0026-895X/89/020327-06$02.00/0

MOLECULAR PHARMACOLOGY, 36:327-332

Radiation Inactivation Reveals Discrete Cation Binding Sites that Modulate Dihydropyridine Binding Sites

GORDON T. BOLGER,[1] PHIL SKOLNICK, and ELLIS S. KEMPNER

Laboratory of Neuroscience, National Institute of Diabetes and Digestive and Kidney Diseases (G.T.B., P.S.) and Laboratory of Physical Biology, National Institute of Arthritis, Musculoskeletal, and Skin Diseases (E.S.K.), National Institutes of Health, Bethesda, Maryland 20892

Received July 11, 1988; Accepted April 17, 1989

SUMMARY

In low ionic strength buffer (5 mM Tris·HCl), the binding of [^{3}H]nitrendipine to dihydropyridine calcium antagonist binding sites of mouse forebrain membranes is increased by both Na^+ and Ca^{2+}. Radiation inactivation was used to determine the target size of [^{3}H]nitrendipine binding sites in 5 mM Tris·HCl buffer, in the presence and absence of these cations. After irradiation, [^{3}H]nitrendipine binding in buffer with or without Na^+ was diminished, due to a loss of binding sites and also to an increase in K_d. After accounting for radiation effects on the dissociation constant, the target size for the nitrendipine binding site in buffer was 160–170 kDa and was 170–180 kDa in the presence of sodium. In the presence of calcium ions, [^{3}H]nitrendipine binding showed no radiation effects on K_d and yielded a target size of 150–170 kDa. These findings suggest, as in the case of opioid receptors, the presence of high molecular weight membrane components that modulate cation-induced alterations in radioligand binding to dihydropyridine binding sites.

Detailed biochemical and pharmacological studies in both peripheral tissues (skeletal, cardiac, and smooth muscles) and the central nervous system have identified the high affinity DHP binding site as a regulatory component of the voltage-dependent calcium channel (1–3). Estimates of the size and composition of DHP calcium antagonist binding sites in a number of tissues have been reported. Digitonin solubilization of the nitrendipine receptor complex from skeletal muscle transverse T-tubules produced three polypeptides of M_r 130,000, 50,000, and 33,000 (4); similar extracts of chick cardiac membranes contained a M_r 170,000 peptide under nonreducing conditions or M_r 140,000, 32,000, and 29,000 peptides under reducing conditions (5). Covalent modification of DHP binding sites of canine cardiac membranes (6) and guinea pig ileal smooth muscle (7) labeled proteins with M_r 32,000 and 45,000, respectively. Radiation inactivation of DHP binding sites in guinea pig skeletal muscle and brain (8–11) yielded target sizes of 136 to 185 kDa. In guinea pig ileal longitudinal smooth muscle, a target size of 278 kDa was obtained for nitrendipine binding sites (7).

Tris·HCl buffer (50 mM) is routinely used to measure radioligand binding to DHP. However, it has been shown that a lower ionic strength buffer (5 mM) resulted in selective effects of mono- and divalent cations on radioligand binding to DHP in rat brain. Thus, Na^+ increased the apparent affinity and Ca^{2+} increased the binding site density of [^{3}H]nitrendipine in brain membranes (12). In the same tissue, mono- and divalent cations block the inhibition of nitrendipine binding by local anesthetics (13). These observations suggest that neuronal DHP calcium antagonist binding sites are linked to mono- and divalent cation-activated effectors (12–15). Using radiation inactivation and target size analysis, we have further explored the interactions of cations with neuronal DHP binding sites in mouse forebrain membranes.

Materials and Methods

Preparation of membranes for irradiation. Male mice (18–22 g; Institute of Cancer Research, Veterinary Resources Branch, National Institutes of Health) were killed by decapitation. The brains were rapidly removed and placed in ice-cold 0.32 M sucrose. The forebrain was isolated by making an oblique cut rostral to the superior colliculus on the dorsal surface to the mammillary bodies on the ventral surface, and a crude synaptosomal fraction of forebrain P_2 was prepared, according to the method of Gray and Whittaker (16). The P_2 pellet was lysed in 30 volumes of 5 mM Tris buffer, pH 7.4, using a short burst (5 sec) with a Polytron (Brinkmann Instruments; setting 6). The membranes were centrifuged at 25,000 × g for 15 min, the supernate was discarded, and the membranes were resuspended and centrifuged once more. Membranes were resuspended with a Polytron in 3 volumes of 5 mM Tris-buffered 0.1 M sucrose, and 0.5-ml aliquots were placed in thin-walled 2-ml glass vials, which were immediately frozen on solid CO_2, sealed with an oxygen-gas flame, and stored for no longer than 1 week at −70°.

Radiation inactivation. Radiation inactivation was performed

[1] Present address: BioMega, Inc., 2100 Rue Cunard, Laval, Quebec, Canada H7S 2G5.

