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Histamine Action on Vertebrate $GABA_A$ Receptors

DIRECT CHANNEL GATING AND POTENTIATION OF GABA RESPONSES[*][S]

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Histamine is not only a crucial cytokine in the periphery but also an important neurotransmitter and neuromodulator in the brain. It is known to act on metabotropic H1-H4 receptors, but the existence of directly histamine-gated chloride channels in mammals has been suspected for many years. However, the molecular basis of such mammalian channels remained elusive, whereas in invertebrates, genes for histamine-gated channels have been already identified. In this report, we demonstrated that histamine can directly open vertebrate ion channels and identified β subunits of $GABA_A$ receptors as potential candidates for histamine-gated channels. In *Xenopus* oocytes expressing homomultimeric β channels, histamine evoked currents with an EC_{50} of 212 μM (β_2) and 174 μM (β_3), whereas GABA is only a very weak partial agonist. We tested several known agonists and antagonists for the histamine-binding site of H1-H4 receptors and described for β channels a unique pharmacological profile distinct from either of these receptors. In heteromultimeric channels composed of $\alpha_1\beta_2$ or $\alpha_1\beta_2\gamma_2$ subunits, we found that histamine is a modulator of the GABA response rather than an agonist as it potentiates GABA-evoked currents in a γ_2 subunit-controlled manner. Despite the vast number of synthetic modulators of $GABA_A$ receptors widely used in medicine, which act on several distinct sites, only a few endogenous modulators have yet been identified. We show here for the first time that histamine modulates heteromultimeric $GABA_A$ receptors and may thus represent an endogenous ligand for an allosteric site.

Ligand-gated ion channels mediate the fast responses of cells to neurotransmitters (1). A universal feature of ligand-gated ion channels subunits is a common topology, comprising four membrane-spanning segments (M1-M4) and a huge N-terminal extracellular domain with a hyperconserved cysteine loop motif. In vertebrates, this "Cys loop" family of phylogenetically related genes codes for anion and cation channels activated by acetylcholine and serotonin (cation channels) or GABA[3] and glycine (anion channels) (1, 2). Despite the many years of intensive research on such ion channels, recent reports revealed unexpected new findings about this channel family. In vertebrates, a gene for zinc-gated ion channels was recently discovered (3). In insects, new classes of ligand-gated chloride channels gated by histamine or pH and cation channels gated by GABA were reported (4–7).

Histamine is a neurotransmitter and neuromodulator acting on the metabotropic H1-H4 receptors (8). In addition, a direct activation of mammalian anion channels has been postulated (9). Histamine-containing neurons are found exclusively in the tuberomamillary nucleus of the hypothalamus (10). The GABA-synthesizing enzyme, glutamate acid decarboxylase, and GABA are also seen in most tuberomamillary neurons, and many neurons in the tuberomamillary nucleus contain both GABA and histamine (10). The molecular structure of a potential mammalian histamine-gated channel is still elusive, but in invertebrates, two genes for histamine-gated channels have been identified (4, 5, 11, 12). In addition, GABA and histamine can activate the same ion channel (13). Therefore, we hypothesized that a subtype of mammalian GABA receptors may be a good candidate mediating the effect of histamine in vertebrates. GABA is the major inhibitory neurotransmitter in the central nervous system. Most of the rapid inhibitory neurotransmission in the central nervous system is mediated by the GABA type A receptors (14). $GABA_A$ receptors are heteropentameric proteins constructed of various subunits (α, β, γ, δ, θ, ϵ, and π). The most prominent native receptors are heteromultimers of α, β, and γ subunits (14), but at least in recombinant systems, functional homomultimeric receptors composed of β or γ subunits alone exist (15–19). Such homomultimeric ion channels differ in many aspects from the conventional, heteromultimeric $GABA_A$ receptors and were therefore candidates for receptors with unexpected new properties.

EXPERIMENTAL PROCEDURES

$GABA_A$ cDNAs and RNAs—Rat α_1 and β_1 cDNAs were cloned by PCR-based methods using standard molecular biology procedures. Rat β_2 cDNA was kindly provided by R. Ruppprecht (Munich, Germany). Mouse γ_{2L} and human β_3 cDNA was obtained from ImaGenes (Berlin, Germany). All cDNAs were subcloned into pSGEM (courtesy of M. Hollmann, Bochum, Germany) or pCDNA3 (Invitrogen, Karlsruhe, Germany) for HEK293 cell expression.

