

## Expression of a cloned rat histamine H<sub>2</sub> receptor mediating inhibition of arachidonate release and activation of cAMP accumulation

(second messengers/phospholipase A<sub>2</sub>/[<sup>125</sup>I]iodoaminopotentidine/Chinese hamster ovary cells)

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**ABSTRACT** A DNA, cloned after screening a rat genomic bank with probes derived from the sequence of a putative dog histamine H<sub>2</sub> receptor [Gantz, I., Schäffer, M., Delvalle, J., Logsdon, C., Campbell, V., Uhler, M. & Yamada, T. (1991) *Proc. Natl. Acad. Sci. USA* 88, 429–433], was used to prepare a probe for Northern blot analysis and to transfect Chinese hamster ovary (CHO) cells. Distribution of the gene transcripts in guinea pig tissues was consistent with that of H<sub>2</sub> receptors. Transfected CHO cells expressed a high density of sites binding [<sup>125</sup>I]iodoaminopotentidine, a selective H<sub>2</sub>-receptor ligand. These sites were characterized as typical H<sub>2</sub> receptors by using a series of competing agents that displayed apparent dissociation constants closely similar to corresponding values at a reference biological system. In transfected cells, histamine stimulated, with high potency and large receptor reserve, the accumulation of cAMP. In addition, in the same cells, histamine potently inhibited the release of arachidonic acid induced either by stimulation of constitutive purinergic receptors or by application of a Ca<sup>2+</sup> ionophore. This inhibition was independent of either cAMP or Ca<sup>2+</sup> levels. The results suggest that a single H<sub>2</sub> receptor may be linked not only to adenylyl cyclase activation but also to reduction of phospholipase A<sub>2</sub> activity. Because H<sub>1</sub> receptors have been reported to stimulate arachidonic acid release, inhibition of this release, an unexpected signaling pathway for H<sub>2</sub> receptors, may account for the opposing physiological responses elicited in many tissues by stimulation of these two receptor subtypes.

Histamine (HA), a ubiquitous cell-to-cell messenger, exerts its numerous actions in the nervous, endocrine, and immune systems through interaction with three pharmacologically distinct receptor subtypes, termed H<sub>1</sub>, H<sub>2</sub>, and H<sub>3</sub> (1, 2). H<sub>1</sub> and H<sub>2</sub> receptors are positively coupled to phospholipase C and adenylyl cyclase, respectively, whereas the intracellular signaling system of the H<sub>3</sub> receptor is still unknown (3–5).

In contrast to membrane receptors for other monoamines, molecular studies of HA receptors have just begun (6). For instance, H<sub>2</sub>-receptor peptides were identified in guinea pig brain by photoaffinity labeling (7) and, recently, canine (8) and human (9) H<sub>2</sub>-receptor genes have been cloned. Transfection of mammalian cells with these intronless genes resulted in the expression of both [<sup>3</sup>H]tiotidine binding sites and HA-sensitive adenylyl cyclase activity. Preliminary pharmacological characterizations of these receptors have been reported (8, 9).

Because molecular biological studies progressively reveal a much greater heterogeneity among receptor subtypes than previously thought (10, 11), we felt it of interest to identify

and characterize receptors related to those described by Gantz *et al.* (8). We have cloned (12), from a rat genomic bank, a DNA encoding a 358-amino acid protein displaying 82% homology with canine H<sub>2</sub> receptor.

We report here that this protein represents a typical H<sub>2</sub> receptor, as indicated by both tissue localization of the gene transcripts and detailed pharmacological analysis in transfected CHO-K1 cells. In addition, we show that this H<sub>2</sub> receptor mediates not only the expected activation of cAMP formation but also a marked inhibition of arachidonic acid ( $\Delta_4$ Ach) release, which is independent of both cAMP and Ca<sup>2+</sup> levels.

