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# BINDING OF [$^3$H]FLUNITRAZEPAM TO THE LM CELL, A TRANSFORMED MURINE FIBROBLAST

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**Abstract**—LM cells have a saturable, high affinity binding site for [$^3$H]flunitrazepam with a $K_D$ of 13 nM and a $B_{max}$ of 19 pmoles/mg protein. The $IC_{50}$ values for Ro 5-4864, flunitrazepam and clonazepam against [$^3$H]flunitrazepam were 6, 23 and 2800 nM, respectively, indicating that this receptor is of the peripheral type. A decrease of 37, 26 and 26% in $B_{max}$ was associated with substituting dimethylethanolamine, monomethylethanolamine or ethanolamine, respectively, for choline in the cell culture medium. These treatments did not change either the $K_D$ of [$^3$H]flunitrazepam binding or the $IC_{50}$ values of the different benzodiazepine drugs. Metastatic cell lines of the LM cell obtained from either athymic or $C_3H$/Hef mice exhibited alterations in the binding parameters of [$^3$H]flunitrazepam. There was a reduction in the $B_{max}$ values of the athymic (34%) and the $C_3H$/Hef (44%) cell lines compared to the LM cell. In both groups there was a 90% increase in the $K_D$. In the $C_6$ astrocytoma, the peripheral type receptor appears to regulate plasma membrane mediated synthesis of phosphatidylcholine from phosphatidylethanolamine. However, this was not observed in the LM cell. Nor did it modulate cyclic AMP metabolism as assessed by measurement of cyclic AMP levels in whole cells after drug treatment.

The benzodiazepines are widely prescribed as anxiolytic agents, sedatives, hypnotics, muscle relaxants and anticonvulsants. There are specific high affinity receptor sites for these drugs in the central nervous system [1–8]. The proposal that the benzodiazepines produce their effect by binding to these receptors is supported by a correlation between their affinities for the binding site and their clinical and experimental efficacy [1, 3, 6, 8]. Receptors for these compounds also have been reported in cell cultures of neural [9–14] and non-neural [2, 15–17] origin. However, the pharmacology of these binding sites is different from the central type [2, 11, 15–17]. Clonazepam is a more potent inhibitor of either [$^3$H]diazepam or [$^3$H]flunitrazepam binding compared to Ro 5-4864 at the central type receptor while the converse is observed at the peripheral type.

A necessary condition for defining a binding site as a receptor is the identification of a response associated with the binding. There is evidence that the peripheral type benzodiazepine binding site stimulates phospholipid methylation in the plasma membrane of $C_6$ astrocytoma cells [18]. The formation of phosphatidylcholine from phosphatidylethanolamine is catalyzed by a two enzyme system asymmetrically distributed between the two halves of the plasma membrane bilayer in several tissues [19, 20]. The enzyme for the first methylation is associated with the inner monolayer and has a high $K_m$ for $Mg^+$ and *S*-adenosylmethionine. The addition of the second and third methyl groups is mediated by an enzyme located in the outer half of the bilayer and has a low $K_m$ for $Mg^{2+}$ and *S*-adenosylmethionine. This methylation system can be regulated by the $\beta$-receptor in rat reticulocytes and $C_6$ astrocytoma cells, the concanavalin A receptor in rat mast cells, and the peripheral type benzodiazepine receptor in $C_6$ astrocytoma cells [18, 21, 22]. In these systems, the potencies of various analogues in stimulating the methylation response correlate with their binding affinities. Thus, the stimulation of phospholipid methylation by benzodiazepine binding might be an important mechanism for the transduction of information by these ligands across the plasma membrane in certain cells. The binding of benzodiazepines appears to stimulate melanogenesis in B16/C3 melanoma cells [17]. An increase in benzodiazepine binding sites in the kidney of the rat is associated with hypertension induced by deoxycorticosterone and salt administration [23, 24]. However, further studies are required to characterize the biological significance and regulation of this receptor.

In this paper, we report the presence and characteristics of a high affinity benzodiazepine binding site of the peripheral type in membranes of the LM cell. This cell is a transformed murine fibroblast which can be grown in a serum-free, lipid-free, chemically defined medium as either a monolayer or suspension cultures [25–27]. We investigated the effect of altering the composition of phospholipid polar head groups by dietary manipulation on benzodiazepine binding and studied possible biological functions of this receptor. In addition, the binding parameters of the receptor were examined in related primary tumors and metastases [28].