Expression of Receptor cRNA in Xenopus Oocytes—cRNAs were synthesized using the AmpliCap T7 high yield message maker kit (Epicenter, Madison, WI), according to the manufacturer's protocol, with PacI-linearized pSGEM plasmids as tem-

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[3] The abbreviations used are: GABA, γ-aminobutyric acid; $GABA_A$, GABA, type A.

plates. *Xenopus laevis* oocytes were prepared by standard methods. After 24 h, stage V–VI oocytes were injected with cRNA (typically 5–25 ng/oocyte), incubated at 16 °C in Barth's solution, and tested for functional expression of $GABA_A$ receptors by two-electrode voltage clamp recording after 3–7 days. Agonists and antagonists were diluted to the concentrations indicated with Frog-Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES, pH 7.2). Recording was done with a two-electrode voltage clamp amplifier (TURBO TEC-03, npi, Tamm, Germany) and pCLAMP software (Axon Instruments, Union City, CA) with typical membrane potential of −40 to −60 mV. The pH of all Ringer's solutions containing histamine was adjusted to pH 7.2 if necessary.

The quality of the water used for solution preparation can be critical for the amount of contamination by traces of Zn^{2+} or Cu^{2+}. Previously it was shown that in native cerebral Purkinje neurons, co-application of histidine enhances GABA-evoked currents by complexing trace amounts of copper ions (20). To exclude that the observed histamine effects depend on the complexation of such divalent cation contaminations, potentiation and dose-response curves experiments were performed in Ringer's solution prepared with ultrapure water (AMPUWA water, Fresenius, Bad Homburg, Germany) as suggested (20).

In experiments targeting the effect of histamine on heteromultimeric receptors, it was vital to ensure the absence of any contaminating population of homomultimeric channels composed of β subunits when expressing $\alpha_1\beta_2\gamma_2$ receptors as such could mimic a histamine effect. In preliminary studies with oocytes injected with a 1:1:1 ratio of $\alpha_1\beta_2\gamma_2$ subunits, in some experiments, small currents were directly evocable by 1 mM histamine, obviously caused by a small, contaminating population of homomultimeric β_2 channels. Therefore, we injected an excess of α_1 and γ_2 over β_2 subunit RNA (ratio α_1:10, β_2: 1, γ_2:2). In these oocytes, 1 mM histamine itself evoked no detectable currents ($\ll$1% of the maximum GABA evoked current), proving the absence of a contaminating population of homomultimeric β_2-subunits. The same is valid for receptors composed of $\alpha_1\beta_2$ subunits, where the α subunit was also injected in a 10-fold excess over β. To ensure the incorporation of the γ_{2L} subunit, oocytes were screened with 10 μM Zn^{2+} in the presence of GABA. Although $\alpha\beta$ subunit combinations are highly sensitive for an inhibition by Zn^{2+}, the $\alpha\beta\gamma$ isoforms are insensitive.

Statistics—For electrophysiological measurements, statistical analysis and curve fitting was done by the Hill equation using SigmaPlot V8.0 (Systat Software, San Jose, CA). All mean values are ± S.E.

Patch Clamp Experiments—HEK293 cells were maintained under standard conditions in a minimum essential medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and streptomycin, and 2 mM L-glutamine. Semiconfluent cells were transfected in 35-mm dishes (BD Biosciences) by using standard calcium phosphate precipitation, 48–72 h after transfection. Recordings were performed using the whole-cell mode of the patch clamp technique. Cells were maintained in an extracellular recording solution containing 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose, pH 7.4. Patch electrodes were pulled from borosilicate glass and fire-polished to 4–6-megaohm tip resistance using a horizontal pipette puller (Zeitz Instruments, Munich, Germany). The pipette solution contained 140 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM EGTA, 10 mM HEPES, pH 7.4 for recordings. Recordings were carried out using a HEKA EPC7 amplifier. Membrane potential was held at −40 mV.

