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Azido- and Isothiocyanato-Substituted Aryl Pyrazoles Bind Covalently to the CB_1 Cannabinoid Receptor and Impair Signal Transduction

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Abstract: 3-Azidophenyl- and 3-isothiocyanatophenyl- and 2-(5′-azidopentyl)- and 2-(5′-isothiocyanatopentyl)pyrazoles were synthesized to determine whether these compounds could behave as covalently binding ligands for the CB_1 cannabinoid receptor in rat brain membranes. Heterologous displacement of [^{3}H]CP55940 indicated that the apparent affinity of these compounds for the CB_1 receptor was similar to that of the parent compound, SR141716A, with the exception of the 3-isothiocyanato derivatives, which showed a 10-fold loss of affinity. The 3-azidophenyl and 3-isothiocyanatophenyl compounds behaved as antagonists against the cannabinoid agonist desacetyllevonantradol in activation of G proteins [guanosine 5′-*O*-(γ-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) binding] and regulation of adenylyl cyclase. The 2-(5′-azidopentyl)- and 2-(5′-isothiocyanatopentyl)pyrazoles were poor antagonists for [^{35}S]GTPγS binding, and both compounds failed to antagonize the cannabinoid regulation of adenylyl cyclase. After incubation with the isothiocyanato analogues or UV irradiation of the azido analogues, the 3-substituted aryl pyrazoles formed covalent bonds with the CB_1 receptor as evidenced by the loss of specific binding of [^{3}H]CP55940. In the case of the isothiocyanato analogues, the log concentration–response curve for cannabinoid-stimulated [^{35}S]GTPγS binding was shifted to the right, indicating that loss of receptors compromised signal transduction capability. These irreversibly binding antagonists might be useful tools for the investigation of tolerance and receptor down-regulation in both in vitro and in vivo studies.
Key Words: Adenylyl cyclase—Desacetyllevonantradol—Forskolin—G protein-coupled receptors—N18TG2 neuroblastoma—WIN55212-2—SR141716A.
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The CB_1 cannabinoid receptor is a G protein-coupled receptor that activates the G_i subfamily of G proteins to inhibit adenylyl cyclase, inhibit K^+ currents, stimulate mitogen-activated protein kinase, and induce expression of certain immediate-early genes (for review, see Howlett, 1995; Childers and Breivogel, 1998). Four classes of agonist ligands can stimulate these responses: ABC-tricyclic cannabinoid ligands, including Δ^9-tetrahydrocannabinol, HU210, and desacetyllevonantradol (DALN); AC-bicyclic cannabinoid ligands, typified by CP55940; aminoalkylindole ligands, including WIN55212-2; and eicosanoid ligands, including arachidonylethanolamide and 2-arachidonylglycerol (for review, see Howlett et al., 1995; Hillard and Campbell, 1997; DiMarzo, 1998). SR141716A, a potent aryl pyrazole antagonist for the CB_1 receptor, was shown to have high affinity for the CB_1 receptor (Rinaldi-Carmona et al., 1996), to antagonize competitively CB_1 receptor signal transduction pathways (Rinaldi-Carmona et al., 1994), and to block cannabimimetic responses in intact animals (Rinaldi-Carmona et al., 1995; Compton et al., 1996; Lichtman and Martin, 1996; Bouaboula et al., 1997; Brodkin and Moerschbaecher, 1997; Griffin et al., 1998; Mallet and Beninger, 1998). This antagonist has also been shown to evoke precipitated withdrawal in cannabinoid-treated rats (Aceto et al., 1995; Tsou et al., 1995). An aryl pyrazole antagonist for the CB_2 receptor has recently been described (Rinaldi-Carmona et al., 1998). Aryl pyrazole analogues of SR141716A are currently being produced for investigations of structure–

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Abbreviations used: DALN, desacetyllevonantradol; GTPγS, guanosine 5′-*O*-(γ-thio)triphosphate; KDMal-Mg buffer, 20 m*M* potassium diethylmalonate (pH 7.5) plus 3 m*M* $MgCl_2$; TME buffer, 20 m*M* Tris-Cl (pH 7.5), 3 m*M* $MgCl_2$, and 1 m*M* EDTA.

