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Direct coupling of opioid receptors to both stimulatory and inhibitory guanine nucleotide-binding proteins in F-11 neuroblastoma–sensory neuron hybrid cells

(dorsal root ganglion neuron/cholera toxin/pertussis toxin/adenylate cyclase/signal transduction)

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ABSTRACT **Evidence is presented for linkage of opioid receptors directly to the stimulatory G protein (guanine nucleotide-binding protein), G_s, in addition to the generally accepted linkage to the inhibitory and "other" G proteins, G_i and G_o, in F-11 (neuroblastoma–dorsal root ganglion neuron) hybrid cells. Treatment of intact F-11 cells with cholera toxin decreased specific binding of the opioid agonist [D-Ala², D-Leu⁵]enkephalin to F-11 cell membranes by 35%, with the remaining binding retaining high affinity for agonist. Under these conditions cholera toxin influenced the α subunit of G_s ($G_s\alpha$) but had no effect on the α subunit of $G_{i/o}$ ($G_{i/o}\alpha$), based on ADP-ribosylation studies. Pertussis toxin treatment decreased high-affinity opioid agonist binding by about 50%; remaining binding was also of high affinity, even though pertussis toxin had inactivated $G_{i/o}\alpha$ selectively and essentially completely. Simultaneous treatment with both toxins had an additive effect, reducing specific binding by about 80%. While opioid agonists inhibited forskolin-stimulated adenylate cyclase activity of F-11 cells as expected, opioids also stimulated basal adenylate cyclase activity, indicative of interaction with G_s as well as G_i. Cholera toxin treatment attenuated opioid-stimulation of basal adenylate cyclase, whereas pertussis toxin treatment enhanced stimulation. In contrast, inhibition by opioid of forskolin-stimulated activity was attenuated by pertussis toxin but not by cholera toxin. It is concluded that a subset of opioid receptors may be linked directly to G_s and thereby mediate stimulation of adenylate cyclase. This G_s–adenylate cyclase interaction is postulated to be responsible for the novel excitatory electrophysiologic responses to opioids found in our previous studies of sensory neurons and F-11 cells.**

Opioid receptors are generally known to be linked to pertussis toxin (PTX)-sensitive guanine nucleotide-binding proteins (G proteins) that mediate inhibition (G_i proteins) of neuronal activity and (via G_i adenylate cyclase (AC) (1–6). However, opioid agonists, at concentrations lower than that required to inhibit neuronal activity, elicit excitatory electrophysiologic effects on dorsal root ganglion (DRG) neurons in culture (3, 7, 8). Considerable circumstantial evidence has implicated G_s, a cholera toxin (CTX)-sensitive stimulatory G protein, and cAMP in these excitatory effects (3, 9). We have also found that for DRG neurons and DRG–spinal cord explants, in addition to the predicted inhibition of forskolin-stimulated AC activity by opioids, basal activity is stimulated by opioids (10) via a receptor-mediated process (11).

In the present study we have utilized F-11 cells to obtain direct biochemical support for mediation of the dual excitatory/inhibitory effects of opioids by coupling of opioid receptors to both CTX- and PTX-sensitive G proteins. The F-11 cell line, a mouse N18TG2 neuroblastoma–rat primary DRG neuron hybrid, retains a number of properties of sensory (DRG) neurons (12, 13). Also, F-11 cells contain an opioid-inhibited AC system (13) and δ- and μ-opioid receptor binding sites (14). Opioids at higher concentrations inhibit electrophysiologic activity of F-11 cells (14) but at lower concentrations produce excitation (S. F. Fan, K.-F. Shen, and S.M.C., unpublished data). We report here that F-11 cells manifest dual regulation (stimulation and inhibition) of AC by opioids. We also report that CTX-sensitive as well as PTX-sensitive G proteins influence opioid receptor binding and opioid-sensitive AC in F-11 cells.

