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Benzodiazepines have high-affinity binding sites and induce melanogenesis in B16/C3 melanoma cells

(diazepam/radioreceptor assay/fluorescence microscopy)

ELIZABETH MATTHEW, JEFFREY D. LASKIN, EARL A. ZIMMERMAN, I. BERNARD WEINSTEIN, KONRAD C. HSU, AND DEAN L. ENGELHARDT

College of Physicians and Surgeons, Columbia University, New York, New York

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ABSTRACT We found that two markers of differentiation, tyrosinase (monophenol, dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) activity and melanin synthesis, are induced by diazepam in B16/C3 mouse melanoma cells. We also demonstrated high-affinity binding sites for [^{3}H]diazepam in these cells by radioreceptor assay, and we visualized binding to the cell surface by fluorescence microscopy with a benzodiazepine analog conjugated to a fluorescein-labeled protein. Our studies also showed that there are differences between the binding characteristics in intact cells and in membrane fractions prepared from these cells. Scatchard analysis of the binding data from membrane fractions gave a linear plot ($K_d = 9.1 \times 10^{-8}$ M). With intact cells, a curvilinear Scatchard plot was obtained. This was resolved into two components defining binding sites with affinity constants of 1.7×10^{-9} M and 4.6×10^{-7} M. Thus, it appears that [^{3}H]diazepam binding in intact cells is more complex than in isolated membranes. Several related benzodiazepines, including flunitrazepam, Ro-5-4864, nitrazepam, oxazepam, lorazepam, Ro-5-3072, chlordiazepoxide, and clonazepam also induced melanogenesis. When these compounds were tested for their ability to inhibit [^{3}H]diazepam binding, flunitrazepam, diazepam, and Ro-5-4864 were found to be the most effective inhibitors. These three compounds were also the most potent in inducing melanogenesis. Our results suggest that the benzodiazepines modulate cell differentiation. The presence of high-affinity binding sites in this homogeneous, easily grown cell line may provide a useful model for studies on the mechanism of action of these compounds.

The benzodiazepine group of drugs are in widespread use today as tranquilizers, hypnotic sedatives, anticonvulsants, and muscle relaxants (1). Current evidence suggests that these compounds act by binding to high-affinity receptors in the nervous system (2, 3). Correlations between the affinities of the various benzodiazepine analogs for binding sites in membrane preparations from brain tissue and the dose of each analog required to produce anticonvulsant effects support this hypothesis (4, 5). Binding sites also have been identified by radioreceptor assay in membrane preparations from a variety of tissues (6–11), including several transformed cell lines (12). However, characterization of pharmacological responses associated with these binding sites has been hampered by the lack of suitable bioassay systems. The present report describes an *in vitro* experimental system in which benzodiazepine compounds produce a clear-cut biological effect. These compounds accelerate the onset of melanogenesis in B16/C3 mouse melanoma cells. These cells were selected for study because we observed that a fluorescein-labeled probe incorporating Ro-5-3072, a benzodiazepine analog, bound to the surface of these cells. We also studied the binding of radiolabeled diazepam to these cells. Our findings suggest that these cells may be a useful biological model for studying this important group of drugs.

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MATERIALS AND METHODS

Maintenance of Cells and Studies of Melanogenesis. B16/C3 mouse melanoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 0.059% sodium bicarbonate (DME medium F) and 10% (vol/vol) fetal calf serum (13). Cells were maintained at 37°C in an incubator with humid 5% CO_2/95% air. For studies on melanogenesis, cells were plated (2×10^4 cells per cm^2) in 35-mm plastic Petri dishes. After 4 hr, the medium was substituted with fresh medium containing diazepam or its analogs. Stock solutions of the benzodiazepines were 25 mM in dimethyl sulfoxide (Me_2SO). Dilutions of these stock solutions in medium resulted in final Me_2SO concentrations of 0.003% or less. In control experiments, cells were grown in medium containing these concentrations of Me_2SO. Cell counts and melanin determinations were performed daily as described (13). Tyrosinase was measured as described by Pomerantz (14) with [^{3}H]tyrosine (specific activity, 40 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) (Amersham). For this assay, cells grown in the absence or presence of diazepam (0.8–30 μM) for 5 days were treated with [^{3}H]tyrosine. Aliquots (0.5 ml) of the medium were removed 24 hr later and assayed for release of tritiated water.