Compound	R1	R2	R3
1	N_3	H	–
2	H	N_3	–
3	NCS	H	–
4	H	NCS	–
5	–	–	N_3
6	–	–	NCS
SR	H	Cl	–

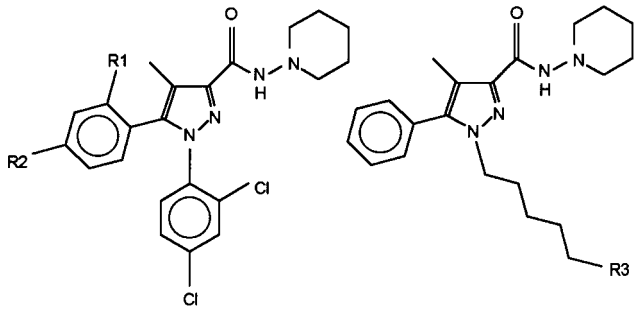

FIG. 1. Structures of the azido and isothiocyanato aryl pyrazole derivatives and SR141716A (SR).

activity relationships (Thomas et al., 1998; Lan et al., 1999).

The molecular mechanism by which aryl pyrazoles such as SR141716A interact with the CB_1 receptor to produce antagonism is unknown. One mechanism could be that SR141716A can compete for binding to the same ligand binding site(s) as the cannabinoid receptor agonists. Previous studies have shown that covalently bound cannabinoid (Charalambous et al., 1992; Guo et al., 1994; Morse et al., 1995), aminoalkylindole (Yamada et al., 1996), and eicosanoid (Deutsch et al., 1997) agonists can preclude the subsequent binding of the radiolabeled cannabinoid agonist [^{3}H]CP55940. These studies suggest that these three chemically distinct ligands share a common binding pocket within the receptor such that the irreversible interaction of the covalently bound ligand with the receptor prohibits any subsequent ligand binding within that pocket.

The hypothesis was proposed that aryl pyrazoles can bind to the CB_1 receptor within the same binding pocket that is shared by the agonist ligands. To test this hypothesis, potential covalently binding analogues of SR141716A were designed and synthesized (Fig. 1). The strategy was to place an azide or isothiocyanate substituent at one of several positions on the molecule. Isothiocyanato derivatives can potentially form covalent bonds with nucleophilic groups on proteins. Azido derivatives must be converted to nitrenes by UV irradiation before covalent bonding is possible. Covalent binding to the CB_1 receptor occurs based on the high affinity that these analogues should have for the CB_1 receptor compared with other proteins. The reaction also depends on the availability of an appropriate amino acid in close proximity within the binding pocket, e.g., amines in Lys or Arg, sulfhydryls in Cys, for the covalent reaction to occur.

The present study shows that certain aryl pyrazole analogues appear to be useful as potential covalently binding ligands in signal transduction studies. After appropriate pretreatment of rat brain membrane preparations with these analogues, binding of radiolabeled agonist [^{3}H]CP55940 was attenuated, and the ability of the cannabinoid agonist DALN to activate G proteins was reduced, supporting the proposed hypothesis.

MATERIALS AND METHODS

Materials

Aryl pyrazole analogues were synthesized at the University of Connecticut in the laboratory of Dr. A. Makriyannis. The compounds were synthesized in a manner similar to the aryl pyrazoles previously published (Lan et al., 1999). Their structures were confirmed by electron absorption mass spectrometry and NMR analysis (structural data are available on request from A.M.). Compounds were dissolved as 10^{-1} or 10^{-2} *M* solutions in dimethyl sulfoxide and stored at −80°C under N_2 gas in aliquots sufficient for a single experiment. β-Cyclodextrin was purchased from Research Biochemicals International. [^{3}H]CP55940 and guanosine 5′-*O*-(γ-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) were from New England Nuclear, and [^{3}H]SR141716A was from Amersham International. SR141716A and DALN were gifts from Pfizer, and Ro 20-1724 was a gift from Hoffmann–La Roche. WIN55212-2 was purchased from Research Biochemicals International. Forskolin was purchased from Calbiochem. GTPγS and all other reagents were purchased from Sigma Chemical Co.

Radioligand binding assays

The affinity of this series of compounds for the CB_1 receptor was determined by displacement of [^{3}H]CP55940 from receptors in rat brain membranes as previously described (Meschler and Howlett, 1999) with modifications that attempted to diminish the presence of a large excess of amine groups and protein during the approach to equilibrium. Membranes were suspended in 20 m*M* potassium diethylmalonate (pH 7.5) plus 3 m*M* $MgCl_2$ (KDMal-Mg buffer). The aryl pyrazole ligands were initially suspended at 10^{-4} *M* in 5% β-cyclodextrin, followed by subsequent dilution in KDMal-Mg buffer containing 0.05% β-cyclodextrin. Membranes (30 μg) plus [^{3}H]CP55940 and unlabeled ligands were incubated for 1 h at 30°C. Experiments using compounds 1, 2, and 5 were performed in the absence of fluorescent lighting. Samples were filtered over glass fiber GF/B filters using a Tomtec harvester and counted for radioactivity using a Wallac Betaplate counter as previously described (Meschler and Howlett, 1999).