RESULTS

Homomultimeric β Channels—We investigated the action of histamine on homomultimeric $GABA_A$ receptors. Complementary RNAs (cRNA) from β_{2-3} subunits were expressed in *X. laevis* oocytes. 3–7 days after injection, histamine- and GABA-elicited currents were recorded in the voltage clamp configuration. We used the known β channel agonist pentobarbital (15, 16, 18) as a positive control and found that 3 mM histamine could also directly open homomultimeric β_3 channels with an average current amplitude of 536 nA (±95, $n = 15$). When compared with saturating concentrations of pentobarbital, histamine evoked currents were smaller, reaching 67% (±6%, $n = 8$, $p = 0.008$) of the pentobarbital response (Fig. 1*A*). 3 mM histamine also opened homomultimeric β_2 channels, although with lower average amplitudes (132 nA (±21, $n = 7$), data not shown). Dose-response curves show that homomultimeric receptors composed of β_2 or β_3 were similarly sensitive to histamine, with an EC_{50} of 212 ± 29 μM ($n = 4$) and an EC_{50} of 174 ± 14 μM ($n = 11$), respectively (Fig. 1, *B* and *C*). It has been reported that homomultimeric β channels are activated by GABA (15–18). Our experiments expressing β_2 or β_3 confirmed the effect of GABA, but even at high, saturating GABA concentrations (3 mM), the amplitudes were only 10% of the histamine-activated currents (β_3: $I_{GABA\ (3\ mM)}/I_{Histamine\ (10\ mM)} = 0.11 \pm 0.012$ ($n = 10$)). GABA is a weak partial agonist when compared with histamine (Fig. 1*A*). Homomultimeric channels composed of $GABA_A$ β subunits are histamine-gated channels rather than GABA-gated channels. Histamine evoked a current reversing typically at −23 mV under standard ion conditions in oocytes expressing β_3 (Fig. 1*D*), which is consistent with the previously reported chloride selectivity of β channels (15).

The histamine effect is independent on the oocyte expression system. In whole-cell patch clamp experiments on human embryonic kidney 293 (HEK293) cells expressing β_3 channels (Fig. 1*E*), 1 mM histamine evoked currents with an average amplitude of 203 pA (±31, $n = 8$), which was typically 60% of the pentobarbital response (21). The EC_{50} of histamine with 386 μM (±24, $n = 6$) was in the same range as in *Xenopus* oocytes (Fig. 1*F*).

Pharmacology of the Histamine Response—The currents evoked by 1 mM histamine expressed in *X. laevis* oocytes expressing β_3 were completely blocked by 10 μM picrotoxinin, but 100 μM of the GABA-antagonists bicuculline or gabazine were ineffective (data not shown). Histamine analogs such as histidine and *tele*-methylhistamine were also found to be agonists for β_3 channels (Fig. 2*A* and supplemental Figs. 1 and 2), although with lower potencies (EC_{50} 1.14 mM ± 0.15 μM ($n = 5$) and EC_{50} 1.1 mM ± 0.13 μM ($n = 3$), respectively), demonstrating that metabolic precursors and metabolites could also be active agonists. For the histamine-binding sites of metabo-