## MATERIALS AND METHODS

*Cell culture.* The LM cell, a tranformed murine fibroblast dependent on choline supplementation of

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the medium, was obtained from the American Type Culture Collection (CCL 1.2). The cells were cultured in suspension at $10^6$ cells/ml in a serum-free, chemically defined medium [25–27].

*Benzodiazepine binding assay.* The cells were harvested by centrifugation at 200 *g* for 10 min and washed with an equal volume of phosphate buffered saline (PBS) (pH 7.2). A crude particulate fraction was prepared by homogenizing 100–300 × $10^6$ cells in 20 ml of ice-cold 50 mM Tris buffer (pH 8.0 at 25°), with a Tekmar Tissumizer (setting of 90 for 30 sec) and centrifuging the membranes at 49,000 *g* for 10 min. This was repeated a second time. The crude particulate fraction was resuspended in sufficient 50 mM Tris-citrate buffer (pH 7.2) to yield 0.06 to 0.15 mg protein/ml in the assay. Aliquots (970 μl) of membrane suspension were incubated at 4° for 15 min with approximately 0.3 nM [$^3$H]flunitrazepam (80.0 Ci/mmole) and increasing concentrations of unlabeled flunitrazepam. The incubation was terminated by filtration through GF/B glass fiber filters (Whatman Corp.). The filters were washed with 8 ml of ice-cold Tris–HCl (pH 8.0 at 25°), and the radioactivity was determined by scintillation spectroscopy. The data were transformed into a Rosenthal plot of bound/free versus bound [29], and the $K_D$ and $B_{max}$ were determined by least squares linear reggression. $B_{max}$ values were expressed on the basis of protein. The $IC_{50}$ values for diazepam, Ro 5-4864 and clonazepam were determined by incubating a fixed concentration of [$^3$H]flunitrazepam and tissue with eight to eleven concentrations of unlabeled drug.

*Dietary manipulation of polar head group composition.* A chemically defined medium [25] containing 40 μg/ml of either choline (trimethylethanolamine), dimethylethanolamine, monomethylethanolamine or ethanolamine was used to alter the phospholipid composition. The cells were cultured for 3 days and then benzodiazepine binding was determined as described above. We have shown previously that supplementing the LM cell medium with the methyl analogues of choline for the first 3 days is non-toxic as assessed by cell growth, total cell protein, and $Na^+$–$K^+$ ATPase activity [27].

*Primary tumors and lung metastases.* Primary tumors and lung metastases were obtained by the method of Kier and Schroeder [28] by injecting 1 × $10^7$ LM cells s.c. into the left hindquarter of athymic or $C_3H$/Hef mice. After 28 days, solid tumors, removed from the initial site of cell injection, or metastatic tumors, dissected from lungs, were placed in medium containing 10% calf serum, gentamycin (10 mg/ml), fungizone (250 μg/ml), penicillin (10 units/ml), and streptomycin (10 μg/ml). After removing excess tissue, the primary tumors or metastases were transferred to fresh medium and chopped into small pieces. The minced tissue was pelleted at 960 *g* for 10 min, resuspended in fresh medium, and plated. Cells, initially grown as monolayers, were subsequently cultured in the same medium, but adapted to growth in suspension [28]. The cells were weaned from antibiotics and serum, and grown in chemically defined medium for at least 3 weeks and then assayed for [$^3$H]flunitrazepam binding.

