

Sodium Regulation of Agonist Binding at Opioid Receptors.

I. Effects of Sodium Replacement on Binding at μ - and δ -Type Receptors in 7315c and NG108-15 Cells and Cell Membranes

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SUMMARY

The effects of varying the sodium concentration (at constant ionic strength) on opioid binding at μ - and δ -opioid receptors in 7315c and NG108-15 cells has been examined. The binding of [3 H]etorphine to μ -receptors on 7315c cells was increased by replacing the sodium in the incubation medium with potassium or *N*-methyl-D-glucamine. This effect was shown to be attributable to an increase in affinity, with no change in the maximum number of binding sites, both in cell membrane suspensions and in intact 7315c cells. Replacement of sodium with potassium or *N*-methyl-D-glucamine in NG108-15 membrane or intact cell suspensions also resulted in an increase in [3 H]etorphine binding, but in these cells the effect was associated with an increase in the number of binding sites measurable under these experimental conditions. The effects of sodium on opioid inhibition of adenylate cyclase in membrane preparations from 7315c and NG108-15 cells also differed. Sodium reduced apparent agonist affinity in 7315c membranes. In NG108-15 cell membranes, sodium was essential for the demonstration of opioid inhibition of cyclase activity. Increasing the sodium concentration above 0.5 mM resulted in an increase in the fraction of total enzyme activity

inhibited by opioid, but the opioid IC_{50} did not change. In the companion paper, it is shown that the effects of sodium removal on μ - and δ -receptor binding in guinea pig brain neural membranes were similar to those observed in the cell preparations. An increase in intracellular sodium concentration without change in extracellular concentration was effected by incubation of 7315c and NG108-15 cells with the sodium-selective ionophore, monensin. When sodium was present in the extracellular medium, monensin reduced [3 H]etorphine binding by 50% or more, both at μ -receptors in 7315c cells and at δ -receptors in NG108-15 cells. In the absence of sodium, however, monensin treatment produced only a small inhibition of binding. These results suggest that sodium acts at an intracellular site to regulate opioid agonist binding at both μ - and δ -receptors, but that the mode of regulation is not identical at each site. Since a reduction in intracellular sodium concentration by removal of extracellular sodium increases agonist binding, and an increase in intracellular sodium following monensin treatment reduces agonist binding, it is probable that the intracellular sodium concentration is a critical regulator of opioid agonist binding in intact cells.

The sensitivity of opioid-binding sites to sodium was first demonstrated by Simon *et al.* (1) and Pert *et al.* (2). Although all cations have some effects on opioid binding, opioid receptors are much more sensitive to regulation by sodium than by other common monovalent cations (1, 3). The effect of sodium noted in these studies was usually a reduction of opioid agonist affinity (4, 5), brought about largely through an increase in the rate of agonist dissociation (6). However, other studies have suggested that sodium induced changes in the density (number)

of opioid-binding sites in neural membranes (7-9). Resolution of this apparent discrepancy has been complicated by the heterogeneity of opioid-binding sites (10), a heterogeneity that was not appreciated in many of the earlier studies, and by the interactions in receptor regulation between mono- and divalent cations and guanine nucleotides (8, 11, 12).

In this paper we have examined the effects of sodium on ligand binding to homogeneous populations of receptors in NG108-15 neuroblastoma \times glioma hybrid cells and in 7315c pituitary tumor cells. It has been shown that opioid-binding sites in NG108-15 cell membranes behave as a single homogeneous binding site of the δ type (13, 14). Opioid receptors of the μ type which regulate prolactin secretion have recently been shown in the 7315c pituitary tumor cells (15). The use of

The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the view of the Department of Defense or the Uniformed Services University.

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ABBREVIATIONS: BSA, bovine serum albumin; DAGO, Tyr-D-Ala-Gly-N(Me)Phe-Gly-ol; DADLE, [D-Ala²-D-Leu⁵]enkephalin; DSLET, [D-Ser²-Leu⁵]enkephalyl-Thr; EGTA, ethylene glycol-bis(β -aminoethyl ether) *N,N'*-tetraacetic acid; EKC, ethylketocyclazocine; ET, etorphine; HEPES, *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid; K_D , dissociation constant estimated directly from saturation binding measures; K_i , dissociation constant estimated by competition against a labeled ligand; NAL, naloxone; N-MG, *N*-methyl-D-glucamine; U50488H, *trans*-3,4-dichloro-*N*-methyl-*N*-[2-(pyrrolidinyl)-cyclohexyl]benzeneacetamide methanesulfonate hydrate.

homogeneous populations of receptors in cells which can be obtained intact offers several advantages in the study of the sodium effect on opioid binding. The effects of the cation on one class of site are not confounded by interactions at other types of site. It is also possible to obtain presumptive evidence of the cellular location of the sodium-regulatory site by examining opioid binding in intact cells in which the use of the ionophore, monensin, allows the intracellular sodium concentration to be increased without changes in the extracellular concentration (16). In both cell types, opioids also inhibit adenylate cyclase activity (15, 17). It is therefore possible to examine the effects of sodium on opioid inhibition of adenylate cyclase in each cell type. We report here that sodium acts through an intracellular site to reduce the density (number) of δ -type sites detected by agonist binding in NG108-15 cells to 50% of the density observed in cells maintained in the absence of sodium. No change was observed in the affinity of the residual δ -agonist sites. In contrast, sodium reduces the affinity of agonists for the μ -type receptors in 7315c cells without any change in the number of binding sites. In the following paper (18), we report that sodium has similar effects on μ - and δ -receptors in membranes prepared from guinea pig cortex.

Materials and Methods

Chemicals. Chemicals and reagents were obtained from the following sources: [3 H]ET (45 Ci/mmol), [3 H]DADLE (41.8 Ci/mmol), and [3 H]DAGO (57.5 Ci/mmol) from Amersham Corp. (Arlington Heights, IL); [3 H]EKC and [3 H]NAL (38.6 Ci/mmol) from New England Nuclear (Boston, MA); monensin, GTP, ATP, and hypoxanthine-aminopterin-thymidine from Sigma Chemical Co. (St. Louis, MO); DAGO and DADLE from Cambridge Research Biochemicals, Ltd. (Atlantic Beach, NY); β -endorphin from Peninsula Laboratories (Belmont, CA); morphine sulfate from Merck (Rahway, NJ); glutamine and Dulbecco's modified Eagle's essential medium from Biofluids (Rockville, MD); fetal bovine serum from KC Biologicals (Lenexa, KS); Eagle's minimal essential medium containing 0.25% BSA from Gibco Laboratories (Grand Island, NY); cholera toxin from Schwartz/Mann (Orangeburg, NY); BSA (fraction V) from Miles Laboratories, Inc., (Elkhart, IN); U50488H, a gift from The Upjohn Company (Kalamazoo, MI); and levallorphan tartrate, a gift from Sterling Winthrop (Rensselaer, NY). The initial stock of neuroblastoma \times glioma (NG108-15) cells was a generous gift of Dr. Werner Klee (National Institute of Mental Health).

