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Effects of sodium on agonist efficacy for G-protein activation in μ-opioid receptor-transfected CHO cells and rat thalamus

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1 Sodium ions inhibit spontaneous G_i/G_o-coupled receptor activity and promote agonist-induced responses *in vitro*. The effects of sodium on the relative efficacy of opioid agonists for G-protein activation was measured by guanosine-5′-*O*-(γ-^{35}S)-triphosphate ([^{35}S]-GTPγS) binding in membranes from two μ-opioid receptor-containing systems: CHO cells stably transfected with mouse μ receptors (mMOR-CHO cells) and rat thalamus.

2 NaCl inhibited basal [^{35}S]-GTPγS binding in both systems, and this effect was partially mimicked by KCl. In mMOR-CHO membranes, net [^{35}S]-GTPγS binding stimulated by partial but not full agonists was inhibited by NaCl with a potency that was inversely proportional to agonist efficacy. Monovalent cations were required for agonist-stimulated [^{35}S]-GTPγS binding in this system, and increasing NaCl concentrations magnified relative efficacy differences among agonists.

3 In thalamic membranes, which contain a lower receptor:G-protein ratio than mMOR-CHO cells, similar monovalent cation effects were observed, with two exceptions: (1) [^{35}S]-GTPγS binding stimulated by both full and partial agonists was inhibited by NaCl; and (2) monovalent cations were not required to observe agonist-stimulated [^{35}S]-GTPγS binding.

4 Basal [^{35}S]-GTPγS binding stimulated by the absence of monovalent cations resembled that of agonist-stimulated binding and was blocked by pretreatment of mMOR-CHO cells with pertussis toxin.

5 These results indicate that sodium inhibits spontaneous and agonist-occupied μ receptor-mediated G-protein activation in a manner inversely proportional to the efficacy of the agonist, and that spontaneous μ receptor activity and the relative efficacy of partial agonists acting at these receptors are both increased by increases in the stoichiometric ratio of receptors:G-proteins.
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Abbreviations: DAMGO, [D-Ala^2,(N-Me)Phe^4,Gly^5-ol]-enkephalin; DMEM, Dulbecco's modified eagle's medium; FBS, foetal bovine serum; GTPγS, guanosine-5′-*O*-(γ-thio)-triphosphate; PTX, pertussis toxin

Introduction

$G_{i/o}$-coupled receptors can display spontaneous activity in the absence of agonist, as demonstrated by the effects of inverse agonists (negative antagonists) on basal G-protein activity (Costa *et al*., 1990; Hilf & Jakobs, 1992; Tian *et al*., 1994). This inverse agonism is most readily observable in the absence of sodium (Costa *et al*., 1990; Hilf & Jakobs, 1992; Tian *et al*., 1994). Although the structural basis for the inhibitory effect of sodium on receptor-mediated G-protein activation is not known, it may be due to interaction of the cation with a conserved aspartate residue in the second putative transmembrane domain (TM2) of G-protein-coupled receptors (Horstman *et al*., 1990). Mutation of this residue has been shown to result in a loss of sodium sensitivity of high affinity agonist binding, but the effect of these mutations on the ability of receptors to activate G-proteins is dependent on both the receptor type and endpoint (e.g. effector and/or G-protein activity) under investigation (Ceresa & Limbird, 1994; Kong *et al*., 1993a,b; Tao & Abood, 1998).

μ-type opioid receptors, which have a high affinity for opiate-derived analgesics (Corbett *et al*., 1993), are members of the superfamily of G-protein-coupled receptors (Chen *et al*., 1993; Thompson *et al*., 1993) and are primarily coupled to G-proteins of the pertussis toxin (PTX)-sensitive $G_{i/o}$-type (Childers, 1991). Different μ-opioid agonists display varying intrinsic efficacies for a variety of biological responses both *in vivo* (Adams *et al*., 1990; Mjanger & Yaksh, 1991) and *in vitro* (Carter & Medzihradsky, 1993; Yu & Sadee, 1988). Several laboratories have demonstrated that differences in μ-opioid agonist efficacy can be measured at the level of G-protein activation, as determined by their ability to maximally stimulate binding of the hydrolysis-resistant GTP analogue, guanosine-5′-*O*-(γ-^{35}S)-triphosphate ([^{35}S]-GTPγS), to membranes from cultured cell lines (Emmerson *et al*., 1996; Selley *et al*., 1997; 1998; Traynor & Nahorski, 1995) and brain (Selley *et al*., 1997; 1998; Sim *et al*., 1998). Moreover, relative efficacy differences among μ-opioid agonists depend on factors that affect the balance between G-protein activation and inactivation, such as the GDP concentration and the stoichiometric ratio of receptors:G-proteins (R:G ratio) (Selley *et al*., 1997; 1998), in a similar manner to other $G_{i/o}$-coupled receptors (Lorenzen *et al*., 1996; Newman-Tancredi *et al*., 1997; Pauwels *et al*., 1997). Studies from our laboratory have also shown that agonists of high and low intrinsic efficacies differ in their abilities to stimulate a maximal apparent affinity of [^{35}S]-GTPγS binding and to activate a maximal number of G-proteins (Selley *et al*., 1997; 1998). These differences are probably related to differences in the ability of receptors to modulate G-protein affinity for GDP relative to that of [^{35}S]-

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GTPγS when occupied by agonists of different intrinsic efficacies (Breivogel *et al*., 1998; Lorenzen *et al*., 1996; Selley *et al*., 1997).

Mathematical modelling of the inhibitory effect of sodium on receptor-coupled G-protein activity has predicted that relative agonist efficacy should be inversely proportional to the sodium concentration (Costa *et al*., 1992). In the present study, this hypothesis was tested by examining agonist-stimulated [^{35}S]-GTPγS binding using opioid agonists of different intrinsic efficacies in membranes from two μ-opioid receptor-containing systems: CHO cells stably transfected with mouse μ receptor cDNA (mMOR-CHO cells) and which express high receptor levels (4–7 pmol mg^{-1}) (Abood *et al*., 1995; Kaufman *et al*., 1995; Selley *et al*., 1998), and rat thalamus, which contains a moderate number of μ receptors (~0.75 pmol mg^{-1}) with few other opioid receptor types present (Herkenham & Pert, 1982; Selley *et al*., 1998). Furthermore, studies were performed in mMOR-CHO cell membranes to determine whether sodium inhibition of basal [^{35}S]-GTPγS binding was due to inhibition of spontaneous μ receptor activity. These studies examine the mechanisms by which G-protein activity is regulated by receptors and their agonists, as well as by the cellular environment.

