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Activation by β-carbolines of G-proteins in HL-60 Membranes and the Bovine Retinal G-protein Transducin in a Receptor-independent Manner

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ABSTRACT. Naturally occurring β-carbolines are lipophilic compounds which show psychotropic and physiological effects in mammals. They bind to distinct high-affinity binding sites in various mammalian tissues. However, the mechanism by which the β-carbolines affect transmembrane signal transduction processes is still unknown. Since β-carbolines are cationic-amphiphilic substances and since such substances are known to activate heterotrimeric regulatory guanine nucleotide binding proteins (G-proteins) in a receptor-independent manner, we put forward the hypothesis that β-carbolines act directly on G-proteins. Therefore, we investigated the ability of β-carbolines to stimulate high-affinity GTP hydrolysis in membranes of dibutyryl-cAMP differentiated HL-60 cells and of the purified bovine G-protein, transducin (TD). The β-carbolines norharman and harman, stimulated the GTPase in HL-60 membranes with an EC_{50} of 410 μM and 450 μM, respectively, and a maximum effect at 1 mM each. Norharman and harman stimulated the GTPase of TD with an EC_{50} of 60 μM and 300 μM, and a maximum at 1 mM for both compounds. The stimulatory effect of norharman in HL-60 membranes was pertussis toxin-sensitive. Structure/activity characteristics of the β-carbolines showed a specifity of norharman to stimulate the GTPase of TD, because norharman activated GTP hydrolysis in HL-60 membranes approximately 7 times less potently than that of TD. Norharman was a five-fold more potent activator of TD than tetrahydronorharman. Hydroxylation of the β-carboline molecule in position 6 led to a loss of GTPase-activating properties. Our data suggest that naturally occurring β-carbolines are a novel class of receptor-independent G-protein activating substances. This mechanism could contribute to their diverse biological effects. BIOCHEM PHARMACOL **53**;11:1621–1626, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. β-carbolines; G-protein; GTPase; HL-60 cells; pertussis toxin; transducin

Naturally occurring β-carbolines are cationic-amphiphilic substances consisting of a heterotricyclic ring system [1] which are present in various mammalian tissues [2]. The plasma level of the β-carboline, norharman, is elevated in chronic alcoholics, suggesting a role in alcohol addiction [3]. β-carbolines exert psychotropic effects in humans [4]. They bind with high affinity to distinct binding sites in rat brain membranes [5], the adrenal medulla [6] and other peripheral tissues, e.g. in the rat liver [7]. Moreover, natural β-carbolines bind to receptors for 5-hydroxytryptamine, dopamine and benzodiazepines with an affinity in the micromolar range [8–11]. In the latter case, β-carbolines act as inverse agonists [12]. However, the molecular mechanism by which natural β-carbolines affect signal transduction remains to be elucidated.

Several cationic-amphiphilic substances [13–23] are known to stimulate heterotrimeric regulatory guanine nucleotide binding proteins (G-proteins)* in a receptor-independent manner. As β-carbolines also have a cationic-amphiphilic character, we wondered whether β-carbolines were also able to stimulate G-proteins in a receptor-independent manner. Because there is no detectable high-affinity binding site for β-carbolines in HL-60 cells* and because HL-60 cells are an established model system for the study of receptor-independent G-protein activation [13, 14, 18], we characterized the effects of β-carbolines on high-affinity GTP hydrolysis [EC 3.6.1.-] in HL-60 membranes. To further relate the effects of β-carbolines on GTPase to receptor-independent G-protein activation, we also assessed the direct activation of the purified bovine retinal G-protein, transducin (TD). TD is directly activated by various cationic-amphiphilic substances and has the advantage that reconstitution into membranes is not required for measurement of GTPase [17, 23].

Here we demonstrate that naturally occurring β-carbo-

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Abbreviations: G-proteins, heterotrimeric regulatory guanine nucleotide binding proteins; PTX, pertussis toxin; TD, the bovine retinal G-protein, transducin.

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lines are a novel class of substances which activate pertussis toxin (PTX)-sensitive G-proteins in HL-60 membranes and TD. Moreover, the effects depend on characteristics of the chemical structure of β-carbolines.

