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Differential Effects of Agonists on Adenylyl Cyclase Superactivation Mediated by the κ Opioid Receptors: Adenylyl Cyclase Superactivation Is Independent of Agonist-induced Phosphorylation, Desensitization, Internalization and Down-regulation

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ABSTRACT

Prolonged activation of opioid receptors followed by agonist removal leads to adenylyl cyclase (AC) superactivation. In this study, we examined in CHO cells stably expressing the human or rat κ opioid receptor (hkor or rkor) whether agonists had differential abilities to induce AC superactivation and whether the hkor and rkor exhibited differential AC superactivation. Pretreatment of the hkor with U50,488H induced AC superactivation in a time- and dose-dependent manner, reaching a plateau at 4 h and 0.1 μM. The extents of AC superactivation following a 4-h pretreatment of the hkor with saturating concentrations of agonists were in the order of the full agonists U50,488H, dynorphin A(1-17), (+/-)ethylketocyclazocine, etorphine and U69,593 > the high-efficacy partial agonist nalorphine > the low-efficacy partial agonists nalbuphine, morphine and pentazocine. Interestingly, the full agonist levorphanol caused much lower AC superactivation than other full agonists and reduced the AC superactivation induced by U50,488H and dynorphin A(1-17) in a dose-dependent fashion. The order of relative efficacies of agonists in causing AC superactivation mediated by the rkor was similar to that mediated by the hkor and the extents of AC superactivation were slightly lower. Since the rkor does not undergo U50,488H (1 μM)-induced phosphorylation, desensitization, internalization and down-regulation in these cells, the degree of AC superactivation is independent of these processes. This is among the first reports to demonstrate that relative efficacies of agonists in causing AC superactivation generally correlated with those in activating G proteins and a full agonist reduced AC superactivation induced by another full agonist.

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Activation of opioid receptors (at least μ,κ,δ) produces a variety of effects including the remarkable analgesic effects and mood changes. Chronic administration of opioid compounds leads to tolerance and dependence, which is shown to be mediated by the opioid receptors. Opioid receptors are coupled to pertussis toxin-sensitive G proteins, which in turn, affect down-stream effectors, including inhibit adenylyl cyclase (AC), increase K^+ conductance, decrease Ca^{++} conductance and stimulate p42/p44 mitogen activated protein kinases [for a review, see (Law et al., 2000)]. μ,κ,δ receptors have been cloned from several species [for reviews, see (Kieffer, 1995; Knapp et al., 1995)]. Protein analysis reveals that the opioid receptors have seven transmembrane domains and belong in the rhodopsin subfamily of G protein-coupled receptors (GPCR).

κ opioid receptors mediate many effects of opioid drugs, including analgesia (VonVoigtlander et al., 1983; Dykstra et al., 1987), dysphoria (Pfeiffer et al., 1986; Dykstra et al., 1987) and water diuresis (VonVoigtlander et al., 1983; Dykstra et al., 1987). κ agonists may be useful as analgesics (VonVoigtlander et al., 1983), water diuretics (Reece et al., 1994) and antipruritic drugs (Gmerek and Cowan, 1988) and against cocaine craving in addicts (Shippenberg et al., 2001). Repeated administration of the selective κ opioid agonist U50,488H [(trans)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide methanesulfonate] leads to tolerance to its analgesic and hypothermic effects (VonVoigtlander et al., 1983; Bhargava et al., 1989). Following repeated U50,488H administration, naloxone precipitated withdrawal signs that are distinctly different from those mediated by the μ opioid receptor (Murray and Cowan, 1988).

Chronic opioid treatment followed by removal of the drug leads to enhanced forskolin-stimulated AC activity. The change was first observed in cultured NG108-15 neuroblastoma

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× glioma hybrid cells that express the δ opioid receptor (Sharma et al., 1975) and later confirmed in several cell systems [for example, (Yu et al., 1990; Law et al., 1994; Avidor-Reiss et al., 1995b; Blake et al., 1997a)]. This phenomenon, referred to as AC superactivation, AC sensitization or AC supersensitization or cAMP overshoot, has been used as a cellular hallmark of opioid withdrawal. The term "AC superactivation" is used in this paper. AC superactivation has also been demonstrated following *in vivo* opioid administration in neurons in several regions of the rat brain including the locus coeruleus (Nestler and Aghajanian, 1997).

Prolonged treatment of cells stably transfected with the human or rat κ opioid receptor (hkor or rkor) with U50,488H or U69,593 has been shown to result in AC superactivation (Avidor-Reiss et al., 1995b; Li et al., 2003). It has been previously reported that there are species and agonist differences in agonist-induced regulation of the κ opioid receptors. When expressed in CHO cells, HEK293 cells or NG108-15 cells, the human κ opioid receptor undergoes U50,488H (1 μM or less)-induced phosphorylation, desensitization, internalization and down-regulation, but the rat or mouse κ receptor does not, using agonist-induced [^{35}S]GTPγS binding or inhibition of forskolin-stimulated adenylyl cyclase as the functional endpoint (Blake et al., 1997b; Li et al., 1999; Jordan et al., 2000; Li et al., 2000; Li et al., 2002; Zhang et al., 2002; Schulz et al., 2002). In AtT-20 cells or HEK293 cells transfected with the rat κ receptor – green fluorescence protein, McLaughlin et al. (2003) found that the rat κ receptor was desensitized by 0.1 μM U50,488H with agonist-enhanced K^+ current as the functional endpoint, phosphorylated by 1 μM U50,488H and internalized by 10 μM U50,488H. In Xenopus oocytes expressing the rat κ receptor and Kir3.1 and Kir3.4 potassium channels, desensitization of κ agonist-induced K^+ current was modest without expression of GRK3 or GRK5 and arrestin3 (Appleyard et al., 1999). In addition, while U50,488H, U69,593, dynorphin A(1-17), (+/-)ethylketocyclazocine and

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tifluadom cause internalization of the human κ receptor in CHO cells, etorphine and levorphanol do not (Li et al., 1999; Li et al., 2003). Dynorphin peptides promote internalization and down-regulation of the rat κ opioid receptor in CHO cells, but nonpeptide agonists do not (Jordan et al., 2000). U50,488H and dynorphin A(1-17) enhance phosphorylation of the human κ receptor to greater extents than etorphine, whereas levorphanol does not increase phosphorylation (Li et al., 2003). Furthermore, etorphine and levorphanol reduce U50,488H- or dynorphin A(1-17)-promoted internalization and phosphorylation (Li et al., 2003).

In this study, we investigated whether there were agonist and species differences in AC superactivation following activation of the rat or human κ opioid receptor stably expressed in Chinese hamster ovary (CHO) cells.

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MATERIALS AND METHODS

Materials

[^{3}H]cAMP (30-40 Ci/mmol) and [^{3}H]diprenorphine (~50 Ci/mmol) were purchased from Perkin Elmer Life Sciences (Boston MA). (-)U50,488H and U69,593 were provided by Upjohn Co. (Kalamazoo, MI). Dynorphin A (1-17) was purchased from Phoenix Pharmaceuticals, Inc. (Belmont, CA). Other opioid receptor agonists were provided by the National Institute on Drug Abuse. Naloxone HCl, 1-methyl-3-isobutylxanthine (IBMX), forskolin and cAMP were obtained from Sigma-Aldrich Co. (Louis, MO). Geneticin was purchased from Mediatech Co. (Herndon, VA). Cell culture reagents were obtained from Invitrogen Co. (Carlsbad, CA).