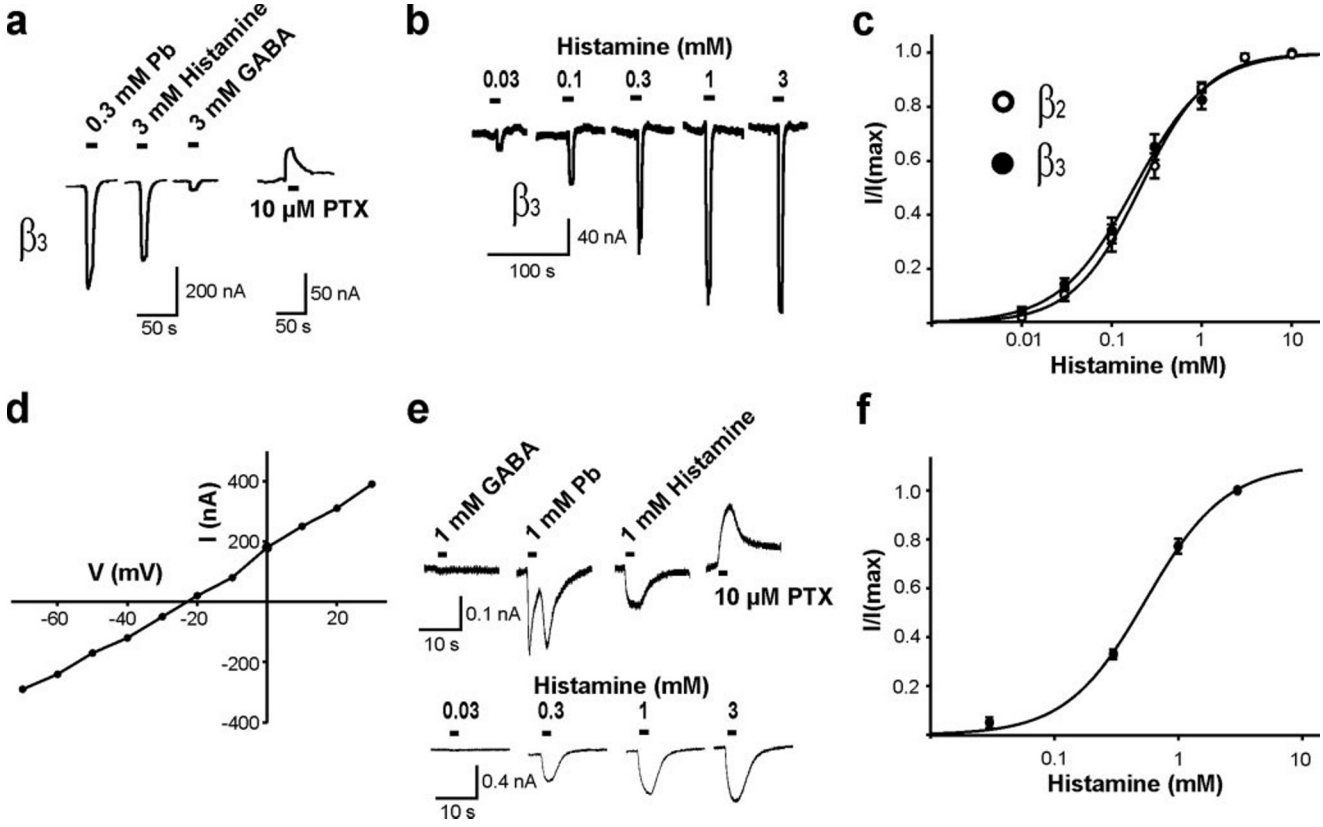

FIGURE 1. **Action of histamine on homomultimeric β channels.** *a–d*, homomultimeric β receptors are activated by histamine. β-Expressing oocytes were voltage-clamped at potentials between −40 and −60 mV, and various histamine concentrations (10 μM–10 mM) were bath-applied. *a*, current induced by histamine, pentobarbital (*Pb*), and GABA in *Xenopus* oocytes expressing β_3 receptors; 10 μM Picrotoxinin (*PTX*) blocked the population of spontaneously open channels, which is typical for β channels (15–18). *b*, dose dependence of β_3 activation. *c*, histamine dose-response curves for β_{2-3}. *d*, I/V relationship of currents induced by 1 mM histamine in an individual oocyte expressing β_3 receptors. *e*, whole cell currents induced by histamine, pentobarbital, and GABA in HEK293 cells expressing β_3 receptors (*upper trace*) and by histamine in increasing concentrations (*lower trace*). The off response after pentobarbital application is typical for saturating concentrations. *f*, histamine dose-response curves for β_3 in HEK293 cells.

tropic H1–H4 receptors, specific agonists and antagonists are known (22). We investigated whether the histamine-binding site of GABA$_A$ β subunits match one of those pharmacological profiles. In oocytes expressing β_3 channels, current evoked by 300 μM histamine was effectively blocked by the H3/4 antagonist thioperamide. Higher concentrations of thioperamide additionally blocked a fraction of spontaneously open channels as indicated by the apparent "outward" current; it thus behaves like an "inverse agonist" (Fig. 2*B*). The IC$_{50}$ was 7.2 ± 0.7 μM ($n = 5$) in the absence of histamine (Fig. 2*D*). In the presence of 300 μM histamine, the IC$_{50}$ for thioperamide was 32 ± 3.8 μM ($n = 5$) determined by a four-parameter Hill fit of the complete blocking curve, but it would be in the range of 10 μM if one would relate it only to the histamine evoked current. Therefore, the found value is only a rough estimate as the population of open channels interferes with the analysis. Next, we tested whether thioperamide and histamine might compete for the same binding site and found that at 10 mM histamine, thioperamide was a far less effective blocker, a clear indication for a competitive mechanism (Fig. 2*C*). The H2 antagonist famotidine (IC$_{50}$ = 154 ± 21 μM ($n = 5$)) and the H1/2 agonist histamine trifluoromethyl toluidide (IC$_{50}$ = 162 ± 19 μM ($n = 5$)) were also found to be blockers of the histamine-evoked current as well as the population of open channels (supplemental Fig. 3).

