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Regulation of μ-Opioid Receptor in Neural Cells by Extracellular Sodium

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Abstract: SH-SY5Y neural cells expressing μ- and δ-opioid receptors were maintained viable in isotonic, sodium-free buffer in vitro. Intracellular sodium levels were manipulated by various methods, and ligand binding to intact cells was studied. In physiological buffer containing 118 m*M* sodium, [^{3}H]Tyr-D-Ala-Gly-(Me)Phe-Gly-ol ([^{3}H]-DAMGO) and [^{3}H]naltrexone bound to μ receptor with K_D values of 3.1 and 0.32 n*M* and B_{max} values of 94 and 264 fmol/mg of protein, respectively. Replacement of sodium by choline decreased the affinity of the antagonist and increased B_{max} for [^{3}H]DAMGO, without significantly affecting the other corresponding binding parameters. Depolarizing concentrations of KCl (34 m*M*) in physiological buffer decreased the intracellular sodium levels by 67%, but this did not decrease the [^{3}H]DAMGO binding to the cells. Incubation of cells with monensin and ouabain increased the intracellular sodium levels dramatically (from 78 to 250 and 300 nmol/mg, respectively), with no changes in agonist binding parameters. Ethylisopropylamiloride inhibited [^{3}H]DAMGO and [^{3}H]naloxone binding to intact cells with EC_{50} values of 24 and 3,600 n*M*, respectively. Adenylyl cyclase activities measured in intact cells, at different concentrations of sodium, showed the physiological significance of this ion in signal transduction. Potency of DAMGO in inhibiting the forskolin-stimulated adenylyl cyclase activity was significantly higher at lower concentrations of sodium. However, inhibition reached the maximal level only at 50 m*M* sodium, and typical sigmoidal dose–response curves were obtained only in the presence of 118 m*M* sodium. Furthermore, even at low or high intracellular sodium levels, DAMGO inhibition of cyclic AMP levels was normal. These results support a role for extracellular sodium in regulating not only the ligand interactions with the receptor, but also the signal transduction through the μ receptor. **Key Words:** Sodium—Opioid receptor—Potassium—Amiloride—Monensin—Cyclic AMP.
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The potent modulation of opioid receptor binding in isolated neural membranes by sodium has been well described (e.g., Pert et al., 1973; Simon et al., 1975), and the mechanism by which sodium enhances the binding of opioid antagonists and reduces that of agonists has been studied (Fischel and Medzihradsky, 1981). In many studies (e.g., Pert and Snyder, 1976), the effective concentrations of sodium (100–150 m*M*) that influenced ligand binding to opioid receptors in isolated membranes pointed to extracellular modulation by this cation. In contrast, maximal modulation of opioid receptor binding was observed at sodium concentrations typical for the intracellular milieu (10 m*M*) (e.g., Nijssen and Childers, 1987) in some studies. In 7315c cells, Puttfarcken et al. (1986) showed an intracellular sodium site by using ligand binding to the μ receptor. In the course of our work on characterization of ligand binding to intact cells, however, we observed that changes in intracellular levels of sodium ($[Na]_i$) under various conditions had no effect on ligand binding parameters. This report thus reexamined the site of sodium action influencing the agonist/antagonist binding and opioid agonist inhibition of adenylyl cyclase activity in intact neural cells. It is particularly important to study a homogeneous population of receptor in view of the differential effects of sodium on μ, δ, and κ receptors (Werling et al., 1986). Thus, all the studies presented in this report were carried out in SH-SY5Y cells, which express predominantly the μ receptor, and the results were confirmed in C6μ5 glial cells that are stably transfected with the cloned μ receptor.

MATERIALS AND METHODS

Materials

[^{3}H]Tyr-D-Ala-Gly-(Me)Phe-Gly-ol ([^{3}H]DAMGO; 60 Ci/mmol) was obtained from Amersham, and [^{3}H]-naltrexone (11 Ci/mmol) was kindly provided by the National Institute on Drug Abuse. [^{3}H]Naloxone (57.5 Ci/

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Abbreviations used: DAMGO, Tyr-D-Ala-Gly-(Me)Phe-Gly-ol; EIPA, ethylisopropylamiloride; $[Na]_i$, intracellular sodium concentration; $[Na]_o$, extracellular sodium concentration.

mmol) was from New England Nuclear, and ethylisopropylamiloride (EIPA) was from Research Biochemical International. Ouabain, monensin, DAMGO, and other biochemicals were purchased from Sigma Chemical Co. Dulbecco's modified Eagle's medium and fetal bovine serum were from GIBCO Laboratories (Grand Island, NY, U.S.A.), and cyclic AMP kits were from Diagnostic Product Corp.