Methods

Materials

Male Sprague-Dawley rats (200 g) were purchased from Zivic-Miller (Zelienople, PA, U.S.A.). [^{35}S]-GTPγS (1250 Ci mmol^{-1}) was purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). mMOR-CHO cells were generously provided by Drs Lawrence Toll and Christopher J. Evans. ScintiSafe Econo-1 scintillation fluid was obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). [D-Ala^2,(N-Me)Phe^4,Gly^5-ol]-enkephalin (DAMGO), naloxone, penicillin-streptomycin and pertussis toxin (PTX) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other non-peptide opioid agonists were obtained from the N.I.D.A. drug supply program (Research Triangle Institute, Research Triangle Park, NC, U.S.A.). Foetal bovine serum (FBS) and Geneticin (G-418) were purchased from Gibco/BRL (Grand Island, NY, U.S.A.). Guanosine-5′-*O*-(γ-thio)-triphosphate and guanosine-5′-diphosphate were purchased from Boehringer Mannheim (New York, NY, U.S.A.). All other chemicals (reagent grade) were obtained from Sigma Chemical Co. or from Fisher Scientific.

Cell culture

mMOR-CHO cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in 50% DMEM and 50% F-12 Nutrient Mixture (Ham) containing 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 5% FBS (culture media). Cells were harvested by replacing the culture media with cold phosphate-buffered saline containing 0.04% EDTA, incubated for 5 min followed by agitation, and collected by centrifugation at 345 × *g* for 10 min. For cell culture treatments, cells were incubated in culture media without serum in the presence and absence of 100 ng ml^{-1} PTX or 5 μM DAMGO for 18–24 h.

Membrane preparation

Rats were sacrificed by decapitation and the thalamus was dissected on ice. mMOR-CHO cells or rat thalami were homogenized in 20 volumes of ice-cold 50 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EGTA, pH 7.4 (membrane buffer) with a Tissuemizer (Tekmar, Cincinnati, OH, U.S.A.). The homogenate was centrifuged at 48,000 × *g* at 4°C for 10 min, resuspended in membrane buffer, centrifuged again at 48,000 × *g* at 4°C for 10 min and finally resuspended in 50 mM Tris-HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, pH 7.4 (assay buffer).

[^{35}S]-GTPγS binding assays

Agonist-stimulated [^{35}S]-GTPγS binding was assayed as previously described with slight modifications (Selley *et al*., 1997; 1998). mMOR-CHO cell (25 μg protein) or rat thalamic (10 μg protein) membranes were incubated for 1 h at 30°C in a final volume of 1 ml. The incubation mixture included 0.05-0.1 nM [^{35}S]-GTPγS and 10 μM (mMOR-CHO) or 30 μM (rat thalamus) GDP with and without maximally effective concentrations of various agonists, in assay buffer. Unless otherwise indicated, agonist concentrations were: 100 nM levallorphan, 50 nM buprenorphine, 10 μM morphine, and 5 μM (mMOR-CHO) or 10 μM (rat thalamus) DAMGO. In some experiments, the NaCl or GDP concentrations were varied or omitted from the reaction mixture. In other experiments, the total monovalent cation concentration was maintained at 120 mM by replacing NaCl with equimolar concentrations of KCl. In all experiments, basal binding was assessed in the absence of drug, and nonspecific binding was measured in the presence of 10 μM unlabelled GTPγS. For GTPγS saturation analyses, mMOR-CHO cell membranes were incubated in assay buffer (1 ml final volume) with 0.08–0.1 nM [^{35}S]-GTPγS and 0.02–2000 nM unlabelled GTPγS in the presence of 10 μM GDP, with and without various drugs, for 1 h at 30°C. All incubations were terminated by rapid filtration under vacuum through Whatman GF/B glass fibre filters, followed by three washes with 3 ml ice-cold 50 mM Tris-HCl, pH 7.4. Bound radioactivity was determined by liquid scintillation spectrophotometry at 95% efficiency for ^{35}S, after overnight extraction of the filters in 5 ml ScintiSafe Econo-1 scintillation fluid.

Receptor binding assays

mMOR-CHO cell membranes (50 μg protein) were incubated for 1 h at 30°C in assay buffer with 0.8 nM [^{3}H]-naloxone and 0–50 nM unlabelled naloxone. Nonspecific binding was determined with 10 μM unlabelled levallorphan. Reactions were terminated by rapid filtration through glass fibre filters, and the filters were rinsed three times with ice-cold Tris buffer. Bound radioactivity was determined by liquid scintillation spectrophotometry at 45% efficiency for ^{3}H, after overnight extraction of the filters in scintillation fluid.

Data analysis

Unless otherwise indicated, data are reported as mean values ± s.e. of at least three separate experiments that were each performed in triplicate. Net stimulated [^{35}S]-GTPγS binding is defined as stimulated binding minus basal binding. Per cent stimulation is defined as net stimulated binding divided by basal binding (× 100%). Non-linear regression analysis of concentration-effect curves was performed with JMP (SAS Institute, Cary, NY, U.S.A.) using an iterative model. Statistical significance of the data was determined by analysis of variance followed by the non-paired 2-tailed Student's *t*-test or Dunnett's test, also performed with JMP.

IC_{50} values of GDP were determined by log-logit (Hill) analysis. Saturation analyses of absolute and net-stimulated [^{35}S]-GTPγS binding were conducted using EBDA and LIGAND, as previously described (Selley *et al*., 1997; 1998). Because the presence of GDP precludes determination of absolute K_D and B_{max} values from [^{35}S]-GTPγS saturation analyses, these values are termed apparent K_D and B_{max} values.

Results

Effects of sodium on relative agonist efficacy

To examine the effects of sodium on the relative efficacy of μ-opioid agonists, saturating concentrations of agonists were assayed for stimulation of [^{35}S]-GTPγS binding in membranes prepared from mMOR-CHO cells (Kaufman *et al*., 1995). Agonists were chosen on the basis of different relative and intrinsic efficacies for G-protein activation: DAMGO, sufentanil, morphine, buprenorphine and levallorphan (Emmerson *et al*., 1996; Selley *et al*., 1997; 1998 Traynor & Nahorski, 1995). Agonist-stimulated [^{35}S]-GTPγS binding was measured with excess (10 μM) GDP and varying concentrations of NaCl. Sodium inhibited basal [^{35}S]-GTPγS binding with an IC_{50} value of 30 ± 5.7 mM (Figure 1A). No significant stimulation of [^{35}S]-GTPγS binding was observed with any agonist in the absence of NaCl. However, the potency of NaCl in inhibiting [^{35}S]-GTPγS binding was decreased in the presence of agonists. Levallorphan increased the concentration of NaCl required for half-maximal inhibition by approximately 2.5 fold, whereas buprenorphine increased it by approximately 4 fold. These potency values could only be estimated because maximal effects of sodium could not be observed in the presence of these partial agonists. The high efficacy agonists, DAMGO, sufentanil and morphine, eliminated NaCl inhibition of [^{35}S]-GTPγS binding up to 125 mM NaCl. Figure 1B shows net agonist-stimulated [^{35}S]-GTPγS binding as a function of NaCl concentration. For the partial agonists levallorphan and buprenorphine, agonist-stimulated [^{35}S]-GTPγS binding increased as a function of NaCl concentration up to 25 and 50 mM, respectively. Net [^{35}S]-GTPγS binding stimulated by levallorphan and buprenorphine was decreased by 100–125 mM NaCl ($P<0.05$ versus 25 and 50 mM NaCl, respectively). With the higher efficacy agonists, DAMGO, sufentanil and morphine, net agonist-stimulated [^{35}S]-GTPγS binding increased with NaCl concentrations up to 75 mM, but was not significantly inhibited by NaCl concentrations up to 125 mM. When the data were calculated as per cent stimulation over basal [^{35}S]-GTPγS binding (Figure 1C), stimulation by the high efficacy agonists increased with increasing NaCl concentrations up to approximately 100 mM, due in part to the decrease in basal [^{35}S]-GTPγS binding. However, per cent stimulation by levallorphan and buprenorphine increased to a maximum at 50 and 75 mM NaCl, respectively, and decreased with 125 mM NaCl. ($P<0.05$ versus 50 and 75 mM NaCl, respectively). Thus, relative agonist efficacy for G-protein activation *via* μ-opioid receptors was dependent on the sodium concentration, with increasing sodium concentrations magnifying relative efficacy differences.