MATERIALS AND METHODS

Materials. [D-Ala², D-Leu⁵]Enkephalin (DADLE) and [D-Ala², *N*-Me-Phe⁴, Gly⁵-ol]enkephalin (DAGO) were from Sigma, levorphanol and dextrorphan were from Hoffman–LaRoche, naloxone was from Endo Laboratories (New York), PTX and CTX were from List Biological Laboratories (Campbell, CA), and trans(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide (U50,488H) was from Research Biochemicals (Natick, MA). [³H]Diprenorphine (56 Ci/mmol; 1 Ci = 37 GBq), [³H]DADLE (40 Ci/mmol), and [³H]DAGO (54.4 Ci/mmol) were from New England Nuclear.

Cell Culture Conditions. F-11 cells (12, 13) and NCB-20 cells [a mouse neuroblastoma–Chinese hamster embryo brain explant hybrid cell line (15)] were grown on plastic dishes in Dulbecco's modified Eagle's medium (high glucose) with 10% (vol/vol) heat-inactivated fetal calf serum under 95% air/5% CO_2 at 37°C.

Opioid Receptor Binding. After removal of culture medium, cells were scraped into 50 mM Tris buffer (pH 7.5), and membranes were prepared as described (16). For binding assay, aliquots containing ≈40 μg of membrane protein were incubated in triplicate at 25°C for 40 min in 50 mM Tris buffer containing 0.1% bovine serum albumin. For displacement studies, labeled ligand was present at ≈0.2 nM. Data were analyzed by the LIGAND computer program (16, 17).

Adenylate Cyclase (AC) Activity. Particulate (membrane) fractions were prepared (10), assay incubations (with 50 μM GTP and with or without 40 mM NaCl) were carried out in triplicate at 30°C for 15 min, and the cAMP formed was

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Abbreviations: DRG, dorsal root ganglion; CTX, cholera toxin; PTX, pertussis toxin; DADLE, [D-Ala², D-Leu⁵]enkephalin; DAGO, [D-Ala², *N*-Me-Phe⁴, Gly⁵-ol]enkephalin; AC, adenylate cyclase; G protein, guanine nucleotide-binding protein; G_s, G_i, and G_o proteins, stimulatory, inhibitory, and "other" G proteins.

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measured as described (10). Specific activity is expressed as pmol of cAMP formed per mg of membrane protein (18).

Toxin-Catalyzed ADP-Ribosylation. Membrane suspensions were prepared as for the AC assay and then centrifuged; the pellets were resuspended for ADP-ribosylation assays (19). Aliquots (65 μl) containing 125–250 μg of membrane protein were incubated with dithiothreitol-activated CTX (5 μg) or PTX (2.5 μg) or without toxin (control) and with [^{32}P]NAD at 23–60 Ci/mmol under conditions previously described for *in vitro* ADP-ribosylation (19). Samples were processed and solubilized in 0.1% sodium dodecyl sulfate for electrophoresis on 10% polyacrylamide gels (19). Gels were stained with Coomassie blue and analyzed by autoradiography.

RESULTS

Opioid Receptor Characterization in F-11 Cells. Cross-displacement studies of F-11 membranes with labeled and unlabeled DADLE and DAGO indicated the presence of both δ and μ receptors in an ≈6:1 ratio, with K_d values for DADLE of 2.7 ± 1.5 and 14 ± 5.8 nM for δ and μ sites, respectively, and K_d values for DAGO of 1.16 ± 0.16 and 283 ± 7.2 nM for μ and δ sites in a two-site model (15) (Fig. 1). B_{max} values were 327 ± 33 fmol/mg of protein for δ sites and 55 ± 4.4 fmol/mg of protein for μ sites (mean values ± SEM; $n = 3$). With the antagonist [^{3}H]diprenorphine as ligand (data not shown), K_i values for DADLE and DAGO were in the low nanometer range, confirming the presence of δ and μ receptors. K_i values for levorphanol for both [^{3}H]DADLE and [^{3}H]diprenorphine sites were ≈5 nM, whereas dextrorphan caused no displacement (K_i > 10,000). U50,488H, an opioid agonist selective for certain κ receptor subtypes (20), failed to displace [^{3}H]diprenorphine binding at concentrations up to 0.1 mM.