Preparation of NG108-15 membranes and cells for binding assays. NG108-15 cells were grown in 150-cm² plastic tissue culture flasks containing Dulbecco's modified Eagle's essential medium, 0.1 μ M hypoxanthine, 10 μ M aminopterin, 17 μ M thymidine, 2 nM glutamine, 0.1 M glucose, and 10% fetal bovine serum. The cells were maintained in a 37° humidified atmosphere of 10% CO₂ and 90% air. Cells were removed from the flasks by discarding the growth medium and rinsing the flask with the appropriate buffer. The cell suspension was then decanted into plastic tubes and centrifuged at 450 rpm (40 \times g) for 2 min. For studies on intact cells, the cells were rinsed twice in the appropriate buffer before resuspension at the required dilution. For studies with cell membranes, the 450 rpm cell pellet was disrupted by homogenization in the appropriate modified Krebs buffer, using a Teflon-glass homogenizer (four passes at speed setting 70 on T-Line laboratory stirrer; Thomas Scientific, Philadelphia, PA). The suspension was then washed twice by resuspension and centrifugation at 10,000 \times g for 15 min.

Preparation of 7315c cells and membranes for binding assays. Approximately 5 g of tumor tissue were excised from the peritoneal cavity of a rat carrying the 7315c tumor, and cells were prepared as previously described (15). The cells were rinsed twice in the appropriate buffer before resuspension at the required concentration. Mem-

branes were prepared from cells by resuspension in 20 volumes of a solution containing 6 mM Tris-HCl (pH 7.4), 2 mM EGTA, 1 mM MgSO₄, and 250 mM sucrose and disrupted with two to three 5-sec bursts of a Polytron generator (Brinkman Instruments, Westbury, NY). The homogenate was centrifuged at 120 \times g for 10 min, and the supernatant fluid was decanted into a fresh tube and centrifuged at 25,000 \times g for 30 min. The resulting pellet was resuspended in 6 mM Tris-HCl (pH 7.4), 2 mM EGTA, 1 mM MgSO₄, and 10% glycerol. These membranes were stored under liquid nitrogen for later use. Prior to use the membranes were thawed and rinsed three times in the appropriate modified Krebs buffer before resuspension at the required concentration.

Determination of adenylate cyclase activity. Adenylate cyclase activity was assayed as described previously (15, 19, 20). NG108-15 or 7315c cells were incubated for 3 hr in Eagle's minimal essential medium containing 0.25% BSA at 37° under an atmosphere of 95% O₂ and 5% CO₂ at a density of 10⁶ cells/ml in the presence of cholera toxin (30 nM). Treatment of 7315c cells with cholera toxin (30 nM) was found previously to increase adenylate cyclase activity by more than 300% and to improve quantitation of opiate-mediated inhibition of adenylate cyclase activity (15). The cAMP level was determined using a protein-binding assay (20).

Opioid binding assays. Receptor binding in cells and membranes was carried out by incubating the cell or membrane suspensions in a 1-ml (NG108-15) or 0.5-ml (7315c) final volume. For binding in the presence of sodium, modified Krebs buffer (25 mM HEPES, 118 mM NaCl, 4.8 mM KCl, 1.2 mM MgCl₂, 2.5 mM CaCl₂, pH adjusted to 7.4) was used. In some experiments, an equimolar concentration of either N-MG or potassium replaced sodium, as indicated in the text. Nonspecific binding was defined as the fraction of radioligand that remained bound in the presence of 1 μ M unlabeled levallorphan. Radioligand binding was estimated as described previously (21). All saturation experiments employed 12 radioligand concentrations (between 0.05 and 25 nM) of the radioligand. Protein concentrations were determined by a modification of the Lowry procedure (22).

Analysis of data. Saturation data were analyzed by the computer program LIGAND (23). This program utilizes a nonlinear least squares curve-fitting algorithm and assumes the simultaneous contribution of one or more independent binding sites. Data sets from replicate experiments have been modeled together. All possible models, in which parameters (i.e., K_D or B_{max}) were either assumed to be independent, or were constrained to be equal, in the presence and absence of sodium, were considered for each radioligand. The model best fitting the experimental data was selected on the basis of F test comparisons of the residual variances, and a runs test examining the sequences of positive and negative differences between the actual data points and the estimated binding curve. If the estimates for a parameter were not significantly different ($p > 0.05$) in the presence and absence of sodium, estimates for the model in which this parameter was constrained to be equal under the two experimental conditions are reported. The estimated reliability of a reported parameter value is indicated as a standard error of the parameter estimate, calculated by the LIGAND program.

Results

Characteristics of opioid-binding sites in NG108-15 and 7315c cell membranes. Opioid-binding sites on NG108-15 cells have previously been shown to behave as a homogeneous population of sites with properties characteristic of δ -receptors (13). In the present experiments, in which binding has been measured at 37° in the presence of appropriate cations, this result has been confirmed. NG108-15 cell membranes and intact cell suspensions showed high affinity displaceable binding of [3 H]DADLE and [3 H]ET, ligands with good affinity for δ -receptors, but did not show significant high affinity displaceable binding of [3 H]DAGO, a μ -receptor-selective ligand, or

[³H]EKC, a ligand with high affinity for κ -receptors and intermediate affinity for μ -receptors but with negligible affinity for δ -receptors under these experimental conditions (21). In both intact NG108-15 cell suspensions (Fig. 1A) and membrane suspensions (data not shown), binding of [³H]ET reached equilibrium by 20 min. Addition of a large excess of unlabeled DADLE after 30 min incubation produced almost complete dissociation of the labeled ligand within a further 60 min (Fig. 1A). Saturation analysis of [³H]ET binding to intact NG108-15 cells generated linear Scatchard plots (Fig. 2A), a result consistent with binding to a single class of binding sites.

Under our incubation conditions, 7315c cell membranes showed high affinity displaceable binding of [³H]DAGO and [³H]ET, ligands with high affinity for μ -receptors, but did not display any high affinity displaceable binding of [³H]DADLE or [³H]EKC (both ligands used in the presence of unlabeled DAGO to block binding to μ -receptors). Binding of [³H]ET by 7315c cell membranes was completely inhibited by DAGO, with a calculated K_i for inhibition of 9 nM (Fig. 3). The κ -selective agonist, U50488H, produced no inhibition at concentrations up to 1 μ M. DSLET inhibited [³H]ET binding to 7315c membranes with a K_i of about 90 nM, a value very similar to its estimated K_i for μ -receptors in guinea pig brain membranes under similar incubation conditions (21). These results suggest that the high affinity binding of [³H]DAGO and [³H]ET by 7315c membranes was to μ -type receptors.