MATERIALS AND METHODS

Materials

Mastoparan was from Saxon Biochemicals (Hannover, Germany). Harman, harmin, harmalin, harmol, harmalol, norharman, PTX, tryptamine and 5-hydroxytryptamine were from Sigma Chemie (Deisenhofen, Germany). Tetrahydronorharman, 6-hydroxy-tetrahydronorharman and 6-hydroxynorharman were synthesized according to Vejdelek et al. [24]. Stock solutions of the compounds (10 mM each) were prepared in distilled water and in the case of 6-hydroxynorharman (10 mM), harman and norharman (100 mM each) in DMSO (100%, v/v) and were than stored at −20°C. Mastoparan (1 mM) was prepared in 1 mM sodium acetate, pH 5.0, and stored at −20°C. Carrier-free [^{32}P]P_i was obtained from Dupont/New England Nuclear (Bad Homburg, Germany). [γ-^{32}P]GTP synthesis was performed according to the protocol described by Walseth et al. [25]. All other chemicals were of highest purity and purchased from standard commercial sources.

Cell Culture and Membrane Preparation

HL-60 cells were grown in suspension culture at 37°C and were differentiated towards neutrophil-like cells with dibutyryl-cAMP (0.2 mM) for 48 hr [26]. To determine high-affinity GTPase-activity, HL-60 membranes were prepared as follows. Cells were centrifuged for 20 min at 1600 × g and 4°C. The pellets were suspended in a buffer containing 140 mM NaCl and 10 mM triethanolamine/HCl, pH 7.4, and centrifuged for 20 min at 1600 × g and 4°C. Then cells were suspended in a buffer containing 100 mM NaCl, 0.5 mM EDTA and 50 mM KH_2PO_4, pH 7.0, at a concentration of 1.0–10.0 × 10^7 cells/mL and disintegrated by 25 bar nitrogen cavitation for 30 min at 4°C. EDTA (2.5 mM) and β-mercaptoethanol (12.5 mM) were added to the broken cells. The nuclei were removed by centrifugation of the suspension for 3 min at 1000 × g and 4°C. The supernatant was centrifuged for 15 min at 30000 × g and 4°C. The resulting pellet fraction was resuspended in 10 mM triethanolamine/HCl, pH 7.4, and is subsequently denoted "membranes". The membrane fraction was stored at −80°C.

PTX (100 ng/mL) or its carrier (control) were added to cell cultures 24 hr before membrane preparation. Under these conditions, more than 95% of G_i-protein α-subunits are ADP-ribosylated [26].

TD Purification

All procedures were performed under standard laboratory light. Bovine rod outer segment disk membranes from approximately 160 eyes were prepared according to Papermaster and Dreyer [27]. TD was eluted from membranes with 100 μM GTP as described [28]. The TD-containing supernatant fluid was removed and concentrated in an Amicon concentration chamber (PM 10 membrane, Amicon, Witten, FRG). Thereafter, concentrated TD was diluted with GTP-free hypotonic buffer containing 0.1 mM EDTA, 1 mM dithiothreitol and 10 mM Tris/HCl, pH 7.5. This concentration/dilution procedure was repeated four times to remove free GTP and to generate GDP-liganded TD-α-subunits. The purity of TD was >98% as assessed by SDS-PAGE and silver staining. TD (50–60 μM) was stored at −80°C.

GTPase Assay

GTP hydrolysis was determined as described [14]. Reaction mixtures (100 μl) contained washed membranes from dibutyryl-cAMP-differentiated HL-60 cells (2.9 μg–11.5 μg of protein per tube), 0.5 μM [γ-^{32}P]GTP (0.1 μCi/tube), 0.5 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM ATP, 1 mM adenosine 5′-(βγ-imido)triphosphate, 5 mM phosphocreatine, 40 μg of creatine kinase, 1 mM dithiothreitol and 0.2% (w/v) bovine serum albumin in 50 mM triethanolamine/HCl, pH 7.4. After 3 min of preincubation at 25°C, reactions were initiated by addition of [γ-^{32}P]GTP and conducted for 15–25 min. Low-affinity GTPase activity was determined in the presence of 50 μM GTP and was <5% of total GTPase activity. Under the conditions employed, GTP hydrolysis was linear with respect to protein concentration and time.

GTP hydrolysis of transducin was performed as described above for HL-60 membranes except that the reaction mixtures contained 250 nM of TD, 0.1 μM [γ-^{32}P]GTP (0.1 μCi/tube), 0.5 mM $MgCl_2$, 0.1 mM EGTA, 1 mM dithiothreitol and 0.2% (w/v) bovine serum albumin in 50 mM triethanolamine/HCl, pH 7.4.

