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Proton sensitivity of the $GABA_A$ receptor is associated with the receptor subunit composition

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1. Modulation of $GABA_A$ receptors by external H^+ was examined in cultured rat sympathetic neurones, and in *Xenopus laevis* oocytes and human embryonic kidney (HEK) cells expressing recombinant $GABA_A$ receptors composed of combinations of $\alpha 1$, $\beta 1$, $\beta 2$, $\gamma 2S$ and δ subunits.
2. Changing the external pH from 7·4 reduced GABA-activated currents in sympathetic neurones. pH titration of the GABA-induced current was fitted with a pH model which predicted that H^+ interact with two sites (pK_a values of 6·4 and 7·2).
3. For $\alpha 1\beta 1$ $GABA_A$ receptors, low external pH (< 7·4) enhanced responses to GABA. pH titration predicted the existence of two sites with pK_a values of 6·6 and 7·5. The GABA concentration–response curve was shifted to the left by low pH and non-competitively inhibited at high pH (> 7·4).
4. $\alpha 1\beta 1\gamma 2S$ receptor constructs were not affected by external pH, whereas exchanging the $\beta 1$ subunit for $\beta 2$ conferred a sensitivity to pH, with predicted pK_a values of 5·16 and 9·44.
5. Low pH enhanced the responses to GABA on $\alpha 1\beta 1\delta$ subunits, whilst high pH caused an inhibition (pK_a values of 6·6 and 9·9). The GABA concentration–response curves were enhanced (pH 5·4) or reduced (pH 9·4) with no changes in the GABA EC_{50}.
6. Immunoprecipitation with subunit and epitope-specific antisera to $\alpha 1$, $\beta 1$ and δ subunits demonstrated that these subunits could co-assemble in cell membranes.
7. Expression of $\alpha 1\beta 1\gamma 2S\delta$ constructs resulted in a 'bell-shaped' pH titration relationship. Increasing or decreasing external pH inhibited the responses to GABA.
8. The pH sensitivity of recombinant $GABA_A$ receptors expressed in HEK cells was generally in accordance with data accrued from *Xenopus* oocytes. However, rapid application of GABA to $\alpha 1\beta 1$ constructs at high pH (> 7·4) caused an *increased* peak and reduced steady-state current, with a correspondingly increased rate of desensitization.
9. Modulation of $GABA_A$ receptor function was apparently unaffected by the internal pH. Moreover, pH values between 5 and 9·5 did not significantly affect the charge distribution on the zwitterionic GABA molecules.
10. In conclusion, this study demonstrates that external pH can either enhance, have little effect, or reduce GABA-activated responses, and this is apparently dependent on the receptor subunit composition. The potential importance of H^+ sensitivity of $GABA_A$ receptors is discussed.

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter present in the mammalian CNS, a role that is reliant upon the almost ubiquitous distribution of 'type A' GABA receptors. Previous molecular cloning studies have established that $GABA_A$ receptors are hetero-oligomeric proteins composed of assemblies of different subunit polypeptides. On the basis of amino acid sequence identities, these subunits are characterized into four

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discrete subunit families, the majority possessing multiple members, designated as: $\alpha(1-6)$, $\beta(1-4)$, $\gamma(1-3)$ and $\delta(1)$ (Sieghart, 1995). Despite detailed knowledge regarding the primary amino acid sequences for individual subunits, the composition of any native $GABA_A$ receptor is unknown; although, receptor expression studies indicate that combinations of α, β and γ subunits form functional ion channels capable of emulating many of the pharmacological and physiological properties observed previously for native neuronal $GABA_A$ receptors (Sigel, Baur, Trude, Mohler & Malherbe, 1990; Verdoorn, Draguhn, Ymer, Seeburg & Sakmann, 1990).

$GABA_A$ receptors represent an important locus for the action of many drugs (e.g. benzodiazepines and barbiturates) that modulate receptor function by binding to numerous allosteric sites on the receptor protein (Macdonald & Olsen, 1994; Smart, 1995). Most of these modulatory sites have been uncovered by the use of exogenous agents, for which there are as yet no clear examples of endogenous counterparts (with the possible exception of the pregnane- and androstane-based steroids; Macdonald & Olsen, 1994; Smart, 1995). However, an important feature of the pharmacological regulation of $GABA_A$ receptors is their sensitivity to modulation by cations normally present *in vivo*, including, Zn^{2+} (Smart, Xie & Krishek, 1994) and H^+ (Kaila, 1994). Zn^{2+} inhibits GABA-activated responses in a variety of neuronal preparations (Smart *et al.* 1994; Harrison & Gibbons, 1994) with IC_{50} or K_d values ranging from approximately 11 to 320 μM (Mayer & Vyklicky, 1989; Nakagawa, Wakamori, Shirasaki, Nakaye & Akaike, 1991). These inhibitory actions are not observed on all neuronal preparations, particularly brain slices (Smart *et al.* 1994). Moreover, responses to GABA recorded from recombinant $GABA_A$ receptors are differentially sensitive to Zn^{2+} depending on the subunit composition. Receptors composed of $\alpha1\beta1$ or $\alpha1\beta2$ subunits are substantially inhibited compared with $\alpha1\beta1\gamma2S$ or $\alpha1\beta2\gamma2S$ subunit combinations (Draguhn, Verdoorn, Ewert, Seeburg & Sakmann, 1990; Smart, Moss, Xie & Huganir, 1991; Smart *et al.* 1994).

Protons are also potential endogenous modulators of $GABA_A$ receptors, with a ubiquitous distribution in the CNS compared with Zn^{2+}. At present, by increasing the extracellular H^+ concentration, responses to GABA recorded from dorsal root ganglia can be enhanced (Gallagher, Nakamura & Shinnock-Gallagher, 1983), whereas inhibition has been reported for spinal or reticulospinal neurones and sympathetic ganglia (Groul, Barker, Huang, MacDonald & Smith, 1980; Smart, 1992). In contrast, H^+ appeared to have little effect on responses to GABA recorded from hippocampal neurones (Tang, Dichter & Morad, 1990). These differential effects of H^+ could conceivably result from heterogeneity in the $GABA_A$ receptor structure. The present study addresses this issue by using heterologous expressions systems to elucidate the variety of effects H^+ may have on $GABA_A$ receptors and postulates further, from titration analyses, the pK_a values of the potential H^+ binding sites. These pK_a values have been used to tentatively identify which amino acid residues may be responsible for conferring a sensitivity to H^+ upon the receptor structure.

METHODS

Cell preparation

***Xenopus laevis* oocytes.** *Xenopus laevis* were anaesthetized by immersion in a 0·2% (w/v) solution of ethyl-*m*-aminobenzoate (Tricaine). Stage IV–VI *Xenopus* oocytes were surgically removed and microinjected with murine cDNAs corresponding to $\alpha1$, $\beta1$, $\beta2$, $\gamma2S$ and δ $GABA_A$ receptor subunits and cultured in a modified Barth's medium (MBM) comprising (mM): 110 NaCl, 1 KCl, 2·4 $NaHCO_3$, 7·5 Tris-HCl, 0·33 $Ca(NO_3)_2$, 0·41 $CaCl_2$, 0·82 $MgSO_4$; plus 50 $\mu g\ ml^{-1}$ gentamicin; pH 7·6; as previously described (Krishek, Xie, Blackstone, Huganir, Moss & Smart, 1994). All the *Xenopus* oocyte experiments were performed at room temperature (18–23 °C). Cells were used after 2 days and possessed membrane potentials of −30 to −60 mV and input resistances of 1–5 MΩ.

Human embryonic kidney (HEK) cells. HEK cells (ATCC CRL1573) were grown in Dulbecco's modified Eagle's medium and Ham's F12 medium (Gibco) supplemented with 10% fetal calf serum, 0·12% (w/v) $NaHCO_3$, 100 u ml^{-1} penicillin G and 100 $\mu g\ ml^{-1}$ streptomycin at 37 °C in 95% air–5% CO_2 (Smart *et al.* 1991). Exponentially growing cells were subjected to calcium phosphate co-transfection (Krishek *et al.* 1994) using equimolar ratios of cDNAs, corresponding to murine $GABA_A$ receptor subunits $\alpha1$, $\beta1$, $\gamma2S$ and δ. HEK cells were used for whole-cell recording from 18 to 72 h after transfection and had membrane potentials ranging from −20 to −65 mV.