### MATERIALS AND METHODS

**Materials.** Na<sup>125</sup>I (2000 Ci/mmol; 1 Ci = 37 GBq), [<sup>3</sup>H] $\Delta_4$ Ach (202 Ci/mmol), [<sup>3</sup>H]choline (76 Ci/mmol), and *myo*-[2-<sup>3</sup>H]inositol (17.4 Ci/mmol) were from Amersham. [<sup>125</sup>I]iodoaminopotentidine (<sup>125</sup>I-APT) was synthesized as described (7). The drugs and their sources were as follows: cimetidine, burimamide, metiamide, and dimaprit (Smith Kline & French); famotidine (Merck Sharpe & Dohme); tiotidine (ICI); and ranitidine (Glaxo). 5-Amino-2-(3-[1-(1-pyrrolidinyl)ethyl]phenoxy)propyl)amino-1,3,4-thiadiazole (PPAT) stereoisomers (13) and APT were generous gifts from W. Schunack (Institute of Pharmacy, Berlin). ATP was from Boehringer Mannheim and other reagents from Sigma.

**Northern Blot Analysis.** Poly(A)<sup>+</sup> mRNAs, isolated from tissues of male Hartley guinea pigs (14), were subjected to agarose gel electrophoresis (1% agarose containing 1 M HCHO), blotted onto nitrocellulose, and immobilized by heating at 80°C for 2 h. Prehybridization was at 42°C for 2 h in 40% (vol/vol) formamide/2× Denhardt's solution/50 mM Tris-HCl, pH 7.4/4× standard saline citrate (SSC)/0.1% sodium pyrophosphate/1% SDS/denaturated salmon sperm DNA (100 μg/ml)/yeast tRNA (50 μg/ml). Hybridization was carried out overnight at 42°C, in prehybridization solution containing a probe (15 × 10<sup>6</sup> dpm/ml) corresponding to nucleotides 10–644 of the NuA<sub>2</sub> clone (12) <sup>32</sup>P-labeled by nick-translation. Blots were washed three times in 2× SSC/0.1% SDS at 42°C for 10 min, once in 0.2× SSC/0.1% SDS at 42°C for 25 min, and once in 0.2× SSC/0.1% SDS at 55°C for 20 min.

**Expression in CHO Cells.** The expression vector pSVH<sub>2</sub> was derived from the pSVD<sub>2</sub> plasmid (15) in which a *Hind*III–*Bgl* II restriction fragment was replaced by an ≈1.3-kilobase *Apa* I–*Bgl* II fragment of the NuA<sub>2</sub> clone, comprising the full

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Abbreviations: APT, aminopotentidine; <sup>125</sup>I-APT, [<sup>125</sup>I]iodoaminopotentidine; HA, histamine;  $\Delta_4$ Ach, arachidonic acid; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; [Ca<sup>2+</sup>]<sub>i</sub>, intracellular free Ca<sup>2+</sup> concentration; K<sub>B</sub>, apparent dissociation constant.

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1074-base coding sequence of the putative rat H<sub>2</sub> receptor (12); a *HindIII*-*Apa* I oligonucleotide adaptor was used. CHO-K1 cells, deficient in dihydrofolate reductase, were transfected with this plasmid using Lipofectin (Bethesda Research Laboratories). Stable transfectants were selected in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum, without hypoxanthine and thymidine, and tested for H<sub>2</sub>-receptor binding.

**H<sub>2</sub>-Receptor Binding.** Membranes were incubated for 90 min at 25°C with <sup>125</sup>I-APT alone (total binding) or in presence of 1 μM tiotidine (nonspecific binding) or with various drugs, and incubations were terminated by filtration (7).

**cAMP Accumulation.** Cells (96-well plates) were washed twice (10 min at 37°C) and incubated with the appropriate drugs in DMEM for 10 min at 37°C. cAMP was extracted and analyzed by radioimmunoassay (New England Nuclear).