*Lipid methylation.* Methylation was assayed by the procedure of Strittmatter *et al.* [18], with some modification. LM cells, at 1 × $10^6$ cells/ml, were suspended in 1 ml of fresh medium which contained 0.3 mM methionine and 30 μCi [Me-$^3$H]methionine (80.0 Ci/mmole). All samples were preincubated for 30 min at 37° to allow uptake of the [$^3$H]methionine into the cell. Subsequently, the cells were treated with drug or drug vehicle only (5 mN HCl) for 30 min at 37°. The reaction was terminated by centrifuging the cells in a Beckman Microfuge B for 15 sec at 4°. The samples were washed twice and centrifuged with 1 ml of cold PBS and once with 1 ml of 10% trichloroacetic acid. The lipids were extracted by the method of Bligh and Dyer [30], as described by Ames [31], and spotted on 250 μm silica gel G thin-layer chromatography plates preactivated with acetone. The lipids were separated by 2-dimensional chromatography using chloroform/methanol/water (65/25/4, by vol.) in the first direction and *n*-butanol/acetic acid/water (6/2/2, by vol.) in the second direction [25]. The identity and relative mobility of the phospholipids were previously determined in our laboratory [27] according to the procedure of Glaser *et al.* [25] using the following phospholipid standards: phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, phosphatidylinositol, phosphatidylserine, lysophosphatidylethanolamine, cardiolipin, phosphatidic acid, phosphatidylglycerol, lysophosphatidylcholine, phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*,*N*′-dimethylethanolamine. Visualization of the spots by $I_2$, elution of the phospholipids from the spots, and detection by the phosphate assay of Marzo *et al.* [32] and Ames [33] yielded the same results as radioautographic detection by $^{32}$P-labeled phospholipids. Nonpolar lipids were separated as a single spot located at the far corner opposite from the origin. The nonpolar lipids of the LM fibroblast and derivative cell lines contained desmosterol, the sterol synthesized by LM cells [34] as well as neutral glycerides, desmosterol esters, fatty acids and fatty acid methyl esters resolved as described earlier [35]. Ubiquinone and benzothioazoles which could be components [36] were not detected. In the experiments reported herein, the spots were visualized by exposure to $I_2$ and scraped into scintillation vials for counting. Any counts found in the phosphatidylethanolamine spot were subtracted as background. The rate of incorporation of methyl groups into specific lipid fractions was calculated as pmoles/mg protein for 60 min.

*Cyclic AMP.* LM cells at a density of 2.5 × $10^6$ cells/ml in 800 μl of fresh medium were preincubated for 1 hr at 37° with the benzodiazepines or drug vehicle. Samples received 200 μl of phosphate buffered saline (pH 7.2) with or without prostaglandin $E_1$ and were incubated for an additional 6 min. The reaction was terminated by adding 250 μl of 25% trichloroacetic acid, sonicating for 60 sec, and removing the protein by centrifugation. The supernatant fraction was neutralized with calcium carbonate and centrifuged [37]. Cyclic AMP was assayed in the resulting supernatant fraction by radioimmunoassay [38, 39].

*Protein determination.* Protein was determined by the method of Lowry *et al.* [40] with bovine serum albumin as the standard.

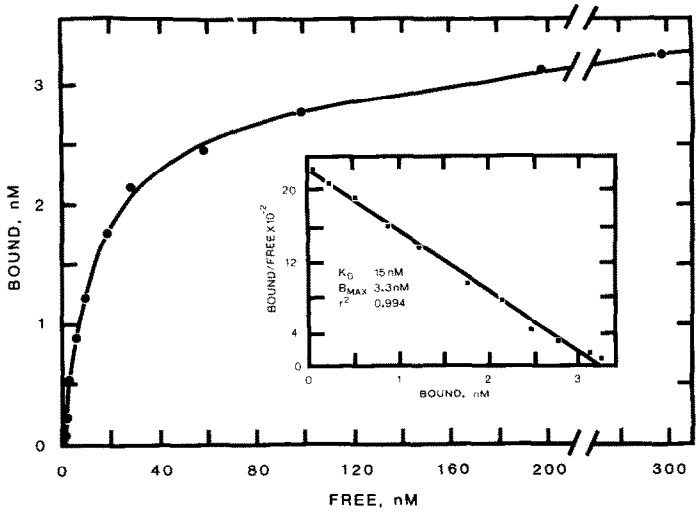


Fig. 1. Binding of [³H]flunitrazepam to a crude membrane preparation of the LM cell. The cells were maintained at $10^6$ cells/ml in a chemically defined medium containing 40 μg/ml of choline (trimethylethanolamine). The crude particulate fraction used in the assay was prepared by homogenization and centrifugation of whole cells. Key: (●) plot of specifically bound drug versus free; and (■) Rosenthal transformation of saturation data.

*Statistics.* Statistical comparisons were made by using Student's *t*-test.

