Cell. Signal. Vol. 10, No. 9, pp. 667–674, 1998

ISSN 0898-6568/98 $19.00
PII S0898-6568(98)00013-8

Differential Receptor–G-Protein Coupling Evoked by Dissimilar Cannabinoid Receptor Agonists

*Devin B. Houston† and Allyn C. Howlett**

Department of Pharmacological and Physiological Science, Saint Louis University School of Medicine, 1402 South Grand Boulevard, St. Louis, Missouri 63104, USA

ABSTRACT. Multiple affinity states are revealed by agonist competition for radioantagonist [^{3}H]SR141716A binding to rat brain CB_1 cannabinoid receptors. Desacetyllevonantradol (DALN), a tricyclic cannabinoid, and WIN55212-2, an aminoalkylindole, both bound in two discrete affinity states (30% high affinity), but the ratios of the IC_{50} revealed distinct differences. Other affinity-state differences between the agonists were: Na^+ reduced the affinity for the membrane-bound receptor by 10-fold for DALN but minimally for WIN55212-2; a non-hydrolysable GTP analogue decreased the fraction of high-affinity WIN55212-2 binding but not that of DALN unless Na^+ was also present. Detergent solubilisation increased the fraction of high-affinity binding for both agonists but eliminated any effect of Na^+ on the agonist affinities. In detergent solution, the GTP analogue reduced the WIN55212-2 high-affinity fraction but not that of DALN, even though the IC_{50} values increased for both DALN and WIN55212-2. The differential modulation of CB_1 receptor–G-protein coupling by Na^+ and guanine nucleotides is dependent upon the cannabimimetic agonist bound. CELL SIGNAL 10;9:667–674, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. Aminoalkylindole drugs, Cannabinoid drugs, Cation-binding sites, Desacetyllevonantradol, G-protein-coupled receptors, SR141716A, WIN55212-2

INTRODUCTION

The biologically active compound in marihuana, Δ^9-tetrahydrocannabinol, promotes specific central nervous system effects in humans. These actions are conveyed through specific membrane CB_1 cannabinoid receptors found in the brain and result in the well-known psychoactive effects of marihuana such as sedation, euphoria and loss of short-term memory as well as analgesic, antiemetic and anticonvulsant actions (reviewed in [1, 2]). At the cellular level, cannabimimetic agonists are known to inhibit the activity of adenylate cyclase and to alter the activity of potassium and calcium ion channels (see [3] for review). These interactions are transduced through the guanine nucleotide binding proteins G_o and G_i. However, it is unclear which subtypes of these proteins (G_{o1}, G_{o2}, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$) are implicated in effector activation.

Most of the data regarding CB_1 cannabinoid receptor interactions with G proteins have been generated by using agonist radioligand binding assays. The high-affinity synthetic cannabinoid agonist [^{3}H]CP55940 has been used to characterise the CB_1 cannabinoid receptor [4, 5]. The aminoalkylindole agonists belong to a second class of compounds having a cannabimimetic profile identical with that of the cannabinoid ligands *in vivo* and *in vitro*—reviewed in [6]. [^{3}H]WIN55212-2 is the agonist radioligand developed from this class of compounds [7]. [^{3}H]CP55940 binding to rat brain membranes revealed that, like many other G-protein-coupled receptors, cannabinoid agonist binding could be affected by the presence of Na^+ or guanine nucleotides, indicating that different states of binding affinity exist [4, 5]. These binding states are indicative of the degree of receptor interaction with G proteins. The interaction of CB_1 receptors with G proteins is thought to be tight, because high-affinity agonist binding is maintained after detergent solubilisation from membranes and is diminished by 75% with the addition of guanine nucleotides [5]. Unlike some receptor systems, complete inhibition of radioagonist binding was not achieved, even in the presence of high (100 μM) concentrations of the non-hydrolysable GTP analogue guanosine 5′-*O*-(3-thio)triphosphate (GTPγS) [5].

Recently, the characterisation of SR141716A, a CB_1 selective antagonist, was reported [8]. The tritium labelling of SR141716A provides a new tool for the analysis of cannabinoid receptor signal transduction. The present study examines the displacement of [^{3}H]SR141716A binding to CB_1 cannabinoid receptors in rat brain membranes and detergent-solubilised receptor preparations by cannabinoid desacetyllevonantradol (DALN) and aminoalkylindole

*Author to whom all correspondence should be addressed. E-mail: howletta@sluvca.slu.edu
†Present address: National Enzyme Company, Forsyth, MO 65653, USA.
Received 18 September 1997; and accepted 9 February 1998.

WIN55212-2. The effects of Na^+ and GTPγS in modulating the affinity of these two structurally distinct cannabimimetic agonists are compared.

MATERIALS AND METHODS

Preparation of Membranes and Solubilization

Membranes were prepared from frozen rat brains (Pel-Freez) as previously described [4] with the exception that 100 μM aminoethylbenzenesulphonylfluoride (AEBSF) (CalBiochem) was included in the initial homogenisation. Aliquots were stored in TME buffer comprising 20 mM Tris-HCl, pH 7.5, 5 mM MgCl and 1 mM ethylenediaminetetraacetic acid (EDTA). Protein concentrations were determined by the Bio-Rad DC assay. Detergent solubilisation of cannabinoid receptors was accomplished by adjusting membranes to a protein concentration of 10 mg/mL with TME and a final concentration of 20% glycerol, 10 mM $MgCl_2$ and 0.5 g 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphoxide (CHAPS) (Boehringer Mannheim) detergent per gram of membrane protein. Upon addition of detergent, the membranes were gently stirred on ice for 20 min, followed by centrifugation at 105,000 × *g* for 40 min at 4°C. The supernatant containing solubilised receptor was removed without disturbing the pellet and stored at −70°C.