ABBREVIATION: DHP, dihydropyridine.

with 10 MeV electrons at −135°, as described (17). The irradiated samples were stored at −70° for no longer than 1 week before assay.

[^{3}H]Nitrendipine binding. Following irradiation, frozen membranes were thawed rapidly in a 25° shaking water bath and were diluted with 15 volumes of ice-cold 5 mM Tris·HCl. [^{3}H]Nitrendipine binding was assayed in a total volume of 2 ml, consisting of 0.5 ml of diluted membranes (0.4–0.6 mg of protein), 1.4 ml of Tris·HCl (containing NaCl or $CaCl_2$ at the indicated concentrations), and 0.1 ml of radioligand solution. Nonspecific binding was determined in the presence of 10 μM nifedipine. Incubations were carried out in borosilicate glass tubes under subdued light for 1 hr at 25°. The experiments were terminated by rapid filtration under vacuum on Whatman GF/B glass fiber filters, followed by two 5-ml washes with ice-cold Tris buffer, using a Brandel Cell Harvester (Brandel Co., Gaithersburg, MD). The filters were placed in 8 ml of Ready Solve-MP (Beckman, Fullerton CA) and the radioactivity retained by the filters was determined (LS 330 liquid scintillation counter, Beckman Instruments, Fullerton CA). Specific binding, expressed as the difference between total and nonspecific binding, varied from 80% at 80 pM to 65% at 800 pM [^{3}H] nitrendipine. Storage of frozen membrane preparations at −20° or at −70° for prolonged periods of time (3–5 weeks) resulted in a decrease in specific [^{3}H]nitrendipine binding. Therefore, radiation inactivation was performed 2–3 days after membrane preparation and [^{3}H]nitrendipine binding assays were conducted no later than 2–5 days after radiation inactivation.

[^{3}H]Nitrendipine (specific activity, 78–81 Ci/mmol) was obtained from New England Nuclear-Dupont (Boston, MA). Nifedipine was obtained from Pfizer (Groton, CT). All other reagents were of the highest purity and were obtained from standard commercial sources. Protein was determined according to the Miller modification (18) of the Lowry assay (19).

Target size calculations. Binding activity was expressed as B/F, where B represents the amount of specific binding of [^{3}H]nitrendipine, in fmol/mg of protein, and F represents the concentration of free radioligand. In irradiated samples, each value of B/F was normalized to that observed in nonirradiated controls, $(B/F)_0$ (see Ref. 20). Least squares linear regression was calculated from $\ln[(B/F)_D/(B/F)_0] = k'D$, where D is the dose of radiation in Mrad (17). The rate of radiation inactivation, k', is corrected for its temperature dependence (21) by a factor S_t, which is equal to 2.8 for irradiations performed at −135°.

The simplest form of target analysis assumes an all or none phenomenon such that the recognition site qualities of a receptor are either completely abolished or remain unaffected. Thus, the loss of binding is due to a reduction in B_{max} (maximum binding site capacity) with no changes in K_d (macromolecular dissociation constant). If this assumption is valid, the target size can be calculated from:

$$\text{Molecular mass (in Da)} = 6.4 \times 10^5\, k' S_t$$

However, if there are radiation-dependent changes in K_d, a more complex analysis is required. Because

$$B/F = B_{max}/(K_d + F) \quad (1)$$

then, after a radiation dose, D,

$$(B/F)_D = B_{max,D}/(K_{d,D} + F_D)$$

and

$$(B/F)_D/(B/F)_0 = [B_{max_D}/B_{max_0}]\cdot[(K_{d,0} + F_0)/(K_{d,D} + F_D)] \quad (2)$$

If the loss in B_{max} is a simple exponential function of radiation dose, independent of the changes in K_d, then Eq. 2 becomes

$$(B/F)_D/(B/F)_0 = e^{-k'D}\cdot[(K_{d,0} + F_0)/(K_{d,D} + F_D)] \quad (3)$$

and the radiation inactivation curve will be described by

$$\ln \frac{(K_{d,D} + F_D)}{(K_{d,0} + F_0)} \cdot \frac{(B/F)_D}{(B/F)_0} = -k'D \quad (4)$$

Solution of Eq. 4 requires knowledge of the manner in which K_d changes with radiation exposure. Two simple models were considered; one involved a linear relationship ($K_{d,D} = K_{d,0} + pD$), whereas the other assumes an exponential relationship ($K_{d,D} = K_{d,0}e^{qD}$). It was assumed that the observed changes in K_d are independent of the decrease in B_{max}, and estimates of p or q were determined from the data in Table 1. These values were used to evaluate k' from Eq. 4 and the target size was then calculated as indicated above.

Statistics. Differences between groups were analyzed statistically by Student's t tests.