**[<sup>3</sup>H]Δ<sub>4</sub>Ach Release.** Cells (24-well plates) were labeled by incubation for 2 h at 37°C with 0.5 μCi of [<sup>3</sup>H]Δ<sub>4</sub>Ach in 1 ml of DMEM supplemented with 0.2% bovine serum albumin to trap the released radioactivity. After washing twice with 0.75 ml of DMEM plus bovine serum albumin, cells were preincubated for 10 min in 0.5 ml of the same medium containing appropriate drugs. After addition of 0.5 ml of DMEM plus bovine serum albumin containing ATP (200 μM) or A23187 (4 μM), incubations were performed at 37°C for 30 min. [<sup>3</sup>H]Δ<sub>4</sub>Ach release was determined by liquid scintillation counting. Thin-layer chromatography revealed that free [<sup>3</sup>H]Δ<sub>4</sub>Ach constituted more than 90% of the released radioactivity (16).

**[<sup>3</sup>H]Δ<sub>4</sub>Ach Incorporation.** Cells (35-mm dishes) were incubated in 3 ml of DMEM containing 0.2% bovine serum albumin and [<sup>3</sup>H]Δ<sub>4</sub>Ach at 0.05 μCi/ml for 30 min at 37°C. The medium was removed and cells were washed. Lipids were extracted into hexane/isopropanol, 3:2 (vol/vol), and, after drying the organic phase under vacuum, fractionated by thin-layer chromatography [hexane/ethyl ether/MeOH/AcOH, 60:30:4:1 (vol/vol)]. After visualization with I<sub>2</sub> vapors, bands corresponding to phospholipids and free fatty acids were scraped and radioactivity was determined.

**[<sup>3</sup>H]Inositol Phosphate Formation.** Cells (24-well plates) were labeled by incubation with *myo*-[<sup>3</sup>H]inositol (0.5 μCi/ml) for 20 h in selective culture medium. After washings and preincubation (15 min at 37°C) in DMEM containing 20 mM LiCl, cells were incubated for 30 min in 0.5 ml of the same medium containing the appropriate drugs. Reactions were stopped by addition of 0.5 ml of 10% (vol/vol) HClO<sub>4</sub> and sonication. After a 1-h incubation at 4°C, the medium was neutralized, extracted with chloroform/methanol/water, 2:1:1 (vol/vol), and [<sup>3</sup>H]inositol phosphates were isolated by chromatography (17).

**[<sup>3</sup>H]Choline Release.** Cells (24-well plates) were labeled by incubation with [<sup>3</sup>H]choline (1 μCi per well) for 15 h at 37°C in 1 ml of DMEM supplemented with 10% fetal calf serum. The medium was replaced with DMEM containing choline chloride (1 mM), and cells were further incubated for 2 h. After washing, cells were preincubated for 10 min with or without HA and, finally, incubated for 30 min with HA with or without A23187 (2 μM). Released radioactivity was measured in a 0.5-ml sample of incubation medium (18).

**Intracellular Free Ca<sup>2+</sup> Concentration ([Ca<sup>2+</sup>]<sub>i</sub>).** Cells were trypsinized, washed twice with DMEM plus bovine serum albumin (0.2%), and incubated for 90 min at 37°C in 2 ml of the same medium containing fura-2 acetoxymethyl ester (2 μM). Cells were washed and resuspended in Tyrode's solution, and [Ca<sup>2+</sup>]<sub>i</sub> was measured by using a Hitachi F-2000 fluorescence spectrophotometer (19).

