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Opioid tolerance/dependence in neuroblastoma×glioma (NG108-15) hybrid cells is associated with a reduction in spontaneous stimulatory receptor activity

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Abstract Chronic opioid regulation of stimulatory receptor activity was investigated in neuroblastoma×glioma (NG108-15) hybrid cells stably transfected to express the human β_2-adrenoceptor (β_2-AR). Expressed β_2-ARs are functionally coupled to G proteins and display ligand-independent signalling activity, as demonstrated by the ability of an inverse agonist to attenuate basal adenylyl cyclase (AC) activity. Despite the relative increase in basal AC activity due to the development of tolerance/dependence, chronic morphine treatment was found to completely abolish spontaneous β_2-AR activity by reducing basal receptor/G protein precoupling. A similar chronic opioid effect was observed in transiently transfected COS-7 cells. These results indicate that during the state of opioid tolerance/dependence basal levels of AC activity are no longer under the control of spontaneously active stimulatory receptors.

Key words: β_2-Adrenoceptor; Precoupling; Opioid dependence; Inverse agonist

1. Introduction

Chronic activation of inhibitory opioid receptors (OR) results in the development of tolerance and dependence. The underlying cellular mechanisms are complex and involve adaptations in OR-associated signal transduction pathways [1,2]. Whereas the phenomenon of opioid tolerance is frequently associated with desensitisation of OR activity, e.g. by phosphorylation, internalisation or down-regulation of the binding site [3,4], more down-stream adaptations are implicated in the development of dependence [5]. These regulatory changes develop in response to persistent OR activation and are thought to attenuate or counteract persistent inhibitory signal transduction on post-receptor level [6–8].

One of the best-studied chronic opioid effects on post-receptor level is sensitisation of adenylyl cyclase (AC), which is characterised by a transient increase in cAMP production after drug withdrawal [5,8,9]. Because the AC effector system is under dual control by both stimulatory and inhibitory receptors, we have recently proposed that the increase in AC activity following chronic morphine treatment is due to an increased activity of stimulatory receptor systems [8,10]. Indeed, chronic opioid treatment was found to induce multiple quantitative and qualitative adaptations at the level of stimulatory PGE_1 receptors and guanine nucleotide-binding proteins (G proteins) in both neuroblastoma×glioma (NG108-15) hybrid and neuroblastoma SH-SY5Y cells [8,10,11]. However, these studies also revealed that the increase in AC activity during the state of opioid tolerance/dependence is accompanied by a paradoxical reduction in the maximum binding capacity for [^{3}H]PGE_1 [8,11,12]. Due to the lack of a suitable antagonist radioligand for this receptor species, we were not able to assign this observation to receptor uncoupling or to real down-regulation. Thus, the aim of the present study was to more closely investigate the role of stimulatory receptors in the phenomenon of chronic opioid-induced sensitisation of AC. For this, the human β_2-adrenoceptor (β_2-AR) was stably expressed in NG108-15 cells and the functional properties of this receptor were analysed by a variety of different approaches, including the use of an inverse agonist to evaluate the extent of spontaneous receptor activity [13,14]. The data obtained demonstrate that in stably transfected NG108-15 cells chronic morphine treatment has no effect on overall β_2-AR abundance but substantially reduces the number of spontaneously active, G protein-coupled receptors.

2. Materials and methods

2.1. Materials

[^{125}I]Cyanopindolol (CYP; 2000 Ci/mmol), [5,6(n)-^{3}H]prostaglandin E_1 (PGE_1, 47 Ci/mmol), and [^{125}I]cAMP tracer (2000 Ci/mmol) were from Amersham International. The inverse β_2-AR agonist (±)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methyl-ethyl)-amino]-2-butanol hydrochloride (ICI-118,551), R(−)-isoproterenol (+)-bitartrate, S(−)-propranolol hydrochloride, S(−)-timolol maleate, and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Research Biochemicals International. Anti-cAMP antibody was from BioYeda, goat anti-rabbit peroxidase-conjugated IgG was from Promega. ATP, cAMP, and all other guanine nucleotides were obtained from Calbiochem. Tissue culture reagents and geneticin (G418) were from Gibco BRL Life Technologies. DOTAP transfection reagent was from Boehringer (Mannheim), methotrexate, PGE_1, indomethacin, and all standard laboratory reagents were purchased from Sigma Chemical Company.

