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Comparison of Opiate Inhibition of Adenylate Cyclase Activity in Neuroblastoma N18TG2 and Neuroblastoma × Glioma NG108–15 Hybrid Cell Lines

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SUMMARY

The ability of various opiate agonists to inhibit adenylate cyclase activity in neuroblastoma N18TG2 cells was investigated and compared with opiate activity in a daughter cell line, neuroblastoma × glioma NG108-15 hybrid. A high-affinity (K_{diss} = 1.44 nM) binding site for ^{3}H-labeled D-Ala^2-Met^5-enkephalinamide was observed in the membrane preparations of N18TG2 cells. The density of this binding site was 80% of that determined with the NG108-15 hybrid membrane preparations. Both the basal and the prostaglandin E_1-stimulated increase in adenylate cyclase activity were inhibited by the opioid peptide, Met^5-enkephalin. The opiate regulation of the enzyme activity in neuroblastoma N18TG2 was observed to be stereoselective, naloxone-reversible, and GTP-dependent. A linear correlation between the potencies of various opiate agonists' inhibition of adenylate cyclase activity in N18TG2 and NG108-15 cells could be established, with etorphine being the most potent agonist in both cell lines. There are several dissimilarities in opiate inhibition of adenylate cyclase activity in these two cell lines. The maximal level of opiate inhibition (efficacy) in neuroblastoma N18TG2 was significantly lower than that in the neuroblastoma × glioma NG108-15 hybrid cells. The difference in the efficacies of various opiates tested was observed within the NG108-15 cell line also. The monovalent cation Na^+ attenuated the opiate potency of inhibition of adenylate cyclase activity in NG108-15 cells, but not in the N18TG2 cells. Chronic exposure of these two cell lines to etorphine caused a reduced sensitivity of the cell lines to the opiate agonist. The naloxone-induced increase in adenylate cyclase activity in the NG108-15 cells chronically treated with opiate was not observed in the N18TG2 cells. These observations suggest there are differences in the molecular events which lead to opiate inhibition of the adenylate cyclase activity in these two cell lines.

INTRODUCTION

Recently we demonstrated the presence of a stereospecific, high-affinity opiate binding site in mouse neuroblastoma N18TG2 cells (1), in contrast to reports by Klee and Nirenberg (2) and Blosser *et al.* (3). The apparent K_{diss} value for [^{3}H]naloxone or [^{3}H]dihydromorphine was determined to be 25–31 nM (1). Although the density of these binding sites in N18TG2 was observed to be 20% of the binding sites with similar affinity in the daughter cell line, neuroblastoma × glioma NG108-15 hybrid, the overall characteristics of the opiate binding sites found in the N18TG2 cell membrane preparations were analogous to those reported with the NG108-15 cell membrane and the opiate receptor found in the brain membrane. Nevertheless, with concentrations up to 0.1 mM, morphine had little to no effect on the basal or PGE$_1$[2]-stimulated adenylate cyclase activity in the N18TG2 cells (1, 4). This absence of opiate regulation of the adenylate cyclase could be attributed either to the lower density of the receptor found in N18TG2 or the inability of the opiate alkaloid to induce the receptor conformation required for the inhibition of the adenylate cyclase.

The opiate receptor in the NG108-15 was demonstrated to be enkephalinergic, i.e., Met^5- and Leu^5-enkephalin have the greatest potency for inhibition of the adenylate cyclase (5). Thus, it is probable that the opiate receptor found in the neuroblastoma N18TG2 cell could also be enkephalinergic. Opiate alkaloids, which are μ agonists, might have low activity in a δ receptor system.

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[2] The abbreviations used are: PGE$_1$, prostaglandin E$_1$; IBMX, 3-isobutyl-2-methylxanthine; ZK62711, 4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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Hence, in the present communication the ability of other opiate agonists, in particular the opioid peptides, to regulate adenylate cyclase in the N18TG2 cell is compared with the opiate inhibition of the adenylate cyclase in NG108-15 cells.

METHODS

Cell lines. Mouse neuroblastoma N18TG2 and mouse neuroblastoma × rat glioma NG108-15 hybrid cells were generous gifts of Dr. B. Hamprecht (Physiologischemisches Institut des Universität, Wurzburg, Federal Republic of Germany). Cell lines were cultured in Dulbecco's modified Eagle's medium plus 10% fetal calf serum in a humidified atmosphere of 10% CO_2 and 90% air. For the mutant N18TG2, 0.1 mM 6-thioguanine was present in the medium. For the NG108-15 cells, 0.1 mM hypoxanthine, 10 μM aminopterin, and 17 μM thymidine were present in the medium. The cells were detached from the growing surface by the addition of Saline D for propagation or experimentation as described previously (1). Cells with passage number less than 35 were used in all experiments.

Intracellular cyclic AMP measurement. The effect of various opiates on intracellular cyclic AMP level was measured by the method of Schutz and Daly (6) using [^{3}H]adenine to label the intracellular ATP pools. Cells were inoculated into 17-mm wells containing 1.0 ml of the growth medium and were cultured for 4 days. The medium was completely changed the day prior to the experiment. At the start of the experiment, after removal of the growth medium, 0.3 ml of the incubation medium (Dulbecco's modified Eagle's medium H-16; $NaHCO_3$, 29.3 mM; glucose, 15.3 mM; NaCl, 15.4 mM; pH 7.3) containing the phosphodiesterase inhibitors IBMX (0.5 mM) and ZK62711 (0.1 mM) and 3 μCi of [^{3}H]adenine were added to each well. The cells were then incubated with this mixture at 37° and 10% CO_2 for 60 min. Afterward, the mixture was aspirated and 0.5 ml of the incubation medium containing identical concentrations of the phosphodiesterase inhibitors, various concentrations of opiates, and PGE_1 (10 μM) was added. The incubation was then carried out at 37° and 10% CO_2 for 10 min and then was terminated by removal of the medium and the addition of 0.55 ml of 0.27 N perchloric acid. Fifty microliters of ^{32}P-labeled cyclic AMP (10,000 cpm) were added to each well and the ^{3}H-labeled cyclic AMP that was formed during the 10-min incubation period and released into the perchloric acid was separated from other nucleotides by Dowex 50 and alumina column chromatography as described by White and Karr (7).