Cell culture

FLAG-tagged human and rat κ opioid receptor (FLAG-hkor and FLAG-rkor) in the expression vector pcDNA3 was generated previously (Li et al., 2002) and clonal CHO cells stably transfected with the FLAG-tagged human or rat κ opioid receptor (CHO-FLAG-hkor and CHO-FLAG-rkor cells, respectively) were established previously (Li et al., 2002). CHO-FLAG-hkor or CHO-FLAG-rkor cells were cultured in 100-mm culture dishes in Dulbecco's modified Eagle's medium F12/Ham's medium supplemented with 10% fetal calf serum, 0.2 mg/ml geneticin, 100 units/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere consisting of 5% CO_2, 95% air at 37°C.

κ Opioid receptor saturation binding assay

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Membranes were prepared as described previously (Zhang et al., 2002). Receptor binding was conducted with [^{3}H]diprenorphine in 50 mM Tris·HCl buffer containing 1 mM Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (pH 7.4) as described previously (Zhu et al., 1997). Naloxone (10 μM) was used to define nonspecific binding. Saturation experiments were performed with 6 concentrations of [^{3}H]diprenorphine (ranging from 0.02 nM to 2 nM). Binding was conducted at 25°C for 60 min in duplicate in a volume of 1 ml with 30 to 40 μg of membrane proteins. Bound and free ligands were separated by rapid filtration under reduced pressure over GF/B filters presoaked with 0.2% polyethylenimine and 0.1% bovine serum albumin in 50 mM Tris·HCl (pH 7.4) for 1 h. Binding data were analyzed with Prism 3.0 (GraphPad Software, Inc., San Diego, CA).

Pretreatment with drugs and determination of cAMP level

CHO-FLAG-hkor cells and CHO-FLAG-rkor cells were transferred to 12-well culture plates and cultured overnight prior to experiments. For agonist pretreatment, cells were incubated with a drug or a combination of drugs at 37°C for 4 h or an indicated time period. Medium was then removed and cells were briefly washed three times with 0.1 M phosphate-buffered saline before the cells were detached with pre-warmed (37°C) Versene solution (0.54 mM ethylenediaminetetraacetic acid, 140 mM NaCl, 2.7 mM KCl, 8.1mM Na_2HPO_4, 1.46 mM KH_2PO_4 and 1 mM glucose). Cells were centrifuged, resuspended in the Opti-MEMI reduced serum medium containing 1 mM IBMX, 10 μM forskolin and 10 μM naloxone and incubated at 37°C for 15 min. The reaction was terminated by boiling in a water bath for 10 min. cAMP contents in each sample were determined with the cAMP binding protein method as described previously (Huang et al., 2001). [^{3}H]cAMP (~ 250,000 dpm in 0.02 M citrate phosphate buffer,

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pH 5.0) was added on ice to all sample tubes and tubes containing known amounts of cAMP (from 1.25 to 40 pmole) for generation of a standard curve. cAMP binding protein partially purified from bovine adrenal glands was added to each tube, except the blanks, at an amount which gave 10,000 – 20,000 dpm [^{3}H]cAMP binding in the absence of cold cAMP. The mixture (final volume 170 µl) was incubated 2 h to overnight at 4°C. Bound and free [^{3}H]cAMP were separated by adsorption of free [^{3}H]cAMP by 100 µl charcoal suspension (10% Norit A charcoal, 4% bovine serum albumin, 1% Antifoam A) and centrifugation (1500 x g for 20 min). Radioactivity of bound [^{3}H]cAMP in an aliquot of the supernatant was determined by liquid scintillation counting. The standard curve was analyzed with a logit-log equation and KaleidaGraph 3.5 Program (Synergy Software, Inc., Reading, PA). The amounts of cAMP in samples were calculated based on the standard curve and converted to pmole/well.

Statistical analysis

For comparison of multiple groups, data were analyzed with analysis of variance to determine if there were significant differences among groups using Prism 3.0 (GraphPad Software, Inc., San Diego, CA). If so, Dunnett's *post hoc* test was performed to determine whether there was significant difference between the control and each treatment group. $P<0.05$ was the level of significance in all statistical analyses.

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RESULTS

Clonal CHO-FLAG-hkor cells (FHK2.2), which express 1.35 ± 0.07 pmole/mg protein (n=3) FLAG-hkor, were used in all experiments, except in the rat κ opioid receptor experiments where a CHO-FLAG-rkor cell line (FRK24) expressing 1.07 ± 0.12 pmole/mg protein (n=3) FLAG-rkor was used.

AC superactivation induced by U50,488H treatment: Time course and dose-response relationship

CHO-FLAG-hkor cells (FHK2.2) were incubated with vehicle (control) or U50,488H (1.5 μM), a selective κ agonist, for different time periods. Pretreatment with U50,488H enhanced forskolin-stimulated AC activity, compared with the controls, in a time-dependent manner and reached a plateau at 4 h (Fig. 1). At the plateau level, forskolin-stimulated cAMP level was 250-300% of the controls. Longer incubation time (up to 28 h) did not enhance the AC superactivation level.

CHO-FLAG-hkor cells were pretreated with vehicle (control) or different concentrations of U50,488H for 4 h. U50,488H at 10 nM or higher concentrations induced significant compensatory increase in forskolin-stimulated cAMP accumulation compared to the control. The extent of increase was related to the concentration of U50,488H used in the pretreatment, with an EC_{50} value of 25.2 ± 0. 7 nM (n=3) and the maximal level was reached at ~0.1 μM (Fig. 2).

Agonists acting on the human κ opioid receptor had differential effects on forskolin-stimulated AC activity

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CHO-FLAG-hkor cells (FHK2.2) were pretreated for 4 h with different opioid receptor agonists, each at an indicated concentration that was 500-1000 fold of the EC_{50} of the drug in promoting [^{35}S]GTPγS binding via the human κ opioid receptor (Zhu et al., 1997; Li et al., 2003), except morphine which was used at 10 μM. The degree of forskolin-stimulated cAMP accumulation following agonist pretreatment was in the order of U50,488H, U69,593, (+/-)EKC, dynorphin A(1-17) and etorphine (200~270% of the control) > nalorphine (~190%) > levorphanol, pentazocine, nalbuphine and morphine (136~155%) (Fig. 3). We have shown previously that U50,488H, (+/-)EKC, dynorphin A(1-17), U69,593, etorphine and levorphanol are full agonists, whereas nalorphine is a high-efficacy partial agonist and nalbuphine, pentazocin and morphine are low-efficacy partial agonists, at the human κ opioid receptor in enhancing [^{35}S]GTPγS binding (Zhu et al., 1997; Li et al., 2003) (morphine, unpublished observation). Thus, the degrees of AC superactivation induced by the agonists in general correlate with their efficacies, except levorphanol.

The low level of AC superactivation caused by levorphanol pretreatment may be due to a different time course of action or levorphanol not being displaced from the receptor by naloxone. Experiments were performed to test these possibilities. Pretreatment with levorphanol (10 μM) for 4, 9 or 24 h caused similar levels of AC superactivation, which were about ~150% of the control (Fig. 4A). Levorphanol was added at 0 h and at 10 h for the 24-h incubation. Since levorphanol has a longer half-life than morphine when administered in human (Gutstein and Akil, 2001), it is likely to be relatively stable in cell culture medium. In addition, following incubation with levorphanol for 4 h and removal of the drug by washing, cells were incubated with 10 μM, 30 μM or 100 μM naloxone. As shown in Fig. 4B, increasing naloxone concentration did not further enhance the extent of AC superactivation. These results indicate that the low level of AC

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superactivation induced by levorphanol is not due to incubation time being too short or insufficient displacement of the drug from the receptor by naloxone.

