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Selective Enhancement of [^{3}H]Opiate Agonist Binding by Divalent Cations

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SUMMARY

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Manganese ions enhance binding of the tritiated opiate agonists dihydromorphine and levorphanol 78% and 64%, respectively, while binding of the tritiated antagonists naloxone, levallorphan, and diprenorphine is unaffected. Magnesium and nickel ions also selectively enhance [^{3}H]dihydromorphine binding with no effect on [^{3}H]naloxone binding. By contrast, cupric and ferrous ions lower the binding of [^{3}H]dihydromorphine far more than that of [^{3}H]naloxone. Manganese ions also enhance the ability of unlabeled agonists to inhibit the binding of [^{3}H]naloxone. All these effects are most pronounced in the presence of sodium chloride. Ethylenediaminetetracetic acid inhibits the binding of [^{3}H]dihydromorphine 50% without altering [^{3}H]naloxone binding, while ethylenebis[(oxyethylenenitrilo)]tetraacetic acid and magnesium and manganese complexes of EDTA are ineffective. The EDTA effect can be reversed by subsequent addition of either manganese or magnesium ions. The divalent cations which enhance opiate agonist binding appear to counteract the ability of sodium ions to inhibit agonist binding selectively. The potency of sodium in inhibiting [^{3}H]dihydromorphine binding is reduced 5-fold by the addition of manganese ions. There is a good correlation between the sensitivity of opiate receptor binding to sodium and manganese.

INTRODUCTION

Opiate receptor binding (1–3) correlates closely with the pharmacological activity of opiates (4–6). Binding has been demonstrated in nervous tissue of numerous vertebrates but not invertebrates (7), and in cell cultures of neuroblastoma-glioma hybrids (8). Opiate receptor binding has been characterized in terms of its regional (9, 10) and subcellular (11) distribution in mammalian brain and the influence of opiate addiction (12, 13). The differential influence of sodium (12–14), protein-modifying reagents (15, 16), and enzymatic treatments (17) upon binding of agonists and antagonists is consistent with an allosteric model of receptor functioning, in which the receptor can exist in two interconvertible

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forms with selective affinities for agonists and antagonists, respectively (14, 18, 19). In the present study we describe a selective enhancement of opiate agonist binding by low concentrations of certain divalent cations.

MATERIALS AND METHODS

Drugs were donated by the following companies: Endo (oxymorphone), Lilly [(−)-methadone], Roche (levorphanol, levallorphan), and Winthrop (pentazocine). Nalorphine was purchased from Merck.

Chemicals used in the study were purchased from J. T. Baker (sodium EDTA, cupric chloride, manganous chloride, nickelous chloride) and K & K Laboratories (dimagnesium, disodium calcium trihydrate, and disodium manganous salts of EDTA).

[^{3}H]Naloxone (23.6 Ci/mmole) and [^{3}H]dihydromorphine (46 Ci/mmole) were purchased from New England Nuclear Corporation. [^{3}H]Levorphanol (5.4 Ci/mmole) and [^{3}H]levallorphan (7.5 Ci/mmole) were prepared at New England Nuclear Corporation by catalytic exchange as previously described (12). [^{3}H]Diprenorphine (13 Ci/mmole) was tritiated and purified by Amersham/Searle.[3]

Membranes from male Sprague-Dawley rat brains (minus cerebella) were prepared and assayed as previously described (15). Opiate specific binding is defined as the difference in binding in the presence of 1 μM levallorphan from that in its absence. All values are opiate specific binding expressed as the means of triplicate determinations, which varied less than 15%, unless otherwise indicated. Ions or drugs were always added prior to the addition of the [^{3}H]opiate. Total binding was routinely 3–7 times the binding in the presence of 1 μM levallorphan.