Culture and treatment of cells

As described previously (Carter and Medzihradsky, 1992), human neuroblastoma SH-SY5Y cells were grown to confluency in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The confluent cells were washed with ice-cold phosphate-buffered saline and lifted off the plate by incubation in Ca^{2+},Mg^{2+}-free modified Puck's solution for 10 min. The suspension was pelleted at 200 *g* for 5 min, the cell pellet was washed with choline buffer, and the resulting pellet was suspended in the respective isotonic buffers. The basic physiological buffer (20 m*M* HEPES, 118 m*M* NaCl, 10 m*M* glucose, 3.0 m*M* $MgSO_4 \cdot 7H_2O$, 1.3 m*M* $CaCl_2 \cdot 2H_2O$, pH adjusted to 7.4 with Tris base at 37°C) was modified by replacing sodium with various concentrations of choline chloride in order to prepare isotonic buffers containing 0–118 m*M* sodium. Buffer containing 118 m*M* choline chloride has been referred to as the choline buffer throughout the text. In experiments designed to reduce $[Na]_i$ by depolarization, retinoic acid-differentiated SH-SY5Y cells were suspended in buffer A (128 m*M* NaCl, 2.4 m*M* KCl, 2.0 m*M* $NaHCO_3$, 3.0 m*M* $MgSO_4$, 10 m*M* Na_2HPO_4, 1.3 m*M* $CaCl_2$, 10 m*M* glucose, pH 7.4 at 37°C), and 34 m*M* KCl was added to induce depolarization.

Ligand binding

Ligand binding to intact cells was carried out in physiological buffers containing various concentrations of sodium in different experiments as described in the legends to the figures. Nonspecific binding was determined with either 1 μM DAMGO or naltrexone. The assay medium consisted of 50 μl of cell suspension (SH-SY5Y, 60–120 μg of protein; C6μ5, 30 μg of protein), 50 μl of radiolabeled drug, 50 μl each of ouabain, monensin, and EIPA (in selected experiments), and 300 μl of buffer medium. After ligand binding reached equilibrium at 37°C (50 min), contents of the tubes were quickly filtered by using a Brandel cell harvester, and the washed filters were subjected to scintillation counting.

Adenylyl cyclase assay

SH-SY5Y cells were differentiated with 10 μM retinoic acid for 6 days, collected, and resuspended in isotonic physiological buffers containing 8 m*M* theophylline and 0, 5, 10, 50, and 118 m*M* sodium. Cells were preincubated in the respective buffers at 37°C for 5 min, and the acute inhibition of adenylyl cyclase was assayed as described (Yabaluri and Medzihradsky, 1995). In brief, the assay mixture contained 50 μl of cell suspension (50–100 μg of protein), 20 μl of 50 μM forskolin, 20 μl of DAMGO solution (5–5,000 n*M*), and 10 μl of buffer. In experiments designed to increase $[Na]_i$, the buffer contained 118 m*M* sodium and 30 μM monensin. Cyclic AMP levels in the samples were estimated by using the radioligand binding assay kit from Diagnostic Product Corp.

Measurement of $[Na]_i$

Confluent cells were collected and suspended in buffers containing various concentrations of sodium under different experimental conditions. After incubation at 37°C for 50–60 min, samples (0.6–1.0 mg of protein) were centrifuged, and the cell pellet was washed three or four times with cold choline buffer. Subsequently, the washed cell pellets were digested in LiCl solution (15 mEq/L) with 70% HNO_3 at 70°C. Sodium concentrations in the solutions were determined by flame photometry as described earlier (Fischel and Medzihradsky, 1981).

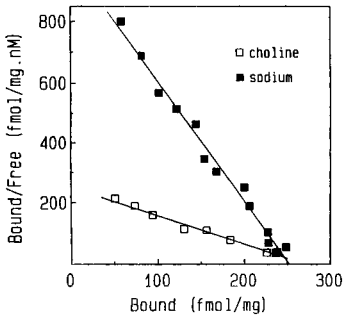

FIG. 1. Antagonist binding to intact SH-SY5Y cells. Cells were washed with isotonic choline buffer and resuspended in physiological buffer containing 118 m*M* sodium (■) or choline (□). [^{3}H]Naltrexone binding was studied at various concentrations (0.1–6.0 n*M*) as described in Materials and Methods. The corresponding binding parameters and data variability are listed in Table 1.

Protein determination

Protein in the samples was solubilized in 1 *M* NaOH prior to estimation by the method of Lowry et al. (1951), with bovine serum albumin as the standard.

Data analysis

The binding data from saturation experiments were analyzed by the statistical program SYSTAT (Wilkinson, 1988). Dose–response curves of DAMGO-inhibited adenylyl cyclase activity were fit and plotted using the nonlinear regression program Graphpad.prism (Graph Pad Software, San Diego, CA, U.S.A.).

RESULTS

SH-SY5Y cells were maintained viable in isotonic buffers containing different concentrations of sodium, the ionic strength being kept constant with the addition of choline chloride. Saturation binding of [^{3}H]-naltrexone to intact cells showed that sodium increases the affinity of the receptor for the antagonists (Fig. 1). The K_D decreased from 0.91 ± 0.12 n*M* to 0.32 ± 0.02 n*M* when sodium replaced choline in the buffer, and there was no change in the maximum number of binding sites, as the B_{max} was 258 ± 6.98 and 264 ± 10.4 fmol/mg, respectively. The increase in affinity was found to be gradual with respect to sodium concentration in the experiments conducted at various concentrations of sodium in the assay buffer; the values are given in Table 1.