## RESULTS

**Northern Blot Analysis.** The probe revealed a single band of ≈4.5 kilobases in various guinea pig tissues. The hybridiza-

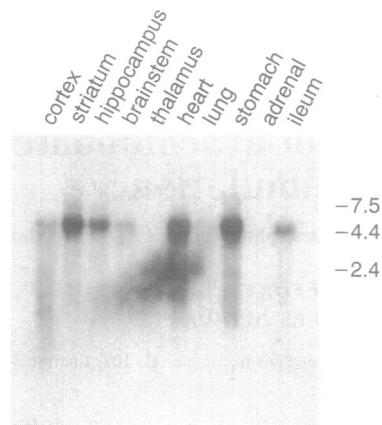


FIG. 1. Northern blot analysis of NuA<sub>2</sub> transcripts in guinea pig tissues. Poly(A)<sup>+</sup> RNA samples (8 μg per lane) were used. Blots were exposed to x-ray films for 6 days at -80°C with intensifying screens. Molecular sizes in kilobases are shown.

tion signal was strongest in striatum, heart, and stomach, whereas it was hardly detectable in thalamus, lung, and adrenals (Fig. 1).

**Expression and Pharmacological Characterization of H<sub>2</sub> Receptors in Transfected CHO Cells.** Stably transfected cells expressed <sup>125</sup>I-APT binding, whereas wild-type CHO cells did not. Among several clones that were isolated on the basis of high H<sub>2</sub>-receptor binding (0.05–2 pmol of sites per mg of membrane proteins), one termed CHO(H<sub>2</sub>) was selected for further studies.

Binding of <sup>125</sup>I-APT to CHO(H<sub>2</sub>) cell membranes was saturable and monophasic ( $n_H = 0.98$ ) with a  $K_d$  of  $0.45 \pm 0.10$  nM and a  $B_{max}$  of  $1.5 \pm 0.5$  pmol/mg of protein (mean  $\pm$  SEM derived from Scatchard analysis of three experiments with triplicate samples and 8–12 concentrations studied; data not shown).

Specific binding at 0.3 nM <sup>125</sup>I-APT represented 90% of the total and was monophasically inhibited by H<sub>2</sub> antagonists with  $K_i$  values highly correlated ( $r = 0.98$ ) with their apparent dissociation constant ( $K_B$ ) values at a reference system (Figs. 2 and 3). HA inhibited <sup>125</sup>I-APT binding biphasically ( $n_H = 0.43 \pm 0.03$ ) with IC<sub>50</sub> values of  $1.1 \pm 0.5$  and  $133 \pm 49$  μM. In presence of 0.1 mM guanosine 5'-[γ-imido]triphosphate, the inhibition curve became nearly monophasic ( $n_H = 0.80 \pm 0.08$ ) and a  $K_i$  value of  $75 \pm 24$  μM was obtained (Fig. 2).

**HA-Induced Responses in CHO(H<sub>2</sub>) Cells.** HA stimulated cAMP accumulation in CHO(H<sub>2</sub>) cells up to 15-fold, and the response was antagonized in a surmountable manner by ranitidine (Fig. 4). Dimaprit (0.1 mM) or impromidine (10 μM), two H<sub>2</sub>-receptor agonists, also enhanced cAMP accu-

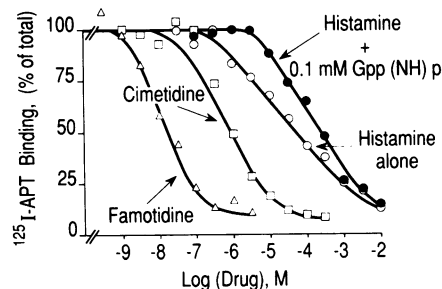


FIG. 2. Pharmacological characterization of <sup>125</sup>I-APT binding to membranes prepared from CHO(H<sub>2</sub>) cells. Membranes were incubated with 0.3 nM <sup>125</sup>I-APT, alone or in presence of competing agents. A representative experiment is shown in which control binding was  $1200 \pm 50$  cpm/μg of protein. Gpp(NH)p, guanosine 5'-[γ-imido]triphosphate.