In intact 7315c cell suspensions, binding of [³H]ET reached

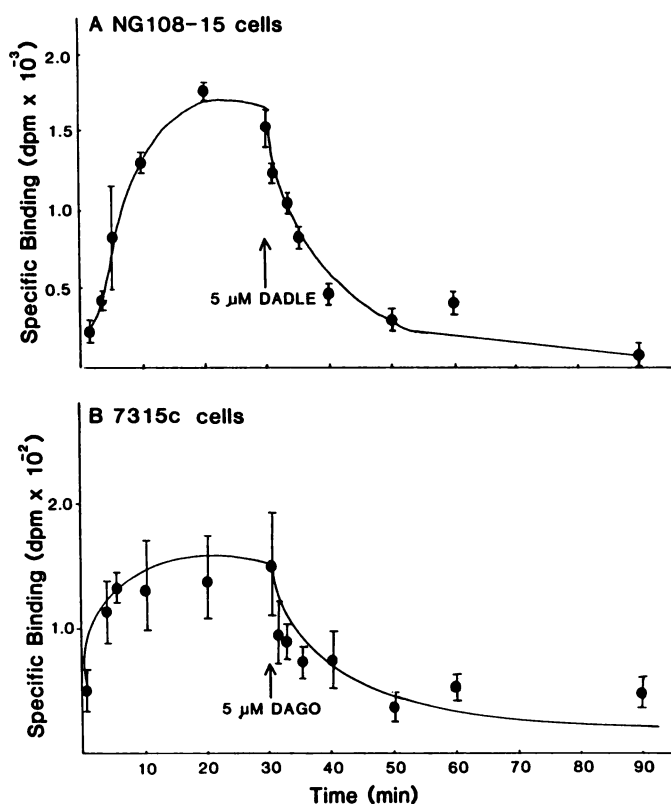


Fig. 1. Time course of binding to 2 nM [³H]ET to δ -receptors in NG108-15 cells (A) and μ -receptors in 7315c cells (B), and dissociation after addition (at arrow) of 5 μ M DADLE (A) or DAGO (B). Aliquots were filtered at indicated times, and specific binding was determined. Presented data are mean values (\pm SE) of triplicate measurements from a single experiment, which was replicated with similar results. A, 245 μ g of protein/assay tube; B, 34 μ g of protein/assay tube.

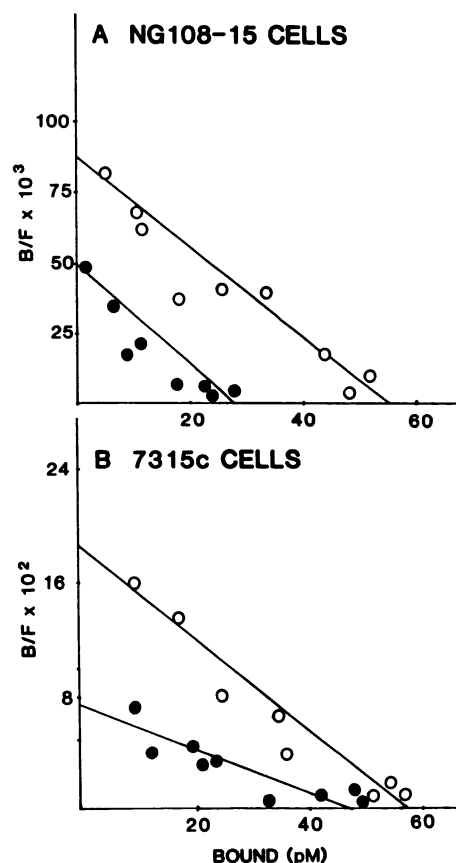


Fig. 2. Scatchard transformation of [³H]ET binding to δ -receptors in NG108-15 cells (A) or μ -receptors in 7315c cells (B) suspended in modified Krebs-HEPES buffer. Specific binding was measured at increasing radioligand concentrations from 0.05 to 25 nM, in the presence of 118 mM sodium (\bullet), or after equimolar replacement of sodium by N-MG (\circ). Data are mean values of triplicate determinations in single experiments, which were each replicated twice with similar results. Estimates of K_D and B_{max} from the combined experiments are listed in Table 1. A, 160 μ g of protein/assay tube, in the presence and absence of sodium; B, 240 μ g of protein/assay tube in the presence of sodium, 300 μ g of protein/assay tube in the absence of sodium. Other details of the incubation conditions are described under Materials and Methods.

equilibrium within 15 min (Fig. 1B). Addition of a large excess of DAGO after 30 min incubation displaced most (about 75%) of the specifically bound [³H]ET within a further 20 min. All other experiments reported here were terminated after 20 min incubation to reduce the extent of possible internalization of bound ligand. The concentration of [³H]ET-binding sites on 7315c cells was found to be considerably lower than their concentration on NG108-15 cells, and variances in binding estimates were generally higher in experiments with 7315c cells and membranes. However, Scatchard plots of [³H]ET binding in 7315c cells and membranes did not deviate significantly from linearity (e.g., Fig. 2B). Thus, our results with both intact 7315c cells and membrane homogenates are consistent with [³H]ET binding to a single class of binding sites with μ -receptor properties in these cells.

Effects of sodium replacement on agonist binding by NG108-15 and 7315c cells and membranes. When sodium in the modified Krebs buffer was replaced by an equimolar concentration of potassium, binding of [³H]DADLE and [³H]ET by NG108-15 cell membranes was increased. Scatchard analysis indicated that the effect was attributable entirely to

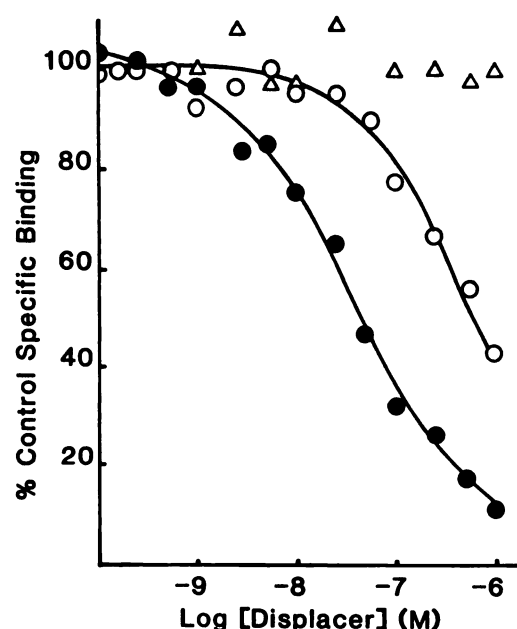


Fig. 3. Inhibition by opioids of specific binding of [3 H]ET, 2 nM, by 7315c membranes. Nonspecific binding was estimated in the presence of 1 μ M levallorphan. Points are the mean values of triplicate determinations: ●, DADLE; ○, DSLET; △, U50488H.

an increase by about 2-fold in apparent B_{\max} , with no significant change in the affinity (Table 1). Replacement of sodium by potassium also increased binding of [3 H]ET in 7315c membranes, although in this tissue the effect was attributable to an increase in affinity of the agonist by a factor of about 2-fold, with no significant change in the number of binding sites (Table 1).