In rat thalamic membranes, previous studies (Selley *et al*., 1997; 1998) showed that morphine and sufentanil acted as partial agonists relative to DAMGO, buprenorphine was a lower efficacy partial agonist, and levallorphan was an antagonist. In this system, NaCl inhibited basal and agonist-stimulated [^{35}S]-GTPγS binding (Figure 2A). The IC_{50} value of NaCl in inhibiting basal [^{35}S]-GTPγS binding was 40 ± 4.4 mM,

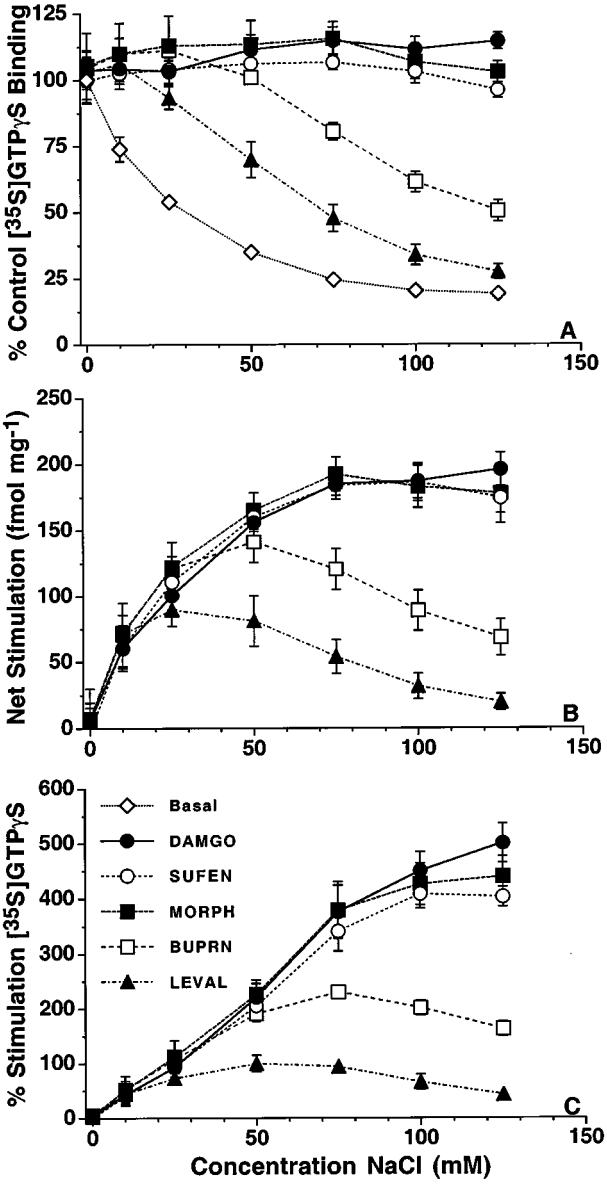

Figure 1 Effect of NaCl on relative agonist efficacy for stimulation of [^{35}S]-GTPγS binding to mMOR-CHO cell membranes. Membranes were incubated with 0.1 nM [^{35}S]-GTPγS and varying concentrations of NaCl in the presence of 10 μM GDP with and without maximally effective concentrations of opioid agonists. Data are mean values $\pm$ s.e. of: (A) per cent of control [^{35}S]-GTPγS binding (measured in the absence of NaCl or agonist); (B) net agonist-stimulated [^{35}S]-GTPγS binding; and (C) per cent stimulation of [^{35}S]-GTPγS binding by agonist (measured at each NaCl concentration). Control [^{35}S]-GTPγS binding was 206 ± 16.9 fmol mg^{-1}.

similar to mMOR-CHO cell membranes. However, in contrast to mMOR-CHO membranes, all agonists stimulated [^{35}S]-GTPγS binding in thalamic membranes by approximately 40% in the absence of sodium, and increasing NaCl concentrations inhibited [^{35}S]-GTPγS binding stimulated by all agonists. Similarly, net agonist-stimulated [^{35}S]-GTPγS binding was inhibited by increasing concentrations of NaCl (Figure 2B). Net levallorphan-stimulated binding was inhibited by all NaCl concentrations tested ($P<0.01$ versus no NaCl), reaching a minimum at 100 mM NaCl, where the compound produced no stimulation. Net buprenorphine-stimulated binding was also inhibited by all NaCl concentrations tested ($P<0.01$ versus no NaCl), while net sufentanil- and morphine-stimulated [^{35}S]-GTPγS binding was decreased by 50–125 mM NaCl ($P<0.05$ versus no NaCl), and net DAMGO-stimulated binding was

only significantly inhibited by 100–125 mM NaCl ($P<0.05$ versus no NaCl). When the data were calculated as per cent stimulation (Figure 2C), stimulation by DAMGO increased with increasing NaCl concentrations, reaching a maximum at 75 mM NaCl. A similar effect was observed with sufentanil and morphine, but the maximal per cent stimulation by these agonists was achieved at 25 mM NaCl, and did not increase further with increasing NaCl concentrations. The per cent stimulation by levallorphan was decreased by all NaCl concentrations ($P<0.01$ versus no NaCl), and was abolished by 100–125 mM NaCl. Similarly, NaCl concentrations of 25–125 mM inhibited the per cent stimulation by buprenorphine ($P<0.05$ versus no NaCl), reaching a minimum at 100 mM NaCl. Thus, increasing sodium concentrations also increased the relative efficacy differences among agonists in rat thalamus.