When the intracellular cyclic AMP level was measured in a cell suspension, the cell lines were cultured in T-75-cm^2 flasks for 5 days with the growth medium as described previously. On the day of the experiment, the growth medium was removed and the cells were washed twice with 10 ml of the incubation medium. Afterward, 10 ml of the incubation medium containing 0.5 mM IBMX, 0.1 mM ZK62711, and 100 μCi of [^{3}H]adenine were added to the flasks. The cells were then incubated with this mixture for 60 min at 37° and 10% CO_2. After removal of the incubation medium, the cells were detached from the surface with Saline D. The cells were then pelleted and the Saline D was removed. Cells from each T-75-cm^2 flask were resuspended into 10 ml of Krebs-Ringer-Hepes buffer (NaCl, 110 mM; KCl, 5 mM; $MgCl_2$, 1 mM; $CaCl_2$, 1.8 mM; glucose, 25 mM; sucrose, 55 mM; and Hepes, 10 mM at pH 7.3). Aliquots (0.25 ml) of the cell suspension were transferred to 13 × 100 mm test tubes and the tubes were then kept at 0° until used. The amount of radioactivity retained by the cells under these conditions was observed to be stable for up to 1 hr. The incubations were initiated by the addition of 0.25 ml of Krebs-Ringer-Hepes buffer containing various concentrations of the opiates and 20 μM PGE_1, 0.5 mM IBMX, and 0.1 mM ZK62711; the mixtures were then placed into a 37° water bath. The incubations were terminated by the addition of 50 μl of a 3 N perchloric acid solution. After the addition of 10,000 cpm of ^{32}P-labeled cyclic AMP, the ^{3}H-labeled cyclic AMP was separated from other radioactive nucleotides as described in the previous section. In the experiments where the effect of monovalent cations was studied, sodium ions in the Krebs-Ringer-Hepes buffer were removed and either replaced with the same concentration of choline chloride or the osmolality was adjusted with sucrose.

Adenylate cyclase measurement. Adenylate cyclase activity in the membrane preparations was determined by measuring the production of cyclic AMP from [α-^{32}P] ATP. Cells were kept at −70° as a pellet until used. Cells were homogenized in a solution of 0.32 M sucrose + 40 mM Hepes + 2 mM ethylene glycol bis(β-aminoethyl ether)-*N*-*N*-*N*′,*N*′-tetraacetic acid buffered at pH 7.6 (homogenizing buffer) with a motor-driven Teflon-glass Elvehjem-Potter homogenizer in a ratio of 1–2 × 10^7 cells per 20 ml of homogenizing buffer. Membranes were pelleted by centrifuging the homogenate at 22,000 × *g* for 20 min at 0°. After removal of the supernatant, the pellets were resuspended in the homogenizing buffer to yield a final concentration of 100–200 μg of protein per 50 μl. Adenylate cyclase activity in the membrane was determined in a 100-μl reaction mixture containing the following final concentrations: 40 mM Hepes (pH 7.7), 5 mM $MgCl_2$, 20 mM creatine phosphate (Na^+), 1 mM cyclic AMP, 1 mM 1,10-*O*-phenanthroline, 12 μM ZK62711, 10 units of creatine phosphokinase, 0.1 mM [α-^{32}P]ATP, and 100–200 μg of membrane protein. When PGE_1 or opiates were added to the reaction mixture, the drugs were dissolved in 25% ethanol solution and 0.155 M NaCl, respectively. Reactions were carried out at 30° for 15 min and were terminated by the addition of 150 μl of 1 N perchloric acid. After the addition of 0.3 ml of water containing 10,000 cpm of ^{3}H-labeled cyclic AMP, the cyclic AMP formed in the reaction was separated from other radioactivity by the same column chromatographic methods as described in the previous section.

When the GTP-dependent opiate inhibition of adenylate cyclase was studied, the nuclei from the crude homogenate were separated from the membrane. The crude cell homogenate was centrifuged at 1,000 × *g* for 10 min to pellet the nuclei. The membrane for the adenylate cyclase assay was then obtained by centrifuging the 1,000 × *g* supernatant at 22,000 × *g* for 20 min. In the GTP-dependent experiments, the membranes were preincubated with the complete reaction mixture minus the ATP

at 30° for 10 min. The reaction was initiated by addition of the radioactive ATP. After incubation at 30° for 20 min, the reaction was terminated by addition of perchloric acid, and the cyclic AMP formed was isolated as described.

Opiate receptor binding. Opiate receptor binding was carried out with the 22,000 × *g* × 20 min membrane pellet in 25 mM Hepes buffer at pH 7.7. Membrane protein (0.6-0.8 mg) was added to the Hepes buffer solution containing various concentrations of radioactive opiates and 5 μM levorphanol to give a final volume of 1.0 ml. Five micromolar levorphanol was present in the reaction mixture in order to determine the nonspecific binding. After incubating at 24° for 90 min, the incubation was terminated by collecting the membrane on a Whatman GF/B filter, and excess radioactivity was removed by washing the filter three times with 5 ml of 25 mM Hepes at 0°. Radioactivity of the filters was determined by liquid scintillation spectroscopy. Specific opiate binding was defined as the difference in the average radioactivity bound to triplicate samples of membranes in the presence or absence of 5 μM levorphanol.

Materials. ^{3}H-labeled D-Ala^2-Met^5-enkephalinamide (46 Ci/mmole), ^{3}H-labeled cyclic AMP (40 Ci/mmole), [^{3}H]adenine (16 Ci/mmole), and [α-^{32}P]ATP (17 Ci/mmole) were supplied by New England Nuclear Corporation (Boston, Mass.). All other reagents used were supplied by Sigma Chemical Company (St. Louis, Mo.). ZK62711 was a generous gift from Schering (Berlin, Federal Republic of Germany). (+)-Naloxone was obtained from Dr. A. Jacobson (National Institute of Mental Health, Bethesda, Md.).

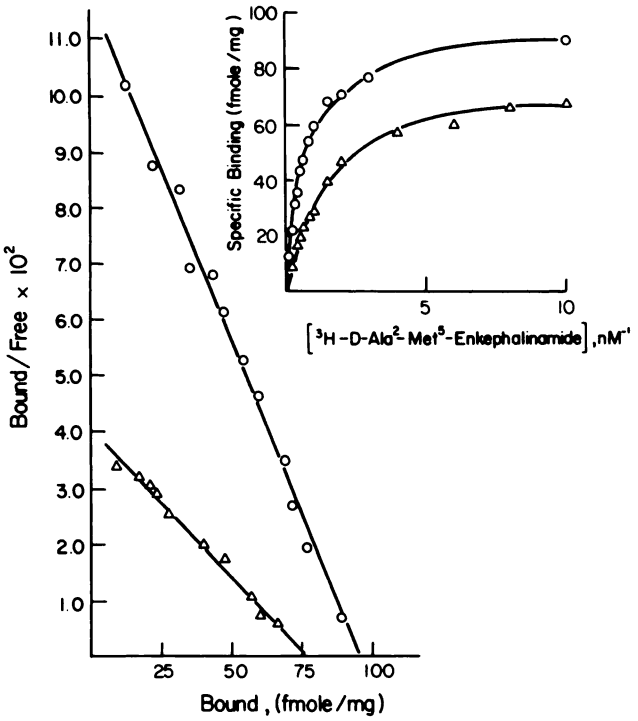

FIG. 1. *Scatchard analysis of specific opiate binding to neuroblastoma N18TG2 and neuroblastoma × glioma NG108-15 membrane preparations*

Specific binding of ^{3}H-labeled D-Ala^2-Met^5-enkephalinamide to N18TG2 (Δ——Δ) and NG108-15 (○——○) was determined as described under Methods. The *insert* shows the amount of enkephalinamide specifically bound to the membrane of the respective cell lines at various concentrations of the opioid peptide.