Binding of [^{3}H]DAMGO to whole cells was sensitive to the presence of sodium in the assay buffer. Sodium reduced the B_{max} of [^{3}H]DAMGO binding sites as presented in Fig. 2. Analysis of the saturation

TABLE 1. *Parameters of [^{3}H]DAMGO and [^{3}H]naltrexone binding in intact cells*

	$[Na]_o$ (m*M*)				
	0	5	10	30	118
[^{3}H]DAMGO					
K_D (n*M*)	4.9 ± 1.5	6.3–8.7	7.1–8.4	7.0–9.8	3.1 ± 0.06
B_{max} (fmol/mg)	332 ± 18	289–315	253–281	220–238	94 ± 2.3
[^{3}H]Naltrexone					
K_D (n*M*)	0.91 ± 0.12	0.51–0.57	0.40–0.42	0.44–0.45	0.32 ± 0.02
B_{max} (fmol/mg)	258 ± 6.98	251–262	252–269	254–273	264 ± 10.4

SH-SY5Y cells were suspended in isotonic physiological buffers containing various concentrations of sodium, and binding of [^{3}H]DAMGO and [^{3}H]naltrexone to whole cells was carried out as described in Materials and Methods. Shown are means ± SEM of four or five experiments or values from two experiments.

binding data obtained in choline buffer yielded a B_{max} of 332 ± 18 fmol/mg, whereas in the presence of sodium it was 94 ± 2.3 fmol/mg (Table 1). It is likely that sodium converted a fraction of high-affinity sites into the low-affinity state, because the K_D increased from a value of 4.9 ± 1.5 to 7.0–9.8 n*M* with the addition of 30 m*M* sodium (Table 1). Previous work carried out in our laboratory estimated a K_D of ~150 n*M* for the low-affinity site (Carter and Medzihradsky, 1992), which cannot be detected by [^{3}H]DAMGO in saturation experiments. However, as multiple affinity states are known for the receptor (Werling et al., 1988), it is possible that DAMGO detects some population of lower-affinity form, in addition to the high-affinity receptor. As a result, the measured K_D, a statistical average of the total population, would be higher at lower concentrations of sodium. The binding affinity in 118 m*M* sodium buffers was again 3.1 ± 0.06 n*M*, possibly as a result of complete conversion of that fraction of receptor sites into low-affinity form that was rendered undetectable in saturation experiments. These effects on binding parameters were selective for sodium, because replacement of sodium with equimolar potassium in the preliminary experiments showed binding indistinguishable from that obtained in choline buffer.

To examine this high-affinity binding further, [^{3}H]-DAMGO binding was displaced by naloxone and morphine in choline and sodium buffers, and the curves are plotted in the inset to Fig. 2. Naloxone showed identical K_i values of 2.3 ± 0.86 n*M* and 2.4 ± 0.66 n*M*, whereas morphine displaced [^{3}H]DAMGO with a slightly lower K_i value of 3.26 ± 0.37 n*M* in sodium buffer in comparison with that observed in choline buffer (4.46 ± 0.55 n*M*). However, these values are not statistically significant. Initial time-dependence studies indicated a period of 50 min as the time required for equilibrium without any loss of specific binding due to the internalization of the receptor.

Agonist binding was studied at various concentrations of extracellular sodium ($[Na]_o$), and $[Na]_i$ was measured in order to examine the site of sodium regulation. As depicted in Fig. 3, $[Na]_i$ dropped to 6 ± 2.3 nmol/mg upon removal of sodium from the incubation buffer and increased progressively with the addition of sodium to the buffer. Maximal levels of $[Na]_i$, 76

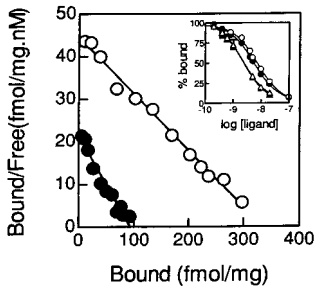

FIG. 2. Saturation binding of [^{3}H]DAMGO to intact cells in buffers containing 118 m*M* sodium (●) or choline (○). Cells were incubated with various concentrations (0.2–16 n*M*) of [^{3}H]-DAMGO at 37°C for 50 min, and the radioactivity bound was measured as described in Materials and Methods. **Inset:** The displacement of [^{3}H]DAMGO (0.6 n*M*) binding by naloxone (triangle) and morphine (circle) in choline (open) and sodium (filled) buffers. The experiments were repeated three or four times. The corresponding binding parameters and data variability are listed in Table 1.