Superior cervical ganglionic neurones. Sprague–Dawley embryonic rats (embryonic day E19–21) were removed from adult female rats previously anaesthetized by halothane inhalation, and killed by exsanguination. Cultured embryonic rat sympathetic ganglion neurones were prepared as described previously (Smart, 1992). Briefly, dissociated neurones were grown on a laminin substratum in Leibovitz's medium (L-15; Gibco) supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 0·6% (w/v) glucose, 0·19% (w/v) $NaHCO_3$, 100 u ml^{-1} penicillin G, 100 $\mu g\ ml^{-1}$ streptomycin and 50 ng ml^{-1} 7S nerve growth factor. Neurones were incubated at 37 °C in 95% air–5% CO_2. Neurones were used after 3–7 days *in vitro* and possessed membrane potentials of −45 to −63 mV and action potential amplitudes of 75 to 110 mV.

Electrophysiology

Intracellular recording. Membrane currents were recorded from *Xenopus laevis* oocytes, retaining their follicular cell envelope, using a two-electrode voltage clamp method. Oocytes were superfused at 8–10 ml min^{-1} (bath volume, 0·5 ml) with an amphibian Ringer solution containing (mM): 110 NaCl, 2 KCl, 5 Hepes, 1·8 $CaCl_2$; pH 7·4. The microelectrodes were filled with 3 M KCl (voltage) and 0·6 M K_2SO_4 (current), providing resistances of 5–10 and 1–2 MΩ, respectively. An Axoclamp 2A amplifier was used in voltage clamp mode and all data were recorded on a Gould 2200 ink-jet pen recorder.

Whole-cell recording. Experiments on HEK cells and cultured neurones were performed using a List EPC7 amplifier in the whole-cell recording mode. Patch electrodes (1–5 MΩ) were filled with a solution containing (mM): 140 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 Hepes, 11 EGTA and 2 adenosine triphosphate. The cells were continuously superfused with a Krebs solution containing (mM): 140 NaCl, 4·7 KCl, 1·2 $MgCl_2$, 2·5 $CaCl_2$, 10 Hepes and 11 glucose. Series resistance compensation of 80% was routinely achieved. Membrane currents were filtered at 10 kHz (−3 dB, 6-pole Bessel filter, 36 dB $octave^{-1}$) and recorded on a Racal store 4D FM tape recorder (DC to 5 kHz) and a Brush-Gould 2200 ink-jet pen recorder.

Analysis of GABA-activated membrane conductance

GABA-induced membrane conductance (ΔG_{norm}) was calculated in *Xenopus* oocytes by subtraction from the resting membrane conductance and normalized to the response induced by 10 μM GABA at pH 7·4. Conductances were determined by briefly stepping the membrane potential from a holding potential range of −30 to −40 mV with a small amplitude voltage command step (1 s duration, −10 mV amplitude, 0·2 Hz frequency) in the absence and presence of GABA. These data were used to construct equilibrium concentration–response relationships for GABA. The data were fitted with a logistic state function of the form:

$$\Delta G/\Delta G_{max} = 1/[1 + (EC_{50} / [A])^{n_H}],$$

where ΔG and ΔG_{max} represent the normalized GABA-induced conductance at a given concentration and the maximum conductance induced by a saturating concentration of GABA, respectively. EC_{50} defines the concentration of GABA ([A]) which induces a 50% of the maximum response and n_H is the Hill coefficient.

Vector construction and immunoprecipitations

The murine α1, β1, β2, γ2S and δ subunit cDNAs were incorporated into the mammalian expression vector pGW1 (British Biotechnology) and have been described previously (Krishek *et al.* 1994). The murine β2 subunit cDNA is identical to that of the rat; this was cloned as an *Eco*RI fragment into pGW1. The 9E10 epitope (EQKLISDEEL; Evans, Lewis, Ramsay & Bishop, 1985) was added between amino acids 4 and 5 of the mature δ subunit by site-directed mutagenesis using the oligonucleotide CATTGCCCT-GGCGCCCAGATCCTCTTCGGAGATCAGTTTCTGCTCATGGT-GCGGCTGCGT. The fidelity of the final expression construct was confirmed by DNA sequence analysis. Exponentially growing HEK cells were transfected with equimolar ratios of plasmid DNA. Forty-eight hours after transfection the cells were labelled with ^{35}S-methionine (0·5 mCi ml^{-1}, ICN Translabel) in methionine-free medium for 4 h. GABA$_A$ receptors were then purified by immunoprecipitation using rabbit polyclonal antisera against the α1 and β1 subunits (Krishek *et al.* 1994), or 9E10 (Evans *et al.* 1985) antiserum directed against the 9E10 tagged δ subunit. Immunoprecipitates were resolved by SDS–PAGE using 8% gels followed by fluorography. In some experiments immunoprecipitates were immunoblotted with 9E10 antiserum with detection enhanced by chemiluminescence (ECL).

Measurement of intracellular pH

Intracellular pH changes were monitored by fluorescence using the pH indicator 2′,7′-bis(carboxyethyl)-5-carboxyfluorescein (BCECF). *Xenopus* oocytes were incubated with a membrane-permeant form of BCECF (BCECF AM, Calbiochem) for at least 1 h prior to recording. In these cells the fluorescent signal was calibrated using an *in vitro* calibration curve for BCECF (Rink, Tsien & Pozzan, 1982). HEK cells were loaded with the free acid form of BCECF by diffusion from the patch pipette. Any changes in the fluorescent signals in the HEK cells were converted to equivalent pH changes using the calibration method of Eisner, Kenning, O'Neill, Pocock, Richards & Valdeolmillos (1989).

Drugs and solutions

Drugs and solutions were bath applied in the *Xenopus* oocyte experiments. For rapid application to the HEK cells and neurones, solutions were applied close to (100–200 μm) the cells using a Y-tube manufactured from glass electrode tubing. Solutions were driven through the Y-tube in response to a vacuum. Drug application was achieved by isolating the vacuum using a solenoid valve, thereby allowing solution to flow out of the Y-tube over the recorded cells by gravity. A complete exchange of solution around the cells was effected within 10–50 ms.

pH model

The pH titration data were fitted according to the following function:

$$\Delta G = f\left(\frac{K_{a1}(nK_{a2} + 2m[H^+]) + l[H^+]^2}{[H^+]^2 + K_{a1}(2[H^+] + K_{a2})}\right).$$

This function was derived by assuming that the receptor protein can be represented as a weak diprotic acid and therefore possesses at least two sites for binding H^+ that influence the GABA-activated conductance. The normalized GABA-activated conductance is then most simply related to the three states of the receptor (uncharged, mono- and divalent anions) as a function of the relative titration of the 'diprotic' receptor protein. The terms l, m and n are the relative contributions each form of the receptor protein (P) makes to the overall titration curve, where l weights the undissociated receptor protein (PH_2), m the monovalent anion (PH^-) and n the divalent anion (P^{2-}). This function provided estimates of the pK_a values (pK_{a1} and pK_{a2}, where p$K_{aj} = -\log K_{aj}$, and K_{aj} represents an acid dissociation constant). The experimental data were fitted with this function using a non-linear least-squares Marquardt–Levenburg routine. The application of acid–base theory to a diprotic acid probably represents an oversimplification of the titration by H^+ of the GABA$_A$ receptor protein. Nevertheless, it demonstrates that different pH sensitivities of GABA$_A$ receptors can be simply accounted for by the binding of H^+ to very few sites.

RESULTS

Proton sensitivity of sympathetic GABA$_A$ receptors

To illustrate the sensitivity of some native neuronal GABA$_A$ receptors to external H^+ and also to assess the appropriateness of a pH model used to fit the experimental data in the pH titration plots (see Methods), GABA-activated membrane currents were recorded from cultured sympathetic neurones and exposed to varying concentrations of H^+. Deviations in the external pH, either side of 7·4, resulted in inhibition of the responses to GABA (Fig. 1*A*). The titration of the 10 μM GABA-induced chloride current against external pH (pH titration relationship), between pH 5·4 and 9·2, yielded a complex relationship described by at least two pK_a values of 6·4 ± 0·02 and 7·2 ± 0·4 (n = 3 neurones; Fig. 1*B*).