Similar experiments were conducted in intact cell suspensions, with the exception that sodium was replaced with an

equimolar concentration of N-MG (24) instead of potassium. Again, removal of sodium resulted in an increase (88%) in apparent B_{\max} with no change in affinity in NG108-15 cells, but a 2-fold increase in affinity with no change in B_{\max} in 7315c cells (Table 1, Fig. 2).

Effects of sodium replacement on binding of antagonist by NG108-15 and 7315c membranes. Binding of the opioid antagonist, [3 H]NAL, by membranes from either NG108-15 or 7315c cells was not substantially affected by replacement of sodium by potassium. In both NG108-15 and 7315c membranes, neither K_D nor B_{\max} was altered significantly (Table 1). In both cell types, the number of [3 H]NAL-binding sites was similar to the number of [3 H]ET-binding sites measured in the presence of sodium.

Effects of monensin on binding of [3 H]ET by NG108-15 and 7315c cells. In order to determine the cellular location of sodium-regulatory sites at δ -receptors in NG108-15 cells, and at μ -receptors in 7315c cells, we have employed the sodium-selective ionophore, monensin, to increase intracellular sodium concentration without changing the extracellular concentration. In intact NG108-15 cells suspended in sodium containing modified Krebs buffer, monensin reduced the specific binding of [3 H]ET by more than 70% (at 50 μ M), the concentration of monensin producing half-maximal effect being between 1 and 5 μ M (Fig. 4A). However, in NG108-15 cells in modified Krebs buffer in which sodium was replaced by N-MG, monensin (1–50 μ M) failed to produce significant inhibition of binding (Fig. 4B). Similar results were obtained in 7315c cell suspensions. Specific [3 H]ET binding was reduced about 60% by monensin in the presence of sodium, with half-maximal reduction of binding occurring at a monensin concentration between 1 and 5 μ M (Fig. 5A). In the absence of sodium, however, no consistent dose-related effect of monensin of [3 H]ET binding was observed (Fig. 5B). Thus, monensin reduced agonist binding in both cell

TABLE 1

Opioid binding by NG108-15 and 7315c cells and cell membranes in the presence and absence of sodium

Cell membranes or intact cell suspensions were incubated for 20 min at 37° with several concentrations of radioligands, in modified Krebs buffer or in buffer in which sodium was replaced by an equimolar concentration of potassium (for membranes) or N-MG (for intact cells). The number of independent replicate experiments under each condition is indicated in the table (n). Results were analyzed by a nonlinear curve-fitting algorithm (23). Under each condition, a single-site model fitted the experimental data better than more complex models. Where no significant differences ($p > 0.05$) in K_D or B_{\max} estimates between the plus and minus sodium conditions were observed, combined estimates of that parameter value are reported, except in the case of NAL binding in NG108-15 membranes. The tabulated values are the estimates of K_D or B_{\max} (\pm standard error of the parameter estimate) from a combined analysis of the results from n independent experiments.

Cell type	Radioligand	Na ⁺ concentration	n	K_D	Percentage ^a	B_{\max}	Percentage ^a
		mM		nM		fmol/mg protein	
Membranes	NG108-15	DADLE	118	2	2.3 \pm 0.7	230 \pm 27	200
		0	2	2.3 \pm 0.7	100	460 \pm 64 ^b	
	ET	118	3	0.67 \pm 0.1	140 \pm 9	330 \pm 19 ^b	236
		0	3	0.67 \pm 0.1	100		
	NAL	118	2	5.2 \pm 2.5	115 \pm 43	146 \pm 56	125
		0	2	11 \pm 5.2	212		
7315c	ET	118	3	1.6 \pm 0.3	26 \pm 2.7	26 \pm 2.7	100
		0	3	0.71 \pm 0.15 ^b	44		
	NAL	118	2	0.25 \pm 0.08	18 \pm 2.1	18 \pm 2.1	100
		0	2	0.30 \pm 0.09	120		
	ET	118	3	0.5 \pm 0.05	195 \pm 11	367 \pm 15 ^b	188
		0	3	0.5 \pm 0.05	100		
Intact cells	7315c	ET	118	3	0.6 \pm 0.14	48 \pm 5.4	48 \pm 5.4
		0	3	0.3 \pm 0.09 ^b	50		

^a K_D and B_{\max} are given as percentage of the value in the presence of sodium.

^b Significantly different ($p < 0.001$) from the value in the presence of sodium.

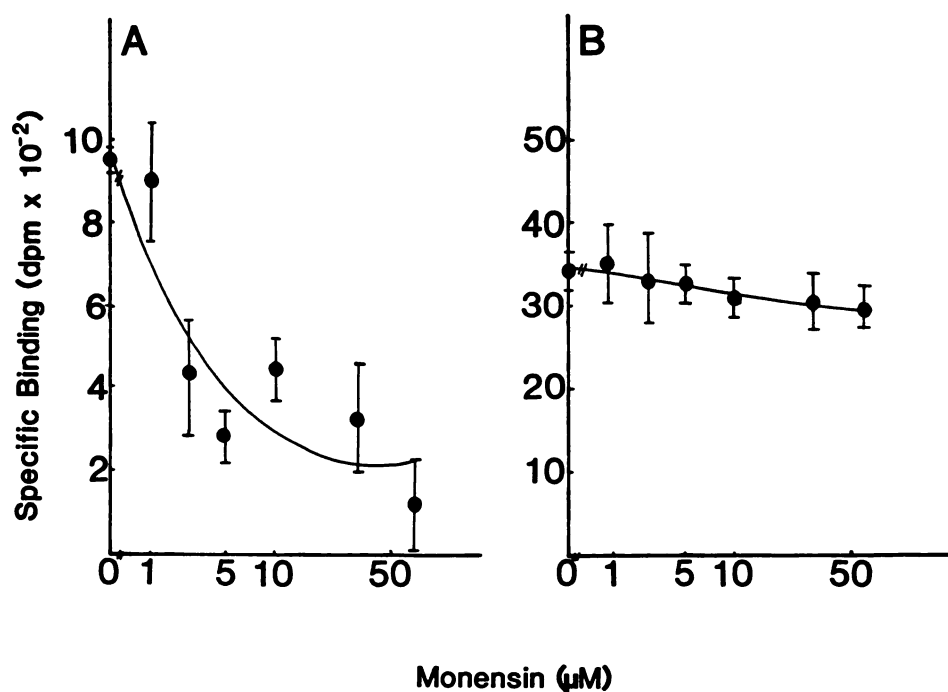


Fig. 4. Effects of monensin on the specific binding of [³H]ET, 2 nM, by δ -receptors in NG108-15 cells: A, in modified Krebs-HEPES buffer containing 118 mM sodium; B, in modified Krebs-HEPES in which sodium was replaced by an equimolar concentration of N-MG. Results are the mean \pm SE of triplicate determinations. Replicate experiments yielded similar results.