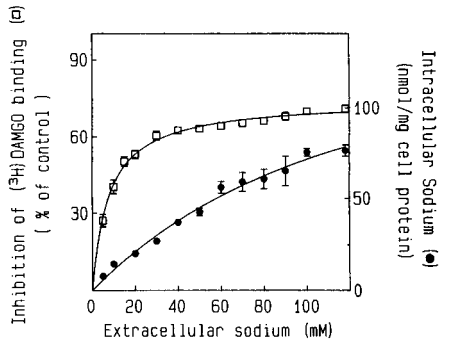

FIG. 3. Agonist binding with varying $[Na]_o$. Cells were incubated at 37°C in isotonic buffers containing 5–118 m*M* sodium and [^{3}H]DAMGO (2 n*M*) binding, and $[Na]_i$ measurements were made as described in Materials and Methods. Values are means ± SEM from six or seven experiments.

± 3.24 nmol/mg [i.e., that may correspond to 30–40 m*M* as measured by others (Motulsky and Insel, 1983)], were attained at 70–80 m*M* $[Na]_o$. In contrast, [^{3}H]DAMGO binding decreased rapidly with the inclusion of sodium (EC_{50} of $[Na]_o$, 15 m*M*) in the assay buffer, and maximal inhibition was observed at 60–70 m*M* $[Na]_o$. Thus, [^{3}H]DAMGO binding curve leveled off at 70–80 m*M* $[Na]_o$, whereas the increase in $[Na]_i$ was almost linear and 50% of the maximal $[Na]_i$ was obtained at 40 m*M* $[Na]_o$.

Reduction in $[Na]_i$

As the inhibition of agonist binding was associated with an increase in both $[Na]_o$ and $[Na]_i$, we decided to manipulate $[Na]_i$ by various approaches. The first strategy was to decrease $[Na]_i$ without changing $[Na]_o$ (Fozzard and Kipnis, 1967). Intact cells were suspended in physiological buffer containing 118 m*M* NaCl, and 34 m*M* KCl was added to decrease $[Na]_i$. As a result, $[Na]_i$ dropped by >60% in comparison with the control cells. Agonist binding was studied under these conditions, and the results are presented in Fig. 4. In spite of the significant reduction in $[Na]_i$, DAMGO binding was not altered. $[Na]_i$ (26 ± 5.0 nmol/mg) was comparable to that obtained in the presence of 20–30 m*M* $[Na]_o$, the concentrations that should have yielded higher binding, if $[Na]_i$ was responsible for the modulation of ligand binding.

Agonist binding at increased $[Na]_i$

$[Na]_i$ could be raised by incubating the cells suspended in physiological buffer containing 118 m*M* sodium in the presence of Na^+,K^+-ATPase inhibitor, ouabain (30 μM), and the sodium-specific ionophore, monensin (30 μM), as reported earlier (Connolly and Limbird, 1983; Puttfarcken et al., 1986). Figure 5a gives the details of [^{3}H]DAMGO binding in the presence of various (10–100 μM) concentrations of ouabain and monensin. Monensin and ouabain increased $[Na]_i$ by 3.5- and 1.5-fold over control values, respectively. However, [^{3}H]DAMGO binding to intact cells did not change at any concentration of monensin or ouabain. Scatchard plots of [^{3}H]DAMGO saturation

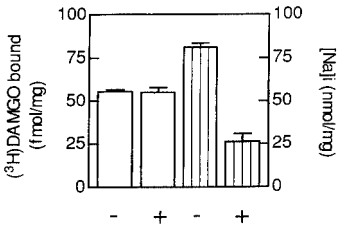

FIG. 4. Agonist binding to intact cells in the presence of depolarizing concentrations of potassium. SH-SY5Y cells differentiated with 10 μM retinoic acid were collected and resuspended in physiological buffers with 3.4 m*M* (−) or 34 m*M* (+) KCl. [^{3}H]-DAMGO binding (2 n*M*) (open bars) and $[Na]_i$ (striped bars) were measured as described in Materials and Methods. Values are means ± SEM from four experiments.

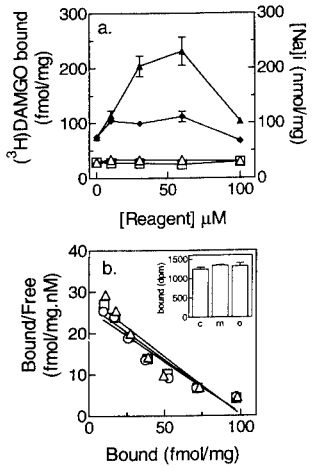

FIG. 5. [^{3}H]DAMGO binding to intact SH-SY5Y cells in physiological buffers containing 118 m*M* sodium in the presence of reagents that increase $[Na]_i$. **a:** $[Na]_i$ (filled symbols) and [^{3}H]-DAMGO binding (open symbols) were measured in the presence of ouabain (□, ♦) or monensin (△, ▲). Values are means ± SEM from three experiments. **b:** Saturation binding of [^{3}H]DAMGO in the absence (○) or presence of 30 μM ouabain (□) or 30 μM monensin (△) in intact SH-SY5Y cells. The data are from a representative experiment that was repeated three times. **Inset:** Agonist binding in C6μ5 cells, glial cells transfected with the cloned μ-opioid receptor. [^{3}H]DAMGO (3.2 n*M*) binding was determined in the presence of 30 μM ouabain (o) or 30 μM monensin (m) as described in Materials and Methods. Ouabain and monensin increased $[Na]_i$ by 300 and 450% over control, respectively. Values are means ± SEM.