RESULTS

Comparison of opiate binding sites in N18TG2 and NG108-15. In the previous studies we reported the presence of [^{3}H]naloxone and [^{3}H]dihydromorphine binding sites with K_{diss} = 21–30 nM in the mouse neuroblastoma N18TG2 cells (1). However, ^{3}H-labeled D-Ala^2-Met^5-enkephalinamide has a greater affinity for the opiate binding sites observed in the N18TG2 membrane (Fig. 1). The K_{diss} value of the ^{3}H-labeled D-Ala^2-Met^5-enkephalinamide binding site in the neuroblastoma N18TG2 cell was determined to be 1.44 nM (1.38–1.49 with a 95% confidence limit by the linear regression analysis of the lines in Fig. 1). The K_{diss} value of the enkephalin binding to the NG108-15 membrane was observed to be 0.60 nM (0.54–0.67). The B_{max} values for ^{3}H-labeled D-Ala^2-Met^5-enkephalinamide in the N18TG2 and NG108-15 membrane preparations were observed to be 77.5 fmoles/mg of protein (77.2–80.1) and 96.9 fmoles/mg of protein (92.1–101.5), respectively. Thus, the neuroblastoma N18TG2 contained 80% of the enkephalin binding sites found in the neuroblastoma × glioma NG108-15 hybrid cells.

Opiate inhibition of adenylate cyclase activity. As reported previously by Sharma *et al.* (4) and Law *et al.* (1), morphine sulfate possessed little to no inhibitory activity toward adenylate cyclase in the neuroblastoma N18TG2 cells. This weak effect of morphine could be due to the δ nature of the opiate receptor found in the neuroblastoma cell lines (8). Thus, the opioid peptides should regulate the adenylate cyclase activity in the N18TG2 cells. As shown in Fig. 2a, Met^5-enkephalin inhibited the PGE$_1$-stimulated increase in the intracellular ^{3}H-labeled cyclic AMP level in a concentration-dependent manner in both cell lines. The maximal inhibitory level of Met^5-enkephalin in N18TG2 cells (38%) was substantially less than that in the NG108-15 cells, which was determined to be 71% (Fig. 2a). Similar to the values reported by Walhström *et al.* (5), the IC$_{50}$ value of enkephalin inhibition of PGE$_1$-stimulated production of cyclic AMP in NG108-15 cells was determined to be 3.0 nM (value obtained from least-squares analysis of the log-logit plot of the data represented in Fig. 2a). The IC$_{50}$ value of enkephalin inhibition in the neuroblastoma N18TG2 cells was determined to be 4.8 nM. There was no cooperativity in the Met^5-enkephalin inhibition in both cell lines since the Hill coefficients for enkephalin inhibition were in the range of 0.99–1.04.

Opiate inhibition of the PGE$_1$-stimulated increase in intracellular cyclic AMP levels in the neuroblastoma N18TG2 was most likely mediated via inhibition of the membrane-bound enzyme adenylate cyclase. The presence of the phosphodiesterase inhibitors, IBMX and ZK62711, in the incubated mixtures excluded the possibility of an opiate effect on the degradation of cyclic AMP. Furthermore, enkephalin could inhibit basal and PGE$_1$-stimulated adenylate cyclase activity in the membrane preparation. Met^5-enkephalin inhibited adenylate cyclase activity in the membrane preparation of NG108-

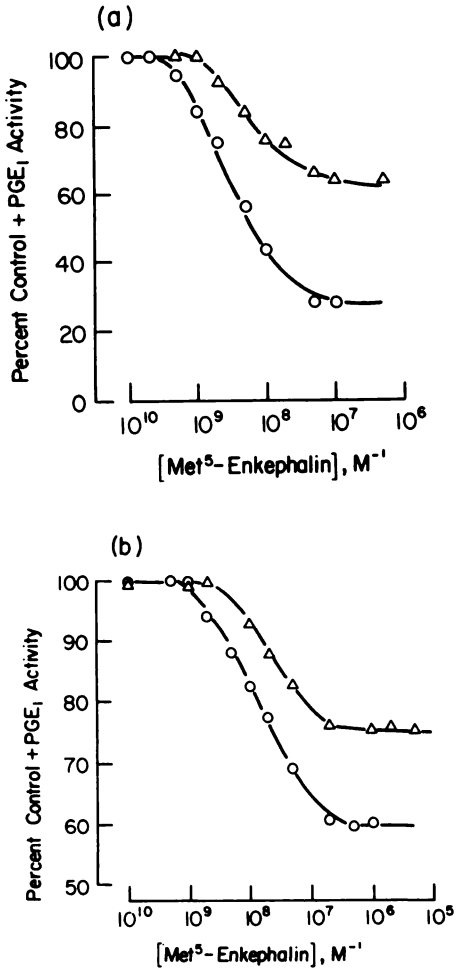

FIG. 2. *Inhibition of adenylate cyclase activity by Met⁵-enkephalin*

a. Inhibition of the formation of intracellular ^{3}H-labeled cyclic AMP in the presence of 10 μM PGE_1 by various concentrations of Met⁵-enkephalin was carried out with N18TG2 (Δ——Δ) and NG108-15 cells (O——O) as described under Methods. In each well 5×10^5 cells were present on the day of the experiment. The amounts of ^{3}H-labeled cyclic AMP formed in the presence of 10 μM PGE_1 were $1.76 \pm 0.03 \times 10^5$ cpm/10^6 cells per 10 min and $5.39 \pm 0.05 \times 10^4$ cpm/10^6 cells per 10 min for NG108-15 and N18TG2, respectively.

b. The effect of various concentrations of Met⁵-enkephalin on PGE_1-sensitive adenylate cyclase activity in membrane preparations of N18TG2 (Δ——Δ) and NG108-15 (O——O). The membranes were prepared and the adenylate cyclase activity was determined according to Methods. The specific activities of the enzyme in the presence of 10 μM PGE_1 were determined to be 54.8 ± 1.5 pmoles/mg of protein per minute and 95.4 ± 4.4 pmoles/mg of protein per minute for N18TG2 and NG108-15 cells, respectively.