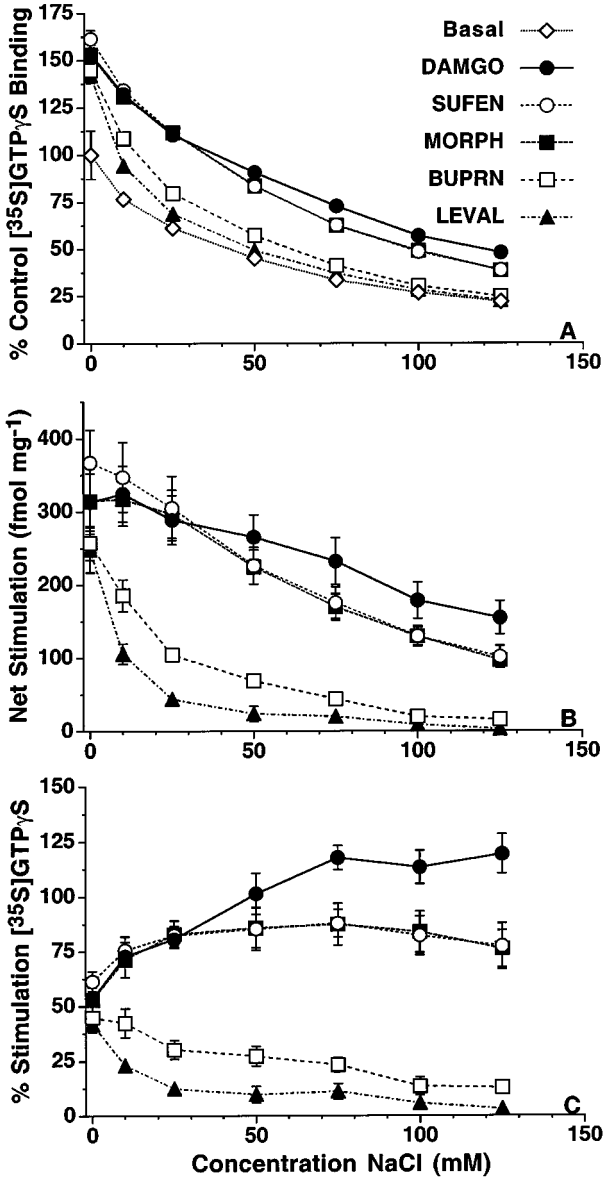

Figure 2 Effect of NaCl on relative agonist efficacy for stimulation of [^{35}S]-GTPγS binding to rat thalamic membranes. Membranes were incubated with 0.1 nM [^{35}S]-GTPγS and varying concentrations of NaCl in the presence of 30 μM GDP with and without maximally effective concentrations of the various opioid agonists. Data are mean values ± s.e. of: (A) per cent of control [^{35}S]-GTPγS binding (measured in the absence of NaCl or agonist); (B) net agonist-stimulated [^{35}S]-GTPγS binding; and (C) per cent stimulation of [^{35}S]-GTPγS binding by agonist (measured at each NaCl concentration). Control [^{35}S]-GTPγS binding was 588 ± 74.9 fmol mg^{-1}.

The inhibitory effect of NaCl on stimulation of [^{35}S]-GTPγS binding by partial agonists was due to decreases in the relative efficacy of these compounds, rather than decreases in their potency (Table 1). In mMOR-CHO membranes, increasing the NaCl concentration from 20 to 120 mM increased the DAMGO and levallorphan EC_{50} values by 150 fold and 6.5 fold, respectively, but also increased the efficacy of DAMGO relative to levallorphan by 10 fold. Similar results were obtained comparing DAMGO and morphine in thalamic membranes, with a 14 fold increase in the EC_{50} value of DAMGO and a 4 fold increase in the EC_{50} value of morphine, while the efficacy of DAMGO increased by 2 fold compared to morphine.

The effect of NaCl was partially selective to sodium, as demonstrated by replacement of NaCl with equal concentrations of KCl. Basal [^{35}S]-GTPγS binding in mMOR-CHO membranes was effectively suppressed by K^+: basal [^{35}S]-GTPγS binding increased only 83% when 120 mM Na^+ was replaced with 120 mM K^+ (Figure 3), in contrast to the 5 fold increase observed in the absence of monovalent cations (Figure 1A). DAMGO- and morphine-stimulated [^{35}S]-GTPγS binding, however, remained unaffected when K^+ was replaced by Na^+ (Figure 3). In contrast, Na^+ decreased the levels of both buprenorphine- and levallorphan-stimulated [^{35}S]-GTPγS binding. These results show that, unlike DAMGO and morphine, the relative efficacies of buprenorphine and levallorphan were sharply decreased by increasing the Na^+:K^+ ratio. Similar results were obtained in rat thalamic membranes (not shown): replacement of Na^+ with K^+ increased basal [^{35}S]-GTPγS binding by 36% (compared to the 5 fold increase in the absence of monovalent cations), and increasing the Na^+:K^+ ratio sharply decreased the relative efficacy of partial agonists, especially buprenorphine and levallorphan. Thus, K^+ only partially substituted for Na^+ with regard to its effects on basal and agonist-stimulated [^{35}S]-GTPγS binding in both mMOR-CHO and thalamic membranes.

Effects of pertussis toxin (PTX) on agonist- and sodium-modulated [^{35}S]-GTPγS binding

To determine whether [^{35}S]-GTPγS binding stimulated by either DAMGO or the absence of NaCl was blocked by uncoupling of $G_{i/o}$ proteins from receptors, mMOR-CHO cells were treated with PTX and assayed for [^{35}S]-GTPγS binding. This treatment completely blocked PTX-catalyzed ADP-ribosylation of G-proteins in mMOR-CHO membranes, as measured with [^{32}P]-NAD^+ (data not shown). In untreated cells, [^{35}S]-GTPγS binding was stimulated approximately 5 fold by either the absence of monovalent cations or by the addition of DAMGO and 100 mM NaCl (Figure 4). Stimulation by DAMGO and by the absence of monovalent cations was not additive. PTX pretreatment eliminated the stimulation of [^{35}S]-GTPγS binding by either the absence of monovalent cations or by DAMGO (with 100 mM NaCl). In untreated cells, naloxone blocked the stimulation of [^{35}S]-GTPγS binding by DAMGO (with 100 mM NaCl), but did not significantly affect basal [^{35}S]-GTPγS binding in the presence or absence of NaCl (data not shown).

Effects of DAMGO, sodium and PTX on competition by GDP for [^{35}S]-GTPγS binding to mMOR-CHO membranes

Agonists stimulate [^{35}S]-GTPγS binding by decreasing the affinity of Gα for GDP, correlating with agonist efficacy (Breivogel *et al*., 1998; Lorenzen *et al*., 1996; Selley *et al*.,

Table 1 Potency and efficacy values of selected partial agonists compared to the full agonist DAMGO in mMOR-CHO cell and rat thalamic membranes

(A) *mMOR-CHO*	*20 mM NaCl*		*120 mM NaCl*	
Agonist	EC_{50} (nM)	E_{max} (%Stim)	EC_{50} (nM)	E_{max} (%Stim)
DAMGO	0.32 ± 0.09^{a}	$136^{b} \pm 10$	48.5 ± 7.8^{b}	632 ± 58^{a}
Levallorphan	0.32 ± 0.07^{a}	$124^{b} \pm 6.8$	2.1 ± 1.1^{a}	61 ± 7.5^{c}
(B) *Rat thalamus*				
DAMGO	33.3 ± 5.8^{a}	49 ± 3.4^{ab}	438 ± 85^{b}	120 ± 6.4^{c}
Morphine	172 ± 25^{a}	45 ± 4.1^{a}	688 ± 138^{b}	64 ± 6.2^{b}

Membranes were assayed for [^{35}S]-GTPγS binding as described in Methods in the presence of the indicated concentration of NaCl and (A) 0.03–3000 nM DAMGO or 0.003–100 nM levallorphan, or (B) 10–30,000 nM DAMGO or 10–30,000 nM morphine. The data were analysed by non-linear regression analysis, and the mean EC_{50} and E_{max} values ± s.e. from four seperate experiments are shown. Pairs of EC_{50} or E_{max} values containing the same letter designations are not significantly different from each other ($P > 0.05$), whereas those not containing any of the same letter designations are $P < 0.05$ different from each other.