isotherms obtained in the presence of 30 μM ouabain or monensin in sodium buffer demonstrate that neither the K_D nor the B_{max} was affected by the dramatic rise in $[Na]_i$ (Fig. 5b). Furthermore, in C6μ5 glial cells that are stably transfected with the cloned μ receptor, ouabain (30 μM) and monensin (30 μM) enhanced the $[Na]_i$ levels by >250 and >490%, respectively, again without any significant effect on agonist binding (inset to Fig. 5b).

However, it is possible that monensin and ouabain failed to show any effect on agonist binding, because the $[Na]_i$ in physiological buffers containing 118 m*M* sodium was already sufficient to cause the maximal regulation by this cation. Inhibition of agonist binding in our experiments was found to be linear up to 20 m*M* $[Na]_o$, which corresponded to ~20 nmol/mg $[Na]_i$ (Fig. 3). It was thought that any changes in $[Na]_i$ in that range might lead to a proportional reduction in agonist binding. In the next experiment, agonist binding to cells was studied in buffer containing 5 m*M* sodium and ouabain (30 μM) or monensin (0.1 μM). Under these conditions, $[Na]_i$ increased from 5–6 to 23–33 nmol/mg. This $[Na]_i$ corresponded to $[Na]_i$ in the presence of 30 m*M* $[Na]_o$. As shown in Fig. 6, however, binding in the presence of these two agents was similar to that in control, whereas 30 m*M* sodium in the extracellular buffer inhibited binding significantly. Monensin is an electrically neutral antiporter

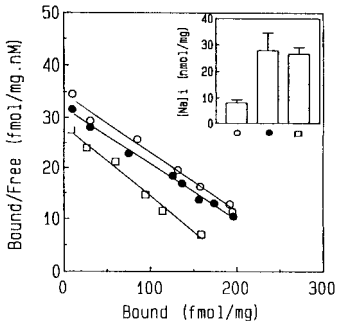

FIG. 6. Agonist binding in the presence of ouabain and monensin at lower $[Na]_o$. SH-SY5Y cells were collected as described in Materials and Methods and resuspended in physiological buffers containing 5 m*M* (○, ●) and 30 m*M* (□) sodium in the presence (●) or absence (○) of 30 μM ouabain and 1 μM monensin. Cells were incubated at 37°C for 50 min with various concentrations of [^{3}H]DAMGO, and binding was measured. **Inset:** $[Na]_i$ estimated under the same conditions. Values are means ± SEM from three experiments.

and couples the transport of sodium with exchange of H^+ or K^+ in a number of systems (Rochdi et al., 1996); these neutral ionophores are also shown to transport cations against the concentration gradients (Harris and Pressman, 1965, and references therein). Under the present experimental conditions, monensin increased $[Na]_i$ with a simultaneous release of potassium ions. At 5 m*M* $[Na]_o$, in the presence of ouabain and monensin, the intracellular potassium levels were 20–25% lower than control (223 ± 13 nmol/mg).

The next step was to study the binding in the presence of agents that are known to interact at the sodium binding sites. Agonist and antagonist binding was studied at various concentrations of EIPA, a potent amiloride analogue, and the results are presented in Fig. 7. EIPA inhibited [^{3}H]DAMGO binding (143 fmol/mg) completely with a K_i value of 14.2 ± 4.26 n*M*, and [^{3}H]naloxone binding (157 fmol/mg) was inhibited with a K_i value of 1.82 ± 0.337 μM.

To explore the effect of sodium further, we decided to study the signal transduction through the μ receptor. Inhibition of adenylyl cyclase activity by DAMGO was studied in intact cells at various concentrations of sodium, and the results are presented in Table 2. Forskolin-stimulated cyclic AMP levels were low in choline buffer (15.37 ± 2.02 pmol/min/mg) and were

TABLE 2. *DAMGO inhibition of adenylyl cyclase in intact cells with varying $[Na]_o$*

$[Na]_o$ (m*M*)	EC_{50} (n*M*)	Maximal inhibition (%)	Forskolin stimulation (% increase over control)
0	~1.1	27 ± 10.2	—
10	4.3 ± 0.308	61 ± 6.1	40 ± 6.80
50	5.8 ± 0.483	83 ± 1.5	107 ± 18.3
118	6.7 ± 0.344[a]	83 ± 1.7	122 ± 8.70

Values are means ± SEM (n = 4). Retinoic acid-differentiated SH-SY5Y cells were collected, washed with isotonic choline buffer, and used to measure DAMGO inhibition of forskolin-stimulated adenylyl cyclase activity. Cells were incubated at 37°C for 15 min in buffers containing different concentrations of sodium, and cyclic AMP levels were estimated as described in Materials and Methods.
[a] $p < 0.05$, compared with 10 m*M* sodium group.

