Protean agonism at histamine H_3 receptors *in vitro* and *in vivo*

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G protein-coupled receptors (GPCRs) are allosteric proteins that adopt inactive (R) and active (R*) conformations in equilibrium. R* is promoted by agonists or occurs spontaneously, leading to constitutive activity of the receptor. Conversely, inverse agonists promote R and decrease constitutive activity. The existence of another pharmacological entity, referred to as ''protean'' agonists (after Proteus, the Greek god who could change shape), was assumed on theoretical grounds. It was predicted from the existence of constitutive activity that a same ligand of this class could act either as an agonist or an inverse agonist at the same GPCR. Here, we show that proxyfan, a high-affinity histamine H_3-receptor ligand, acts as a protean agonist at recombinant H_3 receptors expressed in the same Chinese hamster ovary cells. In support of the physiological relevance of the process, we show that proxyfan also behaves as a protean agonist at native H_3 receptors known to display constitutive activity. On neurochemical and behavioral responses in rodents and cats, proxyfan displays a spectrum of activity ranging from full agonism to full inverse agonism. Thus, protean agonism demonstrates the existence of ligand-directed active states LR* different from, and competing with, constitutively active states R* of GPCRs, and defines a pharmacological entity with important therapeutic implications.

Current models for G protein-coupled receptor (GPCR) activation assume that receptors are in equilibrium between inactive (R) and active (R*) conformations (1–3). R* is promoted by agonists but may also occur in their absence, leading to constitutive activity of GPCRs (4–7). Inverse agonists promote R, thereby decreasing constitutive activity. After the discovery of constitutive activity, another class of ligands, referred to as ''protean'' agonists, was introduced by Kenakin on theoretical grounds (8). The rationale was that the reversal from agonism to inverse agonism (i.e., protean agonism) would occur when an agonist produces an active conformation of lower efficacy than the constitutively active conformation. It was therefore predicted that a same ligand of this class could act either as an agonist or an inverse agonist at the same GPCR, depending on the level of constitutive activity. Apparent protean agonism had been observed so far after modification of recombinant receptors (9–13), such as their desensitization or mutation (10, 12), or their expression in distinct cells or with different Gα-protein subunits (11, 13). However, the process remained to be established for native receptors under physiological conditions.

The histamine H_3 receptor is a GPCR that we identified as an autoreceptor regulating histamine release in brain (14, 15). It was cloned recently (16–18), and the recombinant receptor expressed at physiological densities displays constitutive activity (17, 19). Consistent with the physiological relevance of the process, we showed that high constitutive activity of H_3 autoreceptors regulates histamine neurons in brain (17). Therefore, we have taken advantage of this high constitutive activity of native H_3 receptors (17, 19) to investigate protean agonism experimentally. We have assessed the activity of proxyfan, a high-affinity H_3 receptor ligand (20, 21), at H_3 receptors expressed in Chinese hamster ovary (CHO) cells, and at native H_3 receptors *in vitro* and *in vivo*.

Materials and Methods

p42/p44-Mitogen-Activated Protein Kinase (MAPK) Activity. CHO(H_3) cells were incubated for 10 min with the ligands at increasing concentrations. After washing and lysis of the cells, MAPK activity was measured with a p42/p44-MAPK enzyme assay (Amersham Pharmacia Life Science) and [γ-^{33}P]ATP (22).

[^{35}S]GTPγ[S]-Binding Assay. Membranes of CHO(H_3) cells were pretreated with adenosine deaminase (1 unit/ml) and incubated as described (19) for 60 min with 0.1 nM [^{35}S]GTPγ[S] and the ligands at increasing concentrations.

cAMP Accumulation. CHO(H_3) cells were incubated as described (17) for 10 min with 3 μM forskolin and the ligands at increasing concentrations. cAMP was extracted and measured by RIA (NEN).

[^{3}H]Arachidonic Acid Release. CHO(H_3) cells were incubated as described (17, 19) for 2 h with 0.5 μCi (1 Ci = 37 GBq) of [^{3}H]arachidonic acid in the presence of 0.2% bovine serum albumine. After being washed, cells were incubated for 30 min with 2 μM A23187 and the ligands at increasing concentrations, and [^{3}H]arachidonic acid release was determined by liquid scintillation counting.