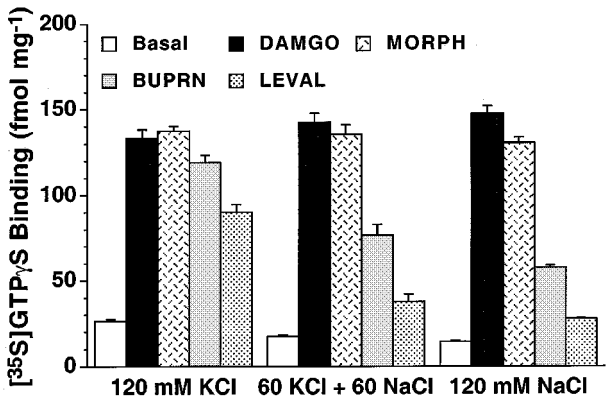

Figure 3 Effect of Na^+:K^+ ratio on relative agonist efficacy for stimulation of [^{35}S]-GTPγS binding to mMOR-CHO cell membranes. Membranes were incubated with 0.05 nM [^{35}S]-GTPγS in the presence of 10 μM GDP with and without maximally effective concentrations of opioid agonists and 120 mM KCl only, 60 mM KCl + 60 mM NaCl, or 120 mM NaCl only. Data are mean fmol [^{35}S]-GTPγS bound per mg membrane protein ± s.e.

Table 2 IC_{50} values for GDP inhibition of [^{35}S]-GTPγS binding in control and PTX-pretreated mMor-CHO cell membranes

	IC_{50} (nM)	
Cell membranes	*Basal*	*DAMGO*
Control + NaCl	73.0 ± 9.7	592 ± 187**
Control − NaCl	511 ± 188**	577 ± 109**
PTX + NaCl	44.2 ± 9.4	49.9 ± 12.4
PTX − NaCl	66.8 ± 18.4	67.2 ± 11.6

Membranes from control and PTX-pretreated mMOR-CHO cells were incubated with 0.1 nM [^{35}S]-GTPγS and varying concentrations (0–100 μM) of GDP in the presence and absence of 100 mM NaCl or 5 μM DAMGO. Data are mean IC_{50} values ± s.e. *$P < 0.05$, **0.01 different from control basal with NaCl present.

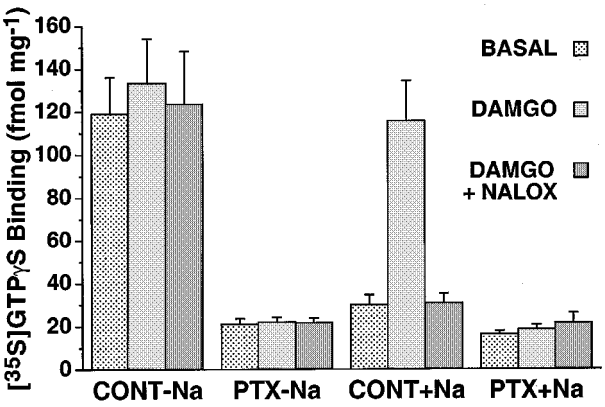

Figure 4 Effect of PTX pretreatment on modulation of [^{35}S]-GTPγS binding by NaCl and agonist. Membranes from control and PTX-pretreated mMOR-CHO cells were incubated with 0.05 nM [^{35}S]-GTPγS and 10 μM GDP in the presence and absence of 100 mM NaCl with and without 5 μM DAMGO and/or 1 μM naloxone present. Data are mean fmol [^{35}S]-GTPγS bound per mg membrane protein ± s.e.

1997). To examine whether [^{35}S]-GTPγS binding stimulated by the absence of monovalent cations produced the same shift in GDP affinity as a full agonist, mMOR-CHO membranes were incubated with varying concentrations of GDP in the presence and absence of NaCl and DAMGO. Results showed that the potency of GDP in competing for [^{35}S]-GTPγS binding was decreased by approximately 8 fold by either the presence of DAMGO or the absence of monovalent cations, compared to binding in the presence of 100 mM NaCl alone (Table 2). DAMGO did not further decrease the potency of GDP in the absence of monovalent cations. Furthermore, the effects of DAMGO and of the absence of monovalent cations on GDP potency were both blocked by prior treatment of the cells with PTX (Table 2).

Saturation analysis of [^{35}S]-GTPγS binding to mMOR-CHO membranes

Previous studies showed that DAMGO stimulated both the apparent affinity and B_{max} values of high affinity (but not low affinity) [^{35}S]-GTPγS binding to mMOR-CHO cell membranes (Selley *et al*., 1997; 1998). To determine whether similar effects are produced by the absence of monovalent cations, saturation analysis of [^{35}S]-GTPγS binding was conducted in mMOR-CHO membranes (Figure 5). These experiments showed that DAMGO decreased the apparent K_D of high affinity [^{35}S]-GTPγS binding by 4 fold, and increased the apparent B_{max} by 2.5 fold, in the presence of NaCl (Table 3). The absence of monovalent cations produced a similar effect, decreasing the apparent K_D and increasing the apparent B_{max} of high affinity [^{35}S]-GTPγS binding by 3 fold. Prior treatment of mMOR-CHO cells with PTX eliminated high affinity [^{35}S]-GTPγS binding in either the absence (Figure 5, Table 3) or presence (data not shown) of NaCl or DAMGO. PTX pretreatment, DAMGO and NaCl all had no affect on low affinity [^{35}S]-GTPγS binding.

Although a slight increase in basal [^{35}S]-GTPγS binding was observed in the absence of monovalent cations without GDP

(data not shown), this effect was not statistically significant ($P=0.16$) and not reversed by PTX. Nonetheless, to rule out a direct effect of sodium on G-protein binding of [^{35}S]-GTPγS, saturation analysis of [^{35}S]-GTPγS binding was conducted in mMOR-CHO membranes in the absence of GDP (not shown). No significant differences in apparent K_D or B_{max} values of high affinity [^{35}S]-GTPγS binding were found in the presence or absence of 100 mM NaCl (2.35 ± 0.23 nM and 21.6 ± 1.89 pmol mg^{-1} versus 1.96 ± 0.18 nM and 22.2 ± 1.86 pmol mg^{-1}, respectively). Similar results were obtained in the presence of DAMGO, which itself had no significant effect on high affinity [^{35}S]-GTPγS binding in the absence of GDP. Thus, similar to effects of agonists, the absence of monovalent cations increased both the apparent affinity and B_{max} of high affinity [^{35}S]-GTPγS binding only in the presence of GDP.

