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DETECTION OF CANNABINOID RECEPTORS BY PHOTOAFFINITY LABELLING

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A novel [^{125}I]-labelled photoaffinity ligand designed to detect cannabinoid binding sites has been used in mouse brain preparations and in cultured S49 mouse lymphoma cells. The ligand, 2-iodo-5'-azido-Δ^8-THC, shows a high affinity for sites in both brain (K_d = 5.60 pM) and whole cell (K_d = 9.38 pM) systems. Photolabelling studies with brain samples revealed the existence of four ligand-protein adducts, of estimated molecular weights 85.5, 62.1, 30.0 and 25.5 kDa, that were diminished by prior exposure to 8 μM THC. A similar study with S49 cells gave adducts with apparent molecular weights of 62.1, 34.4, 16.9 and 13.5 kDa. The ligand produces a typical cannabinoid cataleptic response in mice suggesting that possibly one or more of the binding sites may be involved in some of the receptor mediated actions of THC.

A long sought after goal in the cannabinoid field has been the identification of "the cannabinoid receptor". The last few years have seen much progress towards the attainment of this objective. In an important development, Devane et al. (1) showed the existence of a high affinity binding site in a rat brain membrane preparation that exhibited ligand specificity related to the biological activities of the natural cannabinoids. The ligand used in their study, CP-55940, is a synthetic structural analog of the naturally occurring (-) Δ^9-tetrahydrocannabinol (THC) and shares several of the in vivo activities of THC (2). Using the same ligand, Herkenham et al. (3) were able to map the abundance of binding sites for CP-55940 in various brain regions in rat, rhesus monkey and in man. They concluded that basal ganglia, hippocampus and cerebellum were the regions most densely populated by those sites. Neither of these reports provided any structural information on the putative receptor.

A different approach to the quest for the THC receptor has more recently been reported by Matsuda, et al. (4). A rat cerebral cortex cDNA library was screened using an oligonucleotide probe derived from the bovine substance-K receptor. A novel peptide molecule was obtained by

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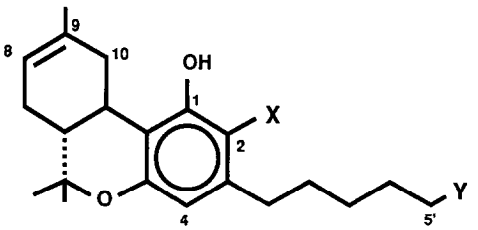

	X	Y
(-) Δ^8 - THC	H	H
(-) 5' - Azido - Δ^8 - THC	H	N_3
(-) 2 - Iodo - 5' - Azido - Δ^8 - THC	I	N_3

Fig. 1. The structure of the ligand (X = I; Y = N_3).

this technique which appeared to be a member of the G-protein coupled receptor family. This new molecule imparted activity to THC-unresponsive CHO cells, similar to that reported by Devane et al. (1) for CP-55940, leading Matsuda et al. to the conclusion that they had cloned cDNA encoding a cannabinoid receptor.

We have adopted yet another approach to the problem of identifying cannabinoid receptors. A derivative of (-) Δ^8-THC (Fig. 1, X = H, Y = H) was synthesized in which a photolabile azido group was introduced at the end of the side chain and an iodine atom was attached ortho to the phenolic group (A. Makriyannis, manuscript in preparation). In this report, we present our initial findings using this ligand as a tool for detecting the presence of cellular components which have a high affinity for THC.

METHODS

Preparation of the ligand

5'-Azido-Δ^8-THC (Fig. 1, X = H, Y = N_3) was prepared from the corresponding 5'-bromo derivative by displacement with azide. Iodine was introduced by treating the azido derivative with potassium iodide in the presence of m-chloroperbenzoic acid to give the ligand (Fig. 1, X = I, Y = N_3). For radiolabelled ligand, a sample of (-) 5'-azido-Δ^8-THC (250 µg) was dissolved in 200 µl of 95% ethanol. To this was added 200 µCi of Na[^{125}I] (specific activity 2200 Ci/mmole) in 25 µl 0.02 M tris-HCl (pH 7.4). The reaction was initiated by the addition of one "Iodo-bead" (Pierce Chemical, Rockford, IL) which is a derivatized form of N-chloro-benzenesulfonamide (oxidative capacity : 0.55 µmole/bead). After 5 min at room temperature, the reaction was terminated by removal of the bead. The ligand, (-) 2-[^{125}I] 5'-azido-Δ^8-THC, was isolated by silica gel thin layer chromatography (20% diethyl ether in petroleum ether; R_f = 0.5). Purity was confirmed by C-18 reverse phase hplc which gave one major peak of radioactivity coincident with unlabelled standard. The calculated specific activity is 2.2µCi/pmol (4.8 x 10^6 dpm/pmol).