enhanced by increasing sodium concentration in the incubation buffer, by 30–40% in buffer containing 10 m*M* and >100% in the presence of 118 m*M* NaCl (33.4 ± 4.89 pmol/min/mg). DAMGO inhibited forskolin-stimulated adenylyl cyclase activity in choline buffer by 20–27% with an EC_{50} of 1.1 n*M*. Inclusion of 50 m*M* sodium brought about the maximal inhibition of forskolin-stimulated adenylyl cyclase activity by DAMGO, which was ~85% under the experimental conditions, and the sigmoidicity of the curves improved with the gradual increments of sodium up to 118 m*M* in the assay buffer (Fig. 8). The EC_{50} of attenuation of cyclic AMP levels by DAMGO increased slightly with increasing sodium concentrations, and the values obtained in buffers containing 0, 10, and 118 m*M* sodium (1.1, 4.30 ± 0.308, and 6.7 ± 0.344 n*M*, respectively) were significantly different ($p < 0.05$). Cyclic AMP levels were also measured in buffer containing 34 m*M* KCl (i.e., under conditions that lead to a reduction in $[Na]_i$), but the dose–response curves of DAMGO were not different from the control, as the EC_{50} and the maximal inhibition were 6.9 ± 3.25 n*M* and 82%, respectively. DAMGO inhibition of forskolin-stimulated cyclic AMP levels in the presence of 30 μM monensin was comparable to control (EC_{50} values of 10.5 ± 1.81 n*M* in control, 13.9 ± 1.19 n*M* with monensin), although forskolin stimulation of cyclic AMP levels increased significantly

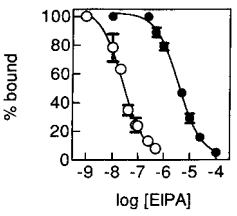

FIG. 7. Effect of EIPA on agonist and antagonist binding to whole cells. Retinoic acid-differentiated SH-SY5Y cells were incubated with various concentrations of EIPA in choline buffer, and [^{3}H]DAMGO binding (4 n*M*; ○) and [^{3}H]naloxone binding (5 n*M*; ●) were measured as described in Materials and Methods. Values are means ± SEM from three or four experiments.

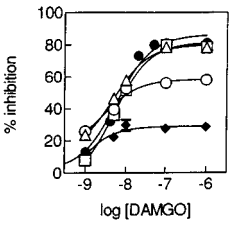

FIG. 8. DAMGO inhibition of forskolin-stimulated adenylyl cyclase activity. Intact cells were incubated at 37°C with various concentrations of DAMGO in physiological buffer containing varying $[Na]_o$: 0 m*M* (◆), 10 m*M* (○), 50 m*M* (△), and 118 m*M* (□). KCl (34 m*M*) was added to the incubation mixture to a set of tubes (●). After 15 min, the incubation was terminated and the cyclic AMP levels were estimated as given in Materials and Methods. The experiment was repeated three times, and the parameters are given in Table 2.

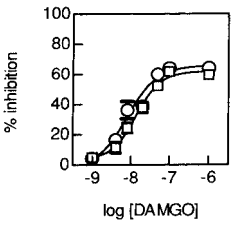

FIG. 9. DAMGO inhibition of forskolin-stimulated adenylyl cyclase activity in intact cells in physiological buffer in the absence (○) or presence of 30 μM monensin (□). Adenylyl cyclase activity was assayed as described in Materials and Methods. Values are from three independent experiments.

(53.7 ± 7.03 pmol/min/mg) (Fig. 9). Cyclic AMP levels measured in the absence of forskolin were negligible in these assays.

DISCUSSION

A number of G protein-coupled receptors are modulated by sodium (Jakobs, 1979). The exact mechanism by which sodium modulates the ligand binding, however, is not completely elucidated. Thus, the present studies were undertaken to study the effect of sodium chemical gradient across the neural membrane on opioid receptor–ligand interactions. These modulations are of particular significance, because the receptors are bathed in extracellular fluids containing high concentrations of sodium and sodium is reported to be essential for receptor-mediated signal transduction (Blume et al., 1979).

In the binding experiments conducted in intact cells, agonist binding and antagonist binding were changed differentially by sodium. Sodium-induced changes in binding were more prominent for the agonists, leading to ~70% reduction in the B_{max} in the presence of 118 mM sodium, whereas antagonist binding showed only an increase in the binding affinity that was not very convenient to measure under different experimental conditions. Hence, all the binding studies reported herein employed DAMGO to study the effect of sodium on receptor–ligand interactions. Agonist binding to cells at various concentrations of sodium suggested that the modulation of sodium in cells was maximal under physiological conditions. Increasing the concentrations of sodium in the incubation buffer progressively raised $[Na]_i$ while inhibiting the agonist binding with an EC_{50} of 15 mM $[Na]_o$ (Fig. 3).