Other ligands for metabotropic histamine receptors, such as cimetidine (H2 antagonist), pyrilamine (H1 antagonist), and dimaprit (H2 agonist), were ineffective in concentrations up to 500 μM (data not shown). Our data reveal that the histamine-binding site of GABA$_A$ β subunits has a unique pharmacology not matching any of the metabotropic receptors.

Histamine analog H2 blockers act on heteromultimeric GABA$_A$ receptors as reported previously (23, 24). Our findings may explain the molecular basis of this observed action as we found that such molecules directly act on β subunits. However, our findings imply that GABA$_A$ receptors are not specifically blocked by H2 antagonists alone but agonists or antagonists of other metabotropic histamine receptors also act.

The Action of Histamine on $\alpha_1\beta_2\gamma_2$ Receptors—The action of histamine on the β subunits resembles that of allosteric GABA$_A$ modulators such as propofol and barbiturates, which also activate currents at homomultimeric β receptors directly (15, 16, 18). At heteromultimeric GABA$_A$ receptors, such modulators potentiate the action of GABA (25). To investigate the action of histamine on heteromultimeric receptors, we investigated recombinant $\alpha_1\beta_2\gamma_2$ receptors, which are the most abundant GABA$_A$ synaptic receptor type in the central nervous system (14). In oocytes expressing $\alpha_1\beta_2\gamma_2$ receptors, 1 mM histamine potentiated the current evoked by 10 μM GABA (about EC$_{30}$) (Fig. 3*A*). The potentiation at 10 μM GABA was 1.5-fold on