[^{3}H]Histamine Release from Synaptosomes. Mouse or rat cortical synaptosomes were incubated for 30 min with [^{3}H]L-histidine (23), and [^{3}H]histamine release was induced as described (24) in the presence of 20 or 55 mM K^+ and, when required, ciproxifan (100 nM), proxyfan (1 μM), or histamine (1 μM). Statistical evaluation of the results was performed by using ANOVA followed by Newman-Keuls test.

Histamine and *tele*-Methylhistamine (t-MeHA) Assays. Histamine levels were evaluated in the medium of depolarized mouse or rat cortical synaptosomes with an enzymoimmunoassay (Immunotech). t-MeHA levels in mouse or rat brain were determined 90 min after oral administration of the drugs with an enzymoim-

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Abbreviations: GPCR, G protein-coupled receptor; MAPK, mitogen-activated protein kinase; t-MeHA, *tele*-methylhistamine; CHO, Chinese hamster ovary.

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munoassay (23). Statistical evaluation of the results was performed by using ANOVA followed by Newman-Keuls test.

Cyclophosphamide-Induced Cystitis. Cyclophosphamide (200 mg/kg, i.p.) was administered to mice 1 h after BP 2-94 (10 mg/kg, orally), proxyfan (0.03 mg/kg, orally), and/or ciproxifan (3 mg/kg, orally). Mice were injected with Evans blue (30 mg/kg, i.v.) 2.5 h after cyclophosphamide, and plasma protein extravasation in urinary bladder was evaluated 30 min later as described (25). Statistical evaluation of the results was performed by using ANOVA followed by Newman-Keuls test.

Sleep-Wake Cycle. Polygraphic recordings were performed after oral (cat) or i.p. (mouse) administration of the drugs and scored as described (26, 27) by 30- or 60-s epochs for each sleep-wake state. Statistical evaluation of the results was performed by using ANOVA followed by two-tailed Student's *t* test. Individual animal served as its own control.

Results and Discussion

We have first assessed the activity of proxyfan at the recombinant receptor expressed at a high density in the same CHO cells. The effects of proxyfan were compared with those of histamine, the natural ligand, and ciproxifan, a potent and selective inverse agonist (17, 19, 26), on various H_3-receptor-mediated responses.

Ciproxifan alone had no significant effect on mitogen-activated protein kinase (MAPK) activity (18, 22), indicating that the H_3 receptor displays no constitutive activity for this response. Proxyfan increased MAPK activity with a maximal response that was ≈30% that of histamine, therefore acting as a partial agonist (Fig. 1*a*). [^{35}S]GTPγ[S] binding to membranes of the same CHO(H_3) cells (17, 19) was slightly decreased (≈−10%) by ciproxifan, indicating a low level of constitutive activity in this system. Proxyfan also behaved as a partial agonist, with an intrinsic efficacy very similar to that observed on MAPK activity (Fig. 1*b*).

On adenylyl cyclase inhibition and arachidonic acid release, two responses in which the H_3 receptor displays high constitutive activity (17, 19), the effect of ciproxifan in the same CHO(H_3) cells was of greater amplitude than on [^{35}S]GTPγ[S] binding (Fig. 1 *c* and *d*). Proxyfan still behaved as a partial agonist and inhibited cAMP formation with an intrinsic activity of ≈60% that of histamine (Fig. 1*c*), but became a partial inverse agonist on [^{3}H]arachidonic acid release with an inverse efficacy of ≈60% that of ciproxifan (Fig. 1*d*).

This spectrum of activity of proxyfan, ranging from partial agonism to partial inverse agonism in the same cells (Table 1), suggested that it could also act as a protean agonist in the brain where native H_3 receptors also display high constitutive activity (17, 19). We assessed this possibility on neurochemical and behavioral H_3-receptor-mediated responses in rodents and cats.