Results

[^{3}H]Nitrendipine binding in frozen membrane preparations. The K_d and B_{max} values obtained for nitrendipine binding to mouse forebrain in membranes before freezing (data not shown) were identical to those obtained in membranes frozen and thawed in 5 mM Tris·HCl/0.1 M sucrose (Table 1) and were consistent with previously reported values (3).

Effects of cations on [^{3}H]nitrendipine binding. Because sodium and calcium ions have differential effects on [^{3}H]nitrendipine binding to rat brain (12), we extended these studies. Aliquots of the same samples used for nitrendipine binding in 5 mM Tris buffer (described above) were also used for the binding of this ligand in the presence of 100 mM sodium or 2 mM calcium. The sodium-dependent decrease in K_d (with no change in B_{max}) and the calcium-dependent increase in B_{max} (with no change in K_d) that were previously observed in rat brain were also present in the mouse forebrain (Table 1, zero radiation dose).

Radiation inactivation of mouse forebrain membranes. After exposure to different doses of radiation, [^{3}H] nitrendipine binding was measured both at 80 and 800 pM in 5 mM Tris buffer. At these two ligand concentrations, the re-

TABLE 1

Scatchard analysis of [^{3}H]nitrendipine binding to mouse forebrain membranes, either in Tris buffer or in Tris buffer with additions (as indicated), following radiation inactivation

Mouse forebrain membranes were prepared as described in Materials and Methods and frozen (−70°). One sample was not irradiated (0 dose) whereas the three remaining samples were irradiated at the doses shown in the table. Scatchard analyses were performed over a [^{3}H]nitrendipine concentration range of 35 to 1500 pM in the presence of Na^+ or Ca^{2+} as indicated. The K_d and B_{max} values were obtained by linear regression analysis (BMDP Software, Los Angeles CA). The data are presented as the mean ± standard error of four experiments.

Radiation Dose	K_d	B_{max}	Increase in B_{max} due to Ca^{2+}
Mrad	*pM*	*fmol/mg of protein*	*fmol/mg of protein*
5 mM Tris·HCl			
0	278 ± 22	260 ± 26	
3	368 ± 27[a]	197 ± 18	
6	379 ± 38[a]	148 ± 19	
12	507 ± 18[b]	88 ± 15	
5 mM Tris·HCl plus 100 mM Na^+			
0	191 ± 30[c]	276 ± 30	
3	250 ± 57	199 ± 6	
6	284 ± 34[a]	163 ± 4	
12	345 ± 49[b]	89 ± 28	
5 mM Tris·HCl plus 2 mM Ca^{2+}			
0	281 ± 12	319 ± 18[c]	59
3	306 ± 17	256 ± 22	59
6	298 ± 24	205 ± 18	55
12	308 ± 20	131 ± 13	43

Significantly different from 0 dose, [a] $p < 0.05$; [b] $p < 0.005$.
[c] Significantly different from 5 mM Tris-HCl, $p < 0.05$.

maining radioligand binding decreased as single (but different) exponential functions of radiation dose (Fig. 1). The simplest form of radiation target analysis assumes an exponential decrease in B_{max} after irradiation, with no change in K_d. However, Scatchard analysis indicated that radiation exposure caused a decrease in [^{3}H]nitrendipine binding that was attributable to an increase in the K_d and a decrease in the B_{max} (Fig. 2 and Table 1). Thus, a more general theoretical analysis (see Materials and Methods) was used for the data in Fig. 1. The calculation of target size accounting for the radiation effects on K_d (Eq. 4) was performed with the linear as well as the exponential assumptions of the K_d dependence on radiation dose, leading to target sizes of 160–170 kDa (Table 2). An approximate target size can also be calculated from the limited values of B_{max} shown in Table 1 (5 mM Tris·HCl), yielding a value of 163 kDa.

Effects of radiation on [^{3}H]nitrendipine binding in the presence of cations. [^{3}H]Nitrendipine binding measured in the presence of Na^+ or Ca^{2+} decreased as a simple exponential function of radiation dose (Fig. 3). As in the case of the radiation inactivation of [^{3}H]nitrendipine binding in the absence of these cations (above), analyses of these data require knowledge of the radiation effects on K_d and B_{max}. In Table 1 it is seen that, in 100 mM Na^+ (as in the case of buffer alone), the K_d for nitrendipine changes with radiation exposure. Under these conditions, target sizes of 170–180 kDa are obtained with the more complex model, comparable to the values determined in Tris buffer. The target size of that portion of [^{3}H]nitrendipine binding attributable to Na^+ stimulation (i.e., the binding in the presence of sodium minus the binding in its absence) was also estimated. Although this determination is subject to large potential error (because it is based on the loss of a 30% increase), the loss appears to be a simple exponential (Fig. 4) and yields an apparent target size of 265 kDa.