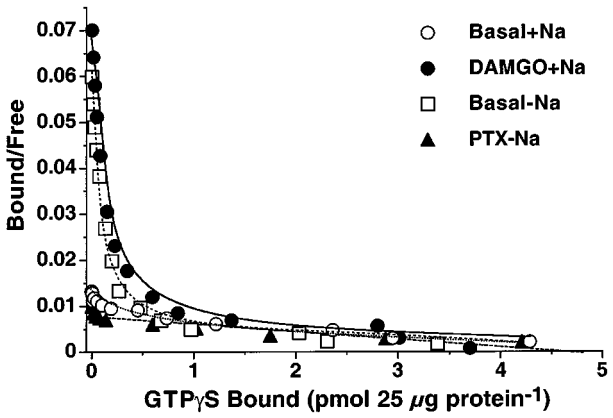

Figure 5 Effect of NaCl and agonist on saturation binding of [^{35}S]-GTPγS presented as a Scatchard plot. Membranes from control and PTX-pretreated mMOR-CHO cells were incubated with 0.1 nM [^{35}S]-GTPγS, 10 μM GDP and varying concentrations of unlabelled GTPγS in the presence and absence of 100 mM NaCl with and without 5 μM DAMGO. Data shown are from a typical experiment that was performed in triplicate and replicated at least three times with similar results.

Effects of chronic agonist treatment of mMOR-CHO cells on modulation of [^{35}S]-GTPγS binding by DAMGO and sodium

Previous research (Breivogel *et al*., 1997) has established that apparent B_{max} values of net agonist-stimulated [^{35}S]-GTPγS binding are decreased by agonist-induced receptor desensitization following chronic agonist treatment of cells. If stimulation of [^{35}S]-GTPγS binding by the absence of monovalent cations was due to spontaneous μ-opioid receptor activity, it might be possible to decrease the apparent B_{max} of these net-stimulated [^{35}S]-GTPγS binding sites by pretreatment of the cells with DAMGO to induce μ receptor desensitization. To examine this possibility, net-stimulated [^{35}S]-GTPγS binding was examined in GTPγS saturation experiments in membranes from control and DAMGO-pretreated mMOR-CHO cells. The results showed that both DAMGO (with 100 mM NaCl) and the absence of monovalent cations, yielded similar populations of net-stimulated [^{35}S]-GTPγS binding sites when basal binding in the presence of 100 mM NaCl alone was subtracted (Table 4). Pretreatment of mMOR-CHO cells for 18 h with 5 μM DAMGO desensitized the DAMGO-stimulated [^{35}S]-GTPγS binding in membranes, resulting in a 64% decrease in the apparent B_{max} of net DAMGO-stimulated [^{35}S]-GTPγS binding, with no significant change in the apparent K_D (Table 4). This attenuation of DAMGO-stimulated [^{35}S]-GTPγS binding was

Table 3 Apparent K_D and B_{max} values from saturation analysis of [^{35}S]-GTPγS binding in mMOR-CHO cell membranes

			High affinity site		*Low affinity site*	
Membranes	*NaCl*	*DAMGO*	K_D (nM)	*B_{max}* (pmol mg^{-1})	K_D (nM)	*B_{max}* (pmol mg^{-1})
Control	+	−	12.1 ± 1.77	2.53 ± 0.27	666 ± 12	192 ± 20
Control	+	+	2.76 ± 0.14**	6.59 ± 0.17**	710 ± 130	214 ± 29
Control	−	−	3.97 ± 0.19**	7.87 ± 0.86**	610 ± 70	175 ± 25
PTX	−	−	N.D.	N.D.	607 ± 60	199 ± 17
Control Net	+	+	2.33 ± 0.17**	5.11 ± 0.42**	N.D.	N.D.

Data are mean apparent K_D and B_{max} values$\pm$s.e., obtained from at least three individual saturation analyses. Representative Scatchered plots are shown in Figure 5. N.D., not detected. Control Net values were obtained from monophasic Scatchard plots of net DAMGO-stimulated [^{35}S]-GTPγS binding in control cell membranes. Net stimulated [^{35}S]-GTPγS binding was determined at each GTPγS concentrations by subtraction of basal [^{35}S]-GTPγS binding (measured in the presence of NaCl) from [^{35}S]-GTPγS binding stimulated by DAMGO. *$P<0.5$, **0.01 different from basal [^{35}S]-GTPγS binding in the presence of NaCl.

Table 4 Apparent K_D and B_{max} values from saturation analysis of net-stimulated [^{35}S]-GTPγS binding in control and DAMGO-pretreated mMOR-CHO cells

	Control cells		*DAMGO pretreated cells*	
Cell line	K_D (nM)	*B_{max}* (pmol mg^{-1})	K_D (nM)	*B_{max}* (pmol mg^{-1})
mMOR-CHO				
DAMGO + NaCl	2.72 ± 0.25^{a}	5.17 ± 0.35^{a}	3.18 ± 0.37^{a}	1.86 ± 0.23^{c}
Basal − NaCl	2.90 ± 0.45^{a}	5.27 ± 0.74^{a}	2.17 ± 0.30^{ab}	3.73 ± 0.39^{b}
Non-trans. CHO				
Basal − NaCl	1.15 ± 0.11^{b}	2.83 ± 0.29^{bc}	1.12 ± 0.10^{b}	3.03 ± 0.36^{bc}

Membranes from control and 5 μM DAMGO-pretreated mMOR-CHO or untransfected CHO cells were incubated with 0.1 μM [^{35}S]-GTPγS, 10 μM GDP and varying concentrations of unabelled GTPγS in the presence and absence of 100 μM NaCl and/or 5 μM DAMGO. Data are mean apparent K_D and B_{max} values$\pm$s.e. derived from analysis of monophasic Scatchard plots of net-stimulated [^{35}S]-GTPγS binding. Net-stimulated [^{35}S]GTPγS binding (measured in the presence of NaCl) from [^{35}S]-GTPγS binding stimulated by either DAMGO or the absence of NaCl. Pairs of apparent K_D or B_{max} values containing the same letter designations are not significantly different from each other ($P<0.05$), whereas those not containing any of the same letter designations are $P<0.05$ different from each other.

not due to a decrease in receptor B_{max} (downregulation), since the B_{max} values of [^{3}H]-naloxone binding ($\sim$4 pmol mg^{-1}) were not significantly different between control and DAMGO-pretreated mMOR-CHO cells (not shown). The apparent B_{max} of net [^{35}S]-GTPγS binding stimulated by the absence of monovalent cations showed a 30% decrease in DAMGO-pretreated cells, with no significant change in apparent K_D (Table 4). These decreases were probably not due to changes in basal (receptor-independent) G-protein activity, since the binding of 0.1 nM [^{35}S]-GTPγS in the presence of 100 mM NaCl was not affected by DAMGO pretreatment (52.6 ± 2.5 fmol mg^{-1} versus 54.3 ± 2.2 fmol mg^{-1} in control and DAMGO-pretreated mMOR-CHO cell membranes, respectively). Thus, DAMGO pretreatment decreased the number of receptor-activated G-proteins measured in either the pesence of DAMGO or the absence of monovalent cations, but the magnitude of this desensitization was about twice as great for DAMGO-stimulated [^{35}S]-GTPγS binding than for that stimulated by the absence of monovalent cations.