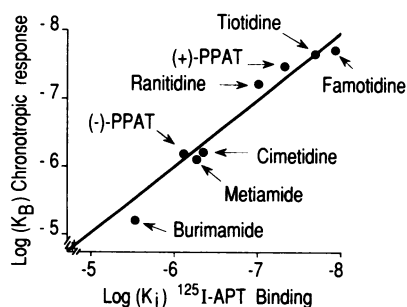


FIG. 3. Compared potencies of  $H_2$ -receptor antagonists as inhibitors of  $^{125}I$ -APT binding to membranes from CHO( $H_2$ ) cells and as antagonists of the  $H_2$ -receptor-mediated chronotropic response at the isolated guinea pig right atrium.  $K_i$  values were calculated (20) from  $IC_{50}$  values derived from two to four experiments as those depicted in Fig. 2.  $K_B$  values are apparent dissociation constants from refs. 13 and 21. PPAT, 5-Amino-2-(3-[3-[1-(1-pyrrolidinyl)ethyl]phenoxy]propyl)-amino-1,3,4-thiadiazole.

mulation in CHO( $H_2$ ). Stimulation corresponded to  $92 \pm 4\%$  and  $88 \pm 5\%$  of the maximal response to HA ( $n = 3$ ).

In CHO( $H_2$ ) cells labeled by incubation with  $[^3H]\Delta_4ACh$ , HA inhibited  $[^3H]\Delta_4ACh$  release evoked by stimulating constitutive purinergic receptors with 0.1 mM ATP (22); inhibition was maximal at 0.5  $\mu M$  HA ( $38 \pm 3\%$ ) and competitively antagonized by ranitidine (1  $\mu M$ ) (Fig. 5). Dimaprit (10  $\mu M$ ) inhibited ATP-induced release of  $[^3H]\Delta_4ACh$  by  $36 \pm 5\%$ , an effect blocked by 10  $\mu M$  ranitidine. In addition, HA inhibited  $[^3H]\Delta_4ACh$  release evoked by the  $Ca^{2+}$  ionophore A23187 (Table 1), to a maximal extent of  $40 \pm 6\%$  and with an  $EC_{50}$  value of  $0.03 \pm 0.02 \mu M$  (data not shown). In contrast, HA did not affect either basal  $[^3H]\Delta_4ACh$  release from CHO( $H_2$ ) cells (Table 1) or stimulated release in wild-type CHO (Table 1). Forskolin and prostaglandin  $E_1$ , which raised cAMP levels in CHO (data not shown), or 8-bromo-cAMP did not mimic the effect of HA on  $\Delta_4ACh$  release (Table 1).

The effect of HA on  $[^3H]\Delta_4ACh$  release was likely produced by inhibition of liberation of  $[^3H]\Delta_4ACh$  rather than by enhancement of  $[^3H]\Delta_4ACh$  reincorporation into phospholipids. In agreement, HA (10  $\mu M$ ) did not affect  $[^3H]\Delta_4ACh$  incorporation into phospholipids. After a 30-min incubation in the presence of  $[^3H]\Delta_4ACh$ , CHO( $H_2$ ) cells incorporated into phospholipids  $3715 \pm 76$  cpm per well. No significant change in labeling was seen when cells were exposed to 10  $\mu M$  HA ( $4020 \pm 109$  cpm per well), 4  $\mu M$  A23187 ( $3325 \pm 79$  cpm per well), or HA plus A23187 ( $3370 \pm 94$  cpm per well) (data from one experiment performed in triplicate; similar results were obtained in one additional experiment).

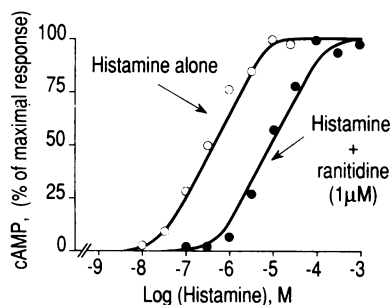


FIG. 4. HA-induced stimulation of cAMP accumulation in CHO( $H_2$ ) cells and its inhibition by ranitidine. cAMP levels in absence and presence of 10  $\mu M$  HA alone were  $0.61 \pm 0.07$  and  $9.7 \pm 1.0$  pmol per well, respectively (mean  $\pm$  SEM of quadruplicates in a single experiment, which was repeated three times). The  $EC_{50}$  values of HA were  $0.2 \pm 0.1$  and  $5.6 \pm 2.4 \mu M$  in the absence and presence of 1  $\mu M$  ranitidine, respectively, leading (20) to an apparent  $K_i$  value of 0.04  $\mu M$ .