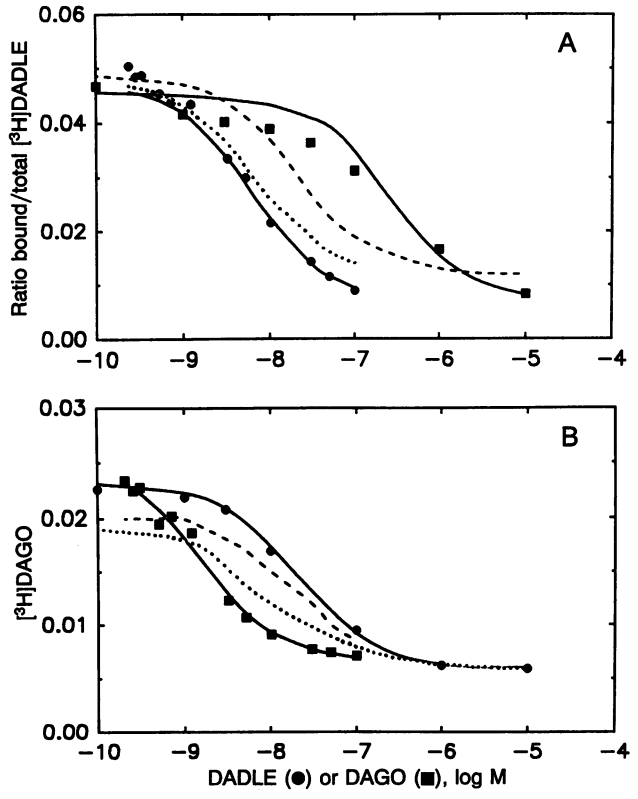

FIG. 1. Self- and cross-displacement studies of opioid agonist binding to F-11 cell membranes: competition by unlabeled DADLE (●) and DAGO (■) for [^{3}H]DADLE (*A*) and [^{3}H]DAGO (*B*) binding sites. Computer-analyzed best fits for two sites are indicated by solid lines; and for one site for unlabeled DADLE, by dotted lines; and for one site for unlabeled DAGO, by dashed lines. Similar results were obtained in two additional experiments.

Influence of CTX and PTX on Opioid Receptor Binding in F-11 Cells. Treatment of F-11 cells for 16–24 hr with 0.5 μg of PTX per ml resulted in about a 50% decrease in high-affinity specific [^{3}H]DADLE binding, with the remaining binding retaining high affinity for DADLE (Fig. 2). Interestingly, treatment with 1 μg of CTX per ml resulted in about a 35% decrease in specific [^{3}H]DADLE binding, with the remaining binding also of high affinity (Fig. 2). Mean values ± SEM for four separate sets of experiments for B_{max} were 300 ± 18, 155 ± 9, and 192 ± 6 fmol/mg of protein for control, PTX-treated, and CTX-treated cells, respectively; corresponding values for K_d were 2.6 ± 0.1, 2.4 ± 0.8, and 2.5 ± 0.4 nM. The changes in B_{max} correspond to a decrease of 145 fmol/mg due to PTX and a decrease of 112 fmol/mg due to CTX. In additional experiments (not shown) neither CTX nor PTX treatment of intact cells influenced high-affinity binding of the antagonist [^{3}H]diprenorphine. Thus, the CTX effect is agonist-selective, consistent with an effect on G-protein-coupling rather than on synthesis or degradation of receptor protein *per se*.

A separate set of experiments confirmed the above results for CTX and PTX and also showed that treatment of cells simultaneously with PTX and CTX resulted in about 80% loss of binding, a loss significantly greater than that caused by either toxin alone ($P < 0.001$) (Fig. 3). The effect of 1 μg of CTX per ml was nearly maximal, since in other studies (data not shown) the effect was dose-dependent, with a maximal decrease of about 40% produced by 10 μg of CTX per ml (see Fig. 2).