Levorphanol reduced U50,488H and dynorphin A(1-17)-caused AC superactivation

CHO-FLAG-hkor cells were pretreated for 4 h with U50,488H (0.1 μM) in the presence or absence of different concentrations of levorphanol for 4 h. Levorphanol significantly reduced U50,488H-induced AC superactivation in a dose-dependent fashion (Fig. 5A). Following U50,488H pre-incubation, forskolin-stimulated cAMP level was ~240% of the control. Levorphanol at 10 μM or 100 μM reduced U50,488H-induced AC superactivation to ~160% and ~120%, respectively, of the control, whereas 1 μM levorphanol had no effect. In addition, levorphanol at 100 μM significantly attenuated dynorphin A(1-17) (10 nM)-induced AC superactivation (from ~250% to ~140% of the control). At 10 μM, levorphanol appeared to decrease AC superactivation caused by dynorphin A(1-17), but the decrease did not reach statistical significance, and levorphanol at 1 μM had no detectable effect (Fig. 5B).

Agonists acting on the rat κ opioid receptor caused different degrees of AC superactivation

We have reported previously that in CHO cell lines expressing the human or the rat κ opioid receptor, including those used in the current study, the human κ receptor undergoes U50,488H-induced phosphorylation, desensitization, internalization and down-regulation but the rat κ opioid receptor does not (Zhu et al., 1998; Li et al., 1999; Li et al., 2000; Li et al., 2002; Zhang et al., 2002). We thus examined if there was also species difference in AC superactivation. CHO-FLAG-rkor cells (FRK24) express 1.07 ± 0.12 pmole/mg protein of the rat κ opioid receptor (n=3), a level similar to that of CHO-FLAG-hkor cells (FHK2.2). CHO-FLAG-rkor

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cells were pretreated for 4 h with different agonists at the same concentrations as described above for CHO-FLAG-hkor and forskolin-stimulated cAMP level was measured after withdrawal of the drugs and addition of naloxone. The order of the degree of forskolin-stimulated cAMP accumulation was: U50,488H, dynorphin A(1-17), U69,593, (+/-)EKC > etorphine, nalorphine > levorphanol, pentazocine, nalbuphine and morphine (Fig. 6). Pretreatment with levorphanol, pentazocine, nalbuphine or morphine showed increased forskolin-stimulated cAMP levels, although the increase did not reach statistical significance. Thus, the relative efficacies of agonists in causing AC superactivation are similar for the human and rat κ opioid receptors, but the level of AC superactivation is slightly lower for the rat κ opioid receptor than for the human κ opioid receptor.

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DISCUSSION

Differential effects of agonists in inducing AC superactivation. We have shown that pretreatment of CHO-FLAG-hkor cells or CHO-FLAG-rkor cells with opioid agonists followed by removal of the agonists and addition of naloxone led to varying degrees of AC superactivation. In general, the relative efficacies of the agonists in promoting AC superactivation are similar to those in enhancing [^{35}S]GTPγS binding, with the exception of levorphanol. To the best of our knowledge, this is the first demonstration of the correlation between the relative efficacies of agonists in causing AC superactivation and those in activating G proteins. AC superactivation following chronic opioid treatment has been shown to be mediated by $G_{i/o}$ proteins since pertussis toxin pretreatment abolished AC superactivation (Avidor-Reiss et al., 1995a). Expression of scavengers of $G_{\beta\gamma}$ subunits prevented AC superactivation following chronic opioid treatment, indicating that the $G_{\beta\gamma}$ subunits play an essential role (Avidor-Reiss et al., 1996). We have shown previously that in CHO cells expressing the human κ opioid receptor, higher-efficacy agonists activate G proteins to higher extents than lower-efficacy agonists (Zhu et al., 1997), as determined by [^{35}S]GTPγS binding. The amounts of $G_{\beta\gamma}$ subunits released by agonists should be proportional to [^{35}S]GTPγS binding, which explains that the efficacies of agonists in promoting AC superactivation generally parallel those in G protein activation.

Levorphanol is an exception. Levorphanol is a full agonist in enhancing [^{35}S]GTPγS binding (Li et al., 2003), but promotes a level of AC superactivation similar to those of low-efficacy partial agonists. The low level of AC superactivation is not due to insufficient pretreatment time or failure of naloxone to displace levorphanol from the receptor. In addition, levorphanol reduces superactivation induced by U50,488H or dynorphin A(1-17). To the best of

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our knowledge, this is among the first demonstrations that a full agonist of the κ opioid receptor significantly reduces AC superactivation induced by another full agonist.

Full agonists acting on the μ receptor were shown to have differential abilities to induce AC superactivation (Blake et al., 1997a). Prolonged pretreatment of HEK 293 cells expressing the μ receptor with morphine and DAMGO resulted in a 3-4-fold compensatory increase in forskolin-stimulated cAMP accumulation, but levorphanol, etorphine, methadone or buprenorphine pretreatment did not, although all acted as full agonists in the system. In addition, levorphanol, etorphine, methadone or buprenorphine decreased the AC superactivation caused by morphine and DAMGO.

Relationship between AC superactivation *in vitro* and withdrawal signs *in vivo*. Following chronic opiate administration, increased AC activity was observed in several brain regions including the locus coeruleus (Nestler and Aghajanian, 1997). The up-regulated cAMP pathway increases the firing rate of the locus coeruleus neurons, which supports the idea that AC superactivation constitutes one of the molecular bases of withdrawal (Nestler and Aghajanian, 1997). In accord with this notion, mice with genetic disruption of one subtype of cAMP-responsive element-binding protein show strongly attenuated symptoms of morphine withdrawal (Maldonado et al., 1996).

After repeated injection of U50,488H, naloxone precipitates distinct withdrawal syndromes (Murray and Cowan, 1988). Whether withdrawal signs occur after chronic administration of other κ agonists has not been examined. Thus, it is not known if the drug with higher efficacy in inducing AC superactivation will result in higher degrees of withdrawal. Although levorphanol, etorphine, methadone or buprenorphine pretreatment of the μ opioid receptor does not cause AC superactivation (Blake et al., 1997a), chronic administration of these

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drugs followed by naloxone *in vivo* has been shown to induce withdrawal signs (Roerig et al., 1985; Gutstein and Akil, 2001). However, all these drugs are non-selective opioid ligands acting on μ, δ and κ receptors, which complicates interpretation of the results. Therefore, whether there is a correlation between AC superactivation in cells *in vitro* and opioid withdrawal signs *in vivo* remains unclear.

Comparison between the human and the rat κ opioid receptors and AC superactivation is independent of agonist-induced phosphorylation, desensitization, internalization and down-regulation: There were no significant differences between the human and the rat κ opioid receptors with regard to the relative efficacies of different agonists in inducing AC superactivation. However, the level of AC superactivation mediated by the rat κ opioid receptor was slightly lower than that mediated by the human κ opioid receptor at a similar receptor expression level. We have reported previously that in CHO cells expressing the human or rat κ opioid receptor (including the cell lines used in this study), U50,488H (1 μM) pretreatment led to phosphorylation, desensitization, internalization and down-regulation of the human κ receptor, but not the rat κ opioid receptor (Zhu et al., 1998; Li et al., 1999; Li et al., 2000; Li et al., 2002; Zhang et al., 2002). Desensitization was demonstrated by agonist-induced [^{35}S]GTPγS binding or inhibition of forskolin-stimulated AC activity (Zhu et al., 1998; Li et al., 2002). Similar findings have been reported by other groups using CHO cells, HEK293 cells or NG108-15 cells expressing the rat or human κ receptor and U50,488H (Blake et al., 1997b; Jordan et al., 2000; Schulz et al., 2002; McLaughlin et al., 2003). Since pretreatment of the rat and human κ opioid receptors with U50,488H led to similar levels of AC superactivation, there is no relationship between the degrees of AC superactivation and agonist-induced receptor phosphorylation, desensitization, internalization and down-regulation.