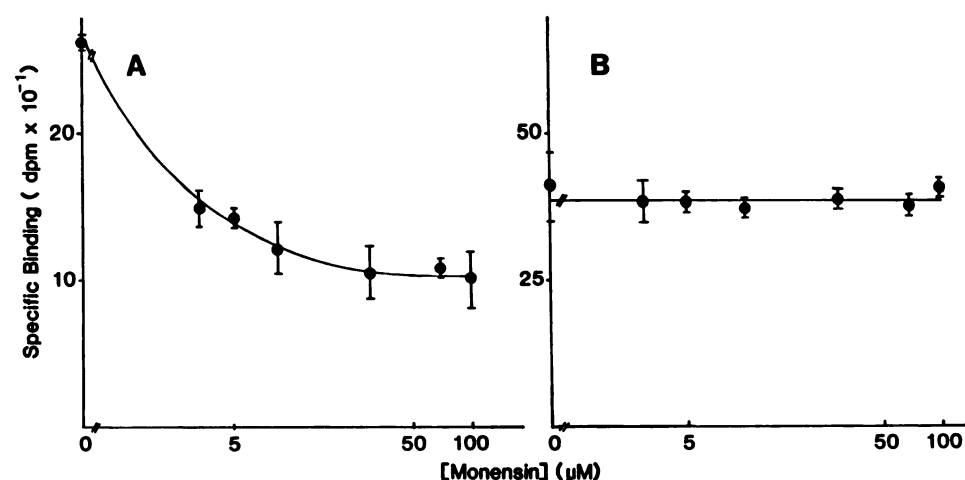


Fig. 5. Effects of monensin on the specific binding of [³H]ET, 2 nM, by μ -receptors in 7315c cells: A, in modified Krebs-HEPES containing 118 mM sodium; B, in modified Krebs-HEPES in which sodium was replaced by an equimolar concentration of N-MG. Results are the mean \pm SE of triplicate determinations. Replicate experiments yielded similar results.

types, provided that sodium was present in the extracellular fluid. In homogenized membrane preparations from NG108-15 and 7315c cells, however, monensin produced only a slight inhibition of [³H]ET binding which was independent of monensin concentration between 3 and 100 μ M and not altered by the removal of sodium (Table 2).

Saturation analysis of the effects of 30 μ M monensin on the specific binding of [³H]ET by intact NG108-15 and 7315c cells in the presence of 118 mM sodium demonstrated that monensin reduced the number of specific binding sites for this agonist in NG108-15 cells from 101 to 54 fmol/mg of protein (data from two independent experiments modeled together) without any change in affinity (combined estimate of K_D , 0.43 nM). Thus, the effect of adding monensin on the number of high affinity binding sites in these cells is similar to the effect of an increase in sodium concentration. In contrast, addition of monensin to 7315c cells did not change the number of binding sites for [³H]ET (two independent experiments, combined estimate of B_{max} , 19 fmol/mg of protein) but reduced affinity (K_D increased from 2.4 to 4.0 nM; $p < 0.01$).

TABLE 2

Effects of monensin on specific binding of [³H]ET by NG108-15 and 7315c membranes

Washed membranes from NG108-15 or 7315c cells were incubated for 20 min with [³H]ET, 2 nM, in modified Krebs buffer containing either 118 mM sodium (+Na⁺) or 118 mM N-MG (−Na⁺), in the presence of varying concentrations of monensin. The table shows the specific binding of [³H]ET in the presence of varying concentrations of monensin, expressed as a percentage (\pm standard error of the mean of triplicate estimates) of the specific binding in the absence of monensin. The experiment was replicated with similar results.

Monensin concentration μ M	Percentage of control binding in absence of monensin			
	NG108-15 membranes		7315c membranes	
	+Na ⁺	−Na ⁺	+Na ⁺	−Na ⁺
3	97 \pm 7	82 \pm 4	93 \pm 1	92 \pm 2
5	88 \pm 3	89 \pm 9	99 \pm 3	100 \pm 11
10	86 \pm 4	93 \pm 9	98 \pm 1	99 \pm 4
30	77 \pm 5	86 \pm 12	99 \pm 2	100 \pm 9
70	81 \pm 10	79 \pm 7	89 \pm 4	91 \pm 2
100	88 \pm 14	79 \pm 8	96 \pm 3	98 \pm 3

Effects of sodium on opioid inhibition of adenylate cyclase activity in NG108-15 and 7315c cell membranes. Opioids have been shown to inhibit adenylate cyclase activity in both NG108-15 and 7315c membranes (15, 17). Sodium has also been shown to be essential in the coupling of opioid receptors to adenylate cyclase in NG108-15 membranes (25). In view of the apparent differences in the manner of sodium regulation of opioid binding in these two membrane preparations, it was of interest to determine whether opioid regulation of adenylate cyclase activity might be differentially affected by sodium in membranes prepared from the two cell types. In these studies, DADLE was used as the agonist, since it has good affinity at both μ - and δ -receptors. The effects of varying the sodium concentration on DADLE regulation of adenylate cyclase was tested. In the absence of opioid, adenylate cyclase activity in membrane incubates from both types of cell was enhanced by increasing the sodium concentration from 0.5 to 100 mM. At the lowest sodium concentration, inhibition of adenylate cyclase in NG108-15 membranes by DADLE (1 nM to 1 μ M) was negligible. However, at 100 mM sodium, significant inhibition of adenylate cyclase was observed, with a maximum inhibition of about 50–60% of control activity (Fig. 6). At an intermediate sodium concentration (30 mM), DADLE induced inhibition of adenylate cyclase, but the maximum inhibition was about 30–40% of control enzyme activity. The IC_{50} for DADLE in NG108-15 cell membranes was about 10 nM both

at 30 and at 100 mM sodium. The sodium dependence of opioid inhibition of adenylate cyclase in NG108-15 membranes was also confirmed in experiments with β -endorphin, where the peptide produced no inhibition of enzyme activity at concentrations up to 10 μ M when the sodium concentration was 0.5 mM. At 100 mM sodium, maximum inhibition of adenylate cyclase by β -endorphin was about 50% of control enzyme activity, and the β -endorphin IC_{50} was about 100 nM. These results are consistent with previous reports of the sodium dependence of opioid inhibition of adenylate cyclase in NG108-15 membranes (25) and show that the maximum inhibition, but not the IC_{50} , is dependent on the sodium concentration.