Modulation of recombinant $GABA_A$ receptors by external pH: expression in *Xenopus laevis* oocytes

To examine what might underlie the variation in the pH sensitivity of GABA-activated responses recorded from different types of native neurones, recombinant $GABA_A$ receptors were expressed in *Xenopus laevis* oocytes by microinjecting the nuclei with equal ratios of murine cDNAs corresponding to the following subunit compositions: $\alpha1\beta1$, $\alpha1\beta1\gamma2S$, $\alpha1\beta2\gamma2S$, $\alpha1\beta1\delta$ and $\alpha1\beta1\gamma2S\delta$. All five prospective receptor subunit combinations resulted in the formation of functional GABA-activated ion channels. Changing the external pH resulted in variations in the resting membrane conductance of the oocytes, which possibly reflects the pH sensitivity of endogenous potassium channels (Woodward & Miledi, 1992). Lowering the pH reduced the resting membrane conductance, whereas increasing the pH produced the converse effect (Fig. 2*A*). However, oscillatory Ca^{2+}-dependent Cl^- currents were not induced by acidic pH perturbations in this series of experiments (cf. Woodward & Miledi, 1992).

In oocytes expressing $\alpha1\beta1$ $GABA_A$ receptor constructs, the whole-cell chloride conductance activated by bath-applied GABA (10 μM) was potentiated by 73·3 ± 10·1% (mean ± S.E.M.) after lowering the pH of the external medium from 7·4 to 5·4. In contrast, increasing the pH from 7·4 to 9·4 resulted in a small reduction (36·3 ± 11·3%) in the response to GABA (Fig. 2*A*). The pH titration relationship for the GABA-activated Cl^- conductance revealed a sigmoidal plot which could be accounted for by assuming two discrete sites were titrated on the $\alpha1\beta1$ subunit combination with apparent pK_a values of 6·6 ± 0·1 and 7·5 ± 0·1 (Fig. 2*B*). The effect of external pH on the interaction of GABA with this recombinant construct was studied using equilibrium concentration–response curves. Lowering the external pH from 7·4 to 5·4 resulted in a leftward displacement in the curve with an increased maximum response to saturating concentrations of GABA (1 mM; Fig. 2*C*). In comparison, increasing the pH to 9·4 produced a small leftward shift with a concurrent non-competitive depression in the curve. For receptors existing *in vivo* as $\alpha1\beta1$ constructs, this suggests that at a

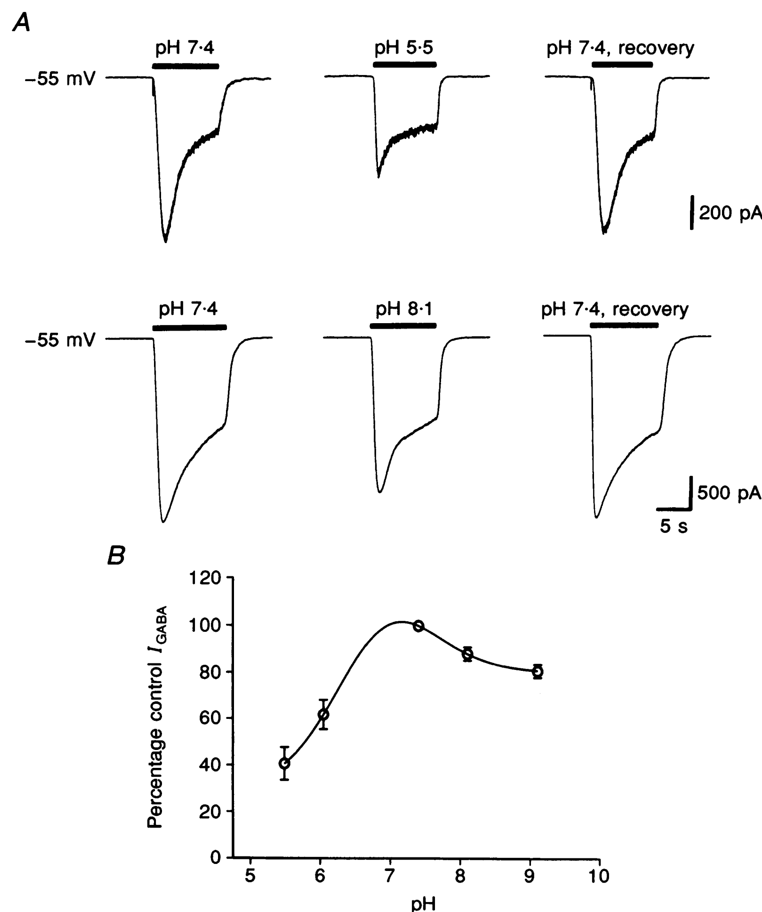

Figure 1. Modulation of GABA-activated currents in cultured rat sympathetic neurones by H^+

A, membrane currents activated by 10 μM GABA (duration indicated by the bar) and recorded under whole-cell voltage clamp at −55 mV. Records are from 2 neurones at pH 7·4 and after exposure to either pH 5·5 (upper trace) or pH 8·1 Krebs solution (lower trace), and following recovery (5 min) at pH 7·4. *B*, pH titration relationship for the GABA-activated inward current from 3 neurones normalized to the response obtained at pH 7·4. The data were fitted with the pH model as described in Methods.

physiological pH of 7·4, GABA$_A$ receptor function is achieving only approximately 50% of its maximum potential 'Cl$^-$ conducting' activity. The apparent pK_a values are close to pK_a values for histidine residues (Mahler & Cordes, 1971), perhaps suggesting a role for these amino acids in modulating the GABA-activated Cl$^-$ conductance. To assess whether H$^+$ ions were affecting the GABA-activated conductance via a voltage-dependent mechanism, current–voltage (I–V) relationships were determined from the steady-state responses to 10 μM GABA at pH values of 5·4 and 7·4. Both I–V relationships displayed outward rectification (Fig. 2C) with low pH reducing the slope or chord conductances in a voltage-insensitive manner.

Whether the pH sensitivity of the α1β1 GABA$_A$ receptor was uniquely dependent on these particular subunits was addressed by adding the short form (Sieghart, 1995) of the γ2 subunit (γ2S) forming the combination α1β1γ2S. Either