Receptor Binding Assay

Dilutions of DALN (Pfizer), WIN55212-2 (Sterling-Winthrop) and SR141716A (BioMol) were made in Regisil-treated (Regis) 12 × 75 mm glass tubes, from 10 mM stock solutions in ethanol or 100 mM in dimethylsulphoxide (DMSO). Ethanol was evaporated under N_2 before re-suspension to 200 μM in TME containing 10 mg/mL fatty-acid free bovine serum albumin (BSA) (Sigma). Subsequent dilutions were into TME containing 1 mg/mL fatty acid-free BSA. Final concentration of [^{3}H]SR141716A (Amersham International TRK1028) in the assay was approximately 1 nM unless otherwise noted. Assays were performed in 96-well plastic assay plates in a total volume of 100 μL. Incubation was initiated by addition of 15 μg membrane protein or 40 μg solubilised receptor protein. Unless specifically mentioned in the figure legends, plates were incubated for 2 h at 24°C on an orbital shaker. Assays were terminated by vacuum filtration (Tomtec 1205 cell harvester) over GF/B-type filter mats (Wallac 1205-404) and washed with approximately 20 mL per well cold TME buffer or TME buffer containing 100 mM NaCl. Scintillant was applied to each filter, and the filter was sealed in a plastic bag and counted in a Wallac Betaplate reader.

[^{35}S]GTPγS Binding Assay

Rat-brain membranes (5 μg of protein) were incubated in 20 mM Tris-Cl buffer, pH 7.5, containing 0.375 nM [^{35}S]GTPγS (New England Nuclear), 10 μM GDP, 1 mM dithiothreitol, 100 mM NaCl and 5 mM $MgCl_2$. Cannabinoid ligand dilutions were prepared as for the radioligand-binding assay. Non-specific binding was determined in the presence of 10 μM GTPγS. Assays were performed in 96-well plates at 30°C for 60 min, terminated by vacuum filtration using a Tomtec harvester and quantified by liquid scintillation counting with the use of a Wallac Betaplate reader.

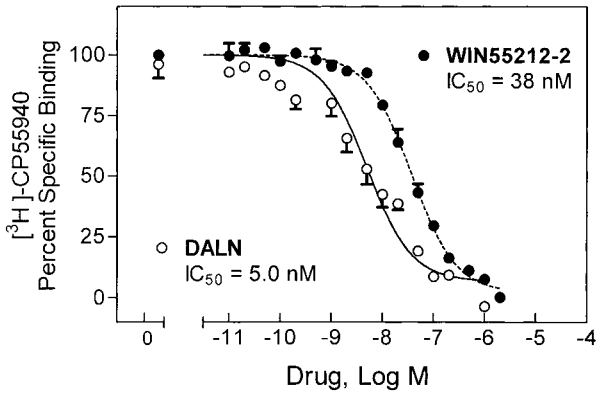

FIGURE 1. Competitive displacement by DALN and WIN55212-2 of [^{3}H]CP55940 binding to rat-brain membranes. Data were calculated as average ± range from two experiments performed with quadruplicate replicates.

Data Analysis

Data obtained from equilibrium binding studies were analysed by using Graphpad Prism to compute non-linear regression values. Comparisons were made of the best fit to either a one- or a two-site binding curve by use of the F test. Saturation-binding analyses were determined by using an equation based on Swillens *et al.* [9] and modified by GraphPad, which describes total binding as a function of the added ligand concentration.

RESULTS

Figure 1 is typical of the binding profile observed for competitive displacement of the radiolabelled cannabinoid agonist [^{3}H]CP55940. DALN and WIN55212-2 represent two structurally different classes of cannabimimetic agonists, known as tricyclic cannabinoids and aminoalkylindoles, respectively. Both agonists bind CB_1 cannabinoid receptors with high affinity; however, WIN55212-2 is less potent than DALN.

Characterisation of SR141716A as a CB_1-selective antagonist revealed the compound to be almost as potent as CP55940 in displacing the cannabinoid agonist [^{3}H] CP55940 binding to rat-brain membranes ($K_i \approx 2$ nM) [8]. The availability of a radiolabelled form of SR141716A allowed further investigation into the receptor affinity of this compound. A comparison of saturation-binding curves for [^{3}H]SR141716A and [^{3}H]CP55940 to rat-brain membranes produced, respectively, $K_d = 1.08 \pm 0.25$ nM and 1.97 ± 0.66 nM and $B_{max} = 1.54 \pm 0.17$ and 1.93 ± 0.15 pmol/mg protein. Guanine nucleotides and Na^+ have been previously shown to decrease radioagonist [^{3}H]CP55940 binding to CB_1 cannabinoid re-

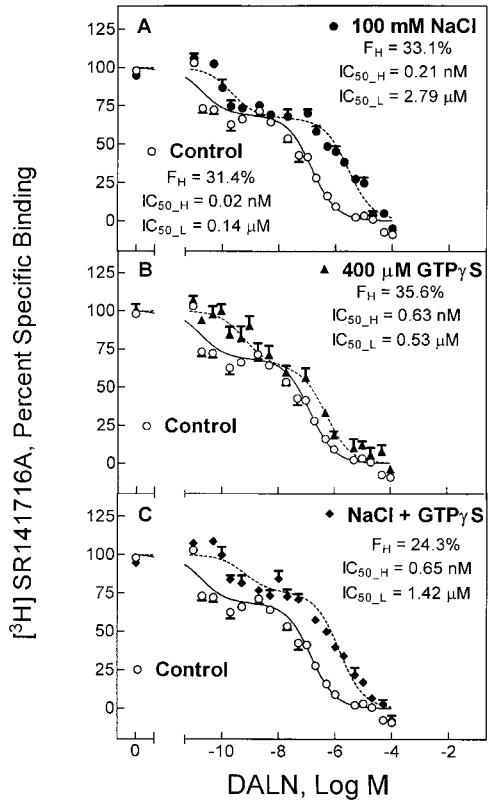

FIGURE 2. Competitive displacement by DALN of [^{3}H]SR-141716A binding to rat-brain membranes in the presence of vehicle buffer or (A) 100 mM NaCl, (B) 400 μM GTPγS or (C) both NaCl and GTPγS. Data were calculated as means ± standard errors from at least three experiments performed with quadruplicate replicates.