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Abbreviations: AC, adenylyl cyclase; β_2-AR, β_2-adrenoceptor; CYP, cyanopindolol; G protein, guanine nucleotide-binding protein; G_s, stimulatory G protein; $G_s\alpha$, α-subunit of G_s; ICI-118,551, (±)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methyl-ethyl)amino]-2-butanol hydrochloride; OR, opioid receptor

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2.2. Methods

2.2.1. Cell culture, transfection, and chronic opioid treatment. Neuroblastoma×glioma (NG108-15) cells carrying endogenous δ-ORs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) foetal bovine serum, 100 μM hypoxanthine, 1 μM aminopterin, 16 μM thymidine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2. A stably β_2-AR expressing cell clone (NGβ20) was generated by co-transfection of the cells with plasmids pBC-dhfr-β_2AR and pSV2-neo using DOTAP transfection reagent. After selection with neomycin (G418; 800 μg/ml), β_2-AR levels were amplified by successive exposure to increasing concentrations of methotrexate (0.01, 0.03, and 0.1 μM) for 14 days each. For experimentation, subconfluent monolayers were rendered opioid tolerant/dependent by chronic exposure to morphine (10 μM) for 3 days [8]. In each case, cells of an identical passage, which were kept in the absence of the opioid, served as controls.

COS-7 cells were grown as described [15]. Cells (10-cm petri dish) were transiently co-transfected with 3 μg each of cDNA's coding for the rat μ-OR, the human β_2-AR, and the rabbit AC type V, all subcloned into plasmid pcDNA3.1 (Invitrogen). The day after transfection, cells were trypsinised, distributed into two 24-well plates, and cultured for another 2 days either in the absence of presence of 1 μM morphine to induce tolerance/dependence.

2.2.2. Radioligand binding assays. These were performed on particulate membrane preparations obtained as described [12]. The final membrane pellets were resuspended in the appropriate assay buffers (see below) and immediately used for experimentation. In case of chronically morphine pre-treated cells, all binding assays were conducted in the presence of 10 μM morphine in order to maintain tolerance/dependence.

The total β_2-AR number was determined from saturation binding experiments using [^{125}I]CYP (0.04–2 nM) as the radioligand [14]. Heterologous binding experiments were performed with [^{125}I]CYP as the tracer (0.08 nM) and either the β_2-AR agonist isoproterenol, the competitive antagonist propranolol or the inverse β_2-AR agonist ICI-118,551 as competitors. Binding reactions (200 μl volume) contained 10–30 μg of membrane protein in 20 mM Tris–HCl buffer, pH 7.4, supplemented with 5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM ascorbic acid, and 0.1 mg/ml BSA. Non-specific binding was defined in the presence of 10 μM propranolol. Binding reactions were conducted to equilibrium (90 min; 25°C) before they were terminated by rapid filtration through Whatman GF/B filters. Saturation isotherms were analysed by the method of Scatchard, yielding estimates for maximum binding capacity (B_{max}) and ligand affinity (K_d). Competition binding data were subjected to non-linear least-squares regression analysis using a two-state model [14]. K_i values were calculated from IC_{50} values according to the equation of Cheng and Prusoff [16].

PGE_1 receptors were measured using a single [^{3}H]PGE_1 tracer (8 nM) concentration in the presence of 10 mM Tris–HCl buffer (pH 7.4), containing 5 mM $MgCl_2$, 100 mM NaCl, 100 μM indomethacin, and 10 mM benzamidine [12]. Binding reactions (200 μl volume) contained 300 μg of membrane protein and were conducted for 90 min at 25°C. Non-specific binding was assessed in the presence of 10 μM PGE_1. The fraction of functionally G protein-coupled PGE_1 receptors was determined in the presence of the stable guanine-nucleotide GTPγS (100 μM). The total number of G protein-coupled receptors was calculated according to the method of DeBlasi et al. [17] using a K_d value of 2.9 nM for the high-affinity binding site [12].