Opioid-Stimulated and Opioid-Inhibited AC Activities of F-11 Cells; Influence of CTX, PTX, and Na$^+$. Levorphanol (Figs. 4 and 5) but not dextrorphan (data not shown) significantly stimulated basal AC of control F-11 cell membranes when assays were carried out in the absence of Na$^+$. Stimulation was concentration dependent and blocked by the opioid antagonist diprenorphine (Fig. 4). Levorphanol also significantly inhibited forskolin-stimulated and basal AC when assays were carried out in the presence of 40 mM NaCl (Fig. 5). CTX treatment of intact cells attenuated the stimulation of basal AC by levorphanol (assays without Na$^+$), while PTX treatment enhanced stimulation by levorphanol (assays without Na$^+$) or resulted in stimulation rather than inhibition by levorphanol (assays with Na$^+$) (Fig. 5). PTX also completely attenuated the levorphanol-induced inhibition of forskolin-stimulated AC, while CTX had no significant

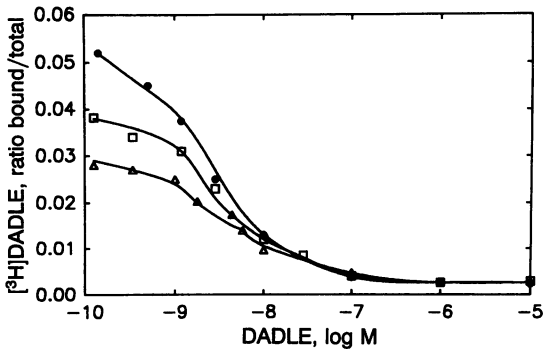

FIG. 2. Influence of treatment of intact F-11 cells for 16–20 hr with CTX or PTX on [^{3}H]DADLE binding: displacement of [^{3}H]-DADLE by unlabeled DADLE with membrane preparations from control cells (●), cells treated with 10 μg of CTX per ml (□), or cells treated with 2.5 μg of PTX per ml (△). Similar results were obtained in three additional experiments (see text for mean values, based on one-site analyses).

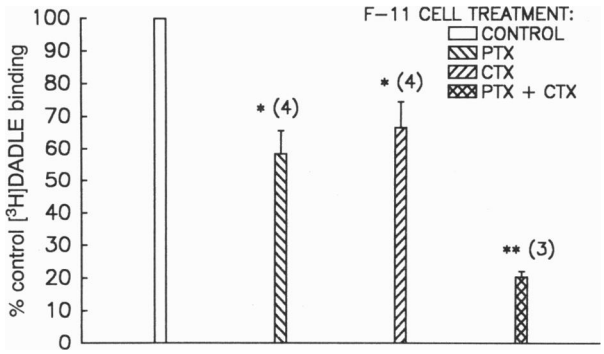

FIG. 3. Additive effects of CTX and PTX treatments of F-11 cells on [^{3}H]DADLE binding to F-11 cell membranes. Cells were treated with CTX (1 μg/ml) or PTX (0.5 μg/ml) or both for 24 hr prior to assay of total specific binding of 0.2 nM [^{3}H]DADLE. Values are means + SEM. (The number of separate experiments is in parentheses.) *, $P < 0.01$ versus control; **, $P < 0.001$ versus CTX or PTX alone.

effect on this inhibition (Fig. 5). In separate experiments, DADLE and etorphine stimulated basal AC and inhibited forskolin-stimulated AC, in the absence and in the presence of added NaCl, respectively.