Miscellaneous

Protein was determined according to Peterson [29].

RESULTS

Fig. 1A shows concentration-effect curves for norharman, harman and mastoparan on high-affinity GTPase in HL-60 membranes. Norharman stimulated GTPase with an EC_{50} of 410 μM and reached a maximum at 1 mM. The corresponding values for harman were 450 μM and 1 mM, respectively. The concentration-effect curve for mastoparan was biphasic (EC_{50} of *ca.* 2 μM; maximum at 10 μM). The three compounds were about similarly effective GTPase activators. Typical Lineweaver–Burk plots of basal- and norharman- (1 mM) stimulated high-affinity GTP hydrolysis at various GTP concentrations between 0.1–10.0 μM showed that norharman increased V_{max} without affecting the K_m of the high-affinity GTPase (0.25 μM) (data

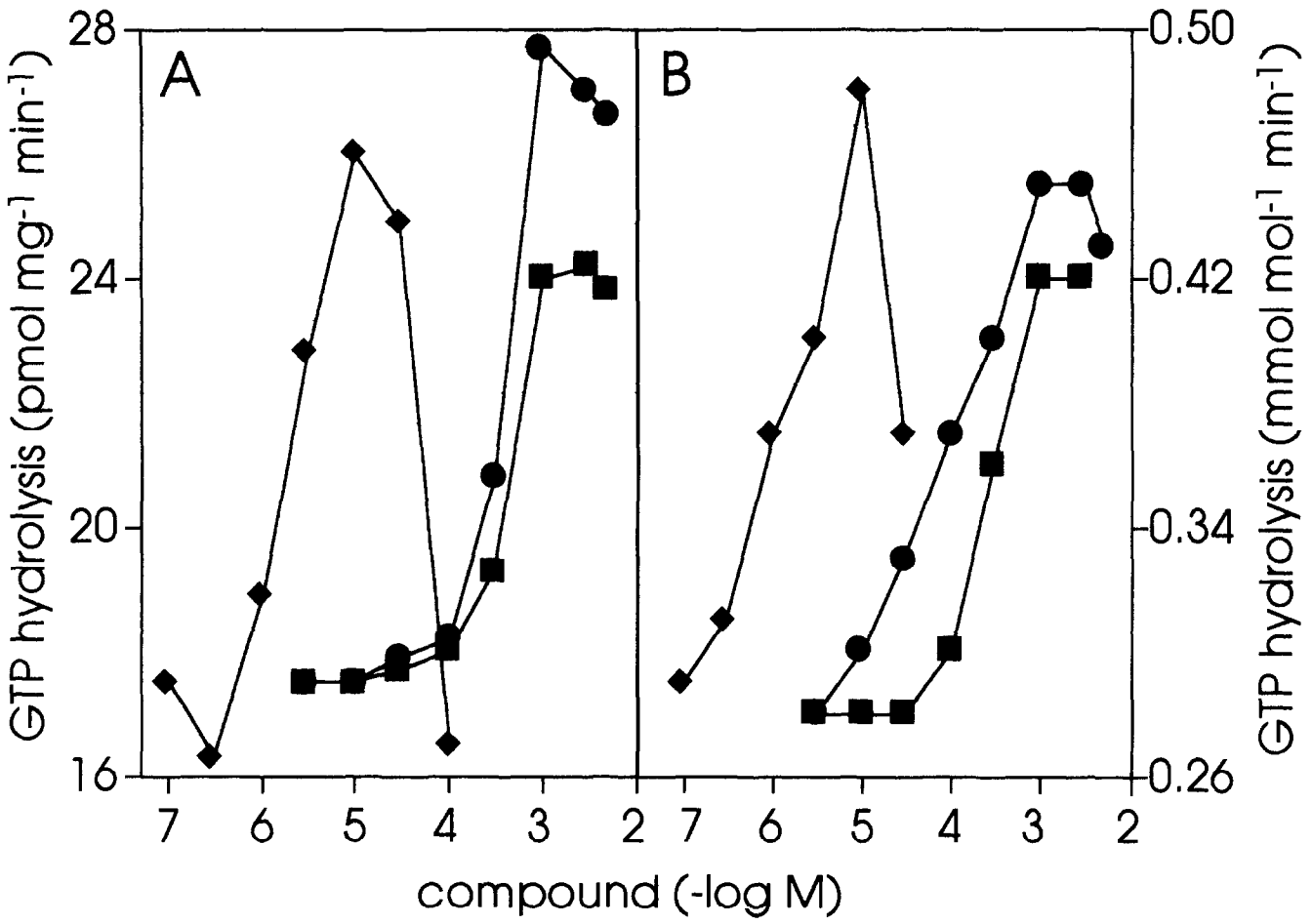


FIG. 1. Concentration-effect curves for norharman, harman and mastoparan on high-affinity GTP hydrolysis in HL-60 membranes and by TD. High-affinity GTP hydrolysis in membranes of dibutyryl-cAMP-differentiated HL-60 cells (A) and by purified TD (B) was determined as described in "Materials and Methods" in the presence of norharman (●), harman (■), and mastoparan (◆) at the indicated concentrations. Data shown are the means of assay quadruplicates. The SD values were generally less than 5% of the means and are, for the sake of clarity of the figure, not shown. Similar results were obtained in at least four independent experiments. In HL-60 membranes, the effects of norharman and harman at concentrations of 300 μM and higher were significantly different from basal activity, while those of mastoparan were significantly different at concentrations of 1 μM and higher ($P < 0.05$, Wilcoxon test). With respect to TD, the effects of norharman and harman were significant ($P < 0.05$, Wilcoxon test) at concentrations of 30 μM and 300 μM, respectively, and higher, and those of mastoparan at concentrations of 1 μM and higher.