2.2.3. AC assays. Enzymatic activity of membrane-bound AC was determined as described [10]. The assay system (100 μl total volume) contained 40 mM Tris–HCl, pH 7.4, 0.2 mM EGTA, 0.2 mM DTT, 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM ATP, 5 μg/ml phosphocreatine, 5 IU/ml creatine phosphokinase, 10 μM GTP, 100 μM IBMX and various ligands as indicated in the text and figure legends. Reactions were started by the addition of 10 μg of membrane protein, kept for 10 min at 32°C, and were stopped with 500 μl of ice-cold 10 mM HCl.

Accumulation of cAMP in intact COS-7 cells was determined as follows: cells grown in 24-well plates were washed three times with 1 ml/well of prewarmed sodium bicarbonate-free DMEM containing 20 mM HEPES (DMEH; pH 7.4), before reactions were started by the addition of 0.25 ml/well of DMEH, supplemented with 1 mM IBMX, 0.01% BSA, and the appropriate receptor ligands as indicated. Chronically morphine (1 μM; 2 days) pre-treated cells were assayed in the continued presence of morphine. Accumulation of cAMP was allowed for 15 min at 37°C and was terminated by the addition of 0.75 ml of ice-cold 50 mM HCl. The amount of cAMP generated was determined in the supernatant fraction by radioimmunoassay [10].

2.2.4. Electrophoresis and Western blotting. Membrane proteins (10 μg/lane) prepared from NG108-15 wild-type and clonal NGβ20 cells were solubilised in Laemmli loading buffer [18], resolved by SDS-PAGE (10%; w/v), and transferred onto PVDF membranes (Immobilon P; Millipore). The blots were probed with subtype-specific anti-G protein antibodies (anti-stimulatory G protein α-subunit ($G_s\alpha$); anti-Gβ) as primary reagent [8] followed by incubation with a peroxidase-conjugated goat anti-rabbit secondary antibody. Immunoreactivity was visualised using enhanced chemiluminescence (Amersham) and quantitated by video densitometry (Herolab E.A.S.Y. RH-3 system).

3. Results and discussion

To investigate the role of stimulatory receptors in chronic opioid-induced sensitisation of AC, the human β_2-AR was stably expressed in NG108-15 cells. Scatchard plot analysis of [^{125}I]CYP saturation binding data (Fig. 1A) revealed that

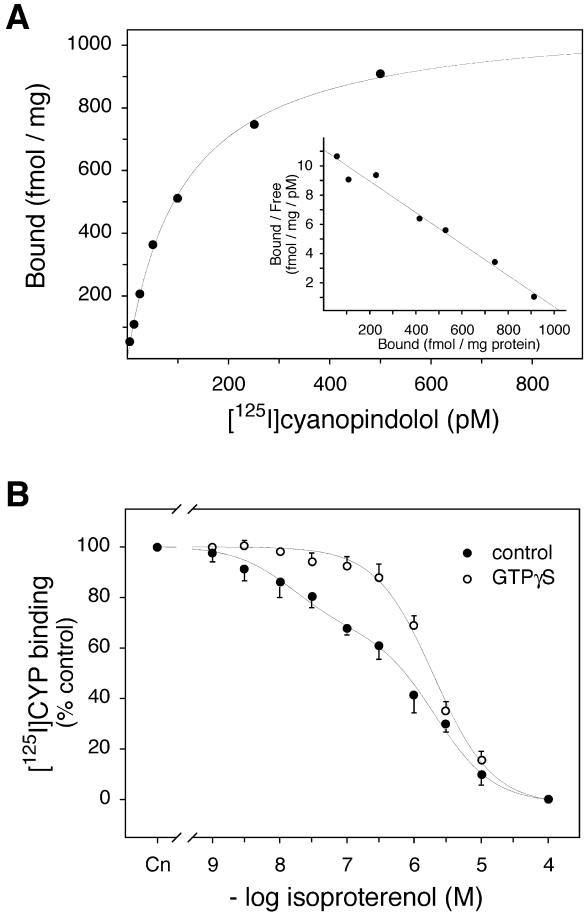

Fig. 1. Functional expression of the human β_2-AR in neuroblastoma×glioma (NG108-15) hybrid cells. A: Saturation binding of [^{125}I]CYP to membranes from clonal NGβ20 cells. The data shown are representative for three similar experiments, done in triplicate. Inset: Scatchard transformation of the data yielded estimates for B_{max} (1.032 ± 62 fmol/mg membrane protein) and K_d (87.6 ± 5 pM) (mean values ± S.D.; $n = 3$). B: Heterologous displacement of [^{125}I]CYP tracer binding with increasing concentrations of isoproterenol. Membranes were assayed either in the absence or presence of the stable guanine nucleotide GTPγS (100 μM). About 35.8 ± 9% of the receptors show high-affinity agonist binding with a K_{iH} value of 3.6 ± 1.2 nM (mean value ± S.D. of $n = 5$ independent experiments) according to a two-state model.