15 with a maximal inhibitory level of 40% instead of the 71% observed with the intact cells (Fig. 2b), as discussed in previous reports (4, 9). The IC_{50} value for enkephalin inhibition of PGE_1-stimulated adenylate cyclase activity in the membrane preparation was determined to be 13.1 nM. In the N18TG2 membrane preparations, Met⁵-enkephalin inhibited PGE_1-stimulated adenylate cyclase activity by 25% (Fig. 2b). The IC_{50} value of Met⁵-enkephalin was determined to be 21.3 nM. The 1.6-fold increase in the enkephalin IC_{50} values in the NG108-15 and N18TG2 membrane preparations was identical with the increase in the IC_{50} values obtained with the whole-cell experiments (Fig. 2a).

The PGE_1-stimulated increase in intracellular ^{3}H-labeled cyclic AMP level was regulated by opiate agonists other than enkephalin. With all of the opiate alkaloids and peptides tested, etorphine possessed the greatest potency in inhibition of PGE_1-stimulated increase of adenylate cyclase activity in both cell lines (Table 1). The opioid pentapeptides were more potent than the longer chain peptides, e.g., α-endorphin, β-endorphin, and dynorphin, in inhibiting adenylate cyclase activity in these two cell lines (Table 1). Conversion of the metabolically labile enkephalin to the stable analogue with D-Ala² substitution did not enhance the potencies of the pentapeptides (Table 1). A correlation between the relative potencies of varous opiates and opioid peptides in inhibiting adenylate cyclase activity in these two cell lines could be established. Linear regression analysis of such a plot produced a straight line with a slope of 1.18 and a correlation coefficient of 0.958.

The maximal levels (efficacy) of opiate inhibition in the N18TG2 cells were significantly less than that in the NG108-15 cells (Table 1). Furthermore, the opiates tested did not attain identical levels of inhibition in the same cell line. Within the same passage number the inhibitory

TABLE 1

Comparison of the potency of various opioids to inhibit adenylate cyclase activity in neuroblastoma N18TG2 and neuroblastoma × glioma NG108-15 hybrid cell lines

The potency of various opioids' inhibition of intracellular increases in cyclic AMP levels produced by PGE_1 was determined as described under Methods. The IC_{50} values of the opioids were determined by linear regression analysis of the log-logit plots of the inhibition produced by 10 different concentrations of the opioids. The maximal inhibitory levels of the opioids were determined with the same passage of cells with 10 μM of opioids with the exception of morphine, dihydromorphine, and levorphanol. A 0.1 mM concentration of these three opioids was used in the assays. The average for N18TG2 cells represents the determinations using four separate wells in the same passage number. The values for NG108-15 represent the averages from three separate passage numbers.

Opioid	IC_{50}		Maximal level of inhibition	
	N18TG2	NG108-15	N18TG2	NG108-15
	nM		%	
Etorphine	3.8	1.5	35.6 ± 0.1[a]	68.6 ± 1.2
Met⁵-enkephalin	4.6	4.0	36.2 ± 0.1[a]	63.4 ± 1.5
Leu⁵-enkephalin	9.9	2.7	36.9 ± 1.7[a]	63.1 ± 2.2
D-Ala²-D-Leu⁵-enkephalin	7.2	2.9	19.2 ± 1.5[a]	63.4 ± 3.5
Cyclazocine	15.1	19.0	33.9 ± 1.4[a]	51.9 ± 8.2
β_h-Endorphin	73.6	49.3	39.6 ± 0.4[a]	64.6 ± 2.1
α-Endorphin	666.0	98.0	38.1 ± 0.7[a]	61.6 ± 3.0
FK-33824[b]	128.0	120.0	35.7 ± 1.0[a]	66.3 ± 2.3
Ketocyclazocine	330.0	116.4	34.9 ± 1.4[a]	66.6 ± 3.6
Levorphanol	1125.0	221.5	15.3 ± 1.0[a]	46.1 ± 5.1[c]
Dynorphine (1–13)	2140.0	220.8	39.1 ± 3.0[a]	63.0 ± 2.2
Dihydromorphine	1650.0	1680.0	28.7 ± 1.6[a]	64.3 ± 4.5
Morphine	—	10140.0	—	45.5 ± 3.1[c]

[a] The unpaired *t*-test analysis of the maximal inhibitory levels of the various opiate agonists in N18GT2 and NG108-15 revealed significant differences ($p \leq 0.01$).

[b] The D-Ala²-*N*-CH_3-Phe⁴-Met⁵ (*O*)al-enkephalin analogue of the enkephalin.

[c] Statistically significantly different as compared with the etorphine efficacy ($p \leq 0.01$).

activity achieved by levorphanol and morphine in the NG108-15 cells was significantly less than that observed with other opiates ($p \leq 0.01$) (Table 1). An analogous situation was observed in the neuroblastoma N18TG2 cells. The notable exception was that the level of morphine inhibition of adenylate cyclase activity in the N18TG2 cells was insignificant (Table 1).

Stereoselectivity and naloxone reversibility of opiate inhibition in N18TG2. In order to illustrate that opiate inhibition of the adenylate cyclase activity in neuroblastoma N18TG2 cells was mediated via the opiate receptor, two receptor characteristics must be observed: there should be a stereoselectivity, and the opiate effect should be reversed by naloxone, an opiate antagonist. Hence, the enantiomeric pairs of opiate ligands, levorphanol/dextrorphan and (−)-/ and (+)-naloxone, were used. As shown in Fig. 3, the active isomers of both pairs were more potent than the inactive isomers in eliciting the opiate effect. The IC_{50} values of levorphanol and dextrorphan in N18TG2 cells were determined to be 1.18 μM and 340 /M, respectively. The concentrations of (−)- and (+)-naloxone required to reverse the 1 μM etorphine inhibition by 50% were observed to be 1.2 μM and 390 μM, respectively. Hence, a 300-fold difference in potencies between the active and inactive enantiomers was observed with both enantiomeric pairs. The experiments also demonstrated that the opiate inhibition of the adenylate cyclase activity was reversed by (±)-naloxone (Fig. 3).