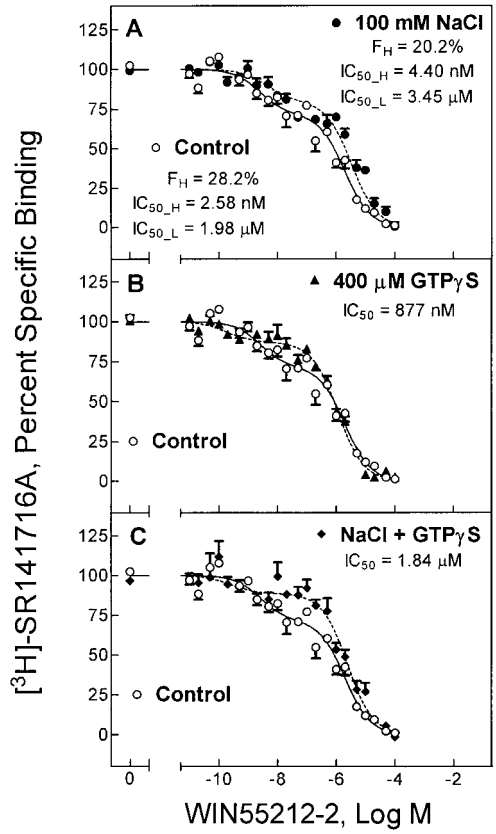

FIGURE 3. Competitive displacement by the aminoalkylindole WIN55212-2 of [^{3}H]SR141716A binding to rat-brain membranes in the presence of vehicle buffer or (A) 100 mM NaCl, (B) 400 μM GTPγS, or (C) both NaCl and GTPγS. Data were calculated as means ± standard errors from at least three experiments performed with quadruplicate replicates.

ceptors [4, 5]. The presence of as much as 500 mM NaCl or 400 μM GTPγS had no significant effect on binding of [^{3}H]SR141716A to CB_1 cannabinoid receptor preparations (data not shown).

To study agonist-induced, high-affinity binding under conditions that can discriminate between multiple affinity states, competition curves for two structurally different cannabimimetic agonists, DALN and WIN55212-2, were constructed versus [^{3}H]SR141716A binding. Figures 2 and 3 demonstrate the agonist competition-binding curves in rat-brain membrane preparations in the presence of NaCl, GTPγS or both, compared with control. Analysis of the curves generated by both ligands revealed binding indicative of differing receptor affinity states. The relative difference in binding for the affinity states can be quantitated by measuring the ratio of low-affinity IC_{50} to high-affinity IC_{50} to produce the value $R_{L/H}$. The $R_{L/H}$ for DALN in the control conditions was 7000, indicating a wide separation of the high- and low-affinity states, and the $R_{L/H}$ for WIN55212-2 was 770 (Figs. 2A and 3A). Both agonist-displacement profiles defined approximately 30% of the binding to be high affinity in control conditions. The IC_{50} value of the high-affinity state for WIN55212-2 was 100 times as great as that shown for DALN. The low-affinity IC_{50} of WIN55212-2 was only 10-fold greater than that of DALN, which accounts for the 10-fold difference in the $R_{L/H}$ values of the two agonists.

In other receptor systems, the presence of Na^+ results in a rightward shift of the agonist-competition curve, indicating a reduction in the affinity of the receptor for agonists [10–14]. When 100 mM NaCl was included in the DALN competition-binding assay, a rightward shift of both the high- and low-affinity components of agonist binding was observed, although the fraction of receptors representing high-affinity binding did not change significantly (Fig. 2A). The $R_{L/H}$ value increased almost 2-fold over control to 13,000, indicating further separation of the two affinity states. This change was primarily due to the 20-fold increase in the IC_{50} value of the low-affinity-binding state, which was twice the increase observed for the high-affinity- binding state. The presence of NaCl had little effect on WIN55212-2 binding, and no change in the $R_{L/H}$ value was noted (Fig. 3A). When heterologous displacement of [^{3}H]SR141716A by agonists was performed in the presence of 100 mM KCl, the competition curves were no different from that of the control, with no indication of a decrease in agonist affinity (data not shown). This would indicate that the changes produced by

NaCl are due to Na^+ rather than the ionic strength conditions in the assay media.

We tested the addition of 400 μM GTPγS to determine the effect of guanine nucleotides on CB_1 receptor binding affinity for both DALN and WIN55212-2 (Figs. 2B and 3B). DALN competition curves in the presence of GTPγS maintained significant two-state binding with no decrease in the fraction of receptors representing high-affinity binding. The IC_{50} value of the high-affinity receptor population increased 30-fold, but the IC_{50} value of the low-affinity population increased only 3-fold. Thus, the presence of GTPγS resulted in a decrease in the $R_{L/H}$ value to 840. Very different results were produced from the aminoalkylindole-binding data (Fig. 3B). GTPγS decreased the fraction of receptors in the high-affinity state such that two-state-binding models could fit the data only at $P > 0.05$. GTPγS clearly had a great effect in converting the receptors into an apparent single-state binding affinity for WIN55212-2.