the cell clone used in this study (NGβ20) carries high levels of the β_2-ARs ($B_{max} = 1.032 \pm 62$ fmol/mg membrane protein; $K_d = 87.6 \pm 5$ pM; mean values ± S.D.; $n = 3$). Heterologous displacement of [^{125}I]CYP binding by the β_2-AR agonist isoproterenol produced a biphasic competition curve with a proportion of 35.8 ± 9% of high-affinity receptors ($K_{iH} = 3.6 \pm 1.2$ nM; mean values ± S.D.; $n = 5$). Uncoupling of the receptors from G_s in the presence of the stable guanine-nucleotide GTPγS (100 μM) resulted in a complete loss of high-affinity agonist binding, yielding a monophasic competition curve with a K_{iL} value of 1.1 ± 0.3 μM (mean ± S.D.; $n = 3$) for the low-affinity receptor state (Fig. 1B). These results demonstrate that expressed β_2-ARs are functionally coupled to G_s.

According to current receptor theory, G protein-coupled receptors exist in equilibrium between two conformations, an inactive (R) and an active (R*) receptor state, that can isomerise spontaneously in a native membrane environment [13,14]. The active receptor state is able to productively interact with its corresponding G proteins, initiating subsequent signal transduction processes. As the absolute number of spontaneously active receptor increases with higher receptor levels [19], overexpression of β_2-ARs in NGβ20 cells was found to significantly increase basal AC activity compared to parental NG108-15 hybrid cells (50.1 ± 1.9 vs. 34.8 ± 2.7 pmol cAMP/mg/min; mean values ± S.E.M. of $n = 12$ and three independent experiments, respectively; $P < 0.001$), confirming previous data [20].

Ligands acting on G protein-coupled receptors may be classified either as agonist, neutral antagonist or inverse agonist, depending on their effect on the equilibrium between both receptor states [21]. Receptor agonists stimulate signal transduction processes by increasing the relative proportion of the activated receptor state due to their higher affinity for this receptor conformation. Neutral antagonists have no effect on the equilibrium between both receptor states and act solely in a competitive manner. In contrast, negative antagonists (inverse agonists) stabilise the inactive receptor state and, thus, are able to reduce the number or spontaneously active receptors [13,21,22]. Therefore, we tested whether the increase in basal AC activity due to β_2-AR overexpression may be reversed by an inverse agonist. As shown in Fig. 2A, inactivation of β_2-ARs by an inverse agonist (ICI-118,551, timolol; 1 μM each) significantly inhibited basal AC activity by some 30%, reaching values comparable to those observed for NG108-15 wild-type cells. Propranolol (1 μM) behaved as a neutral antagonist in this cell system and blocked the inverse agonist effect. Acute activation of inhibitory δ-ORs by morphine (10 μM) attenuated basal AC activity to the same extent as observed for inverse β_2-AR agonists. Concomitant inactivation of stimulatory β_2-ARs as well as activation of inhibitory δ-ORs failed to cooperatively inhibit AC activity. The effects of inverse β_2-AR agonists as well as δ-OR agonists on basal AC activity emphasise the mechanism by which stimulatory and inhibitory G_α subunits regulate enzymatic AC activity. Whereas activated $G_s\alpha$ is the principal stimulator of enzymatic activity, inhibitory G_α subunits represent secondary regulators of AC that are only able to inhibit the stimulated but not the unstimulated enzyme [23]. Thus, termination of spontaneous $G_s\alpha$-mediated stimulation of AC activity by an inverse β_2-AR agonist might explain the failure of concomitant activation of inhibitory δ-ORs to further attenuate basal AC activity.