One explanation for this difference is the presence of other $G_{i/o}$-coupled receptors native to CHO cells, whose spontaneous activity was stimulated by the absence of monovalent cations. To test this possibility, saturation analysis of net [^{35}S]-GTPγS binding was conducted in membranes from untransfected CHO cells. Although DAMGO did not affect [^{35}S]-GTPγS binding in untransfected CHO cell membranes (data not shown), basal [^{35}S]-GTPγS binding was stimulated by the absence of monovalent cations. The apparent B_{max} values from untransfected CHO cells (Table 4) were not significantly different from the apparent B_{max} value obtained in DAMGO-pretreated mMOR-CHO cells in the absence of monovalent cations, but were significantly lower than the apparent B_{max} values obtained in control mMOR-CHO cells in the presence of DAMGO and NaCl or in the absence of monovalent cations. As expected, DAMGO pretreatment had no effect on [^{35}S]-GTPγS binding in untransfected CHO cells. Interestingly, the apparent K_D values of net [^{35}S]-GTPγS binding stimulated by the absence of monovalent cations in untransfected CHO cells were approximately 3 fold lower than those stimulated by DAMGO in control or DAMGO-pretreated mMOR-CHO cells, and than that stimulated by the absence of monovalent cations in control mMOR-CHO cells, but were only 2 fold lower than those from DAMGO-pretreated mMOR-CHO cells in the absence of monovalent cations.

Discussion

The present results support previous studies of sodium effects on spontaneous $G_{i/o}$-coupled receptor activity and relative agonist efficacy (Costa *et al*., 1990; 1992). In both mMOR-CHO cell and rat thalamic membranes, the potency of sodium in inhibiting [^{35}S]-GTPγS binding was inversely related to the agonist efficacy. The ability of sodium to promote agonist stimulation of [^{35}S]-GTPγS binding also varied inversely with agonist efficacy, such that relative efficacy differences between agonists were magnified by increasing sodium concentrations. The effect of sodium on relative efficacy was due to a decrease in maximal stimulation by partial agonists compared to the full agonist DAMGO, rather than to a decrease in the potency of partial agonists. This finding agrees with previous studies of δ opioid and somatostatin sst_5 receptor-stimulated [^{35}S]-GTPγS binding (Szekeres & Traynor, 1997; Williams *et al*., 1997), and agrees with the well-established finding that sodium has a greater effect on binding of full versus partial agonists to opioid receptors in radioligand binding studies (Pert & Snyder, 1974). The present study expands upon previous findings by providing a quantitative examination of the effects of a range of sodium concentrations on the relative efficacy of several full and partial opioid agonists for G-protein activation. Moreover, this is the only report to examine the effects of sodium on agonist efficacy for stimulation of [^{35}S]-GTPγS binding with the μ-opioid receptor, which is primarily responsible for mediating the analgesic, reinforcing and dependence effects of clinically relevant opiates such as morphine (Matthes *et al*., 1996).

The effects of sodium on basal and agonist-stimulated G-protein activation were partially mimicked by potassium. Potassium was nearly as effective as sodium in promoting receptor-mediated G-protein activation compared to the absence of monovalent cations, but was much less effective in reducing the relative efficacy of partial agonists. These results are similar to those previously reported for modulation of low K_m GTPase activity by the δ-opioid receptor in NG108-15 cell membranes (Costa *et al*., 1990). In contrast to these previous results, however, potassium was nearly as effective as sodium in reducing basal G-protein activity in the present study. This difference may be due to different assay conditions, such as the presence of excess GDP in the [^{35}S]-GTPγS binding assay. Indeed, Szekeres & Traynor (1997) reported that in NG108-15 membranes, replacement of sodium with potassium at 100 mM increased basal [^{35}S]-GTPγS binding by 48%, similar to results obtained in this study.

Two important differences between mMOR-CHO cells and rat thalamus may be related to the fact that transfected CHO cells have a higher ratio of μ receptors to activated G-proteins (R:G) than rat thalamus (Selley *et al*., 1997; 1998). First, the ability of sodium to inhibit net agonist-stimulated [^{35}S]-GTPγS binding was greater in the system with the lower R:G ratio; for example, sodium did not inhibit net [^{35}S]-GTPγS binding stimulated by the full agonist DAMGO in mMOR-CHO membranes, compared to nearly a 50% decrease in thalamus. Second, the sodium requirement for agonist-stimulated [^{35}S]-GTPγS binding was greater in the system with the higher R:G ratio. This finding supports the prediction of theoretical models showing that increasing the R:G ratio should increase spontaneous receptor activity (Costa *et al*., 1992), and agrees with experimental evidence that increasing receptor expression increases the spontaneous activity of other G-protein-coupled receptors, including α_2-adrenergic (Tian *et al*., 1994), 5-HT_{1A} (Newman-Tancredi *et al*., 1997) and β_2-adrenergic (Adie & Milligan, 1994).

Interpretation of the present study was affected by the lack of an inverse μ-opioid agonist. Thus, several alternate approaches were utilized to demonstrate that effects of sodium on μ receptor-stimulated [^{35}S]-GTPγS binding were due to an inhibitory effect of the cation on the ability of μ receptors to assume an active (G-protein-activating) conformation. First, pretreatment of mMOR-CHO cells with PTX blocked the stimulation of [^{35}S]-GTPγS binding both by DAMGO (with sodium present) and by the absence of monovalent cations. This finding is important because the mechanism by which PTX-catalyzed ADP ribosylation of $G_{i/o}$-proteins disrupts signal transduction is reportedly due to uncoupling from receptor-stimulated GDP/GTP exchange, and not to inhibition of the basal G-protein activation rate itself (Sunyer *et al*., 1989). Second, the potency of GDP in inhibiting [^{35}S]-GTPγS binding to mMOR-CHO membranes was decreased to the same extent by DAMGO (with sodium) and by the absence of monovalent cations (with or without DAMGO), and the rightward shifts in the GDP curves produced by either agonist or the absence of monovalent cations were both eliminated by

prior treatment of the cells with PTX. Moreover, similar to the effect of agonist, the absence of monovalent cations did not significantly stimulate [^{35}S]-GTPγS binding in the absence of GDP, indicating that sodium did not directly effect G-protein affinity for [^{35}S]-GTPγS. Third, biphasic saturation analysis of [^{35}S]-GTPγS binding revealed that both DAMGO (with sodium) and the absence of monovalent cations increased the apparent affinity and B_{max} values of high affinity [^{35}S]-GTPγS binding without affecting low affinity [^{35}S]-GTPγS binding sites. Moreover, PTX eliminated high affinity [^{35}S]-GTPγS binding both in the absence of monovalent cations and presence of agonist. As with agonist-stimulated [^{35}S]-GTPγS binding, the effect of the absence of monovalent cations on apparent K_D and B_{max} values of high affinity [^{35}S]-GTPγS binding was observed only in the presence of GDP. Fourth, DAMGO (with sodium) or the absence of monovalent cations produced identical apparent K_D and B_{max} values for [^{35}S]-GTPγS binding, while chronic DAMGO-pretreatment of cells desensitized stimulation produced by DAMGO (with sodium) and by the absence of monovalent cations.