Cellular sodium levels are regulated mainly by the activities of Na^+,K^+-ATPase and voltage-dependent sodium channels. The sodium pump maintains a constant electrochemical gradient for sodium by altering $[Na]_i$ in response to changes in membrane potential. In the present studies, addition of depolarizing concentrations of potassium led to >65% reduction in cellular sodium levels. If intracellular sodium has access to the "sodium site" on the receptor, this depletion in sodium levels would enhance the binding of an agonist to the receptor. It is surprising that [^{3}H]DAMGO binding was not increased under these conditions, suggesting that the receptor is rather insensitive to the changes in $[Na]_i$.

In experiments designed to study the agonist binding in cells with higher $[Na]_i$, at unaltered $[Na]_o$, ouabain and monensin were added independently and in combination to the physiological buffer. The presence of monensin brought about a dramatic increase in $[Na]_i$ (from 53 to 240 nmol/mg), whereas ouabain increased $[Na]_i$ to 120 nmol/mg, presumably by blocking Na^+,K^+-ATPase efflux of sodium. However, the increased levels of $[Na]_i$ in the presence of either of these agents did not affect the agonist binding at all. [^{3}H]DAMGO binding measured in cells incubated in the presence of monensin and ouabain at the concentration range of sodium at which agonist binding would be inhibited in a linear fashion (5–10 mM $[Na]_o$) also yielded similar results. These results show the lack of any regulation of agonist binding by intracellular sodium, in contrast to the earlier reports, which proposed a regulatory role for intracellular sodium (Motulsky and Insel, 1983; Puttfarcken et al., 1986).

The role of sodium in the receptor-mediated adenylyl cyclase activity is yet another interesting aspect of the sodium effect. Although it was shown to be essential for the opiate-mediated inhibition of cyclic AMP levels (Lichtshtein et al., 1979), sodium-independent inhibition of adenylyl cyclase activity by the δ receptor was also reported (Isom et al., 1987). In our studies, DAMGO inhibited the activity of forskolin-stimulated adenylyl cyclase by 20–27% in choline buffer, but the maximal effect was attained only in the presence of 50 mM sodium. The potency of DAMGO was higher in 0 and 10 mM sodium buffers, because the precoupled high-affinity receptor is presumably the prevalent form in the absence of sodium (Thomsen et al., 1988; Costa et al., 1989). The agonist potency in inhibiting adenylyl cyclase in these studies ranged from 1.1 to 6.8 nM, and this corresponded to the K_D obtained in binding studies, both measured in intact cells, indicating the importance of the high-affinity state of the receptor in signal transduction. In addition, sodium increased the stimulation of adenylyl cyclase activity by forskolin in these studies, whereas in NG108-15 cells, prostaglandin E_1 and 2-chloro-adenosine stimulation of cyclic AMP levels were reported to be normal in the absence of sodium (Lichtshtein et al., 1979). Forskolin is known to exert its actions directly on adenylyl cyclase, as well as through the mediation of G_s protein (Seamon et al., 1981; Green and Clark, 1982), and perhaps the direct interactions of forskolin with the enzyme are modulated by sodium. The dose–response curves of DAMGO at lower concentrations of sodium were hyperbolic, and a typical sigmoid curve was obtained only in the presence of buffer containing >50 mM sodium. In this respect, the sodium effect on the system can be viewed as that of a negative allosteric modulator that would increase the sensitivity of a system to make it physiologically useful. Furthermore, DAMGO attenuation of cyclic AMP levels did not change when measured in buffers containing 34 mM potassium (low $[Na]_i$) or 30 μM

monensin (high $[Na]_i$). Thus, these results show that signal transduction through the μ receptor is also regulated by extracellular sodium.

To explore the nature of the sodium effect further, EIPA has been employed in the binding assays. Amilorides, in general, are known to bind to sodium binding sites and have been shown to interact with α_2 receptors in plasma membranes from bovine aorta in a competitive, as well as noncompetitive, fashion (Jagadeesh et al., 1990). In the same system, it was also suggested that the allosteric effects caused by amilorides were due to their interaction at the sodium site of action. In the present studies, naltrexone binding was inhibited by EIPA with a K_i of 1.82 μM, which corresponds with the K_i of 1.6 μM for the inhibition of rauwolscine binding by EIPA in the above studies, whereas DAMGO binding was inhibited with a K_i of 14.2 nM. This difference in the sensitivity of agonist and antagonist binding to EIPA corresponds closely to their sodium sensitivities. Sodium increased the affinity of naltrexone by threefold, whereas DAMGO affinity was reduced drastically and was manifested in a reduction in the total number of binding sites. In addition, the results may indicate that the sodium site is located closer to the agonist binding motif and, therefore, the agonist binding is more sensitive to EIPA. Amilorides have been shown to compete for the sodium binding sites in a variety of systems (Vigne et al., 1983), and the effects of EIPA on ligand binding to the whole cells suggest the presence of the sodium site on the external surface of the cell.