Binding to S49 cells

Polypropylene tubes containing 1 x 10^6 cells in one ml of Dulbecco's modified Eagle's medium were incubated with 58,900 dpm (12.3 fmoles) of ^{125}I-ligand for times ranging from 5 to 180 min at room temperature. The cell suspension was sampled (0.25 ml) for the measurement of bound plus free ligand. The remaining suspension was then centrifuged at 3000 g for 10 min and the supernatant sampled (0.25 ml) for the measurement of free ligand. Bound ligand was then calculated as the difference of the two measurements. This procedure eliminates any possibility for

ligand-receptor dissociation during the analysis. Nonspecific binding was measured by prior exposure (5 min) of the cells to THC (8 μM).

Binding to mouse brain preparation

Samples (15 μg protein) of the preparation in buffer (1.0 ml 0.05 M tris-HCl, pH 7.4; 5 mM $MgCl_2$; 1 mM EDTA) were incubated with either the vehicle (10 μl ethanol) or THC (8 μM) for 5 min at room temperature in polypropylene tubes. Radiolabelled ligand in ethanol (10 μl) was then added to all of the samples and the incubation continued for 30 min at room temperature with continuous gentle shaking. After sampling the suspension in duplicate (0.15 ml) the particulate fraction was sedimented by centrifugation at 12,500 g for 20 min. Duplicate aliquots (0.15 ml) of the supernatant were removed for measurement of the free ligand, the bound ligand obtained by subtraction as described for the S49 cells.

Photolabelling procedure

Samples of the brain fraction (30 μg) were equilibrated with ^{125}I-labelled ligand as above with appropriate adjustments in volume. The pellet was washed once with buffer, suspended in 100 μl of buffer and exposed to short wavelength ultraviolet light (9 W) at a distance of 7.5 cm for 35 min at room temperature with periodic agitation. The samples were prepared for PAGE by the addition of 0.1 ml SDS (6.25%) in 0.5 M tris-HCl and heated for 30 min at 50-55°C with occasional mixing after which 0.01 ml DTT (6.5%) in 50% glycerol was added and the mixture heated at 100°C for 5 min. The analysis was done on a 15% slab gel prepared in a manner similar to that of Laemmli (8).

RESULTS

A time course study using S49 cells, shown in Figure 2, indicates that maximum binding is reached in about 30 minutes. Figure 3A shows the binding curves resulting from equilibration of the ligand with a mouse cerebral cortex homogenate fraction. The "total binding" curve was obtained by a direct nonlinear regression fit of the binding data to the Michaelis-Menten expression plus a linear term describing "non-specific" binding. By this method, values for maximum specific binding (B_{max} = 1.32 pmol/mg) and the dissociation constant for specific binding (K_d = 5.60 pM) could be obtained. The curve for binding in the presence of 8 μM THC, that is "non-specific binding", is also shown in Figure 3A. The data were also examined by means of a Scatchard analysis which gave values for K_d of 4.56 pM and B_{max} of 0.85 pmol/mg (r = 0.976). The slope of the best fit line was -0.219 ± 0.02 (confidence limits -0.27 to -0.17). A similar study using

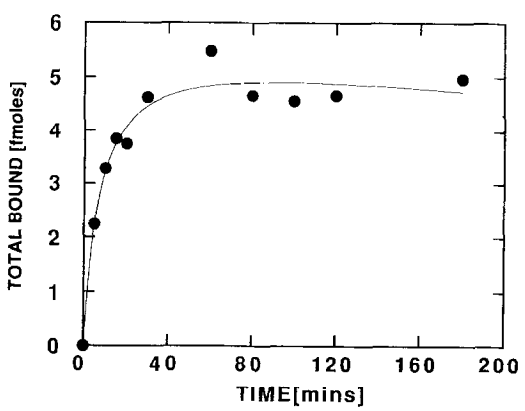

Fig. 2. Time course for binding of ligand to S49 cells. Values shown are the average of two determinations.