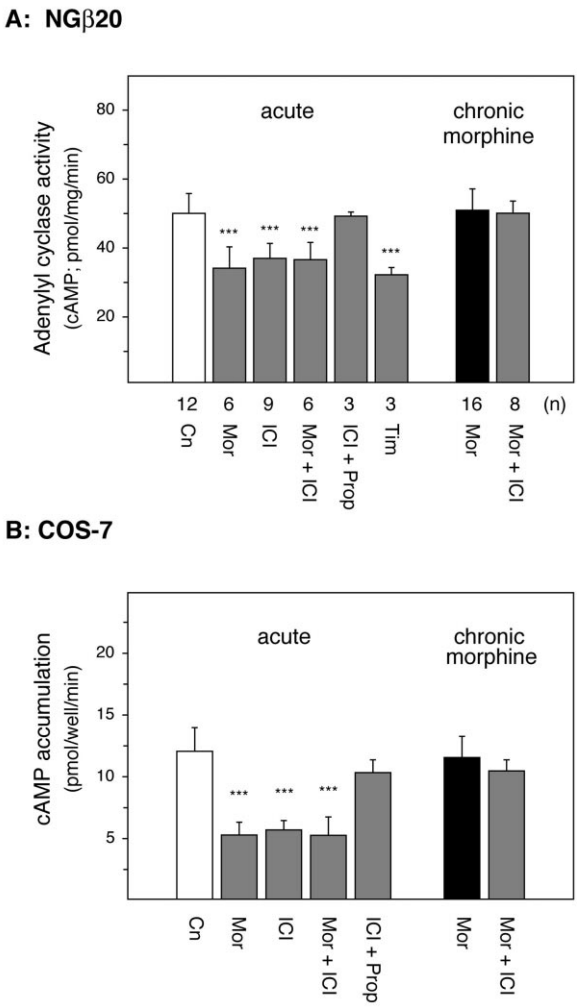

Fig. 2. Effect of inverse β_2-AR agonists on basal AC activity. A: Membranes from naive or chronically morphine (10 μM; 3 days) pre-treated NGβ20 cells were prepared and AC activity was measured in the presence of the following ligands: morphine (Mor; 10 μM), ICI-118,551 (ICI; 1 μM), propranolol (Prop; 1 μM), and timolol (Tim; 1 μM). Membranes from opioid tolerant/dependent cells were assayed in the presence of morphine (10 μM). AC activity is expressed as the amount of cAMP formed per min per mg of membrane protein. The data are mean values ± S.E.M. of the number of experiments indicated. B: COS-7 cells were transiently transfected with cDNA's encoding the β_2-AR, μ-OR, and AC type V, and regulation of basal cAMP accumulation in naive and chronically morphine pre-treated cells was analysed as above. The data shown are mean values ± variation of a representative experiment performed in quadruplicate determination. Two additional experiments yielded qualitatively similar results. ***Significantly different, $P < 0.001$.

The most important finding of the present paper, however, was that chronic morphine treatment (10 μM; 3 days) completely abolished the inhibitory effect of ICI-118,551 on basal AC activity (Fig. 2A). Similar results were obtained in transiently β_2-AR-transfected COS-7 cells co-expressing the rat μ-OR and the rabbit AC type V, indicating that the failure of inverse β_2-AR agonists to attenuate basal AC activity during the state of opioid tolerance/dependence is not restricted to clonal NGβ20 cells but rather represents a more general chronic opioid effect (Fig. 2B).

There are two possibilities for how chronic morphine treatment could block attenuation of basal AC activity by ICI-