not shown). This result is in accordance with data obtained for other cationic-amphiphilic substances [14, 18, 19].

To examine the hypothesis that the effect of β-carbolines on GTPase is due to a receptor-independent activation, we studied the effect of norharman, harman and mastoparan on the stimulation of the GTPase of TD. As shown in Fig. 1B, norharman and harman stimulated its GTPase with EC_{50} values of approximately 60 μM and 320 μM, respectively and a maximum at *ca.* 1 mM. The concentration-response curve for mastoparan was biphasic (EC_{50} 1 μM; maximum at 10 μM).

In order to address the question as to whether the activation of the G-protein-associated nucleoside diphosphate kinase is involved in the G-protein activation by β-carbolines, we examined the effect of the nucleoside diphosphate kinase substrate, GDP, on the stimulatory effects of norharman on high-affinity GTP hydrolysis in HL-60 membranes. It has been shown that GDP augments the stimulatory effect of compounds which activate G-proteins indirectly via the nucleoside diphosphate kinase [30]. GDP (10 μM) inhibited the stimulatory effect of norharman on GTPase in HL-60 membranes (data not shown), arguing against an involvement of the nucleoside diphosphate kinase in β-carboline effects.

Fig. 2 shows the effect of PTX and its carrier on the stimulation of high-affinity GTP hydrolysis in HL-60 membranes by norharman in comparison to mastoparan. The effects of both compounds were abolished in membranes of PTX-treated HL-60 cells. Moreover, norharman inhibited basal GTP hydrolysis in PTX-treated HL-60 membranes.

In Table 1, the structural formulae of selected β-carbolines and indoleamines and their effects on GTP hydrolysis in HL-60 membranes and on TD are compiled. The effects of the compounds were compared to basal GTP hydrolysis at a concentration of 1 mM. The precursor molecules of the biosynthesis of β-carbolines, tryptamine and 5-hydroxy-

