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EFFECTS OF CATIONS AND TEMPERATURE ON THE BINDING OF [^{3}H]SPIPERONE TO SHEEP CAUDATE NUCLEUS

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Abstract—The specific [^{3}H]spiperone binding by sheep caudate nucleus homogenate is increased by divalent cations. The effect of Ca^{2+} or Mn^{2+} (5 mM) is temperature-dependent, and it is optimal at about 37°, but is relatively low below 15° and above 50°. In the absence of added Ca^{2+} or Mn^{2+}, the maximal specific [^{3}H]spiperone binding is observed at about 25°, and the cations shift the optimum to about 37°. Under the experimetal conditions used, the K_D is about 0.6 nM and is not influenced by Ca^{2+} or Mn^{2+}, or by temperature (25 and 37°). In addition to Ca^{2+} and Mn^{2+}, Mg^{2+} and Zn^{2+} also increase the specific [^{3}H]spiperone binding, but to a smaller extent. At the concentrations of Ca^{2+}, Mn^{2-}, Mg^{2+} and Zn^{2+} which produce a maximal increase in the [^{3}H]spiperone binding, the membranes are nearly saturated with the cations which bind about 100 nmoles of Ca^{2+} or Mg^{2+}/mg of protein, 170 nmoles Zn^{2+}/mg of protein and at least 300 nmoles Mn^{2-}/mg of protein. It is suggested that the cations increase the [^{3}H]spiperone binding by either exposing more binding sites, by preventing denaturation or by increasing the solubility of [^{3}H]spiperone in the membrane phase, or by a combination of these processes.

Cations influence the high-affinity binding of many drugs and neurotransmitters. For example, in opiate receptor binding, which has been thoroughly studied in many laboratories, the effects of ions were found to be varied and specific [1–4]. Sodium provided a basis for distinguishing between opiate agonists and antagonists, since the effects are elicited by sodium and to a certain extent by lithium, but not by potassium [2].

The effects of cations upon the binding of [^{3}H]spiperone to dopamine receptors were recently reported by Usdin *et al.* [5]. Both monovalent and divalent cations, and also several chelating agents, were found to increase the number of [^{3}H]spiperone binding sites in rat corpus striatum. The potency of dopamine in inhibiting [^{3}H]spiperone binding is reduced by GTP, and this effect is inhibited by the presence of cations [5, 6]. Furthermore, monovalent and divalent cations themselves alter the competition by dopamine and apomorphine for [^{3}H]spiperone binding [5].

In this communication, we describe the effect of temperature and of divalent cations as a function of temperature on [^{3}H]spiperone binding to the membranes of sheep caudate nucleus.

MATERIALS AND METHODS

Preparation of brain homogenate. Sheep brains were obtained fresh, and the caudates were removed within 1 hr after death. High-affinity binding of [^{3}H]spiperone was determined by the method described by Seeman *et al.* [7]. The tissue, 50 mg wet weight/ml, was homogenized in cold buffer containing 15 mM Tris–HCl (pH 7.4), 1.1 mM ascorbic acid, and 12.5 μM pargyline (TAP buffer). A Teflon pestle in a glass homogenizer, rotating at 500 rpm, was passed up and down 20 times. The crude homogenate was first incubated at 37° for 30 min, and then was stored in 5-ml portions at 20°. Before use, the samples were thawed, resuspended using a glass–Teflon homogenizer, and were centrifuged at 35,000 *g* for 15 min at 40°. The pellet was resuspended in 10 ml of buffer and was rehomogenized using a Polytron homogenizer (Brinkmann Instrument Co.) and a setting of 7 for 20 sec. The protein concentration of the homogenate was adjusted to 4–5 mg/ml, as determined by the biuret method [8].

[^{3}H]*Spiperone binding assay.* The [^{3}H]spiperone binding reaction was carried out in a volume of 0.6 ml of a medium containing 0.2 ml of [^{3}H]spiperone (final concentration of 2.1 nM), 0.2 ml of homogenate suspension containing 0.8 mg of protein, and 0.2 ml of TAP buffer. The samples were incubated for the periods and at the temperatures indicated in the figure legends, and the reaction was terminated by filtering a 0.5-ml portion through glass fiber filters (Whatman GF/B, 24 mm diameter). The filters were washed twice with 5 ml of TAP buffer at room temperature and were counted in 0.8 ml of Triton X-100 scintillation fluid [9]. Specific binding of [^{3}H]spiperone was defined as the difference between the amount of [^{3}H]spiperone bound in the absence and in the presence (non-specific binding) of 10^{-6} M (+)-butaclamol. The amount of [^{3}H]spiperone bound was expressed as fmoles of [^{3}H]spiperone bound/mg of protein. All results were