RESULTS

Influence of divalent cations on binding of [^{3}H]agonists and [^{3}H]antagonists. Manganese chloride (1 mM) increases the receptor binding of [^{3}H]dihydromorphine and [^{3}H]levorphanol by 60–80%, while the binding of the opiate antagonists [^{3}H]naloxone, [^{3}H]levallorphan, and [^{3}H]diprenorphine is essentially unaffected (Table 1). To evaluate the sensitivity and specificity of the influence of divalent cations, we examined a wide range of concentrations of numerous divalent cations (Table 2). As little as 0.025 mM manganese increases the binding of [^{3}H]dihydromorphine by 36% when assayed in the presence of NaCl while causing no significant influence on [^{3}H]-naloxone binding. Maximal enhancement of [^{3}H]dihydromorphine binding occurs at 1 mM $MnCl_2$, which elicits a doubling of binding. Receptor binding of [^{3}H]dihydromorphine is increased less at 5 and 20 mM and is reduced by 85% at 100 mM. When assays are conducted in the absence of sodium the influence of manganese ions is much smaller, with no more than a 30% increase in binding. There is no enhancement of [^{3}H]naloxone binding at any concentration of manganese ions with or without sodium in the incubation mixture, while high concentrations of manganese (20 and 100 mM) lower [^{3}H]naloxone binding to a similar extent whether assayed with or without sodium.

The pattern of effects of magnesium ions on receptor binding of [^{3}H]dihydro-

TABLE 1

Effects of $MnCl_2$ on [^{3}H]opiate binding

Rat brain homogenate was prepared as described in MATERIALS AND METHODS and assayed in the presence of 100 mM NaCl and in the presence and absence of 1 mM $MnCl_2$ and 1 μM levallorphan. The following amounts of [^{3}H]opiate were used per tube: naloxone, 49,000 cpm; levallorphan, 40,000 cpm; diprenorphine, 4300 cpm; dihydromorphine, 66,000 cpm; and levorphanol, 39,000 cpm. Binding values are the means of triplicate determinations, which varied less than 15% and represent specific opiate binding. The experiment was replicated three times.

[^{3}H]Opiate	Binding		
	No $MnCl_2$	1 mM $MnCl_2$	Change
	cpm	*cpm*	*%*
Naloxone	2300	2400	+4
Levallorphan	1900	2200	+16
Diprenorphine	1600	1400	−13
Dihydromorphine	1850	3300	+78
Levorphanol	700	1150	+64

[3] C. B. Pert and S. H. Snyder, manuscript in preparation.

TABLE 2

Effects of divalent cations on binding of [³H]naloxone and [³H]dihydromorphine

Rat brain homogenate was prepared and assayed with and without 100 mM NaCl in the presence and absence of 1 μM levallorphan. Binding in the presence of the ions was divided by binding in their absence to determine the percentage of control values. Binding in the presence of divalent cations and sodium chloride was compared with binding in the absence of divalent cations, but in the presence of sodium chloride. Thus differences in binding are not due to the presence or absence of sodium chloride. All binding values used were opiate specific binding and were determined from the means of triplicate determinations, which varied by less than 10%. Opiate specific control binding of [³H]naloxone and [³H]dihydromorphine was 2700 cpm and 5500 cpm, respectively, in the absence of NaCl, and 3200 cpm and 2500 cpm, respectively, in the presence of NaCl.

Ion	Concentration	Assayed with 100 mM NaCl		Assayed without NaCl	
		[³H]Naloxone	[³H]Dihydro-morphine	[³H]Naloxone	[³H]Dihydro-morphine
	mM	*% control*		*% control*	
$MnCl_2$	0.01	106	115	95	114
	0.025	108	136	117	131
	0.05	119	154	119	132
	0.1	103	154	108	110
	1.0	108	205	99	117
	5.0	94	180	84	91
	20.0	66	129	60	65
	100.0	15	14	0	5
$MgSO_4$	0.05	99	119	113	103
	0.1	117	127	98	123
	1.0	117	158	99	124
	3.0	78	146	105	90
	6.0	110	140	91	111
	10.0	89	156	91	96
	20.0	86	152	85	95
	50.0	78	110	66	77
	100.0	63	78	53	61
$CaCl_2$	0.1	104	89	102	94
	1.0	111	128	86	102
	5.0	89	123	84	90
	10.0	85	128	77	109
	20.0	79	113	79	91
	50.0	69	59	66	81
	100.0	54	31	44	40
	150.0	51	10	32	33
$NiCl_2$	0.0001	82	102	103	92
	0.001	81	101	87	100
	0.005	87	130	100	110
	0.01	87	140	94	112
	0.02	97	163	106	122
	0.05	101	146	73	90
	0.1	77	127	62	59
	1.0	56	120	48	45
	3.0	35	79	23	25
	5.0	20	35	12	20