A protean behavior of proxyfan was first evidenced *in vitro* when its effect was determined at H_3 autoreceptors inhibiting [^{3}H]histamine release from rat or mouse cortical synaptosomes (24). As expected from constitutive activity of the H_3 autoreceptor controlling [^{3}H]histamine release induced by a strong stimulus (55 mM K^+) (17), the amine release was enhanced by ciproxifan acting as an inverse agonist, but remained unaffected by proxyfan, behaving as a neutral antagonist (17) (Fig. 2 *a* and *b*). In the rat, when the release was induced by a low stimulus (20 mM K^+), that is, when the autoreceptor displayed no constitutive activity (17), proxyfan behaved as a partial agonist, inhibiting [^{3}H]histamine release with a maximal effect of ≈40% that of histamine (Fig. 2*a*). In the mouse, this partial agonist effect of proxyfan with the low stimulus was not observed (Fig. 2*b*). This species difference was not due to a much lower affinity of proxyfan for the mouse H_3 receptor (K_i = 5.0 ± 0.7 nM as determined by [^{125}I]iodoproxyfan binding assay (28) vs. 2.9 ± 0.2 nM in the rat (20)). It could also hardly be ascribed to differences in constitutive activity of mouse and rat autoreceptors, as shown with ciproxifan (Fig. 2 *a* and *b*), or in endogenous histamine levels in the medium (4.5 ± 0.4 nM vs. 2.4 ± 0.4 nM in the rat). It more likely reflected a much lower intrinsic efficacy of proxyfan at the mouse autoreceptor, as also shown by the strong inhibition triggered in the same conditions by exogenous histamine (Fig. 2*b*).

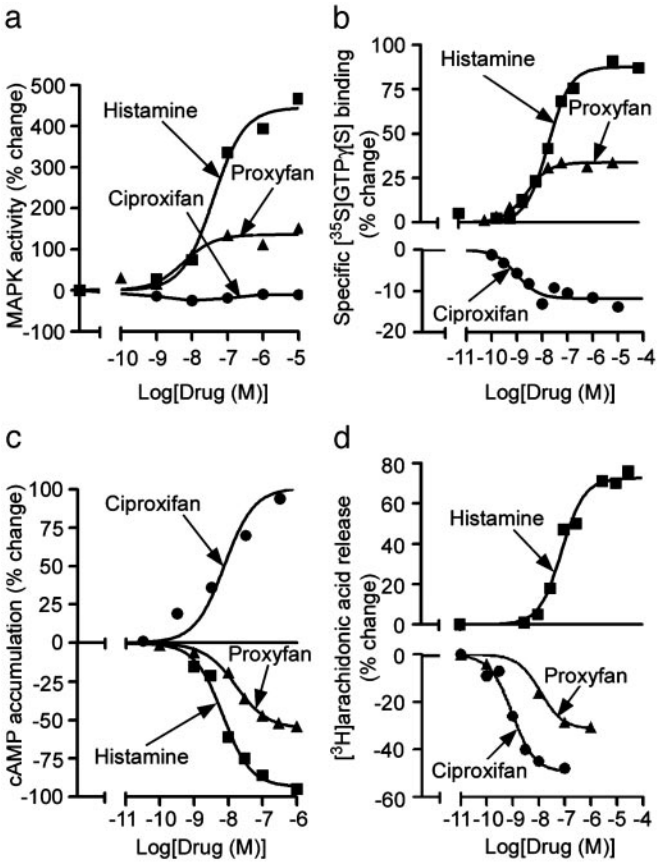

Fig. 1. Effects of H_3 receptor ligands on H_3 receptor-mediated responses in CHO(H_3) cells. The effects of histamine, ciproxifan, and proxyfan were determined in the same CHO($rH_{3\,(413)}$) cells (*17*) expressing 1 pmol per mg protein on MAPK activity (*a*), [^{35}S]GTPγ[S] binding to membranes (*b*), forskolin-induced cAMP accumulation (*c*), and A23187-evoked [^{3}H]arachidonic acid release (*d*). Results, expressed as the percentage change of the response, are means ± SEM of 3–10 determinations from two to three separate experiments.

A protean behavior of proxyfan was also observed at rodent and cat H_3 receptors *in vivo*, when its effect was compared with that of ciproxifan used in each system as a reference inverse agonist. As expected (17), ciproxifan, behaving as an inverse agonist at H_3 autoreceptors, increased histamine neuron activity in mouse and rat brain, and enhanced the levels of the histamine metabolite t-MeHA, a reliable index of this *in vivo* activity (29). In mouse brain, consistent with its neutral antagonist properties (17), proxyfan did not change histamine neuron activity, leaving unaffected t-MeHA levels (Fig. 2*c*). In rat brain, proxyfan given at the same dose (10 mg/kg) markedly enhanced (by ≈70%) t-MeHA levels, therefore acting as an inverse agonist (Fig. 2*c*). This species difference was not due to different levels of constitutive activity displayed by H_3 autoreceptors *in vivo* (17), as shown by the same maximal increase (by 90–100%) in t-MeHA levels induced by ciproxifan in rat and mouse brain (Fig. 2*c*). In addition, the enhancement induced by proxyfan in the rat does not result from the blockade of endogenous