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In accord with this finding, although U50,488H and dynorphin A(1-17) promoted phosphorylation, desensitization, internalization and down-regulation of the hkor, but etorphine caused a low level of phosphorylation and did not induce desensitization, internalization and down-regulation (Blake et al., 1997b; Li et al., 1999; Li et al., 2000; Li et al., 2003), etorphine caused similar degrees of AC superactivation as U50,488H and dynorphin A(1-17). In addition, despite that etorphine and levorphanol did not promote internalization (Li et al., 1999; Li et al., 2003), etorphine caused AC superactivation to a much higher level than levorphanol.

Our results are similar to the findings that DAMGO, but not morphine, caused phosphorylation and internalization of the μ opioid receptor without over-expression of GRK2 [for example, (Keith et al., 1996; Zhang et al., 1998)], but the two drugs induced similar levels of AC superactivation (Blake et al., 1997a). However, our observations are different from those of Finn and Whistler (2001), who, using mutant receptors and various agonists, showed that lack of internalization of the μ opioid receptor resulted in higher degrees of AC superactivation. The reasons for the differences are not clear.

AC superactivation after prolonged activation of the κ opioid receptor: Our results on AC superactivation following U50,488H pretreatment of the human or rat κ receptor are similar to those of Avidor-Reiss et al. (1995b), who reported that incubation of CHO cells stably transfected with the rat κ opioid receptor with 1 μM U69,593 for 4 h resulted in ~250% enhancement of AC activity. In addition, we found that AC superactivation was U50,488H concentration-dependent with an EC_{50} value of about 25 nM, which was about 6 times higher than that for stimulation of [^{35}S]GTPγS binding (Zhu et al., 1997). Avidor-Reiss et al. (1995b) also found that the EC_{50} value of U69,593 for inducing AC superactivation was much higher than that for inhibiting AC. We demonstrated U50,488H (1.5 μM)-induced AC superactivation

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was time-dependent, occurring as early as 1 h and with $T_{1/2}$ of ~2 h. Such a rapid time course suggests that protein synthesis is not required for AC superactivation.

Mechanisms of AC superactivation: In addition to μ,κ and δ opioid receptors, AC superactivation has been observed for several other $G_{i/o}$- coupled receptors following prolonged agonist treatment [for a review, see (Watts, 2002)]. The molecular mechanisms leading to AC superactivation have not been fully elucidated. A model was recently proposed by Watts (2002), in which the withdrawal of drugs after chronic stimulation led to the dissociation of $G\alpha_{i/o}$ from $G_{\beta\gamma,}$ which in turn, induces AC superactivation in a $G\alpha_s$-dependent manner.

AC superactivation following chronic opioid treatment has been shown to be mediated by $G_{i/o}$ proteins (Avidor-Reiss et al., 1995a). Each Giα subunit may play a role (Tso and Wong, 2000; Tso and Wong, 2001) and $G_{\beta\gamma}$ subunits play an essential role (Avidor-Reiss et al., 1996). Prolonged opioid treatment decreased the detergent solubility, and presumably lipid rafts microdomain localization, of $G\alpha_i$ and β_1 subunits, which coincided with AC superactivation (Bayewitch et al., 2000). Chronic opioid treatment resulted in an accumulation of the depalmitoylated form of Gs_α in the plasma membrane, which is associated with AC superactivation (Ammer and Schulz, 1997). AC superactivation required Gs_α-mediated AC stimulation, but it was not a direct Gs_α effect; rather, it involved a secondary regulatory event that requires stimulation of AC by Gs_α(Ammer and Schulz, 1998). Prolonged μ opioid receptor activation induces superactivation of AC types I, V, VI, and VIII, but not of type II, III, IV, or VII, expressed in CHO cells (Avidor-Reiss et al., 1996; Avidor-Reiss et al., 1997). Chronic morphine *in vivo* resulted in upregulation and enhanced protein-kinase C mediated phosphorylation of the AC II isoforms in guinea pig longitudinal muscle/myenteric plexus, leading to a shift in opioid receptor/G-protein signaling from predominantly Giα inhibitory to

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Gβγ stimulatory effect on AC (Gintzler and Chakrabarti, 2000). In addition, there is concomitant enhanced phosphorylation of G protein-coupled receptor kinase 2/3, β-arrestin, and Gβ subunit (Chakrabarti et al., 2001). Prolonged activation of the δ opioid receptor in CHO cells results in Raf-1-mediated enhanced phosphorylation of the AC VI isoenzyme, which contributes in part to AC superactivation (Varga et al., 2002) and calmodulin, protein kinase C and tyrosine kinase pathways appear to converge to activate Raf-1 (Varga et al., 2003).

The mechanisms underlying the differential abilities of full agonists to induce AC superactivation remain to be investigated. It is likely that agonists promote different receptor conformations, which may differentially activate different G proteins, leading to disparate stimulation of down-stream effectors involved in AC superactivation. We have shown previously that U50,488H and dynorphin A(1-17) induce phosphorylation of the hkor to much greater extents than etorphine, while levorphanol does not promote phosphorylation at all, despite that all four are full agonists (Li et al., 2003). Law and co-workers (Chakrabarti et al., 1998) showed that DAMGO and morphine acting on the μ opioid receptor induced different receptor conformations.

In summary, agonists acting on the κ opioid receptors had different efficacies in inducing AC superactivation, which paralleled their efficacies in activating G proteins, except levorphanol. Levorphanol, a full agonist, induced lower AC superactivation than other full agonists and reduced the effects of U50,488H and dynorphin A(1-17) on AC superactivation. For the human and rat κ opioid receptors, the relative efficacies of different agonists in causing AC superactivation were similar and the degrees of AC superactivation following an agonist pretreatment were slightly lower for the rkor than for the hkor. As the human, but not the rat, κ opioid receptor undergoes agonist-induced regulation and agonists induce AC

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superactivation regardless of their abilities to promote internalization, these results indicate that the extents of AC superactivation are not significantly affected by these regulatory processes.

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FOOTNOTES

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FIGURE LEGEND

Figure 1. Time course of U50,488H pretreatment on forskolin-stimulated cAMP accumulation in CHO-FLAG-hkor cells (FHK2.2). Cells were pretreated with vehicle (control) or with U50,488H (1.5 μM) for different time periods and medium aspirated. Cells were washed three times and incubated with IBMX, forskolin and naloxone and intracellular cAMP content was determined as described in **Materials and Methods.** Data are expressed as % of the control group. Each value represents mean ± s.e.m. of 3 independent experiments performed in triplicate.

Figure 2. Dose-dependent effect of U50,488H pretreatment on forskolin-stimulated cAMP accumulation in CHO-FLAG-hkor cells (FHK2.2). Cells were pretreated with vehicle (control) or incubated with different concentrations of U50,488H for 4 hours and medium aspirated. Cells were washed three times and incubated with IBMX, forskolin and naloxone and intracellular cAMP content was determined as described in **Materials and Methods.** Data are expressed as % of the control group. Each value represents mean ± s.e.m. of 3 independent experiments performed in triplicate.