*Materials.* [³H]Methionine (80.0 Ci/mmole) [³H]flunitrazepam (84.8 Ci/mmole), [³²P]ATP (NEG-003), and [³H]cyclic AMP (36.4 Ci/mmole) were purchased from the New England Nuclear Corp. (Boston, MA). Non-radioactive diazepam, flunitrazepam, clonazepam, and Ro 5-4864 were donated by Hoffmann-La Roche Inc. (Nutley, NJ).

## RESULTS

*[³H]Flunitrazepam binding to the LM cell.* LM cells exhibited a saturable, high affinity binding site for [³H]flunitrazepam (Fig. 1). The Rosenthal plot was linear with a $K_D$ of 15 nM and a $B_{max}$ of 17 pmoles/mg protein. Similar to other cells and tissues which contain the peripheral type of benzodiazepine receptor, the $IC_{50}$ values for Ro 5-4864, flunitrazepam, diazepam and clonazepam against [³H]flunitrazepam were 6, 36, 60 and 2500 nM, respectively, which indicates that this receptor is also the peripheral type (Fig. 2). There was no detectable specific binding of any of the following radiolabeled ligands to LM cell membranes: dihydroalprenolol, yohimbine, clonidine, WB-4101, prasozin, quinuclidinyl benzilate, or naloxone.

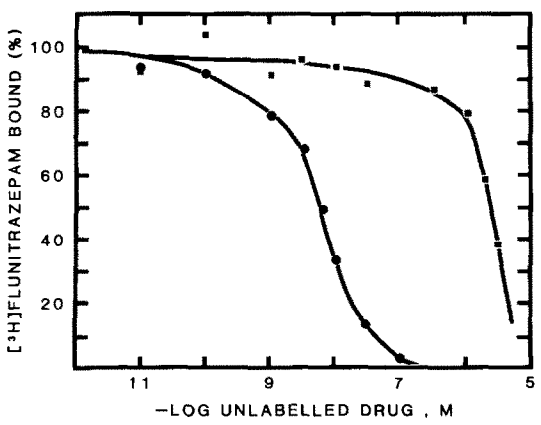


Fig. 2. Inhibition of [³H]flunitrazepam binding by Ro 5-4864 and clonazepam in a crude particulate fraction of the LM cell. Key: (●) Ro 5-4864, and (■) clonazepam.

*Effect of choline analogue supplementation on [³H]flunitrazepam binding.* A change in the binding properties of a receptor might be induced by a covalent modification of a membrane when novel phospholipid polar head groups are exchanged for normal head groups by dietary manipulation. When the choline analogues dimethylethanolamine, monomethylethanolamine or ethanolamine replaced choline in the medium, each was incorporated at 52, 43 and 45%, respectively, of the plasma membrane phospholipids, comparable to results obtained in previous studies [27]. Incorporation of the analogues also occurs in other subcellular fractions: 25–40% of the microsomal phospholipids and 37–49% of the mitochondrial phospholipids contained the analogues [27]. There was a corresponding decrease in phosphatidylcholine and phosphatidylethanolamine in all the subcellular fractions. Decreases of 37, 26 and 26% in $B_{max}$ were associated with the incorporation of dimethylethanolamine, monomethylethanolamine and ethanolamine, respectively, when compared to choline fed control cells (Table 1). Neither the $K_D$ value of [³H]flunitrazepam nor the $IC_{50}$ values of Ro 5-4864 and clonazepam were altered by these treatments (Tables 1 and 2).

*[³H]Flunitrazepam binding in primary and metastatic lines of the LM cell.* Delineating differences in the protein, lipid and carbohydrate composition of metastatic cells compared to either primary tumor cells or normal cells might contribute to a better understanding of their mechanism of formation. Cultured lung metastases from athymic mice had an

Table 1. $K_D$ and $B_{max}$ of [$^3$H]flunitrazepam binding in LM cells cultured in medium containing either choline (trimethylethanolamine), dimethylethanolamine, monomethylethanolamine or ethanolamine*

| Treatment | N | $K_D$ (nM) | $B_{max}$ (pmoles/mg protein) | % Change in $B_{max}$ |
|---|---|---|---|---|
| Choline | 3 | 13.0 ± 1.0 | 19.0 ± 1.0 | |
| Dimethylethanolamine | 3 | 33.0 ± 12.0 | 12.0 ± 2.0 | −37† |
| Monomethylethanolamine | 3 | 17.0 ± 1.0 | 14.0 ± 1.0 | −26† |
| Ethanolamine | 3 | 20.0 ± 2.0 | 14.0 ± 1.0 | −26† |

