higher potency of Δ^9-THC compared to WIN55,212-2 observed in the current study agrees with previous *in vitro* functional assays where Δ^9-THC was a partial agonist compared to other cannabinoid receptor agonists (Breivogel and Childers, 2000; Breivogel et al., 2004). The enhancement of synaptosomal Na^+,K^+-ATPase activity induced by WIN55,212-2 (around 70%) is significantly higher than with other drugs that stimulate Na^+,K^+-ATPase activity such as the agonists of μ-opioid receptors, morphine (30%) (Masocha et al., 2002), and endomorphine-1 (26%) (Horvath et al., 2003), and the agonists of dopamine receptors, bromocriptine (23%) (Hussain et al., 1997) and LY171555 (28%) (Yamaguchi et al., 1996). This response might be explained by the fact that the cannabinoid receptors density is extremely high compared to other G-protein-coupled receptors in brain (Breivogel and Childers, 1998). Thus, even areas that exhibit relatively low levels of cannabinoid receptors contain

relatively high levels compared to other G-protein-coupled receptors. However, agonist-stimulated [^{35}S]GTPγS binding to brain membranes experiments have shown that cannabinoid receptors coupled to G-proteins activate G-protein less efficiently than μ or δ opioid receptors (Sim et al., 1995; Selley et al., 1996). Indeed, μ and δ opioid agonists activate G-proteins 7-fold more per receptor than cannabinoid agonists. Nevertheless, since cannabinoid receptors in certain brain sections outnumber each opioid receptor type by at least 10-fold, the actual density of cannabinoid-activated G-proteins is approximately twice that of either opioid receptor type (Sim et al., 1996; Breivogel and Childers, 1998).

WIN55,212-2 and Δ^9-THC were very potent for the stimulation of Na^+,K^+-ATPase (19 and 1 pM, respectively) compared to their potencies in other *in vitro* functional assays. For example, in [^{35}S]GTPγS stimulation assays in tissue from various rat brain regions, K_s values for WIN55,212-2 were 130–250 nM, while the K_s values of Δ^9-THC were 38–1600 nM (Breivogel and Childers, 2000). This may be explained by a greater receptor reserve for Na^+,K^+-ATPase stimulation than for G-protein activation. Indeed, receptor/effector reserve for the Na^+,K^+-ATPase would be predicted to produce greater apparent potency for some agonists because lower levels of receptor occupancy would be required to obtain the maximal effect. By the other side, while [^{35}S]GTPγS binding measures the first step in the signal-transduction process, an additional signal amplification at subsequent steps in the pathway can increase the apparent potencies and efficacies among agonists at the level of a downstream effector system (for example, Na^+,K^+-ATPase in our study).

Since both, the synthetic cannabinoid agonist WIN55,212-2 and the active component of *Cannabis* Δ^9-THC possess a full spectrum of cannabinoid activity in mice and modulate the Na^+,K^+-ATPase activity, it is reasonable to hypothesize that the cannabinoid receptors should be involved in this interaction. This prompted us to evaluate the effect of the selective CB_1 antagonist AM-251 (Gatley et al., 1997) on the cannabinoid-evoked modulation of the Na^+,K^+-ATPase activity. Our data shows that AM-251 has no effect on Na^+,K^+-ATPase *per se* but antagonizes the stimulation of Na^+,K^+-ATPase activity induced by WIN55,212-2 and Δ^9-THC, which suggests that this stimulatory effect is mediated by cannabinoid CB_1 receptors. AM-251 reduces E_{max} of both WIN55,212-2 and Δ^9-THC without modifying significantly their EC_{50} (at least EC_{50} of WIN55,212-2), suggesting a non-competitive type of antagonism. Our results also show a greater efficacy of AM-251 in blocking the effects of WIN55,212-2 than those evoked by Δ^9-THC, which could be simple the result of the comparison between a synthetic (and selective) cannabinoid agonist and the natural and active constituent of *Cannabis*.

