JPET Fast Forward. Published on March 2, 2004 as DOI: 10.1124/jpet.104.066837

JPET #66837

Dual effects of Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol (DAMGO) and D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP) on adenylyl cyclase activity; implications for μ-opioid receptor G_s coupling

Mária Szücs [‡§], Krisztina Boda[¶] and Alan R. Gintzler[‡]

[‡]Department of Biochemistry, State University of New York, Downstate Medical Center, Brooklyn, New York 11203 (MS and ARG), [§]Institute of Biochemistry, Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary (MS) and [¶]Department of Medical Informatics, University of Szeged, Szeged, Hungary (KB)

JPET #66837

a) running title: Dual effects of DAMGO and CTAP on adenylyl cyclase

b) Corresponding Author: Dr. Alan Gintzler

Box 8, Department of Biochemistry

SUNY Downstate Medical Center

450 Clarkson Ave.

Brooklyn, NY 11203

Tel: 718 270 2129

Fax: 718 270 3316

E-mail: agintzler@netmail.hscbklyn.edu

c) text pages: 23

tables 0

figures: 5

references: 40

abstract: 243 words

introduction: 535 words

discussion: 1791 words

d)Abbreviations: MOR-CHO, Chinese hamster ovary cells stably transfected with μ-opioid receptors; DAMGO, D-Ala-Gly-(NMe)Phe-Gly-ol; CTAP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 ; G protein, heterotrimeric guanine nucleotide binding regulatory protein

JPET Fast Forward. Published on March 2, 2004 as DOI: 10.1124/jpet.104.066837
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

The μ-opioid receptor (MOR) couples to multiple G proteins, of which coupling to G_s has long been debated. As expected, in opioid naïve Chinese Hamster Ovary (CHO) cells expressing recombinant MOR, the predominant action of Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol (DAMGO) is inhibitory. However, inactivation of Gi/Go proteins via pertussis toxin (PTX) unmasks its ability to facilitate forskolin activation of adenylyl cyclase (AC) activity. Tolerance develops to this effect of DAMGO, which can also be attenuated by cholera toxin (CTX). The latter suggests G_s mediation. D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP), previously considered to be a neutral MOR antagonist, also produces a facilitation of FSK activation of AC that is augmented by chronic morphine. Facilitative effects of CTAP in naïve as well as its augmentation in tolerant membranes are both substantially reduced by CTX. This not only suggests G_s mediation but that $G_{s\alpha}$-linked signalling is critical to the chronic morphine-induced enhanced facilitative action of CTAP. Interestingly, the (augmented) CTAP facilitation of FSK-stimulated AC activity that is observed in opioid tolerant (but not naïve) membranes is also sensitive to PTX. This can best be explained by postulating the involvement of G_i-derived $G_{\beta\gamma}$, which would stimulate type 2 ACs, conditional on the presence of activated $G_{s\alpha}$. The emergence of a $G_{\beta\gamma}$ dimension of AC stimulation by CTAP following chronic morphine could explain its ability to augment the stimulatory action of CTAP on AC. These results support putative MOR coupling to G_s and underscore the multifaceted nature and plasticity of MOR G protein coupling.

Traditionally, ligands acting on a G protein-coupled receptor (GPCR) are classified as either agonists, partial agonists or antagonists. Recent findings, however, suggest that several compounds traditionally considered to be competitive (neutral) antagonists, at GPCRs that are physiologically expressed at a low level can exhibit inverse agonist properties, i.e., have negative intrinsic activity (signalling efficacy) when these receptors and/or their cognate G proteins are present at higher levels of expression (Bond et al., 1995; Gether et al., 1995; Barker et al., 1994; Smit et al., 1996; Costa and Herz, 1989; Cetani et al., 1996). Inverse agonists are compounds that can attenuate the ability of GPCRs to activate their cognate G protein in the absence of agonist ligand and thereby produce effects that are opposite those that usually result from receptor activation. Although the concept and existence of such compounds has become very well established and widely accepted, the underlying molecular mechanisms remain unclear.

The discovery of inverse agonists has forced reconsideration of the dynamics that influence receptor coupling to G proteins. Specifically, it has fostered renewed interest in multi-state receptor activation models. In these formulations, receptors can exist in equilibrium between an inactive conformation, unable to couple to G proteins, and an active conformation that is compatible with G protein coupling, which is spontaneously achieved in the absence of agonist (Leff, 1995b; Leff, 1995a; Gintzler and Xu, 1991; Shen and Crain, 1990; Wang and Gintzler, 1997).

All opioid receptors belong to the super family of GPCRs. Most research to date has confirmed coupling predominantly with G_i/G_o, the alpha subunits of which are thought to be the predominant mediators of opioid action. This notwithstanding, there is also pharmacological evidence consistent with opioid receptor coupling to G_s (Gintzler and Xu, 1991; Shen and Crain, 1990; Wang and Gintzler, 1997) but this remains controversial. More recently, the importance of the $G\beta\gamma$ subunit of G proteins to opioid receptor-coupled signaling has become apparent (Chakrabarti et al., 1998a).

The plethora of G protein-coupled signaling strategies and pathways utilized by opioid receptors makes them ideal substrates for the study of inverse agonists (Chiu et al., 1996a; Merkouris et al., 1997; Liu et

JPET Fast Forward. Published on March 2, 2004 as DOI: 10.1124/jpet.104.066837
This article has not been copyedited and formatted. The final version may differ from this version.

al., 2001). Conversely, inverse agonists would appear to be a useful tool to probe opioid receptor functionality and G protein subunit mediators thereof.

Thus far, the study of opioid inverse agonists has been largely confined to interactions with the δ-opioid receptor. The present study investigates inverse agonist properties of a commonly used μ-opioid receptor (MOR) antagonist, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP). Although previously characterized as a neutral antagonist *in vivo* (Bonner et al., 1997; Sterious and Walker, 2003) and in GH_3 cells (Liu and Prather, 2001), the present study reveals that it manifests inverse agonism in Chinese Hamster Ovary (CHO) cells stably transfected with the μ-opioid receptor (MOR-CHO), underscoring the plasticity of inverse agonist responsiveness and its dependence on local cellular factors. Additionally, utilization of two bacterial toxins commonly used to assess receptor G protein coupling, pertussis toxin (PTX) and cholera toxin (CTX) provides further pharmacological evidence for μ-opioid receptor coupling to G_s and augmented μ-opioid receptor coupled signaling via the Gβγ subunit following chronic morphine. The relevance of chronic morphine-induced adaptations to inverse agonist actions is discussed.