Influence of CTX and PTX Treatments of Intact F-11 Cells on Subsequent Abilities of CTX and PTX to ADP-ribosylate Membrane G Proteins. Potentially, CTX treatment of intact cells might influence G_i indirectly by decreasing the amount of G_i or by causing ADP-ribosylation of the α subunits of G_i and G_s ($G_i\alpha$ and $G_s\alpha$). In either case, the amount of $G_i\alpha$ available for subsequent ADP-ribosylation by PTX would then be decreased. In order to measure the remaining G protein still available for *in vitro* ADP-ribosylation after *in vivo* toxin treatment, cells were treated with CTX, and membranes were prepared and then ADP-ribosylated with [^{32}P]NAD in the presence of activated CTX or activated PTX (21). By this criterion, after treatment of cells with 1 μg of CTX per ml, 72% of $G_s\alpha$ had already been inactivated *in vivo* (Fig. 6). The selectivity of the CTX treatment of intact cells for G_s was evidenced by the lack of change in $G_i\alpha$ and $G_o\alpha$ [PTX substrates, measured together ($G_{i/o}$)]. CTX may also catalyze ADP ribosylation of a number of membrane proteins nonspecifically *in vitro* (21). The selectivity of CTX treatment *in vivo* is indicated further by the finding that only the gel bands corresponding to $G_s\alpha$ are altered by *in vivo* CTX treatment (Fig. 6), in agreement with Watkins *et al.* (21). According to these studies, the effects of CTX on opioid binding and on AC are not due to an effect on G_i (or G_o).

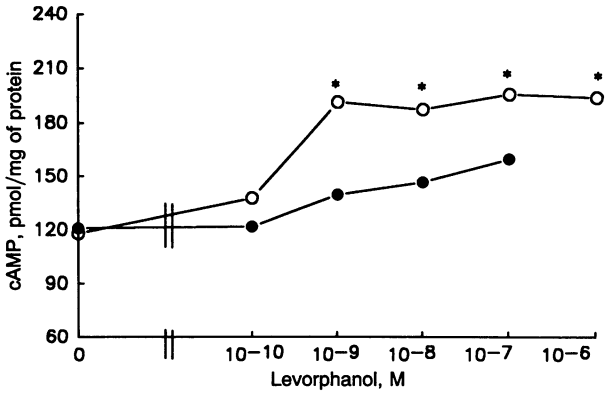

FIG. 4. Stimulation of F-11 cell AC activity by levorphanol (○) and blockade of levorphanol stimulation by 10 nM diprenorphine (●). Assays were in the absence of NaCl. Values are means of three separate experiments. *, $P < 0.01$ versus control.

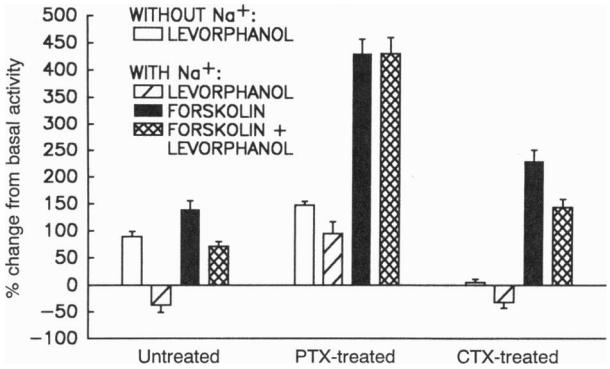

FIG. 5. Influence of CTX and PTX treatments of F-11 cells on dual opioid modulation of AC activity. Cells were treated with PTX (0.5 μg/ml) or CTX (1.0 μg/ml) for 24 hr prior to preparation of membranes for AC assays. Values are expressed as percent change (increase or decrease) from basal activity because of the presence of 2 μM levorphanol or 10 μM forskolin or both and are means of at least four separate experiments for each condition. NaCl was present at 40 mM where indicated. Basal activity values averaged 89, 160, and 171 pmol of cAMP/mg of protein for control, PTX-treated, and CTX-treated cells, respectively (values with and without NaCl were combined since there was no significant effect of NaCl on basal activity).