* LM cells were cultured at $10^6$ cells/ml for 3 days in a chemically defined medium containing 40 μg/ml of either choline (trimethylethanolamine), dimethylethanolamine, monomethylethanolamine, or ethanolamine. The binding parameters were estimated using a crude particulate fraction prepared by homogenization and centrifugation of whole cells. Each value is the mean ± S.E.M.
† $P < 0.05$ as determined by Student's $t$-test.

Table 2. The $IC_{50}$ (nM) of Ro 5-4864, flunitrazepam and clonazepam in LM cells cultured in choline (trimethylethanolamine), dimethylethanolamine, monomethylethanolamine and ethanolamine supplemented media*

| Treatment | Ro 5-4864 | Flunitrazepam | Clonazepam |
|---|---|---|---|
| Choline | 6 ± 2 (2) | 23 ± 6 (6) | 2750 ± 250 (2) |
| Dimethylethanolamine | 8 ± 2 (2) | 36 ± 5 (3) | 6500 ± 3500 (2) |
| Monomethylethanolamine | 5 ± 1 (2) | 22 ± 1 (3) | 2250 ± 250 (2) |
| Ethanolamine | 7 ± 1 (2) | 18 ± 6 (3) | 2950 ± 450 (2) |

* Cells were treated and assayed as described in Table 1. The numbers in parentheses represent the value of N. The values are expressed as the mean ± S.E.M. or ± range.

Table 3. $K_D$ and $B_{max}$ of [$^3$H]flunitrazepam binding in cultured LM cells, $C_3H$/Hef primary tumor cells, $C_3H$/Hef metastasis cells, and athymic mouse metastasis cells*

| Cell type | N | $K_D$ (nM) | $B_{max}$ (μmoles/mg protein) |
|---|---|---|---|
| LM | 7 | 16.0 ± 1.6 | 19.8 ± 2.1 |
| $C_3H$/Hef primary tumor | 3 | 22.1 ± 3.2 | 16.0 ± 2.0 |
| $C_3H$/Hef metastasis | 1 | 30.7 | 11.0 |
| Athymic metastases | 7 | 29.6 ± 2.5† | 13.0 ± 0.8‡ |

* Primary tumors and lung metastases were obtained by injecting $1 \times 10^7$ LM cells s.c. into the left hindquarter of either $C_3H$/Hef or athymic mice. After 28 days, the solid tumors or metastases were removed and adapted to suspension culture. All cells were cultured in the same chemically defined medium for at least 3 weeks prior to the binding assay. Values represent the mean ± S.E.M.
† $P < 0.005$ as determined by Student's $t$-test.
‡ $P < 0.02$ as determined by Student's $t$-test.

Table 4. Effects of Ro 5-4864 and clonazepam on lipid methylation in the LM cell*

| Drug | Concn (mM) | N | FAME | PC |
|---|---|---|---|---|
| Basal | | 2 | 7.0 ± 3.0 | 4.0 ± 1.0 |
| Ro 5-4864 | 30 | 2 | 11.0 ± 4.0 | 5.0 ± 2.0 |
| Clonazepam | 1000 | 2 | 13.0 ± 11.0 | 4.0 ± 1.0 |

* LM cells, at $1 \times 10^6$ cells/ml, were preincubated for 30 min at 37° in fresh medium containing 0.3 mM methionine and 30 μCi [Me-$^3$H]methionine (80.0 Ci/mmole). Subsequently, they were treated for 30 min at 37°. At the end of the incubation, the cells were centrifuged in a Beckman Microfuge B and then washed and centrifuged twice with 1 ml of cold phosphate buffered saline and once with 1 ml of 10% trichloroacetic acid. The lipids were extracted and analyzed by thin-layer chromatography. The rate of methyl group incorporation is expressed as pmoles/mg protein for 60 min and represents the mean ± range. Abbreviations: FAME, fatty acid methyl ester; and PC, phosphatidylcholine.