The apparent affinity of these compounds for the CB_2 receptor in mouse spleen membrane preparations was determined by heterologous competition with [^{3}H]CP55940 as previously described (Lan et al., 1999).

Signal transduction assays

[^{35}S]GTPγS binding to rat brain membranes was performed by a modification of the previously described procedure (Howlett et al., 1998). The assay mixture contained KDMal-Mg buffer, 0.375 n*M* [^{35}S]GTPγS, 10 μ*M* GDP, 1 m*M* dithiothreitol, 100 m*M* NaCl, 5 m*M* $MgCl_2$, 5 μg of rat brain P2

membranes, and the indicated concentrations of compounds that had been diluted in β-cyclodextrin (0.05% final concentration in the assay) in a final volume of 100 μl in 96-well plates. The reaction was incubated at 30°C for 60 min and terminated by vacuum filtration over GF/B filters using a Tomtech harvester. Radioactive [^{35}S]GTPγS was quantitated using a Wallac Betaplate counter.

Assay of the adenylyl cyclase activity in membranes from N18TG2 neuroblastoma cells was modified from the previously described method (Howlett, 1985). Assays were performed in 20 m*M* potassium diethyl malonate (pH 8.0) as the buffer, and compounds were diluted in β-cyclodextrin as described above. Forskolin (10 μ*M*) was present in all assays as the adenylyl cyclase activator, DALN (1 μ*M*) was used as the CB_1 receptor agonist, and compounds in this series were used at the indicated concentrations.

Treatment of membranes with azido- and isothiocyanato-substituted pyrazole derivatives

For azido analogues, rat brain P2 membranes (2 mg/ml) in 20 m*M* potassium diethylmalonate (pH 7.5) were incubated for 1 h at 30°C (final volume, 500 μl) with either 0.05% β-cyclodextrin (vehicle control) or 10 μ*M* compound 1 or 2. After incubation, the samples were placed in a six-well multiwell plate on ice and irradiated while shaking for 10 min with shortwave UV light (254 nm; model UVG-54 Mineralight Lamp) 5 cm from the source. Nonirradiated controls were shaken on ice but not irradiated. After irradiation (or control incubation), 500 μl of ice-cold 50 mg/ml fatty acid-free bovine serum albumin was added, and the samples were sedimented at 13,000 *g* for 10 min. Membranes were resuspended in 2.5 ml of ice-cold 20 m*M* Tris-Cl (pH 7.5), 3 m*M* $MgCl_2$, and 1 m*M* EDTA (TME buffer). Radioligand binding assays were performed in TME buffer as described previously (Meschler and Howlett, 1999). Nonspecific binding was determined by displacement with 1 μ*M* DALN, 10 μ*M* SR141716A, or 10 μ*M* WIN55212-2.

For isothiocyanato analogues, incubations of membranes were performed as described above with vehicle control or 10 μ*M* compound 3 or 4. After incubation for 1 h at 30°C (500 μl), the samples were diluted with 50 mg/ml fatty acid-free bovine serum albumin and immediately sedimented without irradiation. Radioligand binding assays were performed in TME buffer exactly as described previously (Meschler and Howlett, 1999). Nonspecific binding was determined by displacement with 1 μ*M* DALN. [^{35}S]GTPγS binding was determined in TME buffer as described previously (Howlett et al., 1998). The indicated concentrations of DALN were used to stimulate [^{35}S]GTPγS binding.

Data analysis

Radioligand binding assays and [^{35}S]GTPγS binding concentration–response relationships were analyzed by nonlinear regression using Inplot version 4 (Graphpad). Multiple comparisons were performed by one-way ANOVA followed by Tukey's post hoc test. One-way paired comparisons were made using Student's *t* test.

RESULTS

The affinity of the azido derivatives 1, 2, and 5 for the CB_1 cannabinoid receptor was reduced compared with the parent compound SR141716A, although the IC_{50} values remained within an order of magnitude (Fig. 2A). The 3-isothiocyanatophenyl derivatives, 3 and 4, showed a more pronounced reduction in affinity compared with SR141716A, exhibiting a difference in apparent IC_{50} of an order of magnitude or greater (Fig. 2B). Isothiocyanato analogue 6 exhibited about the same affinity as the corresponding azido analogue 5. The isothiocyanato derivatives are subject to covalent reaction with the receptor during the incubation, and thus the apparent affinities will be greater than would be found if covalent binding had not occurred. Because K_i values cannot be calculated for reactions having an infinitely slow off-rate, we have expressed affinity values for all compounds as IC_{50} values.