In parallel studies, treatment of intact cells with PTX resulted in no detectable remaining $G_{i/o}\alpha$ substrates for *in*

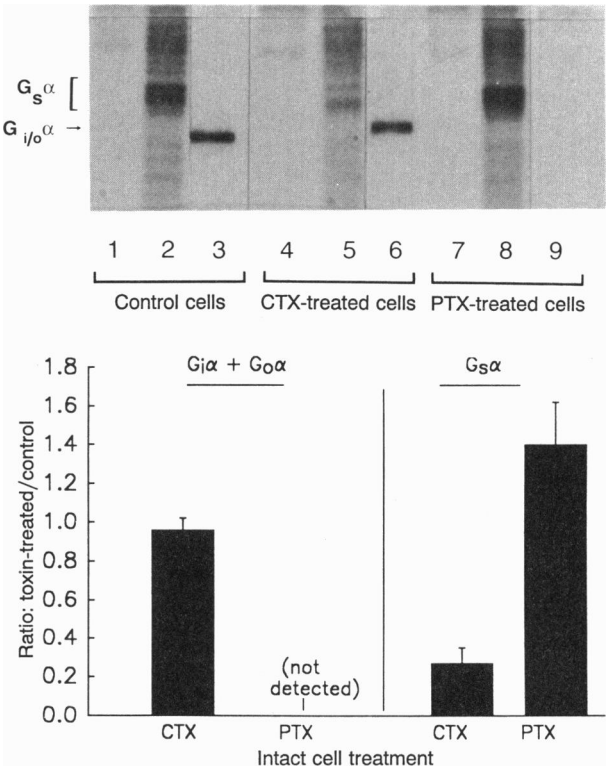

FIG. 6. Influence of PTX and CTX treatments of intact F-11 cells on subsequent PTX- and CTX-dependent ADP-ribosylation of F-11 membrane G protein α subunits. Cells were treated with 0.5 μg of PTX or 1.0 μg of CTX per ml for 24 hr, and membranes were then prepared for *in vitro* ADP-ribosylation. (*Upper*) One of three gel autoradiographs, all qualitatively similar. Lanes: 1, 4, and 7, no added toxin; lanes 2, 5, and 8, CTX (5 μg) added; lanes 3, 6, and 9, PTX (2.5 μg) added to ADP-ribosylation assay incubation. (*Lower*) Mean values ± SEM for three separate experiments determining the ratio of [^{32}P]ADP-ribose incorporation by membranes of toxin-treated cells to that of control cells, based on quantitative analyses of band intensities with a Molecular Dynamics computing densitometer.

vitro ADP-ribosylation by PTX (membranes were assayed with or without lubrol to enhance PTX substrate) (Fig. 6). Despite this essentially complete loss of functional $G_{i/o}$, there was only partial loss of opioid receptor binding after PTX treatment, consistent with association of a separate subpopulation of high-agonist-affinity opioid receptors with another G protein. In addition, after PTX treatment of intact cells, the $G_s\alpha$ membrane substrate for ADP-ribosylation by activated CTX was actually increased by 40% rather than decreased.

Comparative Studies of Opioid Receptors in NCB-20 Neuroblastoma–Primary Brain Explant Hybrid Cell Line; Influence of PTX and CTX. NCB-20 cells, hybrid neuronal cells not of sensory origin (15), were previously found to have an as-yet-uncharacterized opioid-stimulated AC system (stimulated by both DADLE and levorphanol but not by dextrorphan) that was markedly enhanced by PTX treatment of the cells (22). As was found for F-11 cells, CTX treatment of NCB-20 cells caused partial loss of [^{3}H]DADLE binding to δ receptors in NCB-20 cells, and effects of PTX and CTX were additive (Fig. 7); also CTX treatment did not alter high-affinity binding of the antagonist ligand [^{3}H]diprenorphine (data not shown). In NCB-20 cells PTX- and CTX-sensitive binding components may overlap because after treatment with PTX alone, high-affinity agonist binding was nearly all lost (Fig. 7). CTX treatment differed strikingly from PTX treatment with respect to Na^+ sensitivity of the remaining binding: after PTX treatment, Na^+ sensitivity was markedly increased, whereas after CTX treatment, the remaining binding was less sensitive to Na^+ (Fig. 8).