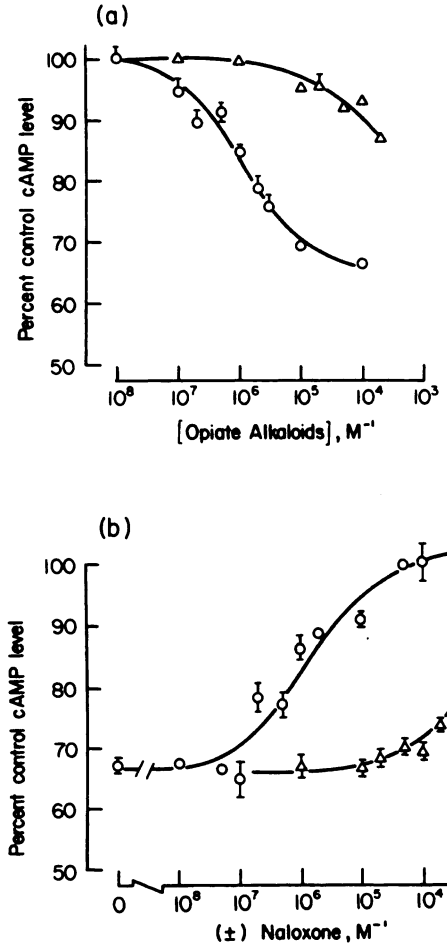

FIG. 3. *Stereoselectivity of opiate regulation of neuroblastoma N18TG2 cells*

a. The ability of various concentrations of levorphanol (○——○) or dextrorphan (△——△) to inhibit formation of the intracellular ^{3}H-labeled cyclic AMP in the presence of 10 μM of PGE_1.

b. The ability of various concentrations of (−)-naloxone (○——○) or (+)-naloxone (△——△) to antagonize the inhibitory effect of 1 μM etorphine on the PGE_1-stimulated increase in the intracellular ^{3}H-labeled cyclic AMP level was studied. The amount of ^{3}H-labeled cyclic AMP formed in the presence of 10 μM PGE_1 and in the absence of any opiates was determined to be 3.99 ± 0.01 × 10^4 cpm/10^6 cells per 10 min.

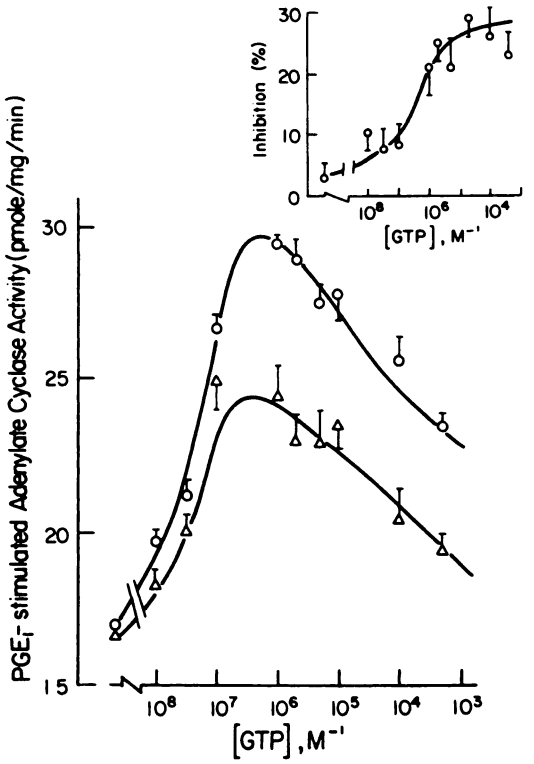

FIG. 4. *The requirement of GTP for opiate inhibition of adenylate cyclase activity in neuroblastoma N18TG2 cells*

The adenylate cyclase activity in the N18TG2 membrane preparation was measured in the presence of 10 μM PGE_1 and various concentrations of GTP in the absence (○——○) or in the presence (△——△) of 5 μM Leu^5-enkephalin. The *inset* represents the percentage of adenylate cyclase activity being inhibited by enkephalin at various concentrations of GTP.

GTP dependence of opiate inhibition. Opiate inhibition of adenylate cyclase activity in neuroblastoma × glioma NG108-15 cells has been demonstrated to be dependent on the guanine nucleotide GTP (9). Opiate inhibition of the basal adenylate cyclase activity in the striatal membrane was also dependent on the presence of added GTP (10). Analogous dependence on GTP was observed with the opiate inhibition of the N18TG2 adenylate cyclase activity. When the N18TG2 membrane was washed with homogenizing buffer and incubated at 30° for 10 min prior to the addition of [^{32}P]ATP, as described by Blume *et al.* (9), enkephalin inhibition of adenylate cyclase activity was not observed in the absence of exogenous GTP (Fig. 4). The PGE_1-stimulated adenylate cyclase activity has a biphasic response to GTP. At guanine nucleotide concentrations of 0.3–1.0 μM, the adenylate cyclase activity was maximally stimulated by PGE_1. At higher concentrations of GTP a de-

crease in enzymatic activity from maximal values (30 pmoles/mg of protein per minute) was observed. Concurrently, Met^5-enkephalin activity was potentiated by exogenous GTP (Fig. 4). The apparent ED_{50} of GTP to potentiate the opiate effect was determined to be 1 μM, which was similar to that reported by Blume *et al.* (9).

Sodium effect on opiate inhibition. Enkephalin inhibition of NG108-15 adenylate cyclase activity has been reported to be dependent on the monovalent cation, Na^+, or the "Na^+ state" of the receptor enzyme complex (9). However, the absolute requirement for sodium in opiate inhibition of striatal adenylate cyclase activity was observed to be dependent on the assay condition (10). Similarly, the absolute dependence on the monovalent cation for opiate inhibition in the neuroblastoma N18TG2 cells was not observed. As shown in Fig. 5, etorphine inhibited the adenylate cyclase activity in the absence of Na^+. When the sodium content in the Krebs-Ringer-Hepes buffer was replaced with an equivalent amount of choline chloride or equiosmal of sucrose, the IC_{50} values of etorphine were determined be be 2.4 nM and 3.0 nM, repectively. In normal Krebs-Ringer-Hepes buffer, an apparent increase in both basal and PGE_1-stimulated adenylate cyclase activity and the etorphine efficacy from the other two media were observed (see legend to Fig. 5). This apparent increase in etorphine