Studies of [^{3}H]Diazepam Binding. [*N-methyl*-^{3}H]Diazepam (83.5 Ci/mmol) was obtained from New England Nuclear. Diazepam, flunitrazepam, nitrazepam, clonazepam, chlordiazepoxide, Ro-5-3072, Ro-5-4864, oxazepam, and lorazepam were donated by Hoffman-La Roche and Wyeth. Stock solutions of ligands (25 mM in Me_2SO) were diluted in binding buffer (DME medium F containing 50 mM 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid, pH 6.8).

The binding assay was performed at 4°C. The specific binding of [^{3}H]diazepam was maximal at 30 min, indicating that equilibrium had been reached between the ligand and the binding sites. As the first step in the assay, confluent cells (2×10^5 cells per cm^2 in 60-mm dishes) were washed three times with binding buffer. The cells were then incubated with 2 ml of binding buffer containing 1.19 nM [^{3}H]diazepam (83.5 Ci/mmol). Nonspecific binding was determined by incubating cells with binding buffer containing the radioligand and excess unlabeled diazepam (100 μM). After 30 min, the reaction was terminated by aspirating the labeled binding buffer off the cells and washing them four times with ice-cold phosphate-buffered saline. The cells then were solubilized with 1.5 ml of lysing buffer (0.01 M Tris·HCl,

Abbreviations: Me_2SO, dimethyl sulfoxide; DME medium F, Dulbecco's modified Eagle's medium supplemented with 0.059% sodium bicarbonate; IC_{50}, concentration that inhibits maximal response by 50%.

pH 7.4/0.1% Na_2DodSO_4/0.01 M EDTA) and scraped off the plates into 15 ml of aqueous scintillation liquid (National Diagnostics, Parsipanny, NJ) for counting. Specific binding was obtained by subtracting nonspecifically bound material from the total. To determine saturability, the assay was performed with binding buffer containing [^{3}H]diazepam (1.19 nM; 83.5 Ci/mmol) and increasing amounts of unlabeled diazepam (0.3–100 μM).

To study dissociation kinetics, the binding was carried out as described. The cells were then washed and placed in 2 ml of [^{3}H]diazepam-free binding buffer with and without an excess of unlabeled diazepam (100 μM). At serial intervals over the subsequent 60-min period, samples were taken and prepared for counting.

Binding assays were performed on synaptosomal fractions from brain tissue of C_{57} BL mice by using methods as described (15, 16) and on membrane preparations from melanoma cells. The membranes were prepared from melanoma cultures grown under the conditions described above. Culture dishes (10^7 cells per 150-mm plate) were washed three times in 5 ml of binding buffer. Cells from 10 dishes were then scraped into 20 ml of binding buffer and disrupted for 15 sec in a Polytron tissue homogenizer. Subsequently, the homogenate was centrifuged at 1000 × *g* for 10 min at 4°C. The supernatant was recentrifuged at 30,000 × *g* for 15 min at 4°C, and the resulting pellet was suspended in binding buffer to give a final concentration of approximately 0.1 mg of protein per ml. Two ml of this membrane fraction was added to 2 ml of binding buffer containing [^{3}H]diazepam and increasing concentrations of unlabeled diazepam. The concentrations of the ligands used were the same as those described for the assay with whole cells. The reaction was terminated by vacuum filtration through Whatman GF/B filters. The filters were then washed three times with 5 ml of Tris·HCl buffer and assayed for radioactivity. Saturable binding did not occur on the plastic tissue culture dishes without the cells or on the glass-fiber filters alone.

Histochemical Studies. Rabbit antibody to sea whelk (*Busycon canniculatum*) hemocyanin, prepared as described by Gonda *et al.* (17), was labeled with fluorescein isothiocyanate. This fluorescein-labeled antibody was conjugated to a benzodiazepine analog, Ro-5-3072. To effect this conjugation, the amino group in the C3 position of the drug was diazotized and linked with tyrosine residues in the anti-hemocyanin antibody (18). Antibody to hemocyanin was used as the protein for conjugation with future ultrastructural studies in mind (17).