80% higher $K_D$ and a 30% lower $B_{max}$ for [$^3$H]flunitrazepam compared to the LM cell (Table 3). The $K_D$ and $B_{max}$ for [$^3$H]flunitrazepam binding in primary tumors from the $C_3H$/Hef mouse were approximately the same as in the LM cell, whereas the $K_D$ and $B_{max}$ in the lung metastases were 90% higher and 40% lower, respectively, as compared to the LM cell.

*Effect of benzodiazepines on phospholipid methylation.* Phospholipid methylation occurs in the plasma membrane of several different cell types [18–22, 41–45]. Stittmatter *et al.* [18] have proposed that the peripheral benzodiazepine receptor is coupled to this system in the $C_6$ astrocytoma. Lipid methylation activity in the LM cell was similar compared to other cell types, but was not stimulated by either Ro 5-4864 or clonazepam (Table 4).

*Effect of Ro 5-4864 on cyclic AMP levels.* The cyclic AMP-adenylate cyclase system serves as the transmembrane mediator of information for a number of hormones and is coupled to a prostaglandin $E_1$ receptor in the LM cell [46]. This system could be either a direct or indirect site of action for benzodiazepines. Ro 5-4864 alone did not affect cyclic AMP levels. Prostaglandin $E_1$ at only 2 $\mu$M maximally increased cyclic AMP levels 6-fold above basal. However, this stimulation was not significantly attenuated or augmented by Ro 5-4864 (Table 5).

## DISCUSSION

The results of this study demonstrate a saturable, high affinity binding site for [$^3$H]flunitrazepam in the LM cell, a transformed murine fibroblast. The $K_D$ for binding was 15 nM and the $B_{max}$ was 17 pmoles/mg protein. Based on the results of the inhibition curves (Ro 5-4864 more potent than clonazepam), this site appears to be of the peripheral type. At 4°, rat kidney membranes bind [$^3$H]flunitrazepam with a $K_D$ of 11 nM while the binding to rat cortex has a $K_D$ of 2 nM [19]. Thus, the $K_D$ for the LM cell is very similar to the $K_D$ obtained for kidney cells, but not cortex. Syapin and Skolnick [11] observed a $B_{max}$ value (pmoles/mg protein) of 1.4 in the $P_2$ fraction of rat cortex, 15.3 in the $P_1$ fraction of C6 glioma cells, 5.1 in the NB41A3 neuroblastoma, 4.7 in BALB/3T3, clone A31 fibroblast, and 1.8 in rat kidney. The high density of [$^3$H]flunitrazepam binding in LM cell membranes is quite comparable to the high density of [$^3$H]diazepam sites in the $P_1$ fraction of C6 glioma cells. [$^3$H]Flunitrazepam binds to differentiating rat cerebral cells in primary culture with a $K_D$ of 1 nM [10]. In these cells, diazepam ($K_i$ = 3 nM) was significantly more potent than Ro 5-4864 in displacing [$^3$H]flunitrazepam. By contrast, the rank order of potencies of these drugs in the LM cell was the reverse of the cerebral cells in culture, but of similar order in the $C_6$ astrocytoma.

Based on *in vitro* studies, it appears that the activities of some membrane associated enzymes are affected by the polar head group composition of the phospholipids [47–51]. It appears also that membrane lipids have a significant role in receptor regulation and function [52]. Choline analogue supplementation was shown to double the low affinity $K_A$ for fluoresceinyl Con A, Con A mediated hemadsorption, and Con A mediated self agglutination. In addition, choline analogue supplementation drastically lowers the temperature breakpoints in Arrhenius plots of LM cell Con-A agglutinability [53]. It has also been observed that basal and prostaglandin $E_1$ adenylate cyclase activities in the LM cell increase when either dimethylethanolamine, monomethylethanolamine or ethanolamine is substituted for choline in the medium [46]. In the present study, the $B_{max}$ for [$^3$H]flunitrazepam binding in the choline analogue fed cells was decreased; however, a statistically significant shift in the $K_D$ was not observed. Previously, it was found that a change in polar head group composition did not affect either the biophysical properties or the activities of some enzymes of the LM cell membranes [27]. Since our binding assay was carried out at 4°, a temperature well below the transition temperatures for most membranes, we would not expect a change in membrane physical properties to be directly responsible for the change in affinity characteristics observed. Culturing cells in a medium containing analogues of choline for 3

