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Modulation of μ_1, μ_2, and δ opioid binding by divalent cations

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The importance of divalent cations on opiate receptor binding was first demonstrated prior to the identification of the various different receptor subtypes [1]. In these studies divalent cations, especially manganese, enhanced the binding of agonist ligands. This action was most pronounced in the presence of sodium chloride, which greatly reduces agonist binding [2]. Since the identification of receptor binding subtypes [3–8], the issue of divalent cations has been explored by a number of laboratories [9–12]. These reports confirmed the earlier report and extended the findings to both μ and δ receptors. Recently we examined the effects of magnesium on μ_1 binding using a selective assay [13]. In this study we also observed a significant potentiation of μ_1 binding in the presence of magnesium. Most of the previous studies examined μ and δ binding sites but did not address the issue of multiple μ receptors. One report noting the ability of magnesium to increase both μ_1 and μ_2 binding did not study other cations [11]. In the present study we have examined the sensitivity of μ_1, μ_2 and δ binding to magnesium, manganese and calcium.

Materials and Methods

All radioligands and Formula 963 scintillation fluor were purchased from the New England Nuclear Corp. (Boston, MA). Fresh calf brains were obtained locally, dissected into the appropriate brain region, and prepared as previously reported and frozen [13]. Stored at −70°, tissue binding is stable for at least 1 month. All binding was performed in potassium phosphate buffer (50 mM; pH 7.0) for 150 min at 25° and assays were filtered over Whatman B glass fiber filters using a Brandel Cell Harvester. Mu_1 binding was determined in thalamic homogenates (2 mL; 15 mg wet weight tissue/mL) using [^{3}H][D-Ala^2,D-Leu^5]enkephalin (DADL; 39.9 Ci/mmol, 0.8 mM) in the presence of [D-Pen^2,D-Pen^5]enkephalin (DPDPE) (10 nM). The inclusion of DPDPE eliminates the δ labeling of [^{3}H]DADL, leaving only μ_1 binding. [^{3}H][D-Ser^2,Leu^5]enkephalin-Thr^6 (DSLET) has a binding selectivity profile very similar to that of [^{3}H]DADL; it also labels μ_1 sites in the presence of DPDPE (10 nM). Mu_2 binding was determined in thalamic homogenates (2 mL; 15 mg wet weight tissue/mL) using [^{3}H][D-Ala^2,MePhe^4,Gly(ol)^{5}enkephalin (DAGO; 30.3 Ci/mmol, 0.8 nM) in the presence of DSLET (5 nM). Since [^{3}H]DAGO labels both μ_1 and μ_2 sites, DSLET is included to inhibit μ_1 binding. DADL also can be used in the assay to inhibit the μ_1 component of [^{3}H]DAGO binding. Delta binding was determined in homogenates of frontal cortex (2 mL; 15 mg wet weight tissue/mL) using the highly δ-selective ligand [^{3}H]DPDPE (51.5 Ci/mmol, 0.5 nM). All determinations were performed in triplicate within an assay, and each experiment was replicated at least three times. Nonspecific binding was determined in the presence of levallorphan (1 μM). Only specific binding is reported. All values are presented as means ± SE. Statistical evaluations were determined using analysis of variance. K_D and B_{max} values were determined by nonlinear regression analysis of the saturation data [13].

Results and Discussion

First, we examined the effects of increasing concentrations of the different cations in the three binding assays. Magnesium sulfate at concentrations ranging from 10 to 50 mM increased μ_1 binding over 3-fold (Fig. 1A), a result similar to that previously reported [13]. Although magnesium sulfate also increased the binding in the μ_2 and δ assays, the increases were far less (approximately 50%). Half-maximal increases by magnesium sulfate were similar for all the assays examined, approximately 1 mM. Previous work has established that magnesium chloride and magnesium sulfate affect opioid binding in a similar manner [1, 13].

Manganese chloride had a similar effect, increasing the binding in the μ_1 assay by over 2-fold with a maximal effect between 0.1 and 1 mM (Fig. 1B), a concentration less than that seen with the magnesium sulfate. However, the increases produced by manganese chloride were far less than those elicited by magnesium ions. Manganese chloride had a far less pronounced action on μ_2 and δ binding than on μ_1 binding. Calcium chloride also increased binding with results quite similar to those observed with manganese chloride (Fig. 1C). Little effect was seen on either the μ_2 or the δ assays, whereas we observed approximately a 75–80% increase in the μ_1 assay.

We next examined the effects of these ions on the affinity (K_D) and the B_{max} of the three assays (Fig. 2). Manganese and magnesium both increased δ binding predominantly through increases in the B_{max}. In contrast, the effects of the different cations were more complicated in both of the μ assays. Of the different ligands examined, magnesium produced the largest increase in B_{max} and affinity.