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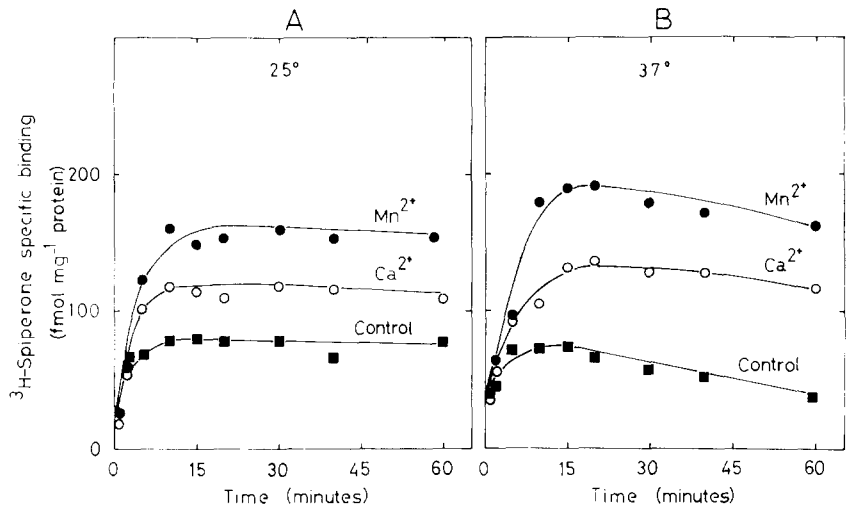

Fig. 1. Time-dependent binding of [^{3}H]spiperone by sheep brain caudate homogenate at 25 (A) and 37° (B) in the presence and in the absence of either Ca^{2+} or Mn^{2+}. Assay conditions were as described in Materials and Methods. The concentration of [^{3}H]spiperone was 2.1 nM and that of protein was 0.8 mg/ml. The concentration of Ca^{2+} or Mn^{2+} was 5 mM.

replicated at least 3 times in independently performed experiments.

Measurement of cations. The cations bound to the homogenate were measured by atomic absorption spectroscopy. After centrifugation of the suspensions containing the homogenate which had been equilibrated with the cation the pellets were extracted with 4% TCA and 1% La^{3+}. The extract, after centrifugation, was used to make the measurements by atomic absorption [10].

Reagents. [^{3}H]Spiperone with a sp. act. of 20 Ci/mmole was obtained from the Radiochemical Center (Amersham, U.K.) and butaclamol was kindly donated by Ayert Research Laboratories (Canada).

RESULTS

Effects of Ca^{2+}, Mn^{2+} and temperature on [^{3}H]spiperone specific binding

The effects of Ca^{2+} and Mn^{2+} on the [^{3}H]spiperone binding at 25 and 37° are depicted in Fig. 1. Either Ca^{2+} or Mg^{2+} increases the specific [^{3}H]spiperone binding, and maximal binding is reached after 15 min of incubation at either temperature. Experiments carried out at lower temperatures show that about 30 min of incubation are necessary to reach equilibrium at 15°, but the period of equilibration is not particularly important at 0–5° since the [^{3}H]spiperone binding remains relatively low even after relatively long periods of incubation (results not shown).

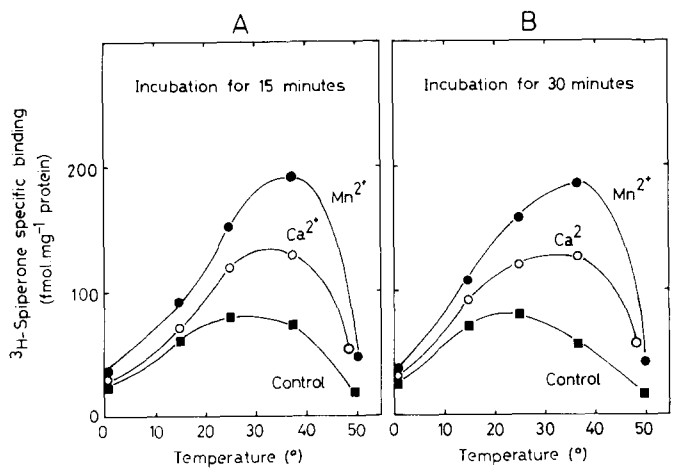

Fig. 2. Influence of temperature and Ca^{2+} or Mn^{2+} on [^{3}H]spiperone binding by sheep caudate homogenate. The protein concentration was 0.8 mg/ml and $CaCl_2$ or $MnCl_2$ were 5.0 mM, when present. The period of incubation was either 15 min (A) or 30 min (B). In these experiments the homogenate was not pre-incubated at 37°.