Materials and METHODS

Materials

[α-^{32}P]Adenosine 5'-Triphosphate ([α-^{32}P]ATP) and [^{3}H]Adenosine 3',5'-cyclic monophosphate ([^{3}H]cAMP) were purchased from New England Nuclear (Boston, MA). Morphine sulphate, Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol (DAMGO), D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 x TFA (CTAP), (+)Naloxone-HCl and ±Naloxone-HCl were supplied by the National Institute on Drug Abuse (Rockville, MD). ATP, cAMP, Guanosine 5'-Triphosphate, (GTP), Phosphocreatine (CP), Creatine phosphokinase (CPK) from rabbit muscle, $MgCl_2$, NaCl, IBMX (3-isobutyl-1-methylxanthine), forskolin (FSK), bacitracin, benzamidine, aprotinin, leupeptin, trypsin-chymotrypsin inhibitor from soybean (STI), HEPES, Na_2HPO_4, KH_2PO_4 and Ethylenediaminetetraacetic acid (EDTA) were from SIGMA (St. Louis, MO).

Cell culture and treatment

MOR-CHO cells (3.63±0.32 pmoles MOR/mg protein assessed with [3H]naloxone) were maintained in Dulbecco's Modified Eagle Medium (DMEM) high glucose with L-glutamine (GIBCO, Carlsbad, CA) supplemented with 10% fetal calf serum (Nova-Tech Inc., Grand Island, NE) 1% penicillin/streptomycin (GIBCO, Carlsbad, CA) and 3.6% geneticin (GIBCO, Carlsbad, CA). Cells were grown at 37° C in humidified atmosphere of 10% CO_2, 90% air. For chronic morphine treatment, MOR-CHO cells at 50% confluency were treated with morphine (1 μM). Morphine was replenished 24 h later with fresh one and cultured for an additional 24 h before harvest. For toxin treatments, cells were treated with either 100 ng/ml pertussis toxin, PTX (List Biological Labs., Inc. Campbell, CA) and/or 1 μg/ml cholera toxin , CTX (List Biological Labs., Inc. Campbell, CA) for the last 24 h in culture unless otherwise stated. At the end of morphine and/or toxin exposure, cells were washed twice with ice-cold phosphate buffered saline (PBS) in the absence or presence of 1 μM morphine for opioid naive and tolerant preparations, respectively. Cells were harvested with PBS containing 1 mM EDTA in the absence or presence of 1 μM morphine for opioid naive and tolerant preparations, respectively. Cell suspension was spun at 2500 rpm for 5 min, after which preparation of cell membranes commenced.

Membrane preparation

Freshly collected cell pellects were homogenized with a Wheaton teflon-glass homogenizer in 10 vols (v/w) of ice-cold homogenization buffer, pH 7.4, composed of 25 mM HEPES, 1 mM EDTA, 0.5 mg/l aprotinin, 1 mM benzamidine, 100 mg/l bacitracin, 3.2 mg/l leupeptin, 3.2 mg/l soybean trypsin inhibitor and 10% sucrose in the absence or presence of 1 μM morphine for opioid naive and tolerant preparations, respectively. Homogenates were spun at 1000 g for 10 min at 4°C, and supernatant collected. Pellets were suspended in half of the original volumes of the homogenization buffer and centrifuged as above. Combined supernatants from the two low speed centrifugation were spun at 20, 000 g for 30 min. Opioid naive and morphine-treated cell pellets were taken up in appropriate volumes of homogenization buffer in the absence or presence of 100 nM morphine, respectively. Aliquotes were stored at -80°C until use. Protein content was determined using bovine serum albumin as a standard with the Bradford assay (Bradford, 1976).

JPET Fast Forward. Published on March 2, 2004 as DOI: 10.1124/jpet.104.066837
This article has not been copyedited and formatted. The final version may differ from this version.

Adenylyl cyclase (AC) assay

AC activity was determined by measuring the synthesis of [α-^{32}P]cAMP from [α-^{32}P]ATP as published (Chakrabarti et al, 1998a and references cited therein) with some modifications. Assay mixture contained 1 mM ATP, 1 μCi/tube [α-^{32}P]ATP, 10 μM GTP, 0.1 mM cAMP, 10 mM $MgCl_2$, 100 mM NaCl, 5 mM creatine phosphate, 30 ug/tube creatine kinase, 1 U/tube myokinase, 1 mM IBMX in 50 mM HEPES buffer, pH 7.4. DAMGO, CTAP, naloxone, FSK (1 μM), and the $G_{\beta\gamma}$ scavanger QEHA peptide (AC2 residues 956-982) were also included where indicated. Reaction was initiated by the addition of appropriate MOR-CHO membrane preparations (50-100 μg protein/tube), incubated for 15 min at 30°C, and terminated by the addition of 0.1 N HCl. After boiling for 2 min, the samples were spun at 5000 x rpm for seconds in an Eppendorf centrifuge and loaded onto columns containing alumina (WN-6 neutral, SIGMA, St. Louis, MO). Columns were eluted with 0.1 N ammonium acetate (SIGMA, St. Louis, MO) and radioactivity determined in UltimaGold XR (Packard Bioscience, Meriden, CT) scintillation cocktail in an LKB Rackbeta (Amersham Pharmacia, Piscataway, NJ) liquid scintillation counter. [^{3}H]cAMP (0.005 μCi/tube) was used as internal standard to correct for column recovery of cAMP.

Data analysis

Unless otherwise stated, AC activity values assessed in the presence of 1 μM FSK, but in the absence of opioid ligands, are defined as 100% for each tissue treatment. Data reported are % FSK values and represent the mean ± S.E.M. for at least three experiments that were each performed in triplicate. When two groups were compared, significance of differences between the means was determined by Student's *t* test. Statistical significance is set at $p \leq 0.05$. DAMGO dose-response curves were analyzed by non-linear regression with the use of the GraphPad Prism program (San Diego, CA) to obtain maximal inhibition and EC_{50} (the concentration of the ligand that elicits half-maximal effect) of FSK-stimulated AC activity.

Effects of CTAP doses (0, 0.1, 1 and 10 μM), condition (naïve and tolerant), bacterial toxins (CTX and CTX+PTX), and interactions thereof were assessed by a three-way repeated measures Analysis of

JPET Fast Forward. Published on March 2, 2004 as DOI: 10.1124/jpet.104.066837
This article has not been copyedited and formatted. The final version may differ from this version.

Variance (ANOVA) using SAS software (SAS Institute, Cary, NC). A mixed model ANOVA was performed supposing first-order autoregressive covariance structure between dose levels to compensate for unequal sample sizes. Post-hoc comparisons were performed for condition and toxin effects based on Estimated Least Squares Means.

Results

Effect of DAMGO on FSK-stimulated AC. As expected, in opioid naive MOR-CHO membranes, the potent and highly selective MOR agonist DAMGO produced a concentration-dependent inhibition of FSK-stimulated AC activity with an EC_{50} = 36.7 ± 17.5 nM and maximal inhibition of 51.8 ± 1.6% (n=4)**.** Following 48 h morphine exposure, the potency of DAMGO did not change significantly (EC_{50} = 52.5 ± 17.4 nM, $p > 0.05$) but its inhibitory effect was significantly attenuated to 31.4 ± 2.2 % (n=4, $p < 0.01$) indicating the development of tolerance (Fig. 1, panel A).