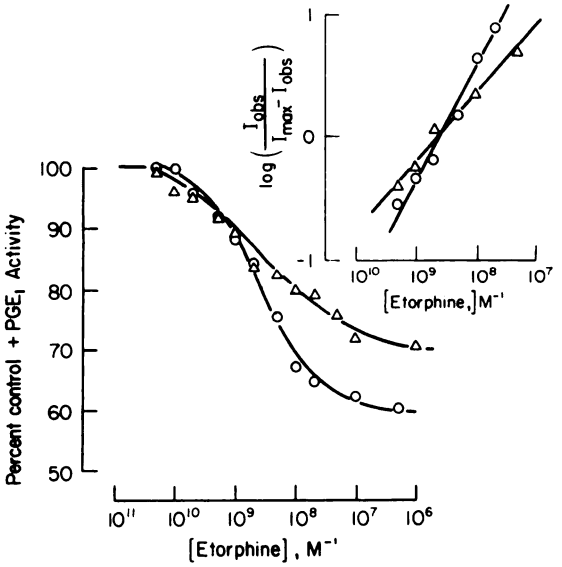

FIG. 5. *Effect of sodium on etorphine inhibition in neuroblastoma N18TG2 cells*

N18TG2 cells were prelabeled with [^{3}H]adenine as described under Methods. After the radioactivity was removed and the cells were washed free of sodium, the effect of various concentrations of etorphine on the 10 μM PGE_1-stimulated increase in intracellular ^{3}H-labeled cyclic AMP was studied in Krebs-Ringer-Hepes buffer (KRB) (O——O) or in KRB in which the sodium content was replaced with an equivalent amount of choline chloride (Δ——Δ). The amounts of intracellular ^{3}H-labeled cyclic AMP formed in the absence of PGE_1 were 580 ± 30 cpm/10^6 cells per 20 min and 490 ± 10 cpm/10^6 cells per 20 min in KRB and choline-substituted KRB, respectively. The amounts of ^{3}H-labeled cyclic AMP formed during the 20-min incubation in the presence of 10 μM PGE_1 were 33,900 ± 100 cpm/10^6 cells and 26,300 ± 380 cpm/10^6 cells in KRB and choline-substituted KRB, respectively. In each assay 1.1 × 10^6 cells were used. The *inset* represents the log-logit plot of etorphine inhibition.

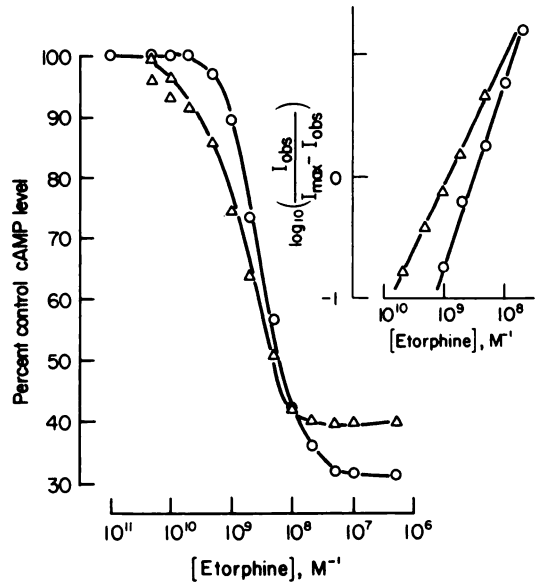

FIG. 6. *Etorphine inhibition of the PGE_1-stimulated increase in intracellular ^{3}H-labeled cyclic AMP in neuroblastoma × glioma NG108-15 hybrid cells*

Ectorphine inhibition was studied in Krebs-Ringer-Hepes buffer (KRB) (O——O) or in sucrose-substituted KRB (Δ——Δ) as described in the legend to Fig. 5 and Methods. The amounts of ^{3}H-labeled cyclic AMP formed during the 20-min incubation periods were determined to be: in normal KRB, basal = 1370 ± 20 cpm/10^6 cells, and plus 10 μM PGE_1 = 95,000 ± 690 cpm/10^6 cells; in sucrose-substituted KRB, basal = 1700 ± 30 cpm/10^6 cells, and plus 10 μM PGE_1 = 89,000 ± 500 cpm/10^6 cells. There were 8 × 10^5 cells in each cell suspension assay. The *inset* represents the log-logit plot of etorphine inhibition of ^{3}H-labeled cyclic AMP formation in NG108-15 cells.

activity could be elicted by 110 mM Li^+, but not by equimolar NH_4^+, Cs^+, or K^+. There was no alteration of etorphine IC_{50} values in the presence of Na^+ (2.5 nM). The apparent cooperativity in etorphine inhibition in the choline-substituted Ringer buffer (Hill coefficient = 1.40) disappeared in the normal Ringer buffer (Hill coefficient = 1.02).

The ability of etorphine to regulate neuroblastoma N18TG2 adenylate cyclase activity in the absence of sodium suggested that the "Na^+ state" of the opiate receptor-adenylate cyclase interaction as described by Blume *et al.* (9) with the NG108-15 membrane preparation does not exist in N18TG2. However, in the current studies, etorphine inhibited adenylate cyclase activity in the NG108-15 hybrid cells in the absence of Na^+ (Fig. 6). The IC_{50} values for etorphine under the Na^+-free condition were determined to be 1.0 nM and 1.3 nM in the sucrose-substituted and choline-substituted Krebs-Ringer-Hepes buffer, respectively. The maximal etorphine inhibitory levels in the Na^+-free media were significantly lower ($p \leq 0.01$, $n = 3$) than that in the sodium Krebs-Ringer-Hepes buffer, 59.7 ± 0.4% and 69.2 ± 0.4%, respectively (Fig. 6). The IC_{50} value of etorphine in normal Krebs-Ringer-Hepes buffer was determined to be 2.9 nM, a 3-fold increase from the IC_{50} values in the Na^+-free medium. This Na^+ shift of opiate inhibitory potencies could be demonstrated further with the NG108-15 membrane preparation. As shown in Fig. 7, when the Tris salts of all of the reagents in the reaction mixtures

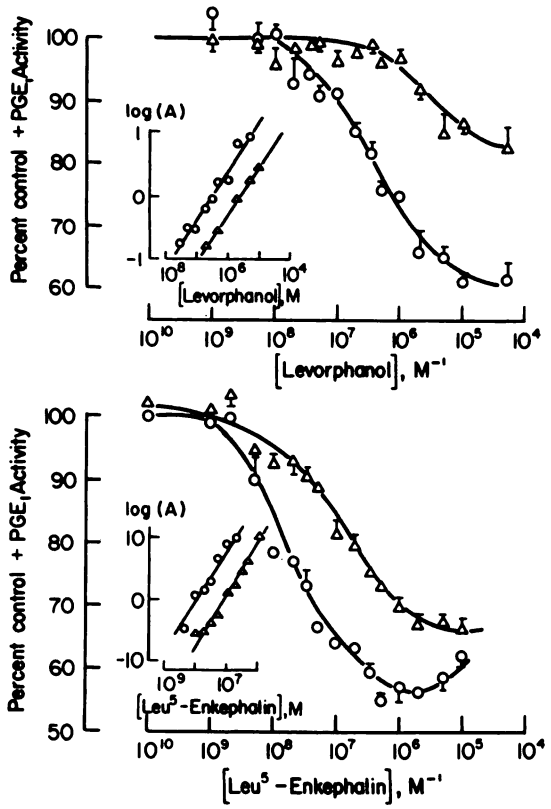

FIG. 7. *Decrease of opiate potency in the NG108-15 membrane preparation by Na^+*