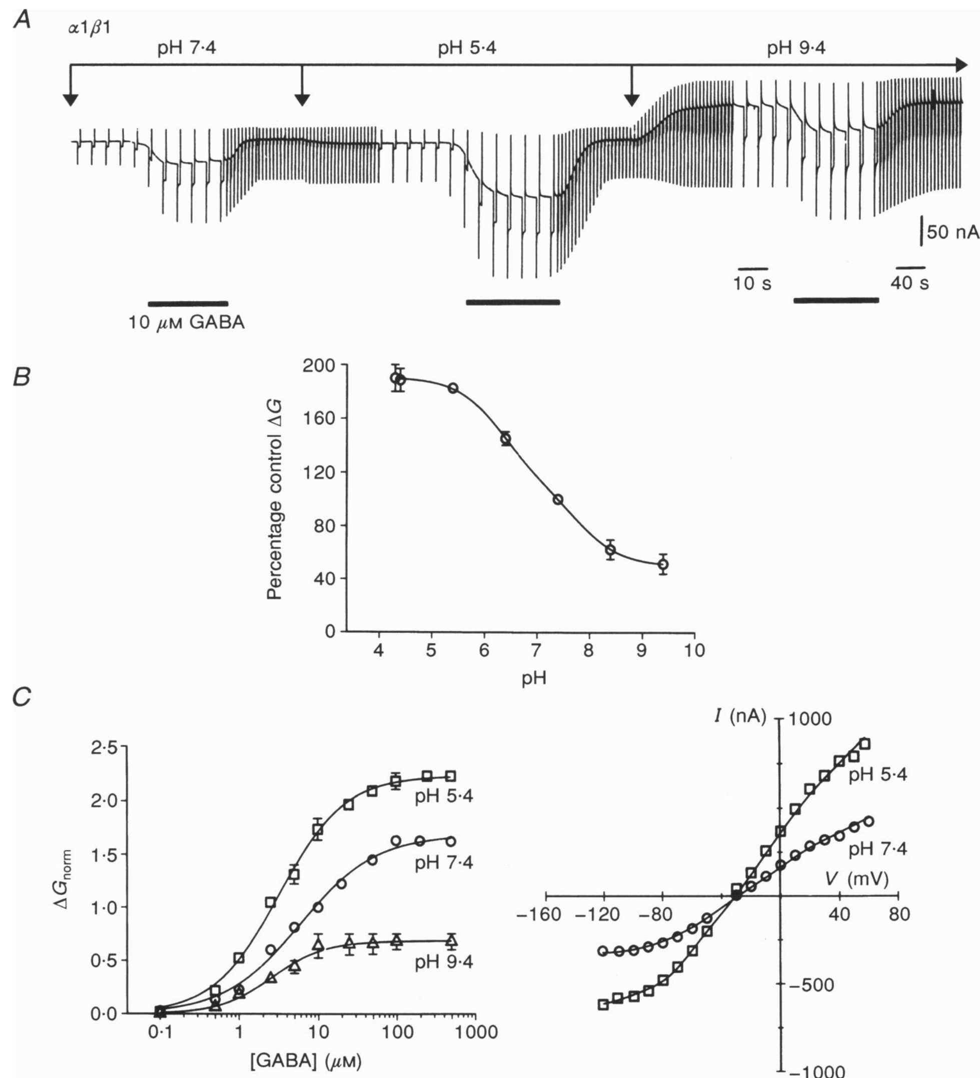

Figure 2. Recombinant α1β1 subunit-containing GABA$_A$ receptors and external pH

A, whole-cell responses to bath-applied 10 μM GABA at external pH values of 7·4, 5·4 and 9·4 in oocytes expressing α1β1 GABA$_A$ receptor constructs. Membrane conductance was monitored by brief hyperpolarizing voltage commands (−10 mV, 1 s, 0·2 Hz) from a holding potential of −40 mV. Note that the resting membrane conductance in all oocytes decreased at low pH and increased at high pH. B, pH titration relationship for the conductance activated by 10 μM GABA and normalized to the conductance at pH 7·4. All points are means ± S.E.M. The data (n = 5) were fitted with the pH model. C, equilibrium concentration–response curves (left) for GABA at pH values of 7·4, 5·4 and 9·4 were normalized to the response induced by 10 μM GABA at pH 7·4. The data were fitted to the logistic equation (see Methods). The EC$_{50}$ values for GABA were: pH 7·4, 5·9 ± 0·6 μM; pH 5·4, 3·2 ± 0·2 μM; pH 9·4, 2·6 ± 0·3 μM (n = 3). Current–voltage relationships at pH 5·4 and 7·4 (right) were determined during the steady-state phases of responses to 10 μM GABA. The data were leak subtracted and the curves were generated using a 3rd order polynomial. The GABA response reversal potential was unaffected by lowering the external pH (pH 7·4, −29·3 mV; pH 5·4, −30·1 mV).

lowering or increasing the pH (range, 4·4–9·4) from the control value of 7·4, did not substantially affect the response to 10 μM GABA (Fig. 3*A* and *C*). Moreover, both the GABA concentration–response curve and the *I–V* relationship were unaffected by external pH over the range 5·4–9·4 (Fig. 3*B*). The principal receptor construct expressed after microinjection of the cDNAs is likely to be $\alpha1\beta1\gamma2$S since responses to GABA could now be enhanced by benzodiazepines (1 μM flurazepam) but were markedly less sensitive to inhibition by 10 μM Zn^{2+}. These properties are features of $\gamma2$ subunit-containing $GABA_A$ receptors (Pritchett *et al.* 1989; Draguhn *et al.* 1990; Smart *et al.* 1991). It is conceivable that homomeric $GABA_A$ receptors, or receptors formed from various binary combinations of subunits, could complicate the interpretation of pH sensitivity of GABA-activated membrane currents. However, this tenet is considered unlikely, as the injection of $\alpha1$, $\beta1$ or $\gamma2$S cDNAs alone, or $\alpha1+\gamma2$S or $\beta1+\gamma2$S binary combinations of cDNAs, routinely failed to produce functional GABA-operated ion channels in our experiments using either *Xenopus laevis* oocytes or HEK cells as the *in vitro* expression system (Angelotti & Macdonald, 1993; Krishek *et al.* 1994; although cf. Sigel *et al.* 1990; Verdoorn *et al.* 1990).

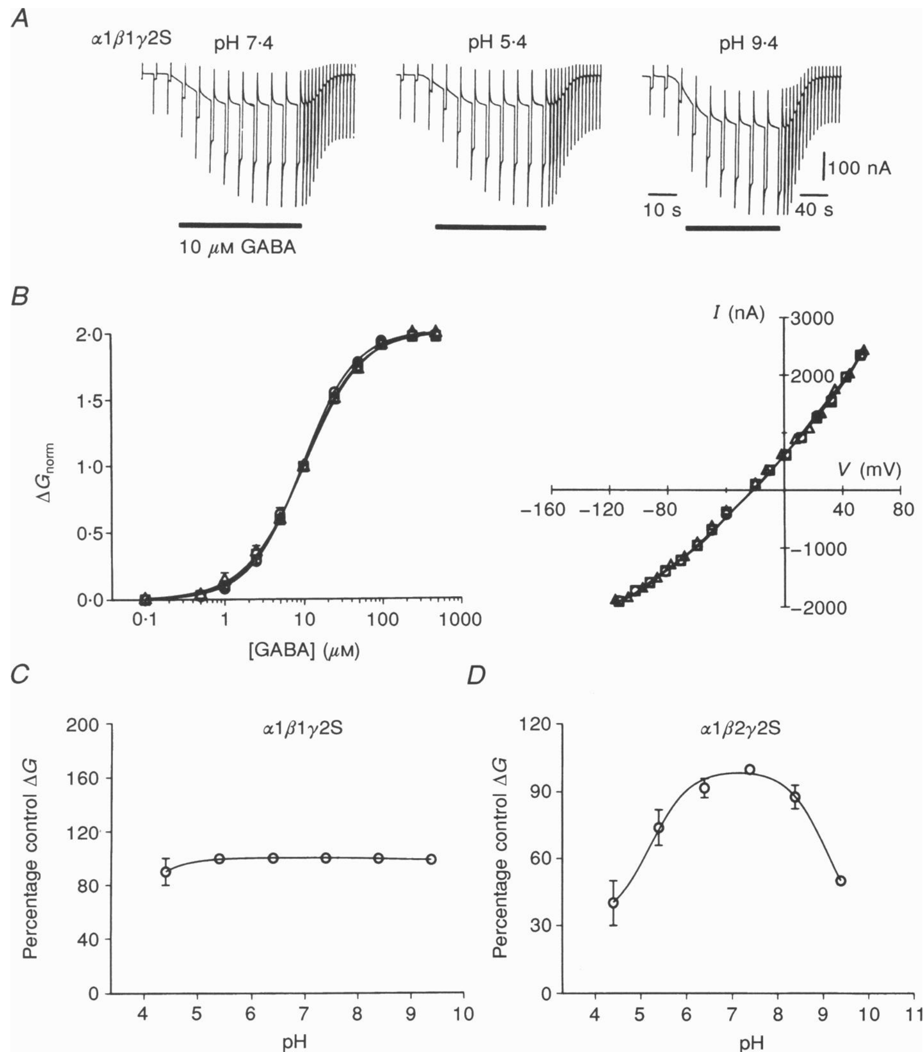

Figure 3. Modulation of $\alpha1\beta1\gamma2$S and $\alpha1\beta2\gamma2$S $GABA_A$ receptors by H^+

A, whole-cell responses to 10 μM GABA at external pH 7·4, 5·4 and 9·4 in oocytes expressing $\alpha1\beta1\gamma2$S $GABA_A$ receptor subunits at a holding potential of −40 mV. *B*, $\alpha1\beta1\gamma2$S constructs. Normalized equilibrium concentration–response curves (left) at pH 5·4 (□), 7·4 (○) and 9·4 (△). The EC_{50} values for GABA determined from the logistic model were: pH 7·4, 9·8 ± 0·3 μM; pH 5·4, 10·1 ± 0·2 μM; pH 9·4, 10·4 ± 0·3 μM ($n = 4$). Current–voltage relationships (right) for the 10 μM GABA response determined at pH 5·4, 7·4 and 9·4, with a reversal potential of −22·5 ± 0·71 mV. *C*, pH titration relationship for the conductance induced by 10 μM GABA normalized to the response at pH 7·4 and fitted according to the pH model for $\alpha1\beta1\gamma2$S subunit-containing receptors ($n = 3$). *D*, normalized pH titration relationship for the GABA conductance (10 μM GABA) recorded from oocytes injected with $\alpha1\beta2\gamma2$S cDNAs. The curve was fitted according to the pH model ($n = 3$).