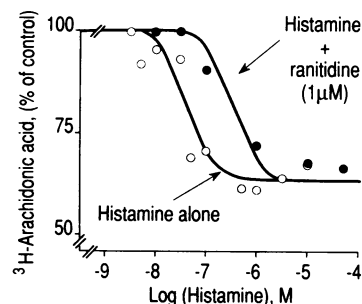


FIG. 5. HA-induced inhibition of evoked  $[^3H]\Delta_4ACh$  release from CHO( $H_2$ ) cells.  $[^3H]\Delta_4ACh$  release, triggered by stimulation of purinergic receptors with 0.1 mM ATP (30-min incubation), was  $2229 \pm 77$  cpm per well. Basal release was  $226 \pm 10$  cpm per well (mean  $\pm$  SEM of quadruplicate determinations from at least three experiments).  $EC_{50}$  values for HA were  $0.04 \pm 0.02 \mu M$  and  $0.3 \pm 0.1 \mu M$  in absence and presence of 1  $\mu M$  ranitidine, respectively, leading (20) to an apparent  $K_i$  value of 0.16  $\mu M$ .

HA (1  $\mu M$ ) did not significantly modify either basal  $[Ca^{2+}]_i$  or  $[Ca^{2+}]_i$  transients induced by 0.1 mM ATP ( $[Ca^{2+}]_i$  was  $388 \pm 30$  nM and  $376 \pm 48$  nM, in the absence and presence of HA, respectively;  $n = 8$ ). HA also failed to affect the basal formation of  $[^3H]$ inositol phosphates ( $178 \pm 4$  cpm and  $183 \pm 6$  cpm in the absence and presence of 3  $\mu M$  HA, respectively;  $n = 4-8$ ). As reported (22), ATP (0.4 mM) stimulated  $[^3H]$ inositol phosphate formation; the effect of ATP was slightly, but significantly ( $+15\%$ ;  $P < 0.01$ ), enhanced by 20  $\mu M$  HA ( $643 \pm 14$  cpm and  $736 \pm 16$  cpm, respectively;  $n = 4-8$ ).

$[^3H]$ Choline release evoked by A23187 (2  $\mu M$ ) remained unaltered in the presence of 10  $\mu M$  HA ( $3836 \pm 97$  cpm and  $4254 \pm 167$  cpm, respectively), as well as basal release ( $2103 \pm 193$  cpm and  $2393 \pm 94$  cpm, respectively;  $n = 4$ ).

## DISCUSSION

The cloned gene encodes a typical  $H_2$  receptor as shown by both tissue distribution of its transcripts and detailed pharmacological characterization.

Northern blot analysis of guinea pig tissues revealed a single band of size similar to that obtained in rat tissues (12). Labeling of this transcript was stronger than in rat, in agreement with the higher density of  $H_2$  receptors in guinea pig revealed with  $^{125}I$ -APT, a highly selective and sensitive probe (7, 23). In brain, regional distribution of the transcript was consistent with that obtained in membrane binding or

Table 1. Effects of HA on  $[^3H]\Delta_4ACh$  release induced by ATP or A23187 in CHO( $H_2$ ) cells