TABLE 2—*Continued*

Ion	Concentration	Assayed with 100 mM NaCl		Assayed without NaCl	
		[^{3}H]Naloxone	[^{3}H]Dihydromorphine	[^{3}H]Naloxone	[^{3}H]Dihydromorphine
	mM	*% control*		*% control*	
$CuCl_2$	0.001	98	76	97	78
	0.003	89	68	71	68
	0.01	73	22	44	37
	0.03	51	21	24	15
	0.1	25	27	9	27
$FeCl_2$	0.005	90	57	86	80
	0.01	96	57	65	72
	0.03	77	35	74	60
	0.1	66	4	50	36
	1.0	16	13	7	11

morphine and [^{3}H]naloxone is qualitatively similar to those of manganese. However, enhancement of [^{3}H]dihydromorphine binding occurs to a lesser extent and requires higher concentrations of magnesium ions than the manganese ions. [^{3}H]Dihydromorphine binding appears to be about 5–10 times less sensitive to magnesium than to manganese, and maximal enhancement of [^{3}H]dihydromorphine binding with magnesium is only about 50–60%.

Calcium is even less efficacious in enhancing [^{3}H]dihydromorphine binding, producing a maximal increase in binding of about 28% at 1–10 mM concentration when assays are conducted with sodium. Calcium, like magnesium and manganese ions, can lower receptor binding of both [^{3}H]naloxone and [^{3}H]dihydromorphine at concentrations in excess of 20 mM. Nickel is extremely potent in enhancing [^{3}H]dihydromorphine binding, producing a 30% increase at 0.005 mM and a maximal increase of 63% at 0.02 mM. While it is more potent than manganese in terms of the lowest concentration which can elicit an increase in [^{3}H]dihydromorphine binding, nickel appears to elicit a smaller maximal response than manganese. As with the other divalent cations, the influence on [^{3}H]dihydromorphine binding is most evident when assays are conducted in the presence of sodium. Higher concentrations of nickel lower both [^{3}H]naloxone and [^{3}H]dihydromorphine binding. However, decreases in receptor binding with nickel occur at concentrations of 3–5 nM, much lower than those required with other divalent cations.

In contrast to the influence of other divalent cations, copper does not enhance [^{3}H]dihydromorphine binding at any concentration, and shows a relatively selective lowering of [^{3}H]dihydromorphine binding relative to [^{3}H]naloxone binding at 0.01 and 0.03 mM, an effect opposite to the previous ions. Again the selective reduction of [^{3}H]dihydromorphine by cupric ions is less apparent when assays are conducted in the absence rather than in the presence of sodium. Ferrous ions appear to act similarly to cupric ions. This pattern, which is opposite that of manganese, resembles the influence of protein-modifying reagents and enzymes upon opiate receptor binding (15–17).

To ensure that the influence of divalent cations is not mediated by the associated anion, we evaluated the influence of eight anions as their ammonium salts upon binding of [^{3}H]naloxone and [^{3}H]-dihydromorphine assayed with or without sodium (Table 3). At 10 mM none of these anions changes receptor binding of [^{3}H]naloxone or [^{3}H]dihydromorphine whether the assays are conducted with or without sodium.