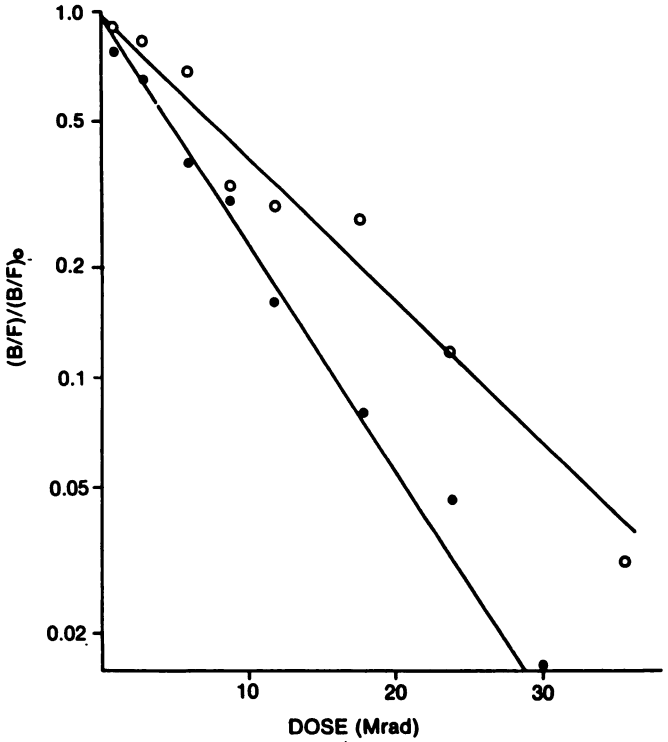

Fig. 1. Radiation inactivation of [^{3}H]nitrendipine binding sites in mouse forebrain membranes; dependence on [^{3}H]nitrendipine concentration. ●, 80 pM; ○, 800 pM [^{3}H]nitrendipine. Data are from a typical experiment, which is representative of four to eight experiments.

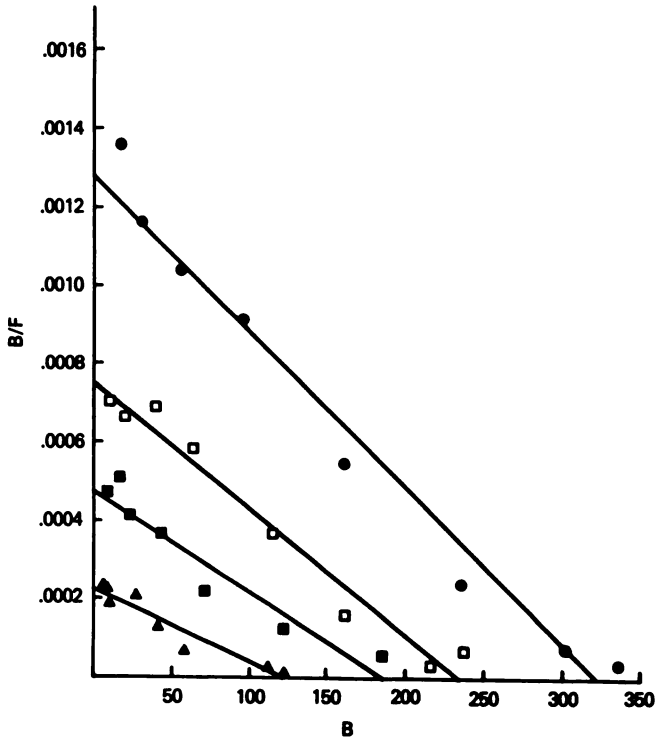

Fig. 2. Scatchard analyses of [^{3}H]nitrendipine binding in nonirradiated and irradiated membranes. Frozen samples of mouse forebrain membranes were irradiated at 0, 3, 6, or 12 Mrad, as described in Materials and Methods. Thawed membrane suspensions were incubated with varying concentrations of [^{3}H]nitrendipine (30–1200 pM). Least squares linear regression was used to determine the apparent K_d and B_{max} values for control (●), 3 Mrad (□), 6 Mrad (■), and 12 Mrad (▲). The mean K_d and B_{max} values from four such experiments are presented in Table 1. *B* is bound, in fmol/mg of protein, and *F* is free, in fM.

In the presence of 2 mM Ca^{2+}, [^{3}H]nitrendipine binding exhibited no radiation-dependent change in K_d (Fig. 5 and Table 1). Thus, the simple form of target analysis could be used, giving sizes of 150–170 kDa (Table 2), all within the range of target sizes seen in buffer alone.

The difference between [^{3}H]nitrendipine binding in the presence and absence of calcium (Table 1) was taken as the calcium-stimulated [^{3}H]nitrendipine binding. Only a very limited decrease was seen after radiation exposure; if a simple exponential loss is assumed, an approximate target size of 80–90 kDa is obtained.

Effects of cations during radiation exposure. The effects of addition of either 100 mM Na^+ or Ca^{2+} to forebrain membranes before radiation exposure were investigated. Neither cation significantly affected the radiation inactivation of nitrendipine binding seen in their absence (Table 2).