Cells grown in culture chambers on glass slides (Lab Tek) were incubated unfixed with this conjugate at dilutions of 1:2.5 and 1:5 in DME medium F for 30–90 min. As a control for specificity, cells were incubated with a mixture of the conjugate, and an excess of unconjugated Ro-5-3072 (100 μM) or diazepam (100 μM). Other controls were provided by incubating the cells with fluorescein-labeled rabbit anti-hemocyanin antibody not conjugated to Ro-5-3072. Incubations were performed at 37°C in order to study the lateral motion, aggregation, and internalization of the ligand receptor complexes (19). After incubation, the glass slides were washed rapidly with DME medium F and examined by fluorescence microscopy.

RESULTS

Melanogenic Effects of Benzodiazepines. The mouse melanoma cell line B16/C3 is amelanotic when grown in culture. However, it will undergo spontaneous melanogenesis after entry into the stationary phase (13, 20). The effect of diazepam on melanin production in these cells was studied on 8 successive days after plating. Individual plates were assayed for melanin content in both the cells and culture medium. In control cultures grown in DME medium F, alone or in the presence of 0.003% or 0.001% Me_2SO, melanogenesis occurred on day 6 after plating (Fig. 1). Melanogenesis occurred 24 hr earlier in cells treated with 10 μM diazepam and 48 hr earlier in cells treated with 30 μM diazepam (Fig. 1). These concentrations did not affect the growth rate of the cells or their final saturation density (Fig. 1 *Inset*). One μM or 3 μM diazepam had no effect on the time of onset of melanogenesis (data not shown). To further define the melanogenic effects of diazepam, we studied another marker of differentiation, tyrosinase activity. When the cells are in stationary phase, just prior to the onset of melanogenesis, there is an increase in the activity of tyrosinase, the rate-limiting enzyme for melanin synthesis (21, 22). We have determined the effect of diazepam on tyrosinase activity in these cells (14). Diazepam affected the oxidation of [^{3}H]tyrosine in a dose-dependent manner in the concentration range of 0.8–30 μM (Table 1); maximal effect was seen at 30 μM.

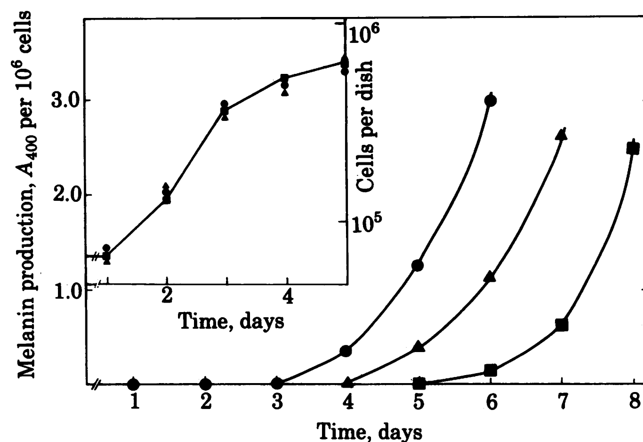

FIG. 1. Effect of 10 μM (▲) and 30 μM (●) diazepam on growth and melanogenesis in B16/C3 melanoma cells. ■, Control. Cells were grown in 35-mm plastic Petri dishes. Daily cell counts and determinations of melanin produced by the cells (from its absorbance at 400 nm) were made. (*Inset*) Effect of diazepam on the growth of the cells.

Analogs of diazepam also were found to accelerate the onset of melanogenesis (Table 2). Under the conditions of this experiment, flunitrazepam and Ro-5-4864 gave results similar to those of diazepam. However, the remaining analogs—chlordiazepoxide, clonazepam, nitrazepam, Ro-5-3072, oxazepam, and lorazepam—required three-fold higher concentrations and an additional 24 hr to induce melanogenesis (Table 2).