The relative insensitivity to pH, apparently conferred on the GABA$_A$ receptor by the γ2S subunit, suggested that pH-induced modulation of GABA$_A$ receptor function may be dependent on the subunit composition. To test this hypothesis, the β1 subunit was substituted by β2, forming $\alpha1\beta2\gamma2S$. The GABA-activated conductance exhibited a sensitivity to external pH, being reduced following pH excursions either side of pH 7·4. The pH titration plot revealed a 'bell-shaped' relationship with pK_a values of 5·16 ± 0·16 and 9·44 ± 0·18 ($n = 3$; Fig. 3*D*). A further test of the hypothesis that the pH sensitivity of GABA$_A$ receptors may be subunit dependent was enabled by reverting to the β1 subunit and exchanging the γ2S subunit with δ, forming $\alpha1\beta1\delta$ GABA$_A$ receptors. Unlike the γ2S subunit, the δ subunit was unable to render the $\alpha1\beta1$ GABA$_A$ receptor complex pH insensitive. Increasing the external H^+ concentration resulted in an enhanced response to 10 μM GABA, whilst reducing the H^+ concentration caused a depression of the response. The pH titration relationship possessed a distinctive biphasic shape with a clear inflection at pH 7·5–8·5 (Fig. 4*A*). At least two discrete sites with pK_a values of 6·6 ± 0·66 and 9·9 ± 0·24 were necessary to account for the shape of the curve, suggesting the involvement of histidine and tyrosine or lysine residues (Mahler & Cordes, 1971). The GABA equilibrium concentration–response curve was enhanced and displaced to the left at pH 5·4 with an increased maximum response. Conversely, at pH 9·4 the curve was non-competitively reduced without any lateral shift in the curve (Fig. 4*B*). With the injection of cDNAs encoding for α1, β1 and δ into oocytes, it is possible that at least two classes of functional receptors are expressed, notably, $\alpha1\beta1$ and $\alpha1\beta1\delta$, with the possibility of other combinations such as $\alpha\delta$, $\beta\delta$ and homomeric δ. Such a heterogeneous mix may cause the inflection in the pH titration relationship; however, a preferred assembly of $\alpha1\beta1\delta$ GABA$_A$ receptors was supported by the unusually slow rate of desensitization of GABA-evoked currents and the long open times of single channel currents (Saxena & Macdonald, 1994), when compared with $\alpha\beta$ or $\alpha\beta\gamma$ subunit-containing GABA$_A$

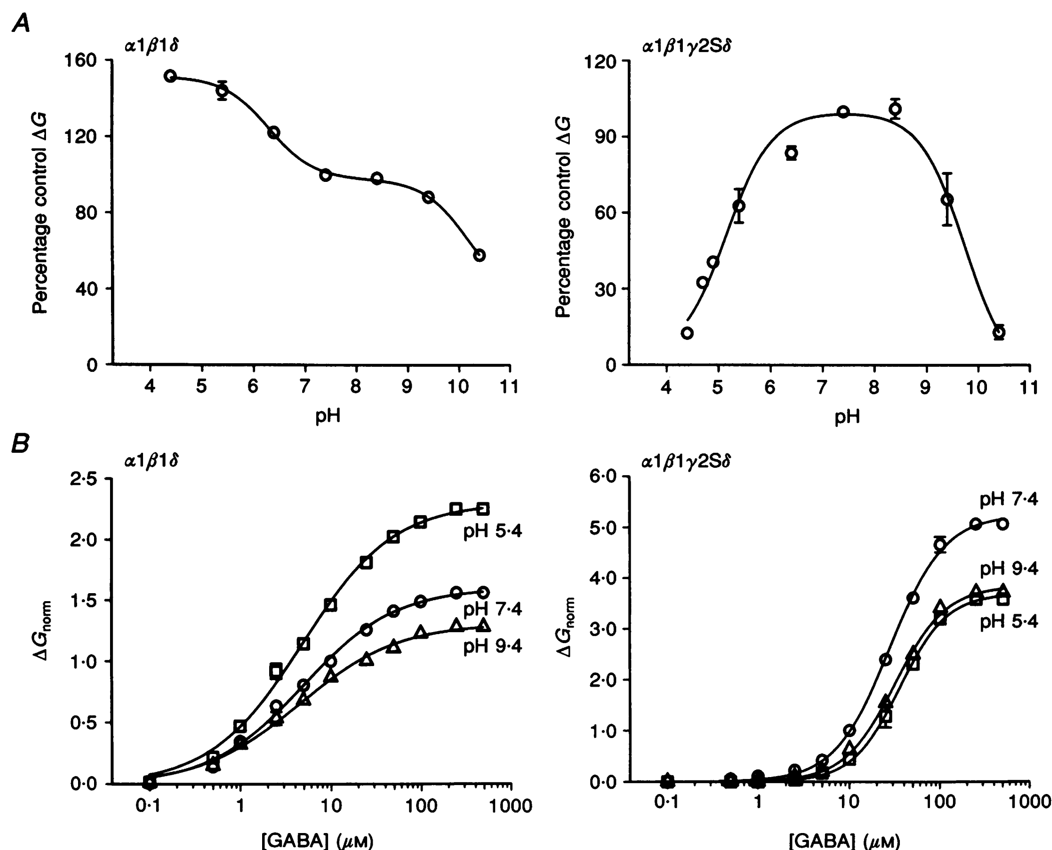

Figure 4. Influence of the δ subunit on H^+-induced modulation of GABA-activated currents

A, pH titration relationships for the GABA$_A$ receptor constructs $\alpha1\beta1\delta$ (left) and $\alpha1\beta1\gamma2S\delta$ (right). The curves describing the data for $\alpha1\beta1\delta$ and $\alpha1\beta1\gamma2S\delta$ GABA$_A$ receptors were generated according to the pH model. *B*, normalized equilibrium concentration–response curves at different external pH values (7·4, 5·4 and 9·4) for the $\alpha1\beta1\delta$ and $\alpha1\beta1\gamma2S\delta$ receptor subunit assemblies. The EC$_{50}$ values for GABA on $\alpha1\beta1\delta$ subunit-containing receptors were determined from the logistic model as: pH 7·4, 4·9 ± 0·4 μM; pH 5·4, 4·9 ± 0·4 μM; pH 9·4, 4·5 ± 0·45 μM. For $\alpha1\beta1\gamma2S\delta$ GABA$_A$ receptors, the EC$_{50}$ values for GABA were estimated as: pH 7·4, 27·7 ± 1 μM; pH 5·4, 35·7 ± 0·98 μM; pH 9·4, 31·7 ± 1·3 μM.

receptors. Moreover, the binary complexes $\alpha\delta$ and $\beta\delta$, and homomeric δ subunit receptors could not be functionally expressed in *Xenopus* oocytes or HEK cells (Saxena & Macdonald, 1994; authors' unpublished observations; cf. Shivers *et al.* 1989) discounting their contribution to the overall pH sensitivity profile which appears mostly, if not completely, due to $\alpha1\beta1\delta$ $GABA_A$ receptors.

Assembly of $\alpha1\beta1\delta$ subunit-containing $GABA_A$ receptors

A correlation of the electrophysiological data regarding the assembly of $\alpha1$, $\beta1$ and δ subunit-containing $GABA_A$ receptors was addressed by using immunoprecipitation with subunit-specific antisera directed against the $\alpha1$ and $\beta1$ subunits. To enable the detection of the δ subunit, an epitope 'tag' consisting of ten amino acids (9E10 epitope, Evans *et al.* 1985) was incorporated between amino acids 4 and 5 into the mature δ polypeptide subunit using site-directed mutagenesis (see Methods). The δ subunit was then resolved using selective antisera for the epitope tag. These studies identified the δ subunit as two bands migrating with apparent molecular masses of 56 and 54 kDa, whilst the $\alpha1$ subunit was detected as two major bands of 52 and 50 kDa, with a minor band at 48 kDa. The $\beta1$ subunit was detected as two bands with molecular masses of 58 and 56 kDa (Fig. 5*A*, lanes 1–4). The multiple species detected for the $\alpha1$ and $\beta1$ subunits appear to arise from proteolysis as described previously (Krishek *et al.* 1994). Immunoprecipitation with the $\beta1$ subunit antiserum followed by Western blotting with the 9E10 antiserum clearly showed the association of the δ subunit with the $\alpha1$ and $\beta1$ subunits suggesting co-assembly of these three subunits (Fig. 5*B* and *C*; cf. Mertens, Benke & Mohler, 1993).