The mechanisms by which Na^+ and GTPγS reduce high-affinity binding are thought to be distinct [15], such that the presence of both modulators should yield additive results over the presence of either one alone. To observe whether this occurs with agonist binding to CB_1 cannabinoid receptors, the heterologous displacement experiments were performed in the presence of 100 mM NaCl plus 400 μM GTPγS (Figs. 2C and 3C). The addition of both modulators to DALN competition assays resulted in a decrease in the fraction of receptors exhibiting high-affinity binding of DALN over that observed in control conditions. As for control, the data were better fit to a two-state-binding model ($P < 0.005$). The high-affinity IC_{50} value was higher than that of the control but was no greater than that observed in the presence of GTPγS alone. The low-affinity IC_{50} value also was increased over that of the control and was intermediate between the values obtained with NaCl alone and GTPγS alone. The $R_{L/H}$ value of 2200 was only 3-fold lower than the control value of 7000, demonstrating that a distinct separation of the high- and low-affinity states was maintained with high concentrations of both modulators of receptor–G-protein coupling. In contrast with the DALN data, WIN55212-2 competition in the presence of NaCl plus GTPγS resulted in complete conversion into one-state binding of low affinity (Fig. 3C). The IC_{50} value of 1.84 μM was similar to that of the low-affinity binding state in control WIN55212-2 binding assays.

When rat-brain membranes are detergent solubilised, high-affinity [^{3}H]CP55940 agonist binding is still present, indicating that any membrane disruption caused by detergent did not disrupt the receptor coupling to G proteins [5]. To determine to what extent the membrane environment may affect receptor–G-protein interactions, we examined the effects of NaCl and GTPγS on DALN and WIN55212-2 displacement of the labelled antagonist in CHAPS-solubilised rat-brain preparations (Figs. 4 and 5). The fraction of receptors in the high-affinity state for both agonists was greater in detergent-solubilised preparations than in membrane preparations. The IC_{50} value for high-affinity binding of DALN was increased 20-fold over the value in membrane preparations, and the IC_{50} value for low-affinity binding was increased 45-fold. This finding would suggest that membrane constraints resulted in a more stable cannabinoid agonist-receptor association in both affinity states. For WIN 55212-2, the IC_{50} value for high-affinity binding was decreased 7-fold over the value in membrane preparations, and the IC_{50} value for low-affinity binding remained about the same. In contrast with the effects observed with the cannabinoid agonist, the effect of the membrane environment observed with the aminoalkylindole agonist was to destabilise the ligand-receptor association in the high-affinity state.

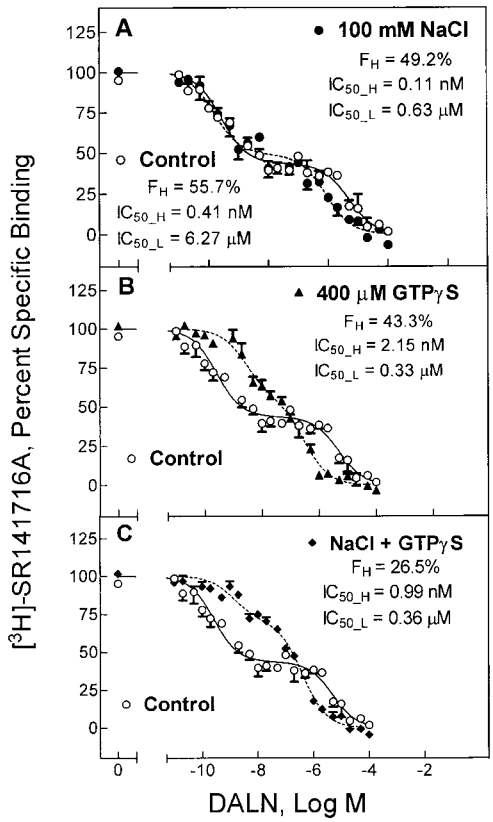

FIGURE 4. Competitive displacement by DALN of [^{3}H]SR-141716A binding in CHAPS-solubilised extracts from rat-brain membranes. Conditions for panels A, B and C, were the same as those described in Figure 2. Data were calculated as means ± standard errors from at least three experiments performed with quadruplicate replicates.

In contrast with the dramatic effects of Na^+ on the affinity of DALN for CB_1 cannabinoid receptors in membranes, 100 mM NaCl added to cannabinoid receptors in detergent solution did not cause a significant change in the high-affinity IC_{50} value compared with that of the control. The change in the low-affinity curve was only a 10-fold reduction in affinity (Fig. 4A). The effect of NaCl on WIN55212-2 competition in detergent solution was slight, differing from observations in the membranes only in the 4-fold increase in the high-affinity IC_{50} value (Fig. 5A).

In detergent solution, the presence of GTPγS increased the DALN high-affinity IC_{50} by 5-fold and decreased the

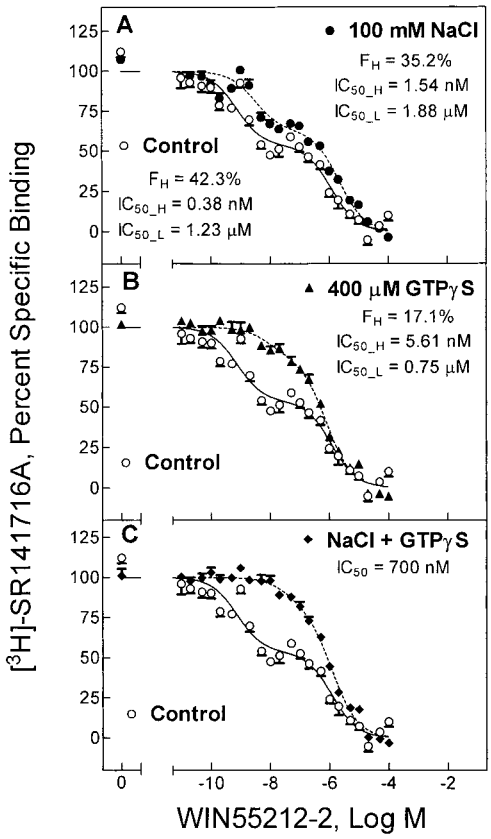

FIGURE 5. Competitive displacement by WIN55212-2 of [^{3}H]SR-141716A binding in CHAPS-solubilised extracts from rat-brain membranes. Conditions for panels A, B and C were the same as those described in Figure 3.