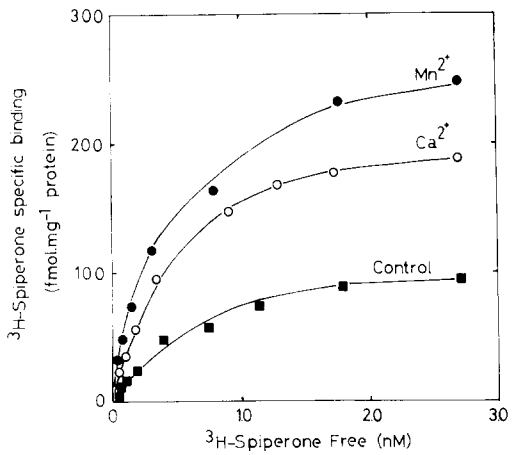

Fig. 3. Saturation curves for [^{3}H]spiperone binding in the absence and in the presence of 5 mM of either $CaCl_2$ or $MnCl_2$. The equilibration was carried out at 37° for 15 min for each of the [^{3}H]spiperone concentrations indicated.

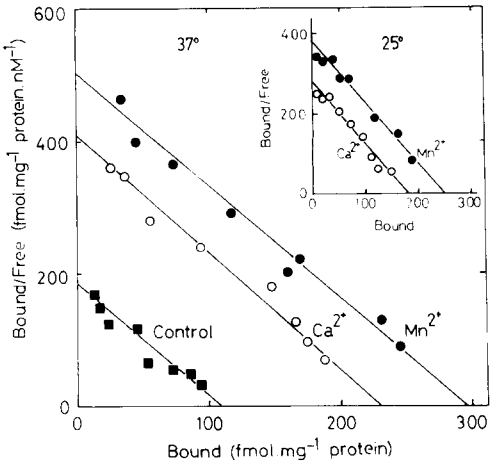

Fig. 4. Scatchard plots of data represented in Fig. 3. The insert is for a similar experiment but carried out at 25°.

At 37°, maximal [^{3}H]spiperone binding decreases when the incubation period is extended beyond 15 min, and this effect is particularly evident when the incubation is performed in the absence of added cations (Fig. 1B). At 50°, the period of equilibration is irrelevant because almost instantaneous loss of activity is observed. Therefore, when carrying out studies in a range of temperature between 0 and 50°, we took into consideration the fact that the [^{3}H]spiperone binding observed is a function of the period necessary for equilibrium to take place, and that at the higher temperatures some receptors are lost, especially if the period of incubation is long.

Figure 2 depicts the [^{3}H]spiperone specific binding after 15 min and 30 min incubation for a range of temperatures from 0 to 50°. The curves in the presence of Mn^{2+} or Ca^{2+} are similar for 15 min and 30 min, and in both cases the divalent cations increase the [^{3}H]spiperone binding over the control value. The maximum binding for the control is about 80 fmoles/mg protein at 25°, but declines above this temperature, especially if the incubation period is 30 min rather than 15 min (Fig. 2B). The optimal binding in the presence of either Ca^{2+} or Mg^{2+} (5 mM) shifts to about 37° at which the value is about 130 and 190 fmoles/mg protein in the presence of Ca^{2+} or Mn^{2+} respectively (Fig. 2).

Effect of Ca^{2+} *or* Mn^{2+} *on maximum binding and on the affinity for* [^{3}H]*spiperone of caudate membranes*

We determined the maximal binding capacities and the binding affinities from Scatchard plots at 25 and 37°. Saturation occurs between 1 and 2 nM (Fig. 3) and the K_D values calculated from the Scatchard plots are about 0.6 nM at both temperatures in the presence and absence of divalent cations (Fig. 4). Therefore, it appears that neither the divalent cations nor the temperature, or a combination of both, influences the affinity of the binding sites for [^{3}H]spiperone. However, both the temperature and the divalent cations can cause an increase in the maximal binding capacity (Fig. 4).