Scatchard analysis of [^{3}H]dihydromorphine binding. Since [^{3}H]dihydromorphine

TABLE 3

Effects of anions on binding of [³H]naloxone and [³H]dihydromorphine

Rat brain membranes were prepared and assayed with the appropriate salt at 10 mM as described in MATERIALS AND METHODS. Control binding of [³H]naloxone and [³H]dihydromorphine was 1900 cpm and 4300 cpm, respectively, in the absence of NaCl, and 2200 cpm and 2400 cpm, respectively, in the presence of NaCl.

Addition	Assayed with 100 mM NaCl		Assayed without NaCl	
	[³H]Naloxone	[³H]Dihydromorphine	[³H]Naloxone	[³H]Dihydromorphine
	% control		% control	
Ammonium fluoride	105	100	100	83
Ammonium chloride	104	86	112	85
Ammonium bromide	110	84	107	88
Ammonium iodide	93	83	91	82
Ammonium sulfate	92	85	88	85
Ammonium thiocyanate	100	91	92	83
Ammonium perchlorate	83	90	85	84
Ammonium formate	115	92	101	86

exhibits the greatest enhancement of binding when assayed with manganese ions, we performed a Scatchard analysis of [³H]-dihydromorphine binding (Fig. 1). Sodium chloride appears to decrease the number of [³H]dihydromorphine binding sites almost 60%. Manganese ions alone show a slight increase (12%) in the number of [³H]dihydromorphine binding sites, but when manganese is added to tissue which already has sodium present, nearly a 2-fold increase in [³H]dihydromorphine binding sites occurs, almost totally reversing the effects of sodium chloride. Thus manganese ions appear to antagonize the sodium-induced decrease in [³H]dihydromorphine binding.

Influence of manganese ions upon receptor interactions of unlabeled opiates. The effects of sodium upon opiate receptor binding of a large number of nonradioactive opiates were studied by examining the ability of sodium to alter the concentration of the nonradioactive drug required to inhibit receptor binding of [³H]naloxone by 50% (ID_{50}). To obtain similar information regarding manganese ions we evaluated the influence of seven opiates upon [³H]naloxone binding measured in the absence of added cations, with manganese ions, NaCl, or both NaCl and manganese ions (Table 4). As reported previously, sodium reduces the ability of agonists to inhibit [³H]naloxone binding to a greater extent than antagonists. However, the shifts in ID_{50} values with sodium are not as pronounced as those reported earlier, because of differences between the present and former incubation conditions. In earlier studies (14) samples were immersed in ice prior to filtration. Recently we observed that

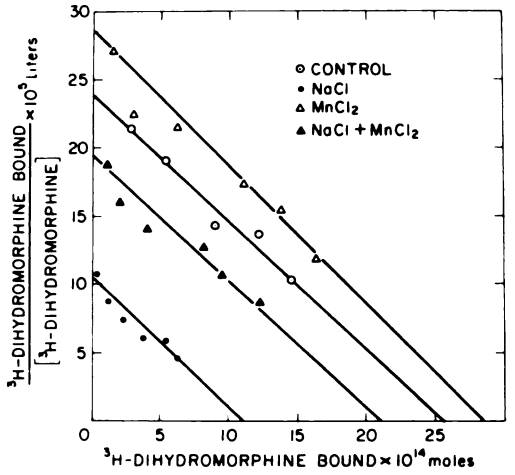

FIG. 1. *Effects of $MnCl_2$ and NaCl on Scatchard analyses of [³H]dihydromorphine binding*

Rat brains were prepared and assayed with various concentrations of [³H]dihydromorphine (0.04–1.2 nM) and either 1 mM $MnCl_2$, 100 mM NaCl, both 1 mM $MnCl_2$ and 100 mM NaCl, or neither, as described in MATERIALS AND METHODS. Opiate specific binding was determined as previously described and plotted according to Scatchard (20). The experiment was replicated three times.