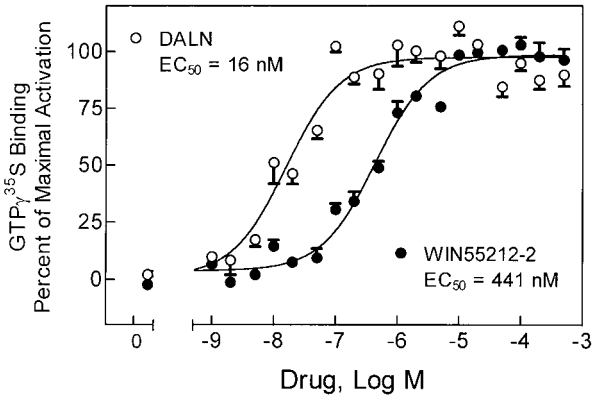

FIGURE 6. Stimulation of [^{35}S]GTPγS binding in rat-brain membranes by DALN and WIN55212-2. The data shown are the average ± range of two experiments performed with quadruplicate replicates.

low-affinity IC_{50} by 20-fold (Fig. 4B). This effect on the low-affinity binding was remarkable in that it had not been observed for receptors in the membranes. Despite this effect, two affinity states were discernible, and GTPγS was only minimally able to decrease the fraction of receptors in the high-affinity state for DALN. As was observed in the membrane binding studies, GTPγS was effective in decreasing the fraction of receptors in the high-affinity state for WIN55212-2 in detergent solution (Fig. 5B).

For DALN binding, a significant fraction of the receptor population was converted into the low-affinity state only when NaCl plus GTPγS were present together in detergent solution (Fig. 4C). Even so, 25% of the receptors maintained high-affinity binding for DALN. The 20-fold decrease in the low-affinity IC_{50} for DALN caused by GTPγS plus NaCl can be attributed to the presence of Na^+ in the reaction (Fig. 4B). NaCl plus GTPγS present in detergent solution caused full conversion of the WIN55212-2 binding-displacement curve into the low-affinity state (Fig. 5C).

G-protein-coupled receptor agonists are known to stimulate the binding of [^{35}S]GTPγS to their respective G proteins in membrane preparations [16, 17]. The increase in binding of [^{35}S]GTPγS in the presence of agonists is a measure of functional agonist activation of receptors and, as such, is able to distinguish ligands of differing efficacy and intrinsic activity. Because the two cannabimimetic agonists DALN and WIN55212-2 are differentially modulated by GTPγS in their affinity for CB_1 cannabinoid receptors, we wished to examine whether the two agonists differed in their abilities to stimulate guanine nucleotide binding to G proteins (Fig. 6). Both agonists were able to maximally stimulate [^{35}S]GTPγS binding 1.8-fold over basal levels, indicating that the two agonists are equal in efficacy. WIN55212-2 required an almost 30-fold greater concentration than DALN to produce the half-maximal effect. This resembled the difference in potencies that these two agonists exhibited in high-affinity displacement of [^{3}H]SR-141716A under similar assay conditions (i.e., containing 100 mM NaCl). Evidence indicates that agonist–receptor high-affinity binding is a requirement for G-protein activation but that the EC_{50} for G-protein activation may be one or two orders of magnitude greater than the IC_{50} value for high-affinity binding [17]. For DALN and WIN55212-2, respectively, the EC_{50} values to stimulate [^{35}S]GTPγS binding were 76-fold and 100-fold greater than the IC_{50} values for high-affinity displacement of SR141716A in membranes in the presence of 100 mM NaCl (see Figs. 2A and 3A).

DISCUSSION

A basic tenet of molecular pharmacology is that agonists, but not antagonists, stabilise an active conformation of the receptor, which in turn promotes effector activation. The ternary complex model for G-protein-coupled receptors equates active receptor with a complex of agonist (A), receptor (R) and G protein (G) and explains multiple agonist–receptor affinity states by the conversion of high-affinity receptors (RG) into low-affinity receptors (R) in the presence of guanine nucleotides [18]. This model was extended by incorporation of an additional receptor state (R*) to accommodate the finding of constitutively active mutant receptors in which agonists exhibit high-affinity binding even under conditions where RG coupling does not occur [19, 20]. According to this allosteric ternary complex

model, alterations in the ratio of unconstrained (R*) to constrained receptors (R) was postulated to be related to the intrinsic activity of the ligand.

To analyse the regulation of multiple affinity states for agonist binding to CB_1 cannabinoid receptors, the CB_1-selective antagonist [^{3}H]SR141716A was used as the radioligand, and the pattern of competition over a wide range of agonist concentrations was examined. The data presented here reveal multiphasic agonist-competition curves in both membranes and detergent extracts, with a plateau clearly defining two apparent affinity states. The cannabinoid DALN and the aminoalkylindole WIN55212-2 both defined a similar proportion of receptors as being in a high-affinity state. When studied in solubilised preparations, a larger percentage of receptors appeared in the high-affinity state. Perhaps solubilisaton allows the receptor to be freed from membrane constraints that might restrict conformational flexibility (R into R* interconversion) or G-protein coupling (R-G association).