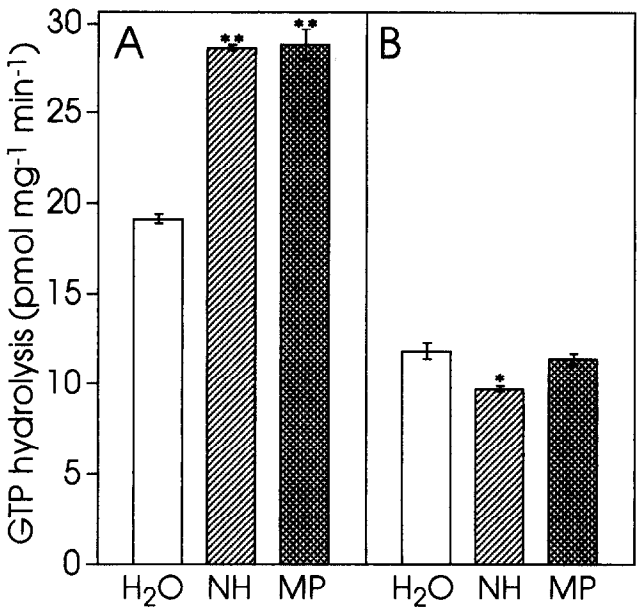


FIG. 2. Effect of PTX on basal, norharman- and mastoparan-stimulated GTP hydrolysis in HL-60 membranes. Treatment of HL-60 cells with carrier (A) or PTX (B) and the determination of high-affinity GTP hydrolysis were performed as described in "Materials and Methods". The concentrations of norharman (NH) and mastoparan (MP) were 1 mM and 10 μM, respectively. Data shown are the means ± SD of a typical experiment performed in quadruplicate. Similar results were obtained in three independent experiments. *P < 0.05, **P < 0.01 (Wilcoxon test).

tryptamine, did not stimulate the GTPase in HL-60 membranes and activated the GTPase of TD only slightly. In HL-60 membranes, norharman and its analogue, tetrahydronorharman (possessing a hydrated pyridine-ring) and harman and its 7-methoxylated analogues, harmin and harmalin, stimulated high-affinity GTP hydrolysis with comparable potency and efficacy. Surprisingly, the 6-hydroxylated analogues of norharman and tetrahydronorharman, 6-hydroxynorharman and 6-hydroxytetrahydronorharman, were without effect on GTPase in HL-60 membranes.

Among all compounds studied, norharman was the most potent activator of the GTPase of TD. Norharman was five-fold more potent in activating the GTPase of TD than its hydrated analogue tetrahydronorharman and in this regard, harmin and harmol were also more potent than harmalin and harmalol, both hydrated in positions 3 and 4. As is true for HL-60 membranes, one of the most remarkable findings concerning TD was the loss of G-protein-stimulatory activity following substitution of position 6 by a hydroxyl group. Substitution of harman with a hydroxyl group in position 7 (harmol) or with a methoxy group (harmin) did not alter the efficacy of these β-carbolines to stimulate TD.

DISCUSSION

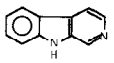
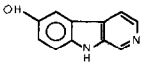
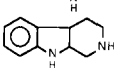
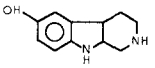
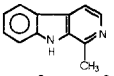
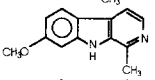
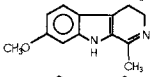
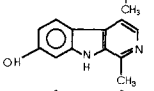
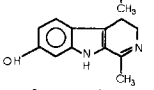
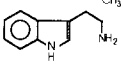
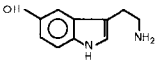
In the present study, we intended to show that naturally occurring β-carbolines activate G-proteins in a receptor-independent manner because they are cationic-amphiphilic substances. Several substances with such biophysical properties are known to activate G-proteins by this mode of action [13–23]. Indeed, β-carbolines stimulated high-affinity GTP hydrolysis in HL-60 membranes in a PTX-sensitive manner, indicative of activation of G_i-proteins (see Figs. 1 and 2). The fact that HL-60 membranes do not possess high-affinity binding sites for β-carbolines* argues against receptors being involved in the responses towards these compounds. In addition, there is no evidence supporting a role of nucleoside diphosphate kinase in β-carboline action in HL-60 membranes. Thus, if the effects of β-carbolines in HL-60 membranes were due to a direct interaction with G-proteins, the compounds should also activate purified TD. This hypothesis was corroborated by the experimental data (see Fig. 1 and Table 1).

Interestingly, norharman led to a slight but statistically significant inhibition of basal GTP hydrolysis in PTX-treated HL-60 membranes (see Fig. 2). First generation H_1-receptor antagonists also inhibit basal GTPase in PTX-treated HL-60 membranes [17]. One possible explanation for this effect would be that β-carbolines and H_1-receptor antagonists inhibit PTX-insensitive G-proteins and that the inhibition of PTX-insensitive G-proteins is not obvious in HL-60 control membranes because the high activity of G_i-proteins masks this inhibitory effect.