Discussion

The regulation of rat brain DHP binding sites by low concentrations of mono- and divalent cations reported using 5 mM Tris·HCl buffer (12) has been confirmed here for [^{3}H]nitrendipine binding to mouse forebrain membranes. Therefore, 5 mM Tris·HCl was used throughout the present study, in contrast to the higher concentration of buffer (50 mM) routinely used by most investigators.

TABLE 2

Target size analysis for [^{3}H]nitrendipine binding to mouse forebrain membranes

Samples were irradiated in 5 mM Tris·HCl (pH 7.4) containing 0.1 M sucrose. As indicated, some samples also contained 100 mM Na^+ or 2 mM Ca^{2+}. After irradiation, samples were thawed, diluted in 15 volumes of 5 mM Tris·HCl, and centrifuged at 24,000 × *g* for 15 min. The final pellet was resuspended in 15 volumes of fresh Tris·HCl, recentrifuged, and resuspended in 15 volumes of Tris·HCl for assay with additions as indicated.

[^{3}H]Nitrendipine	Additions to Irradiation Buffer	Additions to Assay Buffer	Target Size[a]		
			No change	Linear change	Exponential change
pM				kDa	
80				175 ± 39	162 ± 41
800				165 ± 23	157 ± 24
80		100 mM Na^+		181 ± 4	169 ± 4
80		2 mM Ca^{2+}	167 ± 3		
800		2 mM Ca^{2+}	149 ± 9		
80	100 mM Na^+			203 ± 5	192 ± 15
80	100 mM Na^+	2 mM Ca^{2+}	178 ± 4		
800	100 mM Na^+	2 mM Ca^{2+}	160 ± 4		
80	2 mM Ca^{2+}			146 ± 15	133 ± 14
80	2 mM Ca^{2+}	2 mM Ca^{2+}	161 ± 10		
800	2 mM Ca^{2+}	2 mM Ca^{2+}	136 ± 13		

[a] Target sizes (mean ± standard error) from three to nine experiments; analyses included corrections for change in K_d assuming no, linear, or exponential dependence on radiation dose.

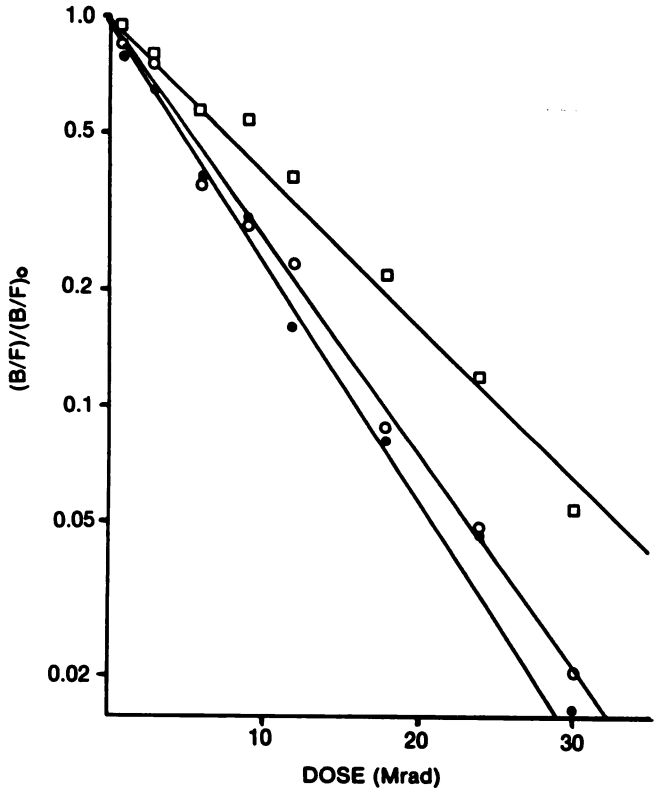

Fig. 3. Radiation inactivation of [^{3}H]nitrendipine binding in mouse forebrain membranes; effects of Ca^{2+} and Na^+. [^{3}H]Nitrendipine (80 pM) binding in the absence of cations (●) or in the presence of 100 mM Na^+ (○) or 2 mM Ca^{2+} (□). The results are from a typical experiment, which is representative of five to eight experiments.