Figure 3. Effects of pretreatment of the human κ opioid receptor with different agonists on forskolin-stimulated cAMP accumulation. CHO-FLAG-hkor cells (FHK2.2) were pretreated with vehicle (control) or treated for 4 hours with U50,488H (1.5 μM), levorphanol (10 μM), etorphine (0.1 μM), dynorphin A 1-17 (0.1 μM), pentazocine (10 μM), nalbuphine (20 μM), morphine (10 μM), (+/-)EKC (0.3 μM), nalorphine (10 μM), U69,593 (1.5μM). Following removal of culture medium, cells were washed three times and incubated with IBMX, forskolin and naloxone and intracellular cAMP content was determined as described in **Materials and**

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Methods. Data are expressed as % of the control group. Each value represents mean ± s.e.m. of 3 independent experiments performed in triplicate.

*$P < 0.05$ compared to the control by one-way ANOVA followed by Dunnett's post hoc test.

Figure 4. Effects of (A) pretreatment time and (B) naloxone concentration on levorphanol-induced AC superactivation. (A) Cells were pretreated with vehicle (control) or with levorphanol (10 μM) for 4, 9 or 24 h, washed three times and incubated with IBMX, forskolin and 10 μM naloxone for 15 min. **(B**) Cells were pretreated with vehicle (control) or with levorphanol (10 μM) for 4 h, washed three times and incubated with IBMX, forskolin and different concentration of naloxone (10 μM, 30 μM or 100 μM) for 15 min. Intracellular cAMP content was determined as described in **Materials and Methods.** All the data are expressed as % of the control. Each value represents mean ± s.e.m. of 3 or 2 (Naloxone 30 μM) independent experiments performed in triplicate.

Figure 5. Effects of levorphanol on (A) U50,488H- and (B) dynorphin A(1-17) induced adenylyl cyclase superactivation in CHO-FLAG-hkor cells (FHK2.2). Cells were pretreated with vehicle (control) or with U50,488H (0.1 μM) or dynorphin A(1-17) (10nM) in the absence or presence of different concentrations of levorphanol for 4 hours and washed three times. Cells were incubated with IBMX, forskolin and naloxone and intracellular cAMP content was determined as described in **Materials and Methods.** Data are expressed as % of the control group. Each value represents mean ± s.e.m. of 3 independent experiments performed in triplicate.

*$P < 0.05$ compared to the control by one-way ANOVA followed by Dunnett's post hoc test.

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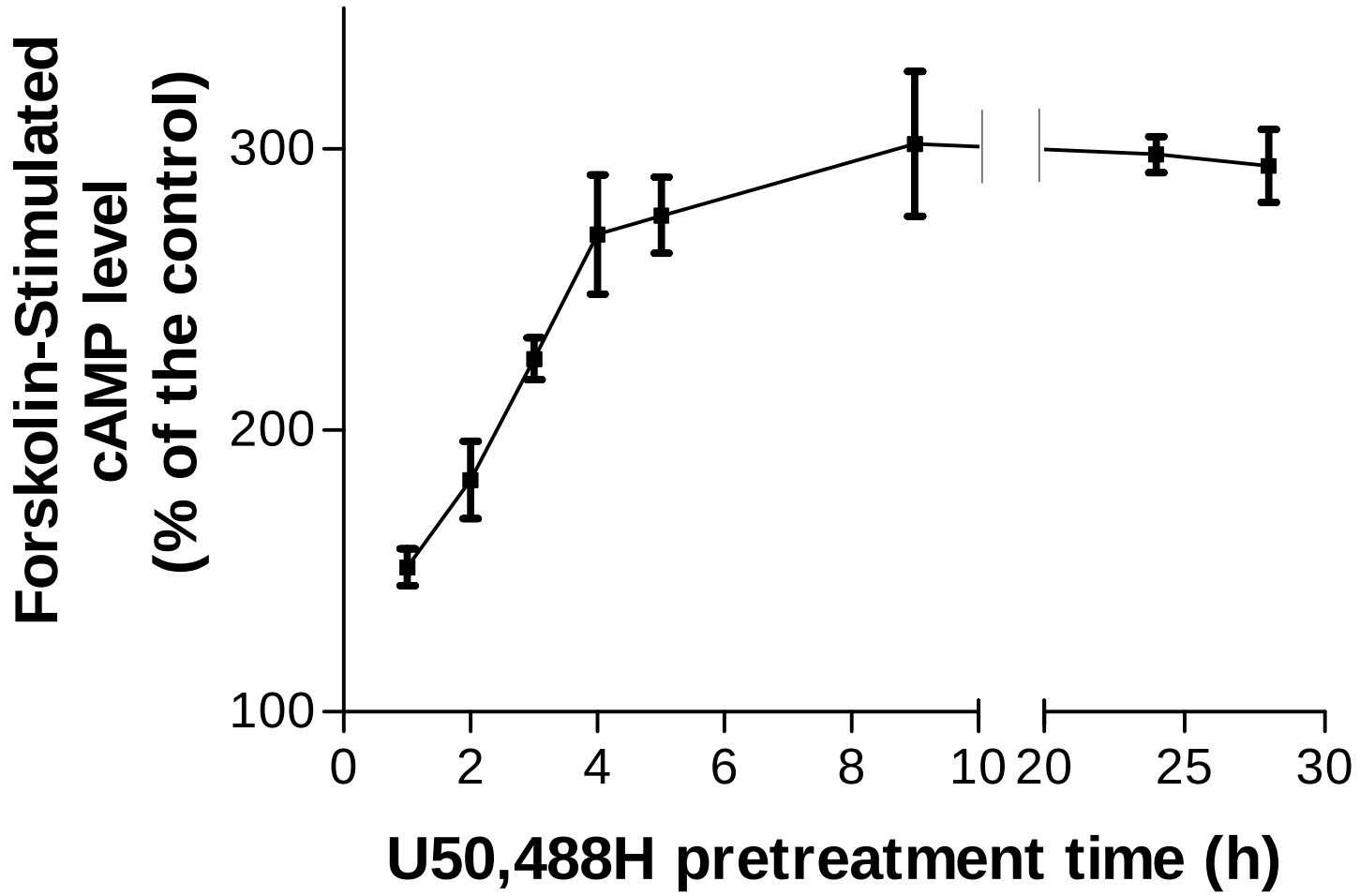
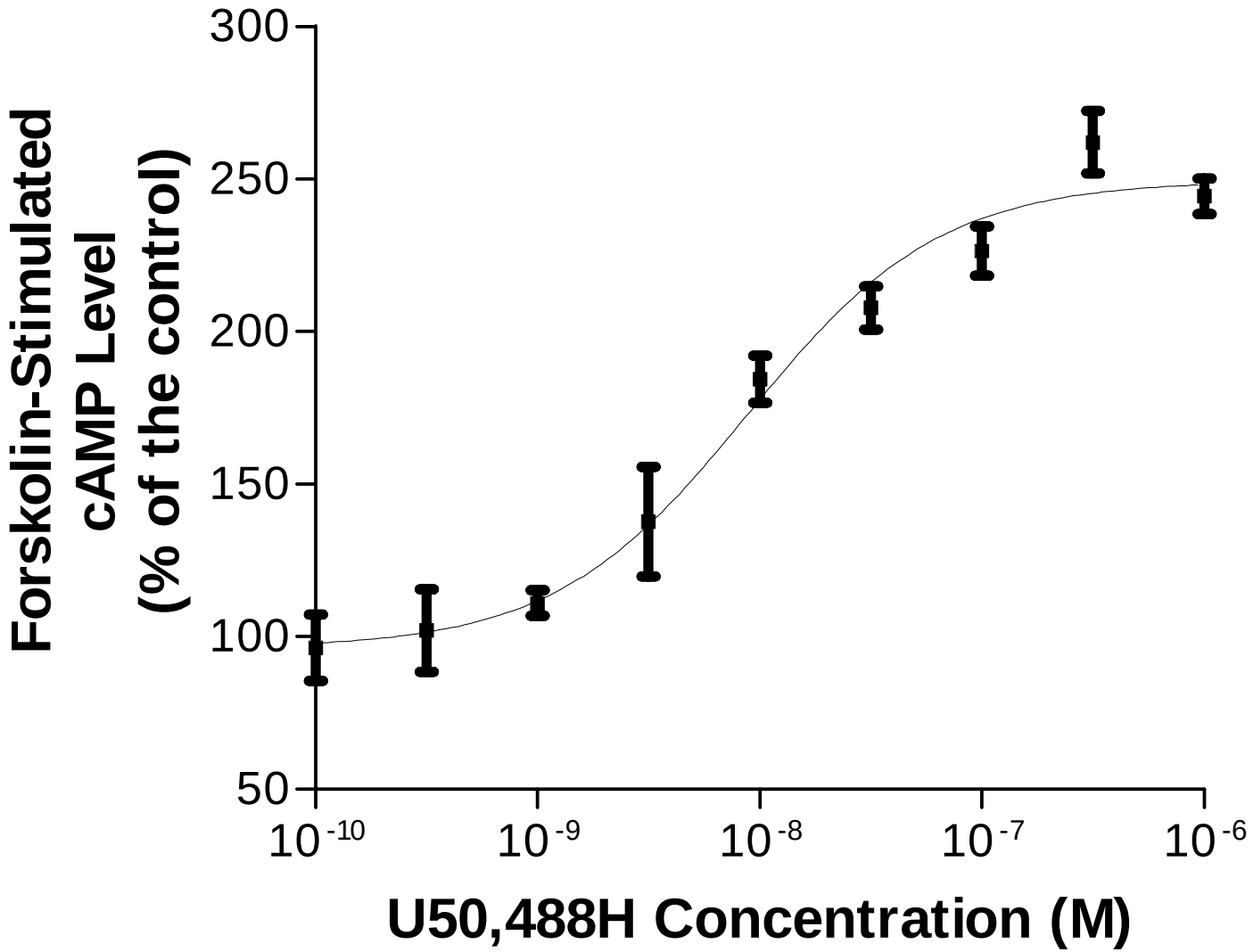
Figure 6. Effects of pretreatment of the rat κ opioid receptor with different agonists on forskolin-stimulated cAMP accumulation. CHO-FLAG-rkor cells (FRK24) were pretreated with vehicle (control) or with U50,488H (1.5 μM), levorphanol (10 μM), etorphine (0.1 μM), dynorphin A 1-17 (0.1 μM), pentazocine (10 μM), nalbuphine (20 μM), morphine (10 μM), (+/-)EKC (0.3 μM), nalorphine (10 μM), U69,593 (1.5 μM) for 4 hours. Cells were incubated with IBMX, forskolin and naloxone following removal of the drug and intracellular cAMP content was determined as described in **Materials and Methods**. Data are expressed as % of the control group. Each value represents mean ± s.e.m. of 3 independent experiments performed in triplicate. *P < 0.05 compared to the control by one-way ANOVA followed by Dunnett's post hoc test.