The attenuation of μ receptor-mediated G-protein activation observed after DAMGO pretreatment of mMOR-CHO cells was apparently due to desensitization rather than downregulation of the μ receptor, as determined by the lack of a significant decrease in the B_{max} of [^{3}H]-naloxone. This observation differed from previous reports of DAMGO-induced downregulation of μ receptors expressed in CHO cells (Kato *et al*., 1998; Pak *et al*., 1996). Variation between the results of the present and previous studies may have been due to differences in cell culture and agonist pretreatment conditions, species of cloned μ receptor or method of membrane preparation. The desensitization observed in the this study was likely due to a homologous uncoupling of μ receptors from G-proteins, rather than a decrease in basal G-protein activity or levels, because no difference in basal [^{35}S]-GTPγS binding was observed in the presence of 100 mM NaCl. This conclusion is supported by a previous report that chronic pretreatment of μ receptor-containing neuroblastoma cells with DAMGO did not affect the levels or basal activity of $G_{i/o}$-proteins (Ammer & Schulz, 1993).

The smaller magnitude of desensitization observed in the absence of monovalent cations was probably due to other $G_{i/o}$-coupled receptors in CHO cells, whose spontaneous activities were also stimulated by the absence of monovalent cations. Indeed, sodium inhibition of spontaneous receptor activity has been reported for other $G_{i/o}$-coupled receptors (Costa *et al*., 1990; Hilf & Jakobs, 1992; Mullaney *et al*., 1996; Szekeres & Traynor, 1997; Tian *et al*., 1994; Wenzel-Seifert *et al*., 1998). This possibility was supported by data in untransfected CHO cells showing that apparent B_{max} values of net [^{35}S]-GTPγS binding in the absence of monovalent cations were similar to the apparent B_{max} value in the absence of monovalent cations in DAMGO-pretreated mMOR-CHO cells, but were lower than in control mMOR-CHO cells. Furthermore, in the present study, there was no increase in the apparent B_{max} of activated G-proteins in the absence of monovalent cations compared to the value obtained and DAMGO with sodium, suggesting that there were enough μ receptors present to fully activate the $G_{i/o}$-protein pool without additional receptor activity. These results are consistent with the hypothesis that high relative efficacies of partial agonists in mMOR-CHO membranes are due to the high R:G ratio; i.e., there is a 'ceiling effect' on the maximal number of G-proteins that can be activated by μ receptors in this system (Selley *et al*., 1997; 1998), since the number of μ receptors in mMOR-CHO membranes exceeds the number of receptor-activated G-proteins (Selley *et al*., 1998). In support of this interpretation, several opioids that were full agonists in mMOR-CHO membranes demonstrated receptor reserve for G-protein activation in this system, but not in rat thalamus (Selley *et al*., 1998). One finding inconsistent with this interpretation is that the B_{max} value of high affinity [^{35}S]-GTPγS binding measured in the absence of GDP was 3 fold higher than that measured with GDP in the absence of monovalent cations or presence of DAMGO and NaCl. However, not all high affinity [^{35}S]-GTPγS binding sites are sensitive to the modulatory effects of $G_{i/o}$-coupled receptors on GDP affinity. High affinity [^{35}S]-GTPγS binding sites such as $G\alpha_s$, $G\alpha_{q/11}$, $G\alpha_{12/13}$ or other GTP-binding proteins in CHO cell membranes may remain occupied by GDP, while GDP dissociates from receptor-activated $G\alpha_{i/o}$.

The finding that the apparent K_D values of net [^{35}S]-GTPγS binding stimulated by the absence of monovalent cations in untransfected CHO cells were lower than those in mMOR-CHO cells may indicate small differences in the ability of different receptor types to decrease the affinity of receptor-coupled G-proteins for GDP. The apparent K_D value obtained in DAMGO-pretreated mMOR-CHO cells in the absence of monovalent cations was intermediate between the value obtained in untreated mMOR-CHO and untransfected CHO cells, further suggesting that [^{35}S]-GTPγS binding stimulated by the absence of monovalent cations in DAMGO-pretreated mMOR-CHO cells was due to spontaneous activity of non-opioid $G_{i/o}$-coupled receptors along with the remaining (undesensitized) μ receptors.

What is the physiological role of sodium in modulating the activation state of $G_{i/o}$-coupled receptors? Previous studies with the δ-opioid inverse agonist ICI-174,864 suggest that spontaneous receptor activity is minimal in intact NG108-15 cells (Costa *et al*., 1992), but that δ-opioid receptors display significant spontaneous activity in transfected cells with high levels of δ receptors (Chiu *et al*., 1996; Merkouris *et al*., 1997). Even if spontaneous receptor activity is minimal in most physiological systems due to the lower naturally-occurring R:G ratios than transfected cells, the present study indicates that sodium can significantly modulate relative agonist efficacy by its ability to produce non-equivalent suppression of the responses stimulated by agonists of varying intrinsic efficacies. This effect of sodium was observed even when high monovalent cation concentrations were maintained with potassium, indicating that the sodium:potassium ratio was a significant factor in the determination of relative efficacy. However, the question remains whether intracellular or extracellular sodium concentrations regulate receptor activity. If intracellular sodium is more important, then changes in the sodium:potassium ratio at the internal membrane surface (such as those occurring transiently during a neuronal action potential) may regulate agonist efficacy *in vivo*. Alternatively, if the primary site of sodium action on the receptor is extracellular, then the minimal fluctuations in the extracellular sodium:potassium ratio that would be expected to occur in mammals may mean that sodium would not significantly modulate agonist efficacy *in vivo*. Unfortunately, the literature has been conflicting with regard to this question (Puttfarcken *et al*., 1986; Yabaluri & Medzihradsky, 1997). Considering previous findings that sodium sensitivity of receptor-coupled G-protein activity varies among different brain regions (Pacheco *et al*., 1994), it will be important to further examine the effects of both intracellular and extracellular sodium on receptor-coupled G-protein activity and agonist efficacy.

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