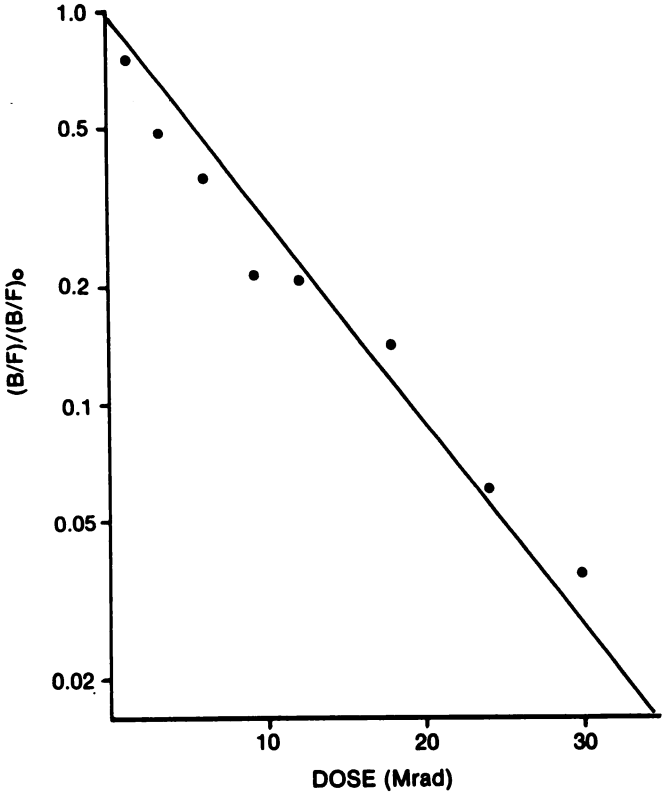

Fig. 4. Radiation inactivation of Na^+-stimulated [^{3}H]nitrendipine binding in mouse forebrain membranes. Na^+-stimulated [^{3}H]nitrendipine binding is defined as specific [^{3}H]nitrendipine binding in the presence of 100 mM Na^+ minus the specific binding of [^{3}H]nitrendipine in the absence of Na^+. The unadjusted target size for Na^+-stimulated binding is 265 ± 41 kDa. Each data point represents the average of five independent radiation experiments.

Unexpectedly, the radiation inactivation of DHP calcium antagonist binding sites on mouse forebrain membranes in low ionic strength buffer was dependent on the concentration of [^{3}H]nitrendipine in the assay. Scatchard analyses revealed that the dependence was due to a radiation-induced change in the apparent K_d of [^{3}H]nitrendipine for DHP binding sites. Thus, the decreases in [^{3}H]nitrendipine binding observed after radiation exposure are due to both a destruction of binding sites and a decrease in the apparent affinity of the ligand for remaining sites.

The simplest form of target theory ascribes radiation inactivation to the destruction of active units; all surviving structures are deemed to be unaltered. Therefore, the radiation loss of binding sites/receptors or enzyme activity is due to a decrease

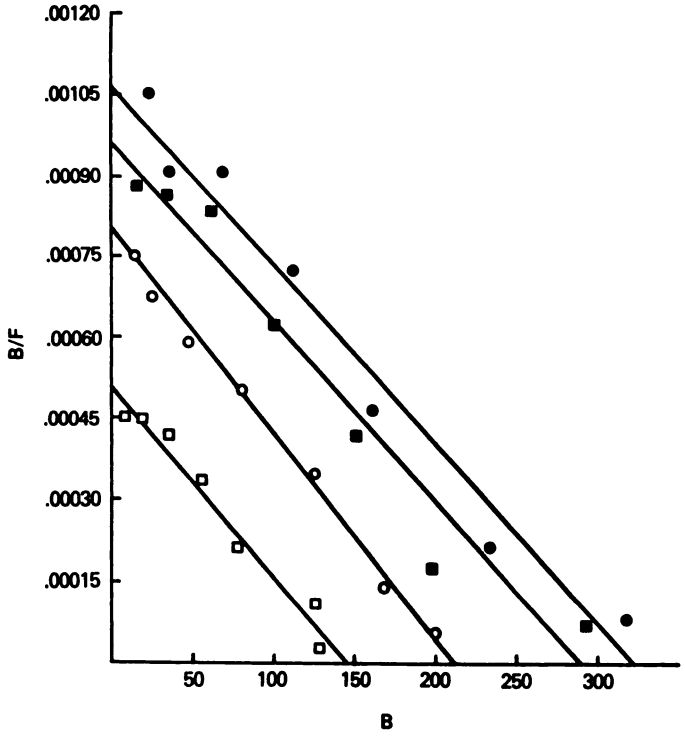

Fig. 5. Scatchard analysis of [^{3}H]nitrendipine binding in nonirradiated and irradiated membranes; effects of Ca^{2+}. Frozen mouse forebrain membranes were irradiated at 0 (●), 3 (■), 6 (○), or 12 (□) Mrad, as described in Materials and Methods. Conditions for [^{3}H]nitrendipine binding were as described in the legend to Fig. 2, but with the addition of 2 mM Ca^{2+}. The mean K_d and B_{max} values from four such experiments are presented in Table 1. *B* is bound, in fmol/mg of protein, and *F* is free, in fM.