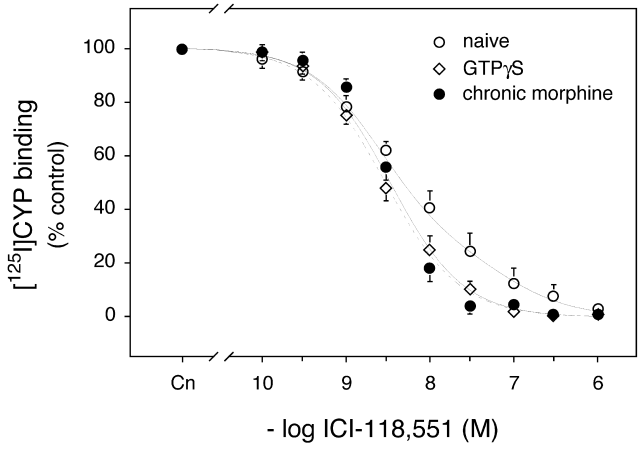

Fig. 3. Chronic morphine treatment alters inverse β_2-AR agonist binding properties. Membranes from naive and chronically morphine (10 μM; 3 days) pre-treated NGβ20 cells were subjected to heterologous binding experiments using [^{125}I]CYP (0.08 nM) as the tracer and increasing concentrations of the inverse β_2-AR agonist ICI-118,551 as competitor. In membranes from naive cells, ICI-118,551 recognised two binding sites with the lower affinity state being sensitive to the stable guanine nucleotide GTPγS. Chronic morphine pre-treatment completely abolished the low-affinity binding site for ICI-118,551, leaving a single site with high-affinity. Data are mean values ± S.D. of four (naive, chronic morphine) and three (GTPγS) separate experiments.

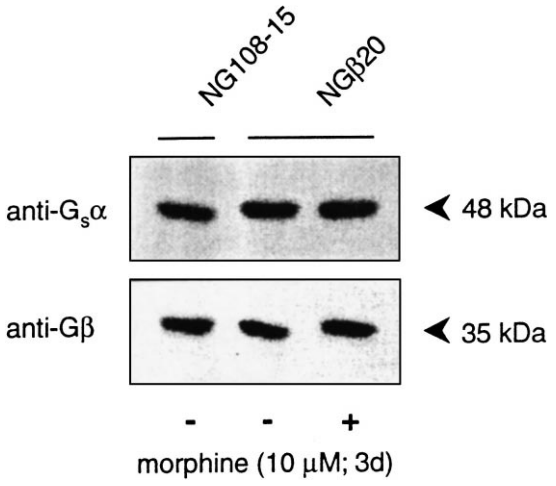

Fig. 4. Effect of β_2-AR expression and chronic morphine treatment on G_s levels. Membrane proteins (10 μg) from NG108-15 wild-type and stably β_2-AR transfected NGβ20 cells were resolved by 10% (w/v) SDS-PAGE and immunoblotted for $G_s\alpha$ and Gβ subunits as described in Section 2. In NG108-15 cells, the C-terminal anti-$G_s\alpha$ antibody recognises a major form of $G_s\alpha$ migrating with a molecular mass of ~48 kDa, whereas the anti-Gβ antibody labels a band at ~35 kDa. Neither stable expression of the β_2-AR nor chronic morphine treatment (10 μM; 3 days) affects G protein abundance.

118,551. First, there is still persistent inhibition of AC via inhibitory G_α subunits associated with the development of tolerance which might obliterate the inverse β_2-AR effect [7] and, second, chronic morphine treatment could reduce the number of spontaneously active stimulatory receptors [20]. Because investigation of inverse β_2-AR activity in membranes from opioid tolerant/dependent cells is excluded in the absence of morphine due to the expression of AC supersensitivity [15], we tried to attack this issue by investigating whether chronic morphine treatment could possibly attenuate spontaneous β_2-AR activity. For this, the property of inverse agonists to bind with high affinity to the inactive receptor conformation was used to evaluate the fraction spontaneously active receptors. In membranes of naive NGβ20 cells, heterologous [^{125}I]CYP binding experiments using the inverse β_2-AR agonist ICI-118,551 as the competitor produced a biphasic displacement curve with about 21.2 ± 3% of the receptors showing low affinity for the competitor (K_{iL} = 32.8 ± 18 nM; mean ± S.D.; n = 4) (Fig. 3). Inactivation of spontaneous receptor activity by receptor uncoupling (GTPγS; 100 μM) yielded a monophasic displacement curve with high affinity for the inverse agonist (K_i = 1.1 ± 0.2 nM; mean ± S.D.; n = 3). Chronic morphine treatment mimicked the GTPγS effect (K_i for ICI-118,551 = 1.2 ± 0.3 nM; mean ± S.D.; n = 4), indicating that spontaneous β_2-AR activity is reduced in membranes from opioid tolerant/dependent NGβ20 cells. Addition of morphine to membranes from naive cells had no effect on ICI-118,551 binding parameters (22.9 ± 7% low affinity receptors; K_{iL} = 43.4 ± 21 nM; mean ± S.D.; n = 2). These results confirm that inverse agonists indeed are able to distinguish between active and inactive receptor forms, and that they display reciprocal binding characteristics compared to agonists [24,25]. Moreover, they also suggest that chronic morphine treatment reduces the number of spontaneously active β_2-ARs as observed after receptor uncoupling. Comparable results were obtained when heterologous [^{125}I]CYP binding experiments were performed using the agonist isoproterenol as a competitor (Table 1).