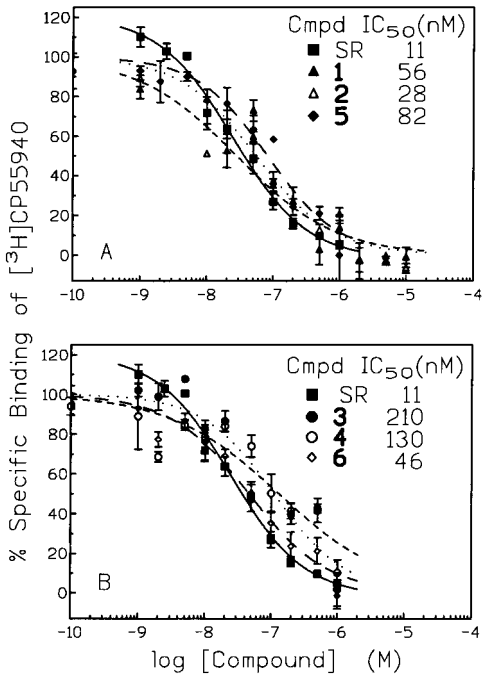

FIG. 2. Relative affinities of aryl pyrazole analogues for the CB_1 cannabinoid receptor: (**A**) compounds 1, 2, and 5 and (**B**) compounds 3, 4, and 6. SR141716A (SR) is shown for comparison. Radioligand binding assays were performed as described in the text using 350 fmol/L [^{3}H]CP55940 as the radioligand and the indicated concentrations of the competing ligands. Data are mean ± SEM (bars) percent specific binding values of [^{3}H]CP55940 from three individual experiments. The curves were produced by nonlinear regression analysis of the sigmoidal curve generated for the averaged data.

These aryl pyrazoles were also tested for their affinity for the CB_2 receptor (Table 1). For compounds 2, 3, and 4, half-maximal displacement of [^{3}H]CP55940 from presumptive CB_2 receptors in mouse spleen membranes was achieved at concentrations that exceeded 1 μ*M*. Compound 1 was not a very potent ligand for the CB_2 receptor either. However, compounds 5 and 6 exhibited affinities for the CB_2 receptor that were similar to those for the CB_1 receptor, suggesting that these compounds have poor subtype selectivity.

The ability of the azido and isothiocyanato aryl pyrazoles to behave as antagonists of the response to the cannabinoid agonist DALN in signal transduction assays

TABLE 1. *Relative affinities of aryl pyrazole ligands for the CB_2 cannabinoid receptor*

Compound	Specific binding IC_{50} (n*M*)
1	394 ± 121
2	1,490 ± 439
3	1,110 ± 161
4	5,970 ± 413
5	20.7 ± 2.3
6	22.6 ± 3.1

Data are average ± range values (n = 2).

was tested. DALN was previously shown to exhibit an EC_{50} of 16 μM for the stimulation of [^{35}S]GTPγS binding to G proteins in rat brain membranes (Houston and Howlett, 1998). Using an agonist concentration expected to produce 96% of the maximal stimulation, all of the compounds were able to antagonize the agonist-mediated stimulation of [^{35}S]GTPγS binding to G proteins in rat brain membranes (Fig. 3). At this agonist concentration, a strictly competitive antagonist would produce a sigmoidal curve having an IC_{50} at 30-fold the concentration of the antagonist equilibrium dissociation constant (Craig, 1993). As observed in Fig. 3, these antagonist log concentration–response curves failed to conform uniformly to a standard Langmuir isotherm, suggesting that the inhibition was more complex than simple competitive antagonism. For compounds 1, 2, and 4, the concentrations estimated to provide half-maximal inhibition of DALN's stimulation of [^{35}S]GTPγS binding were ~10-fold greater than their relative affinities for the CB_1 receptor. Compound 3 was less efficient as an antagonist, yielding a half-maximal inhibition at a concentration that was 50-fold greater than its relative affinity. However, compounds 5 and 6 required ~100-fold greater concentrations for antagonism compared with their relative affinities for the CB_1 receptor. Complexity of the antagonism for compounds 3, 4, and 6 is theoretically the result of both competitive antagonism and the effects of irreversible interaction of the isothiocyanato groups with residues in the receptor binding pocket. However, additional confounds are also obvious from these data. When