Inhibition of adenylate cyclase activity in the membrane preparations of NG108-15 by levorphanol (*upper panel*) and by Leu⁵-enkephalin (*lower panel*) was studied in the presence (△——△) or in the absence (○——○) of added sodium in the reaction mixtures. The Tris salts of all of the reagents were used in the studies. Final Na^+ concentration in the plus-Na^+ mixture was 100 mM. In the presence of 10 μM PGE_1, the specific activities of adenylate cyclase in the presence and in the absence of 100 mM Na^+ were determined to be 89.4 ± 0.98 pmoles/mg per minute and 81.5 ± 0.9 pmoles/mg per minute, respectively. The *insets* represent the log-logit plots of opiate inhibition of adenylate cyclase activity. *Log (A)* = log $(I_{obs}/I_{max} - I_{obs})$.

were used, both levorphanol and Leu⁵-enkephalin inhibited adenylate cyclase activity in NG108-15 membrane preparations with IC_{50} values of 320 nM and 13.3 nM, respectively. When 100 mM Na^+ was added to the reaction mixtures, the IC_{50} values of levorphanol and Leu⁵-enkephalin were determined to be 2320 nM and 63.0 nM, respectively, a 5- to 7-fold increase of the Na^+-free IC_{50} values. Therefore, opiate regulation of adenylate cyclase activity in the neuroblastoma × glioma NG108-15 hybrid preparations exhibited the "classical" sodium shift of receptor activity as demonstrated by the receptor-ligand binding studies (1, 3, 11).

Chronic treatment of cell lines with etorphine. It has been reported that chronic exposure of NG108-15 cells to opiate agonists produced a cellular adaptation to the opioid ligands (12, 13). A "tolerance" response, defined by Sharma and co-workers (12) as the decrease in opiate inhibitory activity, and a "dependence" resonse, defined as the increase in adenylate cyclase activity in chronically opiate-treated cells upon the addition of naloxone, were observed with the NG108-15 cells treated with opioid agonists for a minimum of 12–24 hr (14). Using enkephalin as the ligand, Brandt *et al.* (13) demonstrated a rapid development of the "tolerance" and "dependence" responses in NG108-15 cells within 1 hr of opiate treatment. This rapid cellular adaptation was observed also when the NG108-15 cells were preincubated with etorphine in the present studies (Fig. 8a). The opiate receptor in NG108-15 cells was desensitized to etorphine after a 2.5-hr exposure to the agonist with a concurrent increase in adenylate cyclase activity.[3] These NG108-15 cellular adaptations to the etorphine treatment were rapid. The "tolerance" response developed within 30 min after the addition of etorphine and reached steady-state level after 1 hr of treatment. The increase in adenylate cyclase activity developed 1 hr after the addition of etorphine and reached a maximal level after 4 hr of treatment. Further incubation with etorphine did not enhance the increase.

When neuroblastoma N18TG2 cells were pretreated with etorphine similarly, the neuroblastoma did not develop the naloxone-induced increase in adenylate cyclase activity (Fig. 8b). Analogous to the experiments with NG108-15 cells, preincubation of the N18TG2 cells with varying concentrations of etorphine for 2.5 hr produced a progressive decrease in the 1 μM etorphine inhibitory activity (*curve I*, Fig. 8b). The appearance of the "tolerance" response occurred within 30 min and the steady state within 60–90 min. The neuroblastoma N18TG2 cells were completely "tolerant" to etorphine after 24 hr of preincubation with the opioid ligand. However, when 0.1 mM naloxone was added to the reaction mixture, there was no measurable increase in adenylate cyclase activity in the N18TG2 cells which were pretreated with etorphine (*curve II*, Fig. 8b). Increase of naloxone concentration in the incubation mixture did not produce an increase in adenylate cyclase activity. Prolongation of the preincubation period with etorphine up to 48 hr did not produce a naloxone-induced increase in enzymatic activity.

DISCUSSION

In the present study, opioid peptides and opiate alkaloids, with the exception of morphine, inhibited the adenylate cyclase activity in the neuroblastoma N18TG2 cells. A stereospecific, naloxone-reversible opiate inhibition of adenylate cyclase activity was observed in the whole cells or membrane preparations of N18TG2 cells. Analogous to opiate inhibition of adenylate cyclase in neuroblastoma × glioma NG108-15 hybrids (9) and other receptor regulation of the adenylate cyclase, GTP was required for the opiate inhibition of the N18TG2 adenylate cyclase activity. The similarity between opiate inhibition in the N18TG2 and NG108-15 cells was illustrated further by the loss of opiate inhibition in both cell lines upon chronic exposure to the opiate agonist (Fig. 8).

However, there are some distinct differences in opiate inhibition of adenylate cyclase activity in the N18TG2 and NG108-15 cells. The potencies and efficacies (the maximal inhibitory level) of several opiates and opioid peptides tested were significantly greater in the NG108-15 cells than in the N18TG2 cells. The monovalent cation Na^+ attenuated the opiate potencies in the NG108-15

[3] P. Y. Law, J. E. Koehler, and H. H. Loh, unpublished observation.

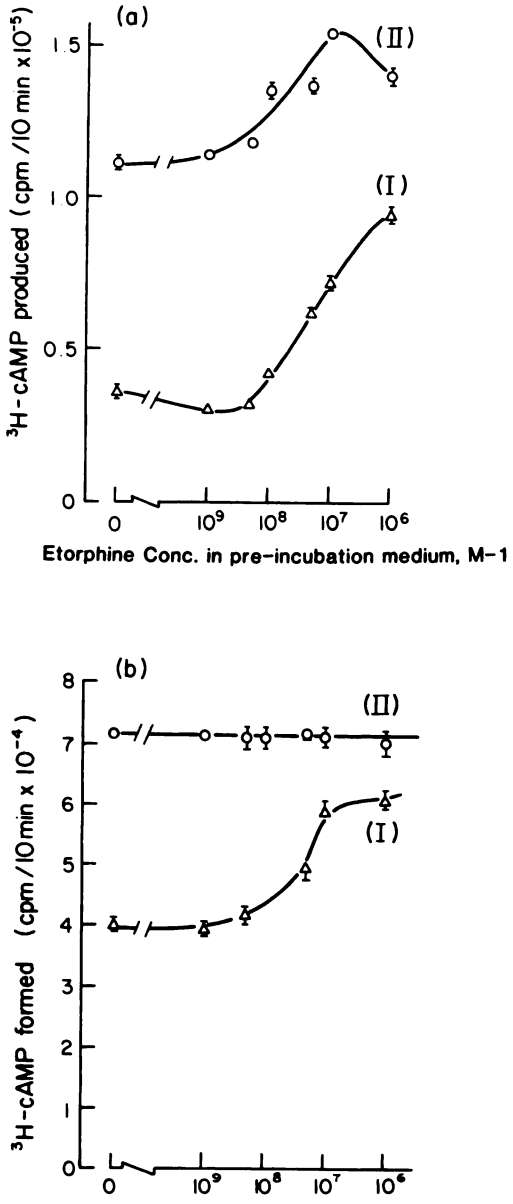

FIG. 8. *Effect of the concentration of etorphine during preincubation (2.5 hr) on the PGE_2-stimulated increase in ^{3}H-labeled cyclic AMP level*