There are several possibilities by which chronic morphine treatment could down-regulate spontaneous β_2-AR activity. In this respect, changes in receptor and G protein abundance have been reported to affect the equilibrium between the active and the inactive receptor conformation [24–26]. Scatchard

Table 1
Regulation of [^{125}I]CYP binding by (−)-isoproterenol in NGβ20 cells

Pre-treatment	Addition	(−)-Isoproterenol binding parameters				
		K_H (nM)	K_L (μM)	R_H (%)	R_L (%)	n
None	–	3.6 ± 1.2	1.3 ± 0.3	32.1 ± 8	67.7 ± 6	5
	GTPγS	NA	1.1 ± 0.3	NA	99 ± 1	3
Morphine	–	3.8 ± 1.6	1.4 ± 0.4	16.7 ± 5[a]	83.0 ± 5[a]	4
	GTPγS	NA	1.2 ± 0.2	NA	98 ± 2	3

Affinity states for isoproterenol were obtained from heterologous binding experiments using [^{125}I]CYP as the tracer and 12 different concentrations of the competitor. K_i values were calculated from IC_{50} values according to the method of Cheng and Prusoff [17]. K_H and K_L designate K_i values of the high (H) and low (L) affinity states. R_H and R_L designate proportion of receptor in high- and low-affinity states. Data are expressed as means ± S.D. Numbers in parentheses indicate percentage reduction of high-affinity β_2-ARs by activation of PGE_1 receptors. NA, not applicable (one-site binding).
[a]Significantly different to controls, $P < 0.001$.

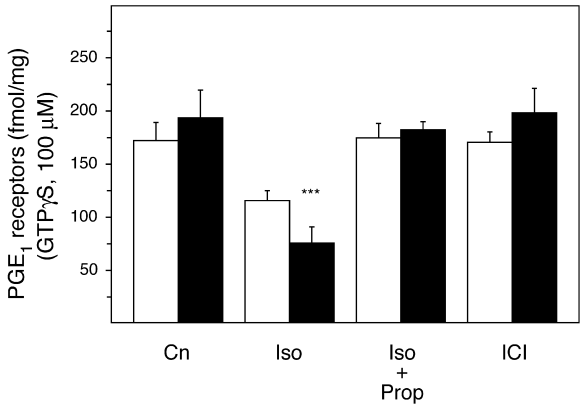

Fig. 5. Chronic morphine treatment limits the functional availability of G_s. Regulation of GTPγS-sensitive PGE_1 receptors in the presence of various ligands for the β_2-AR was employed to determine the functional interaction between both receptor types at the level of G_s. Acute activation of the β_2-AR by isoproterenol (Iso; 10 μM) resulted in a loss of G protein-coupled PGE_1 receptors. Uncoupling of PGE_1 receptors by isoproterenol is significantly greater in membranes from chronically morphine pre-treated (■) than from naive (□) NGβ20 cells and is antagonised by propranolol (Prop; 10 μM). Inactivation of β_2-ARs by ICI-118,551 (ICI; 10 μM) had no opposite effect. Data are mean values ± S.D. of seven independent experiments each performed in duplicate. ***Significantly different between naive and chronically morphine treated membranes, $P < 0.001$.

plot analysis of [^{125}I]CYP saturation binding data failed to detect quantitative changes for β_2-ARs following chronic morphine treatment ($B_{max} = 1.032 \pm 62$ versus 989 ± 74 fmol/mg membrane protein for naive and chronically morphine-treated cells, respectively; mean values ± S.D.; $n = 3$). Likewise, the amount of $G_s\alpha$ and Gβ subunits remained unchanged in membranes from opioid tolerant/dependent cells. As shown in Fig. 4, wild-type NG108-15 cells contain a prominent $G_s\alpha$ isoform with an apparent molecular weight of ~48 kDa. Overexpression of β_2-ARs in NGβ20 cells as well as chronic morphine treatment had no effect on relative $G_s\alpha$ and Gβ concentrations. Thus, the loss of spontaneously active β_2-ARs following chronic morphine treatment is not due to quantitative changes at the level of β_2-ARs and G_s.