It is interesting that SH-SY5Y cells exhibit considerable high-affinity binding of DAMGO in buffers containing physiological concentrations of sodium. Toll (1992) reported DAMGO binding to a single low-affinity site (K_i of 150 nM) in intact SH-SY5Y cells, on the basis of displacement assays using [^{3}H]CTOP (^{3}H-labeled Cys^2,Tyr,Orn^5,Pen^7 amide), a peptide antagonist. This could be the result of using ligand concentrations that were too narrow to discern two affinity states with widely diverging K_D values, because previous studies from our laboratory showed two sites with K_i values of 3.90 and 161 nM for DAMGO in intact SH-SY5Y cells (Carter and Medzihradsky, 1992). In addition, these high-affinity sites have been shown to be coupled to the G protein, as binding to these sites was found to be modulated by the endogenous guanosine triphosphate levels (Yabaluri and Medzihradsky, 1995). Furthermore, it appears that there exist two sets of binding sites for DAMGO, because ~30% of the sites retain their high affinity, whereas 70% of the sites are converted into a low-affinity state in the presence of sodium. It is possible that sodium displaces agonist from these sites, as DAMGO can displace [^{3}H]-naloxone binding completely in physiological buffers, albeit at higher concentrations. Allosteric binding sites for sodium on the receptor protein itself were first suggested for the α_{2A}-adrenergic system (Michel et al., 1980; Nunnari et al., 1986). In addition, site-directed mutagenesis experiments carried out on a variety of G protein-coupled receptors, including opioid receptors, provide important information in this regard. A highly conserved aspartate residue in the second transmembrane domain of the receptors, in general, has been shown to be important in imparting the sodium sensitivity on the $G_{i/o}$-coupled receptors (Kong et al., 1993*a*). It is interesting that this residue (Asp^{95}) was implicated in the high-affinity binding of δ receptor-selective agonists to the cloned δ receptor, which was coupled to G protein and attenuated the forskolin-stimulated adenylyl cyclase activity (Kong et al., 1993*b*). This might suggest that the sodium site is closer to the agonist binding motif, as mentioned earlier in this report. This highly conserved aspartate was identified as a key residue in the agonist-induced receptor activation of not only the μ receptor (Surratt et al., 1994), but also muscaninic acetylcholine receptors (Fraser et al., 1989), β-adrenergic receptor (Strader et al., 1988), α_{2A}-adrenergic receptors (Wang et al., 1991), and lutropin receptor (Ji and Ji, 1991). In the present studies, the affinity of the antagonist was improved by sodium, whereas the number of sites was not changed. Naltrexone may bind to the same set of binding sites as DAMGO (in choline buffer), but sodium interaction may somehow stabilize the antagonist binding, thereby increasing the affinity. In contrast, antagonists may bind to totally different domains and displace agonist binding as a result of a general conformational change (Neve et al., 1990; Kong et al., 1994).

The results presented in this report are contradictory to what has been observed in 7315c cells by Puttfarcken et al. (1986). In these cells, experiments with monensin indicated the presence of the sodium site on the intracellular side. In addition, it was shown that sodium reduced the affinity of the μ receptor without affecting the maximal number of binding sites. The sodium effect on agonist-receptor interactions is known to vary for individual ligands (Pert et al., 1973). Although the radioligand used in our studies ([^{3}H]DAMGO) binds to the high-affinity receptor, [^{3}H]etorphine employed in those studies detects the low-affinity sites. Thus, etorphine binding showed only an increase in K_D as a result of conversion of high-affinity sites into a low-affinity form by sodium, whereas DAMGO failed to detect the low-affinity sites and, as a result, there was a reduction in the total number of binding sites in saturation experiments. However, the reasons for the differences in the experiments using monensin are not clear. The discrepancy could be due to the fact that the 7315c cells used in those studies were not from any stable cell line and were obtained by dispersal of solid tumor masses. In the present studies, the effects observed were reproducible in three cell lines, namely, undifferentiated and retinoic acid-differentiated SH-SY5Y cells and C6μ5 cells. In contrast, these differences may indicate the importance of the ambient environment on receptor–

ligand interactions as reported in the case of cannabinoid receptors (Pacheco et al., 1994).

Although an intracellular site of action was first proposed in platelets (Motulsky and Insel, 1983), the results therein leave many questions unanswered. In the same studies, although the ability of epinephrine to attenuate prostaglandin E_1-stimulated cyclic AMP accumulation was comparable in platelets prepared in the presence or absence of sodium, only the platelet aggregation was shown to be affected by the internal pools of sodium (Connolly and Limbird, 1983). Moreover, this function was independent of G protein activation by pertussis toxin. It is possible that the mechanism of action for the aggregation of platelets is entirely different from the receptor-mediated attenuation of adenylyl cyclase activity. It should also be mentioned that accumulation of sodium per se was shown not to be sufficient to induce platelet aggregation (Feinberg et al., 1977).

Thus, the data presented in this report clearly demonstrate the extracellular site of action for sodium. $[Na]_i$ was manipulated in both directions, and the experiments with monensin, ouabain, and EIPA all point to the regulation of ligand binding by extracellular sodium. These studies also show the importance of the sodium ion in signal transduction through the μ receptor.

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