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Table 1. Effects of proxyfan at histamine H_3 receptors

Response	Property
Recombinant receptor	
MAPK activity (1,000)	Partial agonist (Fig. 1*a*)
[^{35}S]GTPγ[S] binding (1,000)	Partial agonist (Fig. 1*b*)
Inhibition of cAMP formation (1,000)	Partial agonist (Fig. 1*c*)
[^{3}H]Arachidonic acid release (300)	Neutral antagonist (ref. 17)
[^{3}H]Arachidonic acid release (1,000)	Partial inverse agonist (Fig. 1*d*)
Native receptor	
[^{3}H]Histamine release in rat brain	
20 mM K^+	Partial agonist (Fig. 2*a*)
55 mM K^+	Neutral antagonist (Fig. 2*a* and ref. 17)
[^{3}H]Histamine release in mouse brain	
20 mM K^+	Neutral antagonist (Fig. 2*b*)
55 mM K^+	Neutral antagonist (Fig. 2*b* and ref. 17)
[^{35}S]GTPγ[S] binding in rodent brain	Neutral antagonist (refs. 17 and 19)
t-MeHA level in rodent brain (*in vivo*)	
Mouse	Neutral antagonist (Fig. 2*c* and ref. 17)
Rat	Partial inverse agonist (Fig. 2*c*)
Cyclophosphamide-induced cystitis in the mouse (*in vivo*)	Full agonist (Fig. 2*d*)
Sleep-wake cycle (*in vivo*)	
Mouse	Full inverse agonist (Fig. 2*e*)
Cat	Full agonist (Fig. 2*f*)

The level of expression of the recombinant receptor in CHO(H_3) cells is given in parentheses in fmol/mg of protein.

histamine activating H_3 receptors, because a strong increase was similarly induced in rat brain by a low dose (1 mg/kg) of FUB 465, a potent inverse agonist but weak antagonist (17). In the rat, the apparent discrepancy between the inverse agonism and partial agonism/neutral antagonism induced by proxyfan on histamine release *in vivo* and *in vitro*, respectively (Table 1), might reflect its interaction with H_3 receptor isoforms (17, 18) with distinct localizations on histamine neurons (24, 30, 31) or displaying different levels of constitutive activity (17).

Proxyfan induced full agonism at mouse H_3 heteroreceptors located on sensory C fibers and inhibiting neurogenic plasma leakage in urinary bladder *in vivo* (25). On cyclophosphamide-induced cystitis, a very low dose of proxyfan (0.03 mg/kg) attenuated plasma protein extravasation with the same effect ($\approx$-70%) as the agonist BP 2-94 (25). This effect of proxyfan was blocked by ciproxifan, which did not itself affect plasma protein extravasation, indicating the absence of constitutive activity of H_3 receptors in this system (Fig. 2*d*).

Whereas the inverse agonist ciproxifan induced arousal both in the mouse and cat (26, 27), the effect of proxyfan at H_3 receptors modulating the sleep-wake cycle depended on the animal species (Fig. 2 *e* and *f*). In the mouse, proxyfan mimicked the arousing effect of ciproxifan (27), thereby behaving as a full inverse agonist, with a dose-dependent enhancement of wakefulness at the expense of slow wave sleep and paradoxical sleep (half-maximal effective dose $\approx$3 mg/kg, i.p.; Fig. 2*e*). In contrast, in the cat, the effect of proxyfan was similar to that previously reported for the agonist (*R*)-α-methylhistamine (15). Both drugs did not affect wakefulness and light slow wave sleep but significantly increased deep slow wave sleep at the expense of paradoxical sleep (32) (Fig. 2*f*). As expected, ciproxifan decreased the agonist effect of proxyfan.

Altogether, the present findings illustrate how protean agonism can be observed at distinct functional responses within a given cell or a given animal species, as well as at a given response within different animal species (Table 1). Protean agonism has been predicted by Kenakin on theoretical grounds (8). The rationale put forward is that, if an agonist produces an active state LR* of lower efficacy than the constitutively formed one (R*), then the reversal from agonism to inverse agonism (i.e., protean agonism) would be observed. In the absence of constitutive activity (no R*), the ligand would act as an agonist by changing R into LR*. In the presence of a significant amount of R*, the ligand would decrease the efficacy (activity) by changing R* to LR*, leading to an apparent inverse agonism in constitutively active systems (Fig. 3). Therefore, inverse agonism could be achieved not only with drugs promoting the inactive conformation R, as currently stated, but also with partial agonists.