in B_{max} (or V_{max}), with no change in K_d (or K_m). This hypothesis has been experimentally confirmed in many cases (22, 23). Under the present experimental conditions, this analysis leads to apparent target sizes of 232 ± 15 kDa at 80 pM and 190 ± 13 kDa at 800 pM [^{3}H]nitrendipine. However, the present results indicate a more complex system, which cannot be analyzed properly by this simple model. An alternative radiation analysis is given, which involves only the assumption that K_d and B_{max} change independently with radiation. As in the simple model, B_{max} is assumed to decrease exponentially with radiation dose. The nature of the effect of radiation on K_d is not specified in this new analysis. The experimental data presented in Table 1 could be fit with either a linear or an exponential dependence of K_d on radiation dose. Each was utilized in analyzing the nitrendipine binding in irradiated mouse forebrain membranes. Both analyses led to substantially the same results, target sizes of 160–170 kDa. These values are in good agreement with the molecular weight of the DHP receptor complex determined by gel filtration chromatography (4, 5, 24) and the molecular size of guinea pig brain and skeletal muscle [^{3}H]DHP binding sites determined by radiation inactivation (7, 9–11). There have been no reports that the apparent affinity of radioligands for the DHP binding sites in these tissues changes after exposure to radiation, although affinity changes in irradiated opioid receptors have recently been observed (25, 26).

In irradiated samples, [^{3}H]nitrendipine binding in the presence and absence of Na^+ is comparable, displaying radiation effects on both B_{max} and K_d. Target size calculations yielded similar, but slightly different, sizes depending on the assumed nature of the radiation dependence of K_d. The presence of Ca^{2+} in the assay resulted in a simple radiation inactivation of [^{3}H] nitrendipine binding, with no changes in the apparent K_d. After accounting for any complexities, the target calculations for [^{3}H] nitrendipine binding in low ionic strength buffer with or without cations suggest a common unit of 160–170 kDa.

Analysis of the radiation inactivation of the sodium-dependent (or calcium-dependent) component of [^{3}H]nitrendipine binding is complicated. Although the Na^+ effect could be followed to very low survival, the measurement depends on the difference between two large numbers and is thus subject to considerable error. The Ca^{2+}-dependent component of [^{3}H] nitrendipine binding could only be followed over a very limited range; it was not possible to confirm that the effect followed an exponential function of dose, much less obtain an accurate value of the slope. Furthermore, no attempt was made (or could be justified) to treat the Ca^{2+} effects with the complex analysis presented in Materials and Methods. Taken together, it is tentatively estimated that the cation-dependent effects involve target approximately 100 kDa larger (Na^+) or smaller (Ca^{2+}) than the [^{3}H]nitrendipine binding site.

A progressive increase in K_d with radiation dose implies that some molecular structures are being altered, leading to a lower apparent affinity of [^{3}H]nitrendipine. The mass of these putative units must be comparable to or greater than that of the receptor, else the effect would not be seen (the loss of a larger receptor would occur at small radiation doses; thus, the binding would be lost before the smaller regulating unit was damaged). The molecular explanation for the radiation-dependent changes in K_d is highly speculative. However, recent studies by Ott *et al.* (25, 26) of the opioid receptors in rat brain and NG 108-15 cells have shown that 100 mM Na^+ induced a low affinity state of the receptor and this effect was regulated by an allosteric inhibitor "larger than the receptor itself" (26). Although there are obvious differences between DHP binding sites and opioid receptors, the similarities (sodium changes the K_d; K_d also changes with radiation dose; target size for sodium effect is larger than target size for binding site) are so strong as to suggest analogous mechanisms. In this respect, our observation that the presence of calcium removes the radiation dependence of K_d suggests that the divalent cation may antagonize the allosteric inhibitor.

Acknowledgments

The authors acknowledge Kendall Marcus, Caroline Lubeck, and Michael Jackson for their excellent technical assistance and Dr. C. R. Triggle for his helpful comments regarding this manuscript.

References

1. Bolger, G. T., P. Gengo, R. Klockowski, E. Luchowski, H. Siegel, R. A. Janis, A. M. Triggle, and D. J. Triggle. Characterization of binding of the Ca^{2+} channel antagonist, [^{3}H]nitrendipine, to guinea pig ileal smooth muscle. *J. Pharmacol. Exp. Ther.* **225**:291–309 (1983).
2. Triggle, D. J., and R. A. Janis. The 1,4-dihydropyridine receptor: a regulatory component of the Ca^{2+} channel. *J. Cardiovasc. Pharmacol.* **6**:S949–S955 (1984).
3. Triggle, D. J., and R. A. Janis. Calcium channel antagonists: new perspectives from the radioligand binding assay, in *Modern Methods in Pharmacology* (N. Black and S. Spector, eds.). Alan R. Liss, Inc., New York, 1–28 (1984).
4. Curtis, B. M., and W. A. Catterall. Purification of the calcium antagonist receptor of the voltage-sensitive calcium channel from skeletal muscle transverse tubules. *Biochemistry* **23**:2113–2118 (1984).
5. Cooper, C. L., S. Vandaele, J. Barhanin, M. Fosset, M. Lazdunski, and M. M. Hosey. Purification and characterization of the dihydropyridine-sensitive voltage-dependent calcium channel from cardiac tissue. *J. Biol. Chem.* **262**:509–512 (1987).
6. Campbell, K. P., G. M. Lipshutz, and G. H. Denney. Direct photoaffinity labeling of the high affinity nitrendipine-binding site in subcellular membrane fractions isolated from canine myocardium. *J. Biol. Chem.* **259**:5384–5387 (1984).