Magnesium ions greatly prolonged the dissociation of [^{3}H]DADL in the μ_1 binding assay [13]. In the present assay, we examined the effects of calcium and manganese ions on the dissociation of [^{3}H]DADL in a μ_1 selective

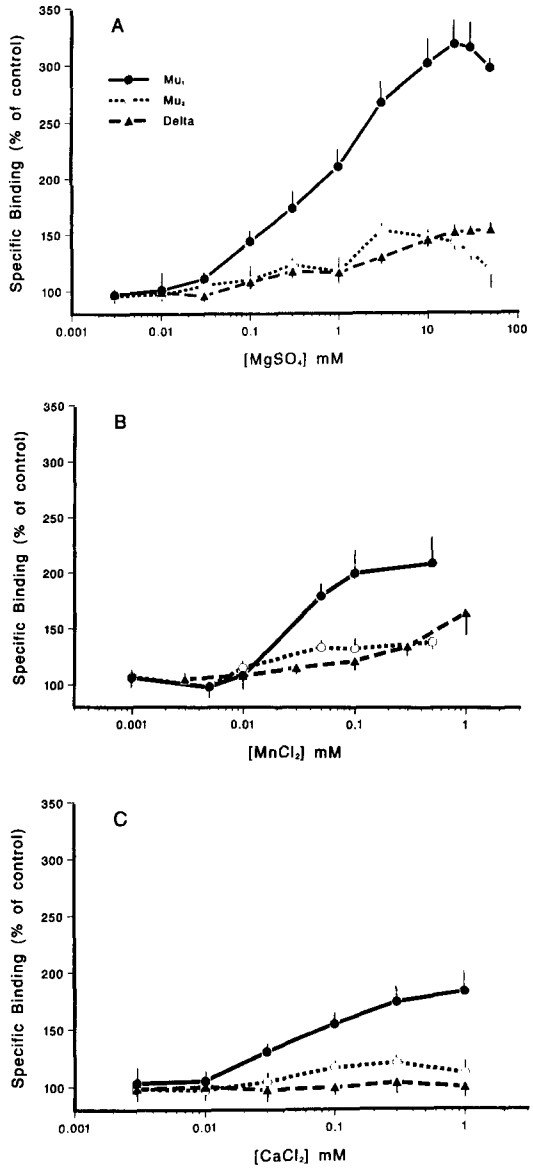

Fig. 1. Effects of increasing divalent cation concentrations on μ_1, μ_2 and δ binding. Binding of μ_1, μ_2 and δ was performed as described in Materials and Methods, and the effects of various concentrations of (A) magnesium sulfate, (B) manganese chloride, or (C) calcium chloride were examined. Binding is expressed as the percentage of control binding and is the mean ± SE of three separate experiments. Control binding (100%) was 570 ± 58 cpm for the μ_1 assay, 1450 ± 87 cpm for the μ_2, and 726 ± 14 cpm for the δ assay.