The active conformation LR* promoted by proxyfan was evidenced by the agonism observed in quiescent systems (no R*) such as MAPK activity in CHO(H_3) cells, histamine release induced from rat synaptosomes by a low stimulus or cyclophosphamide-induced cystitis (Table 1). In constitutively active systems, the competition between the ligand-directed active state LR* and the constitutively active state R* predicted by Kenakin's model (33) accounted for the effects of proxyfan (Fig. 3). In CHO(H_3) cells, when R* displayed a low efficacy ([^{35}S]GTPγ[S] binding), proxyfan still induced agonism. When R* displayed a high efficacy, the effect of proxyfan depended on the efficacy of LR* (Fig. 3). The reversal from agonism (on cAMP formation) to inverse agonism (on [^{3}H]arachidonic acid release) indicated a lower efficacy of LR* on the latter response. This protean behavior of proxyfan observed with high constitutive activity might result from the production of distinct LR* states with different efficacies relatively to R*. In addition, different $G_{i/o}$-proteins may be activated in the two responses, inasmuch as histamine itself was less potent on [^{3}H]arachidonic acid release than on inhibition of cAMP formation (EC_{50} = 82 $\pm$ 15 nM vs. 7 $\pm$ 1 nM). *In vivo*, a low efficacy of LR* coupled to a high efficacy of R* would also yield the inverse agonism observed with proxyfan on t-MeHA levels in the rat and sleep-wake cycle in the mouse (Table 1). Altogether, our data show that inverse agonism can be observed with an agonist when the response displays a high constitutive activity.

An important issue resulting from Kenakin's model (8, 33) is related to neutral antagonism. This pharmacological property is poorly understood and currently attributed to drugs producing