One of the determinants that stabilise the activated receptor conformation in a native membrane environment is the extent of basal receptor/G protein precoupling [13,25]. Thus, uncoupling of β_2-ARs from their associated G_s proteins, as indicated by our radioligand binding studies, might provide a plausible mechanism for the loss of spontaneous β_2-AR activity. However, there are several objections that argue against such a mechanism. Most importantly, disruption of receptor/G protein coupling, e.g. by agonist-mediated phosphorylation, is usually accompanied by desensitisation of receptor function [26]. However, stimulatory PGE_1 receptors in chronically morphine treated NG108-15 wild-type cells remained fully functional following agonist activation [8]. Moreover, agonist-induced desensitisation of β_2-ARs in Sf9 cells has been reported to even increase the potency of inverse agonists to attenuate basal AC activity [27]. Thus, the loss of spontaneously active β_2-ARs as observed in chronically morphine-treated NGβ20 cells appears to be mediated by a regulatory mechanism other than involved in receptor desensitisation.

Recent studies indicated that signal transduction components are localised within highly organised membrane compartments that bring in specificity in transmembrane signalling [28,29]. This is best illustrated with stimulatory receptors that display high specificity in AC stimulation, although they all utilise the same G protein species for transduction [30]. Given the fact that in NG108-15 cells the amount of G_s is in far excess over the number of stimulatory receptors [20], there could be some factors that control the assignment of a particular receptor to its own G protein pool within the plasma membrane [20,30]. To test whether chronic morphine treatment possibly interferes with such a mechanism, the interaction of two stimulatory receptors, i.e. PGE_1 and β_2-ARs, at the level of G_s was examined by evaluating regulation of high-affinity [^{3}H]PGE_1 binding following activation of β_2-ARs. Increasing concentrations of isoproterenol (1 μM) dose-dependently ($IC_{50} = 6.5 \pm 4$ nM) decreased high-affinity [^{3}H]PGE_1 binding to membranes from naive NGβ20 cells to a maximal extent of $33.8 \pm 6\%$ (mean values ± S.D.; $n = 5$), and this effect was blocked by propranolol. The inverse β_2-AR agonist ICI-118,551 had no reciprocal effect, indicating that inactivation of spontaneously active β_2-ARs does not increase the availability of PGE_1 receptors for G_s. Chronic morphine treatment failed to affect the overall amount of GTPγS-sensitive PGE_1 receptors in this cell clone (172.2 ± 28 vs. 193.6 ± 37 fmol/mg membrane protein for control and chronically morphine treated cells, respectively; mean values ± S.D.; $n = 7$). However, it significantly increased the maximum capacity of isoproterenol to down-regulate high-affinity PGE_1 receptors by roughly 80% (Fig. 5). These results indicate that chronic morphine treatment somehow increases the functional interaction between both receptor species at the level of G_s. One possible explanation for this finding could be that the pool of G_s assigned to each receptor species becomes limited during the state of tolerance/dependence. As in NG108-15 wild-type cells the number of G_s is in far excess over the number of receptors [31], limitation in the functional access of stimulatory receptors for G_s might explain the loss of spontaneous β_2-AR activity without affecting parameters of receptor-stimulated AC activity in membranes from chronically morphine-treated NGβ20 cells.

In conclusion, the present study demonstrates that in stably transfected NG108-15 cells chronic morphine treatment reduces the number of spontaneously active β_2-ARs. Thus, the relative increase in basal AC activity due to the development of opioid tolerance/dependence appears to originate from post-receptor mechanisms that directly up-regulate effector activity rather than from an increased stimulatory activity. As stimulatory receptors remain fully functional following chronic morphine treatment, such a direct increase in AC activity might explain the phenomenon of an increased sensitivity towards excitatory receptor ligands as observed during the state of opioid tolerance/dependence.

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