PTX reverses DAMGO inhibition to a facilitation of FSK-stimulated AC activity. PTX treatment of cells slightly enhanced the magnitude of FSK-stimulated AC activity from 36.6±7.3 pmol/mg protein/min to 51.0±4.1 pmoles/mg protein/min. As expected, PTX treatment of MOR-CHO cells abolished the ≈40 and ≈20% inhibitory effect of 1 μM DAMGO on FSK stimulation of AC activity in membranes obtained from opioid naïve or chronic morphine-treated MOR-CHO cells, respectively (Fig. 1B). Unexpectedly, however, in membranes obtained from opioid naïve cells, PTX not only blocked the DAMGO inhibition but also unmasked its ability to facilitate FSK activation of AC (128.9 ± 8.5%; p=0.01; n=8). In membranes obtained from chronic morphine-treated MOR-CHO cells, the DAMGO enhancement of FSK-stimulation of AC activity that was unmasked by PTX was much more modest (111.5 ± 5.7%) and did not achieve statistical significance (p=0.08; n=8) (Fig. 1B). Importantly, the DAMGO stimulation of cAMP formation that was unmasked following treatment of opioid naïve cells with PTX was no longer apparent in membranes obtained from cells that had been treated with a combination of PTX and CTX (Fig. 1C).

JPET Fast Forward. Published on March 2, 2004 as DOI: 10.1124/jpet.104.066837
This article has not been copyedited and formatted. The final version may differ from this version.

Effect of CTAP on FSK-stimulated AC activity. As expected, CTAP markedly reduced the DAMGO inhibition of FSK stimulated cAMP formation in opioid naïve membranes (Fig. 2, bar A). Interestingly, however, in membranes obtained from chronic morphine-treated MOR-CHO cells, CTAP not only blocked the DAMGO inhibition of AC but also significantly enhanced the magnitude of FSK stimulation of AC activity by 134.4±6.1%; $p < 0.05$; n=10; Fig. 2, bar C). In fact, even in the absence of opioid agonist, CTAP increased FSK-stimulated AC activity by 134.8±7.3 and 185±6.8% in opioid-naive and opioid-tolerant membranes, respectively (Figs. 2, bar B and D). These observations suggest that CTAP can behave as an inverse agonist in MOR-CHO cells.

In order to probe the contribution of G_s-coupled signaling to the CTAP facilitation of AC activity and its augmentation by chronic morphine, dose responsiveness and effects of CTX were assessed. As expected, following a 16 h incubation with CTX, FSK-stimulated AC activity was substantially enhanced (34.8 ±4.9 pmoles/mg protein/min, n=10 vs. 430±7.2 pmoles/mg protein/min, n=5 before and following CTX treatment, respectively). ANOVA revealed significant main effects for dose of CTAP ($F_{3,145}$=28.17, $p<0.001$), condition ($F_{1,73}$=3.86, $p=0.05$) and toxin ($F_{2,75}$=47.09, $p<0.05$). The ANOVA also revealed significant dose by condition ($F_{3,145}$=2.81, $p<0.05$) and dose by toxin ($F_{6,146}$=14.97, $p<0.0001$) interactions. This indicates that CTAP dose responsiveness differs among membranes obtained from opioid naïve vs. chronic morphine-treated cells and among membranes obtained from cells grown in the absence vs. the presence of toxin (CTX vs. CTX + PTX), respectively. Furthermore, the significant toxin by condition by dose interaction ($F_{6,146}$=4.15, $p=0.0007$) indicates that toxin treatment (CTX or CTX +PTX) alters the interaction between dose and condition**.**

Post-hoc comparisons revealed that in the absence of toxin (Fig. 3A) the effect of CTAP differs between naive and tolerant membranes at the higher concentrations (1 and 10 μM). In contrast, following treatment with either CTX or CTX + PTX, differences in CTAP stimulatory responsiveness between naïve vs. chronic morphine-treated membranes were no longer discernable (Fig. 3B and 3C). In opioid naive membranes, CTX significantly ($p\leq0.05$) attenuated the inverse agonist effect of 1 and 10 μM CTAP. In contrast, this effect of CTX treatment was manifested at all CTAP concentrations in the tolerant

JPET Fast Forward. Published on March 2, 2004 as DOI: 10.1124/jpet.104.066837
This article has not been copyedited and formatted. The final version may differ from this version.

membranes ($p<0.05$). Pretreatment of MOR-CHO cells concomitantly with PTX as well as CTX produced an additional diminution in CTAP facilitative responsiveness to 1 and 10 μM CTAP in opioid tolerant but not opioid naïve preparations.

PTX abolishes CTAP facilitation of AC in opioid tolerant but not opioid naïve preparations. Since the experiments described in Figure 3 indicate that 1 μM CTAP was optimal for the manifestation of inverse agonism, this concentration was used to probe the contribution of G_i-coupled μ-opioid receptors to the CTAP facilitation of cAMP formation. PTX treatment did not significantly influence the stimulatory effect of 1 μM CTAP in the naïve membranes (Fig. 4, first bar). In contrast, however, treatment of chronic morphine-exposed membranes with PTX abolished the significant facilitative effect of CTAP on FSK-stimulated AC activity (without PTX: 185±6.8, $p<0.001$; with PTX: 111.5±2.4%, $p>0.05$; $n=8$ for both) (Fig. 4, second bar). This implies a role of Gβγ derived from G_i/G_o G proteins in CTAP stimulation of AC activity in the tolerant, but not naive tissue.

In order to explore the generality of the effect of chronic morphine exposure on the manifestation of inverse agonism, the prototypic general opioid receptor antagonist naloxone was also assessed for inverse agonist actions (±)Naloxone, but not its pharmacologically inactive stereoisomer (+)naloxone, enhanced AC stimulation by FSK. The stereoisomeric specificity of the observed effect underscores its mediation via opioid receptors (Fig. 5). In contrast to CTAP, however statistically significant inverse agonism of (±)naloxone was only manifest following chronic morphine treatment, consistent with a previous report (Wang et al., 2001).