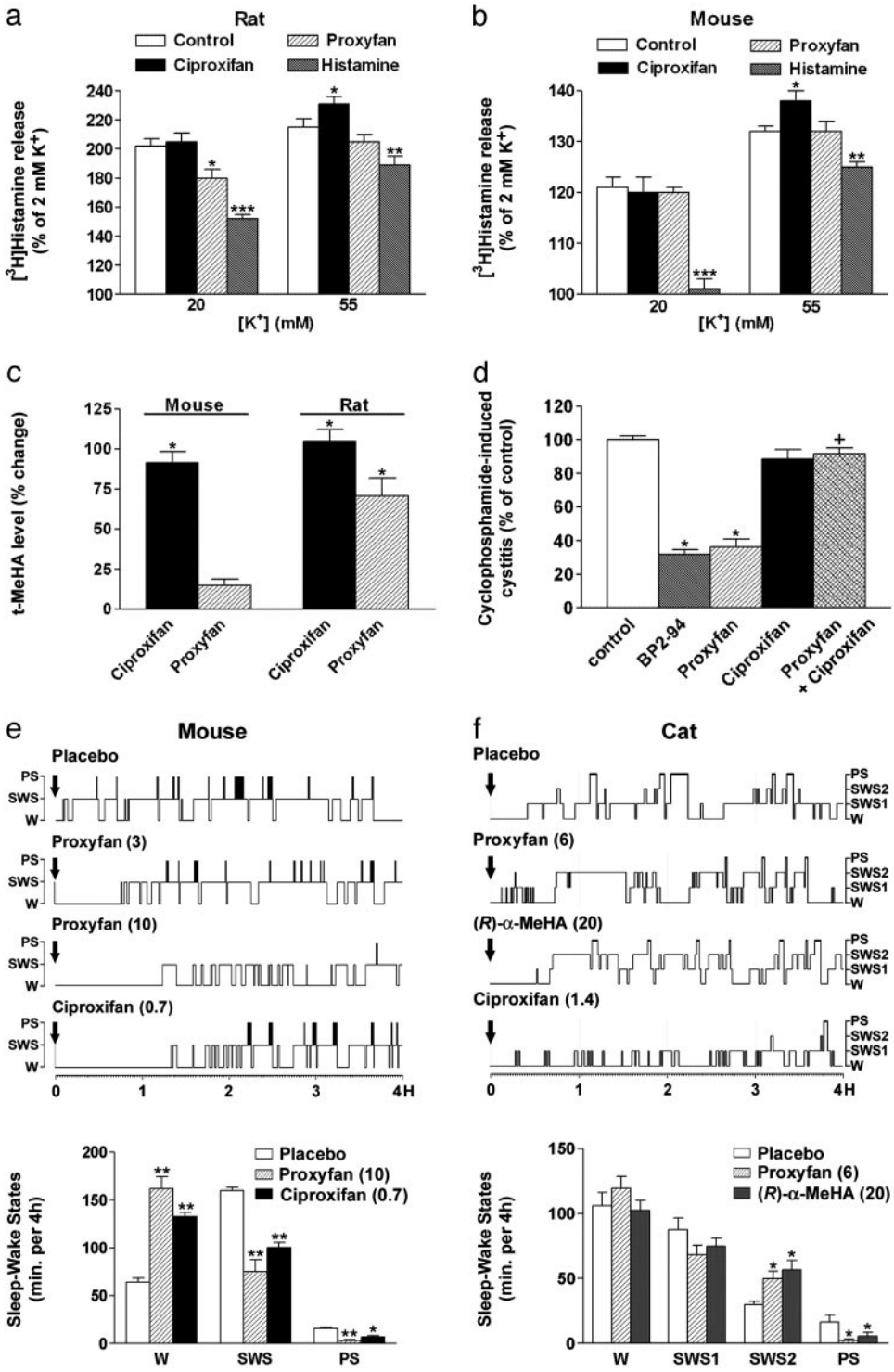

Fig. 2. Effects of H_3 receptor ligands on responses mediated by native H_3 receptors. (*a* and *b*) Effects of histamine, ciproxifan, and proxyfan on [^{3}H]histamine release induced by 20 or 55 mM K^+ from rat (*a*) or mouse (*b*) synaptosomes. Means ± SEM of 9–28 values from three to four separate experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. control. (*c*) Changes in brain t-MeHA levels in mice or rats receiving ciproxifan (3 mg/kg) or proxyfan (10 mg/kg). Means ± SEM of 7–16 values. *, $P < 0.001$ vs. control. (*d*) Effects of H_3 receptor ligands on cyclophosphamide-induced cystitis. Means ± SEM of 10–32 values. *, $P < 0.01$ vs. control; +, $P < 0.05$ vs. proxyfan. (*e* and *f*) Effects of H_3 receptor ligands on the sleep-wake cycle in the mouse (*e*) or cat (*f*). (*Upper*) Representative hypnograms (4 h). (*Lower*) Cumulative time (in min) spent in each sleep-wake stage (W, wakefulness; SWS1, light slow wave sleep; SWS2, deep slow wave sleep; PS, paradoxical sleep). Means ± SEM of five to nine values. The doses in mg/kg are given between parentheses. *, $P < 0.05$; **, $P < 0.001$ vs. placebo.

no effect themselves but binding with identical affinity to inactive and active states of GPCRs, a condition obviously difficult to achieve in terms of thermodynamic modeling. Alternatively, it can be predicted from the competition between LR* and R* for the G proteins, that a partial agonist promoting a state LR* with the same level of efficacy as that of R* would