Expression of $\alpha1$, $\beta1$, $\gamma2S$ and δ $GABA_A$ receptor subunits in *Xenopus* oocytes: a discrete receptor complex

Since the $\gamma2S$ and the δ subunits differentially influence the behaviour of $\alpha1\beta1$ subunit-containing $GABA_A$ receptors, it was of interest to co-inject cDNAs encoding for $\alpha1$, $\beta1$, $\gamma2S$ and δ subunits into the same oocytes, with the aim of expressing $\alpha1\beta1\gamma2S\delta$ $GABA_A$ receptors. This construct was probably assembled (Mertens *et al.* 1993) as another distinctive pH-sensitive profile was exhibited. In support of this conclusion, the only other receptor subunit combinations that may have been assembled following the injection of

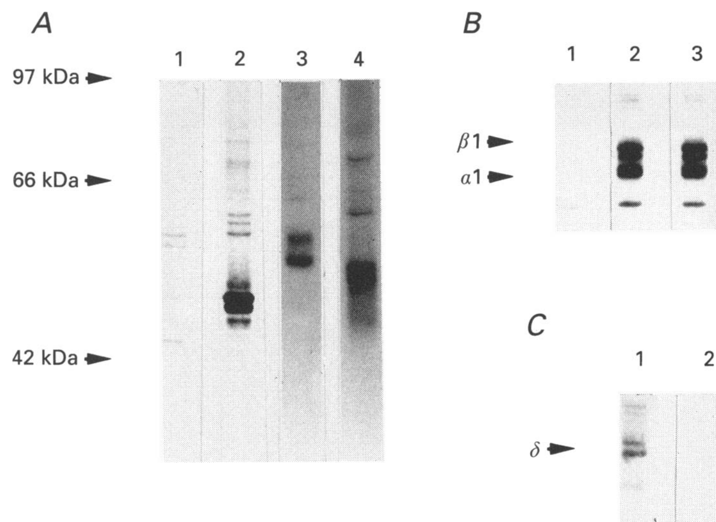

Figure 5. Assembly of $\alpha1\beta1\delta$ subunit-containing $GABA_A$ receptors in HEK cells

A, identification of $GABA_A$ receptor $\alpha1$, $\beta1$ and δ (9E10) subunits in HEK cells (Kraig *et al.* 1983). Cells expressing the $\alpha1$, $\beta1$ and δ (9E10) subunits were labelled with ^{35}S-methionine and precipitated with the 9E10 antiserum, or subunit-specific antisera against the $\alpha1$ and $\beta1$ subunits. Immune complexes were then resolved by SDS–PAGE followed by fluorography. The lanes represent the following (molecular mass markers are shown as arrows): lane 1, untransfected cells immunoprecipitated with a mixture of all three antisera; lane 2, cells expressing the $\alpha1$ subunit alone precipitated with a rabbit polyclonal anti-$\alpha1$ serum; lane 3, cells expressing the $\beta1$ subunit alone precipitated with a rabbit polyclonal anti-$\beta1$ serum; and lane 4, cells expressing the δ (9E10) subunit alone, immunoprecipitated with murine 9E10 antiserum. *B*, assembly of $GABA_A$ receptors composed of $\alpha1\beta1$ and $\alpha1\beta1\delta$ (9E10) subunits. HEK cells expressing these receptor subunit combinations were labelled with ^{35}S-methionine and immunoprecipitated with a rabbit polyclonal antisera against the $\beta1$ subunit. Lane 1, control untransfected cells; lane 2, cells expressing $GABA_A$ receptors composed of $\alpha1\beta1$ and δ (9E10) subunits; lane 3, cells expressing $\alpha1$ and $\beta1$ $GABA_A$ receptor subunits. *C*, detection of the δ (9E10) subunit in association with the $\alpha1$ and $\beta1$ subunits. Cells expressing $GABA_A$ receptors composed of $\alpha1\beta1$ and δ (9E10) (lane 1) or $\alpha1\beta1$ (lane 2) subunits were immunoprecipitated with the anti-$\beta1$ serum. The immunoprecipitates were then probed with murine 9E10 antiserum which is directed against the δ (9E10) subunit by Western blotting.

these four cDNAs, to provide functional GABA-activated receptors, include: $\alpha1\beta1$, $\alpha1\beta1\gamma2S$ and $\alpha1\beta1\delta$ GABA$_A$ receptors. However, mixed populations of these receptor species would be unable to account for the pH profile, presumed to be due to formation of the $\alpha1\beta1\gamma2S\delta$ construct, particularly since low pH resulted in a reduced GABA-activated conductance (Fig. 4*A*), a feature not observed with any of the other alternative constructs. The pH titration relationship revealed that the peak GABA-activated conductance occurred at pH 7·4, and either increasing or decreasing the physiological pH resulted in a reduction of the response to GABA. This complex 'bell-shaped' pH titration relationship could be accounted for by assuming that H^+ bound to two sites with pK_a values of 5·5 ± 0·2 and 9·4 ± 0·2 (Fig. 4*A*) which may also depend on the presence of histidine and tyrosine or lysine residues (Mahler & Cordes, 1971). Thus for $\alpha1\beta1\gamma2S\delta$ subunit-containing GABA$_A$ receptors, pH 7·4 appears to be optimum for maximizing the GABA-activated Cl^- conductance. The concentration–response relationships to GABA revealed non-competitive depressions in the curves at pH 5·4 and 9·4 relative to 7·4 (Fig. 4*B*). The *I–V* relationships for responses to GABA recorded from $\alpha1\beta1\delta$ and $\alpha1\beta1\gamma2S\delta$ constructs revealed unchanged GABA reversal potentials at pH 5·4 relative to 7·4 (−20 ± 1·8 mV). Furthermore, the modulations of the GABA-activated