incubations at low temperatures, even in the absence of sodium, selectively depress agonist and enhance antagonist binding.[4] Thus the previous pronounced effects of sodium upon ID_{50} values of drugs for [^{3}H]naloxone binding represented influences of both sodium and lowered temperature. In the present study incubations were conducted at 25° and samples were maintained at this temperature for filtration so that the sodium effect could be studied exclusively. In the absence of sodium, manganese decreases the ID_{50} values and increases the potency of all drugs for inhibiting [^{3}H]naloxone binding except for pentazocine. In the absence of sodium, therefore, there does not appear to be any pronounced differentiation between agonists and antagonists. However, when assayed with sodium, manganese ions decrease the ID_{50} concentrations of the opiate agonists levorphanol, morphine, oxymorphone, and methadone 3–6-fold. By contrast, the ID_{50} values for the opiate antagonists nalorphine and levallorphan are reduced only 1.7- and 1.2-fold, respectively, by manganese when assays are conducted with sodium. The mixed agonist-antagonist pentazocine displays a 2.7-fold reduction in its ID_{50} value in the presence of manganese.

Influence of chelating agents upon divalent cation interactions with opiate receptor binding. The selective influence of low concentrations of certain divalent cations upon [^{3}H]dihydromorphine binding suggests that the endogenous sources of these cations may play a physiological role in regulating the opiate receptor. If endogenous divalent cations are involved in opiate receptor binding, treatment of brain membranes with chelating agents should produce effects in a direction opposite from that of divalent cations such as manganese. Concentrations of EDTA between 0.1 and 10 mM reduce [^{3}H]dihydromorphine binding when assayed with sodium by about 50%, with no marked change in the extent of reduction over this concentration range (Table 5). The influence of EDTA on [^{3}H]dihydromorphine binding is much less when assayed in the absence of sodium, while EDTA does not significantly alter [^{3}H]naloxone binding under any conditions. While EDTA chelates manganese, magnesium, and calcium, EGTA[5] is a chelating agent with selectivity for calcium. Accordingly, it is striking that 1–10 mM concentrations of EGTA have no significant effect on [^{3}H]dihydromorphine binding. Thus, if EDTA reduced [^{3}H]-dihydromorphine binding by removing an endogenous divalent cation which normally stimulates binding, this cation is probably not calcium. Such a conclusion is reinforced by experiments involving the addition of divalent cation-complexed EDTA. Complexing magnesium or manganese to EDTA attenuates the ability of EDTA to reduce [^{3}H]dihydromorphine binding, while the calcium derivative of EDTA lowers [^{3}H]dihydromorphine binding to the same extent as EDTA added as the sodium salt. If calcium were the endogenous divalent cation whose removal by EDTA had lowered [^{3}H]dihydromorphine binding, the addition of calcium together with the EDTA should have prevented the effect of EDTA upon receptor binding. The observation that calcium-complexed EDTA reduces binding to the same extent as the sodium salt of EDTA indicates that calcium is not likely to be the endogenous divalent cation involved in opiate receptor regulation, while magnesium and manganese may be likely candidates.

To determine whether the removal of the endogenous divalent cation regulator of opiate receptor binding by chelating agents is a reversible process, we performed the following experiment. After treatment of brain membranes with and without EDTA, the treated membranes were washed free of EDTA, and divalent cations were added to preparations which were than assayed in the presence of 100 mM NaCl (Fig. 2). Even after the EDTA has been washed away, brain membranes treated with EDTA exhibit a 70% reduction in [^{3}H]dihydromorphine binding. In

[4] I. Creese, G. W. Pasternak, C. B. Pert, and S. H. Snyder, manuscript in preparation.

[5] The abbreviation used is: EGTA, ethylenebis[(oxyethylenenitrilo)]tetraacetic acid.

TABLE 4

Effect of manganese ions on ID_{50} of unlabeled opiates

Rat brain membranes were prepared and assayed with [^{3}H]naloxone in triplicate with five concentrations of the appropriate opiates. [^{3}H]Naloxone was present at 1 nM, and control binding was 3000 cpm, 3400 cpm in the presence of 1 mM $MnCl_2$, 3400 cpm in the presence of 100 mM NaCl, and 3400 cpm in the presence of both 1 mM $MnCl_2$ and 100 mM NaCl. ID_{50} values were determined by log probit analysis. The experiment was repeated three times.