On the other hand, naloxone did not significantly modify the effect of WIN55,212-2 and completely failed to modify the effect of Δ^9-THC on Na^+,K^+-ATPase activity, which suggests that the stimulatory effect does not seem to be mediated by opioid receptors. This lack of effect cannot be traced to any methodological failure, since a previous study using the same dose and method demonstrated that naloxone antagonizes morphine-induced stimulation of synaptosomal Na^+,K^+-ATPase activity (Masocha et al., 2002). Despite convincing data supporting a functional interaction between the opioid and the cannabinoid systems in antinociception (Pugh et al., 1997; Welch, 1997) and drug addiction (Tanda et al., 1997), as well as other less studied pharmacological effects such as hypothermia (Welch et al., 1998), the nature of such interactions has not yet been fully elucidated. Nevertheless, common anatomical location and similar post-receptorial signalling mechanisms may be of assistance. In this respect, if opioids and cannabinoid CB_1 receptors co-localize (and then interact) only in selected areas (*i.e.* cerebellum, substantia nigra pars reticulata, globus pallidus and hippocampus), a whole brain preparation may mask the naloxone (unspecific antagonism)-mediated effect at a discrete site within the brain. Alternatively, considering that GTP-binding proteins of the G_i/G_o subtypes are the first site at which interactions between opioids and cannabinoids may take place, a competition for the same pool of G-proteins may prevent subsequent signal-transduction mechanisms of these G-protein-coupled receptors, thus affecting the efficiency or their activation/inactivation on Na^+,K^+-ATPase. However, it seems unlikely, because previous studies in N18TG2 neuroblastoma cells expressing both opioid and cannabinoid receptors suggest that these receptors are coupled to their own G-proteins (Shapira et al., 1998). Nevertheless, Devane et al. (1986) found no additivity between opioid and cannabinoid inhibitory effects on adenylyl cyclase in N18TG2 cells, indicating a common pool of adenylyl cyclase influenced by both drugs. In this particular case, a dominant activity of WIN55,212-2-activated cannabinoid CB_1 receptors on the increase in Na^+,K^+-ATPase activity may therefore to control the signalling pathway (*i.e.*, cAMP pathway) and blunt other competitive and non-selective biological signals incoming from closely interacting receptors. It is difficult to explain the finding that in Fig. 3 the WIN55,212-2 + naloxone curve appears to start at around 20% and have a much shallower slope than without naloxone. It could be speculated that, at levels of concentrations in the pM range naloxone increase the dominant cannabinoid activity on Na^+,K^+-ATPase by blocking opioid receptors, suggesting a competitive interaction between cannabinoid and opioid system only at very low concentrations. Subsequently, although in a non-significant way, this competitive interaction would be transformed into a cooperative interaction in greater concentrations.

It is known that both opioid and cannabinoid drugs are coupled to similar intracellular signalling mechanisms, mainly to a decrease in cAMP production through activation of $G_{i/o}$ proteins (Shapira et al., 1998; Manzanares et al., 1999). However, whether the $G_{i/o}$ protein plays a role in the stimulation of Na^+,K^+-ATPase induced by cannabinoid agonists is unknown. Bearing in mind this fact, we tested the effect of pertussis toxin on WIN55,212-2-induced activation of synaptosomal Na^+,K^+-ATPase. Preincubation *in vitro* of the brain synaptosomes with pertussis toxin produced a concentration-dependent non-competitive antagonism of the effect of WIN55,212-2. Our results demonstrate that pertussis toxin-sensitive $G_{i/o}$ proteins participate in the WIN55,212-2-induced increase in Na^+,K^+-ATPase activity in the brain. Earlier studies have shown $G_{i/o}$ proteins to be involved in the regulation of Na^+,K^+-ATPase by agonists of several $G_{i/o}$ protein-coupled receptors such as dopamine D_2 (Yamaguchi et al., 1996; Hussain et al., 1997), angiotensin AT_1 (Bharatula et al., 1998), serotonin 5-HT_{2A} (Rhoden et al., 2000), peptide C (Ohtomo et al., 1996), and μ-

opioid receptors (Masocha et al., 2002). Taken together, these findings are consistent with the hypothesis that the activation of $G_{i/o}$ proteins may represent a general mechanism of regulation of activity of cellular Na^+,K^+-ATPase.

Recent evidence suggests that intracellular messengers can affect Na^+,K^+-ATPase activity. Depending on the tissue, intracellular Ca^{2+} levels, protein phosphatases, activation of protein kinases, and other second messengers can induce an increase or decrease in Na^+ pump (Blanco and Mercer, 1998; Therien and Blostein, 2000). Agonists of cannabinoid receptors are able to modulate several intracellular effector mechanisms such as intracellular Ca^{2+}, cAMP pathway, mitogen-activated protein kinase, *etc.* (Shen and Thayer, 1998; Steffens et al., 2004; Bouaboula et al., 1995). Our data do not specifically address the mechanism by which activation of brain cannabinoid receptors leads to stimulation of Na^+,K^+-ATPase activity, but we think it might be due to the modulation by cannabinoid drugs of these effector mechanisms. Therefore, future efforts must begin to explore the role of each of these molecular substrates in the effect of cannabinoids on synaptosomal Na^+,K^+-ATPase.

The stimulation of Na^+,K^+-ATPase in the brain by the cannabinoids may be correlated with the pharmacological effects of the cannabinoids in mice. It is possible that the stimulation of the sodium pump by WIN55,212-2 and Δ^9-THC *via* CB_1 receptors underlies, at least in part, the cannabinoid-induced inhibition of neurotransmitter release at presynaptic sites and other neuronal mechanisms. The functional significance of this stimulation is unclear; however, given the well-known relationship between opioids and Na^+,K^+-ATPase in analgesia (Masocha et al., 2003; Horvath et al., 2003), we hypothesize that cannabinoid-induced increase in Na^+,K^+-ATPase may be responsible for mediating some antinociceptive properties of the cannabinoids.

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