Wang, et al. Fig. 1



Wang, et al. Fig. 2



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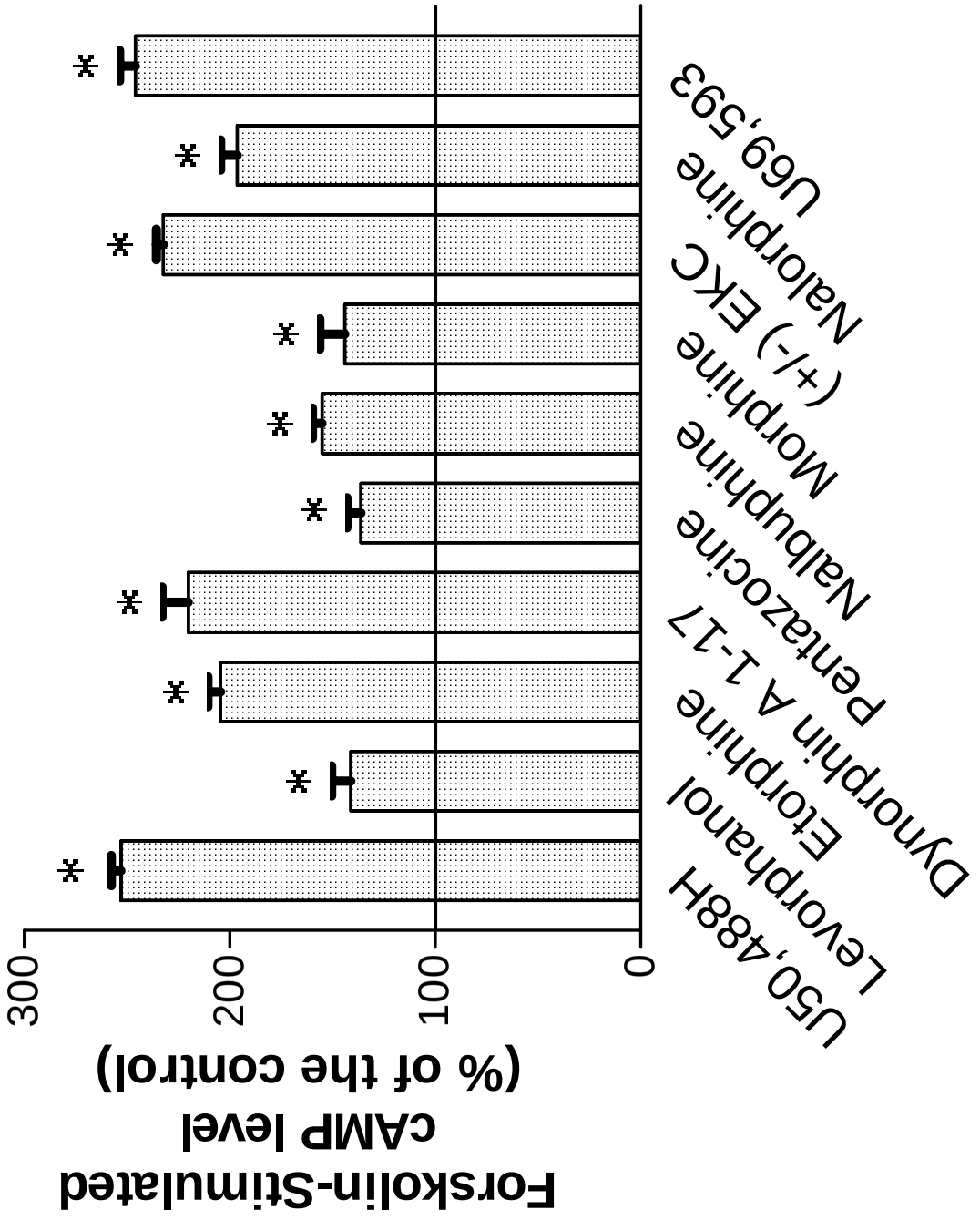
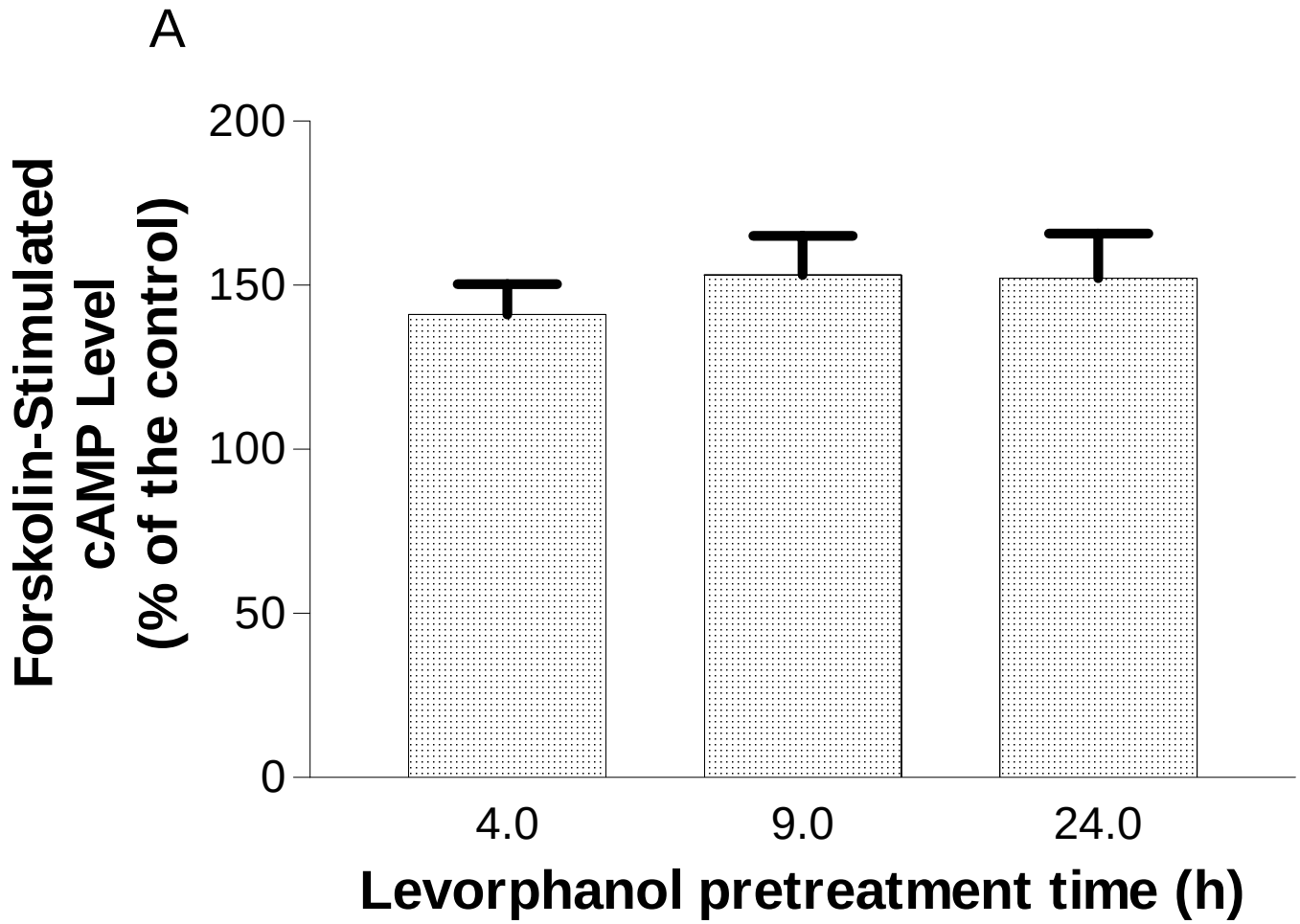
Wang, et al. Fig. 3