Assay conditions	ID_{50}				ID_{50} NaCl/ ID_{50} NaCl + $MnCl_2$
	Absence of sodium		With 100 mM sodium		
	Control	$MnCl_2$	Control	$MnCl_2$	
	nM		*nM*		
Methadone	4.5	1.9	65	11	5.9
Oxymorphone	2.0	1.0	19	4.2	4.5
Morphine	2.5	0.6	29	7	4.1
Levorphanol	0.9	0.5	6.4	2.5	2.7
Pentazocine	16	18	95	35	2.7
Nalorphine	3.9	2.2	11	6.6	1.7
Levallorphan	0.75	0.57	1.5	1.2	1.2

TABLE 5

Effects of chelators on binding of [^{3}H]naloxone and [^{3}H]dihydromorphine

Rat brain tissue was prepared and assayed with the specified amounts of the appropriate EDTA complex as described in MATERIALS AND METHODS. Control binding of [^{3}H]naloxone and [^{3}H]dihydromorphine was 3900 cpm and 6100 cpm, respectively, in the absence of NaCl, and 4000 cpm and 2600 cpm, respectively, in the presence of 100 mM NaCl. Values represent opiate specific binding and are means of triplicate samples, which varied less than 15%. The experiment was replicated three times.

Addition	Concentration	Assayed with 100 mM NaCl		Assayed without NaCl	
		[^{3}H]Naloxone	[^{3}H]Dihydro-morphine	[^{3}H]Naloxone	[^{3}H]Dihydro-morphine
	mM	*% control*		*% control*	
EDTA	0.1	109	40	111	90
	1.0	83	51	83	82
	5.0	90	46	82	69
	10.0	83	49	74	67
EGTA	1.0	97	88	95	91
	5.0	89	85	86	91
	10.0	95	90	103	104
$MgNa_2$ EDTA	1.0	93	82	109	85
$MnNa_2$ EDTA	1.0	95	83	112	98
$CaNa_2$ EDTA	1.0	94	58	105	70

the absence of EDTA, manganese treatment almost doubles [^{3}H]dihydromorphine binding; moreover, the EDTA-induced reduction of [^{3}H]dihydromorphine binding is reversed by manganese. Magnesium enhances [^{3}H]dihydromorphine binding 40% in membranes not treated with EDTA. As observed with manganese, magnesium also reverses the EDTA-induced lowering of [^{3}H]dihydromorphine binding, restoring receptor binding to levels of membranes treated with magnesium alone.

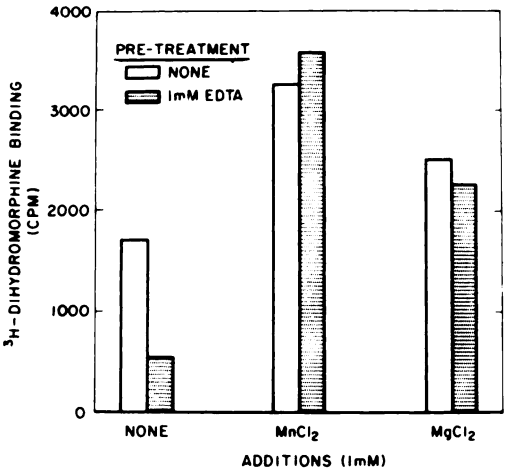

FIG. 2. *Influence of $MnCl_2$ and $MgCl_2$ on [^{3}H]dihydromorphine binding after treatment of rat brain membranes with EDTA*

Rat brain membranes were prepared as described in MATERIALS AND METHODS and treated with EDTA (1 mM) for 20 min at 25°. After the membranes had been centrifuged and the supernatant containing the EDTA discarded, the membranes were assayed with the appropriate ion at 1 mM and [^{3}H]dihydromorphine (0.9 nM) with 100 mM NaCl as described in MATERIALS AND METHODS. All values are the means of triplicate determination and represent opiate specific binding. The experiment was replicated three times.