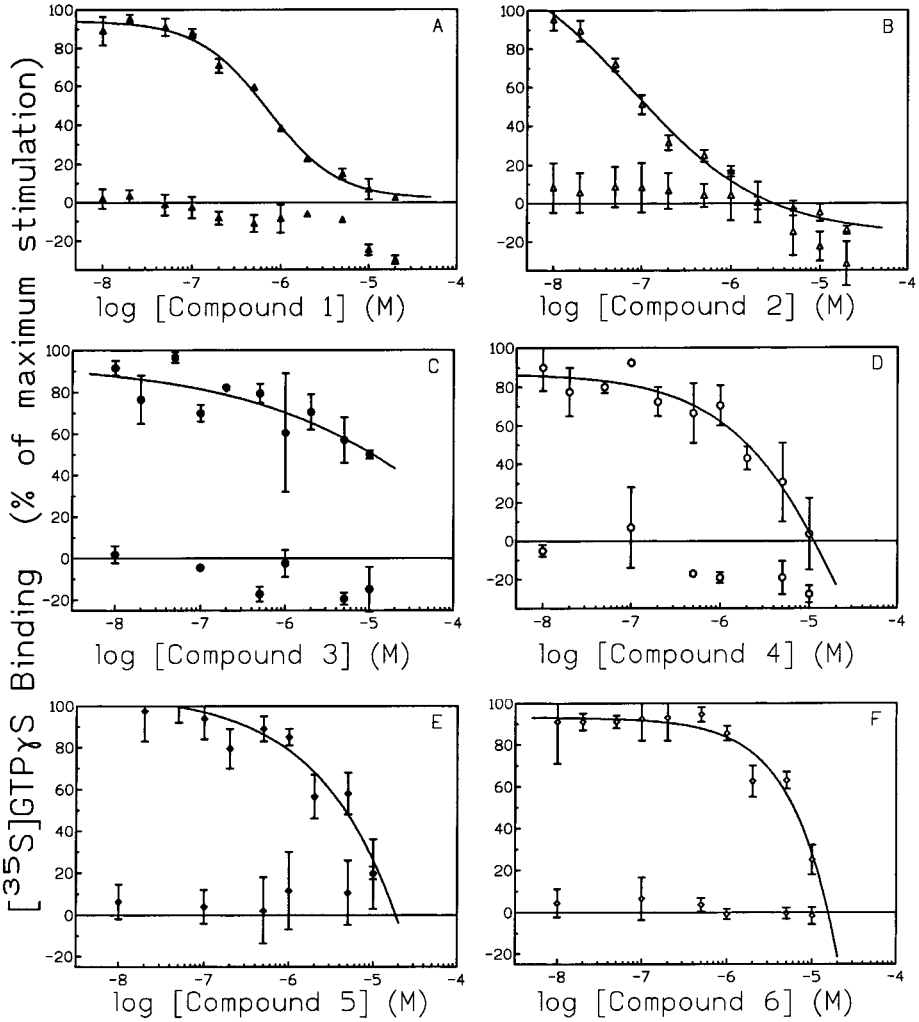

FIG. 3. A–F: Effects of azido and isothiocyanato aryl pyrazole analogues to antagonize the stimulation of [^{35}S]GTPγS binding to G proteins by the CB_1 receptor agonist DALN. [^{35}S]GTPγS binding was examined at the indicated concentrations of analogues in the presence of vehicle (line at 0%) or 500 n*M* DALN (curve beginning at 100%). Data are mean ± SEM (bars) values in percentages of the DALN-stimulated value from three individual experiments, and curves are drawn as nonlinear regression analyses of either sigmoidal (A and B) or exponential decay (C–F).

TABLE 2. *Effects of azido and isothiocyanato aryl pyrazoles on adenylyl cyclase activities in N18TG2 neuroblastoma membranes*

	Adenylyl cyclase	
Compound	Stimulation[a]	Inhibition[b]
Forskolin	100	—
1	107 ± 4.4 (3)	12 ± 4.3 (3)[c]
2	116 ± 2.3 (3)[d]	12 ± 7.7 (3)[c]
3	122 ± 3.6 (3)[d]	18 ± 4 (2)[c]
4	123 ± 6.6 (3)[d]	13 ± 8 (2)[c]
5	97 ± 3.9 (4)	32 ± 6.9 (4)
6	81 ± 11 (2)	36 ± 4 (2)
5 (10 μM)	79 ± 7.6 (4)[e]	—
6 (10 μM)	55 ± 5 (2)[e]	—
DALN	—	32 ± 1.9 (9)

Adenylyl cyclase activity was determined in the presence of 10 μM forskolin (stimulation) or in the presence of 10 μM forskolin plus 1 μM DALN (inhibition), and aryl pyrazole analogues were included in the reaction at 1 μM except where indicated. Data are mean ± SEM values (n).

[a] Values were calculated as pmol/min/mg of protein, and the forskolin response was assigned a value of 100%. The stimulation by the aryl pyrazole compounds is reported in comparison with the forskolin value for each experiment.

[b] The percent inhibition of forskolin-activated adenylyl cyclase by DALN was determined. The antagonism of the DALN response was determined in the presence of 1 μM aryl pyrazole compound, and data are expressed as percent inhibition of forskolin-stimulated adenylyl cyclase.

[c] Significantly different from the inhibition by DALN compared by an unpaired Student's t test at the $p < 0.05$ level.