As indicated in Table 1, 5 mM Ca^{2+} at 37° increases the [^{3}H]spiperone maximal binding capacity (B_{max}) as calculated from the Scatchard plots, from 118 to 234 fmoles/mg protein, whereas 5 mM Mn^{2+} increases the maximal binding to a value of 280 fmoles/mg protein (Fig. 4). Similar results are obtained at 25° (insert of Fig. 4), except that the

Table 1. Effect of cations and temperature on the equilibrium dissociation constant (K_D) and maximal binding capacity (B_{max}) of sheep caudate nucleus for [^{3}H]spiperone

Temperature (°)	Cation (5 mM)	K_D (nM)	B_{max} (fmoles/mg protein)
25	Ca^{2+}	0.66 ± 0.03	174 ± 10
	Mn^{2+}	0.69 ± 0.04	248 ± 13
37	None	0.59 ± 0.02	118 ± 8
	Ca^{2+}	0.59 ± 0.09	234 ± 19
	Mn^{2+}	0.65 ± 0.09	280 ± 22

Experimental conditions were those specified for Figs. 3 and 4, and the values were calculated from the data of those figures, which summarize the data of a representative experiment, and from two other independent experiments.

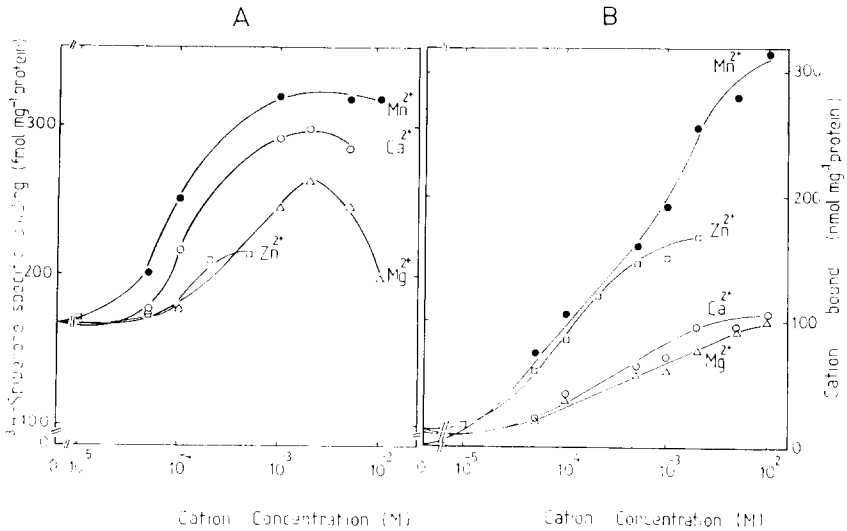

Fig. 5(A). Effect of Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} on [^{3}H]spiperone specific binding by sheep caudate homogenate. Caudate homogenate (0.8 mg/ml of protein), [^{3}H]spiperone (2.08 nM), TAP buffer, and various concentrations of divalent cations ranging from 0 to 10 mM, except for $ZnCl_2$ (0–1 mM), were incubated in a final volume of 0.6 ml. Incubations were conducted at 37° for 15 min, in the presence and absence of (+)-butaclamol. (B) Binding of Ca^{2+}, Mg^{2+}, Mn^{2+} (0–10 mM) and Zn^{2+} (0–1 mM) by sheep brain homogenate. Suspensions (0.4 mg/ml of protein) were incubated for 15 min at 37° in a medium (10 ml) containing TAP buffer and each of the divalent cations. The suspensions were centrifuged and the pellets were washed once in 0.3 M sucrose (10 ml). The pellets were extracted in 4% TCA, and 1% La^{3+} was added to the extracts. Cations were determined by atomic absorption spectrometry.

maximal binding capacities are lower than those obtained at 37° (Table 1).