Discussion

The present work demonstrates that inactivation of Gi/Go proteins via PTX treatment unmasks the ability of DAMGO to stimulate AC activity in opioid naïve CHO cells expressing recombinant MORs. The ability of CTX to abolish PTX-induced reversal of DAMGO inhibition to facilitation of FSK activation of AC strongly suggests μ-opioid receptor coupling to G_s, which has long been debated. Opioid receptors have

generally been thought to signal predominantly via a PTX-sensitive mechanism involving the $G_{i\alpha}/G_{o\alpha}$ subunit of G proteins. This notwithstanding, CTX-sensitive, presumably $G_{s\alpha}$-mediated, excitatory opioid modulation of transmitter (methionine-enkephalin) release and cAMP formation has been reported in the guinea pig ileum myenteric plexus preparation (Gintzler and Xu, 1991; Wang and Gintzler, 1997) as has opioid modulation of cAMP formation in F-11 (neuroblastoma-dorsal root ganglion neuron) hybrid cells (Cruciani et al., 1993). The present findings are consonant with these reports. The observation that chronic morphine exposure alters $G_{s\alpha}$ levels in a time-dependent fashion in subcellular fractions of rat brain further supports the putative relevance of G_s to opioid receptor-coupled signaling (Fábián et al. 2002). Present results are also reminiscent of the PTX-induced reversal of opioid inhibition to a CTX-sensitive facilitation of electrically stimulated cAMP formation that has been reported in the guinea pig myenteric plexus (Wang and Gintzler, 1997). Notably, in the present study, abolition by CTX of facilitative effects of DAMGO on cAMP formation that are unmasked by PTX would eliminate putative contributions of G_q-coupled μ-opioid receptors to facilitative actions of DAMGO. Collectively, these data reinforce putative coupling of μ–opioid receptors to G_s as well as G_i. Thus, responses to DAMGO in the absence of PTX represent the net effect of two opposing forces, the predominant effect of which is inhibition and whose elimination is required for stimulatory effects to be manifest. The failure to observe AC stimulation by DAMGO in membranes obtained from MOR-CHO cells treated chronically with morphine and PTX indicates that tolerance develops to stimulatory as well as inhibitory actions of DAMGO.

The other notable finding of the present work is that CTAP, previously considered to be a neutral μ–opioid receptor antagonist based on *in vivo* and in vitro observations utilizing other systems (Bonner et al, 1997; Sterious and Walker, 2003; Liu and Prather, 2001), enhanced FSK stimulated AC activity (Figs. 2-4). Thus, in MOR-CHO cells, CTAP behaves not only as a μ-opioid receptor antagonist, but exhibits properties characteristic of inverse agonists as well. Notably, consistent with these findings, CTAP has been reported to possess a pharmacology that significantly differs from that of traditional opioid antagonists (Hawkins et al., 1989; Sterious and Walker, 2003). Inverse agonistic effects of CTAP on stimulated accumulation of cAMP is analogous to observations made for ICI174864 in δ-opioid receptor

expressing rat-1 fibroblasts (Merkouris et al., 1997) and human embryonic kidney cells stably expressing the δ-opioid receptor (Chiu et al., 1996).

Sensitivity to CTX, a bacterial toxin that is frequently used to distinguish G proteins involved in receptor-second messenger coupling, was used to reflect involvement of G_s in CTAP inverse agonism signal transduction. CTX catalyzes the ADP ribosylation of the α subunit of the G_s protein on arginine. As a consequence, the receptor-coupled activation/deactivation of the G_s protein cycle is interrupted. Of particular relevance to the current study, pretreatment with CTX has been associated with altered G_s-mediated signal transduction, i.e., decreased ability of guanine nucleotides to induce a decrease in receptor affinity for agonist via the destabilization of the high affinity agonist-receptor ternary complex. For example, in frog erythrocyte membranes, CTX treatment substantially decreased (>10-fold) the potency of GTP or 5'guanylylimidodiphosphate to regulate the affinity of the β-adrenergic receptor for agonists (Stadel and Lefkowitz, 1981). In contrast, in the same membranes, CTX increased to a far lesser extent the ability of GTP to activate AC activity, consistent with observations made in turkey erythrocyte membranes (Cassell and Selinger, 1977) and illuminated bovine rod outer segment membranes (Wieland et al., 1994). Collectively, these observations support the use of CTX sensitivity to reflect G_s mediation. CTX attenuated the ability of CTAP to enhance FSK stimulated cAMP formation in opioid naïve cells. Moreover, it eliminated the chronic morphine-induced increment in the inverse agonistic action of CTAP (Fig. 3B). This suggests that facilitative effects of CTAP on cAMP accumulation require $G_{s\alpha}$ signaling and furthermore, that augmentation of $G_{s\alpha}$-linked events underlies the enhancement of the facilitative effects of CTAP following opioid tolerance formation.

In contrast to CTX, PTX did not modify the stimulatory effect of 1 μM CTAP in opioid naive MOR-CHO membranes. It did, however, abolish the inverse agonistic actions of CTAP in membranes obtained from cells that had been chronically pretreated with morphine (Fig. 4). Thus, in opioid naïve cells, facilitative effects of CTAP on cAMP formation are mediated via $G_{s\alpha}$ whereas an additional component is induced by chronic morphine. PTX sensitivity of a process resulting in the enhancement of cAMP formation can best be explained postulating the involvement of G_i-derived $G_{\beta\gamma}$. This would stimulate ACs of the type two

family (II, IV, VII), conditional on the presence of activated $G_{s\alpha}$ (Tang and Gilman, 1991). Importantly, the mRNAs encoding AC II, IV and VII are present in MOR-CHO cells (Regec and Gintzler, unpublished observations) and have been shown to be upregulated in the longitudinal muscle myenteric plexus preparation following chronic morphine (Rivera and Gintzler, 1998; Chakrabarti et al., 1998a). The chronic morphine-induced emergence of a (PTX-sensitive) $G_{\beta\gamma}$ component of CTAP inverse agonistic effects (PTX does not alter CTAP inverse agonism in naïve membranes), which would enhance $G_{s\alpha}$ stimulation of AC (Rivera and Gintzler, 1998; Chakrabarti et al., 1998a; Chakrabarti et al., 1998b; Chakrabarti and Gintzler, 2003), could, in fact, explain the ability of chronic morphine to augment the inverse agonist actions of CTAP and unmask inverse agonist actions of ±naloxone (Fig. 5), as was initially observed in HEK293 cells (Wang et al., 2001). This formulation could also account for the apparent limited concentration range of CTAP that manifests augmented inverse agonism following chronic morphine since AC stimulation by $G_{\beta\gamma}$ occurs at relatively high concentrations of this G protein subunit (Birnbaumer, 1992; Tang and Gilman, 1992). Moreover, the dose-response relationship for CTAP facilitation of AC activity in tolerant cells would thus be multifactorial, depending not only on the dose relationship for CTAP-induced generation of G_i-derived $G_{\beta\gamma}$ but also on its dose relationship for stimulating AC**.** The ability of chronic morphine to amplify the inverse agonist effects of naloxone, a general opioid receptor antagonist, as well as CTAP underscores the generality of this sequela of opioid tolerance.