In 7315c membranes in the presence of 0.5 mM sodium, DADLE reduced adenylate cyclase activity, with a maximum effect of about 50% inhibition at 10 μ M. At higher sodium concentrations, enzyme activity increased by about 20%, and all of the increased activity was inhibitable by DADLE (Fig. 7). The IC_{50} for DADLE also increased as the sodium concentration was raised, from about 4.5 nM at 0.5 mM sodium, through 45 nM at 30 mM sodium, to about 130 nM at 100 mM sodium. In another experiment, the IC_{50} for morphine inhibition of adenylate cyclase activity in 7315c membranes increased from 40 nM in 0.5 mM sodium, through 60 nM in 10 mM sodium, 300 nM in 30 mM sodium, to 2.5 μ M in 100 mM sodium. Thus, in 7315c membranes, in contrast to NG108-15 membranes, agonist potency was reduced by an increase in sodium concentration.

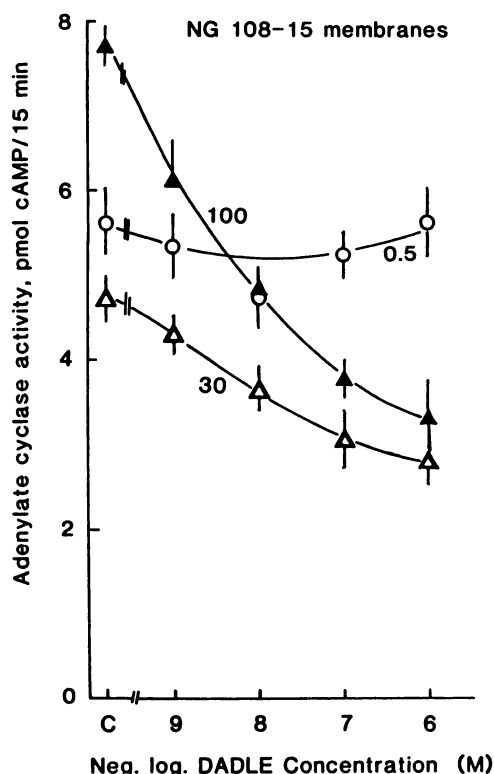


Fig. 6. Effects of sodium on opioid inhibition of adenylate cyclase in NG108-15 cell membranes. Membranes from NG108-15 cells were incubated with increasing concentrations of DADLE, as described in Materials and Methods, at three sodium concentrations: \circ , 0.5 mM sodium; Δ , 30 mM; \blacktriangle , 100 mM. Vertical bars indicate the standard errors of the mean estimates of triplicate determinations. No opioid inhibition of enzyme activity was observed at 0.5 mM sodium; increasing opioid inhibition was seen as the sodium concentration was increased to 100 mM, but the opioid IC_{50} did not change.

Discussion

Binding sites for opioids in NG108-15 and 7315c cells have different properties. Our results support previous studies (13)

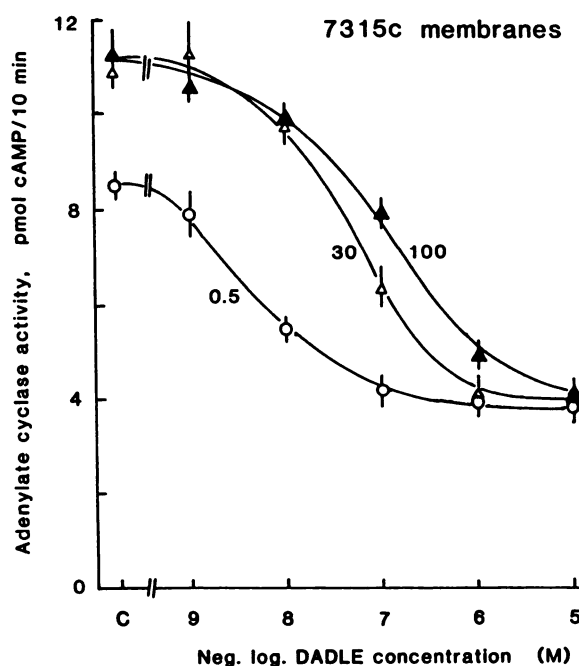


Fig. 7. Effects of sodium on opioid inhibition of adenylate cyclase in 7315c cell membranes. Membranes from 7315c cells were incubated with increasing concentrations of DADLE, as described in Materials and Methods, at three sodium concentrations: \circ , 0.5 mM; Δ , 30 mM; \blacktriangle , 100 mM. Vertical bars indicate the standard errors of triplicate determinations at each concentration of DADLE. The IC_{50} for DADLE increased with increasing sodium concentration.

indicating that the radiolabeled ligands we have used bind to a homogeneous population of δ -opioid-binding sites in NG108-15 cells. In both membranes and intact cell suspensions, binding reached an apparent equilibrium within 20 min. The ability of an excess of unlabeled agonist to displace previously bound ligand suggests that substantial internalization of receptor-bound ligand had not occurred after 30 min of incubation. In subsequent studies, incubation was limited to 20 min.

The 7315c cells and membranes showed high affinity binding of [3 H]ET which was readily inhibited by the μ -selective agonist, DAGO, and unaffected by the κ -selective agonist, U50488H. DSLET inhibited [3 H]ET binding to 7315c membranes with an affinity comparable to its affinity at μ -receptors. These results, together with previous functional studies (15), suggest that the binding site labeled by [3 H]ET in 7315c cells has μ -receptor properties, although further studies are needed to characterize this binding site fully. After 30 min incubation of intact 7315c cells with [3 H]ET, about 75% of the specifically bound ligand could be displaced by unlabeled DAGO. It is not at present clear whether the undisplaced ET was bound to another set of binding sites with very low affinity for DAGO (i.e., non- μ sites) or reflected internalized receptor-bound ligand. Since an apparent binding equilibrium was reached in these cells in about 15 min, all subsequent incubations were limited to 20 min to reduce the extent of possible receptor-ligand internalization. In 7315c cells and membranes, Scatchard plots of specific [3 H]ET binding were linear. Our results indicate that most specifically bound [3 H]ET in intact 7315c cells was reversibly bound to cell surface receptors with μ -receptor characteristics.

It should be noted that the 7315c cells were obtained by dispersal of solid tumor masses. There was some variability between different tissue preparations with regard to the number of binding sites in this tissue, expressed relative to tissue protein. Since cells in the tumor are not necessarily derived from a single clone, variations between preparations in the levels of receptor expressed might be anticipated. It is also possible that the extent of infiltration of the tumor with other tissues varies between preparations. However, when comparisons of treatments were made within experiments using a single cell preparation, the effects of changing the sodium concentration on opioid binding were consistent.

The major finding in these studies is that sodium regulates opioid agonist binding at δ -receptors in NG108-15 cells in a manner different from its regulation of agonist binding at μ -receptors in 7315c cells. In 7315c cell membranes, replacement of sodium by potassium resulted in an increase in agonist affinity by about 2-fold. No change was seen in the number of binding sites following sodium removal. The effects of sodium removal cannot be attributed to a change in ionic strength, since ionic strength was maintained constant in these studies. Similar results were obtained in intact cell suspensions when sodium was removed from the extracellular medium [a treatment which reduces intracellular sodium concentration (16)], indicating that the effect of sodium on agonist affinity was not dependent on fragmentation of the cell membrane. These results also indicate that the effect of sodium removal is not attributable to the action of a particular replacement ion. Thus, sodium appears to reduce the affinity of agonists at μ -receptors in 7315c cells.