The experiments concerning the structure-activity relationship of various β-carbolines to their stimulating effects on the GTPase in HL-60 membranes and of TD produced some interesting findings (see Table 1). The precursors of the β-carbolines, tryptamine and 5-hydroxytryptamine, were without effect and with only a slight effect on GTPase in HL-60 membranes and of TD, respectively. Thus, the pyridine ring is of importance for the GTPase-activating properties of the naturally occurring β-carbolines. The hydration status of the pyridine ring influenced the potency of β-carbolines to activate the GTPase of TD. In this regard, norharman was five-fold more potent than tetrahydronorharman. In contrast to TD, no clear differences in potency between norharman and tetrahydro-norharman were observed in HL-60 membranes. This leads to the assumption that the interaction of norharman with TD is more specific than its interaction with G_i-proteins in HL-60 membranes. Thus, it will be interesting to address the question of whether the interaction of β-carbolines with other purified G-proteins reveals additional pharmacodynamic differences. Substitution of the β-carboline ring with a 6-hydroxyl group led to a dramatic reduction in efficacy of compounds, whereas the corresponding substitution at position 7 had less significant consequences. Such differences in efficacy between closely related compounds point to the specificity of the interaction of β-carbolines with G-proteins.

Activation of G-proteins by β-carbolines occurs at concentrations above 1 μM. The *in vivo* concentrations of naturally occurring β-carbolines are in the picomolar range in the majority of brain regions [2]. However, the substantia

TABLE 1. Effect of β-carbolines and indoleamines on GTP hydrolysis in HL-60 membranes and on GTP hydrolysis by TD

Compound	Formula	GTP hydrolysis (HL-60 membranes) rel. eff. (%)	GTP hydrolysis (HL-60 membranes) EC_{50} (μM)	GTP hydrolysis (TD) rel. eff. (%)	GTP hydrolysis (TD) EC_{50} (μM)
Norharman		54	410	63	60
6-hydroxynorharman		0	n.a.	9	210
Tetrahydronorharman		56	510	57	300
6-hydroxytetrahydronorharman		0	n.a.	18	300
Harman		42	450	52	320
Harmin		41	500	41	370
Harmalin		40	520	31	420
Harmol		n.d.	n.d.	66	300
Harmalol		n.d.	n.d.	27	490
Tryptamine		0	n.a.	13	350
5-hydroxytryptamine		0	n.a.	10	370

High-affinity GTP hydrolysis in membranes of dibutyryl-cAMP-differentiated HL-60 cells and by TD was determined in the presence of compounds (1 μM–3 mM each) or solvent (control) as described in "Materials and Methods". Basal GTP hydrolysis in HL-60 membranes was 15.6 ± 0.6 pmol mg^{-1} min^{-1}. Basal GTP hydrolysis by TD was 0.24 ± 0.01 mmol mol^{-1} min^{-1}. EC_{50} values of compounds were calculated from concentration-response curves. The relative efficacy (rel.eff.) was determined by comparing the stimulatory effects of compounds at 1 mM each and is expressed in % stimulation of GTP hydrolysis above basal. Data are the means of three independent experiments performed in quadruplicate. The SD values were generally <5% of the means and are, for the sake of clarity of the Table, not shown. n.d., not determined; n.a., not applicable.

nigra contains β-carbolines at micromolar levels [31]. This raises the crucial question as to whether the shown effects are of physiological relevance. However, it should be noted that measurement of high-affinity GTP hydrolysis is an *in vitro* method and that *in vivo*, i.e. in compartimented membranes of mammalian cells, where G-proteins are located, the concentration of such lipophilic substances can be much higher than those detected in tissue homogenates. The neuroleptic drug, chlorpromazine, can reach a concentration of 20 mM within the membrane phase under conditions in which in the aqueous phase the concentration is only 1–10 μM [32]. Since β-carbolines are, like chlorpromazine, lipophilic substances, they may reach concentrations in brain membranes which are sufficient to stimulate high-affinity GTP hydrolysis. Another argument supporting a physiological role of β-carbolines in G-protein activation is the fact that with TD, stimulatory effects of the compounds are already observed at 10 μM. It is possible that the predominant neuronal G-protein, G_o, is even more sensitive to stimulation by β-carbolines than TD. Moreover, we cannot exclude the possibility that even a small G-protein activation by β-carbolines may be relevant for the induction of long-term biological effects as those seen in chronic alcoholism.

In conclusion, our data suggest that naturally occurring β-carbolines activate G-proteins in a receptor-independent manner, which may contribute to their biological effects. Their activity depends on the chemical structure of the β-carboline molecule.

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