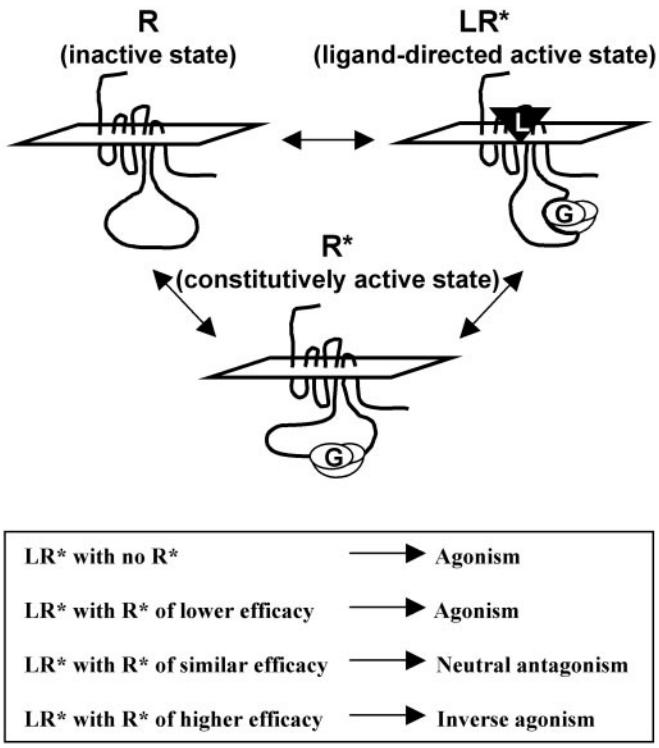

Fig. 3. GPCRs are allosteric proteins that can adopt various conformations in equilibrium. On the contrary to the inactive states (R), the active states can interact with G proteins to initiate response. These active conformations occur either spontaneously (R*), leading to constitutive activity of GPCRs, or through ligand binding (LR*). The competition between LR* and R* for the G proteins leads to agonism, neutral antagonism, or inverse agonism.

appear as a neutral antagonist (Fig. 3). Then, neither agonism nor inverse agonism should be expected to occur with the drug alone. However, the drug would progressively antagonize both full agonists (by producing a state LR* of lower efficacy) and inverse agonists (by changing R into a state LR* with an efficacy similar to that of R*) (Fig. 3). In agreement with these theoretical expectations, proxyfan acted as an inverse agonist at a high receptor density (Fig. 1*d*) but did not affect [^{3}H]arachidonic acid release in CHO(H_3) cells expressing a lower receptor density (300–500 fmol/mg protein) (17), that is, when the R*/G protein stoichiometry was decreased (19). In addition, proxyfan then inhibited the effects of both inverse agonists (such as ciproxifan) and full agonists [such as imetit (23)], thereby acting as a neutral antagonist (17). An equilibrium between active states R* and LR* displaying similar efficacies also accounts for proxyfan behaving as a neutral antagonist on [^{3}H]histamine release induced from synaptosomes by a high stimulus (17). In agreement, when the rat autoreceptor displayed no constitutive activity, that is, by using a low stimulus (17), proxyfan became a partial agonist. The inhibition induced by histamine itself was higher with the low stimulus (Fig. 2*a*). This inverse relationship between the efficacy of LR* and the strength of the stimulus may therefore not only reflect the effect of calcium influx on stimulus-release coupling (30, 34), as has been assumed so far (24), but also the competition of R* with LR* for G proteins, leading in the absence of R* (low stimulus) to a higher LR*/G protein stoichiometry (i.e., higher inhibition of release; Figs. 2*a* and 3).

Our observation that a single drug may belong to all of the classical classes of ligands (full agonists, partial agonists, neutral antagonists, partial inverse agonists, and full inverse agonists) indicates that protean agonism should be added to the general principles of receptor classification and analysis. Protean agonists such as proxyfan are powerful tools to investigate active receptor conformations not only in cells but also in tissues. The existence of ligand-directed active states LR* different from, and competing with, constitutively active states R* may account partly for the apparent pharmacological heterogeneity of GPCRs frequently observed amongst tissues in binding or functional studies and traditionally attributed to the existence of different receptor subtypes. In addition, the existence in tissues of distinct active states LR* and R* of GPCRs may lead to reconsideration of the classical drug classifications used in pharmacology and mainly based on the physiological response produced by a drug. First, protean agonism indicates that a given drug may produce different physiological responses that do not simply reflect its intrinsic property (LR*) but are dependent on constitutive activity (R*) of the system (Table 1 and Fig. 3). Conversely, our findings indicate that the same physiological response may be produced by ligands interacting with different states of the GPCR. Whereas partial or full agonism requires the production of LR*, an apparent antagonism is expected to occur either when R is favored in the absence of R* or when LR* is produced with R* of similar efficacy. An apparent inverse agonism will be observed either when R is favored in the presence of R*or when LR* is produced with R* of higher efficacy. Therefore, the present data on protean agonism not only extend our previous studies indicating that constitutive activity (R*) regulates responses mediated by GPCRs in physiological systems (17, 19), but also show that constitutive activity plays a major role in regulating drug activity.

The therapeutic interest of protean agonists is hard to predict. They might be helpful to maintain a constant level of stimulation of the receptor, but their effect may vary among systems and patients and/or lead to paradoxical responses. Moreover, our data suggest that conditions creating protean agonism can be theoretically achieved for many drugs. Therefore, the process should be taken into account in toxicity studies and drug development.

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