The effect of sodium removal on opioid binding in NG108-

15 cells was not identical to its effects in 7315c cells. In NG108-15 membranes, an increase by a factor of about 2 in the number of observed specific binding sites was noted, with no change in the affinity of the binding sites. This effect occurred both with a peptide agonist, DADLE, and with an alkaloid derivative, ET, and with two different replacement cations, potassium and N-MG. Removal of sodium from the incubation medium also increased the number of [3 H]ET-binding sites in intact NG108-15 cells. It is therefore unlikely that the effect of sodium removal was associated with a particular ligand or replacement ion, or the integrity of the cell membrane. Thus, in contrast to its effects on μ -receptors in 7315c cells, sodium reduces the number of δ -binding sites measurable by saturation binding.

These results clearly indicate that the consequences of sodium regulation of ligand affinity are different at μ - and δ -receptors. This difference in sodium regulation of opioid binding is probably not a function of different membrane organization or composition in two different types of cell, since we show in the following paper (18) that similar differences in the consequences of sodium regulation of agonist binding at μ - and δ -receptors are apparent in a membrane preparation from guinea pig cortex in which both receptor types can be examined. This demonstration, that the effects of sodium on agonist binding at μ and δ sites differs, may provide a resolution of the early, apparently conflicting, reports on the nature of the sodium effect on opioid agonist binding (4-9).

The ability of sodium to reduce the number of high affinity δ -binding sites requires further consideration. It is improbable that binding sites are destroyed by exposure to sodium since the reduction in binding induced by sodium was reversible (all cells were initially prepared in sodium-containing buffer). Furthermore, the number of binding sites for the antagonist, NAL, was not changed by exposure to sodium. The effect of sodium on δ -binding sites is probably to reduce the affinity for agonists of about half the sites to very low values, leaving these sites undetectable by radiolabeled agonists when binding is determined by the rapid filtration technique. This argument is supported by the studies of Law *et al.* (26). By measuring displacement of radiolabeled antagonist binding at NG108-15 δ -receptors, they were able to demonstrate that 30-40% of the binding sites had very low affinity for DADLE when 100 mM sodium was present. In our experiments with labeled agonists, these very low affinity sites would not be detectable. Thus, a large reduction in agonist affinity at a fraction of the binding sites results in an apparent reduction in the total number of binding sites. In studies of antagonist binding to δ sites in guinea pig cortex membranes, we have recently shown that agonist competition curves were biphasic. Removal of sodium from the medium resulted in a significant increase in the ratio of high to low affinity binding sites, thus confirming our hypothesis that sodium produced a substantial reduction in the agonist affinity at a fraction of the δ -binding sites (18). It is probable that the sodium-dependent reduction in the number of δ high affinity binding sites is associated with the interaction of the receptor with guanine nucleotide-binding protein(s), since sodium reduction in agonist affinity at δ -receptors is potentiated by ADP ribosylation of the inhibitory guanine nucleotide-binding protein by pertussis toxin (27). Our results confirm earlier observations indicating that δ -receptors in NG108-15 cells are functionally coupled to adenylate cyclase in a sodium-dependent manner (25).

The cellular location of the site at which sodium induces regulation of agonist binding has not been established. We have employed the sodium-selective ionophore, monensin, to produce increases in intracellular sodium concentration without changing the extracellular concentration. Monensin has been shown to increase sodium uptake into NG108-15 cells (28) and has been employed in studies of sodium regulation of α_2 -adrenergic receptor binding in platelets (16). Our results show that, in both NG108-15 and 7315c cell suspensions, monensin treatment produced a reduction in opioid agonist binding, provided that sodium was present in the extracellular medium. After removal of sodium from the extracellular medium, monensin had very little effect on opioid binding. Moreover, monensin did not produce a sodium-dependent inhibition of binding in homogenized membranes of NG108-15 or 7315c cells. As an ionophore, monensin is roughly 10-fold selective for sodium relative to potassium and is much less effective in facilitating the trans-membrane transport of divalent cations (29). Monensin also produces a transient increase in intracellular pH in NG108-15 cells, but intracellular pH reverts to normal values within a few minutes following the addition of monensin (28). Thus, it is unlikely that the effects of monensin that we have observed are related to changes in intracellular pH, potassium, or divalent cations.

The simplest interpretation of our results is that monensin reduced agonist binding in NG108-15 and 7315c cells by increasing sodium flux through the cell membrane, allowing the intracellular concentration of this ion in the vicinity of opioid receptors to rise to a level at which agonist binding was partially inhibited. This interpretation is supported by the demonstration that monensin behaved in a manner similar to sodium in reducing agonist affinity at the μ -receptors in 7315c cells, but reducing the number of agonist-binding sites in NG108-15 cells without any change in the affinity of the residual binding sites. Thus, we conclude that, at both μ - and δ -receptors, sodium exerts its regulatory effect at an intracellular site. This suggests that sodium regulation of opioid binding at both μ - and δ -receptors is comparable to sodium regulation of α_2 -adrenergic receptor binding, which appears, on the basis of similar criteria, to be mediated through an intracellular regulatory site (16, 24). Since a reduction of intracellular sodium concentration by removal of extracellular sodium increases opioid agonist binding and an increase in intracellular sodium by monensin treatment reduces opioid agonist binding, in both NG108-15 and 7315c cells, it is probable that the normal intracellular sodium concentration in these cells is critically poised to regulate agonist binding. Agonist affinity will therefore be susceptible to modulation by treatments shifting the intracellular sodium concentration in either direction.

If the opioid-binding sites in NG108-15 and 7315c cells are implicated in the opioid inhibition of adenylate cyclase activity in these cells (15, 25), then sodium should exert different effects on the actions of opioids in each cell type. In NG108-15 cell membranes, opioid inhibition of adenylate cyclase activity was dependent on the presence of a sodium concentration in excess of 0.5 mM. Increasing the sodium concentration increased both the activity of the enzyme and the fraction of cyclase activity that was inhibitable by opioid, but did not change the potency of the opioid as measured by the concentration required to inhibit adenylate cyclase by 50% of the maximum inhibition. These results again suggest that addition of sodium assists in

the coupling of the δ -receptor to guanine nucleotide-binding protein and adenylate cyclase in NG108-15 cells. In 7315c cells, significant opioid inhibition of adenylate cyclase activity was observed in the presence of a very low sodium concentration. Increasing the sodium concentration not only increased enzyme activity but also resulted in an increase in the opioid agonist IC_{50} . This result is consistent with the reduction in agonist affinity observed with increasing sodium concentrations in these cells. Thus, functional studies of opioid effects in the two types of cell support the conclusion from ligand binding studies that sodium regulates agonist binding to μ - and δ -receptors by different mechanisms.