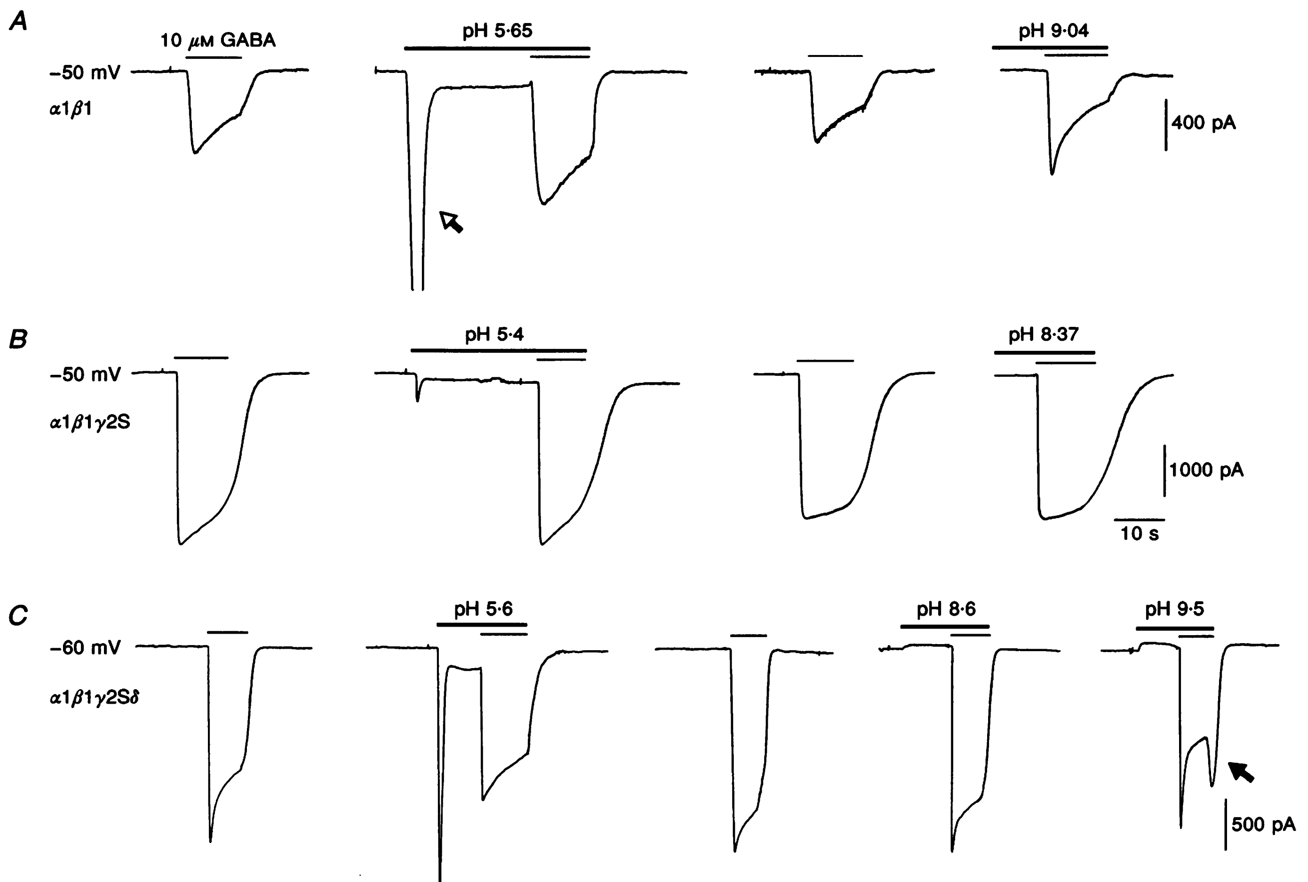

Figure 6. Modulation of recombinant GABA$_A$ receptors expressed in HEK cells

A, membrane currents were activated by 10 µM GABA (duration indicated by the thin line above the traces) and recorded under whole-cell voltage clamp at −50 mV in cells expressing $\alpha1\beta1$ subunit constructs. Cells were exposed to Krebs solution at pH 7·4 throughout unless indicated otherwise. Membrane current was always recovered after GABA application in pH 7·4 Krebs solution. Application of pH 5·65 Krebs solution (thick line) initiated a transient, inactivating current (open arrow). GABA was re-applied at pH 5·65 during the steady-state inward pedestal current and a recovery response was obtained in pH 7·4 Krebs solution. In the same cell, the response to GABA was assessed in Krebs solution at pH 9·04. *B*, for HEK cells expressing $\alpha1\beta1\gamma2S$ subunits, 10 µM GABA was applied in pH 7·4 Krebs solution at −50 mV, and then sequentially in pH 5·4, 7·4 and 8·37 Krebs solution. *C*, GABA-activated responses recorded from cells expressing $\alpha1\beta1\gamma2S\delta$ subunit containing GABA$_A$ receptors at −60 mV. GABA was applied in Krebs solution with differing pH according to the following order: pH 7·4, 5·6, 7·4, 8·6, 7·4 (not shown), 9·5. Note the transient rebound current on recovery with pH 7·4 Krebs solution (filled arrow).

conductances at pH 5·4 were achieved in a voltage-insensitive manner.

pH sensitivity of $GABA_A$ receptors expressed in human embryonic kidney cells

To ensure that the pH sensitivities of recombinant $GABA_A$ receptors were not unduly influenced by the amphibian *Xenopus laevis* oocyte expression system, receptors were accordingly expressed in mammalian HEK cells. This also enabled faster application of GABA to the expressed receptors, providing data on peak and steady-state GABA-activated currents in comparison with the oocyte experiments, which had provided information only on the latter.

As for the *Xenopus* oocytes, HEK cells also exhibited an innate sensitivity to external pH. Application of low pH solutions (< 7·4) to untransfected HEK cells, at a holding potential of −50 mV, resulted in a transient inward current associated with a conductance increase. The current amplitude was reduced at more depolarized holding potentials and approached zero at +20 to +40 mV. A clear reversal potential was not obtained (data not shown). The inward current varied in amplitude between cells, but always rapidly inactivated to leave a constant inward pedestal current invariably less than 5% of the peak current amplitude. In contrast, high external pH solutions (> 7·4) induced a small persistent outward current that did not apparently inactivate. The response of the membrane to external pH was unaffected by the transfection procedure.

GABA-activated membrane currents were recorded from transiently transfected HEK cells in Krebs solutions of differing external pH, after the pH-sensitive membrane currents had attained a steady state. Thus at low pH, GABA was applied during the inward pedestal current after the transient current had inactivated. These responses to GABA were then compared with the corresponding control responses obtained at pH 7·4. In cells expressing $\alpha 1\beta 1$ subunits, GABA-activated responses (peak and steady state) were clearly enhanced when the pH was less than 7·4 (Fig. 6*A*). However, at high pH, the peak response to GABA was slightly enhanced, whereas the steady-state response was reduced with an apparently increased rate of desensitization (Fig. 6*A*). In contrast, cells transfected with $\alpha 1\beta 1\gamma 2S$ cDNAs exhibited $GABA_A$ receptors that were insensitive to low or high pH excursions from 7·4 (Fig. 6*B*). In comparison, the addition of the δ subunit to the preceding complex, forming $\alpha 1\beta 1\gamma 2S\delta$ subunit receptors, changed the pH-sensitive profile for GABA-activated responses. Low pH clearly reduced the peak GABA-induced current but only slightly reduced the steady-state current (Fig. 6*C*), whereas high pH had little effect up to 8·6, but beyond 9·5 the peak response was reduced and the steady-state response clearly inhibited with an increased rate of desensitization (Fig. 6*C*). Interestingly, on recovery from pH 9·5 to 7·4, the GABA response exhibited a rebound transient recovery to the amplitude normally expected for the steady-state current at pH 7·4 (Fig. 6*C*). This may reflect recovery from the effects of high pH prior to the complete removal of GABA from the cell.

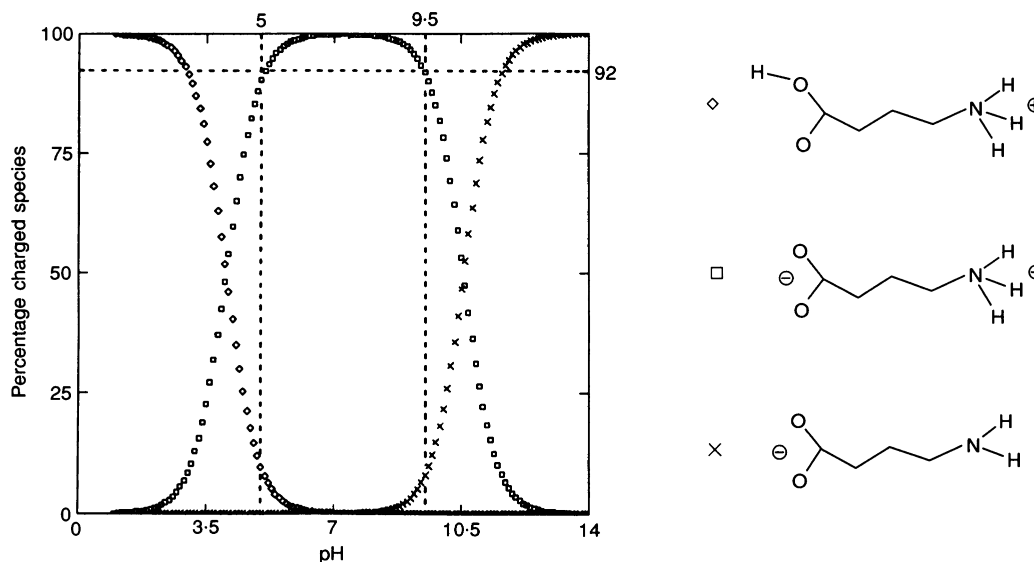

Figure 7. Effect of external pH on the ionization of the GABA molecule

Using the pK_a values of 4 and 10·55, reported for GABA by Takeuchi & Takeuchi (1967), the relative proportions of each of the 3 molecular species of GABA (anionic (×), cationic (◇) and zwitterionic (□)) with respect to the total number of GABA molecules, were calculated at different pH values according to the pH model (see Methods). Between pH 5 and 9·5, never less than 92% of GABA molecules will reside in the zwitterionic form.

pH sensitivity of the GABA molecule

The GABA molecule has two potentially charged centres ($-NH_2$ and $-COOH$) which will be affected by the pH of the Krebs solution. With the amino and carboxyl groups, GABA can exist in up to three different charged forms: anionic, zwitterionic and cationic. The pK_a values for the two charged centres are 4 ($-COOH$) and 10·55 ($-NH_2$; Takeuchi & Takeuchi, 1967). It is thus possible to predict the charge behaviour of the molecule at different external pH values and estimate the percentage of each of the three molecular species in solution. From Fig. 7, it is apparent that over the range of external pH 5–9·5, between 92 and 100% of GABA molecules (mean, 96%) will exist as zwitterions in solution, and only outside this range will the anionic and cationic forms of the molecule constitute a significant proportion of the total number of GABA molecules.