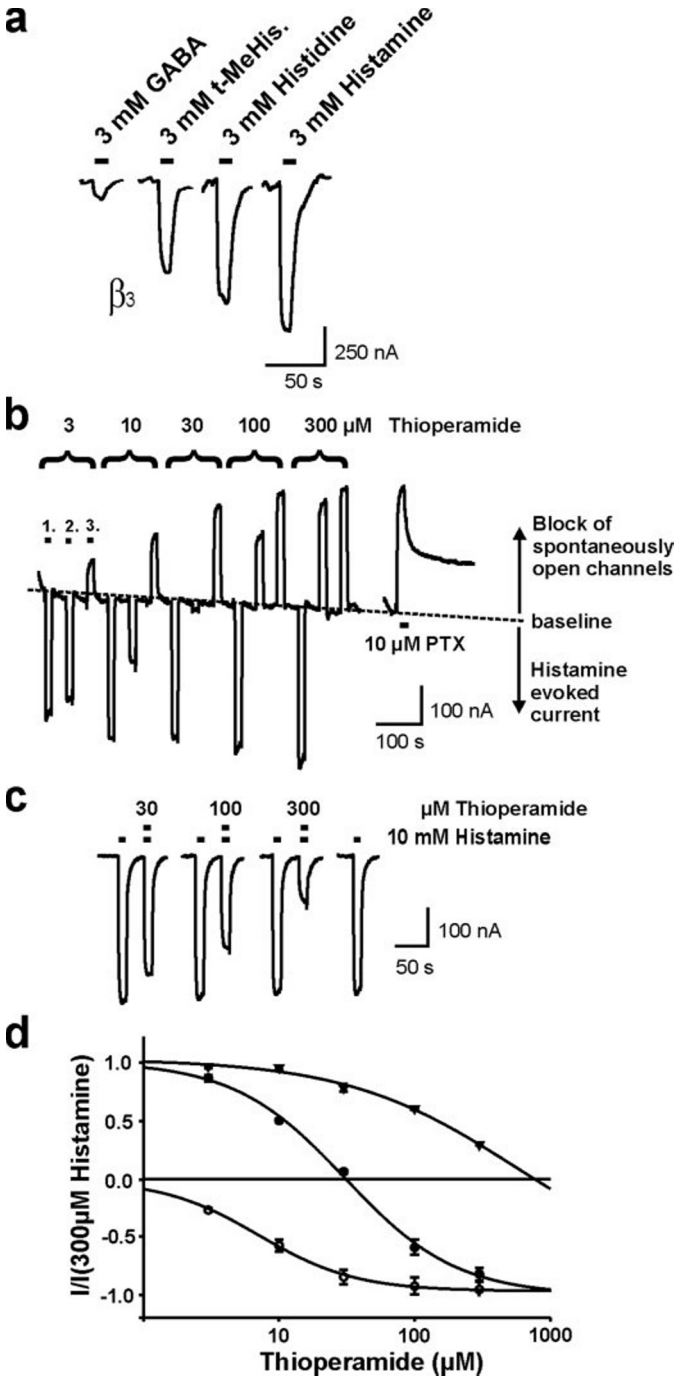

FIGURE 2. *a*, action of histamine analogs on β_3-expressing *Xenopus* oocytes. (*t-MeHis*, *tele*-methylhistamine.) *b*, pharmacology of thioperamide. For each concentration of thioperamide, three consecutive measurements were done as indicated by *1–3* in *b*. *Measurement 1*, 300 μM histamine as control; *measurement 2*, various concentrations of thioperamide co-applied with 300 μM histamine; *measurement 3*, thioperamide alone. Apparent outward currents were caused by block of spontaneously open channels. The amount of spontaneous activity was determined by application of the blocker 10 μM Picrotoxinin (*PTX*). *c*, action of thioperamide on currents evoked by 10 mM histamine. *d*, dose-inhibition curves for thioperamide from measurements as in *b* or *c* (*filled circle*, thioperamide co-applied with 300 μM histamine; *circle*, thioperamide alone; *inverted triangle*, thioperamide co-applied with 10 mM histamine).

average (I(GABA + histamine)/I(GABA) = 1.52 ± 0.43, n = 12) but had a considerable variability, reaching from 0 in few oocytes up to 2.4-fold. In the same set of oocytes, 1 mM histamine was virtually ineffective on the current evoked by saturating concentrations (300 μM) of GABA (1.01 ± 0.06), pointing out that potentiation by histamine was significantly much more effective at submaximal GABA concentrations ($p = 0.0012$, n = 12). This fit to the observation that 1 mM histamine significantly lowered the EC_{50} for GABA from 15.8 ± 2.1 μM to 11.1 ± 1.7 μM ($p = 0.0044$, $n = 5$) (Fig. 3, *A* and *B*). 1 mM histamine itself did not evoke detectable currents (≪1% of the maximum GABA evoked current, Fig. 3*A*).

The histamine potentiation of recombinant $GABA_A$ receptors was independent of the expression system and could also be observed at $\alpha_1\beta_2\gamma_2$ receptors expressed in HEK293 cells. Histamine showed potentiating effects similar to those in oocytes, demonstrating that the effect was not restricted to the oocyte expression system. At cells stimulated with 3 μM GABA, 1 mM histamine evoked an up to 2.0-fold potentiation of the GABA current (1.4-fold potentiation on average; $n = 12$) (Fig. 3*C*), but 1 mM histamine alone never evoked any detectable currents.

The Action of Histamine on $\alpha_1\beta_2$ Receptors—It is known that the presence of a γ_2 subunit modulates the potentiation by allosteric modulators such as benzodiazepines, which are only effective in γ subunit-containing receptors (26). In the case of potentiators acting on β subunits such as propofol, it was reported that the γ_2 subunit alters the mode of potentiation (27). To address the question of whether the presence of a γ_2 subunit alters the potentiation of histamine, we compared the effect of histamine on heteromultimeric $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_2$ receptors. In oocytes expressing $\alpha_1\beta_2$, receptors, 1 mM histamine potentiated currents evoked by GABA. Histamine potentiation was strongly dependent on the GABA concentration but in a different manner as for $\alpha_1\beta_2\gamma_2$ receptors. At $\alpha_1\beta_2$ GABA receptors, 1 mM histamine potentiated best at saturating GABA concentrations (300 μM) but on average did not potentiate at submaximal concentrations of 3 μM GABA (Fig. 3, *D* and *E*). At 300 μM GABA, average potentiation was 1.26-fold (±0.24, n = 14) and significantly greater ($p = 0.013$, $n = 14$) than at 3 μM GABA in the same set of oocytes (1.04 ± 0.07, $n = 14$). Also in these experiments, some oocytes had GABA currents not potentiated by histamine at all. 1 mM histamine itself did not evoke detectable currents (≪1% of the maximum GABA evoked current). The average EC_{50} for GABA was not significantly affected by 1 mM histamine ($p = 0.22$, $n = 4$). These experiments demonstrate that the γ_2 subunit has a vital, modulatory role in histamine potentiation of $GABA_A$ receptors.