It should be noted that, although μ - and δ -receptors regulate adenylate cyclase activity in 7315c and NG108-15 cells, respectively, this may not be their usual function in normal mammalian neurons. The effects we have observed may all be associated with the manner of interaction of each receptor type with guanine nucleotide-binding protein(s). Opioid receptor-GTP-nucleotide-binding protein complexes may regulate different functions in different cells, or under different physiological circumstances.

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References

- Simon, E. J., J. M. Hiller, and I. Edelman. Stereospecific binding of the potent narcotic analgesic [3H]etorphine to rat brain homogenate. *Proc. Natl. Acad. Sci. USA* 70:1947-1949 (1973).
- Pert, C. B., G. Pasternak, and S. H. Snyder. Opiate agonists and antagonists discriminated by receptor binding. *Science (Wash. D. C.)* 182:1359-1361 (1973).
- Pert, C. B., and S. H. Snyder. Opiate receptor binding of agonists and antagonists affected differentially by sodium. *Mol. Pharmacol.* 10:868-879 (1974).
- Simantov, R., S. R. Childers, and S. H. Snyder. The opiate receptor binding interactions of 3H -methionine enkephalin, an opioid peptide. *Eur. J. Pharmacol.* 47:319-331 (1978).
- Simon, E. J., J. M. Hiller, J. Groth, and I. Edelman. Further properties of stereospecific opiate binding sites in rat brain: on the nature of the sodium effect. *J. Pharmacol. Exp. Ther.* 192:531-537 (1975).
- Blume, A. J. Interactions of ligands with the opiate receptors of brain membranes: regulation by ions and nucleotides. *Proc. Natl. Acad. Sci. USA* 75:1713-1717 (1978).
- Pasternak, G. W., and S. H. Snyder. Identification of novel high affinity opiate receptor binding in rat brain. *Science (Wash. D. C.)* 253:563-565 (1975).
- Simantov, R., A. M. Snowman, and S. H. Snyder. Temperature and ionic influences on opiate receptor binding. *Mol. Pharmacol.* 12:977-986 (1976).
- Lee, C.-Y., T. Akera, and T. M. Brody. Effects of Na^+ , K^+ , Mg^{++} and Ca^{++} on the saturable binding of [3H]dihydromorphine and [3H]naloxone *in vitro*. *J. Pharmacol. Exp. Ther.* 202:166-173 (1977).
- Lord, J. A. H., A. A. Waterfield, J. Hughes, and H. W. Kosterlitz. Endogenous opioid peptides: multiple agonists and receptors. *Nature (Lond.)* 267:495-499 (1977).
- Sadee, W., A. Pfeiffer, and A. Herz. Opiate receptor: multiple effects of metal ions. *J. Neurochem.* 39: 659-667 (1982).
- Chang, K.-J., S. G. Blanchard, and P. Cuatrecasas. Unmasking of magnesium dependent high-affinity binding sites for [DAla², DLeu⁵] enkephalin after pretreatment of brain membranes with guanine nucleotides. *Proc. Natl. Acad. Sci. USA* 80:940-944 (1983).
- Chang, K.-J., R. J. Miller, and P. Cuatrecasas. Interaction of enkephalin with opiate receptors in intact cultured cells. *Mol. Pharmacol.* 14:961-970 (1978).
- Chang, K.-J., and P. Cuatrecasas. Multiple opiate receptors: enkephalins and morphine bind to receptors of different specificity. *J. Biol. Chem.* 254:2610-2618 (1979).
- Frey, E., and J. Kebabian. A μ -opiate receptor in 7315c tumor tissue mediates inhibition of immunoreactive prolactin release and adenylate cyclase activity. *Endocrinology* 115:1797-1804 (1984).
- Motulsky, H. J., and P. A. Insel. Influence of sodium on the α_2 -adrenergic receptor system of human platelets: role for intraplatelet sodium in receptor binding. *J. Biol. Chem.* 258:3913-3919 (1983).

17. Sharma, S. K., M. Nirenberg, and W. Klee. Morphine receptors as regulators of adenylate cyclase activity. *Proc. Natl. Acad. Sci. USA* **72**:590-594 (1975).
18. Werling, L. L., S. R. Brown, P. Puttfarcken, and B. M. Cox. Sodium regulation of agonist binding at opioid receptors. II. Effects of sodium replacement on opioid binding in guinea pig cortical membranes. *Mol. Pharmacol.* **30**:90-95 (1986).
19. Cote, T. E., C. W. Grewe, K. Tsuruta, J. C. Stoof, R. L. Eskay, and J. W. Keibarian. D-2 dopamine receptor-mediated inhibition of adenylate cyclase activity in the intermediate lobe of the rat pituitary gland requires guanosine 5'-triphosphate. *Endocrinology* **110**:812-819 (1982).
20. Brown, B. L., J. D. M. Albano, R. P. Ekins, A. M. Sgherzi, and W. Tampion. A simple and sensitive saturation assay method for the measurement of adenosine 3',5' cyclic monophosphate. *Biochem. J.* **121**:561-562 (1971).
21. Werling, L. L., G. D. Zarr, S. R. Brown, and B. M. Cox. Opioid binding to rat and guinea pig neural membranes in the presence of physiological cations at 37°C. *J. Pharmacol. Exp. Ther.* **233**:722-728 (1985).
22. Peterson, G. L. A simplification of the protein assay method of Lowry et al., which is more generally applicable. *Anal. Biochem.* **83**:346-356 (1977).
23. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach for characterization of ligand binding systems. *Anal. Biochem.* **107**:220-239 (1980).
24. Connolly, T. M., and L. E. Limbird. The influence of Na⁺ on the α_2 -adrenergic receptor system of human platelets. *J. Biol. Chem.* **258**:3907-3912 (1983).
25. Blume, A. J., D. Lichstein, and A. Boone. Coupling of opiate receptors to adenylate cyclase: requirement for Na⁺ and GTP. *Proc. Natl. Acad. Sci. USA* **76**:5626-5630 (1979).
26. Law, P.-Y., D. S. Hom, and H. H. Loh. Multiple affinity states of opiate receptors in neuroblastoma × glioma NG 108-15 cells. *J. Biol. Chem.* **260**:3561-3569 (1985).
27. Wuster, M., T. Costa, K. Aktories, and K. H. Jakobs. Sodium regulation of opioid agonist binding is potentiated by pertussis toxin. *Biochem. Biophys. Res. Commun.* **123**:1107-1115 (1984).
28. Lichstein, D., K. Dunlop, H. R. Kaback, and A. J. Blume. Mechanism of monensin induced hyperpolarization of neuroblastoma-glioma hybrid NG 108-15. *Proc. Natl. Acad. Sci. USA* **76**:2580-2584 (1979).
29. Pressman, B. C. Ionophorous antibiotics as models for biological transport. *Fed. Proc.* **27**:1283-1288 (1968).

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