PTX has previously been reported to prevent the inverse agonistic effects of the δ-opioid antagonist ICI174864 on cAMP accumulation in both δ-opioid receptor expressing rat-1 fibroblasts and human embryonic kidney cells expressing either MOR or δ-opioid receptors (Merkouris et al., 1997; Chiu, et al., 1996) The current finding in MOR-CHO cells that pretreatment with CTX or a combination of CTX and PTX substantially attenuates and abolishes, respectively, the ability of CTAP to enhance FSK-stimulated cAMP accumulation underscores the paradoxical prerequisite for functional receptor G protein coupling for inverse opioid agonistic actions. The predominant effect of CTX on CTAP inverse agonism in μ-opioid receptor expressing CHO cells (indicative of G_s mediation) vs PTX on the inverse agonist effects of the δ-opioid receptor antagonist ICI174864 in rat-1 fibroblasts and MOR-HEK cells (indicative of coupling to G_i/G_o proteins) could reflect the plasticity and pleotropic coupling of opioid receptors in different

JPET Fast Forward. Published on March 2, 2004 as DOI: 10.1124/jpet.104.066837
This article has not been copyedited and formatted. The final version may differ from this version.

overexpressing cellular milieus (Chiu et al., 1996a; Merkouris et al., 1997) and/or differences in coupling of overexpressed δ– vs μ-opioid receptors. In this regard, it should be noted that level of receptor expression has been reported to influence specificity of receptor/G protein coupling (Kenakin, 1995) as well as the manifestation of inverse agonistic action (Merkouris et al., 1997). Variations among cell lines in levels of opioid receptor expression, relative abundance of specific G proteins and/or AC isoforms, etc., could underlie behavior of CTAP as a neutral antagonist in GH_3 cells (Liu and Prather, 2001) vs. the present demonstration of an inverse agonist in MOR-CHO cells.

Dependence of inverse agonism on the integrity of opioid receptor G protein coupling remains an enigma that confounds conceptual formulations of opioid inverse agonists. Actions of inverse agonists are usually explained by postulating a two-state model in which there is equilibrium between an inactive conformation of a receptor and a spontaneously active one that can couple to G proteins in the absence of agonists. In this scenario, inverse agonists are thought to abolish spontaneous, agonist-independent, coupling to G_i/G_o by stabilizing the inactive receptor conformation thereby reduce the likelihood of assuming the active, G_i/G_o-coupled state. However, this simplistic model would also predict that the effects of inverse agonists and PTX would be qualitatively similar, which has not proven to be the case (Merkouris et al., 1997; Chiu et al., 1996b). Attempts to account for the paradoxical dependence of opioid inverse agonistic effects on functional opioid receptor G_i/G_o coupling, more complicated models have been proposed, which unlike the two-state model, embodies a role of G proteins in the induction and/or stabilization of various conformational states of the receptor (Samama et al., 1993). In this scenario, receptors would exist in an inactive G protein coupled state, formation of which would be limited by PTX, that transduces inverse agonistic activity, e.g., ICI174864, via their stabilization (Chiu et al., 1996a).

Putative μ-opioid receptor coupling to G_s in MOR-CHO cells as discussed above is consistent with the independent demonstration of PTX reversal of DAMGO inhibition to facilitation of FSK-stimulated cAMP production in the same preparation. The current findings are consistent with a model in which antagonists such as CTAP, in the absence of agonist, can induce a receptor conformation that predisposes coupling to an otherwise less favored G protein, e.g., G_s, that regulates downstream

signaling molecules in a manner opposite that of the G protein to which coupling is induced by agonists at that receptor (G_i/G_o). Differences among antagonists in their ability to induce such receptor conformations could underlie differences in their respective potency as inverse agonists.

The physiological relevance of the current findings remains to be established. The absolute expression levels of GPCRs and the stoichiometry of their associated G-proteins can influence the level of constitutive activity and its detection. Thus overexpression systems are more likely to manifest constitutive activity, i.e. spontaneous coupling of the receptor to G proteins, than their *in vivo* counterparts. This notwithstanding, many naturally occurring GPCR systems do manifest constitutive activity. These include the β-adrenoreceptor (Bond et al., 1995; Gether et al., 1995), the 5-hydroxytryptamine$_{2C}$ receptor (Barker et al., 1994), the histamine H_2 receptor (Smit et al., 1996), the δ-opioid receptor (Costa and Herz, 1989), and the thyrotropin receptor (Cetani et al., 1996). These examples underscore the putative relevance of current findings to naturally occurring *in vivo* opioid systems.

References

Barker EL, Westphal RS, Schmidt D and Sanders-Bush E (1994) Constitutively active 5-hydroxy tryptamine2C receptors reveal novel inverse agonist activity of receptor ligands. *J Biol Chem* **269**:11687-11690.

Birnbaumer L.(1992) Receptor-to-effector signaling through G roteins: roles for βγ dimers as well as α subunits, Cell 71: 1069-1072.

Bond RA, Leff P, Johnson TD, Milano CA, Rockman HA, McMinn TR, Apparsundaram S, Hyek MF, Kenakin TP, Allen LF and et al (1995) Physiological effects of inverse agonists in transgenic mice with myocardial overexpression of the beta 2-adrenoceptor. *Nature* **374**:272-276.

Bonner GG, Davis P, Stropova D, Ferguson R, Yamamura HI, Porreca F, Hruby VJ. (1997) Opioid peptides: simultaneous delta agonism and mu antagonism in somatostatin analogues. Peptides 18: 93-100,

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal.Biochem.* **72**:248-254.

Cassell D and Selinger Z (1977) Mechanism of adenylate cyclase activation by cholera toxin: inhibition of GTP hydrolysis at the regulatory site. *Proc.Natl.Acad.Sci.U.S.A.* **74**:3307-3311.

Cetani F, Tonacchera M and Vassart G (1996) Differential effects of NaCl concentration on the constitutive activity of the thyrotropin and the luteinizing hormone/chorionic gonadotropin receptors. *FEBS Lett* **378**:27-31.

Chakrabarti S and Gintzler AR (2003) Phosphorylation of Gβ is augmented by chronic morphine and

enhances Gβγ stimulation of adenylyl cyclase activity. *Brain Res Mol. Brain Res,* 119: 144-151.

Chakrabarti S, Rivera M, Yan S-Z, Tang W-J and Gintzler AR (1998a) Chronic morphine augments Gβγ/Gsα stimulation of adenylyl cyclase: relevance to opioid tolerance. *Mol. Pharmacol.* **54**:655-662.

Chakrabarti S, Wang L, Tang W-J and Gintzler AR (1998b) Chronic morphine augments adenylyl cyclase phosphorylation: relevance to altered signaling during tolerance/dependence. *Mol. Pharmacol.* **54**:949-953.

Chiu TT, Yung LY, and Wong YH (1996) Inverse agonistic effect of ICI-174,864 on the cloned delta-opioid receptor: role of G protein and adenylyl cyclase activation. *Mol Pharmacol* **50**:1651-1657.

Costa T and Herz A (1989) Antagonists with negative intrinsic activity at delta opioid receptors coupled to GTP-binding proteins. *Proc Natl Acad Sci U S A* **86**:7321-7325.

Cruciani RA, Dvorkin B, Morris SA, Crain SA and Makman MH (1993) Direct coupling of opioid receptors to both stimulatory and inhibitory guanine nucleotide-binding proteins in F-11 neuroblastoma-sensory neuron hybrid cells. *Proc.Natl.Acad.Sci.U.S.A.* **90**:3019-3023.