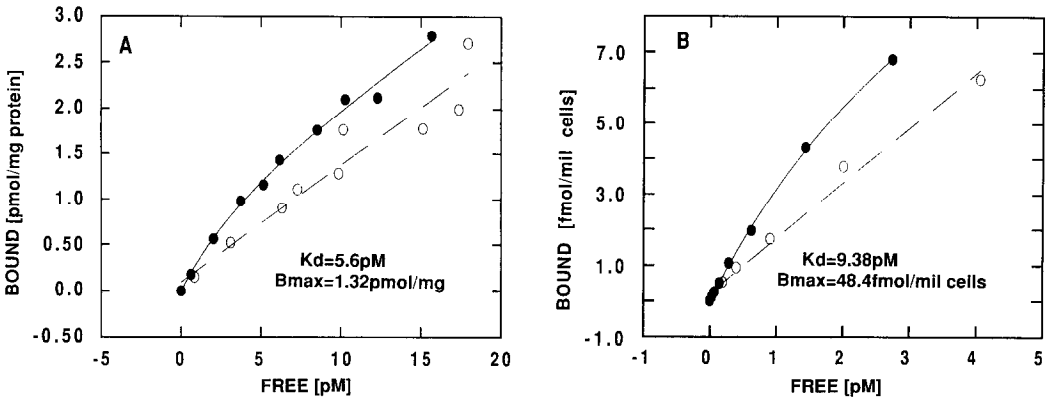

Fig. 3. Equilibrium binding studies. (A) Mouse cerebral cortex 12,000 g pellet. (B) S49 mouse lymphoma cells. Open circles, vehicle pretreated; closed circles, THC (8μM) pretreated.

intact S49 cells was carried out and the results are given in Figure 3B. A comparison of the S49 dissociation constant (K_d = 9.38 pM) with that obtained for cerebral cortex shows that the values are close to each other. These results indicated that this THC derivative binds tightly to sites in both systems and that it would be suitable for further evaluation as a photolabelling ligand.

For photoaffinity labelling, the ligand was equilibrated with samples of the brain preparation for 35 minutes followed by exposure to ultraviolet light. The samples were then analyzed by SDS-PAGE and the radiolabelled molecules detected by autoradiography. The results of a typical experiment, shown in Figure 4, indicate that several protein components have formed a

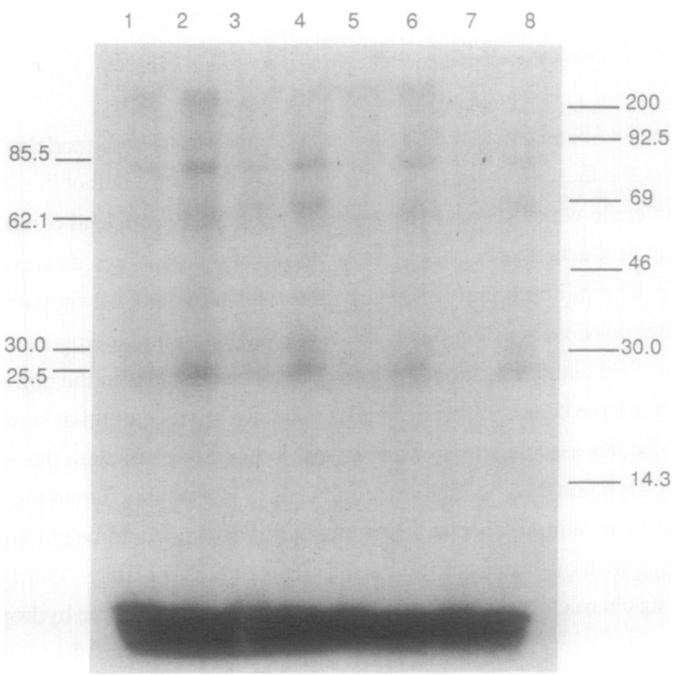

Fig. 4. Photoaffinity labelling experiment. Lanes 1,3,5 and 7 done in the presence of 8μM THC; lanes 2,4,6 and 8 were vehicle treated. Values shown are in kDa.

TABLE 1
DECREASE IN PHOTOAFFINITY LABELLING OF CANNABINOID BINDING SITES BY PRIOR INCUBATION OF BRAIN PREPARATION WITH 8 μM THC†

Band M.W. (kDa)	Band intensity (densitometer units ± S.D.)	
	Control (vehicle)	THC treated (8μM)
85.5	64,820 ± 11,440	36,380 ± 11,800*
62.1	76,150 ± 21,560	30,900 ± 6130*
30.0	63,330 ± 10,420	30,070 ± 6870*
25.5	79,500 ± 20,660	27,990 ± 3890*

†Samples of the 12,000 g cerebral cortex pellet were treated exactly as in Fig 4. The samples were analyzed by SDS-PAGE followed by autoradiography. The radiolabelled bands were quantitated by laser scanning densitometry and the values represent the means of four replicate determinations.