DISCUSSION

These studies provide evidence that F-11 cell opioid receptors interact not only with PTX-sensitive G proteins but also with a CTX-sensitive G protein. The influence of CTX on opioid binding could not be due to an effect on receptor level or turnover, since the effect of CTX on high-affinity agonist binding occurred in the absence of any effect on antagonist ([^{3}H]diprenorphine) binding. Also, the effect of CTX on binding could not be accounted for by an indirect effect on $G_{i/o}$. The effects of toxins on AC indicate further that there exists a dual regulation of AC by opioids, stimulation *via* a

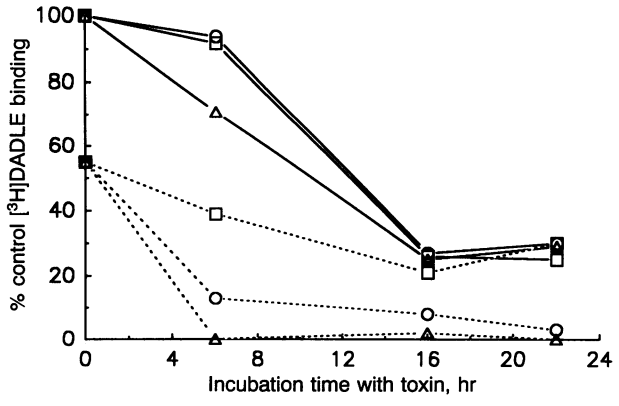

FIG. 7. [^{3}H]DADLE binding after treatment of intact NCB-20 cells with CTX at 1 μg/ml (□), PTX at 0.5 μg/ml (○), or combined CTX/PTX (△): time course for effects of toxins. Binding incubations were with 7 nM [^{3}H]DADLE (2 μM levorphanol used to determine nonspecific binding) in the absence (solid lines) or presence (broken lines) of 100 mM NaCl as indicated. Values have been normalized to the total specific binding of control cells assayed in the absence of NaCl (about 300 fmol/mg of protein). Values are means of three separate experiments. Saturation studies (not shown) indicated that the K_d for DADLE was about 2 nM in control cells and that the K_d for the remaining binding sites after toxin treatment did not change significantly.

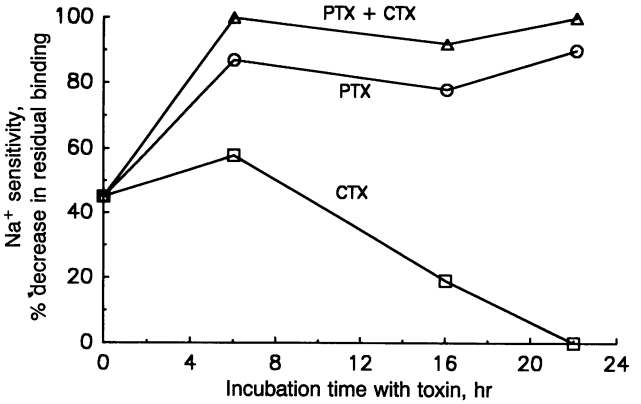

FIG. 8. Influence of CTX (□), PTX (○), and combined CTX/PTX (△) treatments of intact NCB-20 cells on the Na^+-sensitivity of [^{3}H]DADLE binding. Values are derived from Fig. 7 and represent the percent decrease in the post-toxin-treatment residual specific binding that was caused by addition of 100 mM NaCl in the binding assay (NaCl reduced binding by 45% in control cells, as shown).

CTX-sensitive G protein (presumably G_s), and inhibition *via* a PTX-sensitive G protein (G_i). PTX and CTX effects on opioid binding and on opioid-sensitive AC are distinct. It should be of interest to compare the low-affinity agonist receptor state(s) presumably generated by CTX treatment with those generated by PTX.