Table 5. Effect of Ro 5-4864 on basal and $PGE_1$ stimulated cyclic AMP levels in the LM cell*

| Drugs | | | |
|---|---|---|---|
| Ro 5-4864 ($\mu$M) | $PGE_1$ ($\mu$M) | N | Cyclic AMP levels [pmoles·$10^5$ cells)$^{-1}$·(5 min)$^{-1}$] |
| 0 | 0 | 3 | 0.022 ± 0.005 |
| 0.006 | 0 | 2 | 0.022 ± 0.002 |
| 0.03 | 0 | 2 | 0.024 ± 0.003 |
| 0 | 2 | 3 | 0.136 ± 0.017 |
| 0 | 20 | 3 | 0.105 ± 0.021 |
| 0.03 | 2 | 3 | 0.092 ± 0.012 |
| 0.03 | 20 | 3 | 0.097 ± 0.008 |

* LM cells, at 2.5 × $10^6$ cells/ml, were preincubated for 1 hr at 37° with Ro 5-4864 or drug vehicle and incubated for an additional 6 min with either prostaglandin $E_1$ or drug vehicle. The reaction was terminated by the addition of 25% trichloroacetic acid, sonication for 60 sec, and centrifugation of the protein. Calcium carbonate was used to neutralize the sample. Cyclic AMP levels were assayed by radioimmunoassay. Values represent the mean ± S.E.M.

days should permit sufficient time for either a decrease in the rate of synthesis or an increase in the rate of degradation resulting in a decrease in binding site density. Alternatively, the treatment might induce a covalent modification which could cause a masking of binding sites.

Changes in cell surface composition or properties during oncogenic transformation or the formation of metastases might be involved in the mechanism by which tumors and metastases develop [54–56]. An understanding of these processes might lead to more effective chemical approaches to therapy. In the present study, there was a decrease in the $B_{max}$ of metastases from both the athymic and $C_3H$/Hef mice. This reduction in binding site density could reflect a covalent modification of the cell surface associated with the growth of the metastases. The significance of the increase in $K_D$ observed in the metastases compared to the LM cell has yet to be determined. This result is analogous to the previous observations that a higher $K_D$ for the binding of [$^3$H]DHA to the $\beta$-receptor occurs in rat and calf cerebral cortex compared to the cerebellum [57, 58].

The activity of non-polar or fatty acid methyl ester methylation in the LM cells is higher than the level of activity reported in skeletal muscle and retina of the rat, 4–6 fmoles per mg protein per hr [44]. It was reported previously that the LM cell does not synthesize phosphatidycholine by the stepwise methylation of phosphatidylethanolamine [25–27, 59]. However, our results as well as those of Maeda *et al.* [60] suggest that the LM cell can carry on this process at a moderate level of activity, 5 pmoles per mg protein per hr. In contrast, Strittmatter *et al.* [18] reported a basal activity of 40 pmoles per mg protein per hr in the $C_6$ astrocytoma cell. Unlike Strittmatter *et al.* [18], we were not able to find any coupling between the benzodiazepine binding site and the methylation reactions.

The LM cell contains a cyclic AMP-adenylate cyclase system sensitive to prostaglandin stimulation [46]. This system can be modulated by the polar head group composition of the phospholipids. Neither a direct nor indirect role for the peripheral benzodiazepine binding site in the regulation of cyclic AMP-adenylate cyclase has been well characterized. Based on our results, it does not appear that benzodiazepine binding to the peripheral site either directly or indirectly regulates cyclic AMP accumulation in intact LM cells, suggesting that it does not interact with any of the components of this system through a receptor mediated mechanism.

Our results indicate the presence of a high affinity binding site for the benzodiazepines in the LM cell with a pharmacological profile of the peripheral type. It can be regulated by altering the polar head group composition of the phospholipids. The formation of metastatic cell lines also appears to modulate the number and affinity of sites. Our results suggest that the peripheral benzodiazepine binding site in the LM cell does not regulate either phospholipid methylation or cyclic AMP metabolism. However, a lack of response might only reflect an uncoupling of the receptor from the response as the result of cell transformation. Therefore, a function for this binding site has yet to be found in the LM cell.

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