Characterization of [^{3}H]Diazepam Binding Sites. Specific binding constituted 90–95% of the total [^{3}H]diazepam bound

Table 1. Effect of diazepam on tyrosinase activity in B16/C3 melanoma cells

Diazepam content in medium, μM	^3H_2O released, cpm/0.5 ml of medium
Control	163
Control (0.003% Me_2SO)	148
0.8	625
1.0	1258
3.0	1558
10.0	3597
30.0	6233

B16/C3 cells (2 × 10^5) were plated in 60-mm Petri dishes on 5 ml of medium with and without diazepam at the concentrations indicated in the table. [^{3}H]tyrosine (0.25 μCi per dish) was added 120 hr after plating. Aliquots (0.5 ml) of the medium were removed 24 hr later and assayed for the extent of tyrosine oxidation. Assays were performed in triplicate, and the data are mean values (SD ± 10%).

Table 2. Effect of benzodiazepines on melanogenesis and [^{3}H]diazepam binding

Benzodiazepine	Melanin content* at 10 μM drug	Melanin content* at 30 μM drug	IC$_{50}$,† μM
Diazepam	0.322	1.21	0.016 ± 0.002
Flunitrazepam	0.481	1.81	0.012 ± 0.003
Ro-5-4864	0.372	1.53	0.006 ± 0.001
Chlordiazepoxide	0.002	0.221	0.980 ± 0.180
Clonazepam	0.015	0.207	1.5 ± 0.4
Nitrazepam	0.007	0.228	1.7 ± 0.1
Ro-5-3072	0.005	0.314	1.9 ± 0.4
Oxazepam	0.010	0.281	1.8 ± 0.1
Lorazepam	0.003	0.307	1.7 ± 0.2

* The melanin content of B16/C3 cells and the culture media was determined on day 6 after drug treatment at 10 and 30 μM by measuring A_{400} compared with that of standard tissue culture medium. The data are presented as the amount of melanin produced per 10^6 cells.

† Mean ± SD from three determinations. IC$_{50}$ is the concentration of the drug required to produce 50% inhibition of [^{3}H]diazepam binding.

to these cells. Although significant displacement of [^{3}H]diazepam was seen with low concentrations of unlabeled diazepam (1–10 μM), specific binding was saturable only at higher concentrations (100 μM). The specific binding increased in proportion to the number of cells per plate. After a 30-min incubation, binding at 4°C was 3 times greater than that obtained at 37°C. Therefore, all of the binding experiments presented in this paper were performed at 4°C.

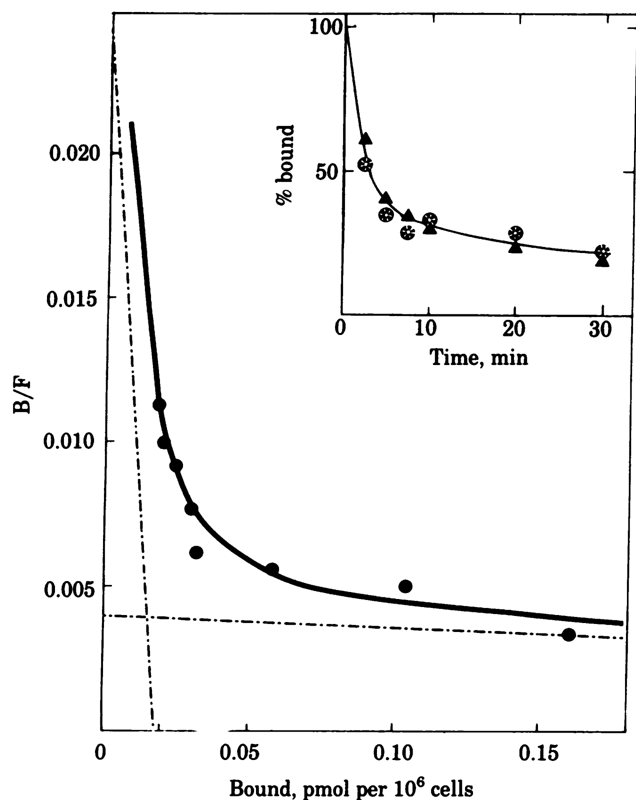

FIG. 2. Scatchard plot of [^{3}H]diazepam binding to B16/C3 cells. Bound (B), pmol of [^{3}H]diazepam bound per 10^6 cells; free (F), pmol of [^{3}H]diazepam bound per ml of binding buffer. The two linear components represent two putative binding sites. (*Inset*) Time course of dissociation of bound [^{3}H]diazepam from B16/C3 cells. The cells were incubated with [^{3}H]diazepam for 30 min, washed, and then incubated in binding buffer with (▲) and without (⊛) 100 μM unlabeled diazepam. At each time point, the amount of [^{3}H]diazepam remaining bound to the cells was calculated as a percentage of the specific binding at zero time.