[d] Significantly stimulated above the forskolin value by a paired Student's t test at the $p < 0.05$ level.

[e] Significantly inhibited below the forskolin value by a paired Student's t test at the $p < 0.05$ level.

compounds 1–4 were tested in the absence of the cannabinoid agonist, [^{35}S]GTPγS binding at concentrations >1 μM declined below the unstimulated value. This might be consistent with compounds 1–4 exhibiting inverse agonist activity. However, this interpretation should be viewed with caution because at a concentration of 10 μM, these compounds were slightly "cloudy" in solution, an indication of limited aqueous solubility. An additional concern is the ability of these compounds to induce membrane perturbation at high concentrations (X. Tian and A. Makriyannis, unpublished data), an effect that may influence signal transduction measurements.

As shown in Table 2, the inhibition of adenylyl cyclase by 1 μM DALN could be partially reversed by 1 μM concentrations of compounds 1–4. When tested alone, compounds 2–4 at 1 μM (and at 0.1 μM; data not shown) increased forskolin-stimulated adenylyl cyclase activity by 15–30%, suggesting some potential for inverse agonist behavior. Higher concentrations failed to antagonize further the response to DALN or to stimulate adenylyl cyclase when present alone (data not shown). This failure to demonstrate dose-dependent effects may be due to poor aqueous solubility of the compounds at these concentrations. Compounds 5 and 6 at 1 μM (or as high as 10 μM; data not shown) were not able to reverse the response to DALN. These 2-(5′-modified pentyl)pyrazole analogues also failed to stimulate adenylyl cyclase at 0.1 (data not shown) or 1 μM (Table 2). At 10 μM concentrations, compounds 5 and 6 both inhibited adenylyl cyclase, suggesting that these compounds may exhibit some agonist potential at high concentrations.

Studies were performed to determine if the 3-azidophenylpyrazole antagonists could form covalent interactions with the CB_1 receptor. If this were the case, one would expect that the binding site for [^{3}H]CP55940 would be occluded and that binding of this radioligand would be compromised. To test this, rat brain membranes were incubated at 30°C for 1 h with vehicle control or compound 1 or 2 at 10 μM, which is 200 times the apparent IC_{50} to displace [^{3}H]CP55940. After equilibration, membranes were irradiated with short-wave UV light in an attempt to generate nitrenes and lead to covalent bonding at or near the binding site within the receptor molecule. Membranes were then diluted with an excess of fatty acid-free bovine serum albumin to bind free ligand and sedimented to remove any unbound ligand from the membranes. Membranes from irradiated and nonirradiated treatment groups were assayed for binding of [^{3}H]CP55940 that could be displaced by the cannabinoid agonist DALN, the aryl pyrazole antagonist SR141716A, or the aminoalkylindole agonist WIN55212-2. The observation that each of these three ligands displaced binding to the same extent (45% of total binding) supports the idea that there are no subpopulations of receptor that prefer one of these chemically distinct classes of ligand. As shown in Fig. 4, the process of UV

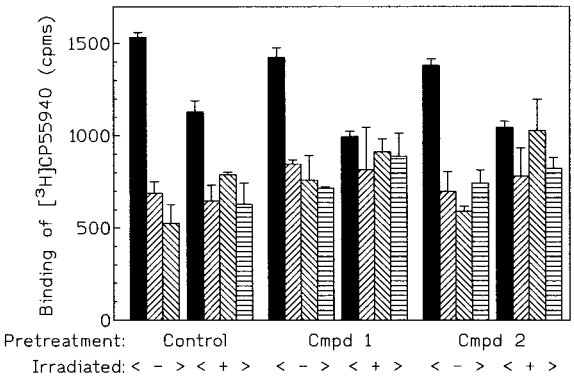

FIG. 4. Ability of 3-azidophenyl pyrazoles 1 and 2 to attenuate irreversibly [^{3}H]CP55940 specific binding to the CB_1 cannabinoid receptor in rat brain membranes. This is a single representative experiment of four, performed as described in the text. Rat brain membranes were equilibrated with vehicle (control) or the indicated compound and then were irradiated (+) or not (−) before washing away the ligand by sedimentation of the membranes. The data are mean ± SEM (bars) cpm values from three replicates of total [^{3}H]CP55940 bound in these membranes (■) or nonspecifically bound as determined by displacement with 1 μM DALN (▨), 10 μM SR141716A (▧), or 10 μM WIN55212-2 (▤). Data were analyzed by ANOVA followed by Tukey's post hoc test. Total binding values were significantly different ($p < 0.05$) from nonspecific binding for all groups except irradiated (+) 1 and (+) 2.