7. Venter, J. C., C. M. Fraser, J. S. Schaber, C. Y. Jung, G. Bolger, and D. J. Triggle. Molecular properties of the slow inward calcium channel. *J. Biol. Chem.* **258**:9344–9348 (1983).
8. Goll, A., D. R. Ferry, J. Streissnig, M. Schober, and H. Glossman. (−)-Desmethoxyverapamil, a novel Ca^{2+} channel probe. *FEBS Lett.* **176**:371–377 (1984).
9. Ferry, D. R., A. Goll, and H. Glossman. Putative calcium channel molecular weight determination by target size analysis. *Naunyn-Schmeideberg's Arch. Pharmacol.* **323**:292–297 (1983).
10. Ferry, D. R., A. Goll, and H. Glossman. Calcium channels: evidence for oligomeric nature by target size analysis. *EMBO J.* **2**:1729–1732 (1983).
11. Goll, A., D. R. Ferry, and H. Glossman. Target size analysis of skeletal muscle Ca^{2+} channels. *FEBS Lett.* **157**:63–69 (1983).
12. Bolger, G. T., and P. Skolnick. Novel interactions of cations with dihydropyridine calcium antagonist binding sites in brain. *Br. J. Pharmacol.* **88**:857–866 (1986).
13. Bolger, G. T., K. A. Marcus, J. W. Daly, and P. Skolnick. Local anesthetics differentiate dihydropyridine calcium antagonist binding sites in rat brain and cardiac membranes. *J. Pharmacol. Exp. Ther.* **240**:922–930 (1987).
14. Wu, K., L. Sachs, R. K. Carlin, and P. Siekevitz. Characterization of a Ca^{2+}/calmodulin-dependent binding of the Ca^{2+} channel antagonist nitrendipine to a postsynaptic density fraction isolated from canine cerebral cortex. *Brain Res.* **387**:167–184 (1986).
15. Hess, P., A. P. Fox, J. B. Lansman, B. Nilius, M. C. Nowcky, and R. W. Tsien. Calcium channel types in cardiac, neuronal, and smooth muscle-derived cells: differences in gating, permeation, and pharmacology, in *Ion Channels in Neural Membranes* (J. M. Ritchie, R. D. Keynes, and L. Bolis, ed.) Alan R. Liss, Inc., New York, 227–252 (1986).
16. Gray, E. C., and V. P. Whittaker. The isolation of nerve endings from brain: an electron-microscope study of cell membrane fragments derived from homogenization and centrifugation. *J. Anat.* **98**:79–85 (1962).
17. Harmon, J. T., T. B. Nielsen, and E. S. Kempner. Molecular weight determinations from radiation inactivation. *Methods Enzymol.* **117**:65–94 (1985).
18. Miller, G. L. Protein determination for large numbers of samples. *Anal. Chem.* **31**:964 (1959).
19. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
20. Harmon, J. T., and J. T. Jamieson. Thrombin binds to a high-affinity ≈900,000-dalton site on human platelets. *Biochemistry* **24**:58–64 (1985).
21. Kempner, E. S., and H. T. Haigler. The influence of low temperature on the radiation sensitivity of enzymes. *J. Biol. Chem.* **257**:13297–13299 (1982).
22. Kempner, E. S., and S. Fleischer. Radiation inactivation of membrane components and molecular mass determination by target analysis. *Methods Enzymol.* **172**:410–439 (1989).
23. Kempner, E. S. Molecular size determination of enzymes by radiation inactivation. *Adv. Enzymol.* **61**:107–147 (1988).
24. Rengasamy, A., J. Ptasienski, and M. M. Hosey. Purification of the cardiac 1,4-dihydropyridine receptor/calcium channel complex. *Biochem. Biophys. Res. Commun.* **126**:1–7 (1985).
25. Ott, S., T. Costa, M. Wuster, B. Hietel, and A. Herz. Target size analysis of opioid receptors. *Eur. J. Biochem.* **155**:621–630 (1986).
26. Ott, S., T. Costa, and A. Herz. Sodium modulates opioid receptors through a membrane component different from G-proteins. *J. Biol. Chem.* **263**:10524–10533 (1988).

Send reprint requests to: Gordon T. Bolger, Ph.D., BioMega, Inc., 2100 Rue Cunard, Laval, Quebec, Canada H7S 2G5.