Na^+ has been shown to alter agonist binding and G-protein interactions with CB_1 cannabinoid receptors [4, 5, 7, 21], as has been shown for other receptors that couple to $G_{i/o}$ [10, 11, 13, 22–24]. The mechanism by which Na^+ perturbs receptor–G-protein interaction has not been clearly defined. Na^+ may directly modify the receptor such that receptor coupling to G proteins is hindered or Na^+ may interact with the agonist-binding site of the receptor to reduce agonist affinity independently of G-protein effects. In the studies presented here, the effect of Na^+ was to cause a parallel rightward shift in the agonist-competition curve for DALN (and to a limited extent for WIN55212-2) such that agonist affinities for both high- and low-affinity components were decreased. This effect of Na^+ on the affinity for DALN was not observed in the solubilised receptor preparation. A similar rightward shift in the agonist-competition curve has been noted with other G-protein-coupled receptors; however, the extent of the shift varied depending on the competing agonist and whether the receptor was membrane bound or in detergent solution [25–27]. Na^+ has been reported to decrease the affinity of the agonist phenylisopropyladenosine for the A_1 adenosine receptor in membranes but not in solubilised preparations [25]. Studies of digitonin-solubilised adrenergic receptors also demonstrated reduced affinity for agonist binding compared with membrane-bound receptors [11]. It is interesting to note that the decreases in affinity for DALN evoked by detergent solubilisation mimic the decreases evoked by Na^+ in the membrane preparation. Additivity of the two effects would not be expected if both procedures resulted in a comparable alteration in conformation of the receptor. Such a change in conformation of the receptor could affect either agonist-receptor interaction or G-protein–receptor interaction.

An alternative explanation for the failure of Na^+ to decrease cannabinoid agonist affinity in detergent solution is that the composition or proportion of G proteins may differ between membranes and detergent extracts. This explanation is consistent with the hypothesis that the mechanism of action of Na^+ is to alter receptor–G-protein interaction and that this effect varies with G-protein subtype and availability in the preparation. Pacheco *et al.* [21] found a brain region-specific effect of Na^+ on cannabinoid CP55940 and aminoalkylindole WIN55212-2 agonist-stimulated GTPase activities and inhibition of adenylate cyclase. Notably, these signal-transduction responses in cerebellar membranes appeared to be refractory to the effects of Na^+. These investigators also compared concentration–effect curves for the CB_1 receptor agonists CP55940 and WIN55212-2 in stimulating GTPase activity and found that Na^+ had no effect on the potency of CP55940 in membranes from rat cerebellum and striatum but decreased the EC_{50} value for WIN55212-2 in cerebellum and increased the EC_{50} in striatum. To explain the observed results, the authors suggested that different G proteins may couple to cannabinoid receptors in different brain regions [21].

Mutagenesis studies of other G-protein-coupled receptors have demonstrated that conserved aspartate residues found in the second and third transmembrane domains are important in mediating the effects of Na^+ on receptor–agonist interactions [14, 24, 28–30]. Sequencing of CB_1 receptor cDNA revealed the presence of an aspartate residue in the second transmembrane domain [31], which may be the target for an allosteric regulatory effect of Na^+ on agonist binding or on coupling to G proteins.

The agonist-competition curves in the presence of a high concentration of GTPγS were quantitatively different from those produced in the presence of Na^+, suggesting that guanine nucleotides alter agonist–receptor–G-protein interactions by a mechanism differing from that of Na^+. GTPγS reduced agonist affinity principally at high-affinity sites for both DALN and WIN55212-2. However, two-state binding was still evident for both agonists, a phenomenon observed in other receptor systems [32–34]. Interestingly, the fraction of receptors in the high-affinity state for DALN was not significantly altered whether in membranes or in detergent extracts. In contrast, GTPγS reduced the fraction of receptors in the high-affinity state defined by WIN55212-2, such that the competition curve was best fit to a single, low-affinity state. Thus, the difference in sensitivity to GTPγS modulation appears to be related to the specific agonist. Studies of other G-protein-coupled receptors have shown that guanine nucleotides differentially regulate the interactions of different agonists for the same receptor [35–38]. The mechanism by which this occurs is not certain. It has been speculated that the agonist–receptor–G-protein ternary complex may comprise two populations, one sensitive to guanine nucleotides and the other insensitive [38]. Consistent with this model, the differential sensitivity to agonists might come about if one agonist (e.g., WIN55212-2) is able to alter the conformation of receptors in both ternary complex populations but other agonists (e.g., DALN) can affect only that population that is sensitive to GTP analogues. To bring this to a greater level of complexity, it is well accepted that different G-protein subtypes having distinct α or βγ proteins or both can interact with the same receptor, allowing many combinations of receptor–G-protein com-