Agent(s)	$[^3H]\Delta_4ACh$ , cpm per well
None	$266 \pm 10$
HA (3 $\mu M$ )	$231 \pm 29$
ATP (100 $\mu M$ )	$2616 \pm 73$
ATP (100 $\mu M$ ) + HA (3 $\mu M$ )	$1844 \pm 53^{**}$
ATP (100 $\mu M$ ) + 8-bromo-cAMP (0.2 mM)	$2926 \pm 100$
ATP (100 $\mu M$ ) + forskolin (0.5 $\mu M$ )	$2652 \pm 100$
ATP (100 $\mu M$ ) + prostaglandin $E_1$ (0.2 $\mu g/ml$ )	$3279 \pm 146^*$
A23187 (2 $\mu M$ )	$4997 \pm 201$
A23187 (2 $\mu M$ ) + HA (3 $\mu M$ )	$3098 \pm 201^{**}$

Cells were preincubated for 10 min in the presence of various agents before the addition of A23187 or ATP and incubated for 30 min. Data are the mean  $\pm$  SEM from at least three experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.001$  as compared to A23187 or ATP alone (Student's  $t$  test). On wild-type CHO, HA did not affect A23187-induced  $[^3H]\Delta_4ACh$  release ( $3809 \pm 89$  vs.  $4010 \pm 112$  cpm per well).

autoradiographic studies with  $^{125}\text{I}$ -APT (7). mRNA was detected in all peripheral tissues where  $\text{H}_2$ -receptor-mediated responses occur (1, 3, 24). Interestingly, the same mRNA was found in both stomach and heart, suggesting that the same molecular species mediates the actions of HA in these tissues. This is consistent with the hypothesis that the marked differences in potency of some  $\text{H}_2$ -receptor antagonists in stomach and heart are related to bioavailability factors rather than to receptor heterogeneity (25, 26). Hence, the absence of corresponding mRNA in dog (8) and rat heart (12) is presumably not due to the existence of a distinct cardiac isoreceptor but rather to the absence of expression of  $\text{H}_2$  receptors in the heart of these two species, as suggested by the lack of HA-induced stimulation of adenylyl cyclase (24).

The pharmacological characterization of  $^{125}\text{I}$ -APT binding sites expressed by CHO cells transfected with pSVH<sub>2</sub> also leaves little doubt about this gene representing that of a typical  $\text{H}_2$  receptor. Thus the  $K_d$  of  $^{125}\text{I}$ -APT at equilibrium and the  $K_i$  values of HA and various antagonists were closely similar to their  $K_B$  values at the reference biological system (1) defining the  $\text{H}_2$  receptor (13, 21) or  $K_i$  values at cerebral  $\text{H}_2$  receptors (7). In agreement, HA and selective  $\text{H}_2$ -receptor agonists markedly enhanced cAMP accumulation in transfected cells, a response blocked competitively by ranitidine with the expected potency. The association of  $\text{H}_2$  receptors with cAMP accumulation (27, 28) and adenylyl cyclase (24, 29, 30) is well established.

The cAMP response elicited by HA in CHO( $\text{H}_2$ ) cells occurred with an  $\text{EC}_{50}$  value about 1000-fold lower than the  $K_i$  value for inhibition of  $^{125}\text{I}$ -APT binding. This large receptor reserve, which likely reflects a high level of expression of  $\text{H}_2$  receptors in these cells, may account for the intrinsic activities displayed by the partial agonists dimaprit and impromidine, which were higher than in brain slices (31). Presence of spare  $\text{H}_2$  receptors has been demonstrated in guinea pig brain slices (7, 32, 33).

Stimulation of cAMP formation is thought to mediate most physiological responses produced by  $\text{H}_2$ -receptor activation (24). We found, unexpectedly, that  $\text{H}_2$ -receptor occupation also resulted in a reduction of evoked  $\Delta_4\text{ACh}$  release from CHO( $\text{H}_2$ ) cells. Maximal inhibition by HA was 40% when [ $^3\text{H}$ ] $\Delta_4\text{ACh}$  release was evoked either by stimulating constitutive purinergic receptors (22) or by increasing [ $\text{Ca}^{2+}$ ]<sub>i</sub> with the  $\text{Ca}^{2+}$  ionophore A23187.