Effect of the concentration of Ca^{2+}, Mg^{2+}, Mn^{2+} *and* Zn^{2+} *on* [^{3}H]*spiperone binding*

In Fig. 5A we depict the relationship between the divalent cation concentration and the [^{3}]spiperone binding at 37°. The binding is highest in the presence of Mn^{2+} at all concentrations of divalent cations studied, and at concentrations about 5 mM maximal binding activity is observed, but higher cation concentrations are inhibitory (Fig. 5A). The half-maximal effect for Mn^{2+} or Ca^{2+} occurs at about 0.2 mM, whereas slightly higher concentrations of Mg^{2+} are necessary to produce the same effect, although in some experiments we observed no difference between Ca^{2+} and Mg^{2+}. The consistent result is that, in the presence of Mn^{2+}, the [^{3}H]spiperone binding is always higher than in the presence of either Ca^{2+} or Mg^{2+}. Zn^{2+} produces a much lower effect than the maximal effect produced by Ca^{2+}, Mg^{2+} or Mn^{2+} (Fig. 5A).

The binding of Ca^{2+} and Mg^{2+} approaches a maximal value at about 100 nmoles Ca^{2+} or Mg^{2+}/mg protein, whereas about 300 nmoles Mn^{2+}/mg protein are bound at 10 mM free Mn^{2+} without reaching saturation (Fig. 5B). The binding of Zn^{2+} is intermediate at about 180 nmoles/mg protein at a free Zn^{2+} concentration of 1.0 mM.

Effect of Ca^{2+} *or* Mn^{2+} *on the* [^{3}H]*spiperone binding by lipids isolated from caudate nucleus microsomes*

Multilamellar liposomes prepared from lipids isolated from microsomes of caudate nucleus of the sheep bind [^{3}H]spiperone, and this binding is increased by either Ca^{2+} or Mn^{2+} from a value of about 308 fmoles/mg lipid in the absence of ions to values of 459 and 427 fmoles/mg lipid in the presence of 5 mM Ca^{2+} or Mn^{2+} respectively (Table 2). The

Table 2. Effect of Ca^{2+} or Mn^{2+} on the [^{3}H]spiperone binding by lipids isolated from caudate nucleus microsomes of sheep

	[^{3}H]Spiperone binding (fmoles/mg lipid)		
Ions added	No ions	Ca^{2+}	Mn^{2+}
[^{3}H]Spiperone bound	308	459	427

Multilamellar liposomes were prepared from lipids isolated from the microsomal fraction of the caudate nucleus [9]. The desired amount of lipid (0.4 mg/test tube), determined by a phosphorus assay according to Bartlett [17], was taken from the chloroform solution and the solvent was evaporated first in a nitrogen stream and then under vacuum for 3 hr. Buffer solutions (TAP, pH 7.4) and several glass beads were added and vortexed for 3 min at room temperature. The same buffer was used in binding assays, performed as previously described in Materials and Methods, in the absence and in the presence of cations (5 mM final concentration), at 37° for 15 min. Data are from a representative experiment. In each of three experiments performed, Ca^{2+} and Mn^{2+} increased the amount of [^{3}H]spiperone retained by the lipid, but the absolute values varied between 180 and 308 fmoles/mg lipid in the absence of ions and between 200 and 459 fmoles/mg lipid in the presence of ions, Ca^{2+} being the most effective cation in each of the experiments. The microsomes correspond to the supernatant of the P_2 fraction of Hájos [18] collected at 39,000 *g* for 15 min.

relevance of these results is presented in the Discussion.

DISCUSSION

The results show that Ca^{2+}, Mg^{2+}, Mn^{2+}, and to some extent Zn^{2+}, increase the specific binding of [^{3}H]spiperone by sheep caudate homogenate.

The number of [^{3}H]spiperone receptor sites is decreased when the homogenate is incubated at 37° for periods over 25 min. Thus, in the control experiments (in the absence of ions), a higher binding is observed at 25° than at 37° (Fig. 2B). However, Ca^{2+} or Mn^{2+} increases the specific [^{3}H]spiperone binding at temperatures between about 15 and 50°, although their effect is greatest at 37° (Fig. 2). Since the effect of the ions is highest at temperatures at which substantial loss of binding already occurs in the absence of cations, the ions may be preventing denaturation of the receptors rather than exposing more receptors at the higher temperatures studied. However, in the absence of ions, an increase in temperature between 0 and 25° also causes an increase in [^{3}H]spiperone binding. This may reflect either a direct effect of temperature on the number of active binding sites available (for example making more receptors accessible), or it may be that the time required for equilibrium is so long that no equilibrium was attained in the time utilized. This latter possibility is difficult to test because long equilibration periods tend to cause loss of specific [^{3}H]spiperone binding. Regardless of whether cations are present or not in the equilibration medium, denaturation of the receptors becomes predominant at the higher temperatures tested, and an increase in available receptors with increasing temperature would no longer be observed.