Fábián G, Bozó B, Szikszay M, Horváth G, Coscia CJ and Szücs M (2002) Chronic morphine-induced changes in mu-opioid receptors and G proteins of different subcellular loci in rat brain. *J. Pharmacol.* Exp. Ther. 302 (2): 774-780.

Gether U, Lin S and Kobilka BK (1995) Fluorescent labeling of purified beta 2 adrenergic receptor. Evidence for ligand-specific conformational changes. *J Biol Chem* **270**:28268-28275.

Gintzler AR and Xu H (1991) Different G proteins mediate the opioid inhibition or enhancement of evoked [5-methionine]enkephalin release. *Proc Natl Acad.Sci (USA)* **88**:4741-4745.

Hawkins KN, Knapp RJ, Lui GK, Gulya K, Kazmierski W, Wan YP, Pelton JT, Hruby VJ and Yamamura HI (1989) [^{3}H]-[H-D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2] ([^{3}H]CTOP), a potent and highly selective peptide for mu opioid receptors in rat brain. *J Pharmacol Exp Ther.* **248(1)**:73-80.

Kenakin T (1995) Agonist-receptor efficacy. I: Mechanisms of efficacy and receptor promiscuity. *Trends Pharmacol Sci* **16**:188-192.

Leff P (1995a) The two-state model of receptor activation. *Trends Pharmacol Sci* **16**:89-97.

Leff P (1995b) Inverse agonism: theory and practice. *Trends Pharmacol Sci* **16**:256.

Liu JG and Prather PL (2001) Chronic exposure to mu-opioid agonists produces constitutive activation of mu-opioid receptors in direct proportion to the efficacy of the agonist used for pretreatment. *Mol Pharmacol* **60**:53-62.

Liu JG, Ruckle MB and Prather PL (2001) Constitutively active mu-opioid receptors inhibit adenylyl cyclase activity in intact cells and activate G-proteins differently than the agonist. *J Biol Chem* **276**:37779-37786.

Merkouris M, Mullaney I, Georgoussi Z and Milligan G (1997) Regulation of spontaneous activity of the delta-opioid receptor: studies of inverse agonism in intact cells. *J Neurochem* **69**:2115-2122.

Rivera M and Gintzler AR (1998) Differential effect of chronic morphine on mRNA encoding adenylyl cyclase isoforms: relevance to physiological sequela of tolerance/dependence. *Mol. Brain Research* **54**:165-169.

Sterious SN and Walker EA (2003) Potency differences for D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2

as an antagonist of peptide and alkaloid mu-agonists in an antinociception assay. J Pharmacol Exp Ther. 304: 301-309.

Samama P, Cotecchia S, Costa T and Lefkowitz RJ (1993) A mutation-induced activated state of the beta 2-adrenergic receptor. Extending the ternary complex model. *J Biol Chem* **268**:4625-4636.

Shen K-F and Crain SM (1990) Cholera toxin A blockade of opioid excitatory effects on sensory neuron action potentials indicates mediation by Gs-linked opioid receptors. *Brain Research* **525**:225-231.

Smit MJ, Leurs R, Alewijnse AE, Blauw J, Van Nieuw Amerongen GP, Van De Vrede Y, Roovers E and Timmerman H (1996) Inverse agonism of histamine H2 antagonist accounts for upregulation of spontaneously active histamine H2 receptors. *Proc Natl Acad Sci U S A* **93**:6802-6807.

Stadel JM and Lefkowitz RJ (1981) Differential effects of cholera toxin on guanine nucleotide regulation of β-adrenergic agonist high affinity binding and adenylate cyclase activation in frog erythrocyte membranes. *J.Cyclic Nucleotide Res.* **7**:363-374.

Tang W-J and Gilman AG (1991) Type-specific regulation of adenylyl cyclase by G protein βγ subunits. *Science* **254**:1500-1503.

Tang W-J. and Gilman AG (1992) Adenylyl cyclases. Cell 70: 869-872.

Wang D, Raehal KM, Bilsky EJ and Sadee W (2001) Inverse agonists and neutral antagonists at mu opioid receptor (MOR): possible role of basal receptor signaling in narcotic dependence. *J Neurochem* **77**:1590-1600.

Wang L and Gintzler AR (1997) Altered μ-opiate receptor-G-protein signal transduction following chronic morphine exposure. *J. Neurochem.* **68**:248-254.

Wieland C, Jakobs KH and Wieland T (1994) Altered guanine nucleoside triphosphate binding to transducin by cholera toxin-catalyzed ADP-ribosylation. *Cellular Signalling* **6(5)**:487-492.

Acknowledgement:

Authors would like to express their appreciation to Dr. Sumita Chakrabarti for excellent technical advice and for helpful editorial suggestions. This work was supported by a grant from the National Institute on Drug Abuse (DA1225) to ARG. MS is supported by the Hungarian Research Foundation OTKA T-033062.

JPET Fast Forward. Published on March 2, 2004 as DOI: 10.1124/jpet.104.066837
This article has not been copyedited and formatted. The final version may differ from this version.

FIG. 1. Inhibitory versus stimulatory effect of DAMGO on AC activities. Panel A. Varying concentrations (10^{-9}-10^{-5} M) of DAMGO were incubated for 15 min at 30˚C with membranes obtained form opioid naive (✸) and chronic morphine-treated (▲) MOR-CHO, n=4. Panel B. Membranes were obtained from opioid naive or chronic morphine-treated cells that had been grown in either the absence (open columns) or presence (crossed-hatched columns) of 100 ng/ml PTX, which was added during the last 24 h in culture. * denotes a significant DAMGO effect on FSK-stimulated AC activity (inhibition or stimulation), # denotes a significant effect of PTX on DAMGO responsiveness; ψ denotes significant difference in DAMGO responsiveness in naive vs chronic morphine-treated membranes; n = 7-11. Panel C. Varying concentrations of DAMGO (10^{-8}-10^{-5} M) were incubated for 15 min at 30˚C with membranes obtained from MOR-CHO cells that had been treated for 24 h with 1 μM CTX and 100 ng/ml PTX. n=3. All curves were fitted with GraphPad Prizm. In each experiment, FSK-stimulated AC activity measured in the absence of opioids, but in the presence of PTX±CTX where appropriate, was defined as 100%. The ability of DAMGO to facilitate AC activation by FSK is unmasked by PTX treatment of opioid naïve

FIG. 2. CTAP reversal of DAMGO inhibition. Membranes from opioid naive (A and B) and morphine tolerant (C and D) cells were incubated with CTAP and DAMGO (1 μM each) for 15 min at 30˚C. Hatched portion of bars A and C indicate the response to DAMGO in the absence of CTAP. The open portion shows the reversal of DAMGO inhibition by CTAP. Bars B and D indicate responses to CTAP (1 μM) alone in naïve and tolerant cells, respectively. Ligand effects are shown as, mean ± S.E.M. % FSK; n = 7-11. ⁕ denotes significantly different from FSK; ψ denotes significant difference between naive and tolerant membranes. CTAP blocks DAMGO inhibition of FSK-stimulated AC activity in membranes obtained from opioid naïve cells. In tolerant membranes, CTAP reverses the inhibition to a facilitation.