The finding that membranes treated with EDTA exhibit a reduction in [^{3}H]dihydromorphine binding even after removal of the EDTA indicates that the lowering of [^{3}H]dihydromorphine binding is not due to the EDTA itself, but presumably to removal of some divalent cation which had been complexed by it.

Scatchard analysis of EDTA effects on [^{3}H]dihydromorphine binding shows changes consistent with a decrease in the number of [^{3}H]dihydromorphine binding sites (Fig. 3). While EDTA elicits a slight decrease in the number of [^{3}H]dihydromorphine binding sites, this decrease is markedly accentuated by sodium ions. Manganese can completely reverse the effects of EDTA alone and nearly reverses the effects of both EDTA and sodium.

Relationship between manganese and sodium effects. The influence of manganese upon opiate receptor binding, which is virtually the opposite of the influence of sodium and is maximized in the presence of sodium, appears to derive from a reversal of the sodium effect. Thus the effects of manganese ions upon receptor binding or radioactive or nonradioactive opiates are most pronounced when assays are conducted in the presence of sodium. To determine whether the manganese and sodium effects on receptor binding are related, we compared the shift in ID_{50} values for opiates upon [^{3}H]naloxone binding produced by sodium with the analogous manganese shift, measured in the presence of sodium (Fig. 4). There is an extremely close correlation between the ability of sodium to decrease the inhibitory potency of unlabeled opiates upon [^{3}H]naloxone binding and the ability of manganese to increase inhibitory potency. The zero-order correlation coefficient between the influences of sodium and manganese is 0.97.

If, as suggested above, manganese acts to a major extent by altering the sensitivity of opiate receptor binding of agonists to

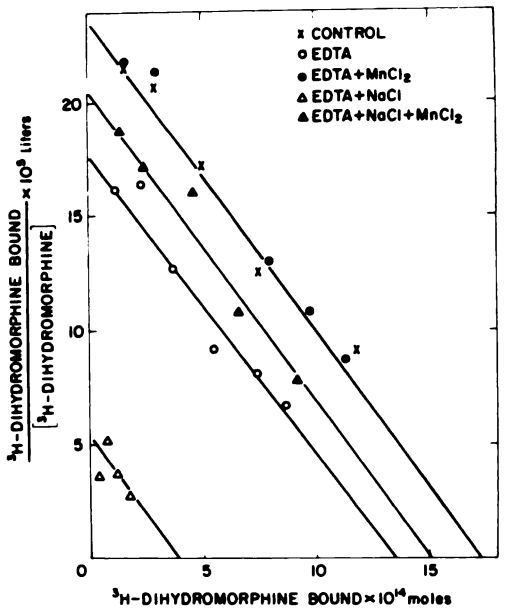

FIG. 3. *Effects of EDTA and $MnCl_2$ on Scatchard analyses of [^{3}H]dihydromorphine binding*

Rat brains were prepared and assayed with various concentrations of [^{3}H]dihydromorphine (0.4–1.2 nM) and either 1 mM EDTA, 1 mM EDTA and 1 mM $MnCl_2$, 1 mM EDTA and 100 mM NaCl, 1 mM EDTA, 1 mM $MnCl_2$, and 100 mM NaCl, or nothing as described in MATERIALS AND METHODS. Data were analyzed as described in Fig. 1. The experiment was replicated twice.

sodium, one might anticipate that manganese would decrease the ability of sodium to reduce agonist binding. Accordingly, we measured the influence of a wide range of sodium concentrations upon receptor binding of [^{3}H]dihydromorphine in the presence of 1 mM manganese, magnesium, and calcium (Fig. 5). In the absence of added divalent cations, the ID_{50} of sodium for [^{3}H]dihydromorphine binding is 27 mM. Manganese markedly decreases the sensitivity of [^{3}H]dihydromorphine binding to sodium, increasing the ID_{50} value of sodium 5-fold, up to 140 m. Magnesium and calcium, which are less active than manganese in enhancing [^{3}H]dihydromorphine binding, increase the ID_{50} of sodium only about 2-fold.