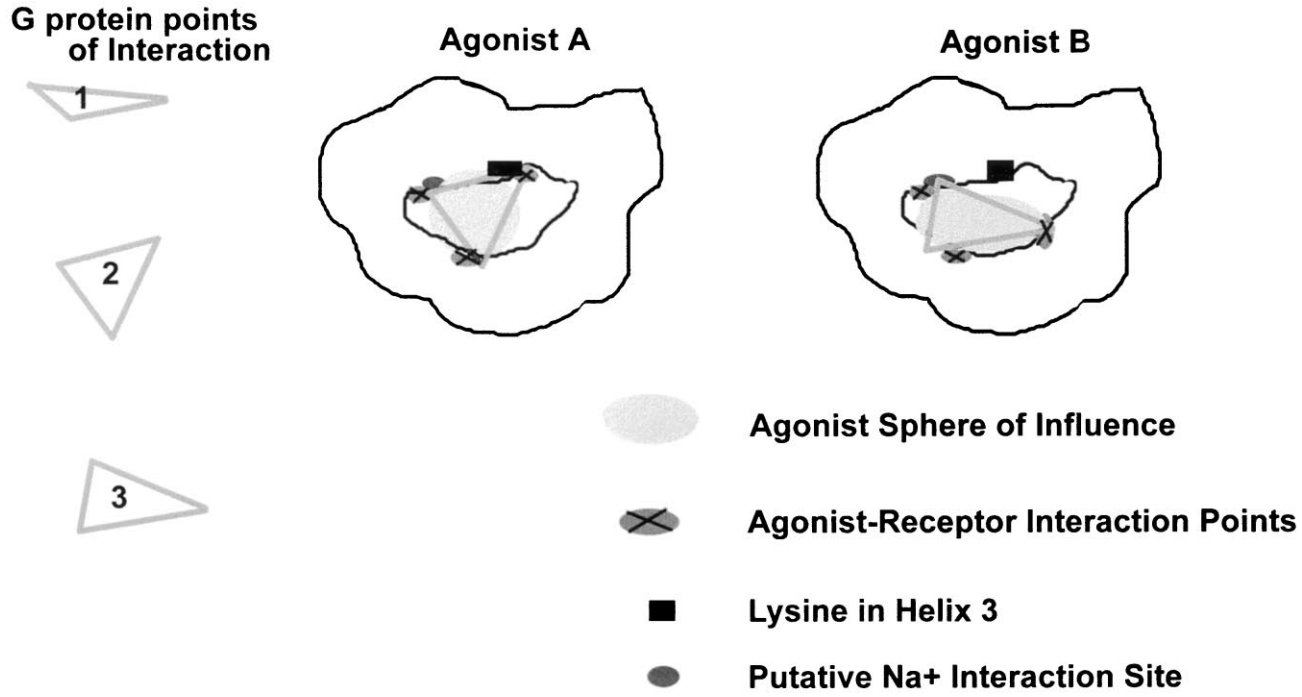

FIGURE 7. Mutually reciprocating relationship between the agonist "sphere of influence" within the receptor binding pocket and the receptor interaction with specific G proteins. A depiction viewed from the cytoplasmic surface of the G-protein-coupled receptor is shown in which agonist A and agonist B can interact with different receptor-binding sites and therefore have a different "sphere of influence" (light gray oval) on the receptor. Three different G proteins (1–3) have distinct sites of interaction with G-protein-coupled receptors; these interaction points are depicted as the points of a triangle, each unique for that particular G protein. Each G protein can interact exclusively with receptor(s) presenting an accommodating pattern of interaction points (dark gray oval with X). Agonist A interacts with the Lys residue (at square), thereby making available a conformation of the receptor allowing three interaction points with G protein 2 but not 1 or 3. Conversely, the positioning of G protein 2 at these points of interaction stabilises the receptor in a conformation that exhibits a high-affinity association with agonist A. The sphere of influence of agonist B does not include interaction with the Lys residue but does include other regions of the receptor-binding pocket. The sphere of influence of agonist B may shift the conformation of the receptor such that the points that accommodate G-protein interaction are now in a somewhat different orientation and may include an interaction site that was not available under the sphere of influence of agonist A. In the presence of agonist B, G protein 2 is no longer able to interact with the receptor; however, G protein 3 can form an adequate interaction. Conversely, a receptor interaction with G protein 3 stablilises the receptor in a conformation suitable for high-affinity association with agonist B, and this conformation may or may not be suitable for a high-affinity interaction with agonist A. G protein 1 cannot interact with this receptor, because of inappropriate interaction sites. The Na^+ site on the receptor (dark gray oval) is depicted to be positioned in a region that can influence at least one point of interaction with the G protein as well as the agonist sphere of influence. The absence or presence of Na^+ at this site may modify either agonist affinity or G-protein interactions.

plexes. Possibly, receptor complexes with certain G-protein subtypes may be more sensitive to modulation by GTP analogues than are others. Receptor interaction with a specific G protein could be dictated by the agonist present or, the converse, agonist interaction with the receptor could be dictated by the G protein associated with that receptor. If this hypothesis is applied to the data presented here, we could argue that those receptor–G-protein complexes activated by WIN55212-2 are less stable and therefore more sensitive to disruption by GTPγS than are those activated by DALN.

One might speculate on the nature of the 25% of the receptor population that maintains a distinct high-affinity state for the cannabinoid agonist DALN even in the presence of high concentrations of GTP analogues or Na^+ or both. Kinetic studies from our laboratory determined that CHAPS-solubilised CB_1 receptors exhibited high-affinity binding of the radiolabelled cannabinoid agonist [^{3}H]CP55940 that was diminished with increasing concentrations of GTP, GppNHp and GTPγS [5]. However, approximately 25% specific binding remained even at maximal concentrations of guanine nucleotide. This population of receptors might exist in a conformation having high cannabinoid agonist affinity irrespective of the state of coupling to G proteins. One can speculate that perhaps post-translational modifications, such as lipidation or phosphorylation, or interactions of the receptor with proteins other than G proteins might contribute to the maintenance of this high-affinity state.

The most important finding of the present investigation is the identification of distinct differences in the nature of the CB_1 receptor interaction with the cannabinoid agonist DALN compared with the aminoalkylindole agonist WIN55212-2. First, the difference between the high- and low-affinity IC_{50} values was much greater for DALN than for WIN55212-2, suggesting that the interconversion between the two states is not by precisely the same mechanism for

both agonists. Second, detergent solubilisation decreased agonist–receptor affinity in either high- or low-affinity states for DALN but enhanced agonist–receptor affinity for WIN55212-2, suggesting that the membrane environment may impose restrictions on the cannabinoid agonist binding that are irrelevant to the aminoalkylindole binding. Third, the manner in which Na^+ and GTPγS modulate the high-affinity-receptor population differs for these two agonists (Fig. 7). Tricyclic cannabinoids such as DALN differ in structure from aminoalkylindoles such as WIN55212-2 and therefore may not be expected to interact with the same pharmacophoric points on the receptor surface. Although these two classes of compounds show competitive-binding interactions at the CB_1 receptor, mutation studies of the ligand binding domain of the CB_1 receptor have identified at least one point of receptor interaction with cannabinoid ligands (a helix-3 lysine) that appears to be irrelevant to the binding of aminoalkylindole ligands [39]. It is possible that the different points of ligand–receptor interaction may promote different receptor conformations, which in turn may result in selective interaction with different G proteins.