FIG. 3. Concentration and toxin-dependence of the inverse agonism by CTAP. Membranes were prepared from opioid naive (◊) and tolerant (■) MOR-CHO cells that were (A) untreated, (B) pretreated with 1 μM CTX FOR 16 h, or (C) pretreated with 1 μM CTX and 100 ng/ml PTX for the last 24 h in culture. Tolerant membranes were maintained in the presence of 1 μM morphine for 48 h. The effect of CTAP (0.1-10 μM) was evaluated on FSK-stimulated AC activities and expressed as % FSK. ψ denotes

significant difference in CTAP responsiveness in naive vs chronic morphine-treated membranes; # denotes a significant effect of CTX on CTAP stimulation, and ⁕ shows significant effect of PTX+CTX pretreatment vs CTX pretreatment. n≥5. Facilitation of FSK stimulation of AC activity by CTAP was augmented following chronic morphine treatment, which could be markedly attenuated by CTX and abolished by the combined pretreatment with CTX and PTX.

Fig. 4. The chronic morphine-induced augmentation of CTAP stimulation of AC activity is substantially reduced by PTX. Opioid naive and morphine tolerant cells were cultured in the presence of 100 ng/ml PTX for the last 24 h in culture followed by preparation of membrane, which were incubated with CTAP (1 μM) for 15 min at 30˚C. The open part of each bar indicates the response to CTAP in the absence of PTX treatment. The hatched (bottom) portion indicates the response to CTAP in the presence of PTX. The effect of ligands is shown as mean ± S.E.M. %FSK; n = 7-11. ⁕ denotes a significant effect of CTAP on FSK stimulation of AC activity. ψ denotes a significant effect of PTX on CTAP facilitative responsiveness; # denotes a significant difference in CTAP responsiveness between naive and tolerant membranes. CTAP facilitation of FSK activation of AC activity is augmented by chronic morphine treatment, which is substantially attenuated by PTX treatment.

FIG. 5. Naloxone (Nx) facilitates FSK stimulation of AC activity in tolerant membranes, which is stereospecific. The effect of (+)Nx or (±)Nx (1 μM) was determined on FSK-stimulated AC activities in membranes obtained from opioid naive and chronic morphine-treated MOR-CHO cells. Data are presented as mean ± SEM % FKS. ⁕ denotes significant facilitation of FSK response; n=2-4. Naloxone facilitation of FSK activation of AC activity is only manifest in opioid tolerant membranes and is stereospecific.