Scatchard analysis produced a curvilinear plot (Fig. 2) (23, 24). Dissociation curves showed that the rate of dissociation was the same in the presence or absence of saturating amounts of diazepam (Fig. 2 *Inset*), which indicates that the curvilinear plot is not the result of negative cooperativity (25). The dissociation curves were biphasic and suggested the presence of at least two types of binding sites with half-times of dissociation estimated to be 2 min and 40 min. The curvilinear Scatchard plot was then resolved into two linear components, each characterizing a putative binding site (26–28). The dissociation constant (K_d) of the high-affinity binding site was $1.7 \pm 0.7 \times 10^{-9}$ M and the number of binding sites per cell was $1.4 \pm 0.4 \times 10^4$. For the binding site with the lower affinity, the dissociation constant was $460 \pm 90 \times 10^{-9}$ M and the number of binding sites per cell was $6.9 \pm 0.2 \times 10^5$. Each of these values represents the mean and SEM of four experiments.

The heterogeneity of the binding sites could be due to the presence of more than one cell type in the population of cultured B16 cells. To test this, we isolated cloned populations of B16/C3 cells by two successive clonal isolations. Studies on [^{3}H]diazepam binding in these subclones showed curvilinear plots identical to that in Fig. 2.

As an index of the relative affinities of the various analogs for binding sites in B16/C3 cells, we compared their IC$_{50}$ values (concentration values that inhibit maximal response by 50%) to their relative potencies as inducers of melanogenesis (Table 2). Flunitrazepam, diazepam, and Ro-5-4864 had IC$_{50}$ values in the range of 6–15 nM and showed a distinct enhancement of melanogenesis at concentrations of 10 μM. The remaining analogs had IC$_{50}$ values of about 1–2 μM and required concentrations of at least 30 μM and an additional 24 hr to produce an enhancement of melanogenesis.

[^{3}H]Diazepam binding in membrane fractions. Studies of [^{3}H]diazepam binding in membrane fractions from a variety of tissues have shown linear Scatchard plots (6–12). In view of the curvilinear Scatchard plot that we obtained with intact cells, we investigated the binding of [^{3}H]diazepam to membrane fractions from B16/C3 cells. We found that the specific binding of [^{3}H]diazepam to B16/C3 cell membrane fractions was saturable

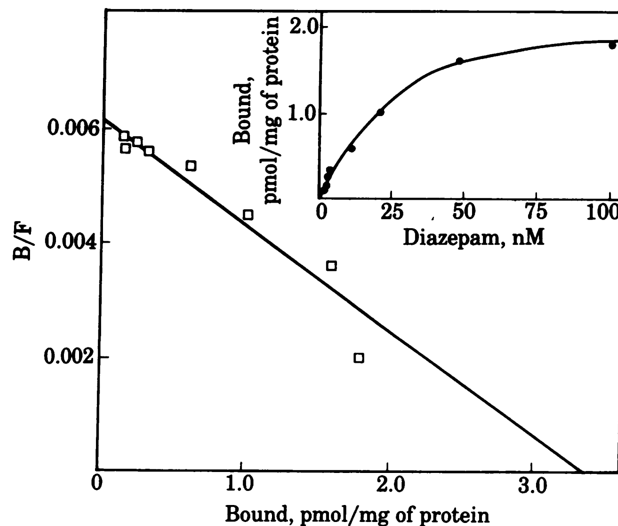

FIG. 3. Scatchard plot of [^{3}H]diazepam binding to B16/C3 cell membrane fractions. Bound (B), pmol of [^{3}H]diazepam bound per mg of protein; free (F), pmol of [^{3}H]diazepam per ml of binding buffer. (*Inset*) Saturability of specific [^{3}H]diazepam binding.