Qualitatively similar results have been reported by Usdin *et al.* [5] for the effects of monovalent and divalent cations. Usdin *et al.* [5] reported that the cations do not affect the affinity of the receptors for [^{3}H]spiperone and that the effect is on the maximal binding capacity. Furthermore, they also observed that there is a time-dependent loss of [^{3}H]spiperone specific binding during incubation at 37° which could be partially reversed by Mn^{2+} or Na^+.

Various types of receptors are influenced by cations [2–5, 11–16], but the mechanism of the effect is not clear. Cations seem to mediate the action of GTP on the potency of dopamine in inhibiting [^{3}H]spiperone binding [5, 6, 11], but this is independent of the effect reported here, since GTP or dopamine were not added. The observation that ions by themselves increase the potency of dopamine in reducing [^{3}H]spiperone binding, Mn^{2+} being the most potent cation [5, 10], suggests a great importance for ions in determining the neuroleptic effect of drugs. On the other hand, the potency of the antagonist chloropromazine is not affected by cations [5]. The effect of cations on [^{3}H]spiperone specific binding under the conditions of our experiments probably reflects a stabilization of the receptors, which tend to be degraded as the temperature is increased, but this does not explain the increase in [^{3}H]spiperone binding at the lower temperatures under conditions where no loss of binding is observed, even in the absence of cations.

We considered another plausible explanation for the effect of cations on the [^{3}H]spiperone binding. Thus, if the cations increase the partition coefficient of [^{3}H]spiperone, one might expect that specific binding sites buried in the lipid bilayer might be in equilibrium with a higher [^{3}H]spiperone concentration in the presence but not in the absence of cations. This presumes that at least some of the [^{3}H]spiperone binding sites (receptors?) are highly hydrophobic structures, and that the bound [^{3}H]spiperone would be in equilibrium with the [^{3}H]spiperone in the lipid phase rather than in the aqueous medium. This raises some question regarding the validity of using the aqueous concentration of [^{3}H]spiperone as the free [^{3}H]spiperone in determining the K_D values. The results reported in Table 2 show that lipids extracted from the microsomal fraction of the caudate nucleus of sheep brain reveal a [^{3}H]spiperone binding of lipid in the absence of cations. The value increases to 459 and 427 fmoles/mg in the presence of 5 mM Ca^{2+} or Mn^{2+}, respectively. This [^{3}H]spiperone binding to the lipid fraction is not stereospecific (data not shown).

Nevertheless, the K_D values calculated in the presence and in the absence of cations are not significantly different, whereas the B_{max} increases. If the cations increased the concentration of free [^{3}H]spiperone in equilibrium with the binding sites in the hydrophobic environment of the membrane, this should yield lower apparent K_D values. Increase in temperature also does not alter the K_D value, although it seems to increase the [^{3}H]spiperone binding within a limited range of temperature. Leysen and Gommeren [14] reported similar results regarding the effect of temperature (25 and 37°) on the K_D value, and also observed an increase in specific [^{3}H]spiperone binding with temperature up to about 25° at 0.1 nM, but not at 1 mM free [^{3}H]spiperone. However these results depend on many parameters, such as buffer type and tissue concentration [13].

The cations, Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+}, which increase the specific [^{3}H]spiperone binding, are bound to the membranes (Fig. 5), and maximal binding is approached at about the concentrations which cause maximal increase in [^{3}H]spiperone binding, except for Mn^{2+} whose binding is not saturated even at 10 mM free Mn^{2+}. These divalent cations also increase the non-specific binding (not shown) of [^{3}H]spiperone probably due to an increase in the partition coefficient of [^{3}H]spiperone (Table 2).

In addition to increasing the non-specific [^{3}H]spiperone binding, a higher concentration of spiperone in the membrane phase could increase the effective concentration in equilibrium with the receptors, as suggested earlier. It is also expected that spiperone, which is lipophilic, should concentrate in monolayers around the membrane miceles to give a higher concentration in this region than in the medium. This would constitute a driving force for interactions with the membrane components [14]. It is expected that this surface phenomenon would be influenced by the composition of the medium, which may explain, in part, the effect of the cations on [^{3}H]spiperone binding. However, it is not possible to specify the mechanism of action.

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