This work was supported by NIH grants R01-DA03690, R01-DA06312 and P01-DA09158.

References

1. Abood M. E. and Martin B. R. (1992) *Trends Pharmacol. Sci.* **13**, 201–206.
2. Dewey W. L. (1986) *Pharmacol. Rev.* **38**, 151–178.
3. Howlett A. C. (1995) Cannabinoid Compounds and Signal Transduction Mechanisms. In: *Cannabinoid Receptors: Molecular Biology and Pharmacology* (Pertwee R. G., Ed.), pp. 167–204. Academic Press, London.
4. Devane W. A., Dysarz F. A. 3d, Johnson M. R., Melvin L. S. and Howlett A. C. (1988) *Mol. Pharmacol.* **34**, 605–613.
5. Houston D. B. and Howlett A. C. (1993) *Mol. Pharmacol.* **43**, 17–22.
6. Howlett A. C., Berglund B. A. and Melvin L. S. (1995) *Curr. Pharm. Design* **1**, 343–354.
7. Kuster J. E., Stevenson J. I., Ward S. J., D'Ambra T. E. and Haycock D. A. (1993) *J. Pharmacol. Exp. Ther.* **264**, 1352–1363.
8. Rinaldi-Carmona M., Barth F., Heaulme M., Shire D., Calandra B., Congy C., Martinez S., Maruani J., Neliat G., Caput D., Ferrara P., Soubrie P., Breliere J. C. and LeFur G. (1994) *FEBS Lett.* **350**, 240–244.
9. Swillens S., Waelbroeck M. and Champeil P. (1995) *Trends Pharmacol. Sci.* **16**, 151–155.
10. Pert C. B. and Snyder S. H. (1974) *Mol. Pharmacol.* **14**, 370–375.
11. Limbird L. E., Speck J. L. and Smith S. K. (1982) *Mol. Pharmacol.* **21**, 609–617.
12. Minuth M. and Jacobs K. (1986) *Naunyn-Schmeidenbergs Arch. Pharmacol.* **333**, 124–129.
13. Neve K., Henningsen R., Kinzie J., DePaulis T., Schmidt D., Keesler R. and Janowsky A. (1990) *J. Pharmacol. Exp. Ther.* **252**, 1108–1116.
14. Horstman D. A., Brandon S., Wilson A. L., Guyer C. A., Cragoe E. J. Jr. and Limbird L. E. (1990) *J. Biol. Chem.* **265**, 21590–21595.
15. Jagadeesh G. and Deth R. C. (1992) *J. Pharmacol. Exp. Ther.* **262**, 775–783.
16. Traynor J. and Nahorski S. (1995) *Mol. Pharmacol.* **47**, 848–854.
17. Lorenzen A., Guerra L., Vogt H. and Schwabe U. (1996) *Mol. Pharmacol.* **49**, 915–926.
18. De Lean A., Stadel J. M. and Lefkowitz R. J. (1980) *J. Biol. Chem.* **255**, 7108–7117.
19. Samama P., Cotecchia S., Costa T. and Lefkowitz R. J. (1993) *J. Biol. Chem.* **268**, 4625–4636.
20. Lefkowitz R. J., Cotecchia S., Samama P. and Costa T. (1993) *Trends Pharmacol. Sci.* **14**, 303–307.
21. Pacheco M. A., Ward S. J. and Childers S. R. (1994) *J. Neurochem.* **62**, 1773–1782.
22. Motulsky H. J. and Insel P. A. (1983) *J. Biol. Chem.* **258**, 3813–3819.
23. Wilson A. L., Seibert K., Brandon S., Cragoe E. J. Jr. and Limbird L. E. (1991) *Mol. Pharmacol.* **39**, 481–486.
24. Neve K. A., Cox B. A., Henningsen R. A., Spanoyannis A. and Neve R. L. (1991) *Mol. Pharmacol.* **39**, 733–737.
25. Stiles G. L. (1988) *J. Neurochem.* **51**, 1592–1598.
26. Jagadeesh G., Cragoe E. G. J. and Deth R. C. (1990) *J. Pharmacol. Exp. Ther.* **252**, 1184–1196.
27. Pike L. J. and Lefkowitz R. J. (1978) *Mol. Pharmacol.* **14**, 370–375.
28. Kong H., Raynor K., Yasuda K., Bell G. I. and Reisine T. (1993) *Mol. Pharmacol.* **44**, 380–384.
29. Ceresa B. P. and Limbird L. E. (1994) *J. Biol. Chem.* **269**, 29557–29564.
30. Befort K., Tabbara L., Kling D., Maigret B. and Kieffer B. L. (1996) *J. Biol. Chem.* **271**, 10161–10168.
31. Matsuda L. A., Lolait S. J., Brownstein M. J., Young A. C. and Bonner T. I. (1990) *Nature* **346**, 561–564.
32. Herrick-Davis K. and Titeler M. (1988) *J. Neurochem.* **50**, 528–533.
33. Harrington M. A. and Peroutka S. J. (1990) *J. Neurochem.* **54**, 294–299.
34. Wiener H. L. and Maayani S. (1991) *Eur. J. Pharmacol.* **208**, 271–272.
35. Childers S. R. and Snyder S. H. (1978) *Life Sci.* **23**, 759–762.
36. Childers S. R. and Snyder S. H. (1980) *J. Neurochem.* **34**, 583–593.
37. Frances B., Moisand C. and Meunier J.-C. (1985) *Eur. J. Pharmacol.* **117**, 223–232.
38. Mahle C. D., Wiener H. L., Yocca F. D. and Maayani S. (1992) *J. Pharmacol. Exp. Ther.* **263**, 1275–1283.
39. Song Z.-H. and Bonner T. I. (1996) *Mol. Pharmacol.* **49**, 891–896.