Liberation of  $\Delta_4\text{ACh}$  from membrane phospholipids mainly occurs through either activation of intracellular phospholipases A<sub>2</sub> (PLA<sub>2</sub>) (34) or by hydrolysis of 1,2-diacylglycerol, produced by activation of phosphatidylinositol-specific phospholipase C (35). Transfected  $\text{H}_2$  receptors may reduce evoked  $\Delta_4\text{ACh}$  release by inhibiting PLA<sub>2</sub> activity. In agreement, we found that HA did not affect the incorporation of [ $^3\text{H}$ ] $\Delta_4\text{ACh}$  into phospholipids or the activation of either phosphatidylinositol-specific or phosphatidylcholine-specific phospholipase C (18).

The mechanism of  $\text{H}_2$ -dependent inhibition of  $\Delta_4\text{ACh}$  release, described here, remains unknown. The results suggest, however, that the response was independent of changes in cAMP because it could not be mimicked by agents known to enhance cAMP levels. Furthermore, activation of dopamine D<sub>1</sub> receptors enhances cAMP levels in transfected CHO cells without inhibiting ATP- or A23187-induced [ $^3\text{H}$ ] $\Delta_4\text{ACh}$  release (36).

Receptor-dependent activation of PLA<sub>2</sub> is often associated with elevation of [ $\text{Ca}^{2+}$ ]<sub>i</sub>, even though these two events may be independent (22).  $\text{H}_2$  receptors do not appear to modulate  $\Delta_4\text{ACh}$  release by reducing agonist-induced [ $\text{Ca}^{2+}$ ]<sub>i</sub> increases. (i) HA failed to inhibit  $\text{Ca}^{2+}$  transients evoked by stimulating purinergic receptors. (ii) HA-induced inhibition was also observed when [ $^3\text{H}$ ] $\Delta_4\text{ACh}$  release was evoked by the  $\text{Ca}^{2+}$  ionophore A23187, which increases [ $\text{Ca}^{2+}$ ]<sub>i</sub> bypassing receptor

stimulation. Activation of transfected  $\text{H}_2$  receptors may reduce  $\Delta_4\text{ACh}$  release through a direct inhibition of PLA<sub>2</sub>—i.e., by a mechanism (perhaps mediated by a guanine nucleotide binding protein) similar to that responsible for receptor-linked inhibition of adenylyl cyclase. Thus, the results support the hypothesis that PLA<sub>2</sub> activity may be subjected to both stimulatory and inhibitory control by membrane receptors, as earlier suggested by *in vitro* studies on retinal tissue (37).

The physiological role of  $\text{H}_2$ -receptor-mediated inhibition of  $\Delta_4\text{ACh}$  release remains to be established. The potential importance of this response is indicated by its associated receptor occupancy, which is similar to or lower than that required to affect cAMP levels. Because  $\Delta_4\text{ACh}$  release is the limiting step in eicosanoid biosynthesis, its inhibition by HA is expected to result in decreased formation of these biologically active lipids.

The existence of a signaling pathway for  $\text{H}_2$  receptors, independent of cAMP, might account for a variety of unexplained observations. (i) In brain, the regional distribution of  $^{125}\text{I}$ -APT binding sites does not parallel that of  $\text{H}_2$ -receptor-linked adenylyl cyclase (7). (ii) Denervation hypersensitivity to HA is observed on electrophysiological but not on cAMP responses (38). (iii) The opposing effects of  $\text{H}_1$ - and  $\text{H}_2$ -receptor stimulation observed in many systems are not likely to result from interactions at the level of cAMP generation where, instead, a synergism occurs (32). These opposing actions may well be accounted for by interactions at the level of  $\Delta_4\text{ACh}$  release since, contrary to  $\text{H}_2$  receptors,  $\text{H}_1$  receptors may be positively coupled to release of this fatty acid (39, 40).

In conclusion, the present study does not substantiate the hypothesis of the existence of multiple  $\text{H}_2$  receptors but underscores the emerging view that a single receptor subtype can control several signaling pathways in the same cell.

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