Wang, et al. Fig. 4



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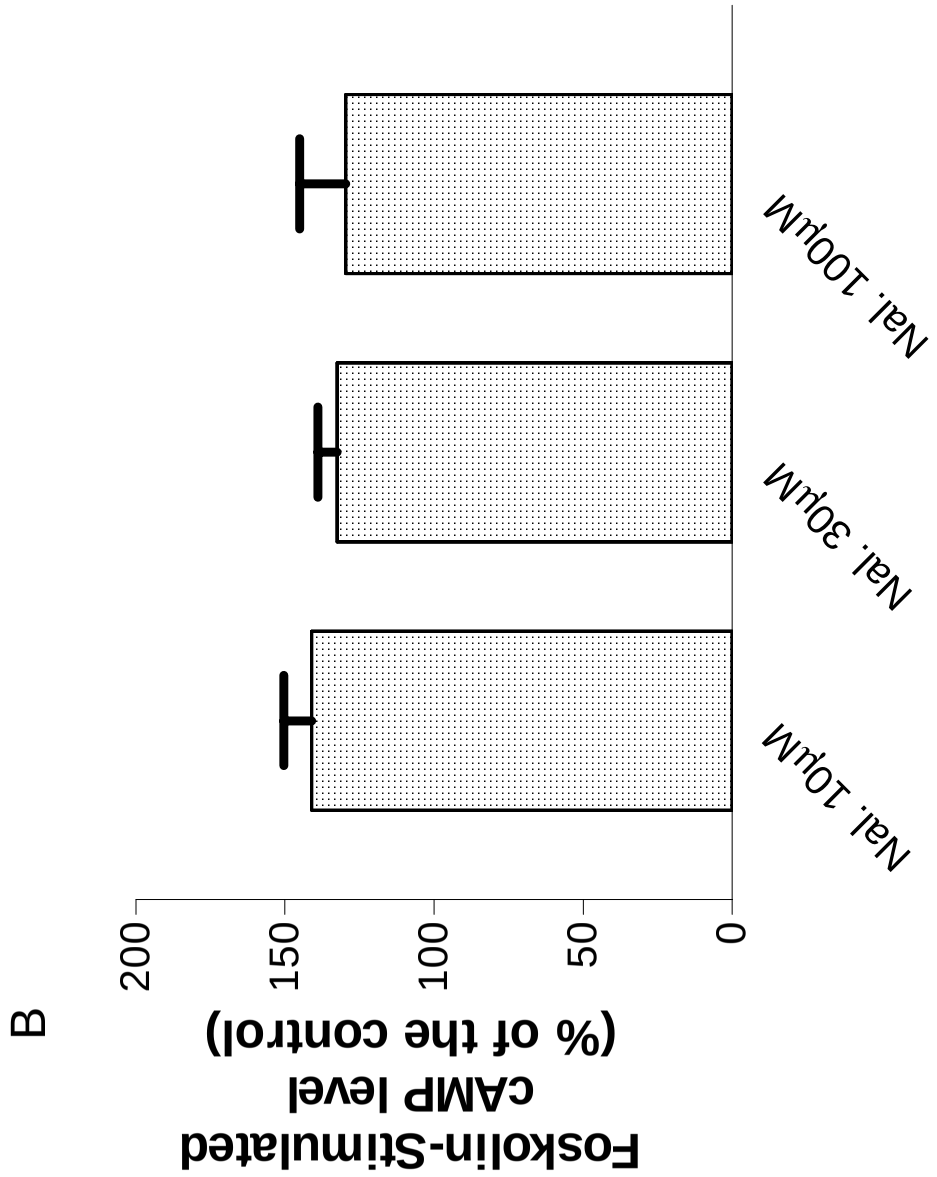
Wang, et al. Fig. 4