irradiation alone reduced the [^{3}H]CP55940 binding ability (control), possibly owing to an effect on receptor protein conformation or receptor–G protein association. For membranes treated with compound 1 or 2 followed by UV irradiation, total binding was reduced, and no displacement by DALN, SR141716A, or WIN55212-2 was observed, indicating that "specific binding," as defined by each of these classes of chemically distinct ligands, had been eliminated. Nonspecific binding was not altered. Compounds 1 and 2 were equilibrated but not UV-irradiated, to monitor for excess compounds in the assay that had not been washed away. After pretreatment with these compounds, only ~5% reduction in [^{3}H]CP55940 binding was observed compared with the control. These studies demonstrate that the placement of an azide moiety on either of two positions on the 3-phenyl ring could form an irreversible interaction with the CB_1 receptor that precludes subsequent binding of the agonist [^{3}H]CP55940. Signal transduction studies could not be performed using irradiated membranes because the UV irradiation itself prevented [^{35}S]GTPγS binding and severely attenuated adenylyl cyclase activity. Irradiation for only 1 min was sufficient to reduce basal and eliminate agonist-stimulated [^{35}S]GTPγS binding in these preparations.

3-Isothiocyanatophenylpyrazole antagonists were also able to attenuate [^{3}H]CP55940 binding in rat brain membranes (Fig. 5A). Membranes were treated with concentrations of compounds 3 or 4 that were 50–75-fold greater than the IC_{50} values for binding to the receptor. After washing these compounds from the membranes by sedimentation, the binding of [^{3}H]CP55940 was determined. Specific binding as defined by the cannabinoid agonist DALN was reduced to 30–50% of the control value by the 3-isothiocyanatophenylpyrazoles. Although this loss of receptor binding was not complete under these conditions, the ability of the agonist DALN to stimulate [^{35}S]GTPγS binding to G proteins was affected (Fig. 5B). A rightward shift in the log concentration–response curve for DALN of one order of magnitude was observed in those membranes that had suffered a diminished CB_1 receptor binding capacity.

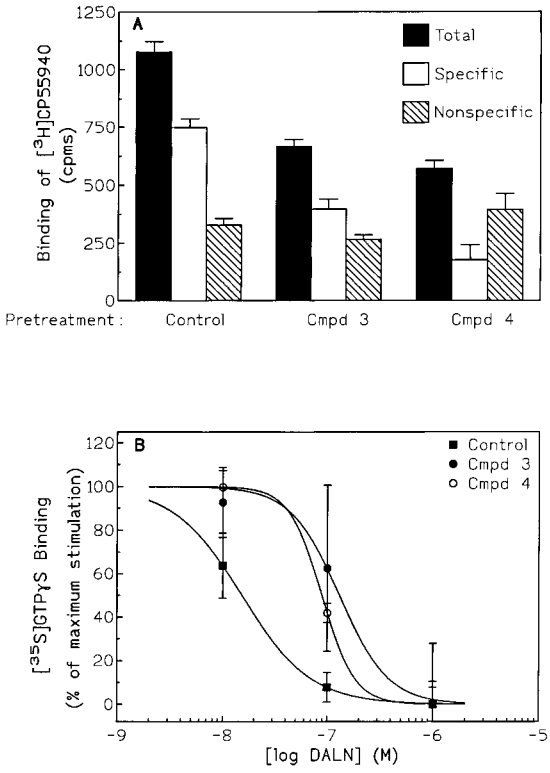

FIG. 5. Ability of 3-isothiocyanatophenyl pyrazoles 3 and 4 to attenuate irreversibly [^{3}H]CP55940 specific binding to the CB_1 cannabinoid receptor and diminish signal transduction via G proteins in rat brain membranes. Rat brain membranes were equilibrated with either vehicle or 10 μM compound 3 or 4, followed by removal of the ligands by sedimentation of the membranes as described in the text. **A:** Membranes were assayed for [^{3}H]CP55940 binding shown as total binding (■), specific binding calculated as binding that could be displaced by DALN (□), and nonspecific binding defined by failure to be displaced with DALN (▨). **B:** The same membranes were also examined for the ability of DALN to concentration-dependently stimulate [^{35}S]GTPγS binding. Data are mean ± SEM (bars) values from three individual experiments.