In F-11 cells, separate subsets of opioid receptors may be coupled to G_s and to G_i, since there persists high-affinity opioid binding that is CTX-sensitive, even after PTX treatment has apparently inactivated essentially all of the G_i and G_o ("other") proteins. Possibly in the intact cell a common pool of opioid receptors interacts either with one of several PTX-sensitive G proteins or with G_s, while in the membrane preparation assayed *in vitro*, individual receptors become fixed in their linkage to a particular G protein. The opioid receptor population potentially capable of linkage to G_s may be significantly greater than 35% (Figs. 2 and 3), since CTX treatment of intact F-11 cells resulted in incomplete ADP-ribosylation of G_s under the conditions used and since any excess in G_s relative to receptor would tend to minimize the loss of high-affinity binding because of CTX treatment. In NCB-20 cells at least, the binding studies indicate that a significant population of opioid receptors may be linked either to G_s or to $G_{i/o}$. Dopamine D_1 receptors have recently been reported to be capable of coupling either to G_s or to G_i (23). Our present results indicate that δ receptors, the dominant receptor subtype in F-11 cells, are coupled to G_s as well as to $G_{i/o}$; possible coupling of F-11 μ receptors to G_s remains to be evaluated. It is also not known if different states of an opioid receptor, such as might arise from posttranslational modification, determine whether opioid receptor linkage is to G_s or to $G_{i/o}$.

The present studies of AC together with our recent findings of opioid receptor-mediated stimulation of AC in DRG–cord explant cultures (11) represent the first reports of G_s-linked receptor-mediated stimulation of AC by opioids. In addition to the stimulation by levorphanol reported here, in preliminary studies basal AC of F-11 cells was also stimulated by nanomolar concentrations of DADLE and etorphine, with sensitivity to these agonists enhanced by PTX pretreatment of the cells. The results for NCB-20 cells indicate that the G_s-linked opioid system may occur not only in sensory neurons but also in other types of neurons. In preliminary studies, we did not find evidence for such a system in N18 neuroblastoma cells (parental line of the F-11 hybrid) or in rat brain striatum or amygdala. A PTX-sensitive (G_i-linked) opioid-stimulated AC has been reported to be present in rat olfactory bulb (24). G_s-linked, CTX-sensitive opioid receptor

binding sites have been described in NG-108 hybrid neuroblastoma–glioma cells (25). However, in contrast to our results with F-11 cells, CTX treatment of NG-108 cells attenuated inhibition of AC by opioids, while possible stimulation was not evaluated (26).

The Na^+ sensitivity of agonist binding to G_i-linked receptors but not to G_s-linked receptors and the relationship of ADP-ribosylation of G_i to effects of Na^+ and GTP on binding are well known (4, 21, 27–31). GTP and Na^+ may be required together to maximally increase the rate of dissociation of agonist from G_i-linked receptors, and PTX treatment may replace the requirement for GTP (21). The results for toxin effects on opioid binding in NCB-20 cells (Fig. 8) as well as on AC activity of F-11 cells (Fig. 5) are consistent with this differential influence of Na^+. Also, those results provide evidence, in addition to that from the ADP-ribosylation studies, that CTX action differs qualitatively from that of PTX and hence is not due to a mimicking of the effect of PTX by CTX.

Basal AC of F-11 cell membranes was no longer stimulated by opioids in the presence of 40 mM Na^+ (Fig. 5). However, whether or not opioids stimulate cAMP levels of intact cells in the presence of the usual concentrations of extracellular Na^+ is unclear, since extrapolation from membrane assay conditions to the intact cell is quite tenuous. Studies of intact cells will be required to establish this link between G_s-mediated opioid effects and electrophysiological events. Opioid effects in intact cells will most likely depend on the functional balance among the different G proteins as well as the status of AC activation and inhibition via other receptors and factors such as calmodulin. In view of the present biochemical studies, it seems highly likely that a major (if not the only) mechanism for excitatory bioelectric effects of opioids on sensory and possibly other neurons (3, 7, 8) involves the linkage of opioid receptors via G_s to stimulation of AC. Such a mechanism is consistent with the influences of CTX, PTX, cAMP analogues, and an inhibitor of cAMP-dependent protein kinase (PKA) on excitatory responses of opioids.

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