Histamine potentiates GABA receptors in a dose-dependent manner. At $\alpha_1\beta_2$ receptors, the EC_{50} of potentiation is 965 μM (±306, $n = 4$) (Fig. 3*F*). The potentiation effect requires higher histamine concentrations as the direct action on homomultimeric β_2 channels.

DISCUSSION

Our study shows that histamine directly opens homomultimeric $GABA_A$ receptors that thus can function as histamine-gated channels. The existence of such histamine-gated chloride channels in mammals has been suggested for a long time (4, 9). The putative histamine receptor described by Hatton and Yang (9) in the *nucleus supraopticus* shares some pharmacological similarities with homomultimeric β channels (*e.g.* the PTX sen-

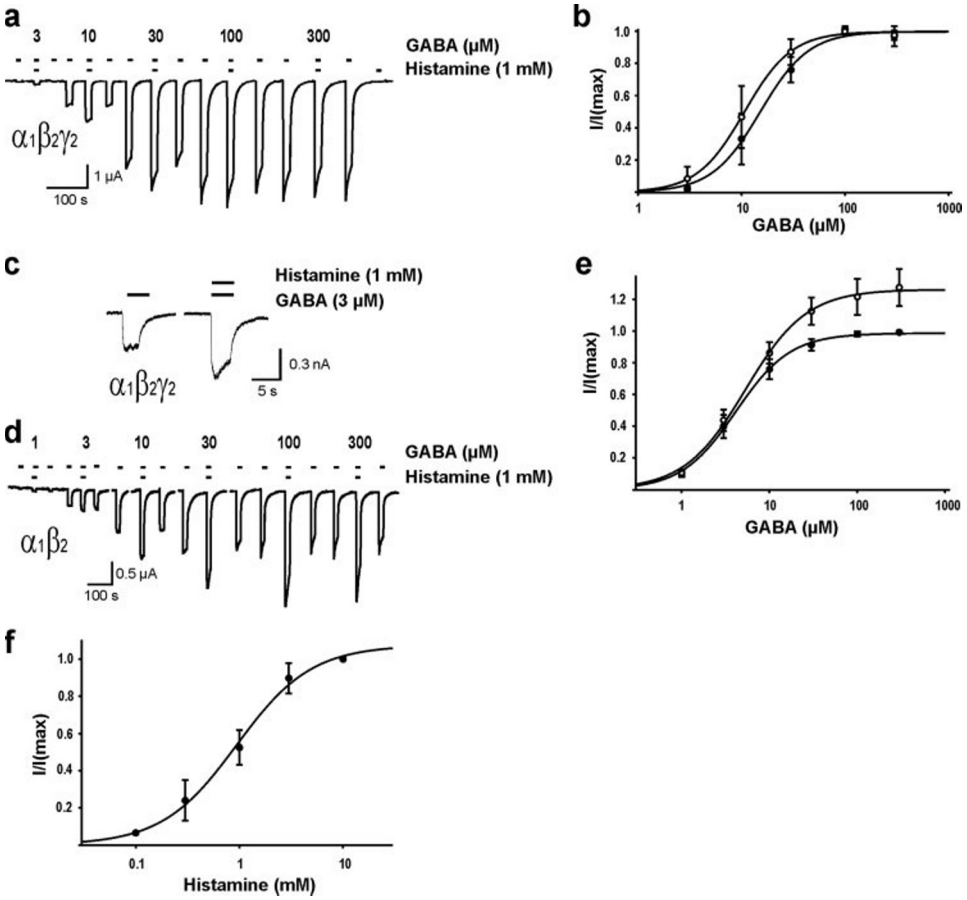

FIGURE 3. **Histamine potentiation of GABA-evoked currents.** The potentiation of GABA responses by histamine was measured. *a*, $\alpha_1\beta_2\gamma_2$-expressing *Xenopus* oocytes were voltage-clamped, and various GABA concentrations with or without 1 mM histamine were bath-applied. *b*, the effect of 1 mM histamine on the dose-response curve for GABA in oocytes expressing $\alpha_1\beta_2\gamma_2$. Data points are the mean of five individual oocytes (*filled circle*, GABA; *circle*, GABA + 1 mM histamine). *c*, potentiation of GABA responses in $\alpha_1\beta_2\gamma_2$-expressing HEK293 cells measured by whole-cell patch clamp experiments. *d*, action of 1 mM histamine on GABA-evoked currents in *Xenopus* oocytes expressing $\alpha_1\beta_2$ receptors. *e*, the effect of 1 mM histamine on the dose-response curve for GABA in oocytes expressing $\alpha_1\beta_2$. Data points are the mean of five individual oocytes (*filled circle*, GABA; *circle*, GABA + 1 mM histamine). *f*, dose-response curve for histamine potentiation of currents evoked by 300 μM GABA in oocytes expressing $\alpha_1\beta_2$. Data points are the mean of four individual oocytes.

sitivity). However, there are also some pronounced differences (*e.g.* the affinity for histamine and the sensitivity to cimetidine), suggesting that the native receptor may need additional components. When compared with the high affinity of metabotropic histamine receptors, the EC_{50} of β channels with ~200–400 μM is quite low, and they are not expected to be activated by typical extracellular histamine concentrations in the brain. However, at synaptic transmission, high enough concentrations could be reached in the synaptic cleft as histamine concentration in synaptic vesicles be as high as 670 mM (28). Nevertheless, our findings support the idea that $GABA_A$ subunits may be vital parts of potential native mammalian histamine-gated channels and could provide the histamine-binding site.