DISCUSSION

We have studied the effects of H^+ on GABA$_A$ receptor function for two principal reasons. First, variations in external pH is a useful method for protonating weak acidic and basic groups contained within receptor proteins which may modulate receptor function. Moreover, protonation and subsequent determination of pK_a values may allow a broad identification of the key amino acid residues that are involved in receptor function. Second, previous studies have demonstrated that changing external pH can differentially influence native GABA$_A$ receptor function. For example, ionophoresing H^+ onto cultured spinal or reticulospinal neurones (Groul *et al.* 1980), or lowering external pH to 6·2 in sympathetic neurones (Smart, 1992) resulted in reduced responses to GABA. In comparison, raising the extracellular H^+ concentration (from pH 7·4) around dorsal root ganglia (Gallagher *et al.* 1983) resulted in enhanced GABA-activated responses. A similar change had little effect on GABA-induced responses in hippocampal neurones, although raising the pH from 7·4 to 8·0 resulted in inhibition (Tang *et al.* 1990). Our present study attempts to offer an explanation for these different results, since we demonstrate that pH modulation of GABA$_A$ receptors is dependent upon the receptor subunit composition. On this basis, neurones containing a preponderance of $\alpha1\beta2\gamma2$ subunits would be expected to display responses to GABA that are relatively insensitive to external pH, whilst reduced amplitude responses at low or high pH could be achieved in cells retaining either $\alpha1\beta2\gamma2$ or $\alpha1\beta1\gamma2\delta$ constructs; however, if multiple subtypes of the GABA$_A$ receptor exist in single neurones, then the pH profile of a neuronal GABA response could also reflect a 'mosaic' of different receptor subunit combinations.

The results obtained with recombinant GABA$_A$ receptors demonstrated that a range of sensitivities to external pH could be observed with a small number of receptor constructs. Throughout this study we chose to keep the α subunit constant in the receptor constructs to limit the number of different receptor combinations to be tested; however, it is possible that changing $\alpha1$ for αj ($j = 2$–6) could result in further changes in the receptor sensitivity to external pH (e.g. changing $\alpha1$ for $\alpha6$ in the $\alpha j\beta1\gamma2$ construct increased the amplitude of responses to GABA at low pH; B. J. Krishek & T. G. Smart, unpublished observations).

There are very few genuinely 'receptor isoform specific' agents that can be used as probes to examine the distribution of particular GABA$_A$ receptor subunits. Such is the distinctive nature of the pH sensitivity of GABA$_A$ receptors that this criterion may aid tentative identification, by serving as a molecular fingerprint for some receptor subunit assemblies throughout the CNS; however, categorical identification will still require alternative approaches including Western blot analyses.

Multiple actions of H^+ on GABA$_A$ receptor function

The complex level of modulation of GABA$_A$ receptors probably reflects an action of H^+ at more than one site (see below). For example, low external pH can result in an enhanced (for $\alpha1\beta1$ or $\alpha1\beta1\delta$ constructs), a reduced ($\alpha1\beta1\gamma2S\delta$), or unaffected ($\alpha1\beta1\gamma2S$) GABA-activated response. This differential modulation is not an aberrant feature of the *Xenopus laevis* oocyte expression system since similar results were observed for steady-state responses to GABA on expressed GABA$_A$ receptors in transfected HEK cells. The rapid application of GABA allowed the resolution of the early peak GABA-induced current before the full onset of desensitization. Under these conditions, for $\alpha1\beta1$ or $\alpha1\beta1\gamma2S\delta$ subunit constructs, it was notable that on increasing the external pH from 7·4 the peak GABA-activated current was either enhanced or unaffected, and the rate of desensitization was increased, resulting in a reduced steady-state current. Due to the slower bath application of GABA, the increased peak current at alkaline pH would not have been detected in the *Xenopus* oocyte experiments, which demonstrated a reduced steady-state current in accordance with the data obtained using HEK cells.

The inhibitory effects of H^+ on some of the recombinant GABA$_A$ receptors appeared mostly non-competitive, suggesting that H^+ ions were not competing for the GABA recognition site. Moreover, since GABA activates an anion-selective conductance, it would appear unlikely that H^+ ions are gaining direct access to the ion channel and causing a blockade. The simplest hypothesis involves H^+ binding to one or more allosteric sites which initiate the inhibitory effects. Indeed, the enhancement of GABA-activated responses by H^+ in $\alpha1\beta1$ and $\alpha1\beta1\delta$ subunit-containing receptors may also be due to H^+ binding to possibly different allosteric sites. Moreover, it is unlikely that the pH dependence of the charge distribution of the GABA molecule underlies the modulation by H^+, since the zwitterion is the most abundant species present from pH 5–9·5.

Multiple locations for the H^+ binding sites?

The transmembrane topography of the $GABA_A$ receptor has been largely predicted from hydropathy plot analyses of individual polypeptide subunits. Each mature subunit contains approximately 400–500 amino acid residues, yielding 2000–2500 residues for a presumed pentameric receptor (Nayeem, Green, Martin & Barnard, 1994). Of these, up to 60% of the total number of residues will be expected to reside extracellularly compared with the remaining 40% which are postulated to be equally divided between the four transmembrane-spanning regions and the two intracellular loops. On this basis and considering that many residues will contain charged side chains capable of dipole interactions, there seems ample opportunity for H^+ to interact with multiple sites on the receptor protein from which receptor function could be affected. Estimation of pK_a values from the pH titration relationships has enabled a tentative identification of some of the residues involved, either directly with the H^+ binding sites and/or with the function of the receptor. However, in a mature protein, accurate measurements of the pK_a values for individual amino acids can be affected by dipoles and the 'charged environment' within the protein, thereby distorting the pK_a value (Antosiewicz, McCammon & Gilson, 1994). It is also conceivable that some of the pK_a values may represent 'compound' values for several sites with similar charge distribution. Thus these estimates can only be used as an approximate guide for future site-directed mutagenesis studies. The implication that multiple sites are involved with the H^+ modulation might also be compatible with the multiple effects of H^+, notably on the EC_{50} for GABA and the extent of $GABA_A$ receptor desensitization, together with the complex pH titration relationships.

Intracellular pH and modulation of the $GABA_A$ receptor

Whilst changing the external pH, it is conceivable that changes to internal pH (pH_i) will also occur (Mellergard, Ouyang & Siesjo, 1992) and this may contribute to the observed modulation of $GABA_A$ receptor function. This issue was addressed by monitoring the changes of intracellular pH, both in *Xenopus* oocytes and HEK cells, while changing the pH of the external solution. Measurements of pH_i were performed using the membrane-permeant fluorescent pH indicator BCECF (see Methods) (Rink *et al.* 1982). Challenging *Xenopus* oocytes with Ringer solution at pH 5·4 or 9·4 for 4 min evoked a very small shift in pH_i (pH 7·4 to 5·4: ΔpH_i, 0·17 ± 0·04; pH 7·4 to 9·4: ΔpH_i, 0·08 ± 0·01; $n = 4$). Similarly, perfusing HEK cells with Krebs solution at pH 5·4 and 9·4, whilst recording membrane currents from cells in the whole-cell configuration, induced negligible pH changes (pH 7·4 to 5·4: ΔpH_i, 0·013 ± 0·013; pH 7·4 to 9·4: ΔpH_i, 0·022 ± 0·02; $n = 4$). Thus it appears likely that the small variations in internal pH are unable to account for the modulation of $GABA_A$ receptor function by extenal pH (cf. Kaila, 1994). Interestingly, invertebrate GABA-gated Cl^- channels in crayfish muscle fibres are unaffected by intracellular pH changes (Pasternack, Bountra, Voipio & Kaila, 1992).

Physiogical implications of H^+ modulation

H^+ ions are involved in many facets of cellular biochemistry and transient changes in extracellular pH can occur during 'normal' synaptic transmission (Chesler & Chan, 1988; Chen & Chesler, 1992) which may affect GABA-mediated synaptic inhibition and therefore cell excitability. Activation of $GABA_A$ receptors alone may also change the external pH following bicarbonate efflux through the integral anion-selective channel (Kaila & Voipio, 1987; Kaila, 1994) and this might directly affect the primary GABA-activated conductance. Changes in external pH are also a feature of pathological processes such as ischaemia, anoxia and epileptiform activity (Urbanics, Leniger-Follert & Lubbers, 1978; Kraig, Ferreira-Filho & Nicholson, 1983; Chesler, 1990; Chesler & Kaila, 1992) and thus, depending on the $GABA_A$ receptor subunit composition in the affected area, inhibitory neurotransmission may be enhanced, unaffected or reduced. Furthermore, the potential for a complex modulation of GABAergic synaptic inhibition, together with the known sensitivity of the *N*-methyl-D-aspartate receptor to external pH (low pH inhibits NMDA receptor-mediated responses; Tang *et al.* 1990; Traynelis & Cull-Candy, 1990; Vyklicky, Vlachova & Krusek, 1990) could have important consequences for neurotoxicity.

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