a. NG108-15 cells (2.3×10^5 cells) were treated with various concentrations of etorphine for 2.5 hr. Sixty minutes before the main incubation period, [^{3}H]adenine was introduced to the media containing varying concentrations of etorphine. The amounts of radioactivity being taken up by the cells in the presence and absence of etorphine were identical. During the main incubation period (10 min at 37°), after removal of the [^{3}H]adenine and preincubation mixture, 10 μM PGE_1 + 1 μM etorphine (*curve I*) or 10 μM PGE_1 + 100 μM naloxone (*curve II*) were added to the 17-mm wells.

b. The neuroblastoma N18TG2 cells were pretreated with etorphine as described in a. The number of cells present in each well was determined to be 9.5×10^5. *Curve I* represents the amount of ^{3}H-labeled cyclic AMP formed during the 10-min incubation period in the presence of 10 μM PGE_1 + 1 μM etorphine. *Curve II* represents the ^{3}H-labeled cyclic AMP formed in the presence of 10 μM PGE_1 + 100 μM naloxone.

cells but not in the N18TG2 cells. Chronic exposure of these two cell lines to opiates produced a naloxone-induced increase in adenylate cyclase activity in the NG108-15 cells but not in the N18TG2 cells. These data suggested that the molecular events after the formation of the opiate ligand-receptor complex and the subsequent inhibition of adenylate cyclase activities in these two cell lines could not be identical.

One possible explanation for the difference in opiate activities between N18TG2 cells and NG108-15 cells could be the difference in opiate receptor affinities and densities. There is unequivocal evidence that opiate receptor density in the N18TG2 cell is significantly lower than that in the NG108-15 cell, but, apparently, opiate regulation of adenylate cyclase in the NG108-15 cells was not altered by the destruction of the opiate receptor. Fantozzi *et al.* (15) reported that the enkephalin inhibition of adenylate cyclase activity was not affected by the reduction of opiate binding up to 95% with chlornaltrexamine, an irreversibly binding opiate ligand. Studies with phospholipase C revealed a reduction of enkephalin potency and efficacy in NG108-15 membrane treated by the enzyme without a concurrent decrease in the number of opiate receptors (16). The binding affinity was decreased by phospholipase C. Furthermore, in the present study opiates have different efficacy in inhibiting adenylate cyclase activity in NG108-15 cells (Table 1). Therefore, the difference in opiate activities in N18TG2 and NG108-15 could not be accounted for by the difference in receptor densities.

Another possible explanation for the difference in opiate activities in these two cell lines is the difference in the N-component. From genetic studies with the S-49 cells (17), it was concluded that the receptor activity could be regulated by the concentration of the regulatory component of adenylate cyclase, the N-component (18). Since opiate receptor inhibition of adenylate cyclase activity has an absolute requirement for GTP (9, 10) (Fig. 4), the density of the N-component in these two cell lines, N18TG2 and NG108-15, could be a possible site for the differences in opiate activity. In our preliminary studies, using cholera toxin and [^{32}P]NAD^+ to label the N-component of both cell lines, we observed similar density of a 42-K component in N18TG2 and NG108-15 cells. Whether this 42-K component is the regulatory component of the adenylate cyclase activity requires further experimentation. Nevertheless, these data suggested that the density of the N-component in N18TG2 cells might not be the cause for the differences in opiate activity.

Another possibility for the differences in opiate activity in N18TG2 and NG108-15 cells is the difference in the coupling process. It is unequivocally evident that the opiate receptor-adenylate cyclase coupling processes in these two cell lines are dissimilar. Sodium ions have different effects on the opiate potencies in N18TG2 and NG108-15 cells (Figs. 5–7). This is surprising since the opiate agonist receptor interactions in N18TG2 and NG108-15 cells are attenuated by the monovalent cation (1, 19).

A greater difference in the coupling process in these cell lines was revealed by the difference in the adaptation of these cell lines to the chronic opiate treatment. As reported by Sharma *et al.* (12, 14) and Brandt *et al.* (13), the adenylate cyclase activity in NG108-15 cells treated with opiate chronically was increased above the control level upon the addition of naloxone—the "withdrawal" response. Although similar to that in NG108-15 cells,

chronic opiate treatment produced a "tolerance" response in N18TG2 cells, and the "withdrawal" response was absent in this cell line. Since this "withdrawal" response could be observed with the membrane preparations of the NG108-15 cells (12, 14), it is possible that there was an alteration of the membrane components in the NG108-15 cells chronically treated with opiates. Wilkening and Nirenberg (20) reported that this "withdrawal" effect in NG108-15 cells was eliminated by the growth of the NG108-15 cells in delipidized serum, and that the effect could be restored by unsaturated fatty acids. Therefore, the inability of the N18TG2 cells to undergo a "withdrawal" response after the chronic opiate treatment suggested a possible difference in NG108-15 and N18TG2 membrane components other than the opiate receptor and N-component in the coupling of the receptor adenylate cyclase.

In conclusion, the stereoselective, high-affinity opiate receptor observed in the neuroblastoma N18TG2 cell is demonstrated to be coupled to the adenylate cyclase. Basically, opiate inhibition of adenylate cyclase activity in N18TG2 is analogous to that in NG108-15 cells. However, the opiate potency and efficacy observed are significantly lower with the N18TG2 cells. This difference in efficacy could be due to the differences in the opiate receptor adenylate cyclase coupling process. Thus, if the process in N18TG2 could be altered, so would the efficacy of the opiate agonist. As discussed in our subsequent paper (21), manipulation of the coupling process by alteration of the membrane lipid composition was carried out. The effect of membrane lipid modification on opiate receptor binding activity and opiate inhibition of adenylate cyclase activity in neuroblastoma N18TG2 cells will be reported.

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