*$P < 0.05$, t test compared with control.

covalent bond with the ligand. Four of these proteins exhibited reduced binding when the samples were exposed to 8 μM THC prior to equilibration with ligand (Table 1). The estimated molecular weights of the bands were 25.5, 30.0, 62.1 and 85.5 kDa. Incubation times up to 90 minutes gave essentially the same pattern of radiolabelling. A control experiment done without ultraviolet light exposure resulted in no detectable photolabelling.

The equilibration of S49 cells with the ligand followed by photoactivation of the receptor-ligand complex also resulted in bond formation between the two. This was evidenced by SDS-PAGE analysis of the 10,000 g pellet obtained from a homogenate of labelled cells. At least four ligand-protein adducts with molecular weights of 13.5, 16.9, 34.4 and 62.1 kDa were observed (data not shown).

DISCUSSION

The multiplicity of bands obtained by SDS-PAGE analysis of both models raises certain questions. It is possible that the lower molecular weight proteins are in fact breakdown products of the 62.1 kDa molecule which seems to be common to both S49 cells and cerebral cortex. These could be proteolytic products formed during the experimental procedures, however, the observation that longer equilibration times gave a similar pattern does not support the occurrence of proteolysis. Another possibility is that there are several cannabinoid binding sites each with its own functional role. This latter situation has many precedents such as in the opioid field where multiple binding sites have been reported. Finally, it cannot be ruled out that some of the bands may arise from molecules associated with or proximal to the molecules with the binding sites.

The relatively high nonspecific binding seen in Fig. 3 is probably due to the very lipophyllic nature of the ligand. The naturally occurring cannabinoids are known to be among the most hydrophobic of drugs with oil-water partition coefficients of 8000 or more. Our ligand closely resembles THC in its chemical structure and, therefore, shares this extreme hydrophobicity. This property may explain some of the unique pharmacology of THC and attempts to use ligands that are less hydrophobic may not uncover all of the receptors involved in the spectrum of actions produced by THC.

Directly related to the functional significance of the binding sites are the pharmacological activities of the ligand. One of the typical cannabinoid effects in rodents is the induction of a cataleptic state that can be measured with good accuracy in the laboratory (5). Recently, we reported data that showed a second messenger role for eicosanoids in this THC-induced effect which may involve peripheral as well as central sites (6). When tested in the same mouse strain that we used as a source of the brain preparation, the ligand produced a cataleptic response comparable to that of THC. The cataleptic effect of the ligand was measured in female CD-1 mice (25g body weight) as described in Ref. 5. The cannabinoid was administered orally in peanut oil (0.5 mg in 0.05 ml/mouse) N = 9. Immobility was measured after one hour and a value of 30 $\pm$ 13% was obtained. An equimolar dose of THC gave a value of 34 $\pm$ 8% while vehicle treated mice showed a 7 $\pm$ 2% response. In addition, the ligand shows considerable antinociceptive activity in standard animal models for this type of response (A. Makriyannis, manuscript in preparation). Finally, the ligand used here is able to displace [^{3}H]-CP-55940 from its binding site in a rat brain preparation (7). CP-55940 is the ligand used by all three groups mentioned above in their reports on cannabinoid receptors (1,3,4).

Whether our 62.1 kDa receptor candidate is the same as the gene product reported by Matsuda et al. (4) is not known at this time. The calculated molecular mass of their 473 amino acid sequence gives a value of 52.823 kDa for the unprocessed peptide. Given the inaccuracies of molecular weight measurements by PAGE, and the increased mass produced by potential glycosylation and lipidation, our protein is in the right range to be the receptor reported by them. (A preliminary study in our laboratory with a mixture of endoglycosidase F and N- glycosidase F suggested that the 62.1 kDa band, but not the 85.5 kDa band, was glycosylated.) The isolation and characterization of the 62.1 kDa protein will provide an answer to this question. The approach we have described in this report, utilizing a photoaffinity labelling ligand, should facilitate further research into the nature of cannabinoid receptors. In particular, our findings provide evidence that there may be more than one cannabinoid receptor especially in tissues with heterogeneous cell populations such as the mouse cerebral cortex preparation described here.

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