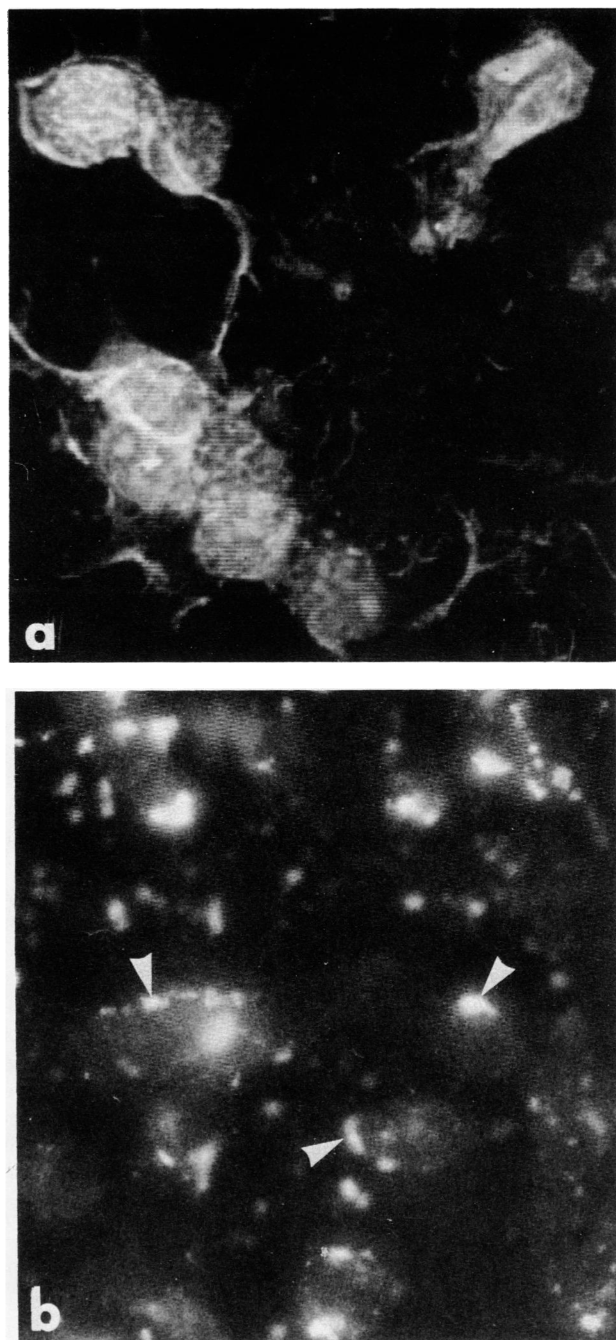

FIG. 4. Photomicrograph of fluorescein-labeled protein conjugate of the benzodiazepine analog Ro-5-3072 bound to B16/C3 cells. (*a*) Cells were incubated with the conjugate for 30 min. Localization of the fluorescent material can be seen on both the cell bodies and processes. Dilution of the label, 1:2.5. (*b*) Sibling cultures, incubated with the conjugate for 60 min. Arrow heads point to aggregates of fluorescent material on the cells ("patching"). (×600.)

(Fig. 3 *Inset*) and, in contrast to the binding data in whole cells, gave a linear Scatchard plot (Fig. 3). The dissociation constant was estimated at $91 \pm 9 \times 10^{-9}$ M with 5.4 ± 0.4 pmol of diazepam bound per mg of protein (three determinations). This is similar to data reported from membrane fractions in neuroblastoma cells (12). The differences in [^{3}H]diazepam binding between whole cells and membrane preparations suggest that the process of preparing membrane fractions disrupts aspects of membrane structure that influence binding in the intact cell.

We also carried out studies of [^{3}H]diazepam binding in homogenates of mouse brain. Our data showed a single population of saturable binding sites with a dissociation constant of 4×10^{-9} M, which is in accordance with other reports with rat brain homogenates (2, 3, 15, 16).