DISCUSSION

3-Azidophenylpyrazole derivatives (1 and 2) were synthesized that exhibited an apparent affinity for the CB_1 receptor within an order of magnitude of the affinity of the parent compound, SR141716A. However, the two 3-isothiocyanatophenylpyrazole analogues (compounds 3 and 4) showed >10-fold loss of apparent affinity. Each of these 3-substituted aryl pyrazole derivatives maintained the property of the parent SR141716A to behave as competitive antagonists against a classical cannabinoid ligand, DALN, in the signal transduction assays of G protein activation and inhibition of adenylyl cyclase. The 2-(5′-azidopentyl) (compound 5) and 2-(5′-isothiocyanatopentyl) (compound 6) analogues exhibited an affinity for the CB_1 receptor within an order of magnitude of the parent SR141716A. These compounds behaved as antagonists to reverse the effects of the cannabinoid agonist DALN in the [^{35}S]GTPγS binding assay, although a relatively greater concentration of these compounds was required to antagonize the response compared with their affinity for the receptor. These compounds failed to manifest antagonism against the cannabinoid agonist in the adenylyl cyclase determinations under conditions in which antagonism was observed for the 3-azidophenylpyrazole derivatives (1 and 2), which exhibited comparable affinities for the receptor. Compounds 5 and 6 appear to exhibit CB_1 receptor agonist properties in the adenylyl cyclase assay at high concentrations but not at all in the [^{35}S]GTPγS binding assay. Thus, we can postulate that the 2-(5′-modified pentyl)pyrazole derivatives might be partial agonists. However, the problems encountered with aqueous solubility and the potential for membrane perturbations at high concentrations temper this interpretation.

The data presented here support the hypothesis that the 3-modified phenylpyrazole antagonists can bind to the CB_1 receptor within the same binding pocket as the agonist ligands. Both the 3-azidophenylpyrazole and 3-isothiocyanatophenylpyrazole derivatives were able to form an irreversible interaction with the receptor that precluded subsequent [^{3}H]CP55940 binding. This would be expected if an appropriate amino acid exists within the binding pocket for covalent bonding, and the presence of the aryl pyrazole anchored in that position obstructs the ability of the cannabinoid ligand to form a high-affinity interaction. One could infer that the binding site for the aryl pyrazole analogues before covalent coupling must also overlap that of [^{3}H]CP55940 at least in part. This evidence for overlap does not necessarily demand that the same amino acids that interact with the pharmacophoric elements of a cannabinoid agonist are also those required for the binding of the aryl pyrazole. Structure–activity relationship studies for the aryl pyrazoles will be necessary to define pharmacophoric elements for this class of CB_1 receptor binding ligands before prediction of common binding sites can be attempted.

The present study is the first evidence showing that the decrement in the number of receptors produced by the irreversible bonding of an antagonist ligand results in the requirement for stimulation of a greater fraction of the remaining receptors to produce the same signal transduction response. This is consistent with studies from Childer's laboratory, which suggest that cannabinoid receptors in the brain are not as efficient at G protein activation as opioid receptors (Sim et al., 1996*b*). The shift in the log concentration–response curve for G protein activation observed for the 3-isothiocyanatophenylpyrazole-treated receptor preparations is consistent with the premise that the loss of a population of receptors can be compensated for by increasing the concentration of the agonist. These data are consistent with the existence of "spare" receptors. The concept of spare receptors presupposes that an excess of receptors is eligible for high-efficiency signal transduction such that depletion of the fraction of the receptors that are considered "spare" will not make a significant difference in the maximal response (Kenakin, 1997). However, depletion of the spare CB_1 receptors in the rat brain membranes decreases the "efficacy" of the stimulation in the receptor-depleted membranes such that a greater percentage of the receptors must be stimulated by agonists to evoke the same degree of activation of G proteins.

Although treatment with the covalently binding antagonists could not deplete a sufficient number of CB_1 receptors to diminish the maximal response, it is evident that signal transduction was compromised by receptor depletion. This experimental depletion of receptors mimics the physiological loss of receptors resulting from down-regulation after chronic exposure to active cannabinoid compounds (Oviedo et al., 1993; DeFonseca et al., 1994; Fan et al., 1996). Irreversibly binding antagonist compound 3 or 4, which shows decrements in signal transduction in vitro and does not require UV irradiation to initiate the bonding, may be a useful ligand in vivo to modulate CB_1 receptor capacity in experimental animal models of tolerance. CB_1 receptor down-regulation in the rat basal ganglia determined by [^{3}H]CP55940 autoradiography correlated with loss of cannabinoid hypoactivity (Oviedo et al., 1993). CB_1 receptor down-regulation in mouse cerebellum homogenates was correlated with a loss of potency of CP55940 to evoke hypoactivity, hypothermia, and catalepsy in mice (Fan et al., 1996). Protocols that result in behavioral tolerance also diminish signal transduction capability as determined by cannabinoid agonist-stimulated [^{35}S]GTPγS binding in rat brain slices (Sim et al., 1996*a*). The 3-isothiocyanatophenylpyrazole antagonists could be useful for quantitatively depleting CB_1 receptors and correlating receptor loss with both behavioral and signal transduction decrements.

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