Fig. 1

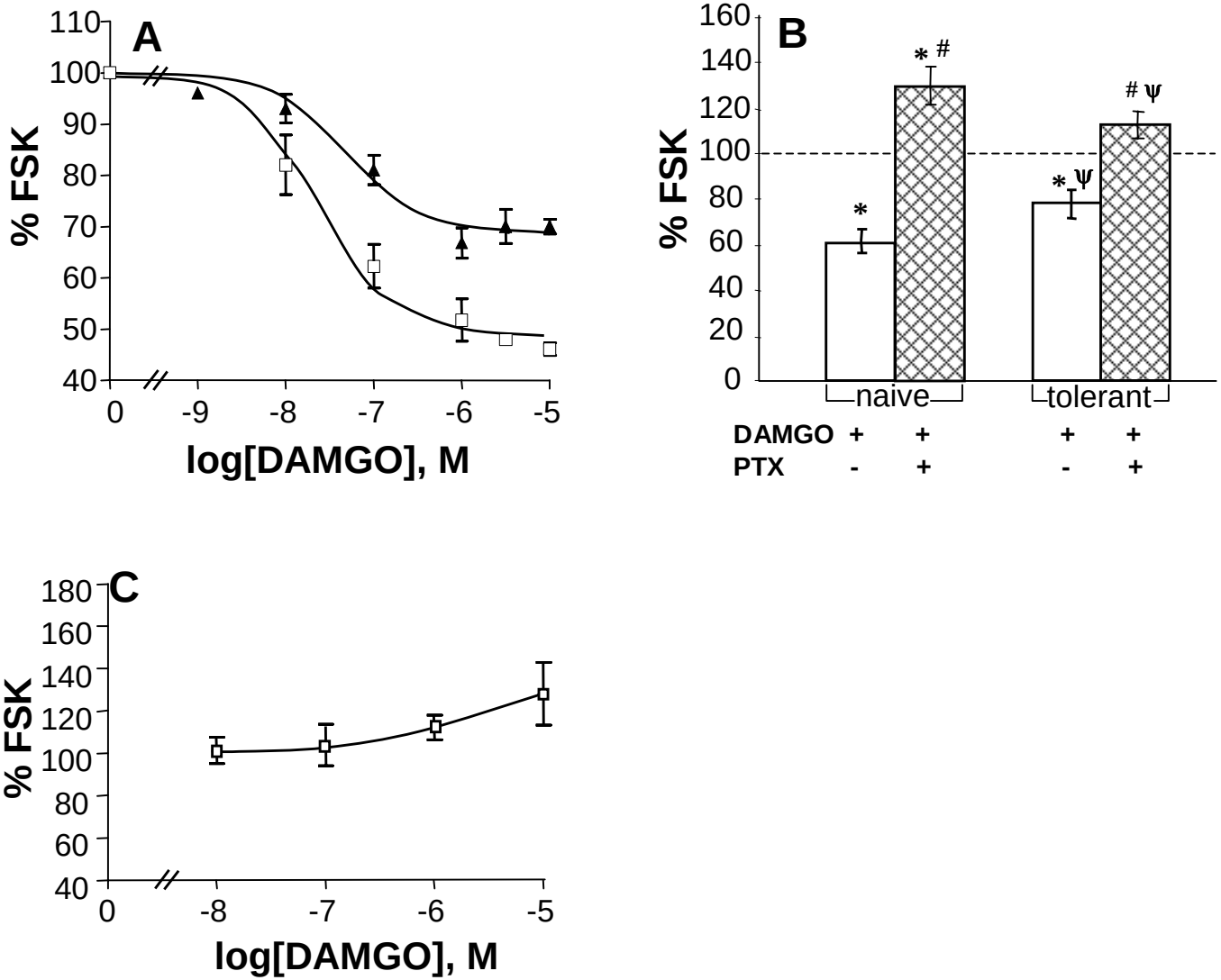
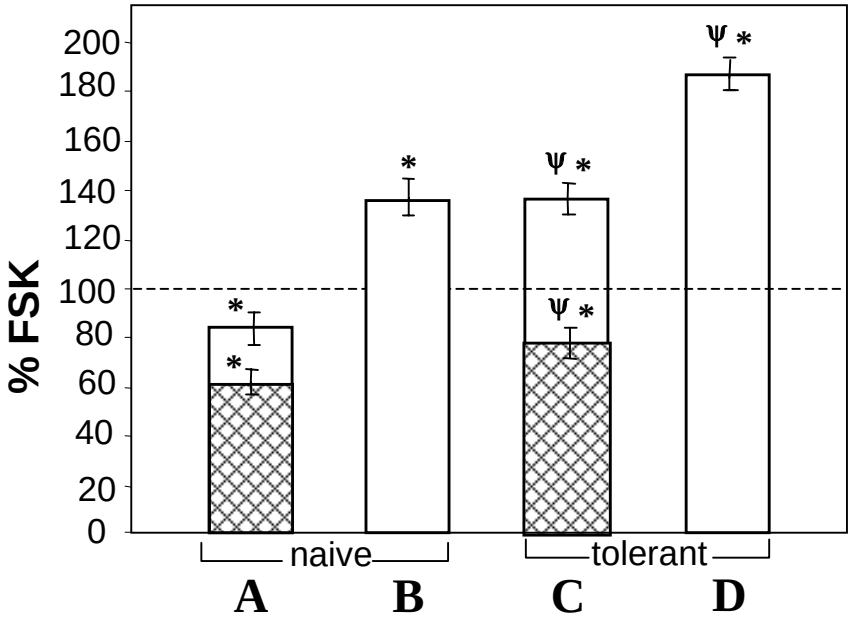
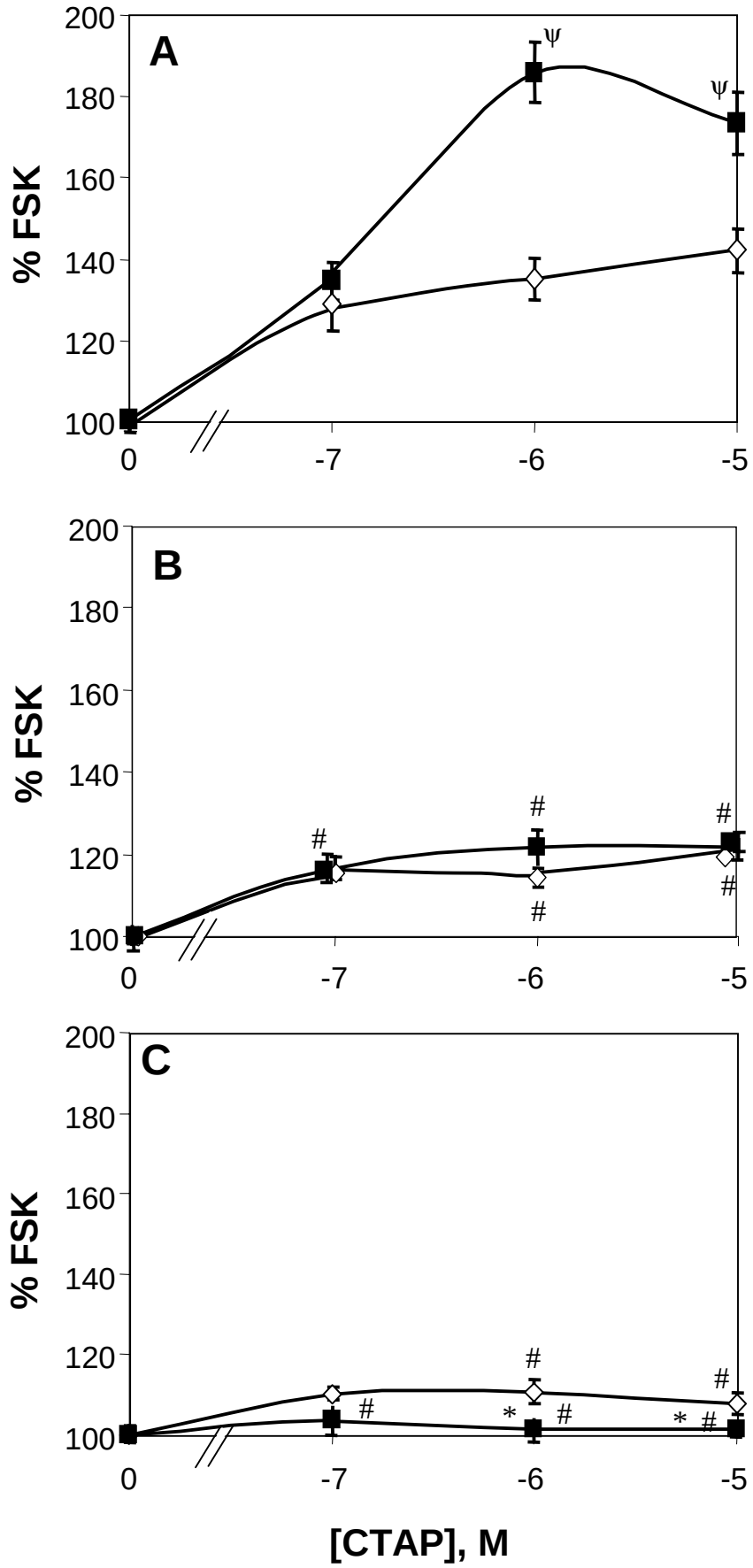


Fig. 2



JPET Fast Forward. Published on March 2, 2004 as DOI: 10.1124/jpet.104.066837
This article has not been copyedited and formatted. The final version may differ from this version.

Fig 3



JPET Fast Forward. Published on March 2, 2004 as DOI: 10.1124/jpet.104.066837
This article has not been copyedited and formatted. The final version may differ from this version.

Fig 4

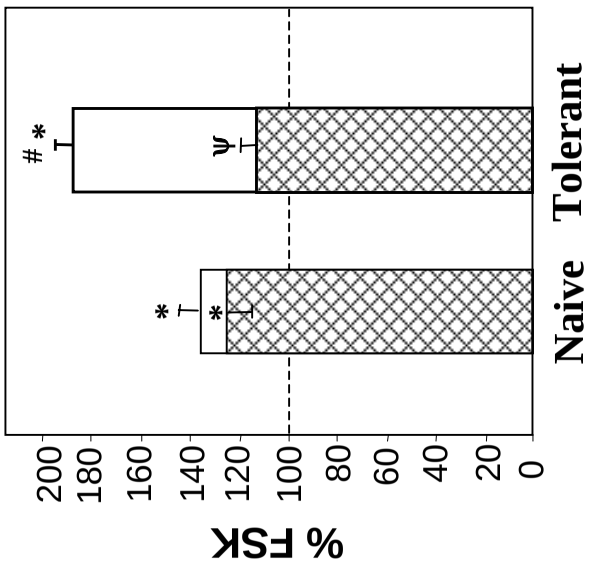


Fig. 5