***In Situ* Localization of Benzodiazepine Binding Sites.** To determine the cellular localization of the benzodiazepine sites, cells were incubated with the fluorescein-labeled probe. A diffuse cell-surface fluorescence was detected within 30 min of addition of the probe (Fig. 4). No fluorescence was observed when the cells were incubated with fluorescein-treated rabbit anti-hemocyanin antibody not conjugated to Ro-5-3072 (not shown). Furthermore, an excess of unlabeled diazepam or Ro-5-3072, when added together with the conjugate, blocked cell-surface fluorescence. With more prolonged incubations (60 min) with the conjugate, aggregations of fluorescent material were observed on the cell surface. This was followed by perinuclear fluorescence (90 min), which suggested internalization of the ligand–receptor complexes. Ro-5-3072 had to be used in these studies because of a free amino group (29), which was essential for the linkage to the fluorescein-labeled probe. Though it is one of the weaker inhibitors of [^{3}H]diazepam binding, it is not "inert" because it also induces melanogenesis. In addition, the fluorescence was blocked by excess diazepam, providing evidence that the binding is specific.

DISCUSSION

These data show that benzodiazepine compounds enhance melanogenesis in B16/C3 melanoma cells and that these cells contain high-affinity binding sites for diazepam and related compounds. The compounds with high affinities for diazepam binding sites were also the most potent inducers of melanogenesis. The doses of benzodiazepine required to induce melanogenesis were in the 10 μM range and the IC_{50}s were in the range of 1 μM to 1 nM (Table 2). We cannot be certain that the high-affinity binding sites mediate the effects on melanogenesis because our data (Table 2) do not show a strict quantitative relationship between these two parameters. The lack of a clear correlation may be due to the fact that the binding assays were done at 4°C after a 30-min incubation, whereas melanogenesis was studied at 37°C after several days, during which there is a significant increase in cell number. It is also possible that the drugs may have to be metabolized or internalized to affect melanogenesis. In this regard, nuclear binding sites for benzodiazepines have been demonstrated (30). In addition, the assay for melanogenesis is not quantitative because it measures the amount of melanin that accumulates in the cells and medium at an arbitrary time rather than at the actual rate of melanin synthesis. Assays for induction of tyrosinase (Table 1) by diazepam appear to be more sensitive than those for melanogenesis. These studies indicate that tyrosinase activity is induced by 800 nM diazepam. This is less than twice the weaker Scatchard constant. Therefore, it is possible that the lower-affinity binding site may be involved in the induction of melanogenesis.

When Scatchard analysis was performed on the binding data, a curvilinear plot was obtained. Such curvilinear plots have been described for the β-adrenergic receptor (31) and also for several peptide hormone receptors (25, 32, 33). Our measurements of dissociation kinetics, in the presence or absence of saturating amounts of diazepam, indicate that the curvilinear nature of the Scatchard plot is not due to negative cooperativity and suggest the presence of heterogeneous binding sites with differing affinities for diazepam. Multiple binding sites also have been postulated in homogenates of bovine retina (34) and rat brain (35). Siegert and Karobath (36) have demonstrated molecular heterogeneity of benzodiazepine receptors with a pho-

toaffinity label, thereby supporting the model that multiple populations of receptors exist.

The histochemical methods described in the present study represent a novel approach to the demonstration of binding sites. Autoradiographic techniques, developed for the study of the opiate receptor, have been used in whole brain sections from man and other mammals (37). However, this precludes the use of living tissue. The use of a benzodiazepine compound directly conjugated to a labeled marking system permits immediate and direct visualization of the binding sites.

Although the significance of benzodiazepine binding sites in B16/C3 cells is not clear, their presence in a transformed cell line is not without precedent. Benzodiazepine binding sites have been identified in a neuroblastoma cell line (12, 38). Both cell types share a common embryonic origin from the neural crest (39), and it may be that they retain some of the characteristics of neural crest cells. The B16/C3 cells, like the neuroblastoma, show a high affinity for the benzodiazepine compound Ro-5-4864 (6 nM) and a low affinity for clonazepam (1500 nM). These features are shared by binding sites in the kidney and are thought to be characteristic of the "peripheral-type" binding site (9). They differ from "central-type" binding sites in brain, which have a high affinity for clonazepam and a low affinity for Ro-5-4864. Recent studies (40) have shown that peripheral-type binding sites are involved in phospholipid methylation, a process which affects a variety of membrane functions, which suggests that these binding sites may be functionally significant. B16/C3 cells present numerous advantages for further studies on the mechanism of action of this class of durgs. These include the presence of high-affinity binding sites, a homogeneous and easily grown cell line, and a readily observed biological response.

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