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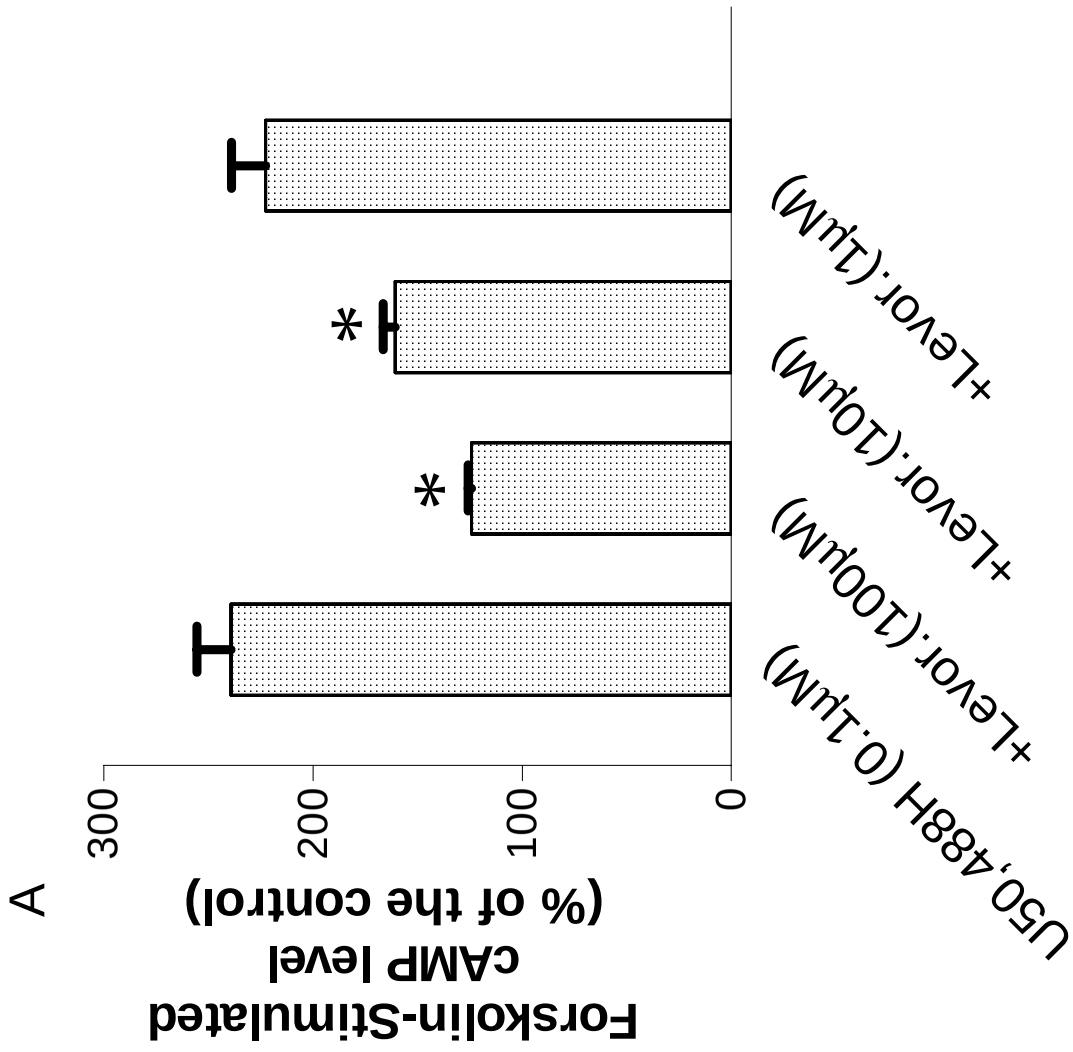
Wang, et al. Fig. 5



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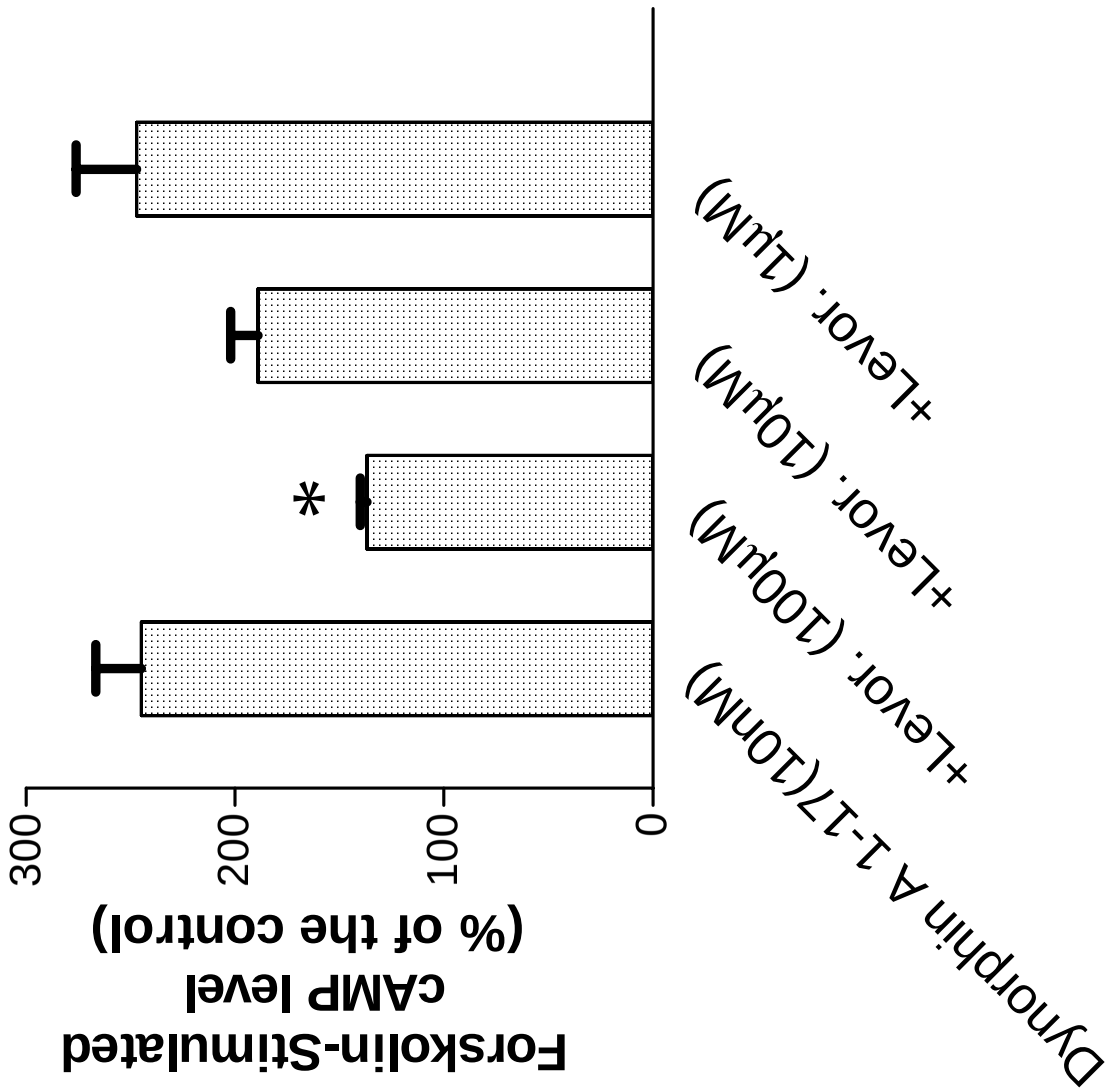
Wang, et al. Fig. 5

B



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Wang, et al. Fig. 6