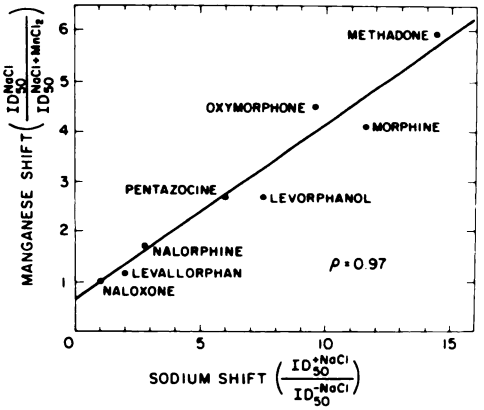

FIG. 4. *Correlation of sodium effect and its reversal by manganese effect*

Assays were performed as described in Table 5, and the ratio of ID_{50} of various opiates in the presence of NaCl to the ID_{50} in the presence of both NaCl and $MnCl_2$ was compared with the ratio of ID_{50} values in the presence and absence of NaCl. The zero-order correlation coefficient (ρ) was calculated on an Olivetti Programma 101 (Program 15.07) and is 0.97.

DISCUSSION

The principal finding of this study is that certain divalent cations at low concentrations selectively enhance opiate receptor binding of agonists. Experiments with chelating agents suggest that some endogenous divalent cation normally serves as a regulator of opiate receptor function. Which might be the best candidates for such an endogenous modulator of the opiate receptor? Manganese produces the most marked influences on [^{3}H]dihydromorphine binding. Magnesium is less potent than manganese but produces similar effects. Endogenous levels of magnesium are about 6 mM throughout the brain, while calcium and manganese levels are about 1.3 and 0.03 mM, respectively (21). The endogenous divalent cation regulator of the opiate receptor need not occur in very high concentrations in the free form. A trace element such as nickel might be the relevant divalent cation and be highly

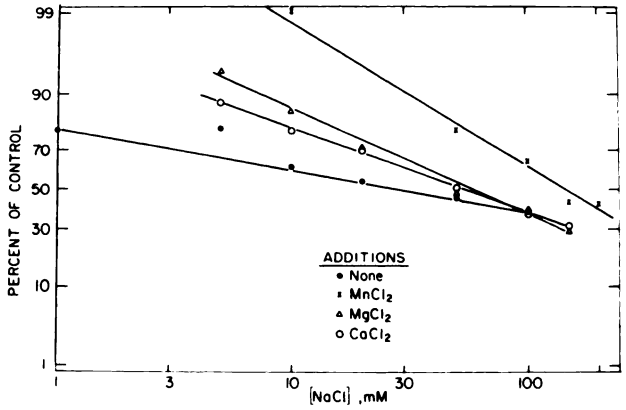

FIG. 5. *Influence of manganese on ID_{50} of NaCl for [^{3}H]dihydromorphine binding*

Rat brains were prepared and assayed with the appropriate ions as described in MATERIALS AND METHODS. All values were determined from triplicate samples and represent opiate specific binding. [^{3}H]Dihydromorphine was present at 0.9 nM, and control binding was 5300 cpm in the absence of manganese and 6200 cpm in its presence. The experiment was replicated three times.

concentrated as a tightly bound component of the opiate receptor. Because of its very weak effects upon opiate receptor binding, the failure of the calcium-specific chelator EGTA to alter opiate receptor binding, and the ability of EDTA complexed with calcium to lower [^{3}H]dihydromorphine binding, it is unlikely that calcium is the endogenous divalent cation which regulates the opiate receptor.

Since the divalent cation effects upon the opiate receptor are exerted best in the presence of sodium and since the ability of manganese to reverse the sodium reduction of opiate agonist binding correlates closely with the influence of sodium itself upon receptor binding, we suspect that manganese acts primarily by decreasing the sensitivity of the opiate receptor to sodium. This conclusion is supported by experiments showing that manganese increases the ID_{50} of sodium for [^{3}H]dihydromorphine binding by almost 5-fold.

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