Further, we demonstrated that histamine potentiates GABA responses in heteromultimeric receptors and thereby identified a new type of allosteric potentiator for $GABA_A$ receptors. The mode of potentiation resembles anesthetics such as propofol; like propofol, it targets β subunits and opens homomultimeric β channels directly but modulates the GABA response at heteromultimeric channels (15, 16, 18, 27, 29, 30). Also the influence of the γ_2 subunit is similar. In receptors composed of $\alpha_1\beta_2$ subunits, both histamine and propofol potentiate effectively at saturating GABA concentrations, increasing the maximally evoked currents. In contrast, at $\alpha_1\beta_2\gamma_2$ receptors, histamine and propofol were noneffective at high concentrations but are shifting the GABA dose-response curve leftwards (30). In our oocyte expression system, we found that at recombinant heteromultimeric receptors, the strength of histamine potentiation was quite variable, an indication that potentiation might be regulated for example posttranscriptionally by a yet unknown mechanism. Such variability of receptor properties in recombinant expression systems is often observed and can have several reasons: for example, differences in expression level (31), receptor clustering (32), and different amounts of γ subunits relative to α or β in $\alpha\beta\gamma$ $GABA_A$ receptors (33), as well as different receptor phosphorylation, just to mention a few. Interestingly, phosphorylation regulates $GABA_A$ receptor potentiation by neurosteroids (34). In contrast to the variability of the histamine effect on heteromultimeric channels, exclusively all measured homomultimeric β channels responded to histamine, and none were found that only respond to GABA or pentobarbital.

For $GABA_A$ receptors, about 10 different sites for allosteric modulators are known including neurosteroids, benzodiazepines, general anesthetics, and ethanol (25, 35). With the exception of neurosteroids, no endogenous modulators have been identified so far. Our findings that histamine potentiates GABA action on $GABA_A$ receptors suggests that it is an endogenous ligand for an allosteric site located on the β subunits. Therefore, our results suggest an additional function for histamine *in vivo*, apart from the action on metabotropic histamine and *N*-methyl-D-aspartic acid receptors (36). All histaminergic neurons in the mammalian brain are found in the tuberomamillary nucleus and send axons to almost all parts of the central nervous system (10). Some of these neurons contain both GABA and histamine. In addition, histamine could diffuse out of a histaminergic synapse by a "spillout" effect as described for GABA-ergic synapses (37) and thus may act on neighboring synaptic or extrasynaptic $GABA_A$ receptors. Further, mast cells in the brain are a source for histamine. Mast cells occur in the central nervous system of many species, and up to 50% of the brain histamine is attributable to the presence of these cells. By

direct gating of channels or by affecting $GABA_A$ receptor currents, histamine should modulate processes in which rapid GABA-evoked currents participate (8).

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