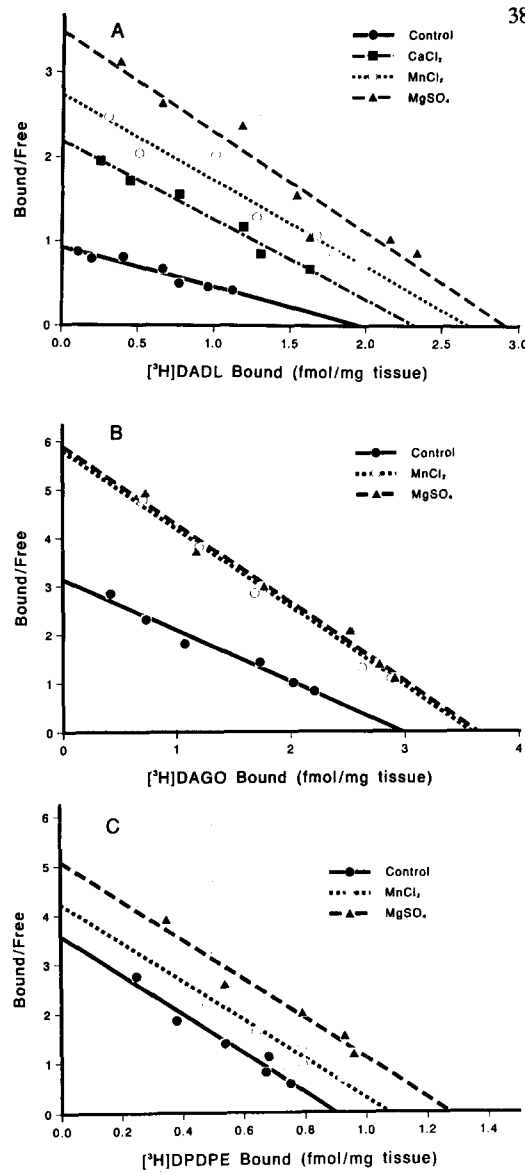

Fig. 2. Effects of divalent cations on saturation analysis of μ_1, μ_2 and δ binding. The saturation curves with the state ligands were analyzed by nonlinear regression analysis to obtain values for both K_D and B_{max}. Scatchard plots of representative experiments are presented. The K_D and B_{max} values are the means ± SE of separate determinations. (A) μ_1 binding of [^{3}H]DADL (0.12 to 2.8 nM) without any divalent cations (K_D 2.7 ± 0.4 nM; B_{max} 2.4 ± 0.2 fmol/mg tissue; N = 8) and in the presence of $CaCl_2$ (0.3 mM; K_D 1.2 ± 0.2 nM, P < 0.05; B_{max} 2.7 ± 0.2 fmol/mg tissue, NS; N = 3), $MnCl_2$ (0.1 mM; K_D 1.4 ± 0.2 nM, P < 0.05; B_{max} 3.1 ± 0.3 fmol/mg tissue, NS; N = 6) and $MgSO_4$ (5 mM; K_D 1.1. ± 0.1 nM, P < 0.05; B_{max} 3.5 ± 0.2 fmol/mg tissue, P < 0.05; N = 6). (B) μ_2 binding of [^{3}H]DAGO (0.17 to 2.7 nM; N = 4) without any divalent cations (K_D 1.1 ± 0.1 nM; B_{max} 3.5 ± 0.2 fmol/mg tissue) and in the presence of $MnCl_2$ (0.1 mM; K_D 0.7 ± 0.1 nM, P < 0.05; B_{max} 4.0 ± 0.2 mol/mg tissue, P < 0.05) and $MgSO_4$ (5 mM; K_D 0.8 ± 0.1 nM, NS; B_{max} 4.2 ± 0.2 fmol/mg tissue, P < 0.05). (C) δ binding of [^{3}H]DPDPE (0.1 to 1.6 nM; N = 3) without any divalent cations (K_D 0.24 ± 0.02 nM; B_{max} 0.91 ± 0.02 fmol/mg tissue) and in the presence of $MnCl_2$ (0.1 mM; K_D 0.27 ± 0.01 nM, NS; B_{max} 1.19 ± 0.05 fmol/mg tissue, P < 0.05) and $MgSO_4$ (5 mM; K_D 0.22 ± 0.02 nM, NS; B_{max} 1.24 ± 0.07 fmol/mg tissue, P < 0.05).

assay (Fig. 3). We observed a dramatic increase in the half-life of dissociation of the radioligand in the presence of either cation, similar to that seen with magnesium ions. This is greater than might have been predicted on the basis of the change in K_D values and probably is explained by the slower rate of association of the ligand in the presence of these divalents (data not shown) [13].

In conclusion, we found that the three divalent cations examined, magnesium, manganese and calcium, differentially affect μ_1, μ_2 and δ binding. Of the three assays, all three divalent cations exerted their greatest effect on μ_1 binding, and magnesium was the most effective. In the μ_1

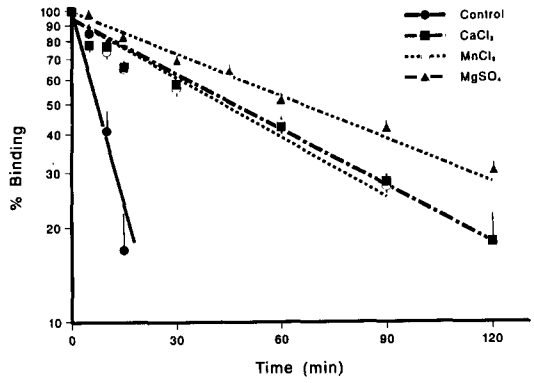

Fig. 3. Dissociation of μ_1 binding in the absence and presence of divalent cations. [^{3}H]DADL was incubated with tissue and either nothing, calcium chloride (0.3 mM), manganese chloride (0.1 mM) or magnesium sulfate (5 mM) for 2.5 hr, after which levallorphan (1 μM) was added and binding was assessed at the stated times. Results are the means ± SE of a typical experiment which has been replicated three times. Binding in these studies was 378 ± 21 cpm for the untreated control, 1081 ± 70 cpm for the calcium control and 1345 ± 140 cpm for the manganese control.

system, these cations exerted a complex effect with an increase both in affinity and in numbers of sites. In the δ assay, the increase in binding appeared to be an increase in the number of binding sites with no significant change in affinity. These differences among the receptor subtypes give further support to the division of μ receptors into μ_1 and μ_2 sites [6] and may help provide an understanding of potential mechanisms of binding. The increased binding elicited by the divalent cations coupled with the sensitivity of opioid agonist binding to sodium ions [2] and GTP and its analogs [9–12] suggests an important role for G-proteins. Although δ receptors are negatively coupled to cyclase, the second messenger system for μ_2 receptors remains unclear, and no system has been implicated in μ_1 actions [14].

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